Development and Validation of Stability Indicating RP-UPLC Method for the Estimation of Pomalidomide in Bulk and Pharmaceutical Dosage Form

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Abstract

The present research work is mainly focussed on development of a novel, rapid, accurate and stability indicating UPLC method by using an advanced separation technique called Ultra Performance Liquid Chromatography, for the quantitative analysis of Pomalidomide in bulk and pharmaceutical dosage forms. This is a modified form of HPLC technique in which there is a significant increase in sensitivity, resolution and rapidity. The operation was carried out on a Waters Acquity UPLC instrument. Data acquisition and processing was done using Empower 2 software. The drug was eluted on an Acquity BEH C18 (50×2.1mm id, 1.7µm) analytical column with mobile phase containing 0.01M Potassium dihydrogen orthophosphate (adjusted to pH 3.5 using 0.1% dilute orthophosphoric acid (30%): Acetonitrile (70%). 0.3ml min^{-1} flow rate was maintained and the study was performed at 225nm using TUV detector. The drug was found to be retained at 1.682min. The number of theoretical plates and asymmetry was also found to be within the acceptance criteria. Method validation was carried out according to the ICH guidelines and the parameters namely- accuracy, precision, linearity, robustness, ruggedness, specificity, LOD and LOQ were studied. It was found that 5-30µg/ml was the linearity range and all other parameters result were found to be within the limits. Pomalidomide drug was stressed to acid, alkali, oxidative, thermal, hydrolytic and photolytic degradation and the results revealed that the developed method was capable to segregate all the degraded products from the active pharmaceutical ingredient. Hence this method can be used for routine quality estimation of drug analysis.

Key words: *UPLC, Method development and Validation and Stress testing.*

Introduction

Nowadays, most of the pharmaceutical industries are mainly focussing to reduce the cost for the development of new drug substances and to improve the selectivity, resolution and rapidity. This can be achieved by an advanced separation technique called Ultra Performance Liquid Chromatography (UPLC) which is the modified form of HPLC. In this system, the particle size is reduced to less than 2.5µm thus, increased the efficiency and also resolution. As the length of the column decreased in UPLC, the solvent consumption, analysis time as well as the cost also decreased which is more advantageous in pharmaceutical industries. The present investigation was carried out on Pomalidomide drug which is chemically 4-amino-2-(2,6-dioxopiperidin-3-yl) isoindole-1,3-dione is an orally bioavailable thalidomide derivative having immunomodulatory, anti-angiogenic and anticancer activities [1]. FDA granted approval to Pomalidomide drug for treating multiple myeloma on Feb 8, 2013. It is also approved by European commission in Aug, 2013 [2]. On May 14, 2020, FDA also accelerated approval to Pomalidomide for treating AIDS related Kaposi Sarcoma. It is available in market as 1mg, 2mg, 3mg, and 4mg capsules [3]. Pomalidomide inhibits TNF-alpha production, increases the activity of T cells and natural killer (NK) cells and antibody dependent cellular cytotoxity. It also inhibits tumour angiogenesis arrest the cell cycle in susceptible tumour cell populations and stimulate erythropoiesis [1].

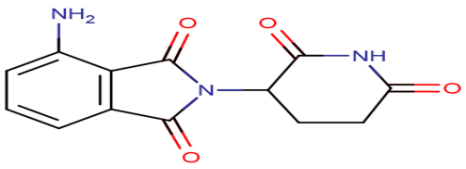

Figure 1: Chemical structure of Pomalidomide

After the detailed literature survey, it was revealed that many authors reported their studies on RP-HPLC [4] [5] [6], Stability indicating RP-HPLC [7], UPLC MS/MS [8] [9], LC-MS [10] [11] [12], and Spectro-fluorometry [13] techniques but till today there was no developed simple stability indicating UPLC method for the analysis of Pomalidomide. Hence it felt worthwhile to develop a simple, new stability indicating UPLC method so that it can applied for the routine laboratory analysis of Pomalidomide.

Materials and Methods

Materials:

Pomalidomide active pharmaceutical ingredient was procured as a gift sample from Spectrum Pharma labs and Pomalid 4 mg capsules formulation manufactured by Natco pharma Ltd. was purchased from a local pharmacy. The chemicals used for the preparation of mobile phase like water, acetonitrile, methanol, orthophosphoric acid and potassium dihydrogen orthophosphate were from Rankem.

Instrument used:

The experiment was performed using a Waters Acquity UPLC instrument and the drug was eluted on an Acquity BEH C18 column. Data processing was done on Empower 2 software. The samples were weighed using Denver electronic balance and all the prepared solutions were dissolved and degassed by using BVK Enterprises ultrasonicator and the pH of the mobile phase was adjusted by using BVK Enterprises pH meter.

Methodology

Preparation of stock and working stock solutions: About 2mg of Pomalidomide was accurately weighed, transferred into a 10ml volumetric flask**,** diluted to volume using diluent, sonicated for 10 min and filtered. From this, 1ml stock solution was pipetted into 10ml volumetric flask, diluted to volume, mixed thoroughly, sonicated and filtered.

Diluent: Acetonitrile and Water taken in the ratio of 50:50%v/v was selected as diluent based on the solubility of the drug.

Preparation of mobile phase: Accurately measured 300ml (30%) of 0.01M Potassium dihydrogen orthophosphate (adjusted to pH 3.5 using 0.1% dilute orthophosphoric acid) and 700 ml of Acetonitrile (70%) were mixed thoroughly, degassed and finally filtered through 0.45 µ filter under vacuum filter.

Preparation of sample solution: Taken 10 capsules, emptied and prepared the sample solution by taking the 115mg weight of the sample equivalent to 4mg and diluted to volume up to 10ml using diluent. Sonicated for 10min and filtered through 0.45µ filter paper. From this, 0.5ml of sample stock solution was pipetted out and diluted to volume up to 10ml using diluent, mixed thoroughly, sonicated and filtered.

Method development

To obtain an optimized method several trials were conducted by injecting the working stock solutions into the UPLC using different analytical columns like STD Hibar C18, STD HSS C18 and Acquity BEH C18 using various mobile phase compositions of 0.1% Ortho phosphoric acid: Acetonitrile (50:50), phosphate buffer (0.01M): Acetonitrile (40:60) and 0.01M Potassium dihydrogen orthophosphate (pH -3.5): Acetonitrile (30:70) by maintaining the flow rate to 0.3ml/min at 225nm UV detection. Finally, on observation it felt that Acquity BEH C18 column and 0.01M Potassium dihydrogen orthophosphate (adjusted to pH -3.5 by dilute orthophosphoric acid): Acetonitrile (30:70) mobile phase composition was found to give accurate and precise results with good peak shape, peak asymmetry and a greater number of theoretical plates. Therefore, it was finalized to be an optimized method and proceeded to carry out method validation.

Method validation

Method validation was proceeded by using various analytical parameters like accuracy, precision, linearity, detection limit, quantitation limit and robustness based on guidelines given by ICH[14] [15] [16].

System suitability: For verifying the system performance, 20µg ml^{-1} concentration standard solution of Pomalidomide was injected for six times and recorded the chromatograms.

Accuracy: Method's accuracy was confirmed by calculating the recovery of the spiked samples at the concentration level of 50%, 100% and 150%. To carry out this, 10µg ml^{-1} ,20µg ml^{-1} and 30µg ml^{-1} sample working stock solutions of Pomalidomide were added to the standard solution of 20µg ml^{-1}. Later injected the solutions in triplicates, recorded the chromatograms and measured the peak responses. Finally, calculated the amount found, percentage recovery and mean percentage recovery values.

Precision: Repeatability was carried out for the evaluation of method's precision. For this, 20µg/ml concentration solution of Pomalidomide sample solution was injected for six times under unchanged operating conditions for a short period of time, measured the peak areas and calculated the % Relative standard deviation.
Inter-day precision was performed by injecting 20µg ml^{-1} concentration sample solution of Pomalidomide for six replicate injections on different days under unchanged operating conditions. Measured the peak responses and calculated the %RSD values.

Linearity: The method's linearity over the range of 5-30µg ml^{-1} was carried out by injecting each standard solution in triplicates into the UPLC system. Recorded the chromatograms, plotted a graph of concentration vs peak areas and calculated correlation coefficient by regression analysis.

Specificity: Method's specificity was evaluated by injecting the blank and placebo (equivalent to the weight of placebo in sample) and recorded the chromatograms.

Robustness: To verify the deliberate changes in the method parameters, the sample was analyzed at 0.25mlmin^{-1} and 0.35ml min^{-1} rather than 0.3ml min^{-1} and recorded the chromatograms. The sample was also analyzed at 25℃ and 35℃ rather than 30℃ and by varying the mobile phase composition of 30:70%v/v of potassium dihydrogen orthophosphate buffer and Acetonitrile to 35:65%v/v and 45:55%v/v in five replicate injections into the UPLC system by maintaining the operating conditions. Recorded the chromatograms and calculated the %RSD.

Assay determination of Pomalidomide: The prepared standard and sample solutions of Pomalidomide were introduced into the UPLC system in six replicate injections, recorded the chromatograms and calculated the % Assay.

Detection limit (LOD) and Quantitation limit (LOQ): Detection limit and Quantitation limit were calculated from the values of standard deviation of intercept and slope of calibration curve.

LOD= 3.3 × σ/ slope and

LOQ = 10 × σ/ slope

Stress testing studies

For proving the stability indicating nature of the developed method, the drug solution was stressed to extreme conditions of Acid, Alkali, peroxide, thermal photolytic and hydrolytic degradation. Recorded the chromatograms and calculated the percentage of degraded amount and active amount.

Acidic degradation: 1ml of 0.1N HCl was added to 0.5ml of 400µg ml^{-1}concentration solution of Pomalidomide and refluxed for half an hour at 60℃. Neutralized the solution by adding 0.1N NaOH and diluted to volume to attain 20µg ml^{-1}concentration solution. Cooled and filtered the solution through 0.45 µ membrane filter and then introduced in to the system, measured the chromatograms and calculated the percentage degraded amount.

Alkaline degradation: 1ml of 0.1N NaOH was added to 0.5ml of 400µg ml^{-1}concentration solution of Pomalidomide and refluxed for half an hour at 60℃. Neutralized the solution by adding 0.1N HCl and diluted to volume to attain 20µg ml^{-1}concentration solution. Cooled and filtered the solution through 0.45 µ membrane filter and then introduced in to the system, measured the chromatograms and calculated the percentage degraded amount.

Peroxide degradation: 1ml of 20% hydrogen peroxide was added to 0.5ml of 400µg/ml concentration solution of Pomalidomide and retained for 30 minutes at 60℃ and diluted to volume to attain 20µg ml^{-1}concentration solution. Cooled and filtered the solution through 0.45µ membrane filter and then introduced in to the system, measured the chromatograms and calculated the percentage degraded amount.

Photolytic degradation: For this study, 400µg ml^{-1} concentration solution of Pomalidomide was exposed to UV light for 7 days. Later, the resultant solution was diluted to 20µg $ml^{-1}$and introduced in to the system, measured the chromatograms and calculated the percentage degraded amount.

Thermal degradation: 400µg ml^{-1} concentration solution of Pomalidomide was retained in an oven for 6 hours at 105℃. Later, the resultant solution was diluted to 20µg ml^{-1}, introduced in to the UPLC system, measured the chromatograms and calculated the percentage degraded amount.

Hydrolysis: For hydrolytic study, 1ml of distilled water was added to the 0.5ml of sample stock solution of Pomalidomide and refluxed for 6hrs at 60℃ temperature. Later, the resultant

solution was diluted to obtain 20µg ml^{-1} and injected into the UPLC system. Recorded the chromatograms and calculated the percentage degraded amount.

Results and discussions

Method development: After so many trial and errors, finally Acquity BEH C18 column and 0.01M Potassium dihydrogen orthophosphate (adjusted to pH 3.5 using 0.1%Orthophosphoric acid): Acetonitrile (30:70) mobile phase composition, detected at 225nm at 0.3ml/min flow rate was confirmed to be specific meeting all the acceptance criteria. The recorded standard chromatogram of Pomalidomide was depicted in **Figure 2**, the optimized chromatographic conditions and the result were reported in **Table 1** and **Table 2** respectively.

Method validation

System suitability: From the chromatographic data, the calculated %RSD was found to be 1.1% which is within the specified limits. Therefore, it was confirmed that the system performance is good and giving precise and accurate results. The measured results are given in **Table 3**.

Accuracy: The measured percentage average recovery at the levels of 50%, 100% and 150% was found to be 99.99% which is within the acceptance limits. The data is reported in **Table 4**.

Precision: The measured %Relative standard deviation of Repeatability and intermediate precision were found to be 0.7 and 1.0 respectively which are within the specified limits. Accordingly, it confirms the method's precision. The data is provided in **Table 5** and **Table 6** respectively.

Linearity: From the linearity graph, it was confirmed that the method is exhibiting linearity over the range of 5-30µg ml^{-1}. The correlation coefficient is 0.999 which is meeting the validation criteria. The plotted graph and linearity data are provided in **Figure 3** and **Table 7** respectively.

Specificity: The method was found to be Specific as there was no interference of blank and placebo peaks with the Pomalidomide peak. The chromatograms of blank and placebo are shown in **Figure 4** and **Figure 5** respectively.

Robustness: From the chromatographic data, it was observed that there was no much variations in the parameters like number of theoretical plates and peak asymmetry upon slight changes in flow rate, temperature and mobile phase composition. The data is provided in **Table 8**.

Assay determination of Pomalidomide: The calculated average %Assay in marketed formulation bearing the label claim Pomalidomide 4mg obtained was 100.16%. The results are provided in **Table 9**.

Detection Limit (LOD) and Quantitation Limit (LOQ): The Detection limit and Quantitation limit values are found to be 0.29µg ml^{-1} and 0.87µg ml^{-1} respectively. The chromatograms of LOD and LOQ are presented in **Figure 6** and **Figure 7** respectively.

Stress testing studies: The degradation of the Pomalidomide in the conducted stress conditions was found to be very less and is within the acceptance limits (5%-20%) specified by FDA. It is also observed that, no degraded peaks are interfered with the Pomalidomide peak thus, proved the stability indicating nature of the developed UPLC method and can be adequately applicable for the separation of the degradation products. The %degraded amount under all the stress conditions is tabulated in **Table 10**. The respective chromatograms obtained under stress conditions of acid, alkali, peroxide, thermal, photolytic and hydrolysis are presented in **Figures 8, 9, 10, 11, 12 and 13**.

From the results obtained it is confirmed that the present established UPLC method is novel, simple, fast, precise and accurate for the quantification of Pomalidomide drug. In comparision to the previously reported RP-HPLC methods, the present method developed by advanced UPLC technique saved the total analysis time and consumption of solvents. The lesser retention time reduced the cost for the quantification of Pomalidomide drug in bulk and pharmaceutical dosage forms along with improving the sensitivity, resolution and rapidity. Thus, made it suitable for the routine laboratory analysis. This method also has ability to separate all the degradation peaks from the Pomalidomide peak. Hence, it can be employed as stability indicating method for the routine quality control analysis and to check the stability of Pomalidomide.

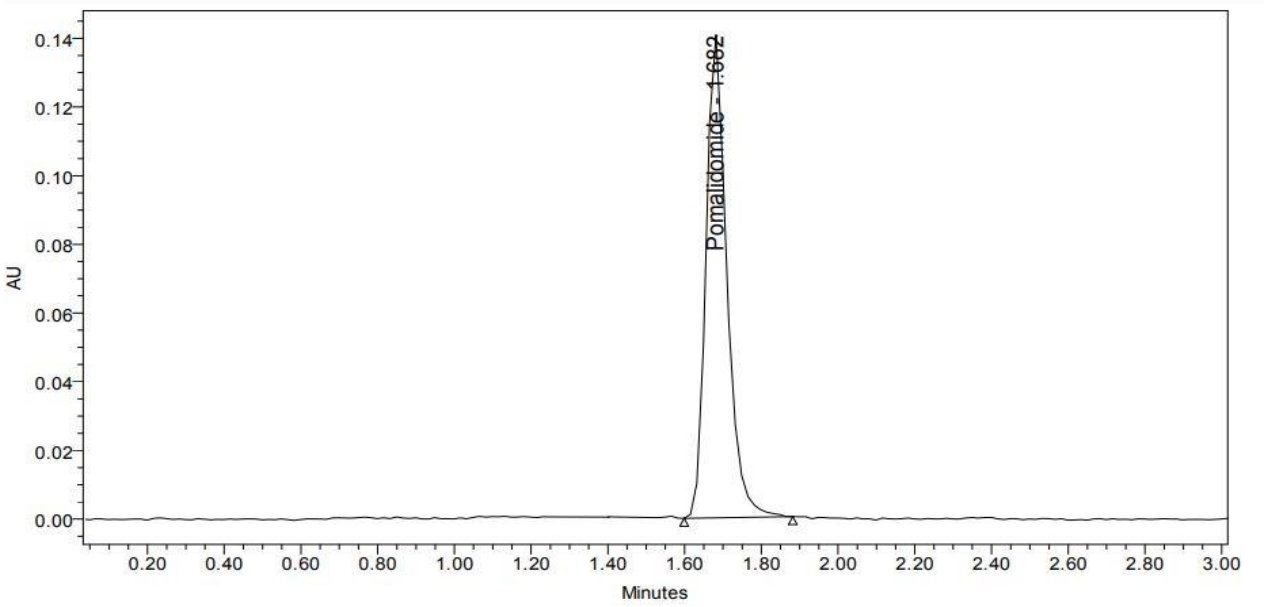

Figure 2. Pomalidomide optimized chromatogram

Table 1. Optimized chromatographic conditions

Mobile phase	Potassium dihydrogen orthophosphate (pH- 3.5): Acetonitrile (30:70) v/v
Column	Acquity BEH C18 (50×2.1mm, 1.7µm)
Flow rate	0.3mL/min
Column temperature	30°C
Wavelength	225 nm
Injection volume	2.0 µL
Run time	3 min
Retention time	1.682min
Elution mode	Isocratic

Table 2. Result of Pomalidomide optimized standard chromatogram

Drug	Pomalidomide
Retention time	1.682
Peak Area	501088
Theoretical plates	4579.7
Peak Asymmetry	1.2

Table 3. System suitability data of Pomalidomide

Injection	**Retention Time(min)**	**Peak area**	**Theoretical plates**	**Peak Asymmetry**
1	1.682	511088	4580	1.28
2	1.682	511088	4580	1.28
3	1.685	520892	4389	1.31
4	1.685	525620	4413	1.29
5	1.685	520383	4452	1.32
6	1.686	518290	4450	1.33
***Mean(n=6)**	1.684	517894	-	-
±Standard Deviation(n=6)	0.0017	5790.9	-	-
%RSD(n=6)	0.1	1.1	-	-

*Mean of six determinations, RSD: Relative Standard Deviation

Table 4. Accuracy data of Pomalidomide

Spiking level	Analyte response	Actual Amount (ppm)	Measured Amount (ppm)	% Recovery	*Mean %Recovery; SD;%RSD (n=9)
50%	774439	10	10.08	100.83	99.99; 0.58; 0.58.
	770745	10	9.94	99.38	
	770993	10	9.95	99.48	
100%	1028016	20	19.99	99.93	
	1030144	20	20.07	100.35	
	1026240	20	19.92	99.59	
150%	1290041	30	30.22	100.73	
	1286600	30	30.09	100.29	
	1279115	30	29.79	99.31	

*Mean of nine determinations; SD-Standard deviation; %RSD-Percentage Relative Standard Deviation

Table 5. Repeatability data of Pomalidomide

Injection	Pomalidomide	
	Retention Time(min)	Analyte response
1	1.680	515945
2	1.682	521858
3	1.683	520653
4	1.685	512080
5	1.686	518656
6	1.687	519180
***Average(n=6)**	-	518062
Standard deviation(n=6)	-	3549.4
%RSD(n=6)	-	0.7

*Mean of six determinations; RSD- Relative Standard Deviation

Table 6. Intermediate Precision data of Pomalidomide

Injection	**Pomalidomide**	
	Retention Time(min)	**Analyte response**
1	1.687	503703
2	1.693	512242
3	1.708	503949
4	1.710	505095
5	1.714	511290
6	1.719	515079
***Average(n=6)**	-	508560
Standard deviation(n=6)	-	4906.4
%RSD(n=6)	-	1.0

*Mean of six determinations; RSD- Relative Standard Deviation

Table 7. Linearity study of Pomalidomide

Pomalidomide				
Concentration (ppm)	**Analyte Response**	***Average Analyte Response(n=3)**	**Standard Deviation(n=3)**	**% RSD(n=3)**
5	130874	134548	627.58	0.46
5	131961			
5	131961			
10	262851	264389	1482.13	0.56
10	264509			
10	265808			
15	389499	390757	1915.33	0.5
15	389810			
15	392961			

20	508193	513470	4589.75	0.89
20	516531			
20	515687			
25	639342	639499	2706.92	0.42
25	636874			
25	642281			
30	771831	775193	2955.71	0.38
30	777384			
30	776363			

*Mean of three determinations; RSD- Relative Standard Deviation

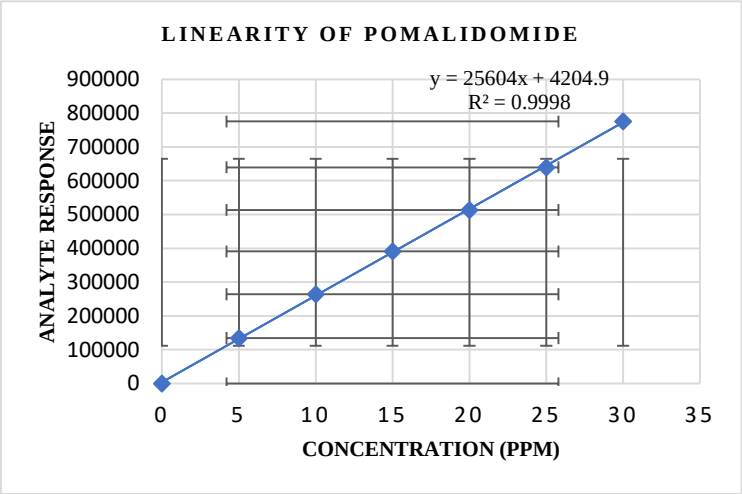

Figure 3. Calibration curve of Pomalidomide

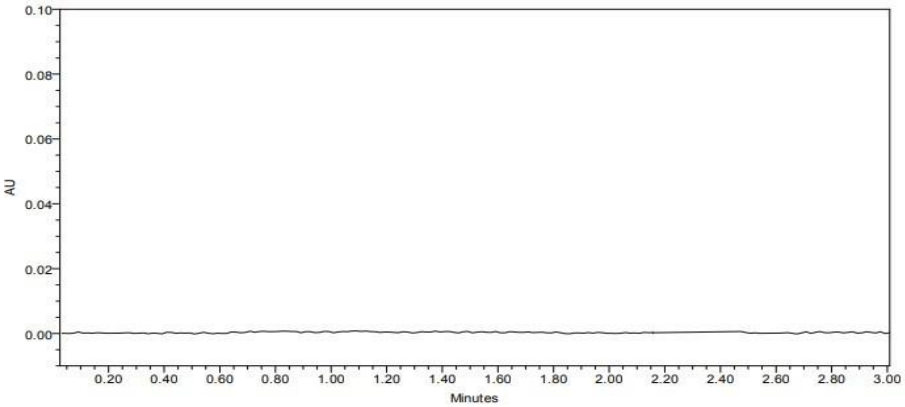

Figure 4. Chromatogram of blank

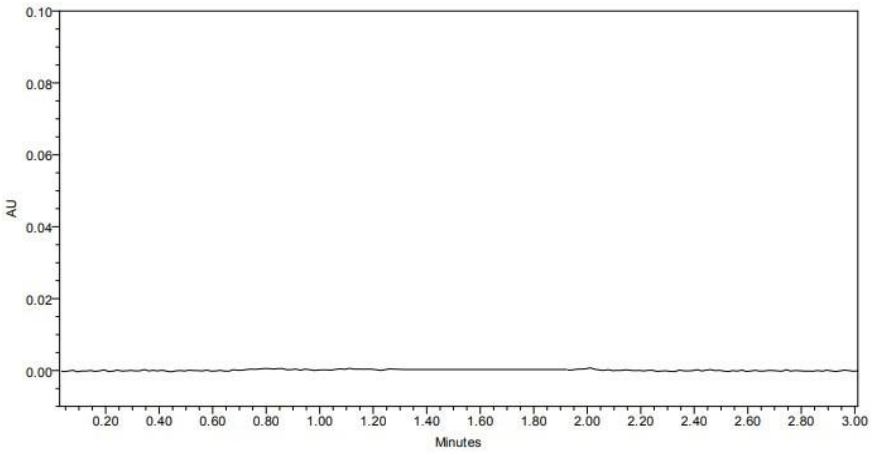

Figure 5. Chromatogram of placebo

Table 8. Robustness data of Pomalidomide

Varied Chromatographic conditions		**%Relative standard deviation**
Flow rate (±0.05 mL/min)	0.25	1.5
	0.35	1.3
Temperature (±5 ℃)	25	0.6
	35	1.2
Mobile phase composition (±5% v/v)	35:65	1
	45:55	0.7

Table 9. Assay data of Pomalidomide

Pomalidomide			
S. No	**Standard Area**	**Sample Area**	**%Assay**
1	511088	516521	99.34
2	511088	521682	100.33
3	520892	518613	99.74
4	525620	526147	101.19
5	520383	524192	100.81
6	518290	517641	99.55
***Mean(n=6)**	517894	520799	100..16
Standard deviation(n=6)	5790.9	3845.7	0.74
%RSD(n=6)	1.1	0.7	0.7

*Mean of six determinations; RSD- Relative Standard Deviation

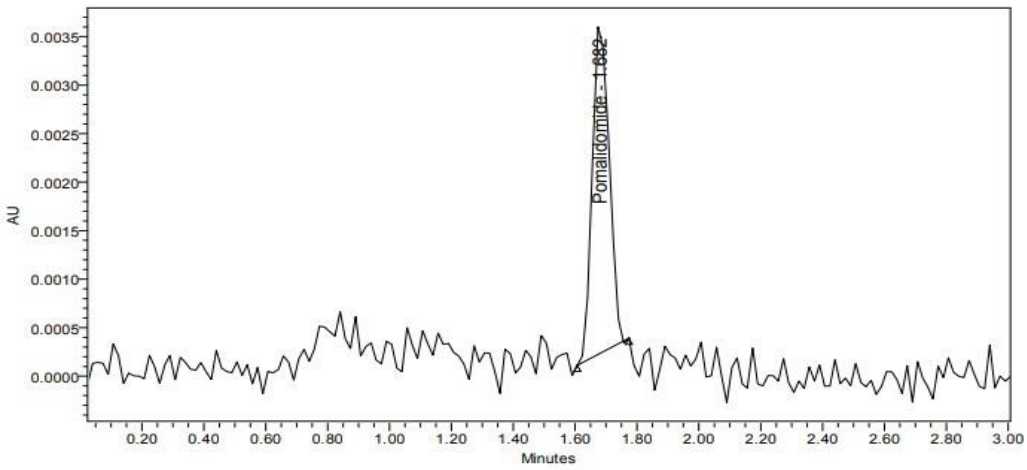

Figure 6: LOD chromatogram of Pomalidomide

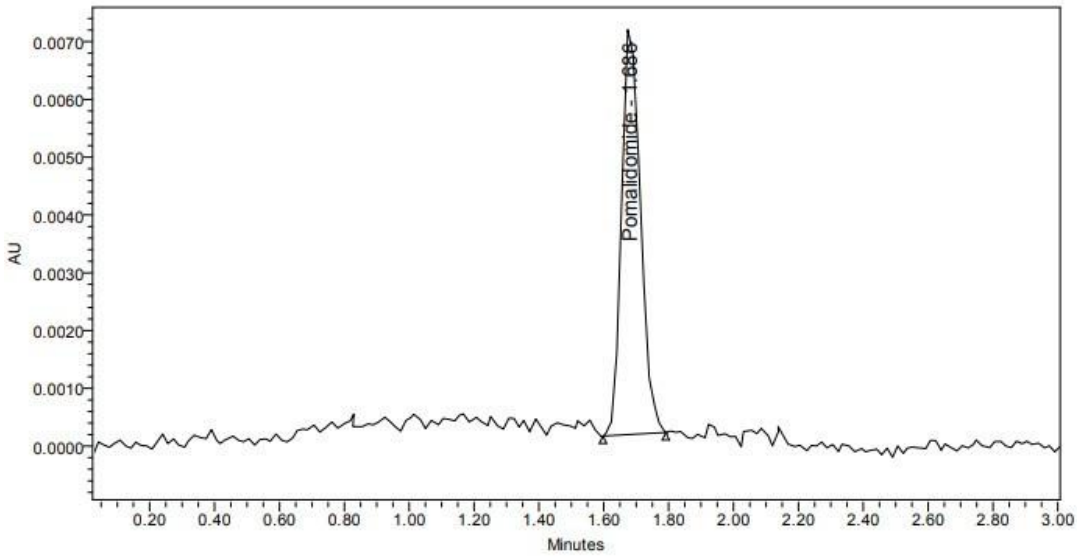

Figure 7. LOQ chromatogram of Pomalidomide

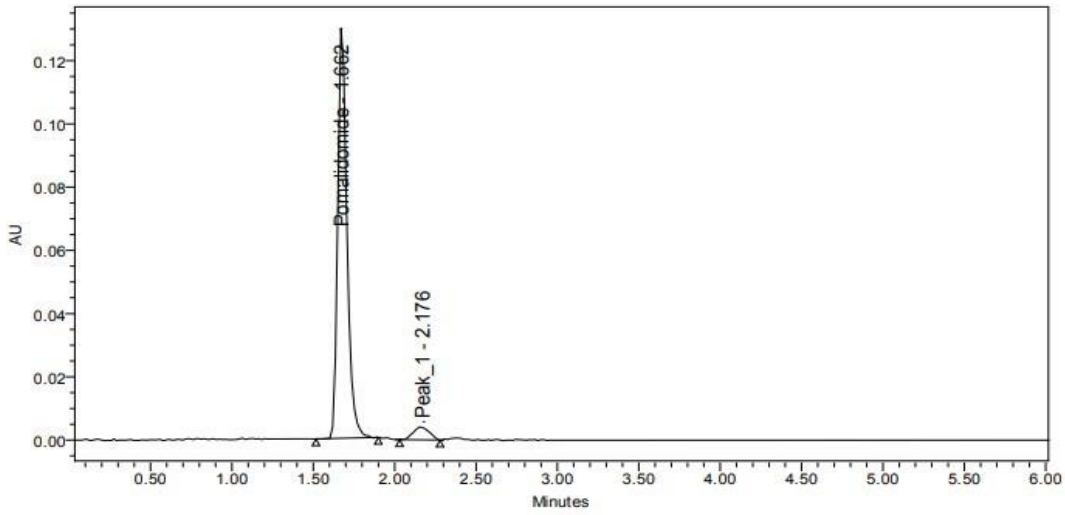

Figure 8. Chromatogram of acid degradation of Pomalidomide

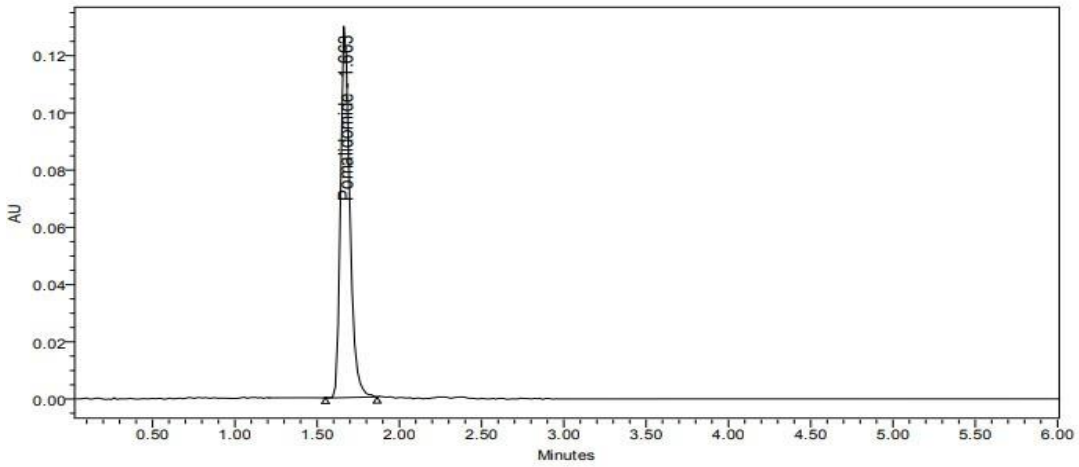

Figure 9. Chromatogram of alkaline degradation of Pomalidomide

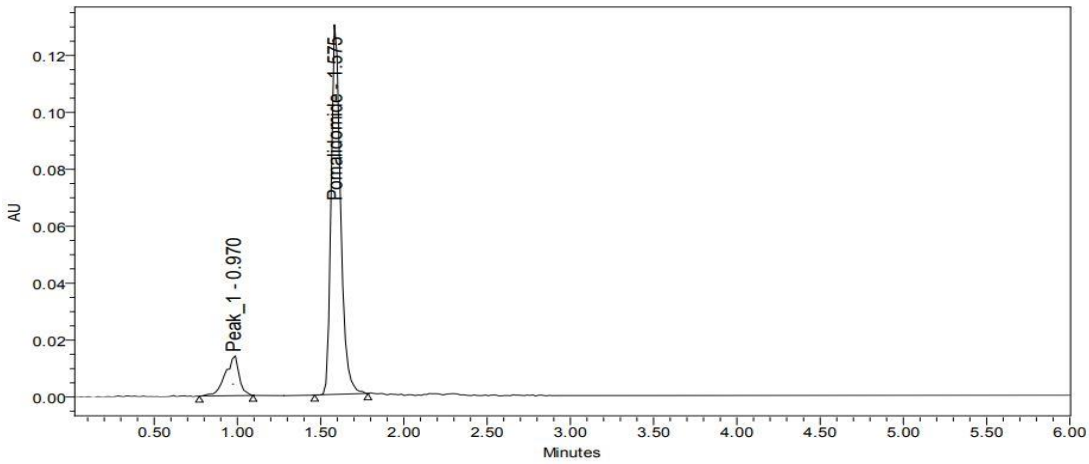

Figure 10. Chromatogram of oxidative degradation of Pomalidomide

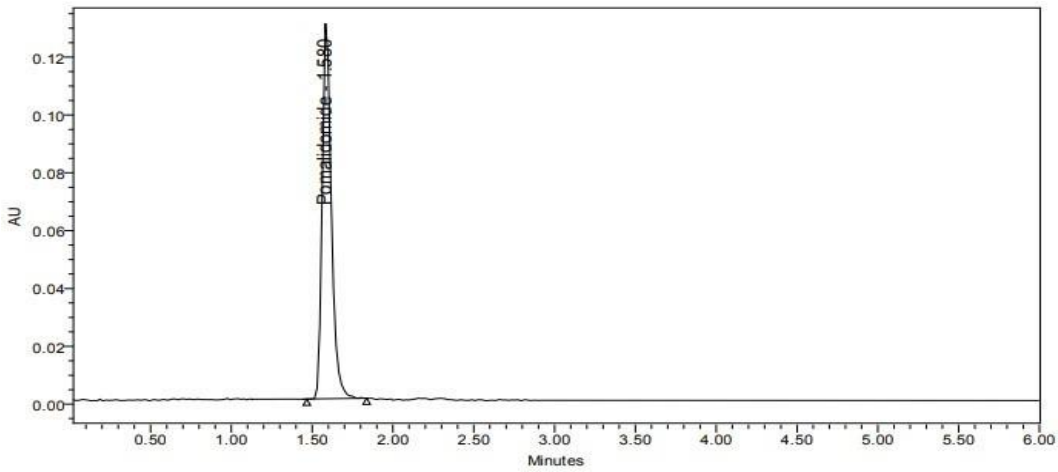

Figure 11: Chromatogram of Thermal degradation of Pomalidomide

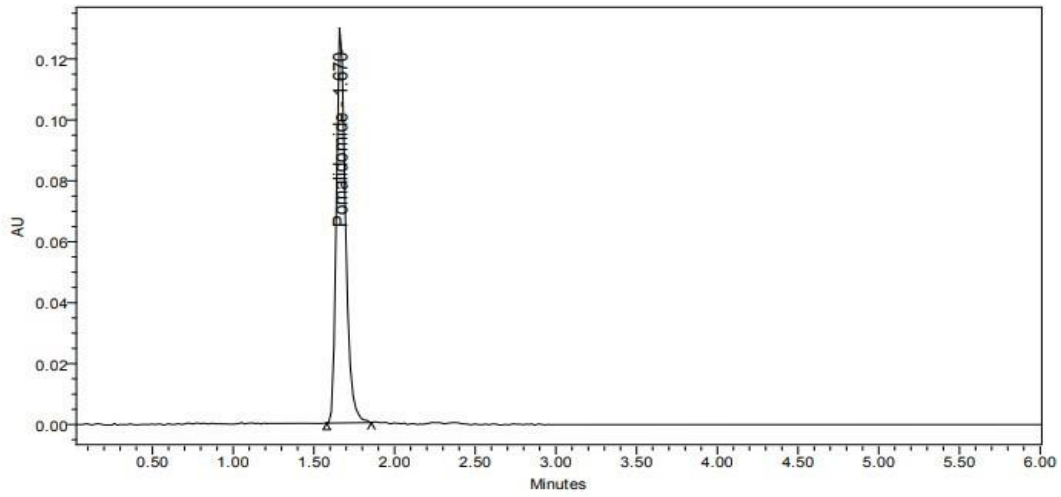

Figure 12. Chromatogram of Photolysis of Pomalidomide

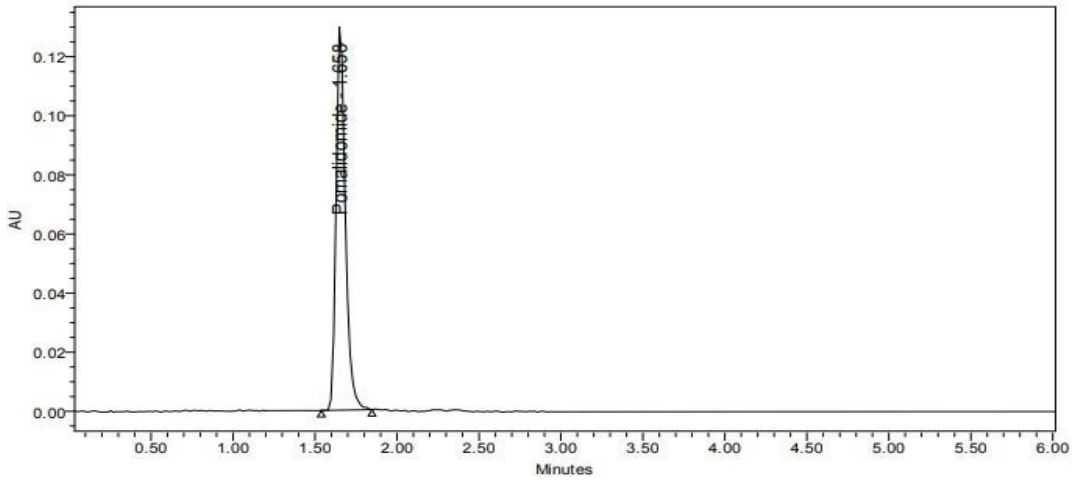

Figure 13. Chromatogram of Hydrolysis of Pomalidomide

Table 10. Stress testing studies data of Pomalidomide

Stress condition	Degraded Peak Area	% of undegraded Amount	% of Degraded Amount
Standard	517894	-	-
Acidic	491418	94.51	5.49
Alkaline	498625	95.89	4.11
Peroxide	493873	94.98	5.02
Photolysis	510311	98.14	1.86
Thermal	508873	97.87	2.13
Hydrolysis	515809	99.20	0.80

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