MARCEL DEKKER, INC. • 270 MADISON AVENUE • NEW YORK, NY 10016



DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY®
Vol. 29, No. 10, pp. 1119–1126, 2003

RESEARCH PAPER

# Stability Indicating HPTLC Determination of Linezolid as Bulk Drug and in Pharmaceutical Dosage Form

**Himani Agrawal, M.Pharm.,**
**K. R. Mahadik, M.Pharm., Ph.D., F.I.C.A., F.I.C.,***
**A. R. Paradkar, M.Pharm., Ph.D., and Neeraj Kaul, M.Pharm.**

Department of Quality Assurance Techniques, Poona College of Pharmacy, Bharati Vidyapeeth Deemed University, Pune, Maharashtra State, India

## ABSTRACT

A simple, selective, precise, and stability-indicating high-performance thin layer chromatographic method of analysis of Linezolid both as a bulk drug and in formulations was developed and validated in pharmaceutical dosage form. The method employed TLC aluminium plates precoated with silica gel 60F-254 as the stationary phase. The solvent system consisted of toluene–acetone (5:5, v/v). This system was found to give compact spots for Linezolid ($R_f$ value of $0.29 \pm 0.01$). Linezolid was subjected to acidic, alkali hydrolysis, oxidation, and photodegradation. The degraded products also were well separated from the pure drug. Densitometric analysis of Linezolid was conducted in the absorbance mode at 254 nm. The linear regression data for the calibration plots showed good linear relationship with $r^2 = 0.997 \pm 0.001$ in the concentration range of 300–800 ng/spot. The mean value of correlation coefficient, slope, and intercept were $0.998 \pm 0.003$, $0.15 \pm 0.009$, and $19.52 \pm 1.66$ respectively. The method was validated for precision, accuracy, ruggedness, and recovery. The limits of detection and quantification were 20 ng/spot and 50 ng/spot, respectively. The drug undergoes degradation under acidic and basic conditions, oxidation and photo degradation. All the peaks of degraded product were resolved from the standard drug with significantly different $R_f$ values. This indicates that the drug is susceptible to acid–base hydrolysis, oxidation, and photo degradation. Statistical analysis proves that the method is reproducible and selective for the estimation of the said drug. Because the method could effectively separate the drug from its degradation products, it can be used as a stability indicating one.

*Key Words:* Linezolid; High-performance thin layer chromatography; Stability indicating; Degradation.

*Correspondence: K. R. Mahadik, M.Pharm., Ph.D., F.I.C.A., F.I.C., Department of Quality Assurance Techniques, Poona College of Pharmacy, Bharati Vidyapeeth Deemed University, Erandwane, Pune-411038, Maharashtra State, India; Fax: +91-20-5439383; E-mail: krmahadik@rediffmail.com.



DOI: 10.1081/DDC-120025869
0363-9045 (Print); 1520-5762 (Online)

www.dekker.com

## INTRODUCTION

Linezolid, chemically, (*S*)-*N*-{[3-[3-fluoro-4-(4-morpholinyl)phenyl]-2-oxo-5-oxazolidinyl]methyl} acetamide (Fig. 1) is a novel oxazolidinone antimicrobial agent effective against infections caused by *S. aureus*, *S. pneumoniae*, and *E. faecium*.[1] It acts by inhibiting bacterial protein synthesis at the initiation phase of translation.[2] In humans, linezolid displayed complete oral absorption with plasma levels of 12–18 μg/mL at the steady state.[3] In the literature, LC-MS method is reported for determination of linezolid in human plasma.[4] Reverse phase HPLC method for determination of linezolid in plasma has also been reported.[5–7] However, the method run time was relatively long (>10 min). Literature survey also reveals isolation and characterization of process related impurities in linezolid[8] and separation of novel oxazolidinone antibacterial agents by supercritical fluid chromatography.[9] None of these methods are stability indicating.

The International Conference on Harmonization (ICH) guideline entitled 'Stability Testing of New Drug Substances and Products' requires the stress testing to be conducted to elucidate the inherent stability characteristics of the active substance.[10] Susceptibility to oxidation is one of the required tests. The hydrolytic and the photolytic stability also are required. An ideal stability-indicating method is one that quantifies the drug per se and also resolves its degradation products. A very viable alternative for stability-indicating analysis of linezolid is high-performance thin layer chromatography (HPTLC). The advantage of HPTLC is that several samples can be run simultaneously by using a small quantity of mobile phase unlike HPLC, thus lowering analysis time and cost per analysis.

The aim of the present work was to develop an accurate, specific, reproducible, and stability-indicating method for the determination of low levels of linezolid in the presence of its degradation products and related impurities for assessment of purity of the bulk drug and stability of its bulk dosage forms. The proposed method was validated as per ICH guidelines.[11]

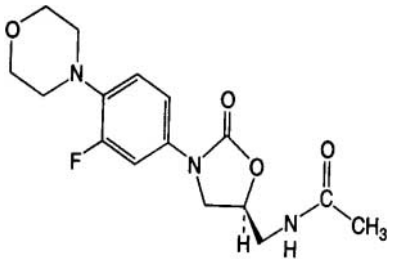


*Figure 1.* Chemical structure of linezolid (oxazolidinone).

## EXPERIMENTAL

### Materials

Linezolid was supplied by Sun Pharma Ltd., India, and tablets (label claim 600 mg/tablet) were procured from the market. Toluene, methanol, and acetone used were of analytical grade (E-Merck Ltd.). All chemicals and reagents used were of analytical grade and were purchased from Merck Chemicals, India.

### HPTLC Instrumentation

The samples were spotted in the form of bands of width 6 mm with a Camag μL syringe on precoated silica gel aluminium plate 60 F—254 (20 × 10 cm with 250 μm thickness; E. Merck, Germany) using a Camag Linomat IV (Switerland). The mobile phase consisted of toluene–acetone (5:5, v/v). The plates were prewashed by methanol and activated at 110°C for 5 min prior to chromatography. Samples were applied as bands 6 mm long, at 5-mm intervals under a stream of nitrogen. The slit dimensions were 5 × 0.45 mm, and sensitivity was kept at auto mode. A constant application rate of 0.1 μL/s and scanning speed 20 mm/s was used. Linear ascending chromatogram development to distance of 8 cm was performed in unsaturated 20 × 10 cm twin trough TLC developing chamber (Camag) at room temperature. Subsequent to the development, TLC plates were dried in a current of air with the help of an air dryer. Densitometric scanning was performed on Camag TLC scanner III in the absorbance mode at 254 nm. The source of radiation used was deuterium lamp.

### Calibration Curves

A stock solution of linezolid (100 μg/mL) was prepared in methanol. Different volumes of stock solution 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 μL of the stock solution were spotted on the TLC plate to obtain concentrations 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 ng/spot of linezolid respectively. The data of peak area vs. drug concentration were treated by linear least square regression

analysis. Linearity was also determined over the range of 300–800 ng/spot to and was selected as working range for the assay and recovery.

## METHOD VALIDATION

### Accuracy and Precision of the Assay

Accuracy was done in terms of recovery studies, and precision was measured in terms of repeatability of measurement and application.

### Ruggedness of the Method

The intraday variation was evaluated in the range of 300–800 ng/spot three times a day. The inter day variation was similarly evaluated over a period of 3 days.

### Limit of Detection and Limit of Quantification

To estimate the limit of detection (LOD) and limit of quantitation (LOQ), blank methanol was spotted six times. The noise level was determined. The limit of detection was calculated to be 3 times the SD whereas 10 times the SD value was considered as limit of quantitation.

### Recovery Studies

The analyzed samples were spiked with extra 50%, 100%, and 150% of the standard linezolid (i.e., 300, 600, and 900 mg respectively), and the mixtures were reanalyzed by the proposed method. The experiment was conducted in triplicate. This was done to check for the recovery of the drug at different levels in the formulations.

### Assay of the Marketed Formulation

To determine the content of linezolid in conventional tablets (label claim: 600 mg/tablet), the tablets were powdered, and powder equivalent to 600 mg of linezolid was weighed. The extraction solvent was methanol. To ensure complete extraction of the drug, it was sonicated for 30 min and volume was made up to 100 mL. The resulting solution was centrifuged at 3000 rpm for 5 min and filtered. Then dilution was made to obtain final concentration of 600 μg/mL. One microliter of the above solution (600 ng/spot) was spotted onto the plate followed by development and scanning. The analysis was repeated in triplicate. The possibility of excipients interference in the analysis was studied.

## FORCED DEGRADATION OF LINEZOLID

### Preparation of Acid and Base Induced Degradation Product

Accurately weighed 100 mg of drug was dissolved in 100 mL of methanol. The drug was subjected to forced degradation under acidic and basic conditions by refluxing with 5 N HCl and 5 N NaOH, respectively, at 70°C for a period of 3 hr and 1 hr, respectively. The forced degradation in acidic and basic media was performed in the dark to exclude the possible degradative effect of light on the drug. The resultant solutions were appropriately diluted and spotted and the chromatograms run.

### Preparation of Hydrogen Peroxide Induced Degradation Product

Accurately weighed 100 mg of drug was dissolved in 100 mL of methanol. Subsequently, 5 mL of hydrogen peroxide 30.0% v/v was added, and the solution was heated in boiling water bath for 1 h until the removal of excess hydrogen peroxide. The solutions were appropriately diluted and spotted and the chromatograms were run. The oxidized product was resolved from pure drug using HPTLC.

### Photochemical Degradation Product

The photochemical stability of the drug was also studied by exposing the drug solution to direct sunlight for 3 days (8 hr/day, overall 24 hr). The resultant solutions were appropriately diluted and spotted (600 ng/spot) and the chromatograms run.

### Detection of the Related Impurities

The related impurities were determined by spotting higher concentrations of the drug to detect and quantify them; 100 mg of linezolid was dissolved

in 10 mL of methanol, and this solution was termed as sample solution (10 mg/mL). One milliliter of the sample solution was diluted to 100 mL with methanol, and this solution was termed as standard solution (0.1 mg/mL). One microliter of both the standard and the sample solution was spotted on the plate and the chromatograms run.

## RESULTS AND DISCUSSION

The TLC procedure was optimized with a view to develop a stability-indicating assay method. Both the pure drug and the degraded products were spotted on the TLC plates and run in different solvent systems.

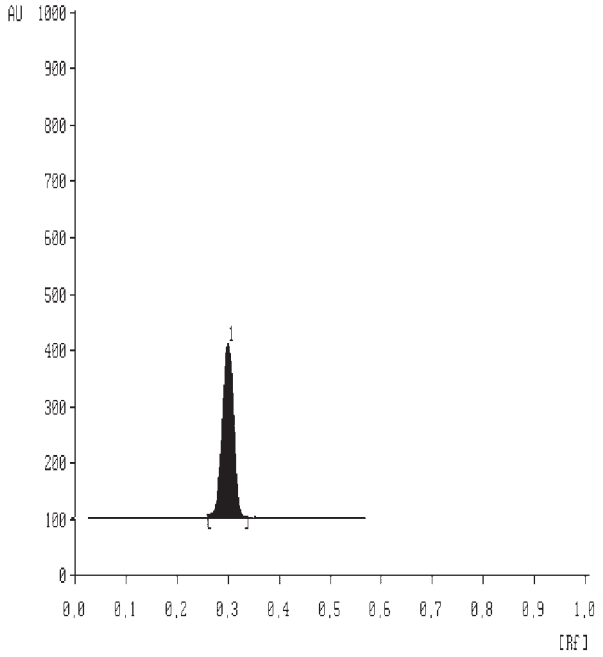


***Figure 2.*** Chromatogram of standard linezolid (1 mg/mL); peak 1 ($R_f$ : 0.29), UV detection at 254 nm, mobile phase toluene:acetone (5:5 v/v).

Initially, toluene–acetone in varying ratios were tried. The mobile phase toluene–acetone (5:5, v/v) gave good resolution, sharp and symmetrical peak with $R_f$ value of 0.29 for linezolid (Fig. 2). The spot for linezolid also was compact and not diffused.

The linear regression data for the calibration curves ($n = 6$) as shown in Table 1 showed a good linear relationship over the concentration range of 300–800 ng/spot as well as over concentration range of 1.0–10.0 μg/spot. No significant difference was observed in the slopes of standard curves (ANOVA; $p > 0.05$).

The precision and accuracy of the developed HPTLC method were expressed in terms of (% RSD). The results depicted revealed excellent accuracy and high precision of the assay method.

The intraday and interday variation were evaluated by comparing the slopes of the calibration curve over the concentration range of 300–800 ng/spot. There was no significant variation in the slope values (ANOVA; $p > 0.05$). The %RSD was found to be 0.59 and 2.90, respectively, for intraday analysis and interday analysis.

The limit of detection, with a signal-to-noise ratio of 3:1, was 20 ng/spot. The limit of quantitation, with a signal-to-noise ratio of 10:1, was 50 ng/spot as shown in Table 2.

The proposed method when used for extraction and subsequent estimation of linezolid from pharmaceutical dosage forms after spiking with 50%, 100%, and 150% of additional drug afforded recovery of 98%–102% as listed in Table 3.

### Stability in Sample Solution

Solutions of two different concentrations (300 ng/spot and 600 ng/spot) were prepared from stock sample solution and stored at room temperature for 24 h and 240, 120, 60, 30, and 10 min and then applied on the same chromatoplate;

***Table 1.*** Linear regression data for the calibration curves.[a]

| Linearity Range (ng/spot) | $r \pm$ S.D. | Slope ± S.D. | Confidence limit of slope[b] | Intercept ± S.D. | Confidence limit of intercept[b] |
|---|---|---|---|---|---|
| 300–800 | $0.998 \pm 0.003$ | $0.15 \pm 0.009$ | $14.28–15.70 \times 10^{-2}$ | $19.52 \pm 1.66$ | 18.19–20.85 |
| 100–1000 | $0.993 \pm 0.62$ | $0.12 \pm 0.005$ | $11.60–12.40 \times 10^{-2}$ | $12.86 \pm 1.25$ | 11.86–13.86 |

[a] $n = 6$.
[b] 95% confidence limit.

after development the chromatogram was evaluated as listed in Table 4 for additional spots if any. There was no indication of compound instability in the sample solution.

***Table 2.*** Summary of validation parameters.

| Parameter | Data |
|---|---|
| Linearity range | 300–800 ng/spot |
| Correlation coefficient | $0.998 \pm 0.003$ |
| Limit of detection | 20 ng/spot |
| Limit of quantitation | 50 ng/spot |
| Accuracy ($n = 3$) | $99.54 \pm 0.42$ |
| Precision (%RSD) | |
| Repeatability of application ($n = 7$) | 1.80 |
| Repeatability of measurement ($n = 7$) | 0.10 |
| Interday ($n = 3$) | 2.90 |
| Intraday ($n = 3$) | 0.59 |
| Specificity | Specific |

***Table 3.*** Recovery studies.[a]

| Excess drug addedto the analyte (%) | Recovery (%) | RSD (%) | SE[b] |
|---|---|---|---|
| 0 | 99.80 | 0.86 | 0.35 |
| 50 | 99.25 | 2.30 | 0.85 |
| 100 | 98.98 | 0.76 | 0.28 |
| 150 | 99.11 | 1.90 | 0.68 |

[a]$n = 6$.
[b]SE = standard error.

***Table 4.*** Stability of linezolid in sample solutions ($n = 3$).

| Actual (ng) | Mean | Range | RSD (%) | SE[a] |
|---|---|---|---|---|
| 300 | 299.35 | 294.12–305.72 | 1.96 | 3.39 |
| 600 | 595.43 | 593.06–598.85 | 0.51 | 1.75 |

[a]Standard error.

### Stability on the Solvent Layer Prior to Development (Spot Stability)

The time the sample is left to stand on the solvent prior to chromatographic development can influence the stability of separated spots and are required to be investigated for validation.[12] Two-dimensional chromatography using same solvent system was used to find out any decomposition occurring during spotting and development. If decomposition occurs during development, peak(s) of decomposition product(s) shall be obtained for the analyte both in the first and second direction of the run. No decomposition was observed during spotting and development.

A single spot at $R_f$ of 0.30 was observed in the chromatogram of the drug samples extracted from conventional tablets. There was no interference from the excipients commonly present in the conventional tablets. The drug content was 99.41% with %RSD of 0.86. Therefore, it may be inferred that degradation of linezolid had not occurred in the marketed formulations that were analyzed by this method as shown in Table 5. The low %RSD value indicated the suitability of this method for routine analysis of linezolid in pharmaceutical dosage forms.

The chromatogram of the acid and base degraded samples for linezolid showed additional peak at $R_f$ value of 0.12 and 0.19, respectively (Figs. 3 and 4). In both cases, the concentration of the drug was changing from the initial concentration, indicating that linezolid undergoes degradation under acidic and basic conditions. The samples degraded with hydrogen peroxide (Fig. 5) showed additional peak at $R_f$ value of 0.15. The spot of degraded product was well resolved from the drug spot. The photo degraded sample showed additional peak at $R_f$ value of 0.24 (Fig. 6). This indicates that the drug is susceptible to acid–base hydrolysis, oxidation, and photodegradation. The lower $R_f$ values of degraded components indicated that they were less polar than the analyte itself. The results are listed in Table 6.

***Table 5.*** Applicability of the HPTLC method for the analysis of the pharmaceutical formulaions ($n = 6$).

| Drug | Label claim | Drug content (%) | RSD (%) | SE | $t^a$ | $F^a$ | $t^b$ | $F^b$ |
|---|---|---|---|---|---|---|---|---|
| Linezolid | 600 mg | $99.41 \pm 0.55$ | 0.86 | 0.35 | 0.49 | 1.10 | 2.44 | 9.27 |

[a]Calculated values of $t$ and $F$.
[b]Theoretical values for $t$ and $F$.

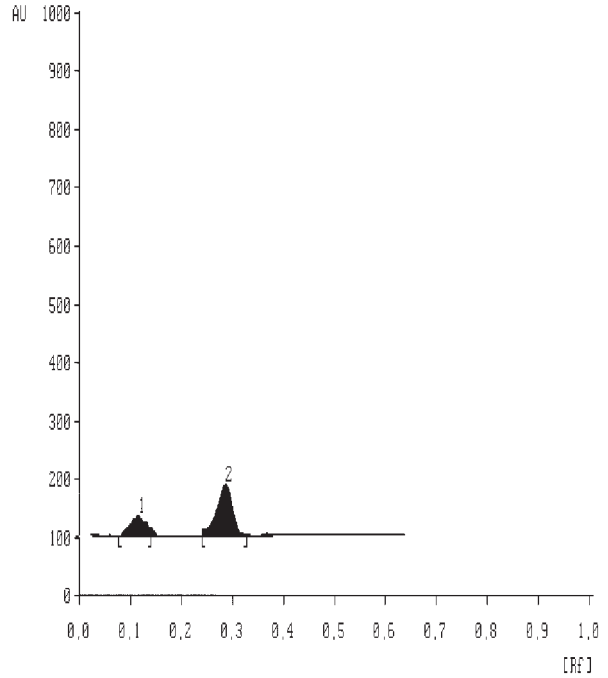


***Figure 3.*** Chromatogram of acid (5 N Hcl, reflux for 3 hr, temp 70°C) treated linezolid; peak 1 (degraded) ($R_f$: 0.12), peak 2 (linezolid) ($R_f$: 0.29).

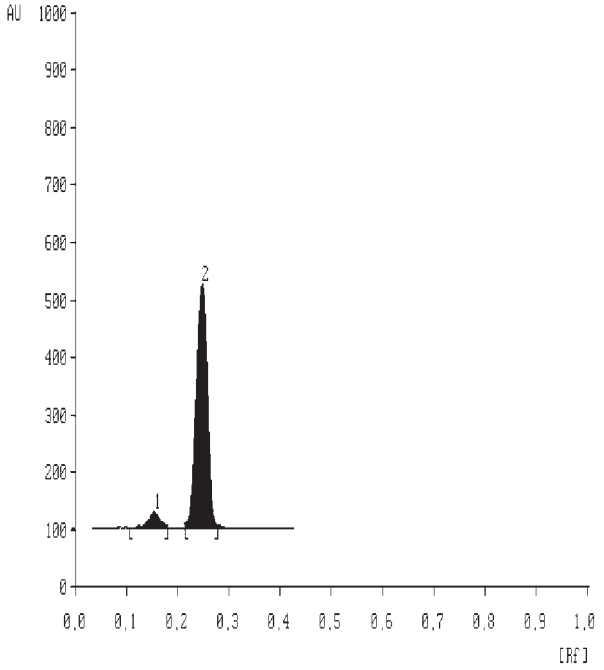


***Figure 5.*** Chromatogram of hydrogen peroxide (30% w/v, reflux for 1 hr, temp 70°C) treated linezolid; peak 1 (degraded) ($R_f$: 0.15), peak 2 (linezolid) ($R_f$: 0.29).

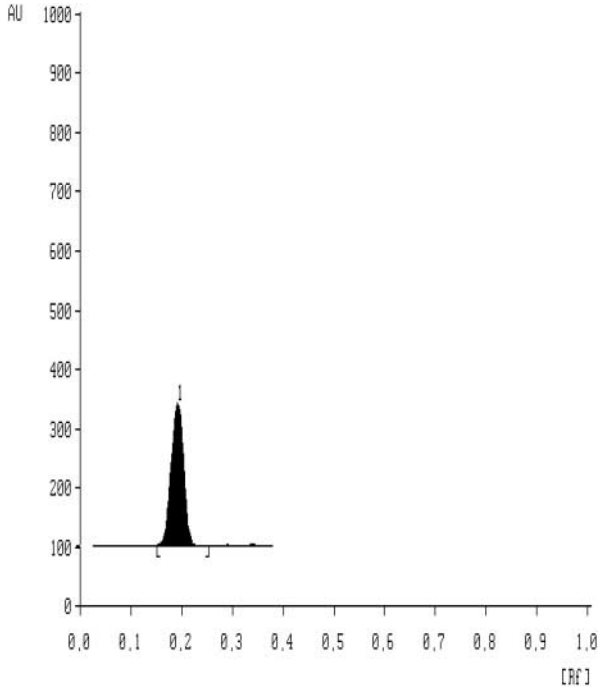


***Figure 4.*** Chromatogram of base (5 N NaOH, reflux for 1 hr, temp 70°C) treated linezolid; peak 1 (degraded) ($R_f$: 0.19).

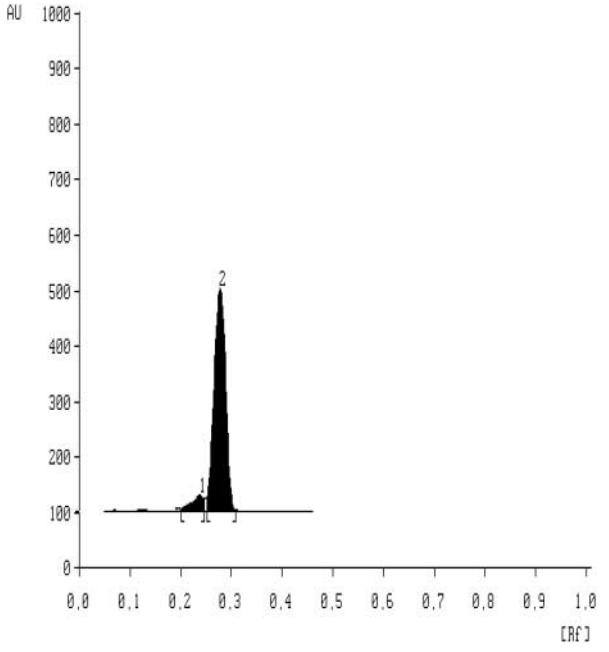


***Figure 6.*** Chromatogram of photodegraded (24 hr) linezolid; peak 1 (degraded) ($R_f$: 0.24), peak 2 (linezolid) ($R_f$: 0.29).

***Table 6.*** Degradation of linezolid.

| Condition | Time (hr) | Recovery (%) | $R_f$ value of degradation products |
|---|---|---|---|
| Acid 5 N HCl (ref)[a] | 3 | 40 | 0.12 |
| Base 5 N NaOH (ref) | 1 | 0 | 0.19 |
| $H_2O_2$ 30 vol (ref) | 1 | 80 | 0.15 |
| Day light (8 hr/day for 3 days) | 24 | 75 | 0.24 |

[a]Refluxed.

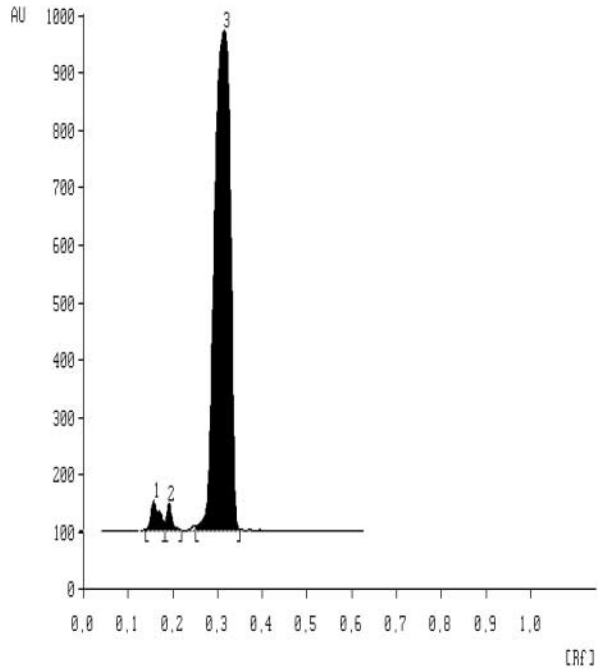


***Figure 7.*** Chromatogram of linezolid and its impurity; peak1 (impurity) ($R_f$: 0.16), peak 2 (impurity) ($R_f$: 0.19), peak3 (linezolid) ($R_f$: 0.29).

The spots other than the principal spot and the spot of the starting point from the sample solution were not intensethan the spot from the standard solution. The sample solution showed two additional spots at $R_f$ of 0.16 and 0.19, respectively (Fig. 7). However, the area of these spots, were found to be much less than the standard solution as indicated in Table 7.

## CONCLUSION

The developed HPTLC technique is precise, specific, accurate, and stability-indicating one.

***Table 7.*** Related impurities.

| Concentration of drug | $R_f$ Value | Area |
|---|---|---|
| 100 ng/spot (standard solution) | 0.29 | 1198.1 |
| Related impurity 10,000 ng/spot (sample solution) | 0.16 | 430.5 |
| | 0.19 | 203.9 |
| Total area | | 634.4 |

Statistical analysis proves that the method is reproducible and selective for the analysis of linezolid as bulk drug and in pharmaceutical formulations. The method can be used to determine the purity of the drug available from various sources by detecting the related impurities. It may be extended to study the degradation kinetics of linezolid and also for its estimation in plasma and other biological fluids. Because the method could effectively separate the drugs from their degradation products, it can be used as a stability-indicating one.

## ACKNOWLEDGMENTS

The authors thank Sun Pharma Ltd., India for gift sample of linezolid. K. R. Mahadik and A. R. Paradkar are thankful to UGC (University Grant Commission, New Delhi) and AICTE (All India Council for Technical Education, New Delhi), respectively for financial support for the project.

## REFERENCES

1. Ford, C.; Hamel, J.; Stapert, D. Oxazolidinones: a new class of antimicrobials. Infect. Med. **1999**, *16*, 435–445.
2. Clemett, D.; Markham, A. Linezolid. Drugs **2000**, *59*, 815–827.
3. Peng, G.W.; Stryd, R.P.; Murata, S. Determination of linezolid in plasma by reversed-phase high-performance liquid chromatography. J. Pharm. Biomed. Anal. **1999**, *20*, 65–73.
4. Oludotun, A.P.; Mohammed, E.A. Determination of linezolid in human plasma by LC-MS-MS, Analyst **2001**, *126*, 609–6014.
5. Tobin, C.M.; Sunderland, J.; White, L.O.; McGowan, A.P. A simple, isocratic, high-performance liquid chromatography assay for linezolid

in human serum. J. Antimicrob. Chemother. **2001**, *48* (5), 605–608.
6. Borner, K.; Borner, E.; Lode, H. Determination of linezolid in human serum and urine by high-performance liquid chromatography. Int. J. Antimicrob Agents **2001**, *18* (3), 253–258.
7. Ehrlich, M.; Trittler, R.; Daschner, F.D.; Kummerer, K. A new and rapid method for monitoring the new oxazolidinone antibiotic linezolid in serum and urine by high performance liquid chromatography-integrated sample preparation. J. Chromatogr. B. Biomed. Sci. Appl. **2001**, *755* (1–2), 373–377.
8. Reddy, K.V.S.R.; Rao, S.M.; Suresh, T. Isolation and characterization of process-related impurities in linezolid. J. Pharm. Biomed. Anal. **2002**, *30*, 635–642.
9. Alasandro, M. Separation of diastereoisomers of DuP 105, a novel oxazolidinone antibacterial agent by supercritical fluid chromatography in a chiral OD column. J. Pharm. Biomed. Anal. **1996**, *14*, 807–814.
10. ICH, Q1A. *Stability Testing of New Drug Substances and Products*, International Conference on Harmonization, Geneva, October 1993.
11. ICH, Q2B. *Validation of Analytical Procedure: Methodology*, International Conference on Harmonization, Geneva, March 1996.
12. Sethi, P.D. *High Performance Thin Layer Chromatography*: *Quantitative Analysis of Pharmaceutical Formulations*, CBS Publishers, 1996; 65–85.