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ESTIMATION OF LENALIDOMIDE IN BULK AND ITS DOSAGE FORM USING UV SPECTROPHOTOMETRIC AND RP-HPLC METHODS

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ABSTRACT

The present work includes a simple, economic, rapid, accurate and precise UV spectrophotometric and isocratic RP-HPLC method development for estimation of Lenalidomide in bulk and its formulation. Estimation was done at 250nm which was found to be λ_{max} of Lenalidomide. The simple, selective, isocratic RP-HPLC method for Lenalidomide was developed on reverse phase C18 waters column (4.6 mm x 250 mm, 5µm) with a mobile phase of acetate buffer (20 mM) (pH 5): Methanol = 85:15 at a flow rate of 1ml/min and detection wavelength 250nm. The developed methods were validated successfully according to ICH Q2 (R1) guidelines. Both the spectrophotometric and chromatographic methods showed a good linear response with r^2 values of 0.998 and 0.9986 respectively. The percentage relative standard deviation for both methods was found to be less than two, indicating that the methods were precise. The mean percentage recovery for UV method was between 99.2% and for RP-HPLC method was 99.6% respectively. From the results it could be concluded that both the developed methods were specific, selective and robust. The methods could be successfully applied for analysis of capsule formulation of Lenalidomide.

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INTRODUCTION

Lenalidomide (LND) is a derivative of thalidomide with better biological activity[1]. Chemically it is, 3-(4- amino-1-oxo 1, 3-dihydro-2H-isoindol-2-yl) piperidine- 2, 6-dione [2]. Lenalidomide is used in the treatment of multiple myeloma but now also shown to be therapeutically efficient in treating class of hematological disorders known as myelodysplastic syndromes (MDS) [3]. It acts through three main mechanisms *viz;* direct anti-tumor effect, inhibition of angiogenesis and immunomodulatory activity. In vivo, lenalidomide induces tumor cell apoptosis directly and indirectly by inhibition of bone marrow stromal cell support, by anti-angiogenic and anti-osteoclastogenic effects and by immunomodulatory activity. Liu, Qing MS *et al* have reported that a highly sensitive liquid chromatography/mass spectrometry method for simultaneous quantification of Lenalidomide and flavopiridol in human plasma [4]. S. Gananadhamu *et al* have reported that fluorometric estimation of Lenalidomide in pharmaceutical formulations [5]. S. Gananadhamu *et al* have reported that new spectrophotometric methods for estimation of Lenalidomide in pharmaceutical formulations using Azo dye. Two sensitive spectrophotometric methods were developed for the estimation of Lenalidomide in pharmaceutical formulations [2]. However, to best of our knowledge, a simple UV spectrophotometric and isocratic RP-HPLC method for estimation of Lenalidomide and its marketed formulation have not been reported. Hence, there is a need to develop simple and precise method for Lenalidomide estimation in bulk and dosage form. The objective of present work is to develop a simple but accurate and precise UV spectrophotometric and isocratic RP-HPLC method for estimation of Lenalidomide in bulk and its marketed formulation.

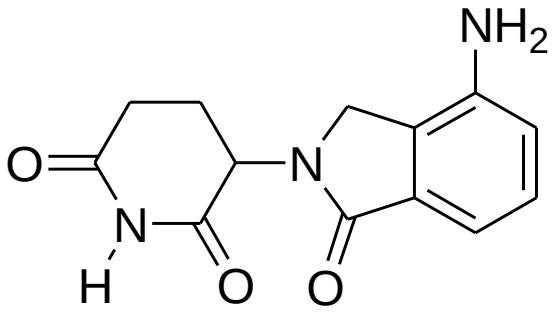

Fig 1. Chemical Structure of Lenalidomide (LND).

MATERIALS AND METHODS

Chemicals and Reagents:

LND an active pharmaceutical ingredient was obtained as a sample gratis from Apicore Pharmaceuticals Ltd. Vadodara, India. Marketed formulation of Lenalidomide lenangio™ (labelled claim 5mg) capsules by manufactured by Dr Reddy's Laboratories, India, was procured from a local pharmacy store. Methanol analytical reagent grade (Rankem, Mumbai, India) was used as the solvent and diluent for UV spectrophotometric method. For chromatographic method, HPLC grade Methanol was procured from Rankem Pvt. Ltd., Mumbai. All the required solutions were prepared in HPLC grade water. Other reagents like ammonium acetate buffer and acetic acid were procured from Loba Chemie Pvt. Ltd., Mumbai, India. All solutions were filtered through a 0.2 µm Ultipor®N66® Nylon 6, 6 membrane filter (Pall Life Sciences, USA) prior to injecting into HPLC.

Instrumentation:

UV Spectrophotometer:

Estimation of LND was carried out using Shimadzu UV-1700 double beam spectrophotometer equipped with Shimadzu UV Probe 2.10 software. Shimadzu UV- 1800 double beam spectrophotometer was also used for ruggedness study. Matched quartz cuvettes of 1 cm was utilized to hold drug solution to be analyzed and UV spectrum was recorded over range of 200-400 nm. All the samples were weighed on electronic analytical balance (A×120, Shimadzu).

HPLC System:

Chromatographic separation was performed on Shimadzu LC system (Shimadzu Corporation, Kyoto, Japan) equipped with Shimadzu LC-20AT pump and Shimadzu SPD-20AV detector and Rheodyne 7725 injector with fixed loop of 20 µL. Data acquisition and integration was performed using Spinchrome software (Spinco biotech, Vadodara). Stationary Phase used was waters C-18 column, (Column dimensions: 250 mm x 4.6 mm, 5µm).

Preparation of Stock Solution and working solutions: (Common for UV and HPLC methods)

10mg of LND was weighed accurately and transferred into a 10mL volumetric flask containing 1 ml methanol. Volume was made up to the mark using methanol to produce a stock solution of LND with concentration of 1000 µg/ml. Working standard solution of LND (100 µg/mL) was prepared by transferring 2mL of aforementioned prepared stock solution to 20 mL volumetric flask and making up the volume up to the mark with methanol.

Preparation of Calibration Curve of Standard LND:

From the working standard solution of LND (100 µg/mL), aliquots of 0.5 mL, 1 mL, 1.5 mL, 2 mL, 2.5 mL and 3.0 mL were withdrawn and transferred accurately into separate 10 mL volumetric flasks. Methanol was added to individual flasks to make up volume up to the mark so as to produce solutions containing 5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL, 25 µg/mL and 30 µg/mL of LND respectively. Prepared solutions were used for UV spectrophotometric method.

From the working standard solution of LND (100 µg/mL) for RP-HPLC aliquots of 0.4 mL, 0.8 mL, 1.2 mL, 1.6 mL, 2.0 mL and 2.4 mL were withdrawn and transferred accurately into separate 10 mL volumetric flasks. Mobile phase was added to make up the volume up to the mark so as to obtain solutions containing 4 µg/mL, 8 µg/mL, 12 µg/mL, 16µg/mL, 20µg/mL, 24µg/mL of LND respectively. Table 1 displays the calibration sets prepared for LND for UV and RP-HPLC methods.

Table 1: Construction of calibration sets of LND by UV spectrophotometric and RP-HPLC method.

UV METHOD		RP- HPLC METHOD	
Sr no	**Concentration of LND(µg/mL)**	**Sr no**	**Concentration of LND(µg/mL)**
1	5	1	4
2	10	2	8
3	15	3	12
4	20	4	16
5	25	5	20
6	30	6	24

Preparation of Mobile Phase for HPLC:

1.54 g of Ammonium acetate was added to 1000 ml of water to prepare 20 mM Acetate buffer. The pH of Buffer solution was adjusted to 5 with acetic acid. Then solution was finally filtered with 0.2 µm Nylon membrane filter. In 850 ml of buffer, 150 ml of methanol was added to prepare 1000 ml of mobile phase. Mobile phase was prepared freshly every day and premixed, filtered and degassed by using sonicator prior to use.

Selection of Analytical wavelength and calibration curve:

Standard solutions of LND were scanned between 200-400nm in UV spectrophotometer for wavelength selection and the solutions showed maximum absorbance at 250nm wavelength as shown in Figure 2. Hence 250 nm was selected as the analytical wavelength.

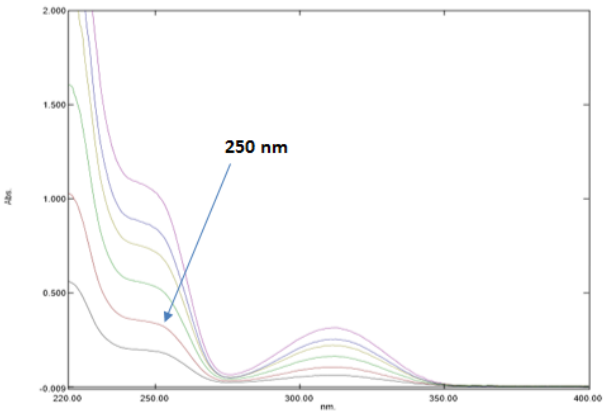

Figure 2: Overlay spectra for LND with analytical wavelength at 250nm.

Prepared calibration standards for both the methods were analyzed at 250nm wavelength.

Stability of solution:

Prepared stock solution of LND was stored at room temperature for 24 hours. After 24 hour, sample was re-injected in HPLC. The chromatogram showed no additional peaks and the %Relative standard deviation of peak area of LND was lower than 1%.This shows that solution of LND was stable in Methanol.

Analysis of formulation by the developed methods

The developed UV method was applied for analysis of LND formulation available in market. "Lenangio" capsule formulation manufactured by Dr Reddy Laboratories was procured from local pharmacy. 5 mg of sample formulation in 5 ml of methanol was prepared (1000µg/mL). 0.2 mL of the sample formulation was withdrawn in a 10mL volumetric flask and diluted up to the mark using methanol to produce a clear solution. The resulting solution was analyzed at 250nm and absorbance was recorded. Concentration of Lenalidomide was then calculated from the calibration graph. Six replicate samples were used for analysis.

For HPLC method, 5 mg of sample formulation in 5 ml of methanol was prepared (1000µg/mL) and centrifuged at 8000 rpm, from this 0.16 mL was withdrawn in a 10 mL volumetric flask and diluted up to the mark using mobile phase to produce a clear solution of concentration 16 µg/mL. Six such replicate solutions were prepared and injected. The optimized HPLC method conditions are reported in Table 2.Their peak areas were calculated. Results of formulation analysis by both the methods are reported in Table 3.

Table 2: Optimized chromatographic conditions for estimation of LND.

Method Parameter	Optimized condition
Stationary Phase	Waters C18
Mobile Phase	Acetate buffer: methanol
Retention time	7.9 min
Detection wavelength	250 nm
Flow rate	1 mL/min
Temperature	Ambient

Table 3. Formulation analysis by HPLC and UV.

Method	Actual conc. of LND(mg)*	Amount of LND found(mg)*	%Assay
UV	5	4.94	99.2 ±0.07
RP HPLC	5	4.981	99.6 ±1.5

*Values represent the average of six determinations.

Validation of the developed methods

Developed spectrophotometric method and RP-HPLC method were validated according to ICH Q2 (R1) guidelines and data obtained was complying with the standards [6]. The linearity of method was evaluated in triplicates by analyzing prepared concentrations of LND in range of 5-30 µg/mL for UV method and respective solutions of LND from 4-24 µg/mL for RP-HPLC method. Linear regression equation was obtained over the concentration range (y = mx+c). Limit of Detection (LOD) and Limit of Quantification (LOQ) were calculated from standard deviation of response and slope of calibration curve. Intra-day and inter-day precision was performed analyzing six replicates followed by %RSD calculation. Accuracy of method was performed by calculating %recovery of LND from its formulation. Selectivity of the methods was confirmed by the absence of any absorbing or interfering excipients in UV spectrophotometric as well as RP-HPLC method.

RESULTS AND DISCUSSION

Developed and optimized UV spectrophotometric and chromatographic methods were validated for various parameters discussed in following sections.

Linearity and Range:

Linearity solutions ranging from concentration of containing 5 - 30 µg/mL (for UV methods) and 4-24 µg/mL (for HPLC method) LND were prepared and analyzed in triplicate at a constant injection volume of 20 µL. Calibration curve and r^2 value for UV methods (Figure 3) and HPLC method (Figure 4) were generated. Figure 3 and Figure 4 show the calibration graph of LND by UV and RP-HPLC method respectively. A good linear relationship was observed with correlation coefficients 0.998 and 0.998 in UV and HPLC method respectively. Figure 5 shows the overlain chromatogram of LND by RPHPLC using the above optimized conditions.

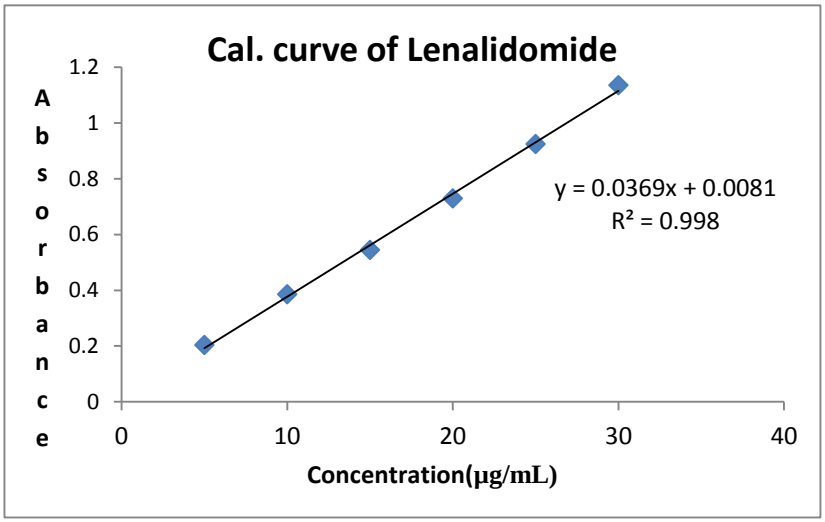

Figure 3: Calibration graph of Lenalidomide in Methanol by UV method (5-30 µg/mL).

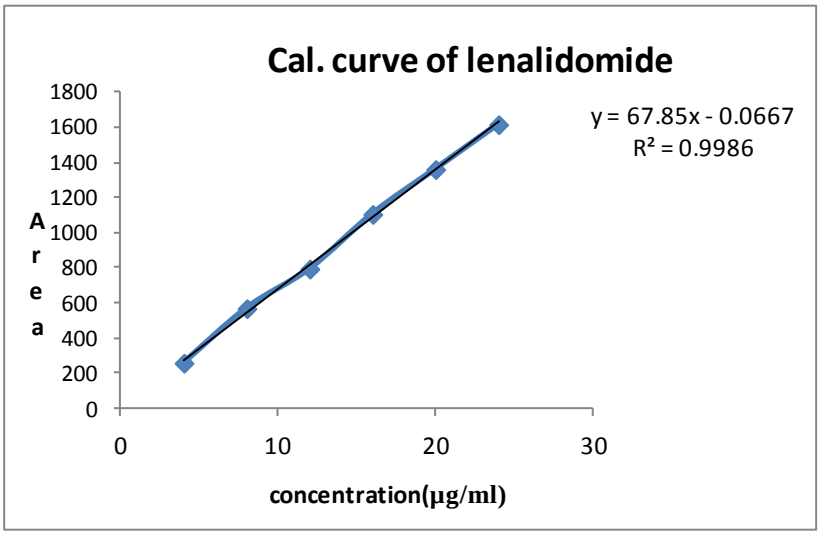

Figure 4: Calibration graph of LND obtained by HPLC (in Acetate buffer: Methanol=85:15).

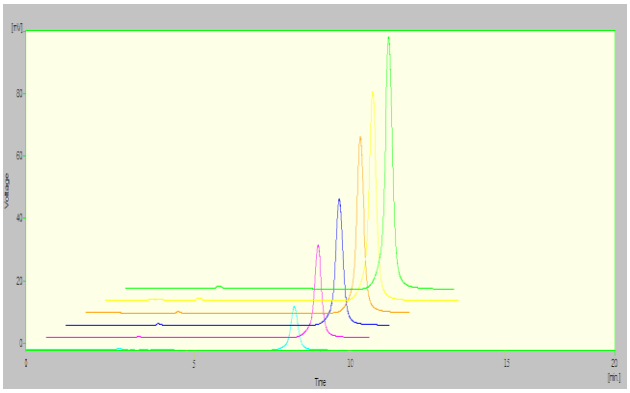

Figure 5: Overlain chromatogram of LND (4-24µg/mL) with Acetate buffer: Methanol= 85: 15 as mobile phase.

LOD and LOQ (Limit of Detection and Limit of Quantitation)

LOD and LOQ of both UV and HPLC methods were determined using standard deviation of response and slope of calibration curve approach. Six replicates of the drug sample with lowest detectable and quantifiable concentration were injected and %RSD was determined. Results of these are given in Table 4.

The results obtained from the validation parameters by both methods are indicated in Table 4.

Table 4: Summary of validation parameters of LND by the developed methods.

Parameter	UV method	RP-HPLC METHOD
Analytical wavelength	250 nm	250 nm
Linearity Range	5-30 µg/mL	4-24µg/mL
Slope	0.0369	67.85
Intercept	0.0081	-0.0667
Correlation coefficient	0.998	0.998
Limit of Detection(LOD)	0.2311µg/mL	0.040µg/mL
Limit of Quantification(LOQ)	0.7005µg/mL	0.122µg/mL

The lowest limit of analyte that could be detected accurately and precisely (LOD) by UV and RP-HPLC method was found to be 0.2311µg/mL and 0.040 µg/mL respectively. This indicates that the methods were very sensitive. Moreover, the limit of Quantitation (LOQ) was reported to be 0.7005 µg/mL and 0.122 µg/mL for UV spectrophotometric and RP-HPLC method.

Precision

Reproducibility of the methods was checked by performing intra-day precision (three times a day) and inter-day precision (repeated triplicates for three consecutive days). Results for RP-HPLC and UV method are expressed in terms of standard deviation and percentage Relative standard Deviation (%RSD). The intra-day and inter-day precision in terms of %RSD for UV method was 0.429% and 0.626 % respectively, while for RP-HPLC method it was 0.224% and 0.175% respectively. Thus, it can be seen from the results in Table 5 that the developed UV spectrophotometric and RP-HPLC method were very precise.

Table 5: Results of Interday and Intraday precision of LND by UV Spectrophotometry and HPLC.

Method	Precision	Standard Deviation	%RSD/%CV(Coefficient of variance)	S.E(Standard Error)
UV	Intra day	0.002	0.429	0.0008
	Inter day	0.0025	0.626	0.0010
RP-HPLC	Intra day	1.187	0.224	0.4864
	Inter day	1.16	0.175	0.4754

Accuracy (% Recovery):

To check the accuracy of the developed methods, recovery studies were carried out from pre-analyzed sample at three different level of standard addition 80%, 100% and 120%. Percentage Recovery was the average of three determinations at each standard addition level. Percentage Recovery was found to be between 99%-101% which prove that the methods were accurate.
% Recovery was found between 99-101% and 99-101% for UV and HPLC methods respectively as shown in Table 6. High percentage recovery values showed that the methods were free from interference of the excipients used in the formulation.

Table 6: Results of Recovery studies of LND by UV Spectrophotometry and RP-HPLC.

Method	%Spiking	Actual concentration of LND(µg/mL)	Amount of LND added (µg/mL)	Amount of LND Recovered (µg/mL)	%Recovery + SD
UV	80%	20	16	16.17	101.15±0.105
	100%	20	20	19.80	99.0±0.003
	120%	20	24	24.06	100.25±0.301
RP-HPLC	80%	20	16	16.20	101.66±1.52
	100%	20	20	19.86	99.30±0.577
	120%	20	24	24.36	101.54±1.67

(SD= Standard Deviation) (*Average of three determinations at each standard addition level).

No interfering peaks were found in the chromatogram indicating that excipients used in the formulation did not interfere with the estimation of drug by the proposed RP-HPLC method.

Ruggedness and Robustness:

Robustness of UV spectrophotometric method was performed by checking the effect of variation in solvent characteristics on the absorbance of the method. Absorbance of solutions in plain methanol, methanol with 5% water and methanol with 10% water as solvent was recorded. Ruggedness of the method was confirmed by measuring absorbance of solutions using two different instruments- Shimadzu UV-1700 double beam spectrophotometer and Shimadzu UV-1800 double beam spectrophotometer.

Both the developed methods were quite robust. Results of robustness and ruggedness for UV method, in terms of standard deviation and %RSD are depicted in Table 7. Method showed 1.767% RSD when concentration of methanol as solvent was varied by adding water up to 10%. The difference in absorbance of resulting solutions measured by two different instrument models of Shimadzu- 1700 and 1800 showed an acceptable variation of 0.383 % RSD.

Table 7: Robustness and Ruggedness of developed UV method.

ROBUSTNESS*				STATISTICS		
Concentration of LND	Absorbance of solution Methanol as solvent	5 % water in Methanol	10% water in Methanol	Mean	Standard Deviation	%Relative Standard Deviation
20 µg/Ml	0.731	0.745	0.758	0.744	0.013	1.767%
RUGGEDNESS*				**STATISTICS**		
Concentration of LND	Absorbance of solution UV-Vis 1700 (Instrument 1)	UV-Vis 1800 (Instrument 2)		Mean	Standard Deviation	% Relative Standard Deviation
20µg/Ml	0.730	0.734		0.730	0.0028	0.383%

(*Data consists of average results obtained in triplicates)

The developed RP-HPLC method was also found to be quite robust when small changes were induced in the method parameters which can be seen in Table 8. Flow rate, Mobile Phase Ratio and pH were varied. Asymmetry, Theoretical plates and retention time were noted standard deviation % RSD was calculated.

Table 8: Results of Robustness study of the developed RP-HPLC method.

Factors	Retention time(min)	Asymmetry	Area(Mv.s)
A. Flow rate			
0.9	8.12	0.78	1149
1.0	7.99	0.82	1154
1.1	7.89	0.87	1162
Mean±SD	8±0.115	0.8033 ±0.015	1162 ±6.5
%RSD	1.44%	0.82%	0.56%
B.% of Methanol			
13	7.86	0.81	1143
15	7.9	0.82	1154
17	8.15	0.83	1177
Mean±SD	7.97±0.15	0.82±0.01	1158 ±17.3
%RSD	1.97%	1.21%	1.49%
B. pH			
4.8	8.1	0.80	1164
5.0	7.93	0.82	1154
5.2	7.84	0.83	1189
Mean±SD	7.95±0.13	0.8166±0.015	1169 ±18.027
%RSD	1.65%	1.87%	1.54%

System suitability test:

The results of System suitability parameters are reported in Table 9. Data obtained from six replicate injections is shown here. It can be seen that number of theoretical plates is greater than 2000. Asymmetry of peak indicated by tailing factor less than 1.5 indicates that all the system suitability parameters are successfully passed.

Table 9: System suitability parameters of the developed RP-HPLC method.

Parameter	Result
Area ± SD	1106.422± 0.729
Theoretical plate ± SD	6687.83± 0.02
Tailing factor ± SD	0.865± 1.86

CONCLUSION

The developed spectrophotometric and UV methods were inexpensive, less time-consuming and did not involve any lengthy sample preparation and extraction steps. Both methods are simple, rapid, accurate, precise and specific. The % recovery for both methods was in good agreement with their respective label claims, which suggested noninterference of additives in its estimation. Hence, the developed methods could be successfully applied for estimation of LND in bulk and its capsule formulation. The method can be further applied in plasma for bioanalytical studies for LND.

LIST OF ABBREVIATION:

LND : Lenalidomide
RP-HPLC : Reverse phase High performance liquid chromatography
LOD : Limit of detection
LOQ : Limit of quantitation
RSD : Relative standard deviation

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CONFLICT OF INTERESTS:

The authors declare they have no conflict of interests.

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