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A rapid and simple liquid chromatography-tandem mass spectrometry method for the determination of linezolid in human serum

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Abstract

Background: Linezolid is an antibiotic that is reserved as the last therapeutic line of defence when resistant micro-organisms are present.

Method: A sensitive, robust, simple and rapid method was developed for the analysis of linezolid in serum. The method was based on the precipitation of proteins in human serum using a precipitation reagent containing an internal standard (cyanoimipramine) and subsequently, using high-performance liquid chromatography (HPLC) analysis and tandem mass spectrometry (MS/MS) detection in an electron positive mode. The method validation included selectivity, linearity, accuracy, precision and stability.

Results: The calibration curve of linezolid was linear in a range of 0.05 mg/L to 40.0 mg/L, with within-run coefficients of variation (CVs) and between-run CVs in the range of 0% to 7.1%. Linezolid was stable in human serum after three freeze-thaw cycles and for 36 hours at 4°C, at room temperature and, after sample preparation, in the autosampler without loss of product.

Conclusion: The developed method is a linear, selective, accurate and precise liquid chromatography-tandem mass spectrometry (LC-MS/MS) method and can be applied to therapeutic drug monitoring of linezolid in patients with resistant micro-organisms. The serum samples from the patient are in the range of the linearity of the method.

Keywords

LC-MS/MS, linezolid, nocardia, TDM (therapeutic drug monitoring), pharmacokinetics

Introduction

Linezolid is from the oxazolidinone class of antibiotics and has shown good activity against Gram-positive micro-organisms and mycobacteria [1-3]. Its molecular structure is shown in Figure 1. It inhibits the formation of 70S subunits in bacterial ribosomes, which are essential components in the translation procedure [1]. Because of this unique mechanism of action, linezolid can be used for the treatment of resistant micro-organisms, including meticillin-resistant Staphylococcus aureus (MRSA), vancomycin-resistant enterococci (VRE) and multidrug resistant Mycobacterium tuberculosis [1-3].

Worldwide resistance to first-line antibiotics is an increasing problem. Therapeutic drug monitoring (TDM) can be used for the prevention of resistance to antibiotics which are reserved as the last line of defense, such as linezolid [4, 5]. Preferably, in the Netherlands, linezolid is only used after microbiological examination or identification of the minimal inhibition concentration (MIC) of the

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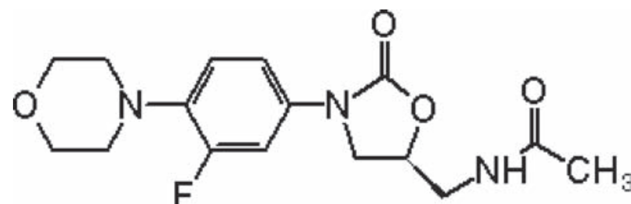
Figure 1: Chemical structure of linezolid



micro-organism. An area under the concentration-time curve (AUC), over 24 hours in steady state, divided by the MIC (AUC/MIC ratio) above 100 and percentage time above the MIC (% of time >MIC) above 85 are good predictors of the development of resistance to linezolid [4, 5]. Inter-patient differences in pharmacokinetics and the frequently associated toxicity of linezolid after prolonged use makes TDM strongly recommended [3, 6]. Therefore a good analytical method for linezolid in serum is necessary.

LC-MS/MS is a commonly used method in laboratories nowadays for the analysis of many different compounds, and can also be used for the determination of linezolid in human serum. Several determinations of linezolid in human fluids using a HPLC-UV method have been published [5, 7-9]. In one paper, a LC-MS/MS method was described. That method required solid phase extraction cartridges for extraction and two calibration curves (from 0.1-5.0 µg/mL and 5-20 µg/mL, respectively) [10]. Solid phase extraction is an expensive method and takes a lot of time.

The objective of this study was to develop a simple, rapid and validated LC-MS/MS analysing method for an easily manageable analysis of linezolid, in human serum, without solid phase extraction. Our method was based on the precipitation of proteins in human serum with the precipitation reagent containing the internal standard (cyanoimipramine), with subsequent HPLC analysis and MS/MS detection of the fragment ions of linezolid and the internal standard.

Materials and methods

Materials

Linezolid was obtained from Pfizer (New York, US). The generally used internal standard, cyanoimipramine, was supplied by Roche (Woerden, the Netherlands).

Acetonitrile for LC/MS and water for LC/MS were provided by BioSolve BV (Valkenswaard, the Netherlands). Methanol Lichrosolv and the other chemicals used were of HPLC or analytical grade and purchased from VWR (Amsterdam, the Netherlands).

The precipitation reagent consisted of 0.04 mg/L cyanoimipramine, the internal standard, dissolved in a mixture of methanol and acetonitrile (4:21 respectively).

Pooled human serum samples were made available according to the protocols of the University Medical Center, Groningen.

Sample preparation

In a 2.0 mL autosampler vial, 10 µL serum sample (blank serum, calibration standard, quality control sample or patient sample) and 750 µL precipitation reagent containing the internal standard were vortexed for one minute, stored at –20°C for 30 minutes to promote protein precipitation, and subsequently centrifuged at 11,000 g for five minutes. From the clear upper layer 5 µL was injected into the LC-MS/MS system.

LC-MS/MS analyses

All experiments were performed on a Thermo Fisher (San Jose, US) triple quadruple LC-MS/MS with a Finnigan Surveyor LC pump and a Finnigan Surveyor autosampler, which was set at a temperature of 20°C. After sample preparation, 5 µL of the clear upper layer were injected into a 100 x 2.1 mm C_{18}, 5-µm analytical column (HyPurity Aquastar, Interscience Breda, the Netherlands) for chromatographic separation. The column temperature was set at 20°C. The mobile phase had a flow of 0.2 mL/minute and consisted of an aqueous buffer (containing ammonium acetate 10 g/L, acetic acid 35 mg/L and trifluoroacetic anhydride 2 mL/L water), water and acetonitrile. The mobile phase operated as follows: 0-1 minute: buffer 5%, water 95% and acetonitrile 0%; 1-4 minutes: buffer 5%, water 95% at one minute to 0% at four minutes, and acetonitrile 0% at one minute to 95% at four minutes; 4-6 minutes: buffer 5%, water 0% and acetonitrile 95%.

The Finnigan TSQ Quantum Discovery mass selective detector operated in electrospray positive ionisation mode and performed selected reaction monitoring as scanning mode. The following mass parameters were used at a scan width of 0.5 m/z: linezolid m/z 338.1 to m/z 296.1 (collision energy 19 eV) and cyanoimipramine m/z 306.2 to m/z 218.0 (collision energy 39 eV). The ion source spray voltage was set at 3,500 V, the sheath and auxiliary gas pressure at 35 arbitrary units (arb) and 5 arb respectively, and the capillary temperature at 350°C. Xcalibur software version 1.4 SR1 (Thermo Fisher, San Jose, US) was used for peak height integration for all components.

Method validation

In accordance with the US Food and Drug Administration's *Guidance for Industry Bioanalytical Method Validation*, such validation includes the five criteria: selectivity, linearity, accuracy, precision and stability [11]. Therefore, on each analytical day, a single calibration curve was obtained and the quality control (QC) samples were

analysed for all five criteria. Furthermore, six pools of blank human serum were analysed in triplicate.

For the preparation of the calibration standards and the QC samples, two stock solutions of linezolid (stock A1 and B1) were prepared by dissolving linezolid in water (1,000 mg/L). Secondary working stock solutions (stock A2 and B2) were prepared by diluting stock A1 and B1 with water.

Stock A1 and A2 were used for preparing the calibration samples by diluting them with pooled human serum to the concentrations: 0.05, 0.25, 0.50, 2.50, 10.0, 15.0, 25.0 and 40.0 mg/L, respectively.

For QC samples, concentrations of 0.05 mg/L (lower limits of quantification LLOQ), 0.50 mg/L (low), 15.0 mg/L (medium) and 30.0 mg/L (high) were prepared by spiking stock B1 and B2 with pooled human serum.

The calibration standards and QC samples were prepared on day zero and stored at –20°C. The QC samples for determining the freeze/thaw stability were freshly prepared from stock B1 and B2 on the first day of analysis.

Pharmacokinetic profile

From a patient with the rare nocardia infection, who received linezolid 600 mg twice daily by mouth, six serum samples were analysed at 0, 1, 2, 4, 8 and 12 hours at steady state after four doses of 600 mg linezolid. The AUC/MIC ratio was calculated with the equation: $(AUC_{0\text{-}12\,h} \times 2)/MIC$.

Results

During the development of this method, carryover was found when high concentrations of linezolid in human serum were analysed. (Sample carryover is defined as sample from a previous injection detected during the analysis of the subsequent injections.) The carryover was about 0.05% to 0.10%, determined in the next blank serum. Therefore the decision was taken to reinject all samples containing a concentration lower than 0.50 mg/L and also the calibration samples. The method of reinjection was also used during the validation for QC-level LLOQ. The reinjection run was only accepted when no peak of linezolid was observed in the last blank sample before the QC samples.

Chromatogram characteristics

Linezolid and cyanoimipramine had a mean retention time of 2.7 minutes and 4.1 minutes, respectively. An example of the chromatographic results is shown in Figure 2.

Selectivity

The selectivity of this method was evaluated by analysing six lots of pooled human serum in comparison with LLOQ samples. No peaks were observed in any of the pooled human serum samples at the retention time of linezolid nor at the retention time of the internal standard. Therefore the pooled human serum is considered as blank.

Ion suppression

No ion suppression was observed by analysing six lots of pooled human serum and simultaneous direct infusion of a stock solution containing linezolid and cyanoimipramine by a syringe pump.

Figure 2: Chromatogram of linezolid (a + c) and cyanoimipramine (internal standard) (a + b)

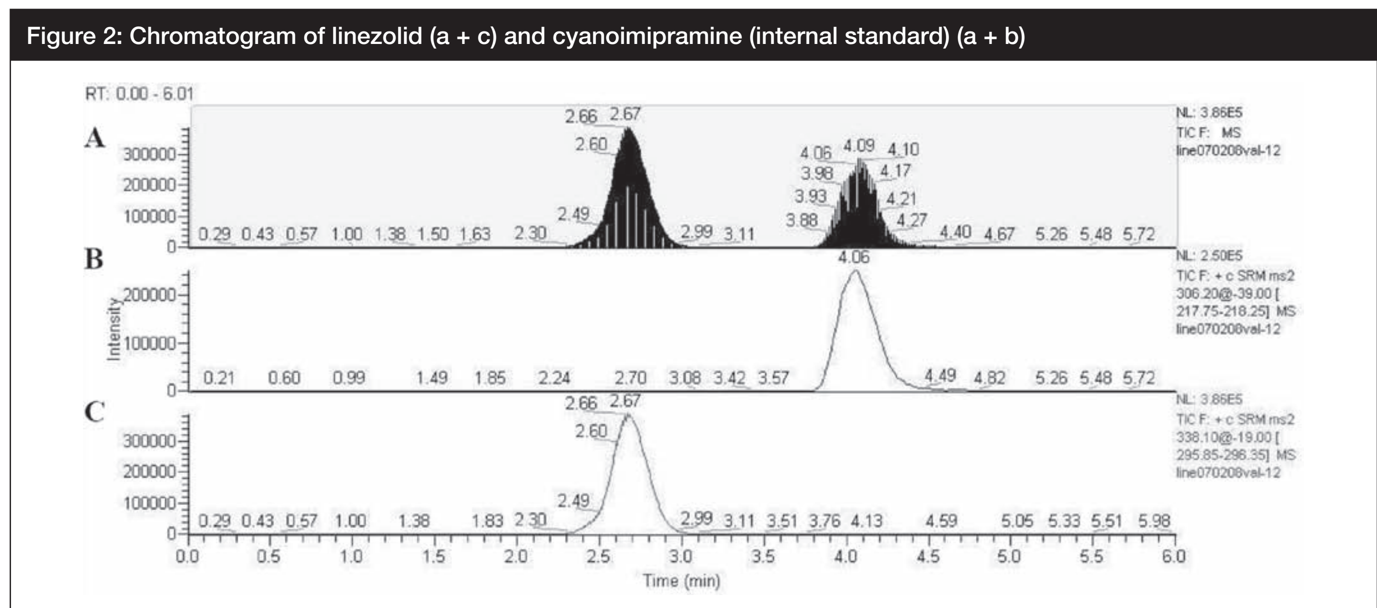


Table 1: Results of precision and accuracy

	QC levels of linezolid			
	LLOQ	**Low**	**Medium**	**High**
Mean concentration (mg/L)	0.05	0.54	15.0	29.0
Bias (%)	8.1	8.5	–0.2	–3.7
Within-run CV (%)	7.1	2.7	2.7	2.5
Between-run CV (%)	1.5	3.3	1.0	0

Key: QC: quality control, LLOQ: lower limits of quantification, CV: coefficient of variation

Recovery
The recovery of linezolid and the internal standard was calculated by comparing the peak heights of low, medium and high control samples in serum (n = 5) with spiked control samples prepared in a mixture of methanol and acetonitrile (4:21 respectively) corresponding to the same concentrations (n = 5). The recovery of linezolid for the low, medium and high control samples was 91.0%, 90.0% and 91.9% respectively. The mean recovery of the internal standard was 85.4%.

Linearity
Over a range of 0.05 to 40.0 mg/L the calibration curve was linear with a mean regression equation of:

$y = 0.590 (\pm 0.00726) x + 0.00220 (\pm 0.00101)$ by using a weight factor of $1/x^2$. The mean correlation coefficient (r) was 0.998.

Accuracy and precision
The accuracy and precision were determined by analysing QC samples fivefold at four concentrations (LLOQ, low, medium and high) on three different days. These results were statistically analysed by using one-way ANOVA (analysis of variation) and are listed in Table 1. The within-run CV was in a range of 2.5% to 7.1% and the between-run CV was in a range of 0% to 3.3%. The mean measured concentration was within 96.3% to 108.5% of the theoretical concentration.

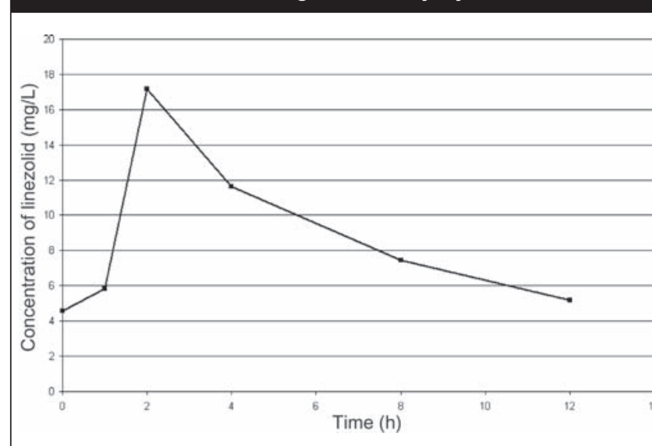
Sample stability
After three cycles of freeze-thaw, the concentration of linezolid was 104.8% of the mean concentration of the fresh prepared samples for QC-level low (CV 2.4%) and 105.5% for QC-level high (CV 2.3%).

In addition, the stability of linezolid in human serum was measured after 36 hours at 4°C, at room temperature and, after sample preparation, in the autosampler. For QC-level low the concentrations of linezolid were 91.5%, 87.7% and 95.5% of the concentration at t = 0, respectively. For QC-level high the concentrations of linezolid were 93.6%, 97.2% and 91.2% respectively.

Pharmacokinetic profile
The measured serum samples from a patient with a linezolid-sensitive nocardia infection are shown in Figure 3. The concentrations of linezolid are between 4.58 mg/L and 17.2 mg/L with a t_{max} of two hours, the AUC was 109.1 The MIC of the nocardia was 0.5 mg/L. The AUC/MIC ratio was 436.4.

Discussion
The aim of this study was to develop a simple and rapid method in which only one calibration curve and no solid phase extraction needed to be used [10]. The results of the validation demonstrated that the method was selective and linear in the range of 0.05 mg/L to 40.0 mg/L. Our results were consistent with the US Food and Drug Administration's *Guidance for Industry Bioanalytical Method Validation*, which states that all values for accuracy and precision should be less than 15%, except for LLOQ, in which case a value of 20% can be accepted [11].

Figure 3: Pharmacokinetic profile after four doses of linezolid 600 mg twice daily by mouth



The tests for determining the stability of linezolid in human serum demonstrated that the linezolid samples are stable for at least three freeze-thaw cycles, for 36 hours at the bench top and in the refrigerator at 4°C and, after sample preparation, in the autosampler.

Because of the possibility of a large concentration range in samples taken from patients, there is a risk of carryover when a low concentration is measured after measuring a sample with a high concentration. Carryover is a problem which cannot be totally eliminated, but it should be reduced to an acceptable range [12]. The reinjection procedure is acceptable, because of the expectation that most of the samples contain a concentration higher than 0.50 mg/L. This was confirmed by the measured serum concentrations from the patient with a linezolid-sensitive nocardia infection.

During the validation, cyanoimipramine was used as internal standard. Cyanoimipramine has the advantage that it is never used as a drug in patients. Nevertheless, other suitable internal standards may also be used for the same method, after complete validation.

Conclusion

In summary, a selective, linear, accurate and precise method, which can be used for the determination of linezolid in human serum, has been developed.

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Conflict of interest

Authors declare no conflict of interest.

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