



ORIGINAL ARTICLE

A validated stability-indicating LC method for the separation of enantiomer and potential impurities of Linezolid using polar organic mode

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Abstract Although a number of methods are available for evaluating Linezolid and its possible impurities, a common method for separation of its potential impurities, degradants and enantiomer in a single method with good efficiency remain unavailable. With the objective of developing an advanced method with shorter runtimes, a simple, precise, accurate stability-indicating LC method was developed for the determination of purity of Linezolid drug substance and drug products in bulk samples and pharmaceutical dosage forms in the presence of its impurities and degradation products. This method is capable of separating all the related substances of Linezolid along with the chiral impurity. This method can also be used for the estimation of assay of Linezolid in drug substance as well as in drug product. The method was developed using Chiralpak IA (250 mm × 4.6 mm, 5 μm) column. A mixture of acetonitrile, ethanol, n-butyl amine and trifluoro acetic acid in 96:4:0.10:0.16 (v/v/v/v) ratio was used as a mobile phase. The eluted compounds were monitored at 254 nm. Linezolid was subjected to the stress conditions of oxidative, acid, base, hydrolytic, thermal and photolytic degradation. The degradation products were well resolved from main peak and its impurities, proving the stability-indicating power of the method. The developed method was validated as per International Conference on Harmonization (ICH) guidelines with respect to specificity, limit of detection, limit of quantification, precision, linearity, accuracy, robustness and system suitability.

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1. Introduction

Linezolid (Zyvox), (S)-N-((3-(3-fluoro-4-morpholinophenyl)-2-oxooxazolidin-5-yl) methyl) acetamide, is a synthetic antibiotic belonging to a new class of antimicrobials called the oxazolidinones [1]. Linezolid is effective for the treatment of nosocomial infections involving gram positive bacteria. The oxazolidinones possess a unique mechanism of bacterial protein synthesis inhibition [2]. Linezolid acts by inhibiting the initiation of bacterial protein synthesis, a mechanism of action distinct from that of any other antibiotics that are commercially available. Linezolid was launched in May 2000 for the treatment of patients with infections caused by gram-positive bacteria [3].



The daily dosage of Linezolid is 1200 mg/day. As per the ICH guidelines all the impurities (known and unknown) have to be controlled below 0.08% in drug substance w.r.t. Linezolid.

In the literature a number of methods have been described for the determination of Linezolid and its impurities employing various techniques such as HPLC [4–11], chiral HPLC [12–14], capillary electrophoresis [15–18] and NMR [19]. The major objective of the present work is to develop a single method for the separation of chiral and nonchiral impurities of Linezolid in drug substance and drug product and also the method should have low LOD and LOQ values. The same method was used for the estimation of assay of the drug substance and drug product. Further the use of immobilized chiral HPLC column ensures the column life unlike glycoprotein-based columns. So far to our knowledge, no HPLC method has been reported for simultaneous quantitative determination of the chiral impurity and related substances in bulk drug and formulation of Linezolid. Therefore, in prevision of a racemic switch, it would be useful and imperative to develop a simple and suitable method for the measurement of Linezolid and related substances including its enantiomer in bulk drug and formulations.

Hence a reproducible stability-indicating HPLC method was developed for the quantitative determination of Linezolid and its six impurities, namely Imp-A, Imp-B, Imp-C, Imp-D, Imp-E and Imp-F (Fig. 1). This method was successfully validated according to the International Conference Harmonization (ICH) guidelines (Validation of Analytical Procedures: Test and Methodology Q2).

2. Experimental

2.1. Materials and reagents

Active pharmaceutical ingredient standards and samples were supplied by Dr. Reddy's Laboratories Limited, IPDO, Hyderabad, India. Commercially available Zyvox tablets from Novartis Pharmaceuticals Corporation, USA, were used for the dosage form analysis. The HPLC grade acetonitrile and analytical grade trifluoro acetic acid and ethanol were purchased from Merck, Darmstadt, Germany. N-butyl amine was purchased from Spectrochem, Mumbai, India.

2.2. Equipment

The Waters HPLC system (Waters, Milford, USA) used consists of a pump, auto sampler and a PDA detector. The output signal was monitored and processed using empower-2 software. Cintex digital water bath was used for hydrolysis studies. Photo stability studies were carried out in a photo stability chamber (Sanyo, Leicestershire, UK). Thermal stability studies were performed in a dry air oven (Cintex, Mumbai, India).

2.3. Chromatographic conditions

The chromatographic separation was achieved on Chiralpak-IA (250 mm × 4.6 mm, 5 μm) column using a mobile phase containing acetonitrile–ethanol–n-butylamine–trifluoro acetic acid (96:4:0.10:0.16, v/v/v/v). The mobile phase was filtered through a nylon membrane (pore size 0.45 μm) filter. The flow rate of the mobile phase was 0.8 mL/min. The column temperature was

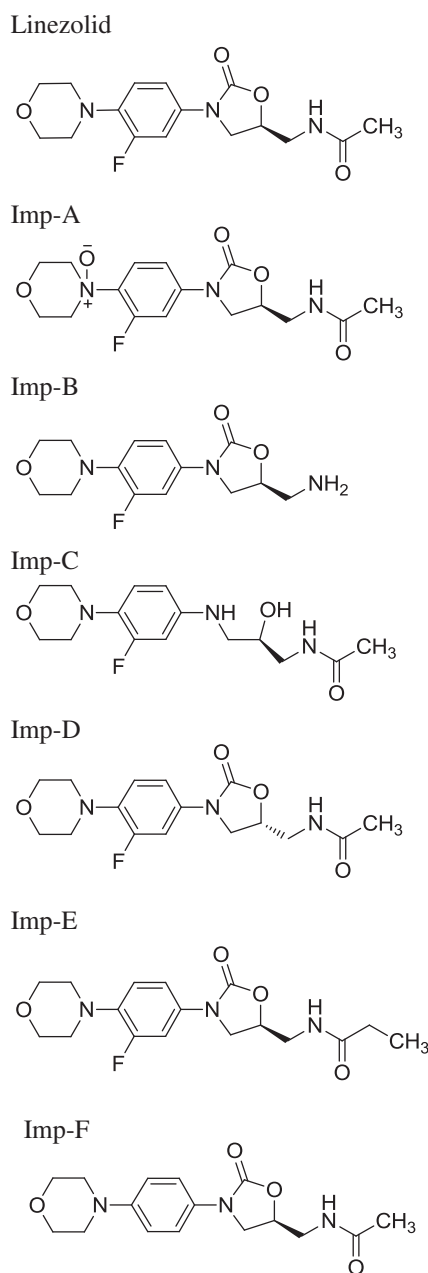


Figure 1 Structures of Linezolid and its six impurities.

maintained at 40 °C and the wavelength was monitored at 254 nm. The injection volume was 5 μL. A mixture of acetonitrile and ethanol in the ratio of 96:4 (v/v) was used as diluent during the standard and test samples preparation.

2.4. LC-MS/MS conditions

LC-MS/MS system (Agilent 1200 series liquid chromatograph coupled with Applied Biosystem 4000 Q Trap quadrupole mass spectrometer with analyst 1.4 software, MDS SCIEX, USA) was used for the identification of unknown compounds formed during forced degradation studies. Chiralpak-IA (250 mm × 4.6 mm, 5 μm) column was used as stationary phase. Acetonitrile–ethanol–n-butylamine–trifluoro acetic acid (96:4:0.10:0.16, v/v/v/v) was used as mobile phase. The flow

rate was 0.8 mL/min and the column temperature was maintained at 40 °C. The analysis was performed in positive electro spray ionization mode. Ion source voltage was 5500 V. Source temperature was 450 °C. GSI and GS2 are optimized to 30 and 35 psi respectively. Curtain gas flow was 20 psi.

2.5. Preparation of stock solutions

A stock solution of Linezolid (1.0 mg/mL) was prepared by dissolving appropriate amount of drug in diluent. Working solutions of 500 and 100 µg/mL were prepared from the above stock solution for the related substance determination and assay determination respectively. A stock solution of impurities (mixture of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E and Imp-F) at 50 µg/mL was prepared in diluent.

2.6. Preparation of sample solution

To prepare the sample stock solution, twenty ($n=10$) tablets of Zyvox, each containing 600 mg of Linezolid, were accurately weighed and crushed to a fine powder. An appropriated amount was transferred into an individual 100 mL volumetric flask, diluted to volume with diluent and sonicated for 10 mins., obtaining the final concentration of 2 mg/mL of the active pharmaceutical ingredient. 25 mL of this solution was diluted to 100 mL with diluent to give a solution containing 500 µg/mL. 20 ml of this solution was diluted to 100 mL with diluent to give a solution containing 100 µg/mL. These solutions were filtered through a 0.22-µm Nylon membrane filter.

2.7. Stress studies

Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities [20]. The specificity of the developed LC method for Linezolid was carried out in the presence of its six impurities. Stress studies were performed at an initial concentration 500 µg/mL of Linezolid on tablets to provide an indication of the stability indicating property and specificity of the proposed method. Intentional degradation was attempted to stress conditions of UV light (254 nm), heat (70 °C), acid (2 M HCl at 70 °C), base (0.01 M NaOH at 25 °C), hydrolytic (70 °C) and oxidation (6.0% H₂O₂ at 25 °C) to evaluate the ability of the proposed method to separate Linezolid from its degradation products. For heat, light studies, study period was 10 days whereas for hydrolysis and oxidative degradation study it was found to be about 48 h. For base and acid degradation, the study period was 2½ and 3½ h respectively. Peak purity test was carried out for Linezolid peak by using PDA detector in stress samples.

Assay of stressed samples was performed (at 100 µg/mL) by comparison with qualified reference standard and the mass balance (% assay+% impurities+% degradation products) for stressed samples was calculated. Assay was also calculated for Linezolid sample by spiking all six impurities at the specification level (i.e. 0.08%).

3. Method validation

The method has been validated for assay and related substances by HPLC as per ICH guidelines [21].

3.1. Precision

The precision of the method was verified by repeatability and by intermediate precision. Repeatability of the related-substance method was checked by (Waters HPLC system with PDA detector, Milford, USA) injecting six individual preparations of Linezolid real sample (600 mg tablets) spiked with 0.08% of its six impurities (0.08% of impurities with respect to 500 µg/mL Linezolid). The RSD of peak area for each impurity was calculated. The intermediate precision of the method was also evaluated using different analysts, different instruments and different columns and performing the analysis on three different days.

Assay method precision was evaluated by carrying out six independent assays of real sample of Linezolid at 100 µg/mL level against qualified reference standard. The intermediate precision of the assay method was evaluated by different analysts.

3.2. Limit of detection (LOD) and quantification (LOQ)

The LOD and LOQ for Linezolid and its impurities were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. Precision study was also carried out at the LOQ level by injecting six ($n=6$) individual preparations and calculating the RSD of the area.

3.3. Accuracy

The accuracy of the assay method was evaluated in triplicate ($n=3$) at the three concentration levels of 50, 100 and 150 µg/mL (50%, 100% and 150%) of drug product, and the recovery was calculated for each added (externally spiked) concentration.

For all impurities, the recovery was determined in triplicate for 50%, 100% and 150% (i.e., 0.20, 0.40 and 0.60 µg/mL respectively) of the analyte concentration (500 µg/mL) of the drug product, and the recovery of the impurities was calculated.

3.4. Linearity

Linearity test solutions for the assay method were prepared from Linezolid stock solutions at five concentration levels from 50% to 150% of assay analyte concentration (50, 75, 100, 125 and 150 µg/mL). The peak area versus concentration data was treated by least-squares linear regression analysis. Linearity test solutions for the related substance method were prepared by diluting stock solutions to the required concentrations. The solutions were prepared at seven concentration levels from LOQ to 150% of the specification level (LOQ, 0.04, 0.06, 0.08, 0.10, 0.12 and 0.16%) with respect to the normal sample concentration (500 µg/mL). The correlation coefficients, slopes and Y-intercepts of the calibration curve were determined.

3.5. Robustness

The robustness of an analytical procedure is a measure of its capability to remain unaltered by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

To determine the robustness of the method the experimental conditions were deliberately changed. The resolution of

Linezolid and the six impurities and the tailing factor for Linezolid were evaluated. The mobile phase flow rate was 0.8 mL/min; to study the effect of flow rate on resolution it was changed to 0.64 and 0.96 mL/min. The effect of column temperature on resolution was studied at 35 °C and 45 °C (instead of 40 °C). The effect of the percent organic strength on resolution was studied by varying ethanol by –10% to +10% from the initial composition.

3.6. Stability of solution and mobile phase

The stability of Linezolid in solution was determined by leaving test solutions of the sample and reference standard in tightly capped volumetric flasks at room temperature for 48 h during which they were assayed at 12 h intervals. Stability of mobile phase was determined by analysis freshly prepared sample solutions at 12 h intervals for 48 h and comparing the results with those obtained from freshly prepared reference standard solutions. The mobile phase was prepared at the beginning of the study period and not changed during the experiment. The % assay of the results was calculated for both the mobile phase and solution-stability experiments.

The stability of Linezolid and its impurities in solution for the related substance method was determined by leaving

spiked sample solution in a tightly capped volumetric flask at room temperature for 48 h and measuring the amounts of the eleven impurities at every 12 h. The stability of mobile phase was also determined by analysis freshly prepared solution of Linezolid and its impurities at 12 h intervals for 48 h. The mobile phase was not changed during the study period.

4. Results and discussion

4.1. Method development and optimization

The main objective while developing this method was to have a single method for separation of impurities A,B, C, E and F along with the separation of enantiomer of Linezolid (impurity-D) from Linezolid. As the separation demands the resolution of chiral and non-chiral impurities together, chiral stationary phases based on carbohydrate like cellulose and amylose were chosen as first choice. Initially the separation was attempted in both normal phase and reverse phase. None of the normal phase and reverse phase-based chiral columns was able to separate all the impurities. Further trials have been carried out using a mixture of polar organic solvents

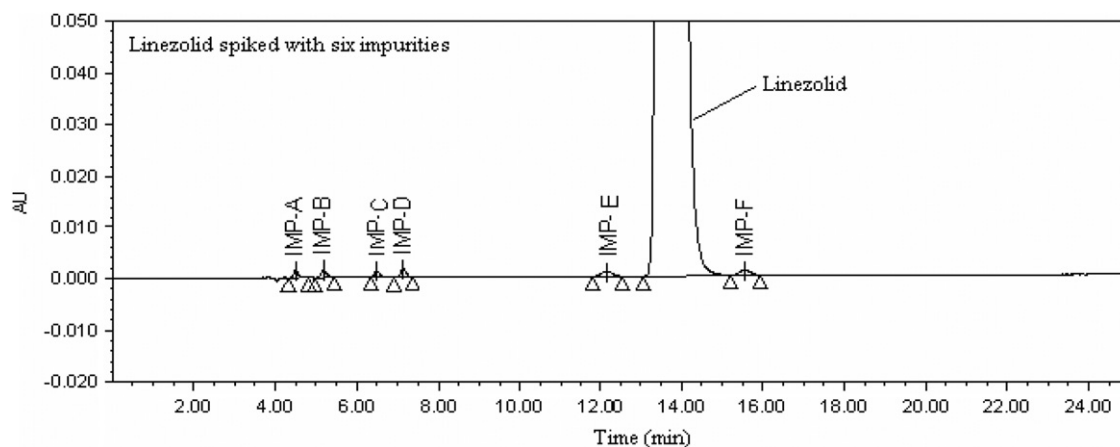


Figure 2 Linezolid spiked chromatogram.

Table 1 LOD, LOQ, regression and precision data.

Parameter	Linezolid	Imp-A	Imp-B	Imp-C	Imp-D	Imp-E	Imp-F
LOQ ($\mu\text{g/mL}$)	0.120	0.085	0.080	0.095	0.075	0.120	0.130
LOD ($\mu\text{g/mL}$)	0.035	0.025	0.025	0.030	0.025	0.035	0.040
0.5							
Regression equation (y)							
Slope (b)	40897	35011	38094	33697	38450	40624	41462
Intercept (a)	350.12	-7.03	-46.40	30.13	126.50	376.79	-57.55
Correlation coefficient	0.9996	0.9998	0.9998	0.9998	0.9999	0.9997	0.9998
Y-intercept at 100% level	2.1%	-0.1%	-0.3%	0.2%	0.8%	2.3%	-0.3%
R square value	0.9995	0.9996	0.9997	0.9997	0.9998	0.9994	0.9996
Precision (%RSD)	2.1	2.9	1.6	2.0	1.7	2.2	2.3
Intermediate precision (%RSD) ^a	2.3	2.2	2.1	2.4	2.3	1.6	2.7

Linearity range is LOQ-200% with respect to 500 $\mu\text{g/mL}$ Linezolid for impurities; Linearity range is 50–150% with respect to 100 $\mu\text{g/mL}$ of Linezolid for assay.

^aSix determinations using LOQ solution for impurities and 100 $\mu\text{g/mL}$ for assay of Linezolid.

along with small quantities of trifluoro acetic acid and n-butyl amine. No sign of separation between the enantiomers have been seen in Chiral pak-IB and Chiral pak-IC columns. Chiral pak-IA column was found to be showing excellent separation of all the impurities using acetonitrile and ethanol along with the combination of trifluoro acetic acid and n-butyl amine in mobile phase. The mobile phase composition was fixed as acetonitrile, ethanol, trifluoro acetic acid and n-Butyl amine in 96:4:0.16:0.10 (v/v/v/v) ratio with an isocratic elution. The

flow rate of the mobile phase was fixed as 0.8 mL/min. A mixture of acetonitrile and ethanol in the ratio of 96:4(v/v) was chosen as the diluent, as the blank chromatogram was clean without any interference with analyte peak and the impurity peaks. Also no interference from the excipients was observed. Under optimized conditions Linezolid and its six impurities were well separated with resolution greater than 2.5; typical retention times were approximately 14.20, 4.53, 5.23, 6.60, 7.29, 12.66, 15.58 min for Linezolid, impurity-A,

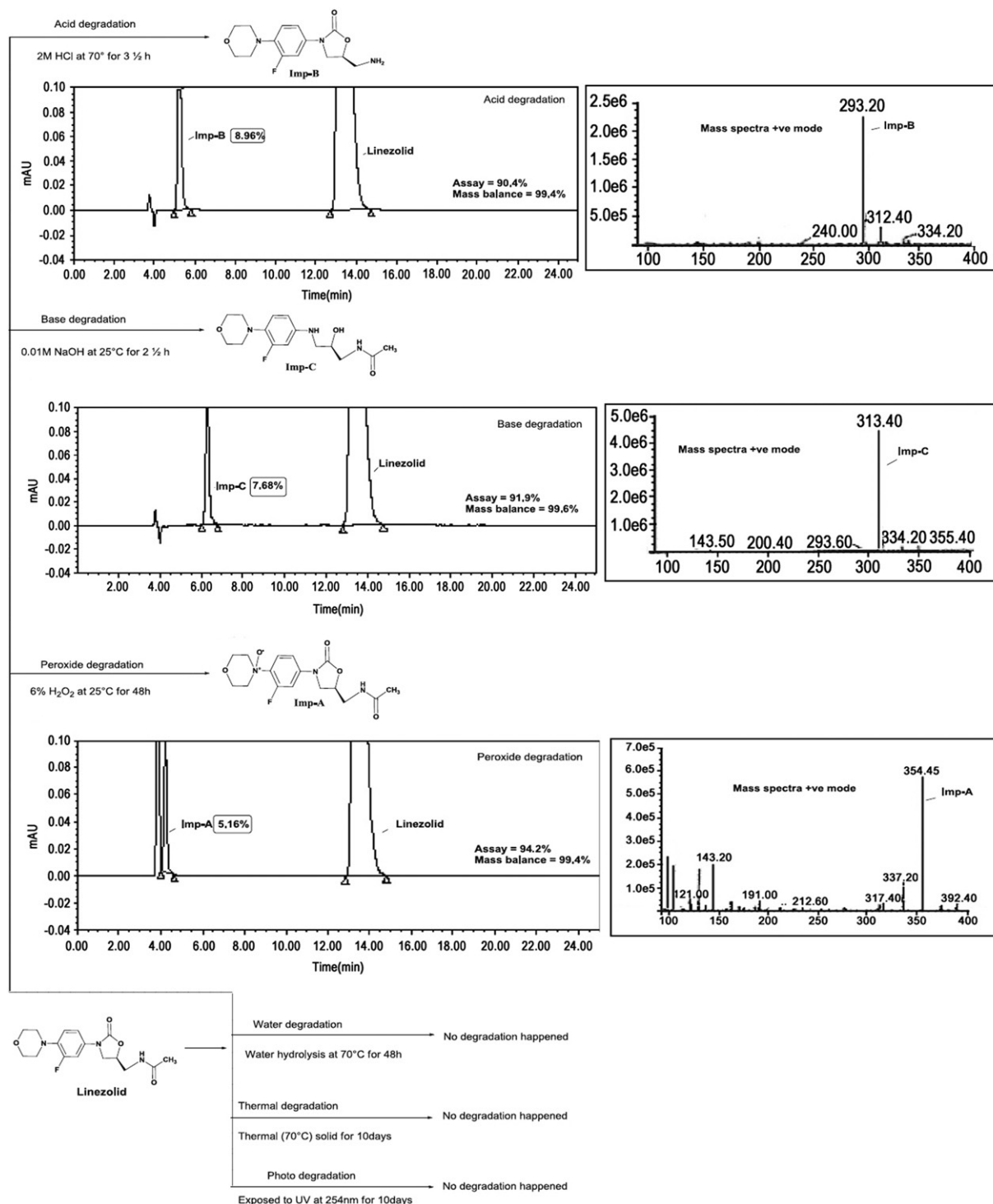


Figure 3 Complete degradation profile of Linezolid along with the % impurity formation and corresponding mass nos. of impurities.

impurity-B, impurity-C, impurity-D, impurity-E and impurity-F, respectively. System suitability parameters were evaluated for Linezolid and its six impurities. Tailing factor for all six impurities and Linezolid was found to be less than 1.2

4.2. Validation of the method

4.2.1. Precision

The RSD of assay of Linezolid during the assay method repeatability study was 1.3% and the RSD for the area of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E and Imp-F in related substance method repeatability study was within 6.6%. The RSD of the assay results obtained in the intermediate precision study was within 1.2% and the RSDs for the area of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E and Imp-F were well within 3.80%, conforming good precision of the method. These results confirmed that the method was highly precise (Fig. 2).

4.2.2. Limits of detection and quantification

The limit of detection, limit of quantification and precision at LOQ values for Linezolid and its six impurities are shown in Table 1.

4.2.3. Accuracy

Recovery of Linezolid from pharmaceutical dosage forms ranged from 96.4% to 99.6%. Recovery of the six impurities from pharmaceutical dosage forms ranged from 96.9% to 103.2%.

4.2.4. Linearity

The linearity calibration plot for the assay method was obtained over the calibration ranges tested, i.e. 50–150 µg/mL and correlation coefficient obtained was greater than 0.999. The result shows that an excellent correlation existed between the peak area and concentration of the analyte.

Linear calibration plot for the related substance method was obtained over the calibration ranges tested, i.e. LOQ to 0.30% for impurities. The correlation coefficient obtained was greater than 0.999. The above result shows that an excellent correlation existed between the peak area and the concentration of Imp-A, Imp-B, Imp-C, Imp-D, Imp-E and Imp-F.

4.2.5. Robustness

In all the deliberate varied chromatographic conditions (flow rate, column temperature and composition of organic solvent), all analytes were adequately resolved and elution orders remained unchanged. The resolution between critical pairs was greater than 2.0 and tailing factor for Linezolid and its impurities was less than 1.2. The assay variability of Linezolid was within $\pm 1\%$. The variability in the estimation of Linezolid impurities was within $\pm 8\%$.

4.2.6. Stability in solution and in the mobile phase

RSD (%) for assay of Linezolid during solution stability and mobile phase stability experiments was within 1.2%. No significant changes in the amounts of the six impurities were observed during solution stability and mobile phase stability experiments when performed using the related substances method. The results from solution stability and mobile phase stability experiments confirmed that standard solutions and

solutions in the mobile phase were stable for up to 48 h during assay and determination of the related substances.

4.2.7. Results from forced degradation studies

All the results of forced degradation studies are mentioned in Fig. 3. Assay studies were carried out for stress samples (at 100 µg/mL) against qualified Linezolid reference standard. The mass balance (% assay + % sum of all compounds + % sum of all degradants) results were calculated for all stressed samples and found to be more than 99.4%. The purity and assay of Linezolid was unaffected by the presence of its impurities and degradation products, thereby confirming the stability-indicating power of the developed method.

5. Conclusion

The simple isocratic LC method developed for quantitative analysis of Linezolid and the related substances in bulk samples and pharmaceutical dosage forms is precise, accurate, linear, robust and specific. Satisfactory results were obtained from validation of the method. The method is stability-indicating and can be used for routine analysis of production samples and to check the stability of samples of Linezolid in bulk drugs and in pharmaceutical dosage forms.

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