Rapid and Sensitive Determination of the Antibiotic Linezolid in Low Plasma Volumes by High-Performance Liquid Chromatography

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Abstract

A rapid and sensitive method for the determination of linezolid by high-performance liquid chromatography (HPLC) with UV detection (251 nm) is presented. Linezolid is an important antibiotic against severe infections caused by multi-resistant bacterial pathogens. Scientific efforts continue investigating its effectiveness in different conditions and patient populations including children and newborns. Because plasma samples in a pediatric setting or from animal models are usually collected in low volumes, there is a necessity for a reliable and precise analytical method that is reliable and precise even at sample volumes below 50 µL. The presented method is suitable for plasma sample volumes of 20 µL and can be performed with basic HPLC equipment. Linezolid is extracted from plasma with 10% methanol–90% dichloromethane at neutral conditions and separated isocratically on a microbore ODS column using ammonium acetate buffer (pH 4.4, 0.5%, w/v) and acetonitrile (84:16, v/v) as the eluent. The method exerts linearity from 0.05–40 mg/L and meets commonly accepted specifications regarding accuracy and precision.

Introduction

Linezolid (N-[[(5S)-3-[3-fluoro-4-(morpholin-4-yl) phenyl]-2-oxo-1,3-oxazolidin-5-yl] methyl] acetamide; $C_{16}H_{20}FN_3O_4$) (Figure 1) is currently the only approved member of the class of oxazolidinones and represents an important antibiotic agent in the armamentarium against severe infections caused by methicillin- or vancomycin-resistant gram-positive bacteria (1,2). Linezolid shows favorable pharmacological and pharmacokinetic characteristics such as rapid absorption from the gut following oral administration, a plasma elimination half-life of approximately 4–6 h, excellent tissue penetration properties, and an acceptable safety profile (3). Although linezolid has been approved and marketed for almost 10 years, scientific efforts continue investigating its effectiveness in different conditions and patient populations including children and newborns. Because plasma samples collected in a pediatric setting or from animal models are usually collected in low volumes, there is a necessity for an analytical method that is reliable and precise even at sample volumes of less than 50 µL.

Several methods for the determination of linezolid in biological fluids have been described in scientific literature. Apart from methods utilizing tandem mass spectrometry (4) or capillary electrophoresis (5), which require equipment not readily available in many laboratories, high-performance liquid chromatography with UV detection (HPLC–UV) is the most widely used technique (6–13). The described sample preparation procedures include solid-phase extraction (6,11), on-column extraction with automated column-switching (9), or precipitation of plasma proteins with organic solvents and/or acids (7,8,10,12,13).

The aim of the present work was to develop an HPLC method that can be performed with basic HPLC equipment, is not laborious, and enables selective measurements within a run-time of 15 min maximum. Moreover, mild acidic chromatographic conditions are to be preferred for silica-based stationary phases to ensure a proper lifetime of the analytical column. In preliminary experiments, however, with mobile phases at pH 4–5.5, plasma samples treated with acetonitrile or trifluoroacetic acid for protein precipitation, as previously suggested, produced several late eluting peaks, which interfered with subsequent injections and lowered the sensitivity of the assay. To avoid gradient elution, we developed the herein presented liquid–liquid extraction procedure, which enables rapid processing of plasma samples at volumes of 20 µL.

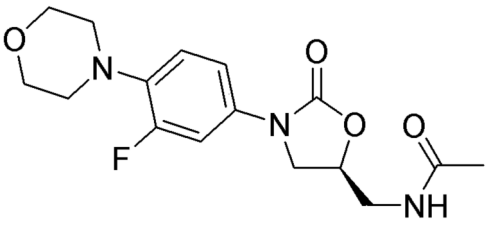

Figure 1. Chemical structure of linezolid.

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Experimental

Chemicals

Linezolid reference standard (purity 99.8%) was kindly provided by Pfizer (Vienna, Austria). All other compounds and solvents (HPLC-grade) were purchased from Sigma Aldrich (Steinheim, Germany).

Preparation of calibration standards and quality control samples

A stock solution of linezolid of 10 mg/mL was prepared by solving 100 mg of accurately weighed reference standard into 10 mL of a water–methanol mixture (70:30, v/v). The stock solution was further diluted with a water–methanol mixture (90:10, v/v) to prepare working solutions at concentrations of 0.5, 1, 5, 10, 50, 100, and 400 mg/L. Working solutions were prepared separately for calibration standards and quality control (QC) samples. Ninety microliter aliquots of pooled plasma from healthy blood donors were spiked with 10 µL of the appropriate working solution to achieve final concentrations of 0.05, 0.1, 0.5, 1, 5, 10, and 40 mg/L. Calibration standards and QC samples from pooled rat plasma were prepared in analogous manner. All calibration standards and QC samples were stored at approximately –75°C until use.

Optimization of the sample preparation

A 20-µL aliquot of plasma sample was transferred into a 1.5-mL standard polypropylene vial and diluted with different volumes of water (0, 20, 40 µL). Then 100, 200, or 300 µL of 10% methanol–90% dichloromethane (MeOH–DCM) was added to each set of samples and thoroughly vortex-mixed for at least 10 s. The mixture was allowed to rest for 10 min. Alternatively, the mixture was centrifuged at 7000 × *g* for 5 min at room temperature. Subsequently, a defined aliquot of the organic phase (80% of the initial added volume) was transferred into another vial and evaporated to dryness simply by keeping the opened vials under the fume hood in a heating block at 50°C for approximately 6 min. The dry residue was re-dissolved with 30 µL mobile phase. Twelve microliters was injected into the HPLC system.

Chromatographic conditions

The HPLC system LaChrom Elite (VWR International, West Chester, PA) equipped with a variable wavelength UV/Vis detector was used. The detection wavelength was set to 251 nm. Separation was carried out on a Symmetry C_{18} column (150 × 2.1 mm, 5 µm; Waters, Milford, MA) thermostatted at 25°C. The eluent consisted of 0.5% ammonium acetate, adjusted to pH 4.4 with acetic acid, and 16% acetonitrile. Before use, the eluent was filtered through a 0.45-µm membrane filter. The flow rate was set at 0.4 mL/min.

Validation

Selectivity was tested by analyzing blank samples of pooled human and rat plasma (from eight and seven sources, respectively) and clinical specimens from intensive care patients. Within-day and between-day inaccuracy and imprecision from QC samples at nominal concentrations of 0.05, 0.5, 1, and 10 mg/L were determined in quadruplicate (each injection from a separately prepared sample) on three different days. Inaccuracy (relative error) was calculated by the formula: Inaccuracy (%) = (mean concentration $[C]_{measured} - C_{nominal})/C_{nominal} \times 100$. Imprecision was expressed as the relative standard deviation (RSD%) of multiple measurements. The lower limit of quantitation (LLOQ) was defined as the lowest concentration, which can be measured with an inaccuracy and imprecision of ≤ 20% (14). For determination of the extraction recovery, the respective linezolid peak areas were related to that obtained from samples of mobile phase spiked at equal nominal concentrations using the formula: Recovery (%) = Area in plasma/area in mobile phase × 100.

Results and Discussion

The herein described liquid–liquid extraction procedure gave clean chromatograms without the need for gradient elution. As expected, the extraction recovery of linezolid was dependent on the proportion of aqueous and organic medium in the extraction mixture. The highest recovery values ranging between 94.1–98.8% were obtained for the ratio of approximately 0.1 for the volumes of undiluted plasma to MeOH–DCM. Thus, for further experiments, 20 µL undiluted plasma was extracted with 200 µL MeOH–DCM. Linezolid was not detected in the aqueous phase of this mixture.

When the extraction mixture was allowed to rest for 10 min, the plasma proportion formed a milky, semi-solid top layer, and 160 µL of the clear organic phase beneath could be easily collected and transferred to another vial. In contrast, high-speed centrifugation of the extraction mixture caused the plasma to decompose into a sticky proteinaceous membrane and a liquid portion, which made the collection of the organic phase more cumbersome. An advantage of the sample extraction with MeOH–DCM is that the small percentage of MeOH dissociates linezolid from plasma proteins and accelerates its distribution into the organic phase. Moreover, the evaporation takes place in a few minutes without the need of a vacuum.

In a previous experiment, we found the retention time of linezolid (pKa 1.8) to be nearly constant at mobile phase pH values 4–7. The peak height, however, was inversely related to the pH of the mobile phase. Consequently, for the present method, we chose pH 4.4, which is a mild condition for the silica-based sta-

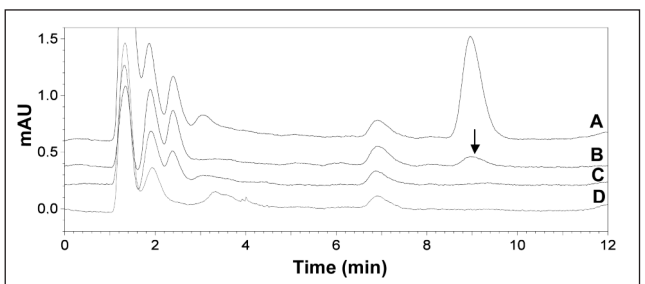

Figure 2. Representative chromatograms of linezolid in human and rat plasma: spiked human plasma containing 0.5 µg/mL linezolid (A); spiked human plasma containing 0.05 µg/mL (LLOQ) linezolid (B); blank human patient plasma (C); blank rat plasma (D). Linezolid is marked with an arrow.

tionary phase and enabled the selective measurement of linezolid in human and rat plasma with an LLOQ of 0.05 mg/L. Representative chromatograms are pictured in Figure 2.

Endogenous matrix components as well as drugs frequently administered to intensive care patients, such as cefpirome, ceftazidime, ceftriaxon, meropenem, ciprofloxacin, levofloxacin, voriconazole, fluconazole, ganciclovir, propofol, midazolam, sufentanil, diazepam, and pantoprazole, did not interfere with linezolid detection in the presented assay.

Linear regression of three calibration curves (peak area versus concentration) gave a slope of 197964 ± 4081, a *y*-intercept of 11855 ± 6073, and correlation coefficients (R) of ≥ 0.9992. Within-day and between-day inaccuracy ranged from 1.6% to 13.6% and from 2.3% to 13.0%, respectively. The corresponding within-day and between-day imprecision ranged from 1.0% to 13.9% and from 1.6% to 10.2%, respectively. Data are shown in Table I. Very similar results were found for rat plasma. The validation data achieved with external standard quantification met previously defined acceptance criteria (14). The inclusion of an internal standard (fluconazole; retention time 5.9 min) did not significantly improve the validation coefficients. These results indicate that the extraction procedure did not add considerable variance to the overall measurement variance.

The stability of linezolid including freeze-thaw stability for three cycles and long-term stability was repeatedly demonstrated by other authors (6–12). Likewise, in our study, linezolid concentrations were found to be unchanged in stock and working solutions, in plasma, and in prepared samples at room temperature, and at storage temperatures from 2 to 8°C for at least 72 h.

The major advantage of the present method is the 2–4 times higher sensitivity compared to previous published HPLC–UV methods using sample volumes of 50–300 µL (7,8,10,12,13). The most sensitive assay for plasma sample volumes of 50 µL (LLOQ 0.01 mg/L) was described by Peng et al. (6). We are aware of the fact that for samples derived from clinical studies, liquid chromatography–tandem mass spectrometry (LC–MS–MS) has become the technique of choice because it provides unique selectivity. Phillips and co-workers described a method employing solid-phase extraction and LC–MS–MS. They reported an LLOQ of 0.1 µg/mL with an assay run-time of less than 3.5 min (4). However, even in analytical laboratories, mass spectrometers are still not standard equipment. Many researchers have to answer their analytical questions by exploiting the capabilities of conventional HPLC detectors. In this situation, the use of narrow-bore columns (2.1 mm i.d. or less) helps to gain assay sensitivity with the beneficial side effect of considerable solvent savings compared with columns of "standard" dimensions.

Table I. Within- and Between-Day Inaccuracy and Imprecision of Linezolid in Human Plasma

Nominal Conc. (µg/mL)	Measured conc. (µg/mL) (Mean ± SD)	Inaccuracy (RE) %	Imprecision (RSD) %
Within-day (n = 4)			
0.05 (LLOQ)	0.043 ± 0.006	–13.6	13.9
0.5	0.487 ± 0.008	–2.7	1.6
1	1.093 ± 0.027	+9.3	2.5
10	10.163 ± 0.106	+1.6	1.0
Between-day (n = 12)			
0.05	0.044 ± 0.004	–13.0	10.2
0.5	0.481 ± 0.011	–3.8	2.4
1	1.083 ± 0.053	+8.3	4.9
10	10.234 ± 0.166	+2.3	1.6

Conclusion

In summary, the herein described method for the determination of linezolid is suitable for plasma volumes as low as 20 µL is not technically demanding and has proved to give reliable and precise results in the desired concentration range of 0.05–40 mg/L. The method is ready for application in pediatric and animal studies.

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