METHOD DEVELOPMENT AND VALIDATION FOR DETERMINATION OF LENALIDOMIDE IN API FORM AND MARKETED FORMULATION BY RP-HPLC

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ABSTRACT: *A new analytical, simple, rapid, accurate, precise, robust RP-HPLC method has been developed and validated for estimation of Lenalidomide in bulk and pharmaceutical dosage forms. The method involves separation on Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm. The optimized mobile phase consists of Phosphate Buffer and Methanol in the ratio of 46:54% v/v (pH-3.2) with a flow rate of 1.0ml/min and UV detection at 206mn. Retention time of Lenalidomide was found to be 3.622min. Linearity range was 20-60ug/ml of Lenalidomide. Accuracy was in the range of 98-102% for Lenalidomide drug. The % RSD for Method Precision was found to be 0.609%. LOD and LOQ are 0.8µg/ml and 0.24µg/ml for Lenalidomide respectively. The method developed is more sensitive, accurate and precise than the methods reported earlier. Retention time and run time were also less and hence the method is economical. When applied for tablet assay, drug content was within 98 -102% of labeled content. The proposed method was found to be simple and sensitive for routine quality control application of Lenalidomide used in bulk form and pharmaceutical tablet dosage forms.*

Key Words: *Lenalidomide, RP-HPLC, Method Development, Validation, Accuracy, Robustness.*

I. INTRODUCTION

Lenalidomide[1] is a dicarboximide that consists of 1-oxoisoindoline bearing an amino substituent at position 4 and a 2, 6-dioxopiperidin-3-yl group at position 2. Inhibits the secretion of TNF-alpha. It has a role as an angiogenesis inhibitor, an antineoplastic agent and an immunomodulator. It is a member of isoindoles, a dicarboximide, a member of piperidones and an aromatic amine. In hematological malignancies, the immune system is deregulated in the form of altered cytokine networks in the tumour microenvironment, defective T cell regulation of host-tumour immune interactions, and diminished NK cell activity. Lenalidomide[2] is an immunomodulatory agent with antineoplastic, antiangiogenic, and anti-inflammatory properties. Lenalidomide exerts direct cytotoxicity by increasing apoptosis and inhibiting the proliferation of hematopoietic malignant cells. It delays tumour growth in nonclinical hematopoietic tumour models in vivo, including multiple myeloma. Lenalidomide[3] also works to limit the invasion or metastasis of tumour cells and inhibits angiogenesis. Lenalidomide acts by a novel drug mechanism—modulation of the substrate specificity of the CRL4CRBN E3 ubiquitin ligase. In multiple myeloma, lenalidomide induces the ubiquitination of IKZF1 and IKZF3 by CRL4CRBN. Subsequent proteasomal degradation of these transcription factors kills multiple myeloma cells. The IUPAC Name of Lenalidomide is 3-(7-amino-3-oxo-1H-isoindol-2-yl) piperidine-2, 6-dione. The Chemical Structure of Lenalidomide is as following

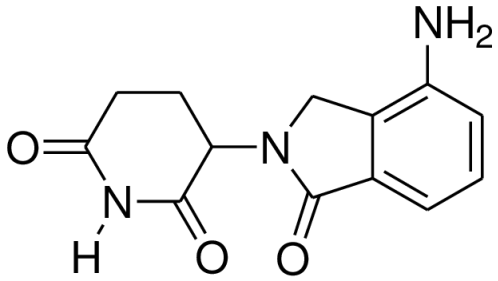

Fig.1. Chemical Structure of Lenalidomide

The literature survey[31-34] shows that there are few methods for the determination of Lenalidomide individually in bulk and pharmaceutical dosage forms by using various analytical instruments like UV-Vis spectrophotometer, HPLC, RP-UPLC, and LC-MS/MS. So, the attempt has been made to develop a new validated RP-HPLC[4] method

for estimation of Lenalidomide in bulk and pharmaceutical dosage form as per International Conference on Harmonization (ICH) guidelines[30].

II. EXPERIMENTAL

Instruments Used

Table-1: List of Instrument used

S. No.	Instruments/Equipments/Apparatus
1.	**HPLC** with Empower2 Software with Isocratic with UV-Visible Detector (Waters).
2.	**T60-LAB INDIA** UV – Vis spectrophotometer
3.	Electronic Balance **(SHIMADZU ATY224)**
4.	Ultra Sonicator **(Wensar wuc-2L)**
5.	Thermal Oven
6.	Symmetry ODS RP C_{18},5µm, 15mm x 4.6mm i.d.
7.	P^H Analyzer **(ELICO)**
8.	Vacuum filtration kit **(BOROSIL)**

Chemicals / Reagents Used

Table-2: List of Chemicals used

S.N.	Name	Specifications		Manufacturer/Supplier
		Purity	Grade	
1.	Doubled distilled water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
2.	HPLC Grade Water	99.9%	HPLC	Sd fine-Chem ltd; Mumbai
3.	Methanol	99.9%	HPLC	Loba Chem; Mumbai.
4.	Hydrochloric Acid	99.9	A.R.	Sd fine-Chem ltd; Mumbai
5.	Acetonitrile	99.9%	HPLC	Loba Chem; Mumbai.
6.	Sodium Hydroxide	99.9	A.R.	Sd fine-Chem ltd; Mumbai
7.	Ethanol	99.9	A.R.	Sd fine-Chem ltd; Mumbai
8.	Octanol	99.9	A.R.	Sd fine-Chem ltd; Mumbai

2.1. Method Development and Its Validation for Lenalidomide by RP-HPLC

Selection of Wavelength

The standard & sample stock solutions were prepared separately by dissolving standard & sample in a solvent in mobile phase diluting with the same solvent.(After optimization of all conditions) for UV analysis. It scanned in the UV spectrum[5] in the range of 200 to 400nm. This has been performed to know the maxima of Lenalidomide, so that the same wave number can be utilized in HPLC UV detector for estimating the Lenalidomide. The scanned UV spectrum is attached in the following page,

Sample & Standard Preparation for the UV-Spectrophotometer Analysis

25 mg of Lenalidomide standard was transferred into 25 ml volumetric flask, dissolved & make up to volume with mobile phase[6]. Further dilution was done by transferring 0.5 ml of the above solution into a 10ml volumetric flask and make up to volume with mobile phase.

Preparation of 0.01M Potassium Dihydrogen Orthophosphate Solution:

About 1.36086grams of Potassium dihydrogen orthophosphate was weighed and transferred into a 1000ml beaker, dissolved and diluted to 1000ml with HPLC Grade water[7]. The pH was adjusted to 3.20 with diluted orthophosphoric acid.

Preparation of Mobile Phase:

460ml of Phosphate buffer (0.05M) pH 3.20 and 540ml of HPLC Grade Methanol were mixed well and degassed in ultrasonic[8] water bath for 15 minutes. The solution was filtered through 0.45 µm filter under vacuum filtration.

Method Validation

Analytical method validation[9-12] establishes documented evidence that the procedure adopted for a test is fit for the intended purpose in terms of quality, reliability and consistency of results.

Specificity

Specificity[13] is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample matrix such as impurities, degradation products and matrix components. It must be demonstrated that the analytical method is unaffected by the presence of spiked materials (impurities and/or excipients).

Linearity

Linearity[14] is the ability of the method to elicit test results that are directly, or by a well-defined mathematical transformation, proportional to analyte concentration within a given range. It should be established initially by visual examination of a plot of signals as a function of analyte concentration of content. If there appears to be a linear relationship, test results should be established by appropriate statistical methods. Data from the regression line provide mathematical estimates of the degree of linearity. The correlation coefficient, y-intercept, and the slope of the regression line[15] should be submitted.

Range

The range of an analytical procedure is the interval between the upper and lower levels of analyte (including these levels) that have been demonstrated to be determined with a suitable level of precision, accuracy, and linearity using the procedure as written. The range[16] is normally expressed in the same units as test results (e.g., percent) obtained by the analytical procedure.

Accuracy

The accuracy of an analytical method is the closeness of the test results obtained by that method to the true value. This is sometimes termed trueness. It is recommended that accuracy[17] should be determined using a minimum of nine determinations over a minimum of the three concentration levels, covering the specified range (3

concentrations/3 replicates each of total analytical procedures).

Precision

The precision[18] of an analytical method is the degree of agreement among individual test results when the method is repeated to multiple samplings of a homogeneous sample. The precision of an analytical procedure is usually expressed as the standard deviation or relative standard deviation (coefficient of variation) of a series of measurements. It is indicated by Relative Standard Deviation, RSD, which is determined by the equation:

$$\%RSD = SD/Average \times 100$$

Generally, the RSD should not be more than 2%.

Detection Limit and Quantitation Limit

The Detection Limit[19] is defined as the lowest concentration of an analyte in a sample that can be detected, not quantified. The Quantitation Limit[20] is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the analytical procedures.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in procedural parameters listed in the procedure documentation and provides an indication of its suitability during normal usage. Robustness[21] may be determined during development of the analytical procedure.

System Suitability Testing

System suitability testing[22-25] is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. They are especially important in the case of chromatographic procedures.

III. RESULTS AND DISCUSSION

Method Development:

Selection of Wavelength:

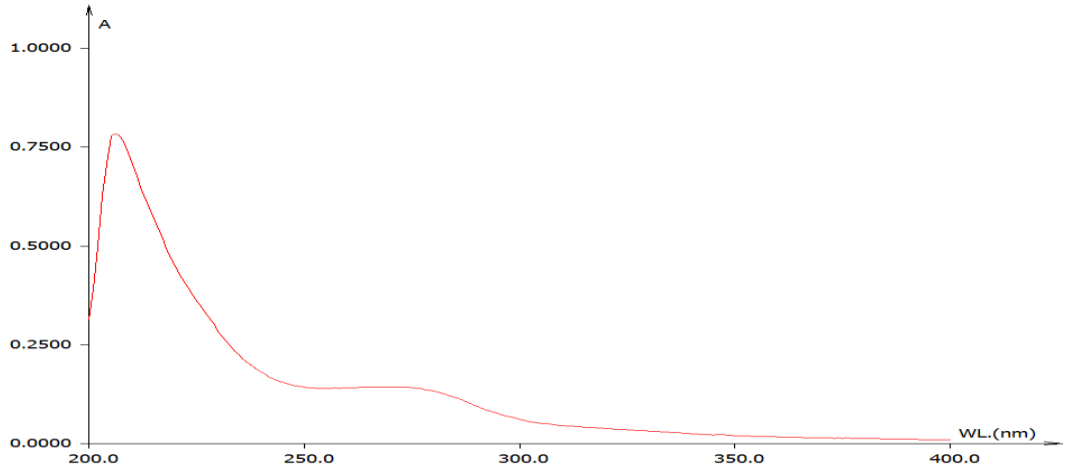

Fig.2. UV Spectrum for Lenalidomide

Observation: While scanning the Lenalidomide solution we observed the maxima at 206nm.

Summary of Optimized Chromatographic Conditions

The Optimum Chromatographic conditions[26] obtained from experiments can be summarized as below:

Table-3: Summary of Optimised Chromatographic Conditions

Mobile phase	Phosphate Buffer : Methanol = 46:54 (pH-3.2)
Column	Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm
Column Temperature	Ambient
Detection Wavelength	206 nm
Flow rate	1.0 ml/ min.
Run time	08 min.
Temperature of Auto sampler	Ambient
Diluent	Mobile Phase
Injection Volume	10µl
Type of Elution	Isocratic
Retention time	3.622 minutes

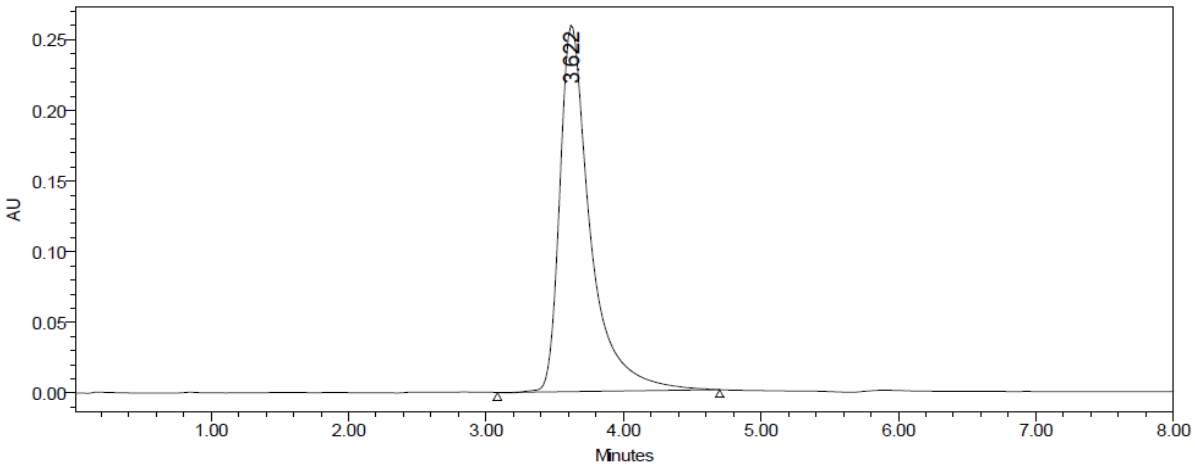

Fig.3. Chromatogram of Lenalidomide in Optimized Condition

Method Validation

The proposed method was validated according to the ICH guidelines for system suitability, specificity, recovery, precision, linearity, and robustness, limit of detection (LOD) and limit of quantification (LOQ). Under the validation study, the following parameters were studied.

1. Accuracy:

Recovery Study:

To decide the exactness of the proposed strategy, recuperation contemplates were completed by including diverse sums (80%, 100%, and 120%) of unadulterated medication of LENALIDOMIDE were taken and added to the pre-examined plan of fixation 100µg/ml. From that rate recuperation esteems were figured. The outcomes were appeared in table-4.

Table-4: Accuracy Readings

Conc. In ppm	Conc. Found	Peak Area	% Recovery
80	80.461	3959294	100.576
80	80.095	3941634	100.118
80	80.194	3946409	100.242
		Avg.	100.312
		S.D	0.236888

		%RSD	0.236151
Conc. In ppm	**Conc. Found**	**Peak Area**	% Recovery
100	100.932	4948323	100.932
100	99.879	4897463	99.879
100	100.030	4904741	100.030
		Avg.	100.2803
		S.D	0.569388
		%RSD	0.567796
Conc. In ppm	**Conc. Found**	**Peak Area**	% Recovery
120	120.019	5870480	100.015
120	119.907	5865040	99.922
120	119.794	5859590	99.828
		Avg.	99.92167
		S.D	0.0935
		%RSD	0.093574

2. Precision:

2.1. Repeatability

The accuracy of every technique was found out independently from the pinnacle regions and maintenance times gotten by real assurance of six recreates of a fixed amount of drug Lenalidomide (API). The percent relative standard deviation[27] was calculated for Lenalidomide are presented in the table-5.

Table-5: Repeatability Readings

HPLC Injection Replicates of Lenalidomide	Retention Time (Minutes)	Peak Area
Replicate – 1	3.639	3948323
Replicate – 2	3.622	3935751
Replicate – 3	3.575	3979135
Replicate – 4	3.525	3971013
Replicate – 5	3.526	3919463
Replicate – 6	3.523	3974741
Average		**3954738**
Standard Deviation		**24108.89**
% RSD		**0.609621**

2.2. Intermediate Precision:

2.2.1. Intra-assay & inter-assay:

The intra & inter day variation[28] of the method was carried out & the high values of mean assay & low values of standard deviation & % RSD (% RSD < 2%) within a day & day to day variations for Lenalidomide revealed that the proposed method is precise.

Table-6: Results of Intra-Assay & Inter-Assay

Conc. of Lenalidomide (API) (µg/ml)	Observed Conc. of Lenalidomide (µg/ml) by the proposed method			
	Intra-Day		Inter-Day	
	Mean (n=6)	% RSD	Mean (n=6)	% RSD
80	79.35	0.88	80.36	0.56
100	100.57	0.65	99.86	0.36
120	119.87	0.93	120.18	0.87

3. Linearity & Range:

The calibration curve showed good linearity in the range of 0 – 140 µg/ml, for Lenalidomide (API) with correlation coefficient (r^2) of 0.999 (Fig-4). A typical calibration curve has the regression equation of y =

48313x + 71968 for Lenalidomide.

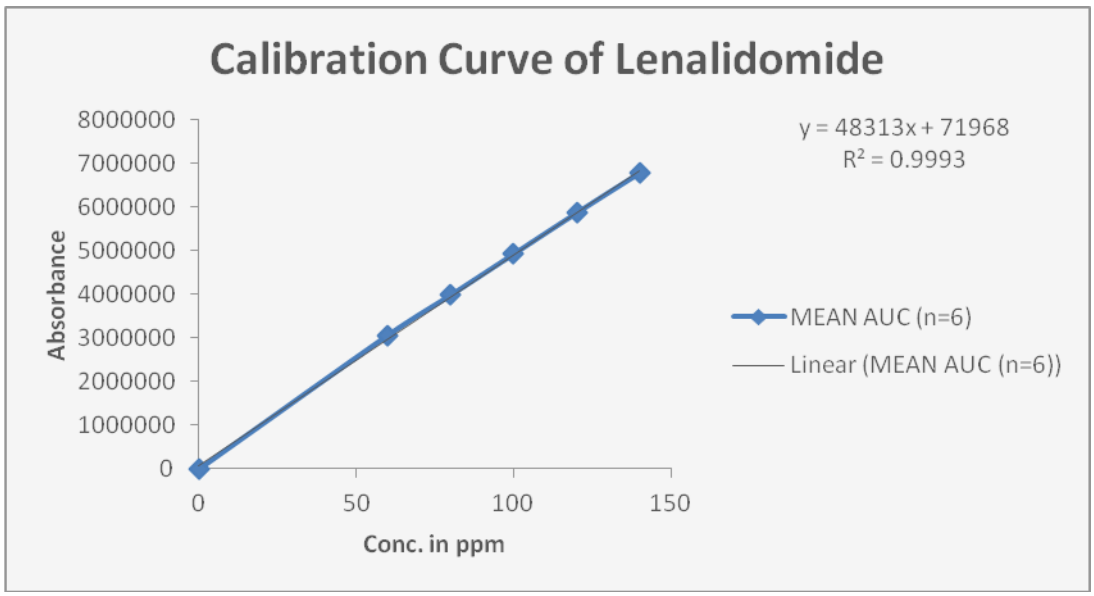

Fig.4. Calibration Curve of Lenalidomide (API)

Table-7: Linearity Results

CONC.(µg/ml)	MEAN AUC (n=6)
0ppm	0
60ppm	3059294
80ppm	3979280
100ppm	4919463
120ppm	5859590
140ppm	6770480

4. Method Robustness:

Impact of little changes in chromatographic conditions, for example, change in Flow rate (± 0.1ml/min), Wavelength of location (±2nm) and organic phase content in mobile phase (±5%) concentrated to decide the Robustness of the technique are additionally for (Table-8, % RSD < 2%) the created RP-HPLC strategy for the examination of Lenalidomide (API).

Table-8: Result of Method Robustness Test

Change in parameter	% RSD
Flow (1.1 ml/min)	0.61
Flow (0.9 ml/min)	0.75
More Organic	0.69
Less Organic	0.81
Wavelength of Detection (208 nm)	0.89
Wavelength of detection (204 nm)	0.99

5. LOD & LOQ:

The Minimum concentration level at which the analyte can be reliable detected (LOD) & quantified (LOQ) were found to be 0.08 & 0.24µg/ml respectively.

6. System Suitability Parameter

Framework appropriateness testing is a necessary piece of numerous explanatory methodologies. The tests depend on the idea that the gear, hardware, logical tasks and tests to be examined comprise a necessary framework that can be assessed thusly. Following framework reasonableness test parameters were built up. The information is appeared in Table-9.

Table-9: Data of System Suitability Parameter

S.No.	Parameter	Limit	Result
1	Resolution	Rs > 2	9.34
2	Asymmetry	T ≤ 2	Lenalidomide=0.16
3	Theoretical plate	N > 2000	Lenalidomide=3065
4	Tailing Factor	T<2	Lenalidomide=1.55

7. Estimation of Lenalidomide in Pharmaceutical Dosage Form

Twenty pharmaceutical dosage forms were taken and the I.P. technique was taken after to decide the normal weight. Above measured tablets were at long last powdered and triturated well. An amount of powder comparable to 25 mg of medications were exchanged to 25 ml volumetric jar, make and arrangement was sonicated for 15 minutes, there after volume was made up to 25 ml with same dissolvable. At that point 10 ml of the above arrangement was weakened to 100 ml with mobile phase. The arrangement was separated through a film channel (0.45 µm) and sonicated to degas. The arrangement arranged was infused in five repeats into the HPLC framework and the perceptions were recorded.

A copy infusion of the standard arrangement was likewise infused into the HPLC framework and the pinnacle zones were recorded. The information is appeared in Table-10.

ASSAY:

Assay % =

$$\frac{AT}{AS} \times \frac{WS}{DS} \times \frac{DT}{WT} \times \frac{P}{100} \times \text{Avg. Wt} = \text{mg/tab}$$

Where:

AT = Peak Area of medication acquired with test readiness
AS = Peak Area of medication acquired with standard readiness
WS = Weight of working standard taken in mg
WT = Weight of test taken in mg
DS = Dilution of Standard arrangement
DT = Dilution of test arrangement
P = Percentage virtue of working standard

Table-10: Recovery Data for estimation Lenalidomide in Lenmid 10

Brand name of Lenalidomide	Labelled amount of Drug (mg)	Mean (± SD) amount (mg) found by the proposed method (n=6)	Assay % (± SD)
Lenmid 10 Tablet (100 mg) (Cipla)	10mg	9.785 (± 0.685)	99.79 (± 0.368)

Discussion: The amount of drug in Lenmid 10 Tablet was found to be 9.785 (± 0.685) mg/tab for Lenalidomide & % assay[29] was 99.79 %.

IV. CONCLUSION

To develop a precise, linear, specific & suitable stability indicating RP-HPLC method for analysis of Lenalidomide, different chromatographic conditions were applied & the results observed are presented in previous chapters. Isocratic elution is simple, requires only one pump & flat baseline separation for easy and reproducible results. So, it was preferred for the current study over gradient elution. In case of RP-HPLC various columns are available, but here Symmetry ODS (C_{18}) RP Column, 250 mm x 4.6 mm, 5µm column was preferred because using this column peak shape, resolution and absorbance were good.

Recognition wavelength was chosen in the wake of checking the standard arrangement of medication more than 200 to 400nm. From the U.V range of Lenalidomide it is apparent that a large portion of the HPLC works can be refined in the wavelength scope of 206 nm helpfully. Further, a stream rate of 1 ml/min and an infusion volume of 10µl were observed to be the best examination. The outcome demonstrates the created strategy is amazingly, one more appropriate technique for test and steadiness related contamination thinks about which can help in the examination of Lenalidomide in various definitions.

A sensitive and particular RP-HPLC technique has been created and approved for the examination of Lenalidomide in API form and marketed pharmaceutical dosage form. Promote the proposed RP-HPLC technique has magnificent affectability, exactness and reproducibility.

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