Development and validation of a high performance liquid chromatography method to determine linezolid concentrations in pig pulmonary tissue

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Abstract

Background: Linezolid is the first synthetic compound of a new group of antimicrobials, the oxazolidinones, which inhibit protein synthesis. It shows a broad spectrum of activity against Gram positive organisms. With respect to its pharmacokinetics, linezolid shows a relatively high volume of distribution and good penetration into inflammatory fluids, bone, fat and muscle.

Methods: A reversed-phase isocratic high-performance liquid chromatographic method for linezolid analysis in piglet pulmonary tissue is described. Tissue samples and controls were prepared in 1X TBE (1 M Tris, 0.9 M boric acid, 0.01 M EDTA). The mobile phase consisted of 20% ultrafiltered water and 80% of (A) 15 mM potassium monohydrogen phosphate buffer (pH = 5) with (B) acetonitrile (80%/20%; v/v). Samples were homogenized and precipitated with HClO4 3% (1/1, v/v). The injection volume was 100 µL. Ofloxacin was used as an internal standard.

Results: The assay was linear over a linezolid concentration range: 1.6–100 µg/mL. The method provided good validation data (n = 15): inaccuracy (3.6%), intra and inter-day variability (4.2% and 5.2%, respectively), recovery (91.8%), limit of detection (0.8 µg/mL) and quantitation (1.6 µg/mL) and acceptable stability within 24 h in the auto-sampler.

Conclusions: The method offers a fast and simple approach to determine linezolid in pulmonary tissue which could be of use in pharmacokinetic studies.

Keywords: analytics; chromatography; high pressure liquid; linezolid; tissue; validation.

Introduction

Linezolid (LNZ) (1) is the first synthetic compound of a new group of antimicrobial drugs, the oxazolidinones (Figure 1) which inhibit protein synthesis early in translation. It shows a broad spectrum of activity against Gram positive organisms including methicillin resistant Staphylococcus aureus (MRSA), penicillin resistant pneumococci and vancomycin resistant Enterococcus faecalis and Enterococcus faecium.

After a dose of 600 mg twice a day, the maximum serum concentration is ~ 20 mg/L. With respect to its pharmacokinetics (PK), linezolid shows a relatively high volume of distribution (40–50 L) and good penetration into inflammatory fluids, bone, fat and muscle (2).

It is well known that antibiotic concentrations at the site of the infection differ greatly from those in plasma. Drug penetration varies depending on the drug, the tissue involved and the infection. Also, protein binding, physical-chemical drug properties, lipid solubility, etc. have some influence on the amount of drug that is able to reach peripheral tissue/compartments. Data on drug penetration for the latest antimicrobial drugs, such as ertapenem, tigecycline, telithromycin or linezolid have recently been published (3–6). Thus, in localized infections including lung infection, meningitis, endocarditis or osteomyelitis, it is extremely valuable to know what fraction of the free drug will be able to cross membranes and barriers and reach the site of the infection. Hence, many reports in the literature have been performed on drug penetration into different tissues. Examples include drug penetration into the epithelial lining fluid (ELF) (7–10) and lung tissue (11).

High performance liquid chromatography (HPLC) can be considered an adequate technique (12) for determining linezolid in pulmonary tissue. The sensitivity and precision of HPLC and its applicability to a wide variety of compounds has led to its use in clinical laboratories for monitoring of a variety of therapeutic agents in hospital settings, and pharmacokinetic and metabolism studies (13, 14).
The goal of this study was to develop a new assay to quantify linezolid in lung tissue obtained from a model of pneumonia in mechanically ventilated piglets treated with linezolid.

Materials and methods

Reagents

Linezolid and ofloxacin, the internal standard (IS), were obtained from Pfizer, S.A. (Madrid, Spain) and Riedel-deHaën Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany), respectively. Perchloric acid 70% PRS and potassium phosphate dibasic, ACS reagent, ≥98% were purchased from Panreac (Barcelona, Spain) and Sigma-Aldrich (Steinheim, Germany). Acetonitrile gradient 240/far-UV-HPLC grade was acquired from Sharlau, S.A. (Barcelona, Spain) and the 10×TBE Buffer Ultrapure (1 M Tris, 0.9 M boric acid, 0.01 M EDTA) from Invitrogen (Paisley, Scotland).

Instrumentation

HPLC was performed using an Agilent 1100 liquid chromatography system (Agilent Technologies Spain, S.L., Madrid, Spain) consisting of a vacuum degasser, a gradient pump, an auto sampler and variable wavelength ultraviolet (UV) detector. Data acquisition was performed using the appropriate software (Agilent Chemstation Rev.B.03.01) for signal treatment.

High-performance liquid chromatography

The HPLC method used in this study is based on the method proposed by Tobin et al. to determine linezolid in plasma (15).

In the present study, the stationary phase was silica based column Novapak® C18 (150×3.9 mm) with pre-column from Waters Corporation (Milford, MA, USA). The mobile phase consisted of 20% ultrafiltered water and 80% of (A) 15 mM potassium monohydrogen phosphate buffer (pH = 5) with (B) acetonitrile (80%/20%; v/v). The flow rate was fixed to 0.7 mL/min and the injection volume after the homogenization process was used Eq. [1] (16).

\[
\frac{((A_{\text{linezolid}}/A_{\text{ofloxacin}})_{\text{molar}} \times V_{\text{real}})}{s} = \frac{s}{n} \times \left(\frac{(L_{\text{linezolid}}/L_{\text{ofloxacin}})_{\text{theoretical}}}{s} \times V_{\text{real}}\right)
\]

where: \(A_{\text{linezolid}}\): linezolid area (mAu); \(A_{\text{ofloxacin}}\): ofloxacin area (mAu); \([L_{\text{linezolid}}/L_{\text{ofloxacin}}]_{\text{theoretical}}\): linezolid theoretical concentration (μg/mL); \([L_{\text{ofloxacin}}]_{\text{theoretical}}\): ofloxacin theoretical concentration (μg/mL); \(s\): slope; \(n\): ordinate at linezolid concentrations equals 0.

Sample collection

Samples were obtained from a model of pneumonia, developed in mechanically ventilated piglets that were treated with linezolid. All tissue samples (standards, quality controls and experimental) were freshly cut into pieces of ~0.5 g, and kept frozen at −40°C until analysis. Prior to analysis, they were homogenized in a manual homogenizer supplied by ANORSA (Barcelona, Spain).

Preparation of standard and quality controls

Buffer solution (1×TBE buffer) A 1×TBE buffer solution was prepared by dilution of 10×TBE (1 M Tris, 0.9 M boric acid, 0.01 M EDTA) in ultrapure water (1/10). This solution was maintained at room temperature.

Stock standard and quality control solutions The same stock solution containing 2 mg/mL of linezolid was used to prepare both standard and quality control material. From this stock solution, three levels of quality control (working standard solutions) at 3.1 μg/mL, 25 μg/mL and 100 μg/mL of linezolid were prepared by appropriate dilutions in 1×TBE. These dilutions were spiked with ofloxacin to achieve a final concentration of 100 μg/mL.

Standard preparation Tissue control specimens (0.5 g) were spiked with the appropriate volume of a stock linezolid solution to attain several standard samples of different linezolid concentrations, and with 70 μL of ofloxacin standard solution (500 μg/mL). Finally, 262.5 μL of 1×TBE was added to obtain a final volume of 350 μL for standards, quality control and unknown samples. These standards were homogenized for 30 s and then extracted and precipitated with 350 μL of HClO₄, 3% (1/1, v/v). Next, they were centrifuged at 8000×g for 10 min. Finally, 100 μL of the supernatant was injected into the chromatographic system.

Sample preparation All tissue samples (0.5 g) were added with 350 μL of 1×TBE spiked with the IS at 100 mg/L. These specimens were homogenized for 30 s, extracted and precipitated with 350 μL of HClO₄, 3% (1/1, v/v). Afterwards, they were centrifuged at 8000×g for 10 min and 100 μL of the supernatant injected into the chromatographic system.

Validation criteria

In accordance with the USA Food and Drug Administration’s Guidance for Industry Bioanalytical Method Validation, and the European Agency for the evaluation of Medicinal Products’ Guideline on Validation of Analytical Procedures, the following seven criteria were evaluated: linearity, accuracy, precision, recovery, limits of
determination and quantitation and stability (17–21). In addition, in this study, quality control samples were analyzed in parallel to establish the suitability of the method.

**Linearity** Calibration curves were constructed by cutting small pieces of control lung tissue (from pigs without antibiotic treatment). A seven-point standard curve of linezolid was constructed by drawing the ratio between the peak area of linezolid and the peak area of ofloxacin vs. the ratio between linezolid concentration and ofloxacin concentration. The concentration of linezolid ranged from 1.6 to 100 μg/mL.

Calculation of the unknown linezolid concentration in tissue samples was performed using the linear regression equation of the peak area ratio against the concentration ratio for the calibration curve. Correction for the tissue experimental weight was taken into account. A standard equation to normalize the weights of pulmonary tissues and the extracted volume after the homogenization process was used Eq. [1] (16).

Linearity was assessed using Pearson’s coefficient (r), the coefficient of correlation (r²), and the (t_critical) for the slope and Pearson’s coefficient.

**Accuracy and precision** Accuracy of an analytical method is defined as the agreement between results obtained by the analytical method to the true value. Precision describes the degree of the similarity (17). The accuracy of the method was performed using a recovery study with 15 drug-spiked samples at three concentrations (100, 25 and 3.1 μg/mL) measured in one run performed in a single day. This allowed us to determine intra-day precision and accuracy.

Analysis of the same drug concentrations over three consecutive days was performed to determine inter-day precision. Accuracy and precision were calculated as follows Eqs. [2, 3], respectively:

\[
\%E_{R} = \frac{[\text{LNZ}_{\text{exp}} - \text{LNZ}_{\text{theo}}]}{\text{LNZ}_{\text{theo}}} \times 100
\]  

where: \(E_{R}\): accuracy; \([\text{LNZ}_{\text{exp}}]\): linezolid theoretical concentration (μg/mL); \([\text{LNZ}_{\text{theo}}]\): linezolid experimental concentration (μg/mL).

\[
\%CV = \frac{(SD/X_{ave})}{X_{ave}} \times 100
\]  

where: CV: coefficient of variation; SD: standard deviation; \(X_{ave}\): average concentration of drug (μg/mL).

For the analysis to be considered acceptable, no more than one-third of the quality control tests were allowed to deviate from the nominal concentration by more than 15%, and at least 50% of the results from each quality control sample had to be within 15% of the nominal concentration.

**Recovery** Recovery was determined by comparison of the analysis between samples with known concentrations of the analyte and extracts, obtained after homogenization of a piece of control pulmonary tissue, spiked with linezolid (plus the IS) at the same concentrations as those prepared in the quality controls. Recovery was calculated as follows:

\[
R(\%) = \frac{A_{\text{LNZ Exp}}}{A_{\text{Oflox Exp}}} \times \frac{A_{\text{LNZ Cont}}}{A_{\text{Oflox Cont}}} \times 100
\]  

where: \(A_{\text{LNZ Exp}}\): linezolid area of the spiked extract (mAu); \(A_{\text{Oflox Exp}}\): ofloxacin area of the spiked extract (mAu); \(A_{\text{LNZ Cont}}\): linezolid area of the control (mAu); \(A_{\text{Oflox Cont}}\): ofloxacin area of the control (mAu).

**Limit of detection (LOD)** Several samples with known concentrations of linezolid (plus the IS) were prepared fresh. The LOD was established by visual evaluation of the minimum concentration at which the linezolid signal can be reliably detected.

**Lower limit of quantitation (LLOQ)** The LLOQ is the lowest amount of analyte in a sample which can be determined with precision and accuracy that is not < ±20%. This parameter was established by analysis of seven samples with known concentrations of linezolid (linearity assay). Bias (Eq.%) and precision (%CV) at the LLOQ were evaluated by a recovery study of five replicates.

**Freeze and thaw stability** Linezolid stability was determined after three freeze (−40°C) and thaw cycles of three drug-spiked samples with concentrations of 100 μg/mL, 25 μg/mL, and 3.1 μg/mL, all measured in one run performed in 1 day.

**Post-preparative stability** The stability of processed samples at room temperature, including the resident time in the auto-sampler was determined using three aliquots with different concentrations. We compared the results obtained with fresh sample with those obtained with these same samples after 24 h (or real work time) in the auto-sampler.

The non-parametric Wilcoxon test was used to compare results and \(p<0.05\) was used for statistical significance. Statistical analysis was performed with SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

**Pig pulmonary tissue samples** Lung tissue samples from a piglet model of pneumonia (22) were analyzed in order to determine linezolid concentrations. Animals were treated with 10 mg/kg (mean dose: 350 mg/12 h) of linezolid twice daily (1) for 96 h, or until death. At euthanasia (at least 12 h after the last dose of linezolid), pulmonary tissue was obtained from several lung segments and kept frozen at −40°C until analysis. Tissue specimens (0.5 g) were spiked with 70 μL of ofloxacin standard solution (50 μg/mL). Finally, 280 μL of 1×TBE was added to obtain a final volume of 350 μL for all samples. These samples were homogenized for 30 s, extracted and precipitated with 350 μL of HClO₄ 3% (1/1, v/v). Afterwards, samples were centrifuged at 8000×g for 10 min. Finally, 100 μL of the supernatant was injected into the chromatographic system.

**Results**

**HPLC assay** Linezolid and the IS were quantitatively extracted from pulmonary tissue after homogenation and precipitation. Under the experimental conditions described in the previous section (Materials and methods: High-performance liquid chromatography), linezolid and the IS were eluted within the retention windows of 11–12 min and 5–6 min, respectively. Representative chromatograms of blank tissue and quality control samples are shown in Figure 2A, B.

**Assay validation** The correlation between drug concentration and peak area was excellent for TBE in tissue across the concentration
range studied (from 1.6 μg/mL to 100 μg/mL). Pearson coefficient (r) and coefficient of correlation (r²) were calculated: r=0.9978 and r²=0.9958. The regression line (y=0.0052 + 2.4395x) was not significantly different from the unity line (p>0.05). Inaccuracy, precision and recovery were assessed by analyzing quality control samples. Results are shown in Table 1.

Table 1 Results for validation parameters.

<table>
<thead>
<tr>
<th>Linezolid</th>
<th>3.1 μg/mL</th>
<th>25 μg/mL</th>
<th>100 μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-day inaccuracy (Eₚ%)(n=5)</td>
<td>-5.1</td>
<td>-7.5</td>
<td>2.1</td>
</tr>
<tr>
<td>Inter-day inaccuracy (Eₚ%)(n=15)</td>
<td>-4.8</td>
<td>-9.7</td>
<td>3.7</td>
</tr>
<tr>
<td>Intra-day precision (CV%)(n=15)</td>
<td>5.1</td>
<td>4.8</td>
<td>2.5</td>
</tr>
<tr>
<td>Inter-day precision (CV%)(n=15)</td>
<td>6.6</td>
<td>6.2</td>
<td>2.8</td>
</tr>
</tbody>
</table>

The LOD was 0.8 μg/mL. The LLOQ was 1.6 μg/mL (bias and precision: Eₚ% = -7.6% and CV% = 7.8%, respectively). Typical chromatograms of the LLOQ are shown in Figure 3A, B.

The mean recovery, calculated from the ratio of the areas between linezolid and the IS (A.LNZ/A.Ofloxo), remained fairly constant (93.6%, 92.5% and 89.5%) at three different concentrations of linezolid (100, 25 and 3.1 μg/mL, respectively). This confirms the use of ofloxacin as an IS.

Results from the stability analysis revealed no loss of linezolid after three freeze (−40°C) and thaw cycles. Results are
shown in Table 2. The IS (ofloxacin) showed degradation after 3 h at room temperature. Thus, quantification ($A_{\text{LNZ}}/A_{\text{IS}}$) was not possible (Table 3). However, no significant differences were seen in the linezolid signal ($A_{\text{LNZ}}$) after maintaining linezolid samples at room temperature for 24 h: ($A_{\text{LNZ}}$)$_{0\ h}$ = 10,331 mAu vs. ($A_{\text{LNZ}}$)$_{24\ h}$ = 9,910 mAu at 100 μg/mL; ($A_{\text{LNZ}}$)$_{0\ h}$ = 1,623 mAu vs. ($A_{\text{LNZ}}$)$_{24\ h}$ = 1,538 mAu at 25 μg/mL and ($A_{\text{LNZ}}$)$_{0\ h}$ = 225 mAu vs. ($A_{\text{LNZ}}$)$_{24\ h}$ = 198 mAu at 3.1 μg/mL.

Pig pulmonary tissue samples

Lung samples (from eight piglets treated with linezolid for 72–96 h) and controls (pulmonary tissues from piglets without treatment) were collected. One sample was obtained 72 h after starting treatment with linezolid (spontaneous death) and 12 h from the last dose. All others were collected at euthanasia after 96 h of treatment, and 12 h after the last drug dose. All were cut in pieces of ~0.5 g and immediately frozen at −40°C until analysis.

Mean (± standard deviation) minimum linezolid concentrations in lung tissue ($n=8$) were $11.0±5.1$ mg/L.

Discussion

A specific HPLC method with adequate specificity, sensitivity, precision and accuracy has been developed to measure linezolid in lung tissue. This method allows rapid determination of linezolid concentrations in pulmonary tissue, offering a highly simplistic approach to sample preparation. It is accurate, reproducible and specific for linezolid.

Several examples of extraction and quantitation of antibiotics in different tissues have been described in the literature. The majority of them are based on homogenization using a homogenator or sonication system as the best technique to extract the drug from the tissue (23–26). Afterwards, samples are centrifuged, washed by filter or solid phase extraction (SPE), evaporated and re-diluted in an
Table 2 Results from the stability analysis of linezolid in lung tissue after three freeze (−40°C) and thaw cycles.

<table>
<thead>
<tr>
<th>Linezolid</th>
<th>ALNZ/AlOflox at baseline</th>
<th>ALNZ/AlOflox after 3 cycles</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 g/mL</td>
<td>2.18</td>
<td>2.17</td>
<td>0.29</td>
</tr>
<tr>
<td>25 g/mL</td>
<td>0.37</td>
<td>0.38</td>
<td>0.18</td>
</tr>
<tr>
<td>3.1 g/mL</td>
<td>0.05</td>
<td>0.05</td>
<td>0.56</td>
</tr>
</tbody>
</table>

ALNZ/AlOflox, linezolid area/ofloxacin area; p < 0.05 to be significant.

Table 3 Results of stability of ofloxacin.

<table>
<thead>
<tr>
<th>Time</th>
<th>Ofloxacin area, mAu x s</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 h</td>
<td>14,079</td>
</tr>
<tr>
<td>30 min</td>
<td>14,271</td>
</tr>
<tr>
<td>1 h</td>
<td>13,789</td>
</tr>
<tr>
<td>2 h</td>
<td>13,846</td>
</tr>
<tr>
<td>2.5 h</td>
<td>14,195</td>
</tr>
<tr>
<td>3 h</td>
<td>14,027</td>
</tr>
<tr>
<td>3.5 h</td>
<td>NA*</td>
</tr>
<tr>
<td>4 h</td>
<td>NA*</td>
</tr>
<tr>
<td>24 h</td>
<td>NA*</td>
</tr>
</tbody>
</table>

*NA, not available. (Double peak).

appropriate mobile phase. With respect to linezolid, there are several HPLC methods that have been published in the literature for measuring this drug in serum or plasma (15, 27–30). However, there are only a few HPLC methods for measuring linezolid quantification in tissues. One is the procedure described by De Jesús et al. (31). They propose an HPLC technique in which 1 g of pulmonary tissue is homogenized in a buffer solution (KH₂PO₄, Na₂HPO₄·12H₂O/0.1% Triton x-100 (pH 7.4)), centrifuged, and 100 µL of supernatant is injected into the chromatographic system. In this method, the column temperature is maintained at 28°C under isocratic conditions. The quantitation limit is 0.25 mg/L and inprecision is <10%. It is noteworthy to consider that these data are similar to our results given that, in our study, the LOD and LLOQ are 0.8 µg/mL and 1.56 µg/mL, respectively. Also, the intra-day and inter-day precision is <6.5% across the entire range of concentrations studied.

Our data are consistent with the results reported by Honeybourne et al. (11). They analyzed linezolid in several tissues including bone, fat and muscle, but not lung, by homogenizing the tissue with acetonitrile. Afterwards, these samples were centrifuged and the supernatant injected into the chromatographic system. The LLOQ is set at 0.1 mg/L and the intra-day and inter-day coefficient of variation was <6.0% and 12.5%, respectively. Our study using pulmonary tissue shows much better results than those reported previously. In our study, intra-day precision (five replicates at three different concentrations) is <5.5% in all cases, and the inter-day precision (five replicates analyzed in three consecutive days) is <6.5%. In our study, the LLOQ was no <1.56 µg/mL. However, it should not have any impact in terms of clinical significance since this value is below the minimum inhibitory concentration of linezolid for MRSA.

Moreover, data from another study (32) which treated lung samples with a mixture of methanol/water (1:1) and lysing buffer, showed results similar to ours with respect to recovery values: 96% and 103% vs. 89.47% and 93.58% in our study.

Our data show lower minimum linezolid concentrations in pulmonary samples than those reported by Luna et al. (33). However, the lack of details on the methodology used to determine linezolid concentrations in pulmonary tissue in their study, and the fact that they used a different dose regimen (300 mg/8 h), makes it very difficult to compare both studies.

A possible limitation of the present study could be the clinical interpretation of the drug concentrations in homogenates related to antibiotic levels at the site of infection. Linezolid shows a low binding protein (31%), and good penetration into skin, blister fluids, bone, muscle, fat, alveolar cells, lung extracellular lining fluid and cerebrospinal fluid (2, 34). It is well known that concentrations in tissue may depend on (i) the amount of blood in the tissue sample, (ii) chemical degradation of the drug during processing and (iii) sample collection. Blood present in the tissue or in its surface is included in the final homogenate. In order to reduce possible bias due to this fact, an adjustment by weight of the real analyzed portion of lung and the extracted volume after homogenation were both taken into account in this analysis. With respect to chemical degradation, calibration with IS (ofloxacin) was used to control any loss of specimen during the analysis. In addition to circumvent any artifact or loss of the drug during sample preparation, all standards were prepared in the appropriate tissue homogenate (lung homogenate). To avoid the drawback related to sample collection, all tissue samples were taken when the distribution phase of the drug had been completed and equilibrium between the vascular space and tissue was complete.

Homogenization implies disruption of cell membranes and mixing of both intracellular and extracellular fluids and solids. However, in this particular case a minor dilutional effect on linezolid concentration could be expected after tissue homogenization since the drug is distributed primarily in the extracellular space. However, one may hypothesize that linezolid concentrations in the biophase might be slightly higher than those observed following tissue homogenization.

The availability of an HPLC method to determine antibiotic concentrations, both in serum and lung, provides a unique opportunity to gain insight into relevant aspects regarding the clinical response to specific treatments. Studies in animal models of pneumonia, where it is possible to obtain lung tissue, offer an exceptional opportunity to learn about the pharmacokinetics and pharmacodynamics (PD) of antibiotics. The combination of PK and PD data might help to further implement optimal antibiotic dosing regimens in humans.

In conclusion, the HPLC assay we developed and reported in this study allows for fast, easy, reliable and cheap analysis of linezolid in pulmonary tissue. Linezolid analysis, per-
formed using an isocratic mode, is completed within 12 min. This method might be helpful to develop future pharmacokinetic studies of linezolid penetration in pulmonary tissue.

Conflict of interest statement

Authors’ conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research funding played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

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