Enantiomeric Separation of Linezolid by Chiral Reversed-Phase Liquid Chromatography

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Abstract

A chiral liquid chromatographic method is developed for the enantiomeric resolution of Linezolid, (S)(–)-*N*-[[-3-(3-fluoro-4-(4-morpholinyl)phenyl]-2-oxo-5-oxazolidinyl] methyl] acetamide, an antibiotic in bulk drugs. The enantiomers of Linezolid are resolved on a Chiralcel OJ-RH column using a mobile phase system containing 150mM di-sodium hydrogen phosphate buffer (pH 4.5)–acetonitrile (86:14, v/v). The resolution between the enantiomers is found to be two. The developed method is extensively validated and proved to be robust. The limit of detection and limit of quantification of (*R*)-enantiomers are found to be 94 and 375 ng/mL, respectively, for 10 µL injection volume. The percentage recovery of (*R*)-enantiomer is ranged from 98.9 to 102.9 in bulk drug samples of Linezolid. Linezolid sample solution and mobile phase are found to be stable for at least 48 h. The proposed method is found to be suitable and accurate for the quantitative determination of (*R*)-enantiomer in bulk drugs.

Introduction

Linezolid, the first approved agent by Food and Drug Administration (FDA), representing a new class of antibiotics, the oxazolidinones (1), is produced as a single isomer and that the (*R*)-isomer could be present as a chiral impurity. Enantiomers of racemic drugs often differ in pharmacokinetic behavior or pharmacological action (2). The development of analytical methods for the quantitative analysis of chiral materials and for the assessment of enantiomeric purity is extremely challenging due to the fact that enantiomers possess virtually identical properties (3). Recently, much work has been reported describing the use of chiral stationary phases, in conjunction with HPLC, as a way to separate and thereby individually quantitate the enantiomers of an enantiomeric pair (4–6).

In the literature, normal-phase chiral methods were reported for the enantiomeric separation of linezolid using an amylose based chiral stationary phase (7,8). The presence of a small amount of water in the mobile phase for some normal-phase separations has been found to be critical and the use of a small amount of water as mobile phase additive can affect enantioseparation of linezolid enantiomers (7). Narayana et al. reported a normal phase chiral HPLC method with reverse elution order for the separation of linezolid (8). To date, no reversed-phase chiral method was reported for the enantiomeric separation of linezolid. In general, the reversed-phase methods are more reproducible when compared to normal-phase methods due to the volatile nature of organic solvents used in the mobile phase of latter methods. In addition, the reversed-phase methods can be useful for bioanalysis. The objective of the present investigation was to develop a simple reversed-phase chiral HPLC method for the enantiomeric separation of linezolid. The developed reversed-phase HPLC method was validated for determination of (*R*)-enantiomer in linezolid.

Experimental

Chemicals

Linezolid (99% ee) and (*R*)-enantiomer (99% ee) were obtained from our R&D (Hyderabad, India). Chemical structures are presented in Figure 1. HPLC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). Di-sodium hydrogen phosphate dihydrate and ortho-phosphoric acid (88%) were purchased from Merck (Mumbai, India). HPLC water from Milli-Q system (Millipore, Bedford, MA) was used. All other chemicals were of analytical grade.

Equipment

The 1100 Series HPLC system (Agilent Technologies, Waldbronn, Germany) is equipped with a G1311A quaternary pump, a G1313A degasser, a G1313A autosampler, a G1316A

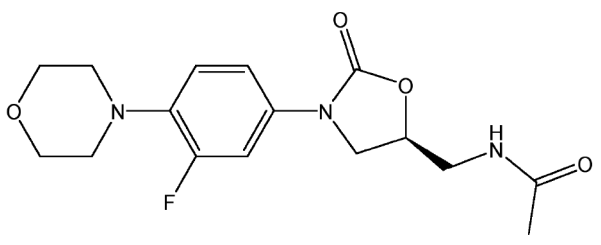

Figure 1. Chemical structure of linezolid [(*S*)-enantiomer].

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thermostatted column compartment, a G1314A UV-detector, and data was processed using a computer program (Chemstation).

Sample preparation

Stock solutions of (*R*)-enantiomer (100 µg/mL) and linezolid (1 mg/mL) were prepared by dissolving the appropriate amount of the substances in mobile phase. The analyte concentration of linezolid was fixed as 300 µg/mL. Working solutions of linezolid and (*R*)-enantiomer were prepared in mobile phase. Standard impurity mixture of linezolid and (*R*)-enantiomer (3 µg/mL each) was prepared using mobile phase.

Chromatographic conditions

The chromatographic conditions were optimized using a chiral stationary phase, Chiralcel OJ-RH (150 × 4.6 mm, 5 µm, Daicel, Japan), which was safeguarded with a guard column Chiralcel OJ-RH (10 × 4 mm). The isocratic mobile phase composition was a mixture of 150mM di-sodium hydrogen phosphate buffer (pH adjusted to 4.5 with ortho phosphoric acid)–acetonitrile (86:14, v/v), which was pumped at a flow rate of 0.5 mL/min. The column was maintained at 35°C temperature, and the eluant was monitored at a wavelength of 220 nm. The injection volume was 10 µL. Different chiral columns were also employed during method development, namely Chiralcel OD-RH (150 × 4.6 mm), Chiral AD-RH (150 × 4.6 mm), all produced by Daicel, Chiral AGP (150 × 4.0 mm, Chromtech, UK), Chiral HSA (150 × 4.0 mm, Chromtech), Chiral CBH (150 × 4.0 mm, Chromtech).

Validation of the method

The intermediate precision was determined over 3 days by performing six successive injections each day. The reproducibility of the method was determined by analyzing six test solutions containing linezolid (300 µg/mL) spiked with (*R*)-enantiomer (1%). LOD and LOQ were achieved by injecting a series of dilute solutions of (*R*)-enantiomer. The precision of the developed chiral method for the (*R*)-enantiomer at was checked by analyzing six test solutions of the (*R*)-enantiomer prepared at the limit of quantitation (LOQ) level and calculating the percentage relative standard deviation of area.

Detector response linearity was assessed by preparing six calibration sample solutions of (*R*)-enantiomer covering from 375 ng/mL (LOQ) to 4500 ng/mL (375, 750, 1500, 3000, 3750, and 4500 ng/mL), in mobile phase from the (*R*)-enantiomer stock solution.

The study was carried out for accuracy in triplicate at 0.5, 1, and 1.5% of the linezolid target analyte concentration. The recovery of the (*R*)-enantiomer was calculated from the slope and Y-intercept of the calibration curve obtained.

To determine robustness of the method, flow rate on the resolution of enantiomers, 0.05 units (± 10%) changed it from 0.45 to 0.55 mL/min. The effect of change in percent acetonitrile (± 2%), the effect of change in pH of the mobile phase (± 0.2 units), and the effect of column temperature (± 5°C) on resolution was studied while the other mobile phase components were held constant as stated in the "Chromatographic conditions" section.

Stability of linezolid in solution at analyte concentration was studied by keeping the solution in tightly capped volumetric flask at room temperature on a laboratory bench for 2 days. The content of the (*R*)-enantiomer was checked for 6 h interval up to the study period. Mobile phase stability was carried out by evaluating the content of the (*R*)-enantiomer in linezolid sample solutions prepared freshly at 6 h interval for 2 days.

Results and Discussion

Optimization of chromatographic conditions

The aim of this work is to separate the enantiomers of linezolid and accurate quantitation of (*R*)-enantiomer. Racemic mixture solution of 3 µg/mL prepared in mobile phase was used in the method development. To develop a rugged and suitable LC method for the separation of Linezolid enantiomers, different mobile phases and stationary phases were employed. The main target of the chromatographic method is to separate the enantiomers of linezolid; various chiral columns, namely: Chiralcel OD-RH, Chiralcel OJ-RH, Chiral AD-RH of Diacel and Chiral AGP, Chiral HSA, and Chiral CBH of Chromtech were employed. A series of 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-RH, Chiral AD-RH, Chiral AGP, Chiral HSA, and Chiral CBH columns using different possible mobile phases. There is an indication of separation on Chiralcel OJ-RH column using a mobile phase consisting of di-sodium hydrogen phosphate (pH 4.0) and acetonitrile (60:40, v/v). The composition of the mobile phase was optimized to enhance the chromatographic efficiency and resolution between the enantiomers. Best separation was achieved on Chiralcel OJ-RH column using the mobile phase composition of 150mM di-sodium hydrogen phosphate (pH 4.5)–acetonitrile (86/14, v/v). Due to better chromatographic results obtained on the Chiralcel OJ-RH column, the method validation was carried out on the same.

In the optimized method, the typical retention times of linezolid and (*R*)-enantiomer were approximately 19.5 and 21.7 min, respectively. The enantiomeric separation of linezolid on Chiralcel OJ-RH column was shown in Figure 2. Baseline separation of linezolid (k_1' = 5.69) and (*R*)-enantiomer (k_2' = 6.46) was obtained with a total run time of 40 min.

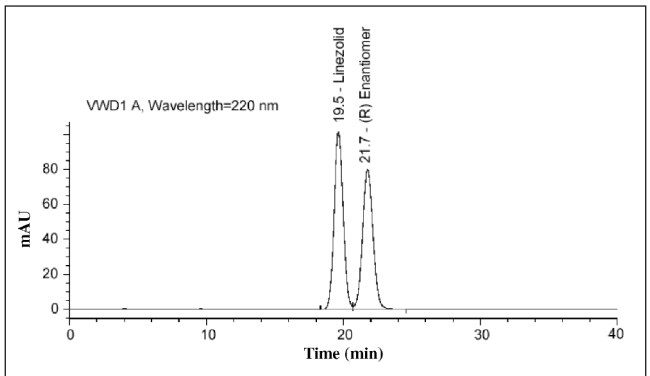

Figure 2. Enantiomeric resolution of Linezolid on Chiralcel OJ-RH column.

Validation results of the method

In the repeatability study, the relative standard deviation (RSD) was better than 0.3% for the retention times of both the enantiomers, 0.4% for linezolid peak area and 0.5% for (*R*)-enantiomer peak area. In the intermediate precision and reproducibility studies, results show that RSD values were in the same order of magnitude than those obtained for repeatability.

The limit of detection (LOD) and LOQ concentrations were estimated to be 94 and 375 ng/mL for (*R*)-enantiomer, when a signal-to-noise ratio of 3 and 10 were used as the criteria. The method precision for (*R*)-enantiomer at LOQ was 0.5% RSD

Good linearity was observed for (*R*)-enantiomer over the concentration range of 375–4500 ng/mL, with the linear regression equation $y = 0.0137x - 0.1161$ (correlation coefficient $r = 0.9998$). Linearity was checked for (*R*)-enantiomer over the same concentration range for three consecutive days. The %RSD of the slope and Y-intercept of the calibration curve were 1.9 and 11, respectively.

The standard addition and recovery experiments were conducted for (*R*)-enantiomer in bulk samples in triplicate at 0.5, 1, and 1.5% of analyte concentration. The recovery was calculated from the slope and Y-intercept of the calibration curve obtained linearity study and percentage recovery was ranged from 98.9% to 102.9%.

The chromatographic resolution of linezolid and (*R*)-enantiomer peaks was used to evaluate the method robustness under modified conditions. The resolution between linezolid and (*R*)-enantiomer was greater than 1.5, under all separation conditions tested, demonstrating sufficient robustness.

No significant change in the (*R*)-enantiomer content was observed in linezolid sample during solution stability and mobile phase stability experiments. Hence, linezolid sample solution and mobile phase are stable for at least 48 h.

Conclusions

A simple, rapid, and accurate reversed-phase chiral LC method was described for the enantiomeric separation of linezolid. Cellulose based chiral column Chiralcel OJ-RH column found to be selective for the enantiomers of linezolid. The method was completely validated, demonstrating satisfactory data for all the method validation parameters tested. The developed and validated method can be used for the chiral purity testing of linezolid. The developed method is also stability indicating and can be used for the quantitative determination of chiral impurity [(*R*)-enantiomer] in bulk materials.

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