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## Development and Validation of Stability-Indicating Method for Assay of Linezolid Immediate Release Tablet by Reversed-Phase HPLC



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### ABSTRACT

A stability indicating reverse-phase high performance liquid chromatographic method was developed for the assay of linezolid as a bulk drug and in pharmaceuticals. LC was carried out by an isocratic LC method using reversed phase technique on a C18 column. Eluents were monitored by UV detection at 251 nm using the mobile phase methanol–ammonium dihydrogen phosphate (0.002 M) (50:50, v/v). The method was statistically validated and yielded good results and included linearity, accuracy, precision, robustness, ruggedness and specificity. The linearity of linezolid peak area responses was demonstrated within the concentration range of 25-75 µg/mL and ( $r^2 = 0.9999$ ). The method was demonstrated to be rapid, robust, linear, precise, accurate, rugged and specific with no interference from the tablet excipients and with good resolution of the drug peak from the peaks of the degradation products (oxidative degradation, acid, base degradation, thermal and humidity degradation). The results indicated that the proposed method is a simple, rapid and useful for linezolid determination in routine quality control and stability assay.



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## 1. INTRODUCTION

Linezolid is a synthetic antibiotic of oxazolidinone class used as antibacterial and anti-infective. It is used for the treatment of serious infections caused by Gram-positive bacteria that are resistant to several antibiotics. It is chemically known as N-[[[(5S) - 3- [3- fluoro- 4 - (4 morpholinyl) phenyl] - 2 - oxo - 5 -oxazolidinyl] methyl] acetamide. It is official in the Indian Pharmacopoeia [1]. Literature Survey revealed that some methods are reported for determination of Linezolid in human serum by HPLC and LCMS/MS [2, 3, 4, 5, 6]. HPTLC, HPLC and UV spectrophotometric method are cited in literature for the determination of linezolid [7, 8, 9]. However, very less work has been reported of a stability indicating method for linezolid by HPLC. The paper describes the development and validation of a stability indicating RP-LC method for the assay of Linezolid as a bulk drug and in its pharmaceutical dosage form [10].

## 2. Experimental

### 2.1. Materials

Methanol of HPLC grade was procured from Spectrochem, Purified Water of HPLC grade was obtained from Lichrosolv- Merck (Milli Q). Ammonium dihydrogen phosphate and Hydrochloric acid 35% of GR grade, Sodium Hydroxide and Hydrogen Peroxide solution (30% v/v) was also used.

### 2.2. Samples

The investigated samples of linezolid IR tablets (B. No. 600/F058) were procured from PTC Zydus Cadila Healthcare Ltd. (Moraiya, Ahmedabad, India). The structure of linezolid was shown in Fig.1.

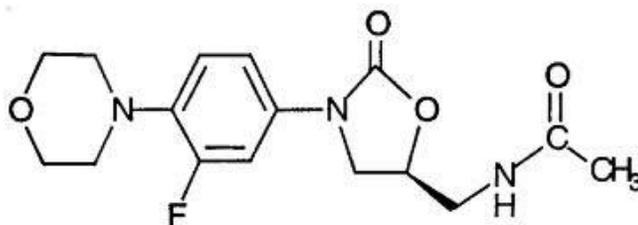


Fig. 1. Structure of linezolid

### 2.3. HPLC instrumentation (Analytical)

A High-Performance Liquid Chromatographic system consisting of a (1200 series- rapid resolution LC) Agilent (Infinity series) equipped with a G1311A quaternary pump, a G1322A degasser, a G1329A ALS, a G1130B FC/ALS Thermo, a G1316A TCC photo diode array UV detector was used for analysis. Data integration was done using EZChrom Elite chromatography software. Other equipments used were weighing balance and sonicator.

### 2.4. Chromatography

An Inertsil ODS-3V column with the dimensions of 150× 4.6 mm (5 µm) i.d. (GL Sciences, Japan) was used for the separations. Mobile phase containing 0.002 M NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> and methanol in a ratio of 50:50 (v/v) was used for separation. The mobile phase was filtered through a nylon membrane (0.45 µm) and degassed by sonication prior to use. Chromatography was performed at 50°C temperature under isocratic conditions at a flow rate of 1.0 mL/min. Detection was done at 251 nm.

### 2.5. Preparation of standard solutions

A stock solution of linezolid (500 µg/mL) was prepared in diluents (Mobile Phase) and diluted further with diluent to obtain a standard solution of 50 µg/mL.

### 2.6. Preparation of sample solutions

Weigh accurately 20 tablets and calculate the average weight. Crush 20 tablets to fine powder. Transfer powder sample equivalent to 100 mg linezolid into 100 mL volumetric flask. Add about 70 mL of diluents and sonicate with occasional shaking for about 30 minutes make volume up to the mark with diluents and mix. Dilute 5.0 mL of this solution to 100 mL with diluent and mix. Filter the solution through 0.45 µm Millipore PVDF filter; collect the filtrate by discarding first 3.0 mL of the filtrate.

### 2.7. Forced degradation of linezolid

Forced degradation studies were performed to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted using

heat, oxidation, acid, base and humidity [11]. For thermal degradation, the drug was exposed for 3 days at 100°C in oven. Oxidative degradation was induced by refluxing on a boiling water bath for 15 minutes, after treating with 2.5 mL 30% H<sub>2</sub>O<sub>2</sub>. Acid degradation was attempted by refluxing linezolid on a boiling water bath for 30 minutes, after treating with 2.5 mL 1N HCl. Base degradation was performed by placing on bench top for 45 minutes, after treating with 5 mL 1N NaOH. Humidity degradation was performed by exposing the drug for 3 days at 40°C and 75% RH.

After completion of the degradation treatments, the samples were allowed to cool to room temperature, neutralized (when required) and injected into the chromatographic system after appropriate dilution with the mobile phase. The degraded samples were analyzed against a control sample (lacking degradation treatment).

## **2.8. Validation of the assay method**

### **2.8.1. Precision**

System precision (repeatability) was evaluated by performing six consecutive injections of a 50 µg/mL linezolid standard solution. Method precision was determined by six repeated assays of the same lot of the tablet formulations. The chromatograph of standard solution of linezolid was shown in Fig.2.

### **2.8.2. Accuracy**

The accuracy (recovery) of the test method was demonstrated by preparing recovery samples. (i.e., spiking placebo with known quantities of API) at level of 50%, 100%, 150%, of target concentration. Prepare the recovery samples in triplicate at each level.

### **2.8.3. Linearity**

Linearity was determined by preparing linearity solutions over the range of 50% to 150% of assay concentration (i.e. 25 ppm to 75 ppm). A standard stock solution was prepared and further diluted to attain concentration of about 50%, 80%, 100%, 120% and 150% of sample concentration. The peak area versus concentration data was treated by least-squares linear regression analysis.

#### 2.8.4. Stability of analyte in solution

The stability of linezolid in the mobile phase was assessed by injecting the standard solution (50 µg/mL) at 0, 6, 18 and 24 h post preparation.

#### 2.8.5. Specificity

To demonstrate the specificity of the method, placebo formulations, containing the tablet excipients other than the drug, were subjected to the sample preparation method as outlined in Section 2.6 and thus the prepared samples were injected into the chromatographic system.

#### 2.8.6. Robustness

The robustness of an analytical method is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters such as (change in flow rate, change in organic phase ratio and change in column temperature) were done to evaluate robustness of the method.

### 3. RESULTS AND DISCUSSION

#### 3.1. Optimization of the Chromatographic Procedure

Primarily Linezolid is metabolized in the liver (50-70%), by oxidation of the morpholine ring, without involvement of the cytochrome P450 system. Metabolites are aminoethoxyacetic acid and hydroxyethyl glycine. Linezolid is non-enzymatically oxidized to inactive derivatives, respectively. The chromatographic conditions were optimized so as to obtain a good separation between the drug and its degradation products. Detection was performed at 251 nm, which was the  $\lambda_{\max}$  of linezolid. The anticipated degradation products were expected to absorb at this wavelength.

A LC and UV spectrophotometric method was reported by S.A. Patel *et al.* [8] using mobile phase in ratio of methanol-water-acetonitrile in ratio of 40:40:20 (v/v/v), the method developed was precise, specific and accurate. C.C.G.O. Lopes *et al.* [10] and H. Agrawal *et al.* [9] reported HPLC and HPTLC method for estimation of linezolid in bulk and pharmaceutical dosage form, method developed was stability indicating. L. Bebawy *et al.* [12] carried out alkaline degradation of linezolid in different percentage. An isocratic method is also reported by Tobin *et al.* [2] for

estimation of linezolid in human serum by HPLC. Rapid and simple HPLC and LCMS/MS method was developed, validated and reported by I.M. Harmelink *et al.* [3], P. Marialuisa *et al.* [4], L.M. Boak *et al.* [5] and T. Friederike *et al.* [6] in human serum and plasma for rapid quantification of linezolid.

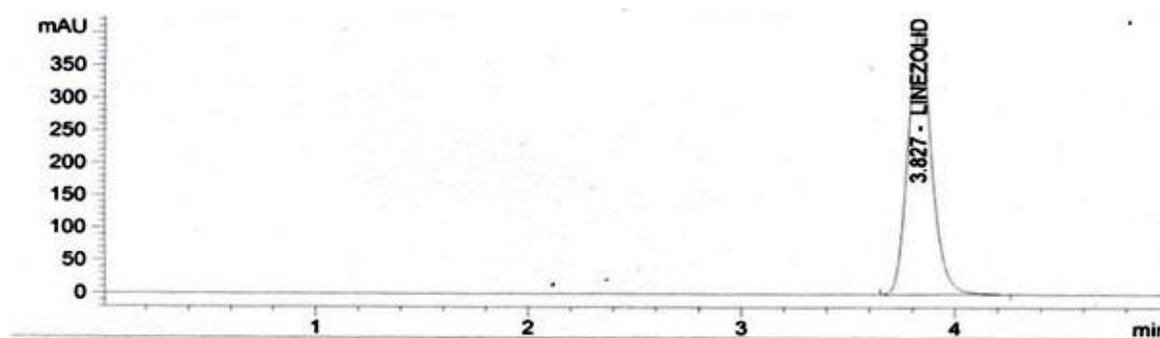
As a preliminary guide to the selection of the mobile phase, the standard solution of linezolid was injected into the chromatographic system and the elution was studied using mobile phases comprising binary mixtures of methanol and  $\text{NH}_4\text{H}_2\text{PO}_4$  (0.002 M) in varying ratios. The results are shown in Table 1. The chromatographic conditions were optimized after taking into consideration the resolution between the drug and its major degradation peaks and the run time for the chromatogram. A mobile phase of methanol–  $\text{NH}_4\text{H}_2\text{PO}_4$  (0.002 M) (50:50, v/v) at a flow rate of 1.0 mL/min was found to well separate linezolid from other degradation peaks with a reasonably short run time. These optimized conditions also afforded separation of the drug from its unknown degradation peaks formed. All of the degradation peaks were well resolved from the drug peak (resolution>2). The percentage of intact linezolid, after forced degradation through various routes, is shown in Table 2.

**Table 1: Optimization of chromatographic condition**

Mobile phase composition	Flow rate ml/min	Temp.	Retention time (min)	Remark
			Linezolid	
Buffer (0.04 M $\text{KH}_2\text{PO}_4$ pH 3.5): ACN: MeOH (20:60:20)	1.5	30°C	--	Peak was not proper; retention time comes under void time.
Buffer (0.02 M $\text{KH}_2\text{PO}_4$ pH 3.5): ACN (35:65)	1.3	45°C	2.500	Peak Shape was not good and shouldering observed; retention time needs to be adjusted.
Buffer (0.02 M $\text{NH}_4\text{H}_2\text{PO}_4$ ): MeOH (40:60)	0.6	45°C	5.587	Peak Shape was not good and broad peak obtained.
Buffer (0.002 M $\text{NH}_4\text{H}_2\text{PO}_4$ ): MeOH (65:35)	1	45°C	8.057	Peak Shape was good and sharp but retention time needs to be reduced.
Buffer (0.002 M $\text{NH}_4\text{H}_2\text{PO}_4$ ): MeOH (50:50)	1	50°C	3.827	Peak Shape was good and sharp with suitable reduced run time 5 min.

**Table 2: Forced degradation of linezolid**

Degradative Conditions	Time	Intact drug (%)	RT of degradation products (min)
Thermal	72 hrs.	98.6	None detected
Humidity	72 hrs.	99.8	None detected
Acid	30 min.	78.6	1.38
Oxidation	15 min.	40.6	1.77, 4.47, 12.58
Alkali	45 min.	85.6	3.5



**Fig. 2. Chromatogram of linezolid**

Linezolid were quantitatively oxidized in 30% H<sub>2</sub>O<sub>2</sub> and was found more susceptible under the conditions employed for oxidation conditions than basic and acidic conditions. As evidenced from the percentage intact drug and difference in hydrolysis and oxidation time. The drug was found to be stable to heat and humidity under the conditions of the study. No degradation was observed upon refluxing the drug with methanol, suggesting that the degradation under oxidation, acidic and basic conditions was the result of oxidation and hydrolysis with no influence of heat and humidity on the degradation.

### 3.2. Validation of assay method

#### 3.2.1. Precision

System precision (repeatability) was evaluated by performing six consecutive injections of the 50 µg/mL standard solution, giving a low RSD value of 0.5% and no change in retention time of the

drug. The linezolid contents found in the tablet formulations using the proposed method are listed in Table 3. The low RSD values indicate that the proposed method is precise.

**Table 3: Precision of the assay method**

Sample No.	Linezolid (% Assay)	Mean	RSD (%)
1	99.4		
2	98.7		
3	97.9	99.0	0.8
4	98.4		
5	99.7		
6	100.0		

### 3.2.2. Accuracy

Recovery data (Table 4) obtained from the study of the tablet formulations ranged from 98–102% with low RSD values (<2%). The percentage (%) recovery at each level, mean percentage (%) recovery and percentage (%) RSD was meeting the established acceptance criteria; the study proves that the method was accurate in the considered range.

### 3.2.3. Linearity

The plot of the drug peak area versus concentration was linear over the concentration range of 25–75 µg/mL. The regression line equation calculated by the least-squares method was  $y = 62184.36368x + 14348.84670$  with a coefficient of correlation of 0.99991.

### 3.2.4. Stability of analyte in solution

Linezolid was found to be stable in solution in the mobile phase, when the standard solution was analyzed at 0, 6, 18 and 24 h post preparation.

### 3.2.5. Specificity

Placebo formulation samples yielded clean chromatograms with no interference from the tablet excipients.

### 3.2.6. Robustness

There was no significant change in peak shape and assay results upon introduction of intentional variations in parameters such as change in flow rate, organic phase ratio and column temperature.

### 3.2.7. Ruggedness

Intermediate precision (ruggedness) linezolid contents was found within the tablet formulations using the proposed method. The low percentage (%) RSD values and low percentage (%) absolute difference in the mean assay value obtained in intermediate precision study and that of in method precision study indicate that the proposed method was precise and rugged.

## 4. CONCLUSION

The RP-HPLC assay method developed for linezolid is rapid, precise, accurate, specific and stability indicating. The method may be used for assessing the stability of linezolid as a bulk drug and in its pharmaceutical formulations. Quantitative determination of the drugs by HPLC was very accurate and also it was found to be simple as compared to other analytical method. Hence, based on the statistical data these methods can be easily and conveniently adopted for routine estimation of assay of Linezolid drug in tablet dosage form.

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