



## **A Simple Development and Validated Reverse Phase-HPLC Method for the Determination of Linezolid in Bulk and Pharmaceutical Dosage Forms**

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### **ABSTRACT**

A convenient, simple, accurate, precise and reproducible RP-HPLC method was developed and validated for the estimation of Linezolid in the bulk drug and pharmaceutical dosage form. The objective was achieved under optimized chromatographic conditions on Welchrom C<sub>18</sub> isocratic column (250 mm × 4.6 mm, 5 μm) with Shimadzu LC-20AT Prominence Liquid chromatograph. The mobile phase was a mixture of acetonitrile: water 50:50 v/v, with apparent pH of 6.8. The separation was attained using an isocratic elution method with a flow rate of 1.2 mL/min at room temperature. The detection was made at a wavelength of 254nm by using UV- Visible detector. The retention time for Linezolid molecule was found to be 2.813. The standard calibration plot was linear over a concentration range of 2-10 μg/mL with  $r^2 = 1$  and the respective linear regression equation being  $y = 177.1x + 0.030$ . The limit of detection and limit of quantification were found to be 0.04 μg/mL and 0.014 μg/mL respectively. The amount of Linezolid presents in the bulk drug 99.81% and in the formulation 99.81% of the stated amount respectively. The method was validated statistically using the %RSD and the values are found to be within the limits. No interference peaks from Excipients and relative retention time indicated the specificity of the Method. The recovery studies were performed and mean percentage recoveries were found to be greater than 99% with RSD less than 1.0%. So the proposed method was found to be simple, specific, linear, robust and reproducible. Hence this method was conveniently and easily applied for routine analysis of Linezolid in bulk drug and tablet dosage form.

**Keywords:** Linezolid, RP-HPLC, Validation.

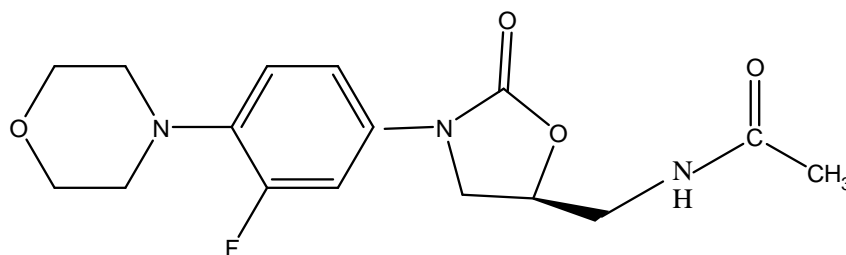
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## INTRODUCTION

Chemical name of Linezolid (LIN) <sup>1</sup>is N-[[[(5S)-3-[3-fluoro-4-(morpholin-4-yl)phenyl]-2-oxo-1,3-oxazolidin-5-yl]methyl]acetamide. It has an empirical formula of C<sub>16</sub>H<sub>20</sub>FN<sub>3</sub>O<sub>4</sub> and a molecular weight of 337.346 g/mol. Discovered in the 1990's and first approved for use in 2000, Linezolid was the first commercially available 1, 3-oxazolidinone<sup>2</sup> antibiotic. It is an antibacterial compound developed by a team at Pharmacia and Upjohn Company<sup>3</sup>. The main indication of Linezolid is used for the treatment of serious infections caused by Gram-positive bacteria<sup>4,5</sup> that are resistant to several other antibiotics. As a protein synthesis inhibitor, it stops the growth of bacteria by disrupting their production of proteins i.e. it is a bacteriostatic agent, not bacteriocidal. Although many antibiotics work this way, the exact mechanism of action of Linezolid appears to be unique in that it blocks the initiation of protein production. In both the popular press and the scientific literature, Linezolid has been called a "reserve antibiotic"—one that should be used sparingly so that it will remain effective as a drug of last resort against potentially intractable infections.<sup>6,7</sup> and Linezolid appears to be as safe and effective for use in children and newborns as it is in adults. Linezolid is official in Indian Pharmacopoeia<sup>8</sup>. Literature survey reveals that several analytical methods have been reported for the estimation of Linezolid in pharmaceutical dosage forms. UV<sup>9</sup>, LC-UV<sup>10</sup>, HPLC-UV<sup>11,12</sup>, RP-HPLC<sup>13,14,15,16</sup> and HPTLC<sup>17</sup> methods. The present research work involves development of a convenient, user friendly, precise, rapid isocratic RP-HPLC method with simple mobile phase for quantitative estimation of LIN in bulk drug and tablet dosage form. The optimized method have been developed and validated as per ICH Q2 (R1)<sup>18</sup> guide lines. The chemical structure of LIN is shown in Figure. 1.



**Figure: 1 Structure of Linezolid**

## MATERIALS AND METHOD

### Materials and Reagents:

The reference sample of Linezolid standard was kindly supplied as gift sample by Hetero Drugs Ltd., Hyderabad, Andhra Pradesh, India. All the chemicals were analytical grade. Potassium

dihydrogen orthophosphate and phosphoric acid from Rankem Ltd., Mumbai, India, while acetonitrile (HPLC grade) and triethylamine (HPLC grade) from Merck Pharmaceuticals Private Ltd., Mumbai, India. Ortho phosphoric acid used was of HPLC grade and purchased from Merck Specialties Private Ltd., Mumbai, India. Commercially available Linezolid tablets(Lizolid-600® tablets from Glenmark pharmaceuticals Limited, Mumbai, India) procured from local market.

**Instrument:**

Quantitative HPLC was performed on a isocratic high performance liquid chromatograph (Shimadzu LC-20AT Prominence Liquid Chromatograph) with a LC-20AT VP pump, manual injector with loop volume of 20  $\mu$ L (Rheodyne), programmable variable wavelength Shimadzu SPD-20A Prominence UV-Vis detector and Welchrom C18 Column (4.6 X 250mm, 5 $\mu$ m particle size). The HPLC system was equipped with Spinchrome software. In addition an electronic balance (Shimadzu TX223L), digital pH meter (Systronics model 802), a sonicator (spectra lab, model UCB 40), UV-Visible Spectrophotometer (Systronics model-2203) were used in this study.

**Chromatographic conditions:**

Linezolid separation was performed on C<sub>18</sub> (250mmX4.6mm, 5 $\mu$ m) column. The mixture of Phosphate buffer (pH adjusted to 6.8with ortho phosphoric acid) and acetonitrile in ratio of 50:50 v/v was selected as mobile phase and UV detection wavelength was 254nm with a flow rate of 1.2 mL/minutes. Injection volume was 20 $\mu$ L, with ambient temperature, run time was 4minutes.

**Preparation of mobile phase:**

Phosphate buffer was prepared by dissolving 1.488 g of potassium dihydrogen orthophosphate and 0.288 g dipotassium hydrogen phosphate in 500 mL of HPLC grade water. pH was adjusted to 6.8 with ortho phosphoric acid. The above prepared buffer and acetonitrile were mixed in the proportion of 50:50 v/v and was filtered through 0.45  $\mu$ m nylon membrane filter and degassed by sonication.(Note: water mixture with buffer should not be added to acetonitrile may it cause precipitation of the entire mobile phase).

**Stock and working Standard Solutions:**

Accurately 10 mg of Linezolid was weighed and transferred to a 10 mL clean, dry calibrated flask and 3mL of mobile phase was added, if necessary sonicate to dissolve. The volume was made upto the mark with mobile phase. This is primary stock standard solution of LIN with concentration of 1000  $\mu$ g/mL. Secondary stock solution having the concentration range 100  $\mu$ g/mL was made by taking 1ml from primary stock solution and made up the volume with buffer to 10mL.Five working standard solutions for calibration were prepared by adding defined

volumes of the secondary stock standard solution and diluting with mobile phase. The eventual concentrations of Pirfenidone were 0.2, 0.4, 0.6, 0.8, 1.0  $\mu\text{g/mL}$  respectively.

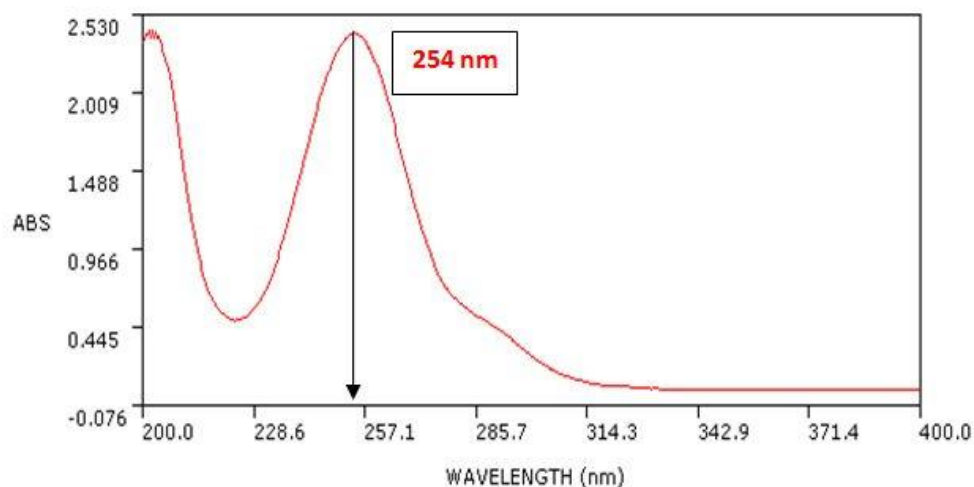
#### Preparation of Sample solution:

The content of twenty tablets were accurately weighed and transferred into a mortar and ground to a fine powder. From this, tablet powder which is equivalent to 10 mg of Linezolid was taken and the drug was extracted in 10 mL of mobile phase. The resulting solution was filtered using Whatman Grade No. 1 filter paper and degassed by sonication. This solution was further suitably diluted for chromatography. The test solutions were injected into the system by filling a 20  $\mu\text{L}$  fixed volume loop manual injector. The chromatographic run time of 4 min. was maintained for the elution of the drug from the column. The elutes were monitored with UV detector at 254 nm. A 20  $\mu\text{L}$  volume of standard and sample solutions were separately injected on HPLC system. From the peak area Linezolid the amount of drug in the sample were computed.

## RESULTS AND DISCUSSION

#### Selection of detection wavelength

The UV spectrum of diluted solutions for various concentrations of Linezolid in mobile phase was recorded using UV spectrophotometer by using systronic double beam SL 2203. The wavelength of maximum absorbance was observed at 254 nm. This wavelength was used for detection of Linezolid. Figure. 2 show absorption maxima for Linezolid.



**Figure. 2 UV spectrum of LINEZOLID (10  $\mu\text{g/mL}$ )**

#### Calibration curve (Linearity):

20  $\mu\text{L}$  of each calibration standard solutions (2, 4, 6, 8, 10  $\mu\text{g/mL}$ ) were injected into the HPLC system to get the chromatograms. The average peak area and retention time were recorded. Linearity curve was constructed by plotting concentration of Linezolid on X-axis and average

peak areas of standard Linezolid on Y-axis and regression equations were computed. The linearity range was found to be 2-10 µg/mL. The results are presented in Table 1. The standard chromatograms of Linezolid calibration standards are depicted in Figure. 3 to Figure. 7. Results show that a phenomenal correlation exists between peak area and concentration of drug within the linearity range. The calibration graph of Linezolid is presented in Figure. 8. The data of regression analysis is presented in Table 2.

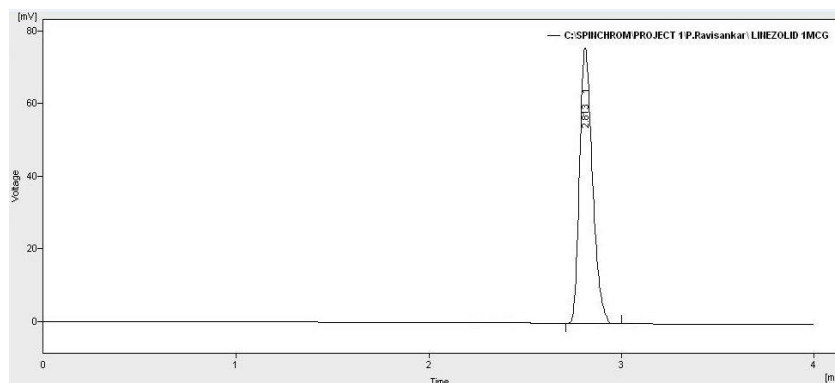
**Table1: Calibration data of the proposed method for the estimation of linezolid.**

S.No	Concentration, µg/ml	Retention time, (t <sub>R</sub> )mi	Peak area, mV.
1.	0	-	0
2.	2	2.813	354.278
3.	4	2.813	708.626
4.	6	2.813	1062.988
5.	8	2.813	1417.662
6.	10	2.813	1771.267

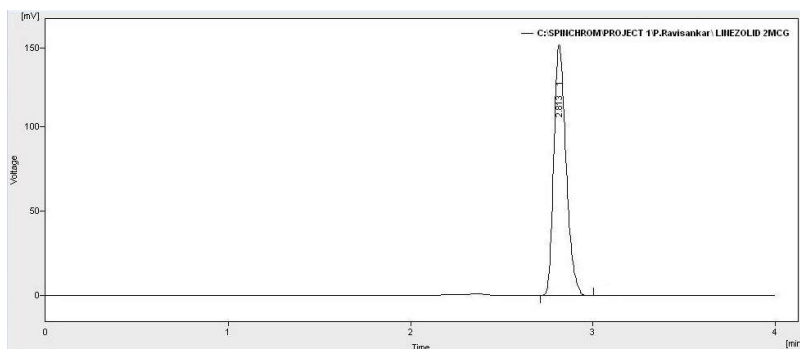
**Table 2: Linear regression data of the proposed hplc method of linezolid.**

Parameter	Method
Detection wavelength( λ <sub>max</sub> )	UV at 254 nm
Linearity range (µg/mL)	2-10 µg/mL
Regression equation (Y = a + bX)	Y = 177.1x + 0.030
Slope(b)	177.1
Intercept(a)	0.030
Standard error of slope (S <sub>b</sub> )	0.183
Standard error of intercept (S <sub>a</sub> )	0.06
Standard error of estimation (S <sub>e</sub> )	0.253
Regression coefficient (R <sup>2</sup> )	0.9999
% Relative standard deviation* i.e.,	0.01752
Coefficient of variation(CV)	
Percentage range of errors* (Confidence limits)	
0.05significance level	0.0140
0.01 significance level	0.0184

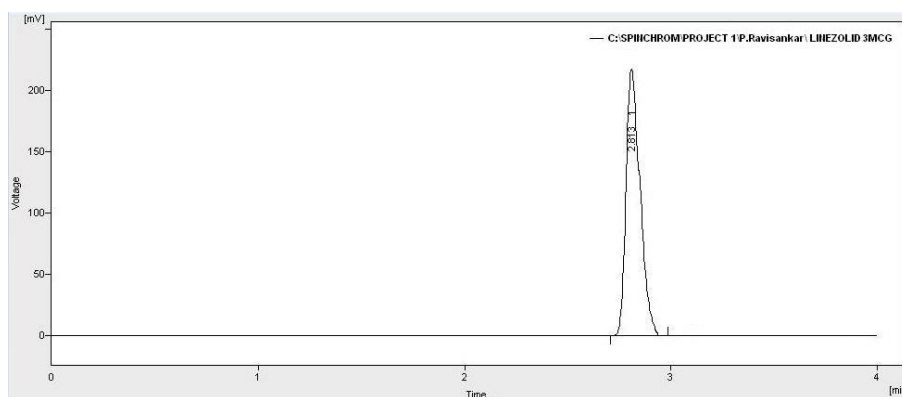
\* Average of 6 determinations; Acceptance criteria < 2.0.



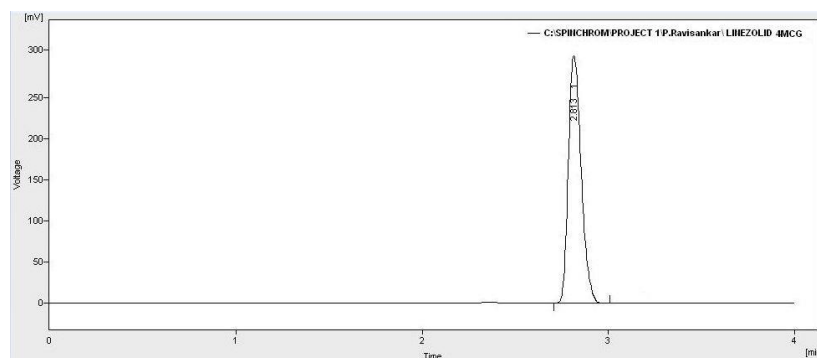
**Figure 3: Standard chromatogram of LINEZOLID (2 µg/ml)**



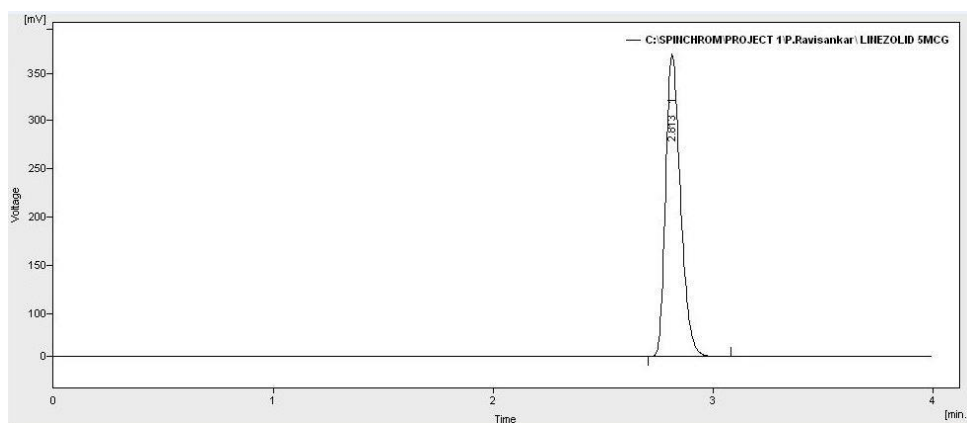
**Figure 4: Standard chromatogram of LINEZOLID (4 µg/ml)**



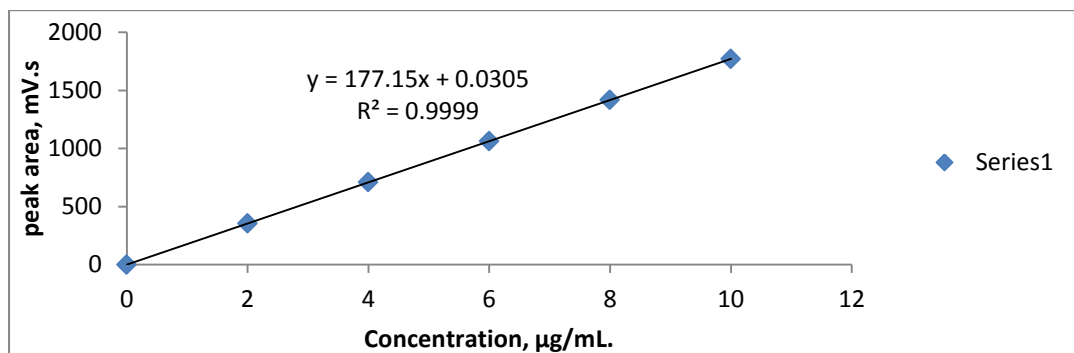
**Figure 5: Standard chromatogram of LINEZOLID (6 µg/ml)**



**Figure 6: Standard chromatogram of LINEZOLID (8 µg/ml)**



**Figure 7: Standard chromatogram of Linezolid (10 µg/ml)**



**Figure 8: Calibration plot of Linezolid**

## VALIDATION OF THE PROPOSED METHOD

The developed method of analysis was validated as per the ICH for the parameters like system suitability, specificity, linearity, precision, accuracy, robustness and system suitability, limit of detection (LOD) and limit of quantitation (LOQ).

### System suitability

The chromatographic systems used for analysis must pass the system suitability limits before sample analysis can commence. Set up the chromatographic system; allow the HPLC system to stabilize for 40 minutes. Inject blank preparation (single injection) and standard preparation (six replicates) and record the chromatograms to evaluate the system suitability parameters like resolution (NLT 2.0), tailing factor (NMT 1.5), theoretical plate count (NLT 3000) and % RSD for peak area of six replicate injections of Linezolid standard (%RSD NMT 2.0). The system suitability data is reported in Table 3.

### Specificity

The specificity of the method was determined by observing interference of any encountered ingredients present in the formulations. The test results obtained were compared with the results of those obtained for standard drug. It was shown that those ingredients were not interfering with the developed method. Furthermore the well-shaped peaks also indicate the specificity of the method. The results for specificity are tabulated in Table 4. The chromatogram for placebo indicating the specificity of developed method is presented in Figure. 9.

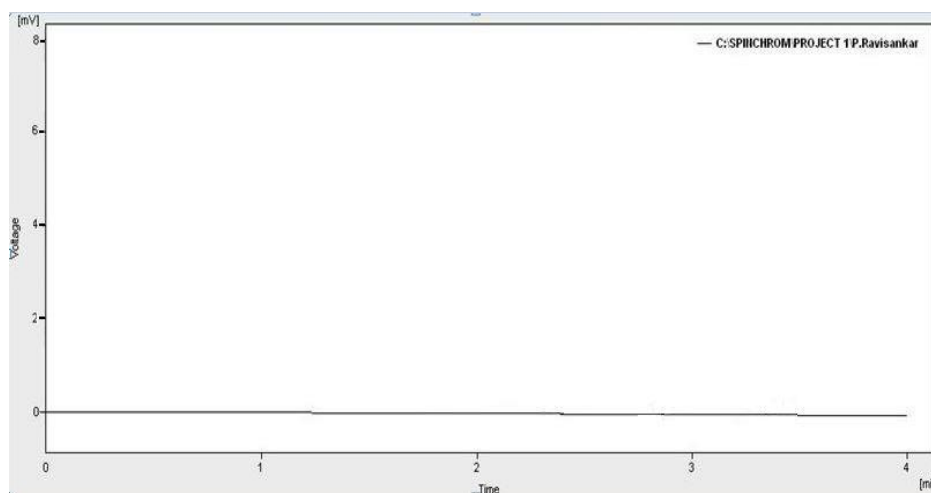
**Table 3: Optimum chromatographic and system suitability data**

Parameter	Chromatographic conditions
Instrument	SHIMADZU LC-20AT prominence liquid chromatograph
Column	WELCHROM C <sub>18</sub> Column (4.6 mm i.d. X 250 mm, 5 µm particle size)
Detector	SHIMADZU SPD-20A prominence UV-Vis detector
Diluents	Acetonitrile : water ( 50 : 50) pH : 3.3 using o-phosphoric acid)

Mobile phase	Acetonitrile : water ( 50 : 50) pH : 3.3 using o-phosphoric acid)
Column modifier	Triethylamine (0.5 mL)
Flow rate	1 mL/min.
Detection wave length	UV at 254nm.
Run time	4 minutes
Column back pressure	111 kgf
Temperature	Ambient temperature(25°C)
Volume of injection loop	20 µL
Retention time (t <sub>R</sub> )	2.813 minutes
Theoretical plates [th.pl]	8154
(Efficiency)	
Theoretical plates per meter [t.p/m]	163080
Tailing factor (asymmetry)	1.360

**Table 4: Specificity Study For Linezolid :**

<b>Name of the solution</b>	<b>Retention time, (t<sub>R</sub>) min.</b>
Mobile phase	No peaks
Placebo	No peaks
linezolid, 10 µg/mL	2.813 min.



**Figure 9: Chromatogram of placebo**

### Precision

Intra-day precision was investigated by replicate applications and measurements of peak area for Linezolid for six times on the same day under similar conditions.

Inter-day precision was obtained from % RSD values obtained by repeating the assay six times on two different days. The percent relative standard deviation (% RSD) was calculated which is within the acceptable criteria of not more than 2.0. The intra-day and inter-day precision results were shown in Table 5 and Table 6 respectively.

**Table 5: Results of Precision Study (Intra-Day) For Linezolid**

Sample	Concentration ( $\mu\text{g/mL}$ )	Injection no.	Peak area (mV.s)	%RSD <sup>#</sup>
Linezolid	10	1	1456.356	0.066
		2	1456.976	
		3	1457.9	
		4	1458.234	
		5	1458.118	
		6	1459.078	

<sup>#</sup> Acceptance criteria < 2.0.

**Table 6: Results of Precision Study (Inter-Day) for Linezolid**

Sample	Concentration ( $\mu\text{g/mL}$ )	Injection no.	Peak area (mV.s)	%RSD <sup>#</sup>
Linezolid	10	1	1458.546	0.049
		2	1458.344	
		3	1457.678	
		4	1456.99	
		5	1459.078	
		6	1458.211	

<sup>#</sup> Acceptance criteria < 2.0.

### Accuracy (Recovery studies)

Accuracy is the degree of agreement between a measured value and the accepted reference value. The accuracy of the method was tested by triplicate samples at 3 different concentrations equivalent to 80%, 100% and 120% of the active ingredient, by adding a known amount of Linezolid standard to a sample with pre-determined amount of Linezolid. The recovered amount of Linezolid, %RSD of recovery, % recovery of each concentration is calculated to determine the accuracy. The recovery results for accuracy study of Linezolid are presented in Table 7.

**Table 7: Recovery Data of for Linezolid:**

Recovery level	Amount taken (mg)	amount added (mg)	Total Amount (mg)	% recovery (mg)	Mean % Recovery	%RSD <sup>#</sup>
80%	8	5	13	12.95	99.61	0.26
100%	10	5	15	14.98	99.86	0.17
120%	12	5	17	16.96	99.76	0.060

<sup>#</sup> acceptance criteria < 2.0.

### Robustness

Robustness is the ability to provide accurate and precise results under a variety of conditions. In order to measure the extent of method robustness, the most critical parameters were interchanged while keeping the other parameters unchanged and in parallel, the chromatographic profile was observed and recorded. The studied parameters were: the composition of mobile phase, flow rate,

detection wavelength. The results of robustness study is shown in Table 8 indicated that the small change in the conditions did not significantly affect the determination of Linezolid.

**Table 8: Robustness Results of Linezolid**

S. no	Parameter	Optimized	Used	Retention time ( $t_R$ ), min	Plate count <sup>\$</sup>	Peak asymmetry <sup>#</sup>	Remark
1.	Flow rate ( $\pm 0.2$ mL/min)	1.0 mL/min	0.8 mL/min	2.763	8172	1.25	*Robust
			1.0 mL/min	2.813	8154	1.36	*Robust
			1.2 mL/min	2.923	8100	1.47	*Robust
2.	Detection wavelength ( $\pm 5$ nm)	315 nm	239nm	2.813	8148	1.34	*Robust
			254nm	2.813	8154	1.36	*Robust
			259nm	2.813	8159	1.36	*Robust
3.	Mobile phase composition (Acetonitrile : Water)	50:50 v/v	55:45, v/v	2.754	8144	1.32	*Robust
			50:50 v/v	2.813	8154	1.36	*Robust
			45:55, v/v	2.921	8165	1.360	*Robust

**Acceptance criteria (Limits):**<sup>#</sup>Peak Asymmetry < 1.5, <sup>\$</sup> Plate count > 3000, \* Significant change in Retention time

### LOD and LOQ

Limit of Detection is the lowest concentration in a sample that can be detected, but not necessarily quantified under the stated experimental conditions. The limit of quantitation is the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy. Limit of Detection and Limit of Quantitation were calculated using following formula  $LOD = 3.3\sigma/S$  and  $LOQ = 10\sigma/S$ , where  $SD$ =standard deviation of response (peak area) and  $S$ = slope of the calibration curve. The LOD and LOQ values are presented in Table 9. The results of LOD and LOQ supported the sensitivity of the developed method.

**Table 9: LOD AND LOQ**

Limit of Detection(LOD)	0.004 $\mu\text{g/mL}$
Limit of Quantitation(LOQ)	0.014 $\mu\text{g/mL}$

### Estimation of Linezolid in bulk and pharmaceutical dosage forms:

The proposed method was successfully applied for the estimation of Linezolid in bulk and pharmaceutical tablets. The assay results were compiled found satisfactory and show there is no interference of the tablet matrix with the drug. Low % RSD shows that this method can be easily applied for the estimation of Linezolid in tablet dosage form. The drug content was calculated as an average of six determinations and assay results are shown in Table 10, 11 and the representative sample chromatogram of Linezolid shown in Fig. 10.

**Table 10: Assay Results of Linezolid ( Bulk)**

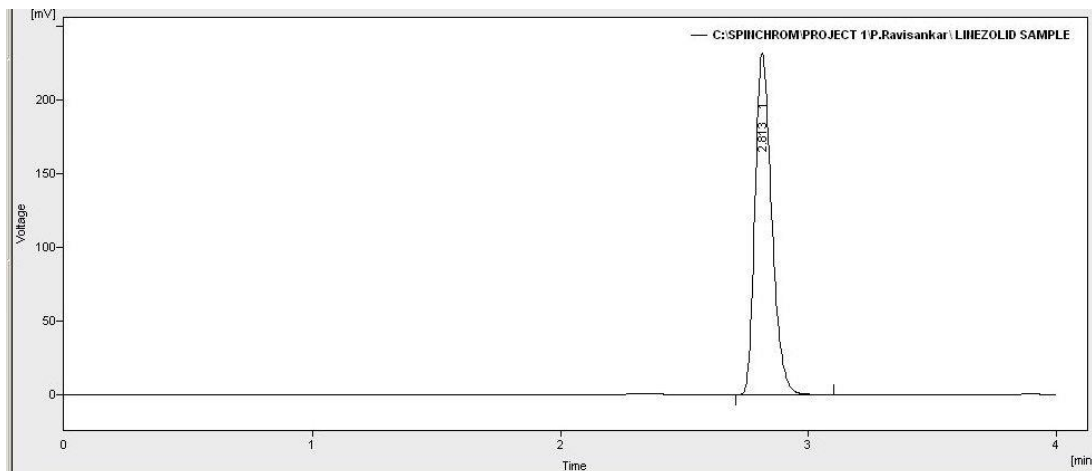
S.NO	Assay	% RSD*
1	99.23	
2	100.31	0.54%
3	99.89	

\* Average of 6 determination

**Table 11: Assay Results of Linezolid Formulation**

S. No	Formulations	Labeled amount	Amount found	% Assay $\pm$ SD*
1	Lizolid®	600 mg	599mg	99.81 $\pm$ 1.195

\* Average of 6 determination

**Figure 10: Chromatogram of marketed formulation LIZOLID**

The present study was aimed at developing a precise, sensitive, rapid and accurate HPLC method for the analysis of Linezolid in bulk drug and in pharmaceutical dosage forms. In order to achieve phenomenal retention time and peak asymmetry, C<sub>18</sub> stationary phase column (250mm X 4.6mm i.d, 5  $\mu$ m particle size) and mobile phase composed of water, acetonitrile (50:50) v/v, with pH adjusted to 6.8 using ortho phosphoric acid and triethylamine as column modifier at a flow rate of 1mL/min was selected. The retention time for Linezolid was found to be 2.813minutes. UV spectra of Linezolid showed that the drug absorbed maximum at 254 nm, so this wavelength was selected as the detection wavelength. The correlation coefficient (0.9999) of regression was found almost equal to 1 in the range of 2-10 $\mu$ g/mL which states that the method was linear to the concentration versus peak area responses. On slight variation in the mobile phase ratio of upto  $\pm$ 5 %, the change in the peak asymmetry, plate count and retention time are within the limits which indicated that the method is robust and also indicating lack of influence on the test results by operational variable for the proposed method. This shows that the method is having phenomenal system suitability parameters under given conditions. The comparison of chromatograms of placebo, standard and sample, there was no interference observed from the

peaks of placebo, standard and sample. It shows that the method is specific. The precision studies were performed and the % RSD of the determinations was found to be 0.0664 for intra-day precision and 0.0497 for inter-day precision which are within the limits which indicates that the proposed method was found to be precise. The accuracy of the method was found to be good with the overall % RSD for recovery at 80%, 100% and 120% levels were all within the limits which indicate that the proposed method was found to be accurate. Method validation following ICH guidelines indicated that the developed method had high sensitivity with LOD of 0.004 $\mu$ g/mL and LOQ of 0.014  $\mu$ g/mL, the assay results of tablets by applying the HPLC method and the proposed analytical methods were contrast using statistical analysis. The Student's t-test was applied and does not reveal significant difference between the experimental values obtained in the sample analysis by this method.

## CONCLUSION

The results of the validation process showed that the proposed method is authenticated and found within predetermined limits, and fitness for purpose. It can be seen that the proposed procedure has good precision and accuracy. Results of the analysis of pharmaceutical formulations revealed that proposed methods are suitable for their analysis with virtually no interference of the usual additives present in the pharmaceutical formulations. Thus, the developed method can be easily used for the routine quality control of bulk and tablet dosage forms of Linezolid within a short analysis time.

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