Introduction

Linezolid (PNU-100766), an oxazolidinone, is a new antibacterial agent recently licensed in Europe and the USA. It has a unique mode of action, inhibiting protein synthesis early in translation. As a result, cross-resistance with other antimicrobial agents is believed to be unlikely. The drug has 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*. Linezolid is available in both iv and oral formulations. Early clinical data indicate that linezolid 600 mg bd is as effective as vancomycin 1 g bd for the treatment of MRSA and hospital-acquired pneumonia. Linezolid peak concentration of 18 mg/L (1–2 h post-dose) and a minimal serum concentration of 4 mg/L after 625 mg bd can be expected. With all new drug therapies it is important to have an assay to individualize therapy, for pharmacokinetic studies and to investigate any possible drug interactions. The aim of this study was to develop such an assay.

Materials and methods

Antibacterial agents

These were as follows: amikacin (Bristol-Myers Squibb, Syracuse, NY, USA); amoxicillin, ampicillin, azlocillin, cefotaxime, cefuroxime, cycloserine, chloramphenicol, gentamicin, norfloxacin, rifampicin, sulfamethoxazole and tobramycin (Sigma, Steinheim, Germany); ceftazidime and aciclovir (GlaxoSmithKline, Greenford, UK); ciprofloxacin and moxifloxacin (Bayer AG, Wuppertal, Germany); flucloxacillin (GlaxoSmithKline, Welwyn Garden City, UK); 5-flucytosine and ganciclovir (Roche, Welwyn Garden City, UK); linezolid (Pharmacia, Kalamazoo, MI, USA); meropenem (AstraZeneca, Södertälje, Sweden); netilmicin (Schering-Plough, Kenilworth, NJ, USA); ofloxacin (Aventis Pharma, West Malling, UK); penicillin-G...
Indianapolis, IN, USA). (Britannia, Redhill, UK); and vancomycin (Eli-Lilly, Indianapolis, IN, USA).

**Solvents**

Acetonitrile, methanol and water (Chromanorm grade) were purchased from Prolabs (Fontenay, France); 1-heptane sulphonic acid was supplied by Sigma; ortho-phosphoric acid and sodium hydroxide by BDH (Analar grade; Poole, UK).

**High-performance liquid chromatography (HPLC)**

The stationary phase was Hypersil 5ODS, 10 cm × 4.6 mm (Waters Corporation, Milford, MA, USA).

The mobile phase was 1% ortho-phosphoric acid, 30% methanol, 2 g/L heptane sulphonic acid, adjusted to pH 5 by the addition of 10 M sodium hydroxide. The pump flow rate was 1.0 mL/min. UV absorbance detection was used (λmax 254 nm). A Gina 50 autosampler was used (Dionex, Macclesfield, UK) and the integrator was a Trilab 2000 (Trivector, Sandy, UK).

**Sample preparation**

Samples were prepared by mixing aliquots (50:50) of the specimen with acetonitrile. The samples were mixed, allowed to rest at ambient temperature for 10 min and centrifuged at 5000 g for 5 min. Twenty microlitres of the supernatant was injected.

**Reproducibility**

Mean peak heights ± S.D. (x ± S.D.) and percentage coefficients of variation (% CV) were calculated. The mean peak height was plotted against linezolid concentration and the percentage recovery calculated [(mean aqueous peak height/mean serum peak height) × 100].

**Linearity**

Linearity was determined with serum samples spiked with 0.0, 0.5, 1.5, 10, 15, 20 and 30 mg/L linezolid. The samples were assayed (n = 6) and peak height measured. The mean peak height was plotted against linezolid concentration and the percentage recovery calculated [(mean aqueous peak height/mean serum peak height) × 100].

**Recovery**

This was investigated with serum and aqueous samples spiked with 0.0, 0.5, 1.5, 10, 15, 20 and 30 mg/L linezolid. The samples were assayed (n = 6) and peak height measured. The mean peak height was plotted against linezolid concentration and the percentage recovery calculated [(mean aqueous peak height/mean serum peak height) × 100].

**Lowest limit of quantification**

The lowest limit of quantification is defined as the concentration equivalent of a peak approximately three times the height of the baseline noise. A linezolid serum sample was prepared to approximately this concentration. The sample was assayed (n = 3) and peak height ± S.D. and % CV calculated.

**Specificity**

The assay was evaluated for any possible interference from other antimicrobial, antifungal and antiviral drugs that may be co-administered with linezolid. The antimicrobial agents for assessment were prepared to contain clinically appropriate concentrations. After sample preparation, the following were assayed: aciclovir 20 mg/L, amikacin 53 mg/L, ampicillin 20 mg/L, amoxicillin 26 mg/L, azlocillin 58mg/L, benzyl-penicillin 20 mg/L, cefotaxime 46 mg/L, cefuroxime 14 mg/L, chloramphenicol 19.4 mg/L, ciprofloxacin 2.6 mg/L, cycloserine 26.2 mg/L, fluocoxacin 74 mg/L, 5-flucytosine 67 mg/L, ganciclovir 20 mg/L, gentamicin 5.7 mg/L, meropenem 15 mg/L, moxifloxacin
HPLC assay for linezolid in human serum

5 mg/L, netilmicin 0.7 mg/L, norfloxacin 5 mg/L, ofloxacin 5 mg/L, rifampicin 5 mg/L, sulfamethoxazole 115 mg/L, tobramycin 9.8 mg/L, trimethoprim 8 mg/L and vancomycin 59.5 mg/L.

The method was also assessed for any interference from unknown endogenous compounds in patient sera. Twelve linezolid-free serum samples (sent to our laboratory for antibiotic assay) were selected at random. The samples were prepared and assayed.

Accuracy using single point calibration

The accuracy of the method was evaluated by the preparation and assay \( n = 6 \) of serum samples spiked to contain 2.5, 8 and 18 mg/L linezolid using a 12 mg/L serum calibrator.

Results

Assay validation

The separated linezolid peak is shown in Figure 1. Assay reproducibility was: intra-day <6%; inter-day <12.5% (Table). The correlation between drug concentration and peak height was good for both aqueous and serum samples across the concentration range \( r = 0.9999 \) for both; Figure 2). Linezolid recovery from serum approached 100% at all concentrations tested. The lowest limit of quantification was calculated to be 0.1 mg/L. The accuracy, expressed as the percentage error \( 100 \times \text{(measured concentration} - \text{target concentration)})/\text{target concentration} \] was 4.0 for the 2.5 mg/L sample, 1.3 for the 8 mg/L sample and 0.0 for the 18 mg/L sample. There was no interference from 23 commonly used antimicrobial agents or from any unknown compound present in linezolid-free patient sera. Benzyl-penicillin eluted close to linezolid. However, this was not considered to be significant since benzyl-penicillin 20 mg/L resulted in a peak equivalent to linezolid 0.4 mg/L. Furthermore, benzyl-penicillin can be removed by \( \beta \)-lactamase.

Drug stability

Linezolid was stable in serum, after sample preparation with acetonitrile, at room temperature over a 24 h period (Figure 3). The drug was stable in serum alone for at least 7 days at both room temperature and 4°C.

The final mean drug concentrations were all c. 100% of the original, apart from 0.4 mg/L kept at 4°C for which the final mean drug concentration was only 86.2% of the original. This was a somewhat aberrant result, particularly since the 0.4 mg/L serum sample kept at room temperature

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<th>Table. Linezolid intra- and inter-day serum reproducibilities assessed by % CV calculations</th>
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<tr>
<td>Linezolid serum concentration (mg/L)</td>
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was stable over the same time period. No drug degradation was found after two heat treatment cycles. The linezolid concentrations were c. 100% of the original concentrations after the first and second cycles. There was little drug loss after two freeze–thaw cycles. The linezolid concentration was 96.4% of the original concentration after the first cycle and 91.7% after the second. Linezolid was stable after storage for 1 year at −70°C; the measured drug concentrations were c. 100% of the weighed-in concentrations.

Discussion

We have demonstrated linezolid to be stable in serum in the short term (≤7 days) at room temperature and at 4°C. This means that samples can be transported without having to use ice or liquid CO₂. The drug is also stable for up to 24 h after treatment with acetonitrile. This means that samples can be prepared and assayed in a batch within a 24 h time period without loss of antibiotic content. Linezolid was also stable after heat treatment. The drug was subjected to two such cycles. Serum samples from high-risk patients may therefore be heat treated before forwarding to a reference laboratory and again upon receipt. Linezolid was stable after storage at −70°C for up to 1 year and after two freeze–thaw cycles. Batches of samples can, therefore, be stored in the long term at −70°C.

The assay is reproducible, accurate and linear across the concentration range. Linezolid was fully recoverable from serum. The lowest limit of detection (0.1 mg/L) is somewhat lower than the MICs for control organisms (0.5–4 mg/L; Linezolid Technical Information, unpublished). The only published HPLC method for linezolid has been validated for dog, mouse, rabbit and rat plasma. This method uses a time-consuming and complicated sample preparation step using solid-phase extraction. With our method, sample preparation is quick and simple, and up to 150 assays can be performed before there is a change in chromatographic performance. With a retention time of only 6.5 min, it is ideal for the processing of clinical samples.

Acknowledgement

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References


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Figure 3. Peak height measurements for 0.4 (●), 25 (■) and 45 (▲) mg/L serum linezolid (after preparation with acetonitrile 50:50) plotted over a 24 h period.