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Journal of Pharmaceutical and Biomedical Analysis
32 (2003) 21–28

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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A validated chiral HPLC method for the enantiomeric separation of Linezolid on amylose based stationary phase

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Received 8 November 2002; received in revised form 7 January 2003; accepted 7 January 2003

Abstract

Two chiral HPLC methods namely method A and method B were developed for the separation of enantiomers of Linezolid. The mobile phases containing hexane, 2-propanol and trifluoro acetic acid (TFA) in the ratio (80:20:0.1, v/v/v); hexane, ethanol and TFA in the ratio (65:35:0.1, v/v/v) were used in method A and method B, respectively. The assay results of the two methods were checked in terms of *F*-test variance ratio and found to be less than the table value, confirming their good precision. The enantiomeric separation of Linezolid on different chiral stationary phases was investigated. The two enantiomers of Linezoild were well resolved on a Chiralpak AD, an amylose based stationary phase. Preparative chiral HPLC was carried out to obtain pure (+) enantiomer of Linezolid from its racemate. The method A was extensively validated and found to be robust. The chiral assay of Linezolid in bulk and pharmaceutical formulations (tablet) were found to be 100.4 ± 0.4 and $101.2 \pm 1.4\%$, respectively at 95% confidence interval. The percentage recovery of (+) enantiomer (chiral impurity) was found to be 99.2 ± 1.9 at 95% confidence interval. The limit of detection and limit of quantification of (+) enantiomer were found to be 123 and 374 ng/ml, respectively for 10 μl injection volume.
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Keywords: Linezolid; Enantiomeric separation; Chiral assay; Preparative chiral HPLC; *F*-test; Limit of detection; Limit of quantification

1. Introduction

Linezolid, S(−)-*N*-[[-3-(3-fluoro-4-(4-morpholinyl)phenyl]-2-oxo-5-oxazolidinyl] methyl] acetamide (Fig. 1), 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-

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doi:10.1016/S0731-7085(03)00031-1

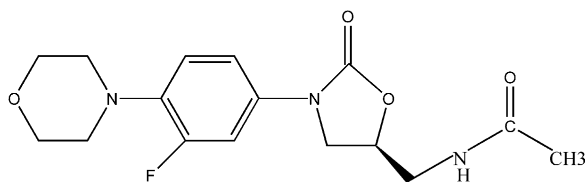

Fig. 1. Chemical structure of Linezolid ((−) enantiomer).

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]. A HPLC method was reported in the literature for the assay of Linezolid in plasma samples using UV detection [4]. Some HPLC methods were cited in the literature for the determination of Linezolid in human serum and urine [5–7]. To our knowledge no chiral HPLC method was reported in the literature. Due to the chiral nature of the drug it is felt necessary to develop a HPLC method for the chiral purity and quantitative determination of enantiomers of Linezolid. The present paper deals with the two chiral HPLC methods and the validation.

2. Experimental

2.1. Chemicals

Samples of Linezolid ((−) enantiomer) and its racemate were received from Process Research Department of Custom Chemical Services of Dr Reddy's Laboratories Ltd, Hyderabad, India. HPLC grade hexane was purchased from s.d.-fine chemicals, India and HPLC grade 2-propanol was purchased from Spectrochem, India. HPLC grade Ethanol Absolute was purchased from Les Alcools De Commerce Inc., Ontario. Trifluoro acetic acid (TFA) of Analytical Reagent grade was purchased from Lancaster, England.

2.2. Equipment

The HPLC system used was a Waters 2690 separations module (Alliance) with 996-photo diode array detector. The output signal was monitored and processed using a millennium 32 chromatography manager software (Waters) on Pentium computer (Hewlett Packard).

The preparative HPLC system used was a Shimadzu LC-8A pump, SCL-8A system controller, SPD-6AV UV-VIS detector, FCV-100B fraction collector and Rheodyne injector model 7725i with 2.0 ml loop.

2.3. Sample preparation

The stock solutions of Linezolid (2.0 mg/ml, (−) enantiomer) and its (+) enantiomer (1.0 mg/ml) were prepared by dissolving the appropriate amounts of the substances in ethanol. Linezolid solutions were prepared from its stock solution by taking 0.25, 0.375, 0.5, 0.625 and 0.75 ml in 5.0-ml volumetric flasks. The solutions were made up to the mark with ethanol.

2.4. Chromatographic conditions (analytical)

The chromatographic column used was a 250 × 4.6 mm Chiralpak AD (Daicel) with 10 μm particles which was accompanied with a 5 cm long guard column. The mobile phase was hexane: 2-propanol: TFA (80:20:0.1, v/v/v) in method A and hexane: ethanol: TFA (65:35:0.1, v/v/v) in method B. The flow rate of the mobile phase was 1.0 ml/min. The column temperature was maintained at 25 °C and the eluant was monitored at a wavelength of 258 nm. The injection volume was 10 μl.

2.5. Chromatographic conditions (preparative)

The preparative column used was a 250 × 10 mm Chiralpak AD with 10 μm particles which was accompanied with a 5 cm long guard column. The mobile phase was hexane: ethanol: TFA (70:30:0.1, v/v/v). The flow rate was set at 2.0 ml/min. Detection was carried out at a wavelength of 258 nm.

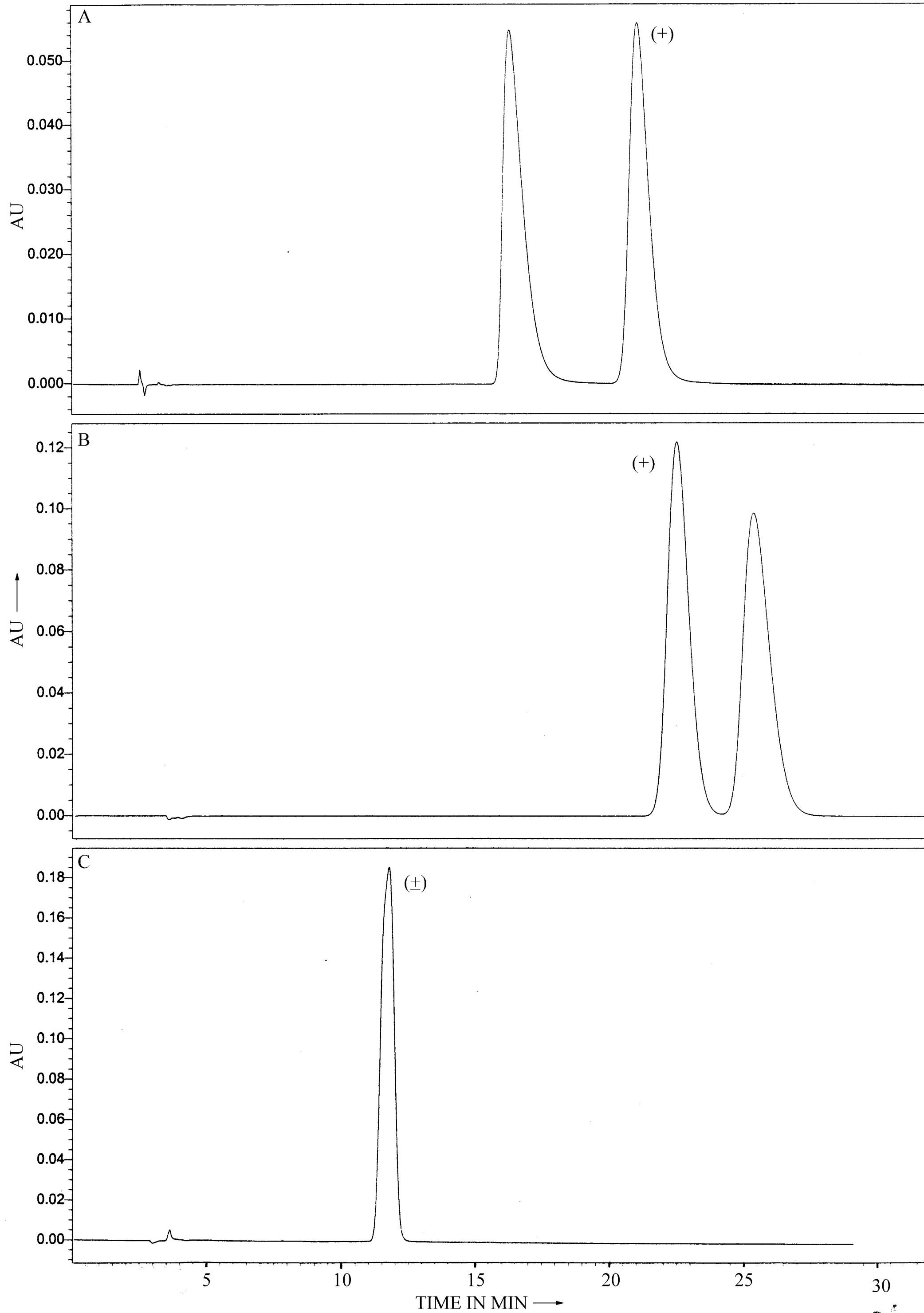

Fig. 2. Enantioselective separation of racemic Linezolid on Chiralpak-AD Column using mobile phase, (A) hexane: 2-propanol: TFA (80:20:0.1, v/v/v), (B) hexane: ethanol: TFA (65:35:0.1, v/v/v), (C) hexane: ethanol: 2-propanol: TFA (70:20:10:0.1, v/v/v).

Table 1
System-suitability report for method A and method B

Method	k'	α	R	N	T
Method A					
Chiralpak AD	15.3	1.30	3.3	2237	2.01
	20.0			3934	1.45
Chiralpak AD-H	18.4	1.32	3.5	2126	2.30
	24.3			4390	1.56
Method B					
Chiralpak AD	21.5	1.13	1.7	3594	1.19
	24.3			2971	1.37
Chiralpak AD-H	21.5	1.24	3.7	6069	1.17
	26.7			4533	1.39

2.6. Method development

The aim of this work was to separate and quantify the enantiomers of Linezolid. The racemic Linezolid sample was used in the method development. To develop a rugged and suitable HPLC method for the separation of the enantiomers different mobile phases and stationary phases were employed. In the method development various chiral columns namely Chiralcel OD, Chiralcel OD-H, Chiralcel-OJ, Chiralcel-OB, Chiralpak AD of Daicel and Whelk-O-1 of Merck were employed. All the columns chosen for the study are of 250 mm length and 4.6 mm internal diameter. Various experiments were conducted to select the best stationary and mobile phases that would give optimum resolution and selectivity for the two enantiomers. No separation was found on Chiralcel-OD, Chiralcel OB and Whelk-O-1 columns. Poor separation was noticed on Chiralcel-OJ and Chiralcel-OD-H columns. Very good separation was achieved on Chiralpak AD column (amylose based chiral column with 10 μm particles) using the mobile phase of hexane: 2-propanol (80:20 v/v). Introduction of TFA reduced the tailing and enhanced the chromatographic efficiency. With the mobile phase consisting of hexane: 2-propanol: TFA (80:20:0.1 v/v/v), the typical retention times of (−) and (+) enantiomers were about 16 and 21 min, respectively (Fig. 2A). This method is referred henceforth as method A.

Ethanol was introduced into the above mobile phase and found drastic decrease in the resolution. Then 2-propanol was replaced with ethanol and checked. Interestingly, reverse elution order with good resolution was noticed. With the mobile phase consisting of hexane: ethanol: TFA (65:35:0.1, v/v/v), the typical retention times of (−) and (+) enantiomers were about 25 and 22 min, respectively (Fig. 2B). This method is referred henceforth as method B. The system suitability results of the two methods were given in Table 1.

Method A and B conditions were checked with Chiralpak AD-H column with 5 μm particles and found still superior separations. The validation was carried out on Chiralpak AD column due to its availability. Method A was extensively validated.

2.7. Collection of (+) enantiomer

To get pure (+) enantiomer from the racemic Linezolid, preparative chiral HPLC was used. With the conditions stated in Section 2.5 the retention times were about 30 and 35 min for (+) and (−) enantiomers, respectively. The prior elution of (+) enantiomer to (−) enantiomer is an added advantage in its collection. The collected fraction was evaporated to dryness in a rotavapor and injected in analytical HPLC using method A and method B conditions. The enantiomeric purity

Table 2
Accuracy in the assay determination of (−) enantiomer.

Taken (mg)	Recovered (mg)	% Recovery	% RSD
0.1605	0.1612	100.4	0.5
	0.1621	101.0	
	0.1606	100.1	
0.2056	0.2068	100.6	0.3
	0.2073	100.8	
	0.2079	101.1	
0.2390	0.2398	100.3	0.5
	0.2374	99.3	
	0.2385	99.8	

Table 3
F-test for the comparison of two methods

Method A assay results	Method B assay results	F-test ($\sigma^2 A/\sigma^2 B$)	Table value ($F_{8,8\ (0.05)}$)
100.4	100.5	1.6	4.4
101.0	100.8		
100.1	100.9		
100.6	100.2		
100.8	99.7		
101.1	100.4		
100.3	99.9		
99.3	99.7		
99.8	99.8		
Mean: 100.4	Mean: 100.2		
S.D.: 0.58	S.D.: 0.46		

was found to be 95%, which is used for the quantification of (+) enantiomer.

2.8. Precision

The precision of the method was checked for injection repeatability and analysis repeatability at target analyte concentration of 0.2 mg/ml.

2.9. Linearity and accuracy

Linearity test solutions were prepared from the stock solution of Linezolid ((−) enantiomer) by taking 0.25, 0.375, 0.5, 0.625 and 0.75 ml in 5-ml volumetric flasks and made up to the volume with mobile phase. The calibration curve was drawn by plotting the peak area of (−) enantiomer versus its corresponding concentration. The accuracy of the method was evaluated by assaying freshly prepared solutions in triplicate at concentrations of 0.16, 0.2 and 0.24 mg/ml. The linearity and accuracy of the method B also checked at the above said concentrations.

2.10. Chiral assay of formulations of Linezolid

The tablets of Linezolid (Zyvox, 600 mg; Pharmacia & Upjohn) were ground to a fine powder. The amount equivalent to 20 mg of active pharmaceutical ingredient (API) was weighed and transferred into 25-ml volumetric flask and extracted into ethanol by ultrasonication. The resultant mixture was filtered through 0.45-μm membrane filter. The filtrate was used as stock solution for preparing the test solutions. The test solutions were prepared in triplicate by taking 1.0 ml of stock solution into 5-ml volumetric flask and making up to the mark with the ethanol. This solution would correspond to the API concentration of 0.16 mg/ml. Similar experiments were carried out to prepare two more test solutions that would contain API at concentrations of 0.20 and 0.24 mg/ml. The chiral assay was checked for all the test solutions.

2.11. Quantification of (+) enantiomer

The Linezolid ((−) enantiomer) sample, provided by Process Research Department of Custom Chemical Services, showed the presence of 0.4% distomer. The UV absorption maximum for Line-

Table 4
Chiral assay results of Linezolid formulations

Taken (mg)	Recovered (mg)	% Recovery	%RSD ($n = 3$)
0.1616	0.1617	100.1	0.3
0.1995	0.2064	103.4	1.3
0.2430	0.2433	100.1	0.8

n, Number of determinations.

zolid was found to be 258 nm. Standard addition and recovery experiments were conducted in the presence of a large enantiomeric excess of the (−) enantiomer to determine accuracy of the present method for the quantification of (+) enantiomer. The study was carried out at 0.4, 0.5 and 0.6 percent of target analyte concentration. The recovery of (+) enantiomer was calculated from the slope and the intercept of the calibration curve, drawn in the concentration range of 0.2–0.7%.

2.12. Robustness

To determine the robustness of the method experimental conditions were purposely altered. The flow rate of the mobile phase was 1.0 ml/min. To study the effect of flow rate on the resolution of enantiomers, it was changed by 0.1 units from 0.9 to 1.1 ml/min. The effect of change in percent 2-propanol on resolution was studied by varying from −1 to +1% while the other mobile phase components were held constant as stated in Section 2.4. The effect of change in %TFA on the resolution was studied by changing from −0.05 to +0.05% while the other mobile phase components were held constant as stated in Section 2.4.

2.13. Stability

Stability of Linezolid ((−) enantiomer) in solution at analyte concentration was studied by keeping the solution in tightly capped volumetric flasks both in refrigerator and on bench top. The samples were analysed at 0 h and after 24 h and areas were compared. The RSD values were found to be below 2.0% in either condition.

3. Results and discussion

The chiral stationary phase (CSP) in Chiralpak AD is 3,5-dimethylphenylcarbamate-amylose derivative coated on silica gel. The separation of enantiomers on Chiralpak AD may be due to the interaction between the solute and the polar carbamate group on the CSP. The carbamate group on the CSP can interact with solute through hydrogen bonding using the C=O and NH groups, which are present in both the CSP and Linezolid. In addition, the dipole–dipole interactions can occur between the C=O group on the CSP and the C=O group on the Linezolid. Wainer et al. reported that solute having aromatic functionalities could provide additional stabilizing effect to the solute–CSP complex by insertion of the aromatic portion into the chiral cavity [8]. This type of stabilization effect may also exist due to the presence of aromatic function in Linezolid. If the chiral discrimination is only due to the above reasons, the Chiralcel OD (3,5-dimethylphenylcarbamate-cellulose derivative coated on silica gel) also should give the baseline separation as it contains carbamate group like Chiralpak AD. Okamoto and co-workers attributed the difference in chiral recognition ability between Chiralcel OD and Chiralpak AD to the conformational difference between them [9]. So the chiral discrimination between the enantiomers of Linezolid on Chiralpak AD is believed to be due to the same reason.

Since the polarity of ethanol is larger than that of 2-propanol, it is expected that the retention with ethanol would be smaller than with 2-propanol, if solvent polarity is the only factor in determining the retention. However, the retention of Linezolid enantiomers in method B is larger than that of method A, suggesting that the polarity of the mobile phase is not the dominating factor. More over, reversal of the elution order of the enantiomers upon changing the alcohol modifier from 2-propanol to ethanol was noticed. The increased retention with the use of ethanol, along with the reversal of elution order, could all be due to an alteration of the steric environment of the chiral cavities by ethanol. As the retention order is reverse for ethanol and 2-propanol, mixture of these solvents in certain proportions should result into a single peak and found with 20% of ethanol and 10% of 2-propanol (Fig. 2C).

In the precision study, the %RSD of injection repeatability for (+) and (−) enantiomer was found to be 1.1 and 0.8, respectively. The %RSD of analysis repeatability for (+) and (−) enantiomer was found to be 1.5 and 0.9, respectively, indicating the good precision of the method.

The coefficient of regression was found to be 0.9991 and 0.9997 for method A and method B,

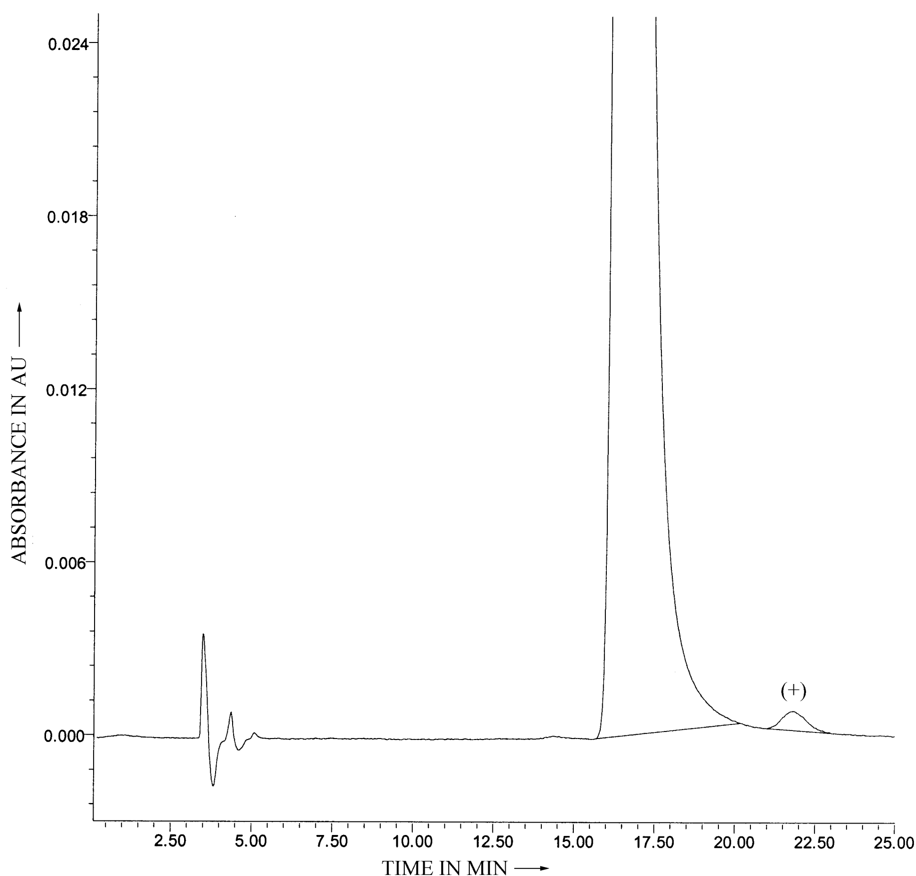

Fig. 3. LC chromatogram of Linezolid spiked with 0.4%(+) enantiomer.

respectively over the concentration range of 0.1 to 0.3 mg/ml. The regression line equation was $y = 33\,620\,190x + 129\,787$ and $y = 34\,790\,900x + 140\,586$ for method A and method B, respectively. The method A percentage recovery was found to be 100.4 ± 0.4 at 95% confidence interval (Table 2). Further more, the method B assay results were compared with method A using F-test. The F-test variance ratio was found to be 1.6 and its table value at 95% confidence interval is 4.4. It indicates that there is no significant difference between the results of method A and method B at 5% significant level. The F-test results were given in Table 3.

Interestingly, (+) enantiomer was not detected in the formulation sample. The chiral assay of Linezolid in the formulation sample was found to be 101.2 ± 1.4 at 95% confidence interval (Table 4). Hence the method is applicable for the determination of chiral assay of Linezolid in its formulation sample.

In the quantification study of the (+) enantiomer the calibration equation was $y = 27.83x - 2461$. The percentage recovery was found to be 99.2 ± 1.9 at 95% confidence interval. The results were shown in Table 5. The spiked chromatogram of the distomer at 0.4% of target analyte concentration was shown in Fig. 3.

The limit of detection (LOD) represents the concentration of analyte that would yield a signal-to- noise ratio of 3 [10]. LOD for (+) enantiomer was 123 ng/ml for 10 μl injection volume. The limit

Table 5
Recovery results of (+) enantiomer

Added (μg)	Recovered (μg)	% Recovery	%RSD ($n = 3$)
0.780	0.773	99.1	2.6
0.992	0.988	99.6	2.7
1.183	1.172	99.0	3.0

n, Number of determinations.

of quantitation (LOQ) represents the concentration of analyte that would yield a signal-to-noise ratio of 10 [10]. LOQ for (+) enantiomer was 374 ng/ml for 10 μl injection volume.

The deliberate changes in the method conditions does not affect the resolution between the enantiomers significantly, confirms the robustness of the method. The solution stability experiments indicate that Linezolid in solution is stable for at least 24 h.

4. Conclusion

Two normal phase chiral HPLC methods with reverse elution order were described for the separation and the quantitative determination of enantiomers of Linezolid in bulk and formulations. The two methods were compared by means of F-test and found that there is no significant difference between the results of the two methods. The method A was extensively validated and it was found to be robust. In addition, preparative chiral HPLC method was also described.

Acknowledgements

The authors wish to thank the management of Dr Reddy's group for supporting this work. We would also like to thank Dr K. Vyas and D. Sreenivas Rao of Analytical Research division of Discovery Research unit for their valuable suggestions in carrying out this work. In addition, we thank Dr G. Om Reddy (Head—Technology Development Center) for his constant encouragement.

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