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Development & validation of RP-HPLC method for estimation of pomalidomide drug in pharmaceutical dosage form

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Abstract
A simple, precise, rapid and accurate RP- HPLC method was developed for the estimation of Pomalidomide (PML) in capsule dosage forms. A Kinetex phenyl Hexyl C_{18}Thermo scientific 250 x 4.6 mm, 5 µm column and the mobile phase, consisting of 0.1 M KH_2PO_4 in water adjusting the pH- 2.5 with O-Phosphoric Acid: Methanol in ratio of 30:70 v/v was used for method development. The flow rate was 1 ml/min and the effluents were monitored at 221 nm. The retention time was found to be 4.7 min for PML. The detector response was linear in the concentration of 15.47- 45.42 µg/mL for PML. The respective linear regression equation being Y = 11771 x - 92623 for PML. The Limit of Detection (LOD) is 0.079& The Limit of Quantification (LOQ) is 0.235 for PML respectively. The % assay of PML was found out to be 100.9%. The method was validated by determining its accuracy, precision and system suitability. The results of the study showed that the proposed RP-HPLC method is simple, rapid, precise and accurate, which is useful for the routine determination of PML in bulk drug and in its pharmaceutical dosageforms.

Keywords: pomalidomide (PML), RP-HPLC, O-phosphoric acid, capsules

Introduction
Pomalidomide (PML) is an immune-modulatory antineoplastic agent, chemically is 4-amino-2-(2,6-dioxopiperidin-3-yl)-2,3-dihydro-1H-isoindole-1,3-dione (Figure 1).The Molecular Formula is $C_{13}H_{11}N_3O_4$ and the Molecular Weight is 273.24 g/mol. The partition coefficient log P value is -1.16. PML has plasma protein-bounding12 - 44% with a half-life of 7.5 hrs. After oral administration, Promalidomide is hepatically metabolized by CYP1A2 and CYP3A4. The metabolites are 26-times less active than the parent compound. "Pomalidomide is the third drug in a class of immunomodulatory agents also acts as anti-angiogenic that incorporates lenalidomide and thalidomide. [1] Pomalidomide has significant anti-TNFα *in vitro* activity and it is 5000-times more potent than thalidomide and 10-times more active than lenalidomide [2].
The parent compound of pomalidomide, thalidomide, was initially discovered to inhibit angiogenesis in 1994 [3]. In light this discovery, thalidomide was taken into clinical trials for cancer, leading to its ultimate FDA approval for various types of myeloma. Further structure activity studies done in Dr. Robert D'Amato's lab at Boston Children's Hospital led to the primary report in 2001[4] that 3-amino-thalidomide was able to directly restrain both the tumor cell and vascular compartments of myeloma cancers. This dual activity of pomalidomide makes it more adequate than thalidomide *in vitro* and *in vivo* [5].
Pomalidomide is made by chemical modification of thalidomide with the aim of increasing therapeutic activity while limiting toxicity. Its mechanism of action is not completely understood but involves anti-angiogenic effects, immunomodulation, an impact on the myeloma tumor microenvironment, and the protein cereblon. It is generally well tolerated, with adverse effects including weakness, neutropenia, neuropathy, and thromboembolic infection. Pomalidomide is a promising new agent in the expanding arms of antimyeloma drugs.[6] Pomalidomide when administered with weekly low-dose dexamethasone seems to be both safe and effective for the treatment of relapsed or refractory multiple myeloma in patients who have had disease progression after completing treatment with bortezomib, lenalidomide, or both [7].
So far, few methods are accounted for the estimation of PML in capsule dosage form and also in biological fluids with different mobile phases, retention time, linearity range conc., and absorption maxima [8, 9, 10]. In the present study, A detailed account of all analytical methods existing for the drug is made to keep away from duplication of the reported methods. The consequences of this work are set forth by developing a simple, precise and accurate reverse-phase HPLC method for the determination of PML in API and in pharmaceutical dosage form i.e., Pomalid® 2 mg capsules (Neon Laboratories Limited, Mumbai).

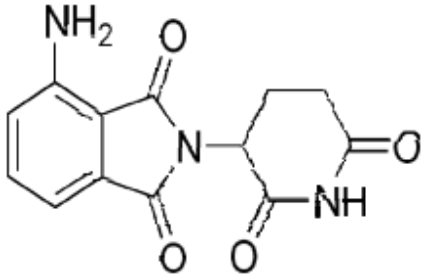

Fig 1: Chemical Structure of Pomalidomide

Materials and methods
Chemicals
Pomalidomide was obtained as a gift sample from Neon

Laboratories Limited, Mumbai. Acetonitrile and water used were of HPLC grade (Merck life science). Commercial available, Pomalid® 2 mg capsules were procured from local market.

Instrumentation

The HPLC system employed was, Waters e 2695, with PDA detector module equipped with automatic injector with injection volume 20 µl, and 2693 pump. An XTerra, RP-C_{18}Column (250x4.6 mm i.d; particle size 5 µm) was used. The HPLC system was equipped with Empower PRO Software. The column was maintained at 30ºC and eluted under isocratic conditions over 7.0 min at a flow rate of 1.0 ml/min.

Determination of working wavelength (λmax)

Preparation of Drug Standard solution: An accurately weighed quantity about 20 mg of Pomalidomide standard was transferred to 200 ml volumetric flask. Add 150 ml of diluent, sonicate to dissolve and dilute up to the mark with diluent and mixed. (100 µg/ml). Take 1 ml from stock solution and make up the volume upto 10 ml (10 µg/ml).This standard solutions were scanned separately between 400nm to 200nm. From the spectrum show high absorbance (fig 2).

Preparation of Diluent: Prepare mixture of water: methanol in the ratio of 70:30 v/v respectively, mix well and degas. It is used as solvent for dissolving API

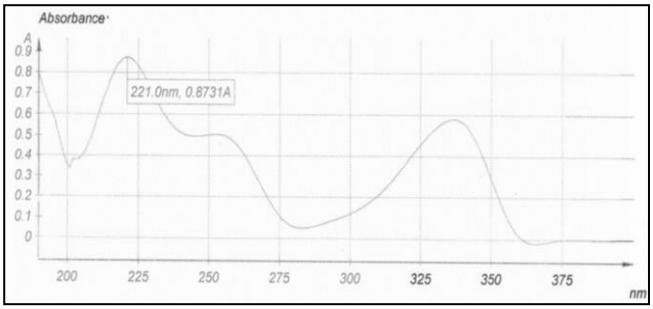

Fig 2: UV spectrum of pomalidomide

Chromatographic conditions

The contents of the Mobile Phase - consisting of 0.1 M KH_2PO_4 in water adjusting the pH-2.5 with O-Phosphoric Acid: Methanol in ratio of 30:70 v/v & 0.1% OPA and Acetonitrile in the ratio of 20: 80(v/v) was used as diluent in the gradient mode.

They were filtered before use, through a 0.45 µm membrane filter, and pumped from the respective solvent reservoirs to the column at a flow rate of 1.0 ml/min. A Kinetex phenyl Hexyl C_{18}Thermo scientific 250 x 4.6 mm, 5 µm column was used. The run time was set at 7.0 min and the column temperature was ambient. Prior to the injection (10 µl) of the drug solution, the column was equilibrated for at least 30 min with the mobile phases flowing through the system. The eluents were monitored at 221 nm.

Standard Prepration

Standard Stock Solution

Weigh and transfer accurately about 30 mg of pomalidomide standard into 100 ml volumetric flask. Add about 15 ml of diluent (2) sonicate to dissolve, cool and dilute up to the mark with diluent (2) and mix.

Working standard solution

Further dilute 5 ml of above solution to 50 ml with diluent (2) and mix. (Concentration of pomalidomide: 30 ppm)

Preparation of Diluent (1): Add 1 ml of Ortho-phosphoric acid in 1000 ml of water and mixed well

Preparation of Diluent (2): Prepare a mixture of 0.1%OPA and Acetonitrile in the ratio of 20: 80 (v/v)

Sample Preparation:

Ten capsules (Pomalid® 2 mg- Neon Laboratories Limited, Mumbai) were taken and the contents were emptied and transferred into a dry watch glass. Capsule powder equivalent to 30 mg of PML were weighed and transferred into a 200 ml of volumetric flask. Add about 50 ml of diluent(2) sonicate for 30 minutes with intermittent shaking, allow it to cool and make up to volume with diluent(1) and mix. Further dilute 5 ml of this solution to 25 ml with diluent (2) and mix. Filter the sample solution through 0.45µ Nylon membrane syringe filter. Discard first 3 or 4 ml of filtrate to get (Concentration of pomalidomide: 30 ppm)

Optimization of method

The mobile phase was chosen after several trials with isopropyl alcohol, acetonitrile, methanol, water and buffer solutions in various proportions.

And at different pH values.

Finally mobile phase consisting of 0.1 M KH_2PO_4 in water adjusting the pH-2.5 with O- Phosphoric Acid: Methanol in ratio of 30:70 v/v selected to achieve maximum separation and sensitivity. (Fig 3)

Table 1: Trials for method development

Trial	Column Used	Mobile Phase	Flow Rate	Wave length	Observation	Retention Time	Result
1.	Hypersil BDS C18 Thermo scientific 250 x 4.6 mm, 5 µm.	100 % Water	0.8 ml/min	221nm	Splitting of peak	No Peak	Method rejected
2.	Hypersil BDS C18 Thermo scientific 250 x 4.6 mm, 5 µm.	Methanol: Phosphate buffer = 20:80	0.8 ml/ min	221nm	Rt was very large	16.4 min	Method rejected
3.	Hypersil BDS C18 Thermo scientific 250 x 4.6 mm, 5 µm.	Methanol: Phosphate buffer = 20:80	1.0 ml/min	221nm	Baseline not proper	11.3 min	Method rejected
4.	Kinetex phenyl Hexyl, 250 x 4.6 mm, 5mm	Phosphate buffer: Methanol = 30:70	1.0 ml/min	221nm	Good sharp peak	4.7 min	Method accepted

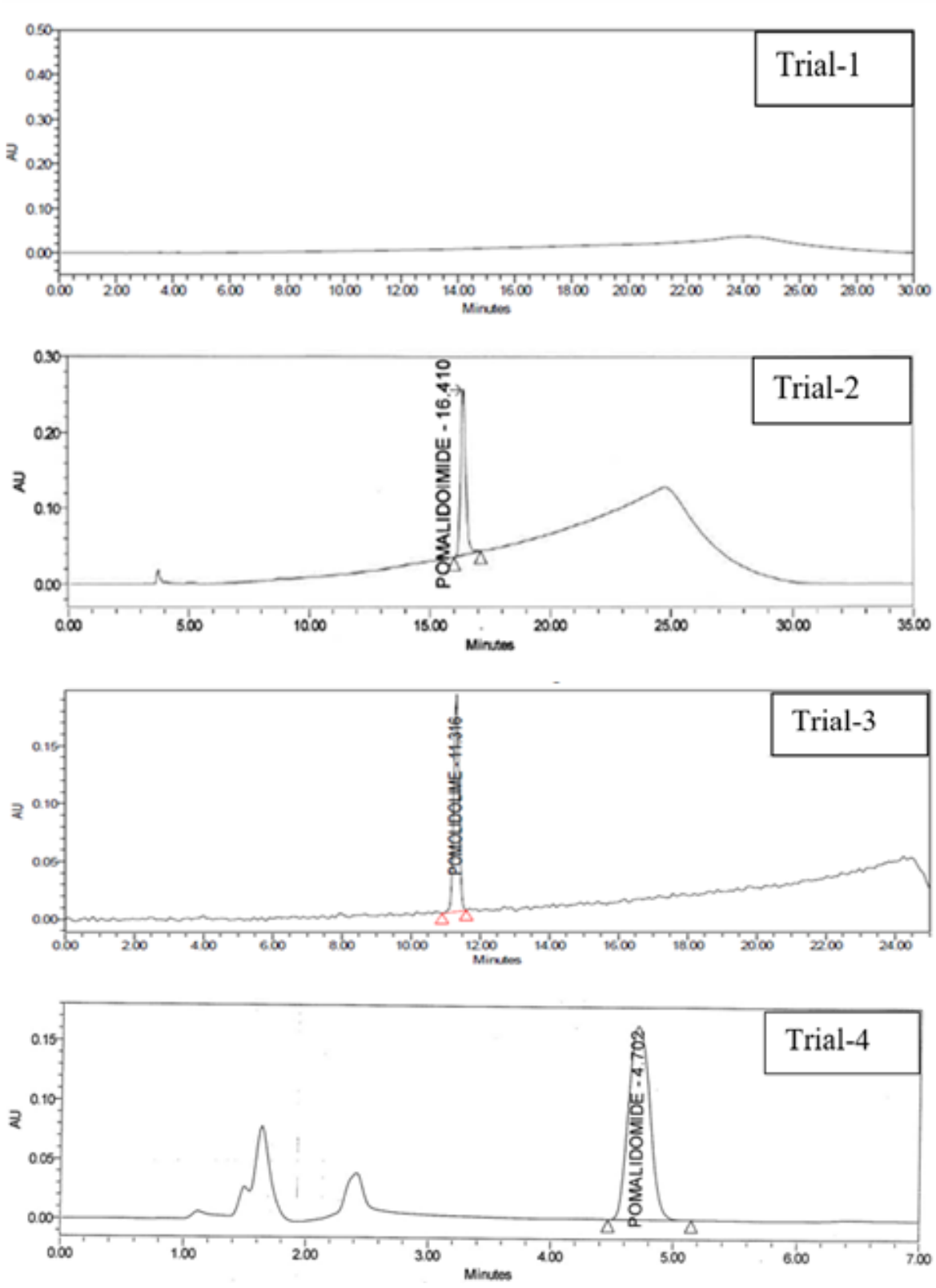

Fig 3: Trials for Method Development

Table 2: Peak Results

Rt	Peak Area	Theoretical Plates	Tailing Factor
4.7	2298653	6560	1.25

Optimized Chromatographic Conditions

Column: Kinetex phenyl Hexyl 250 mm x 4.6 mm, 5µm.
Mobile Phase: 0.1 M O-Phosphoric Acid (pH 2.5): Methanol in ratio of 30:70 v/v
Flow Rate: 1.0ml/minute
Wave length: 221nm
Injection volume: 10µl
Run time: 7.0minutes.
Column temperature: 35 ºc
Auto Sampler Temp: 25ºc
Retention Time: 4.7 min

Results and discussion

The objective of validation of an analytical procedure is to

demonstrate that it is suitable for its intended purpose. According to ICH guidelines, the validation parameters were [11]

System suitability

System suitability test was carried out to verify that the analytical system is working properly to give accurate and precise results. The system suitability tests were carried out on freshly prepared standard stock solution of PML. Solution was injected six times and the chromatograms were recorded. The system suitability parameters were evaluated from standard Chromatograms by calculating the % RSD of retention times, tailing factor, theoretical plates and peak areas. The tailing factors for PML were 1.25 and USP theoretical plates were found to be significantly high around 6560. The system was suitable for use, (Table 3)

Table 3: System suitability test of Pomalidomide

% RSD of six replicate injections of standard	0.68
The Tailing factor for peak	1.25
The theoretical plates for peak	6560
Correlation between 1st standard solution and 2nd standard solution	99.2
S. No.	Area
1	2280560
2	2298653
3	2316352
4	2280639
5	2310937
6	2309238
Mean	2299397
% RSD	0.68

Specificity: (Identification, Interference & Peak Purity)

Specificity was carried out to ensure the absence of known impurities and interference those are likely to be present in pomalidomide Capsules drug product

Preparation of Spike Sample solution:

5 mL from PML sample stock solution was taken and into that added 1 mL of each impurity 5-Amino, Des Amino**,** Nitrodione impurity stock solution (2 ppm each) and make up the volume upto to 25 mL with diluent$_2$ and mix. (Concentration of pomalidomide: 30 ppm). Inject Blank (Diluent), standard solution, impurity Solution, placebo solution and sample solution

For Identification: Results of standard was compared with respect to the retention time of sample. (fig 3 & 4)

In Interference: Blank, Placebo & known impurity does not show any peaks at the Retention time of pomalidomide peak. (Fig 5, 6, 7, 8, 9, 10)

And in Peak Purity: Standard and Sample peak was pure for working concentration level. Purity angle was less than purity threshold. (Fig 3 & 4)

Hence No evidence of signals, in the corresponding times of the chromatogram were monitored as a sign of potential interfering peaks, were found when the pharmaceutical formulations were tested.

Hence, this method can be used reliably for the estimation of respected active pharmaceutical ingredients in a variety of dosage forms. All The data obtained is summarized in (Table 4)

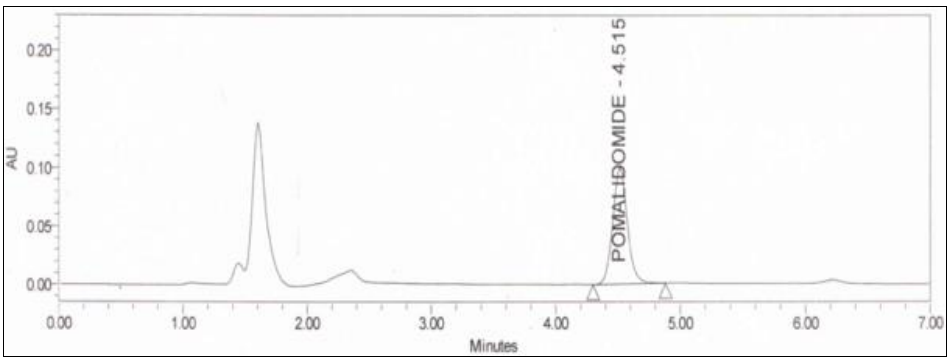

Fig 3: Chromatogram of Standard

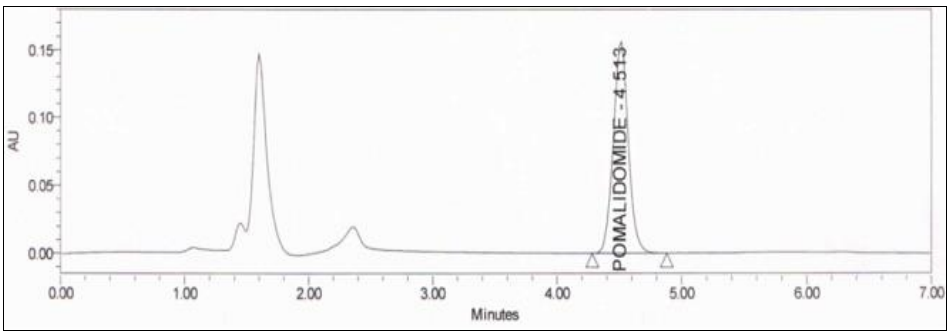

Fig 4: Chromatogram of Sample

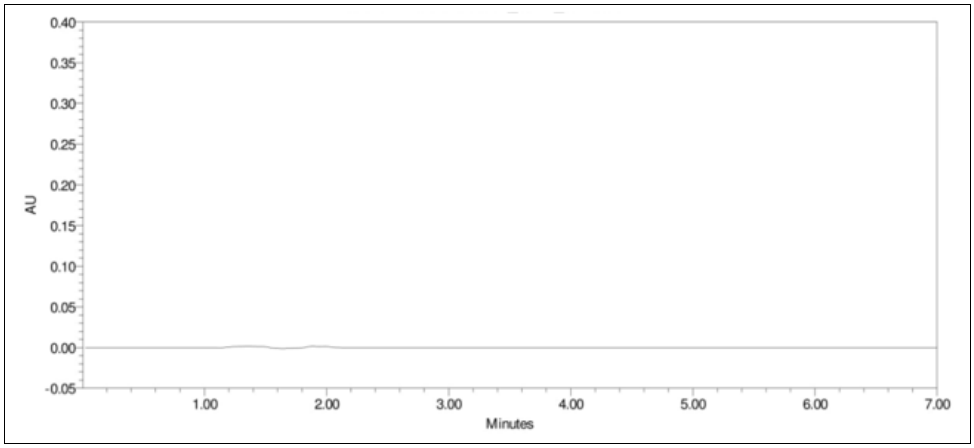

Fig 5: Chromatogram of Blank

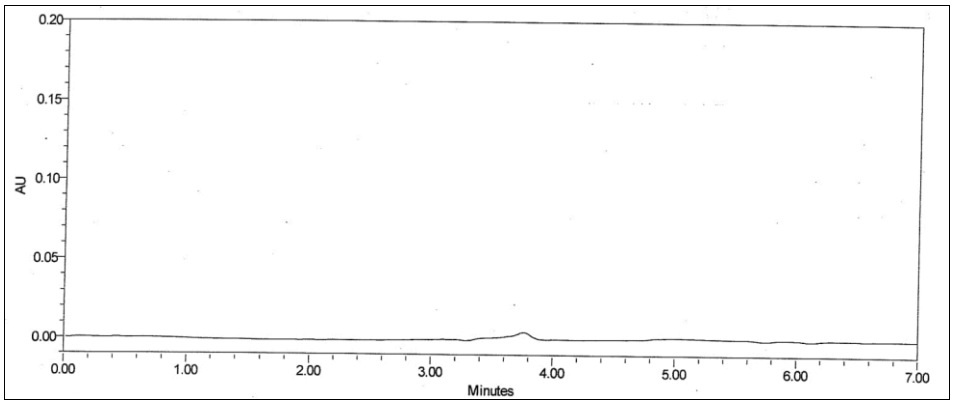

Fig 6: Chromatogram of Placebo

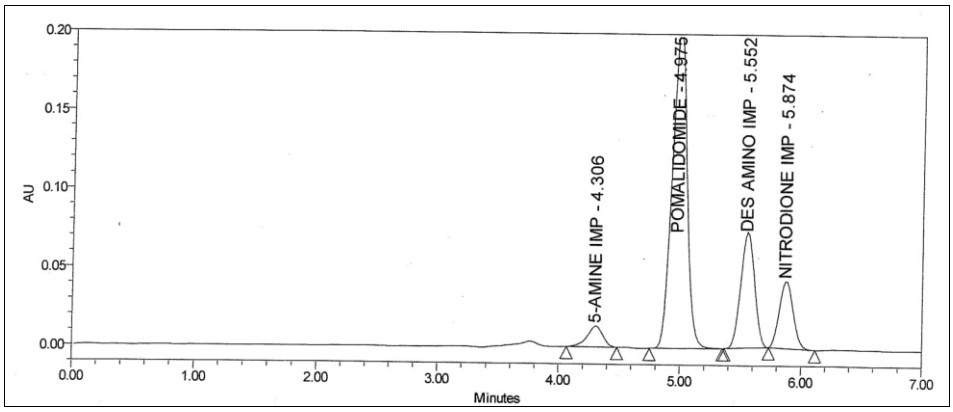

Fig 7: Chromatogram spike sample

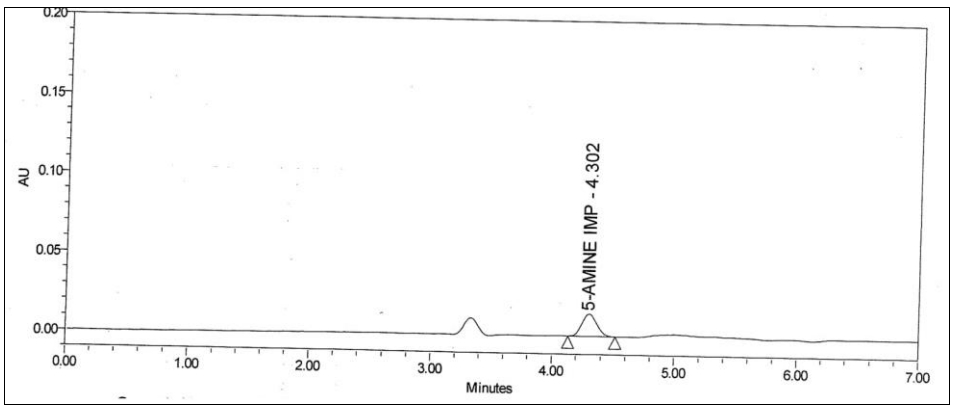

Fig 8: chromatogram of 5-Amino pomalidomide impurity

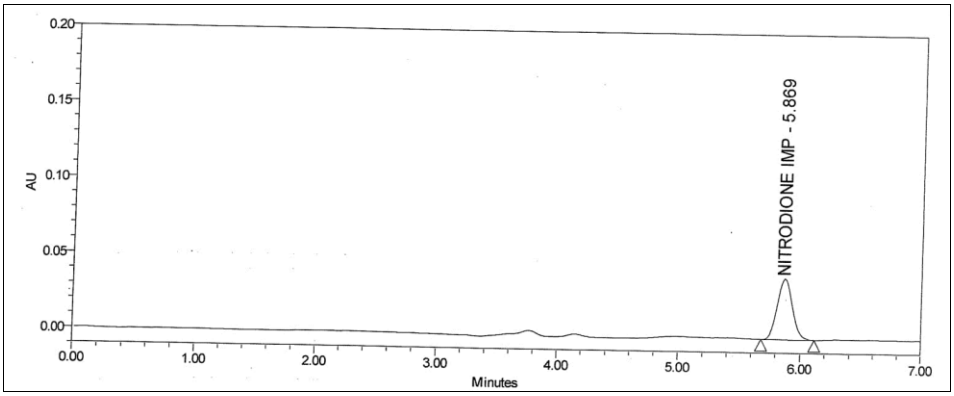

Fig 9: chromatogram of Nitrodione impurity

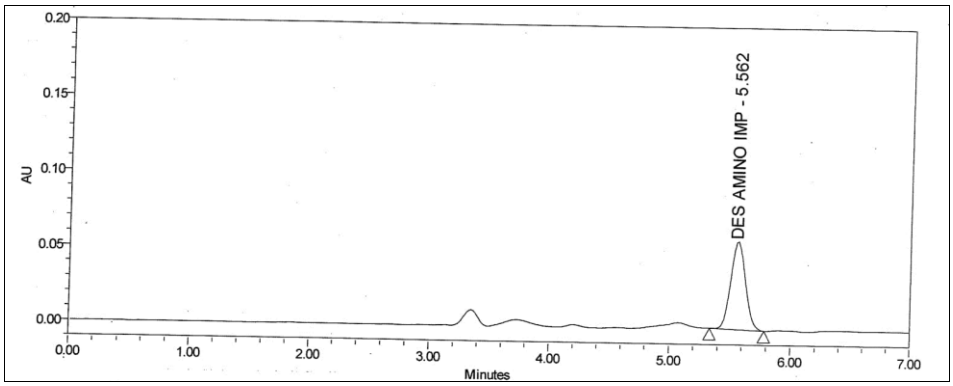

Fig 10: Chromatogram of Des Amino impurity

Table 4: Specificity of Pomalidomide (Identification and Interference)

Component	Retention time (min)	Tailing factor	Theoretical plates	Purity angle	Purity threshold
Blank	-	-	-	-	-
Placebo solution	-	-	-	-	-
Standard solution	4.515	1.06	5060	0.05	0.59
Sample solution	4.513	1.06	4996	0.04	0.27
Spiked sample solution					
Pomalidomide	4.975	1.06	5001	0.05	1.26
5-Amino pomalidomide impurity	4.306	1.02	1517	9.72	36.33
Nitrodione impurity	5.8	1.04	2272	0.21	50.43
Des amino impurity	5.552	1.1	2360	0.57	1.05
Individual Impurity Solution					
5-Amino pomalidomide impurity	4.302	1.04	1457	1.26	1.73
Nitrodione impurity	5.869	1.05	2206	0.38	1.64
Des amino impurity	5.562	1.1	2305	0.55	1.03

Linearity

Linearity was evaluated in the range of 50% to 150% of the working concentration level of PML (30 ppm). Different levels of standard solution were prepared by diluting out known volumes of intermediate stock solution with the diluents [2] to get the the final concentrations of PML was in the range of 15.47- 45.42 µg/mL required analyte concentrations. Each of these drug solutions (10 µL) was injected three times into the column, and the peak areas and retention times were recorded. Evaluation was performed with PDA detector at 221 nm. The calibration graph was obtained by plotting peak area versus concentration in µg/mL of PML. The plot was found to be linear with correlation coefficient of 0.9989. The respective linear regression equation being Y =11771 X - 92623. The % RSD were calculated for slope, intercept and given in (Table 5).

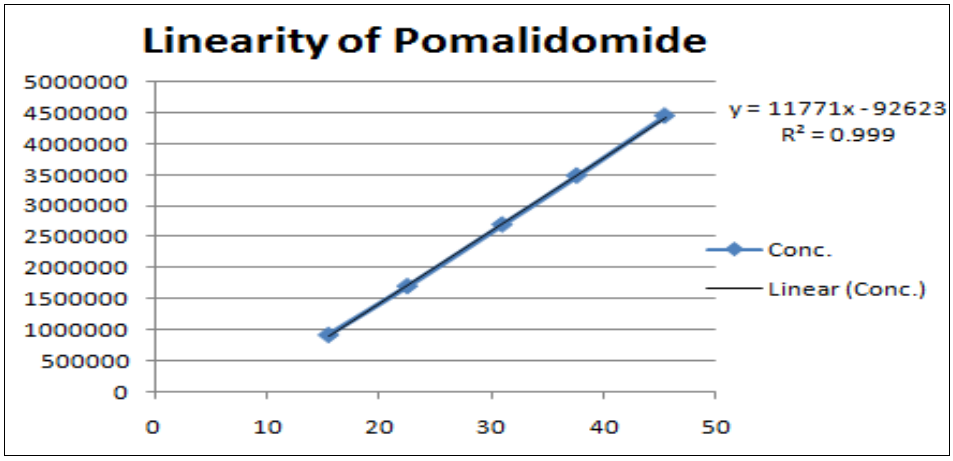

Fig 11: Linearity plot of Pomalidomide

Table 5: Linearity of Pomalidomide

Linearity Level %	Concentration (ppm)	Response		
		1	2	Mean
50	15.47656	919843	923658	1021751
75	22.51484	1705296	1709262	1707279
100	30.95312	2696730	2699107	2597919
125	37.59140	3483804	3483694	3383749
150	45.42968	4447183	4445938	4446561
Correlation coefficient				0.999
Intercept				32136.5265
Slope				33463.1352
Working (Area)				8473721.67
Slope				33463.14
Regression (r2)				0.9989
RRF				1.00
% Y-intercept				0.38

Accuracy (Recovery)

Accuracy was evaluated at three levels 50%, 100% and 150 % of the working concentration level for pomalidomide (30 ppm) in triplicate by addition of three different amounts of PML, to a previously analyzed sample. The amount of drug present per capsule was calculated by comparing the peak area of the sample solution with that of the standard solution. % Recovery of the individual substances at specified concentrations were found to between 100.2% - 101.7 %, % RSD were calculated for amount added which was found always less than 2%. Which indicates that the method is remarkably accurate, produces reliable results shown in (Table 6).

Table 6: Accuracy for Pomalidomide

Level (%)	Concentration added (µg/mL)	Amount Found (µg/mL)	% Recovery	Mean recovery %
50	15.910	16.011	100.7	101.2
	15.936	16.130	101.2	
	15.944	16.149	101.7	
100	30.320	30.633	100.2	100.7
	30.468	30.790	100.7	
	30.948	31.392	101.2	
150	45.104	45.824	101.0	100.8
	45.056	45.798	100.7	
	45.099	45.815	100.9	
Mean recovery				100.9
%RSD				0.26

Precision

Method Precision (Repeatability)

Method precision was performed to indicate whether the method was giving consistent results for a single batch. This was carried out by analyzing six replicate injections of concentration of standard (40ppm). And sample solutions (40ppm) (Pomalid® 2 mg), for the response of peak area. Percentage assay of sample to that of label claim was calculated by comparing the sample solution response to that of standard solution response.

The low value (< 2%) of RSD indicates indicate a considerable degree of precision and reproducibility for the method (Table 7).

Table 7: Method precision Pomalidomide

Sample No.	% Assay
1	101.0
2	101.1
3	101.6
4	101.5
5	101.1
6	101.9
Mean	101.4
% RSD	0.38

Intermediate Precision (Ruggedness)

Intermediate precision was assessed by analyzing the standard solution (40ppm) and sample solution (Pomalid® 2 mg) (40ppm) on different days. Six independent sample preparations were prepared on different day and by different analyst and injected on the HPLC. The data shows that Cumulative % RSD. For % assay of six independent samples preparation of two analysts is within the acceptance criteria and presented in (Table 8).

Table 8: Intermediate Precision for Pomalidomide

	Intermediate Precision	
Parameter	**(Analyst-I)**	**(Analyst-II)**
HPLC Instrument No.	AD/I-008	AD/I-020
HPLC column No.	C_{18}-189	C_{18}-296
Sample No.	% Assay	
1	101.0	100.5
2	101.1	101.2
3	101.6	100.8
4	101.5	100.3
5	101.1	100.5
6	101.9	101.1
Mean	101.4	100.7
RSD	0.38	0.35
Mean (n= 12)	101.0 %	
% RSD (n=12)	0.48	

Robustness

The robustness of an analytical method was carried out to confirm that the method remained unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The standard solution was injected six times for each varied conditions Change in flow rate (±0.1ml/min), Change in wavelength (±2nm), Change in column temperature (±5 °C) then chromatograms were recorded. The variation had no significant effect on the retention time and chromatographic response of the method, indicating that the method was robust. The results are shown in (Table 9)

Table 9: Robustness for Pomalidomide

Changes in parameters	Values	Retention Time Of Pomalidomide	Tailing factor	Theoretical plates	% Assay
Control	As per method	4.9	1.08	5079.1	101.2
Flow rate (± 0.1 mL/min)	0.9 mL/min	4.5	1.180	5628	100.8
	1.1 mL/min	5.0	1.160	4978	101.2
Column temperature (± 5°C)	25°C	4.8	1.170	5262	100.7
	35°C	5.0	1.172	5373	100.9
Change in Wavelength (± 2 nm)	219	4.9	1.170	5213	100.8
	223	4.9	1.172	5171	101.0
Cumulative % RSD					0.18

Limit of detection and Limit of Quantitation

The Limit of Detection (LOD) found was 0.074 and The Limit of Quantification (LOQ) analyzed was 0.222 for PML. These values reflect the high sensitivity of the method, which is of great importance in most studies and also indicating the method can be used for detection and quantification of analytes in a very wide concentration range.

Conclusion

A simple and easily available HPLC method as developed in this study for the quantification of PML in pharmaceutical matrices. The main advantages of this method are its considerably shorter run times, easy-to-use and its simplicity. All of these properties are very important in practice, particularly when a large number of samples are to be analyzed. The absence of additional peaks in the chromatogram indicates non-interference of the common excipients used in the tablets. The results of validation tests were, collectively, indicative for a method with a relatively wide linear range, acceptable precision and accuracy and practically reliable sensitivity. The method enables simple, selective, sensitive, and specific analysis of PML and can be used for routine analysis in pharmaceutical quality control within a shorttime.

A sensitive & selective RP-HPLC method has been developed & validated for the analysis of Pomalidomide API. Promote the proposed RP-HPLC strategy has amazing affectability, accuracy and reproducibility. The outcome demonstrates the created technique is amazingly, one more appropriate strategy for examine, immaculateness and soundness which can help in the examination of Pomalidomide in various details.

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