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Estimation of pomalidomide in capsule dosage form by RP-HPLC

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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. An XTerra RP C_{18}, (250 x 4.6 with 5 microns particle size) and the mobile phase, consisting of 0.03M KH_2PO_4 in water adjusting the pH-3.2 with O-Phosphoric Acid: Acetonitrile in ratio of 20:80 v/v & Acetonitrile HPLC Grade: Water HPLC Grade (50:50 v/v) was used as diluent in the gradient mode. The flow rate was 0.7 ml/min and the effluents were monitored at 220 nm. The retention time was 5.219 for PML. The detector response was linear in the concentration of 7.4 -88.8 µg/mL for PML. The respective linear regression equation being Y (-504143.1912) = 171820.582x + 1730704.1677 for PML. The Limit of Detection (LOD) is 0.074 & The Limit of Quantification (LOQ) is 0.222 for PML respectively. The % assay of PML was found out to be 99.66%. 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 dosage forms.

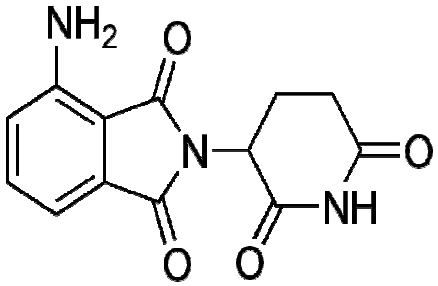
Key words: Pomalidomide (PML), RP-HPLC, Pomalyst, Capsules.

INTRODUCTION

Pomalidomide, chemically is (RS)-4-Amino-2-(2,6-dioxo-piperidin-3-yl)-isoindoline-1,3dione (Figure 1).The Empirical Formula is $C_{13}H_{11}N_3O_4$ and the Molecular Weight is 273.24 g/mol. Pomalidomide is classified as a thalidomide analogue**[1]**, immunomodulatory agent and an anti-angiogenic agent **[2]**. It is used for the treatment of relapsed and refractory multiple myeloma. Dr. Robert D'Amato's labs led to the first report **[3, 4]** stating that, 3-amino-thalidomide was able to directly inhibit both the tumor cell and vascular compartments of myeloma cancers. This dual activity of Pomalidomide makes it more efficacious than thalidomide *in vitro* and *in vivo* **[5, 6]**. Pomalidomide directly inhibits angiogenesis and myeloma cell growth. This dual effect **[7]** is central to its activity in myeloma, rather than other pathways such as TNF α inhibition, by neither inhibiting myeloma cell growth nor angiogenesis. The up & down regulations of Interferon γ, IL-2, IL-10 and IL-6 have been reported for Pomalidomide. These changes **[8]** may contribute to Pomalidomide's anti-angiogenic and anti-myeloma activities. Pomalidomide has a broad range of activities **[9]** that can be exploited to treat many hematologic and solid cancers.
Literature survey reveals a few chromatographic methods **[9-11]** to determine the Pomalidomide in capsule dosage form and also in biological fluids. So far, no assay methods by liquid chromatography were reported for the estimation of Pomalidomide in pharmaceutical dosage forms at the time of commencement of these investigations.

The availability of an HPLC method with high sensitivity and selectivity will be very useful for the determination of Pomalidomide in pharmaceutical formulations. A detailed account of all analytical methods existing for the drug is made to avoid duplication of the method developed. The authors had made some humble attempts, hoping to fulfill and bridge this gap, in succeeding the developing analytical methods, by using HPLC System. The results of this labor of love are set forth by developing a simple, precise and accurate reverse-phase HPLC method for the estimation of Pomalidomide in bulk drug samples and in pharmaceutical dosage forms.

Figure 1: Pomalidomide



MATERIALS AND METHODS

Pomalidomide was obtained as a gift sample from Manus Aktteva Bio Pharma LLP, Gujarat. Acetonitrile and water used were of HPLC grade (Qualigens). Commercial available, Pomalyst® 2 mg capsules were procured from local market.

2.1 Instrumentation:

Quantitative HPLC was performed on liquid Chromatograph, Waters separation 2996, 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 µ) was used. The HPLC system was equipped with Empower 2 Software. The column was maintained at 40^{o} C and eluted under isocratic conditions over 30.0 min at a flow rate of 0.7 ml/min.

2.2 HPLC Conditions:

The contents of the Mobile Phase - consisting of 0.03M KH_2PO_4 in water adjusting the pH-3.2 with O-Phosphoric Acid: Acetonitrile in ratio of 20:80 v/v & Acetonitrile HPLC Grade: Water HPLC Grade (50:50 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 0.7 ml/min. The run time was set at 30.0 min and the column temperature was ambient. Prior to the injection (20 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 220 nm.

2.3 Preparation of the Primary Standard/Stock Drug Solution:

A standard stock solution of the drug was prepared by dissolving 37 mg of PML in 50 ml volumetric flask containing 15 ml of diluent [Acetonitrile: Water (50:50 v/v)], sonicated for about 15 min and then made up to 50 ml with methanol to get standard stock solution of 0.074 mg/mL of PML.

2.4 Preparation of the Working Standard Drug Solution:

5ml of the above stock solution was taken in 50 ml volumetric flask and made up to 50 ml with diluent [Acetonitrile : Water (50:50 v/v)] to get a concentration of 74 µg/mL of PML respectively.

2.5 Preparation of Sample solution:

Twenty capsules (Pomalyst® 2 mg- Manus Aktteva Bio Pharma LLP, Gujarat) were taken and the contents were emptied and transferred into a dry watch glass. Capsule powder equivalent to 37 mg of PML were weighed and transferred into a 50 ml of volumetric flask containing 30 ml of diluents [Acetonitrile: Water (50:50 v/v)] to get standard stock solution of 0.074 mg/ml. The mixture was allowed to stand for 1 hr with intermittent sonication to ensure complete solubility of the drug, and then filtered through a 0.45 µm membrane filter, followed by adding methanol up to 50 ml to obtain a stock solution. 5 ml of the stock solution was taken in 50 ml volumetric flask and made up to 50 ml with diluents to get a concentration of 74 µg/ml of PML.

2.6 Linearity:
Aliquots of standard PML stock solution was taken in different 10 ml volumetric flasks and diluted up to the mark with the mobile phase such that the final concentrations of PML was in the range of 7.4-88.8 µg/mL. Each of these drug solutions (20 µL) was injected three times into the column, and the peak areas and retention times were recorded. Evaluation was performed with PDA detector at 220 nm and the calibration graph was obtained by plotting peak area versus concentration in µg/mL of PML (Figure: 2). The plot of peak areas of each sample against respective concentration of PML was found to be linear in the range of 7.4-88.8 µg/mL with correlation coefficient of 0.998. Linear regression least square fit data obtained from the measurements are given in Table 1. The respective linear regression equation being Y= 171820.582x + 1730704.1677 for PML. The regression characteristics, such as slope, intercept & % RSD were calculated for this method and given in Table 1.

2.7 Accuracy:
Accuracy was evaluated in triplicate by addition of three different amounts of PML, to a previously analyzed sample and comparing the amounts of analytes recovered with the amounts added. The amounts added were equivalent to 80, 100, and 120% of the amount originally present. %Recovery and RSD (%) were calculated for amount added. From the data obtained, it is obvious that the method is remarkably accurate, which ensures that this method produces reliable results as depicted in Table 2.

2.8 Precision:
The precision of the method was ascertained, separately from the peak area obtained by actual determination of six replicas of a fixed amount of the drug and formulation.

The HPLC system was set up, describing chromatographic conditions, mentioned as above and following the system equilibration of the working standard solution containing 74 µg/mL of PML, by injecting six times and recording the response peak areas. The precision was repeated with the formulated sample for the same concentrations by injecting the working sample solutions containing 74 µg/mL of PZP. The sample (Pomalyst® 2 mg- Manus Aktteva Bio Pharma LLP, Gujarat), was processed six times for the response of peak area. The % Relative Standard Deviation (RSD) and % range of error (at 0.05 and 0.01 confidence levels) were calculated and presented in Tables: 3 & 4 respectively.

2.9 Limits of Detection and Quantitation:
Limit of Detection (LOD) of the method was determined as the lowest concentrations of API producing a signal-to-noise (S/N) ratio of about 3. The Limit of Quantitation (LOQ) was determined as the lowest concentrations of active pharmaceutical ingredients capable of being quantified with acceptable accuracy and precision producing signal-to-noise (S/N) ratio of about 10.

2.10 Method Applicability:
The present developed method was evaluated by applying to Pharmaceutical dosage forms for the estimation of PML by our research group.

2.11 Assay:
20 µl of sample solution (Pomalyst® Capsules - 2 mg) was injected into the injector of liquid chromatography. The retention time was found to be 5.219 min for PML. The amount of drug present per capsule was calculated by comparing the peak area of the sample solution with that of the standard solution. The data are presented in Table 2.

2.12 Recovery Studies:
Accuracy was determined by recovery studies of PML; known amount of standard was added to the pre-analyzed sample and subjected to the proposed HPLC analysis. Results of recovery studies are shown in Table 2. The study was done at three different concentration levels.

RESULTS AND DISCUSSION

3.1 HPLC Method Development and Optimization [12]:
In response to lack of simple, reliable and easy-to-use method for the determination of PML concentrations in pharmaceutical matrices, a gradient RP-HPLC method was developed for quantification of above mentioned, API. We examined several HPLC method variables with respect to their corresponding effects on the result of analysis.

To optimize the chromatographic conditions, different combinations of Methanol-Water, Methanol – Acetonitrile, Water – Acetonitrile & 0.03M KH_2PO_4 in water adjusting the pH-3.2 with O-Phosphoric Acid: Acetonitrile were tested. 0.03M KH_2PO_4 in water (pH-3.2 with O-Phosphoric Acid): Acetonitrile (20:80 v/v) was promisingly preferred, because it resulted in greater resolution of API after several preliminary investigatory runs, compared with other mobile phases. The other parameters in this factorial design were different column, temperature, variation in flow rate, detection wavelength, buffer pH variation in mobile phase and volume of injection. Buffer molarity was changed and optimum buffer strength was selected as 0.03M KH_2PO_4 in water adjusted to pH 3.2, on the basis of theoretical plate number. At 220 nm, UV responses of all three active pharmaceutical analytes were good and free form interferences. Under these conditions, the analyte peaks were well defined and free from tailing. Considering the whole body of the data obtained from this extensive study, the set of conditions indicated earlier in this article was selected for further validation. Typical chromatogram (Std & Working Sample) of PML has been shown in Figure: 3 & 4.

The system suitability tests were carried out on freshly prepared standard stock solutions of PML. Parameters that were studied to evaluate the suitability of the system were discussed and presented in Table 4.

3.2 Method Validation Tests:
Recommended method validation characteristics including Method precision (RSD, %), Method accuracy (Recovery % and RSD, %), Linear range (Correlation Coefficient), and LOD & LOQ, were investigated systematically.

3.3 Linearity:
The plots of peak areas of each sample against respective concentrations were found to be linear, in the range of 7.4-88.8 µg/ml for PML with correlation coefficient of 0.998 (Table 1). Linear regression least square fit data obtained from the measurements are given in Table 1**.** The respective linear regression equation being Y= 171820.582x + 1730704.1677 for PML. The regression characteristics, such as slope, intercept, and %RSD were calculated for this method and given in Table 1. The regression characteristics, such as slope, intercept, and %RSD were calculated for this method and given in Table 1**.** These results show that there was an excellent correlation between peak areas and analyte concentration.

3.4 Accuracy:
Recovery of the individual substances at 80%, 100%, and 120% of specified concentrations were between 92.61% - 109.57%, which proves the accuracy of the method. From these data, RSD was always less than 1%, which indicates it is obvious that the method is remarkably accurate, produces reliable results shown in Table 2.

3.5 Precision:
The low value (<1%) of RSD indicates the repeatability of the method. These data indicate a considerable degree of precision and reproducibility for the method both during one analytical run and between different runs (Table 3 & 4).

3.6 Robustness:
Robustness was studied out to evaluate the effect of small but deliberate variations in the chromatographic conditions at three different levels, i.e. –2, 0, +2. To determine the robustness of this method, the experimental conditions were deliberately altered at three different levels and retention time and chromatographic response were evaluated. One factor at a time was changed to study the effect. Variation of the columns with wavelength by ±2 nm (218 nm and 222 nm), mobile phase flow rate by ±2 mL min−1 (0.5 mL min−1 and 0.9 mL min−1), mobile phase pH by ±0.2 units (pH 3.0 and 3.4) 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 5.

3.7 Limit of Detection (LOD) and Limit of Quantification (LOQ): 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.

3.8 Specificity:

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.

Table 1: Linear regression data of calibration curves

Parameter	Pomalidomide
Concentration range(µg/mL)	7.4 - 88.8
Slope (m)	171820.582
Intercept (Y)	504143.1912
Standard error of estimate (c)	1730704.1677
Correlation coefficient (r)	0.998
Linear regression (r^2)	0.997
%RSD	0.34

Table 2: Assay & Recovery accuracy studies of Pomalidomide (PML) in capsule dosage Forms

Tablet formulation	Amount claim (mg/capsule)	Amount Obtained (mg)* by proposed method	** % Recovery by the Proposed method
	PML	PML	PML
1) 120%	2	2.03	109.57
2) 100%	2	1.96	99.26
3) 80%	2	1.83	92.61
Average Mean	2	1.94	100.48

Average of three determinations* * After spiking the sample*

ACCURACY PARAMETER	POMALIDOMIDE	
Assay (120%)	132.59%	
Assay (100%)	108.77%	
Assay (80%)	91.23%	
	Standard	Spiked
% RSD (120%)	0.6	0.3
% RSD (100%)	0.2	0.1
% RSD (80%)	0.6	0.9
	Area	
Standard Deviation (120%)	86049.3	55487.4
Standard Deviation (100%)	25237.5	2140.8
Standard Deviation (80%)	66942.3	120615.0

Table 3: Precision of recommended procedure using API- {Pomalidomide} (PML) & its Formulation Matrice (Pomalyst®)

Sr. No	Inj. No	Name of the Standard Drug & Conc. (74 µg/mL)	Retention time in minutes	Peak Area	Name of the Sample Drug & Conc. (74 µg/mL)	Retention time in minutes	Peak Area
		API (PML)			Formulation Matrice (Pomalyst®)		
1	1	PML	5.469	12122455	Pomalyst®	5.737	12365092
2	2	PML	5.630	12358449	Pomalyst®	5.741	12351188
3	3	PML	5.678	12346026	Pomalyst®	5.603	12244531
4	4	PML	5.661	12354281	Pomalyst®	5.686	12349170
5	5	PML	5.722	12377997	Pomalyst®	5.856	12482186
6	6	PML	5.829	12426266	Pomalyst®	5.736	12339096
7	Mean		5.665	12330912.5	Pomalyst®	5.726	12355210.8
8	Standard Deviation		0.118	106109.8		0.082	75872.1
9	% RSD		2.09	0.9		1.44	0.6

Table 4: Validation summary/system suitability

PARAMETER	POMALIDOMIDE (PML) (Standard API drug)	POMALYST® (Sample drug)
Theoretical Plates(N)	9241.69	9360.21
Tailing factor	1.22	1.23
Retention time(min)	5.219	5.373
Resolution	2.28	2.33
Area	11417696	11666016
% Peak Area	99.48	99.48
LOD (µg/mL)	0.074	0.074
LOQ (µg/mL)	0.222	0.222

Table 5: Results from testing of the Robustness of the method (n=3, 100% of the Working Standard Solution & Sample Solution contains: 74 µg/mL of Pomalidomide (PML)

Condition Studied in Robustness	Modification In OFAT analysis	Parameter Fixation	Mean Peak Area ± S.D	% RSD (Peak Area)	Mean Retention Time (in min) ± S.D	% RSD (Retention time)
			PZP	PML	PML	PML
Column(s) (XTerra RP18)	Hypersil, & Inertsil ODS C18 3V	Std	16938497.0±13566.3	0.1	5.469±0.051	0.71
		Sample	16949840.7±35836.6	0.2	5.737±0.073	1.03
Flow rate (0.7 ml/min)	0.5 ml/min & 0.9 ml/min	Std – Increase	15839219.6±58320.2	0.4	5.630±0.033	0.49
		Std-Decrease	18966766.3±41198.5	0.2	5.678±0.031	0.40
		Sample- Increase	16107848.3±77066.4	0.5	5.603±0.026	0.40
		Sample-Decrease	18890994.4±70374.5	0.4	5.856±0.024	0.31
pH (3.2)	3.0 & 3.4	Std - Increase	17085698.7±59123.4	0.3	5.722±0.009	0.11
		Std- Decrease	17084204.4±52304.9	0.3	5.665±0.013	0.18
		Sample - Increase	17055489.3±58283.0	0.3	5.726±0.011	0.15
		Sample - Decrease	17053343.7±50867.4	0.3	5.741±0.037	0.53

Figure 2: Calibration Curve of the Pomalidomide (PML) by RP-HPLC

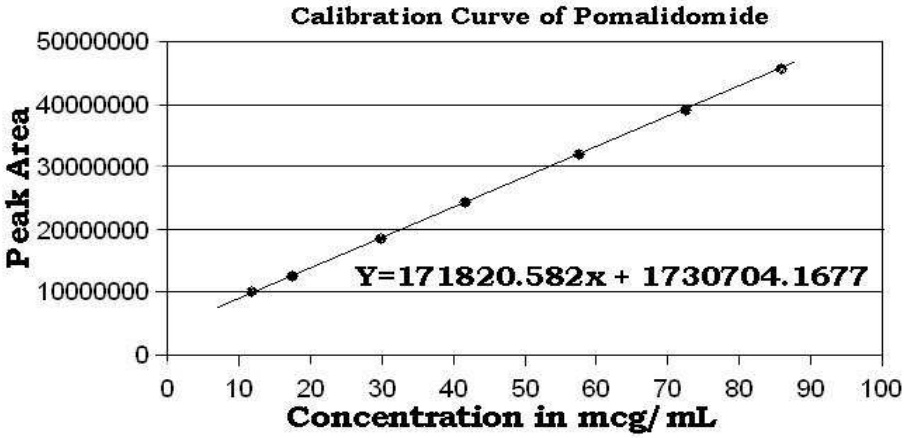


Figure 3: Typical Chromatogram of Pomalidomide Standard by RP-HPLC

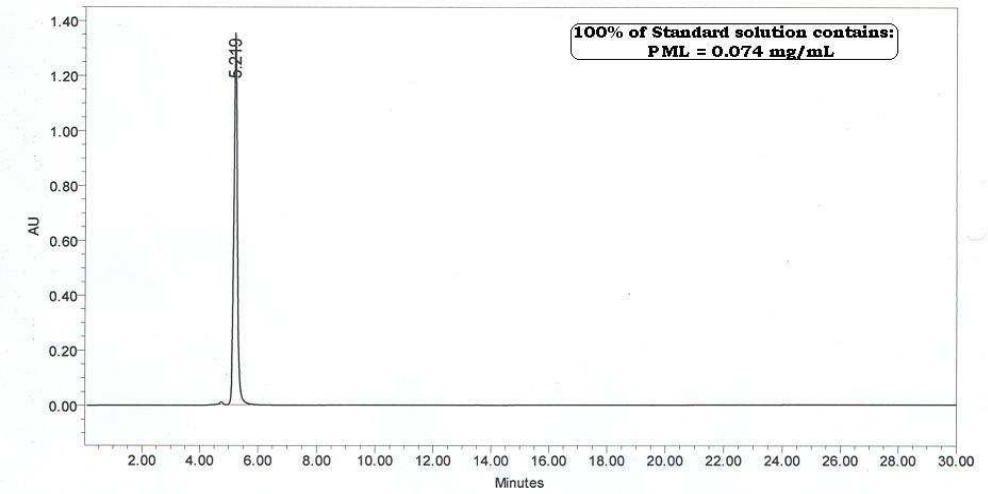
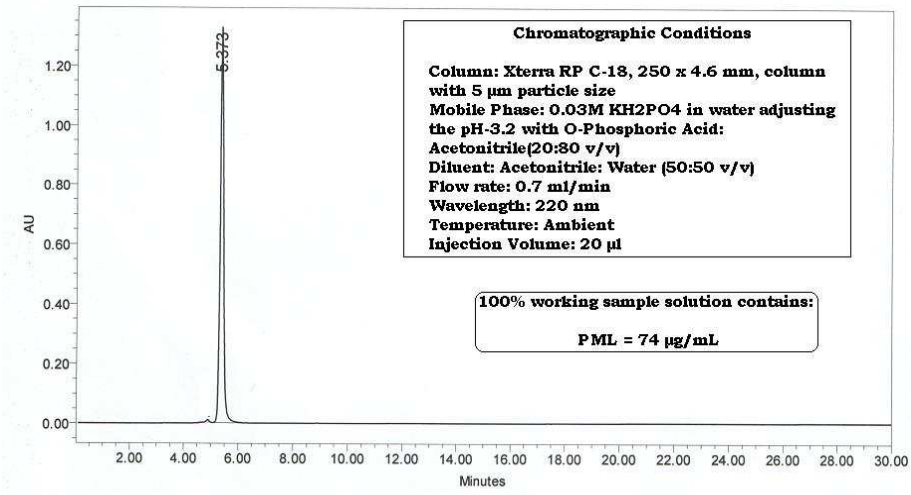


Figure 4: Typical Chromatogram of Pomalidomide Working Sample by RP-HPLC



CONCLUSION

A simple and easily available HPLC method was 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 short time.

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