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Journal of Pharmaceutical and Biomedical Analysis
35 (2004) 847–851
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Simple method for the assay of eperezolid in Brain Heart Infusion broth by high-performance liquid chromatography

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Received 1 April 2003; received in revised form 29 December 2003; accepted 13 February 2004

Available online 15 April 2004

Abstract

A sensitive high-performance liquid chromatography (HPLC) method was developed and validated for quantification of eperezolid in Brain Heart Infusion (BHI) broth. Linezolid was employed as internal standard and sample deproteinization with methanol was used. Calibration standards ranged from 0.10 to 20 mg/l. Recovery was approximately 100% at the concentrations examined. Eperezolid was stable in the autosampler vial for at least 72 h at ambient temperature and in BHI broth for 72 h at 37 °C. The intra- and inter-day accuracy and reproducibility (relative standard deviation, R.S.D.) were less than 12.3%. This assay is rapid and ideal for analysis of a large number of samples.

Keywords: Eperezolid; HPLC; Brain Heart Infusion broth

1. Introduction

Eperezolid (Fig. 1, PNU-100592) is one of the oxazolidinones, a class of novel synthetic antimicrobials with demonstrated bacteriostatic activity against multidrug-resistant Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) [1], vancomycin-resistant enterococci (VRE) [2] and penicillin-resistant pneumococci (PRPN) [3]. Eperezolid binds specifically to the 50S ribosomal subunit at the initial phase of protein synthesis and has no effect on the formation of *N*-formylmethionyl-tRNA, elongation, or termination reactions of translation in bacteria [4,5]. Its significant efficacy has been confirmed in vitro [3,6–9] and in animals [10]. Oxazolidinones do not appear to exhibit cross-resistance with other antibiotics [11,12].

Currently most investigations on oxazolidinones focus on linezolid. Although there are several high-performance liquid chromatographic (HPLC) methods published for linezolid [13–16], there have been none that apply to eperezolid. In this paper a simple HPLC method was developed to quantify eperezolid in Brain Heart Infusion (BHI) broth for in

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doi:10.1016/j.jpba.2004.02.018

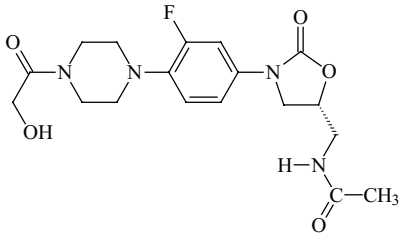

Fig. 1. Structure of eperezolid.

vitro pharmacodynamic studies that may be used to define dosing strategies of eperezolid against emerging multidrug-resistant Gram-positive pathogens.

2. Experimental

2.1. Apparatus

The HPLC system comprised of a LC-10AT*vp* HPLC pump, a DGU-12A degasser, a SIL-10AD*vp* autoinjector, a CTO-10AC*vp* column oven, a SPD-M10A*vp* photodiode-array ultraviolet spectrophotometric detector and a SCL-10A*vp* controller (Shimadzu, Kyoto, Japan) connected to a multi-instrument data acquisition and data processing system (Class-*VP* 6.12 SP2, Shimadzu).

2.2. Chemicals and reagents

Eperezolid and the internal standard linezolid (PNU-100766) were generously supplied by R&D Global Distribution Center of Pharmacia Corporation (MI, USA). Acetonitrile, methanol (Biolab, Vic., Australia) and acetic acid (BDH, Poole, UK) were of HPLC grade. Trichloroacetic acid (analytical grade) was obtained from Sigma-Aldrich (MO, USA). Brain Heart Infusion broth was purchased from Oxoid Australia (West Heidelberg, Vic., Australia). Water was purified by a Millipore Milli-Q system (Millipore Corp., MA, USA).

Two separate eperezolid stock solutions, one for preparation of calibration curve samples and one for quality control samples, were prepared in water at a concentration of 1.0 g/l. From these, two corresponding sets of working solutions were prepared by serial dilution to generate concentrations of 100, 10 and 1.0 mg/l. The solutions were stored at −20 °C. Linezolid (internal standard) was prepared at a concentration of 0.10 g/l in water.

2.3. Sample preparation and HPLC assay

In a 1.5 ml centrifuge tube, 200 μl BHI broth was mixed with 20 μl internal standard (0.10 g/l) and 200 μl methanol was added to precipitate proteins. The contents of the tube were vortex-mixed for 20 s and centrifuged (10,000 × *g*, 10 min). An aliquot (200 μl) of the supernatant was transferred into an HPLC autosampler vial and 20 μl was injected onto a PhenoSphere-*NEXT* C_{18} column (5 μm, 250 mm × 4.6 mm, Phenomenex, NSW, Australia) preceded by a C_{18} guard column (5 μm, 4.0 mm × 3.0 mm, Phenomenex), both of which were contained in the column oven set at 25 °C. The mobile phase consisted of acetonitrile and 2.67 mM acetic acid (25:75, v/v) and was pumped at 1.0 ml/min. The column eluent was monitored for ultraviolet absorbance (signal collected from 190 to 300 nm and processed at 253 nm) and the run time was 10 min.

2.4. Linearity, reproducibility and accuracy

Appropriate volumes of the standard solutions of eperezolid in water (1.0, 10.0 and 100 mg/l and 1.0 g/l) were used to prepare calibration standards in drug-free BHI broth at concentrations of 0.10, 0.50, 1.0, 2.0, 4.0, 8.0, 10 and 20 mg/l. A linear calibration curve was constructed from the relationship between the ratios of the peak area of eperezolid to that of the internal standard and concentrations of eperezolid by using least squares linear regression analysis with a weighting of $1/y$. Reproducibility and accuracy were assessed by (1) the intra-day assay with six separate analyses of quality control samples containing eperezolid at 0.30 and 15 mg/l in BHI broth; and (2) the inter-day assay with three separate analyses of the same quality control samples on separate occasions. The quality control samples were spiked using independently prepared aqueous solutions of eperezolid, as described above. The limit of quantification was determined by measuring the concentration of eperezolid in six samples of BHI broth prepared independently from the calibration standards at a concentration of 0.10 mg/l.

2.5. Recovery

Recovery was investigated with BHI broth and aqueous samples spiked with 1.0, 10 and 20 mg/l eperezolid. An equal volume (200 μl) of methanol, acetonitrile or 5% trichloroacetic acid was added separately ($n = 3$). All the other pre-treatment procedures and HPLC assay conditions were as described above. Recovery was calculated as [(peak area for broth)/(peak area for water)] × 100%.

2.6. Stability of eperezolid in BHI broth at 37 °C and in autosampler vials at ambient temperature

In vitro pharmacodynamic experiments are conducted in BHI broth for incubation periods of up to 72 h and samples collected are analysed in batch mode after completion of the experiment. Thus, the stability of eperezolid under these conditions was investigated. Autoclaved BHI broth (5 ml) was spiked with 1.0 and 10 mg/l eperezolid separately in sterile 10 ml tubes which were placed in a shaking water bath (37 °C, 30 rpm) for 72 h. Aliquots (0.5 ml) were removed aseptically at 0, 24, 48 and 72 h and immediately stored at −20 °C for 7 days pending analysis. All the analyses were performed in duplicate. In a separate study, the stability of eperezolid in HPLC autosampler vials at ambient temperature was investigated by re-assaying the same samples at calibration curve concentrations of 1.0, 10 and 20 mg/l 72 h after the first injection. The autoinjector septa were replaced immediately after the first injection in order to prevent the effect from evaporation. All the analyses were performed in triplicate.

3. Results

Fig. 2 illustrates typical chromatograms obtained for the BHI broth containing 8.0 mg/l eperezolid and a blank sample. Eperezolid and internal standard eluted at approximately 4.1 and 8.7 min, respectively. The calibration curves were linear within the concentration range assayed with high coefficients of determi-

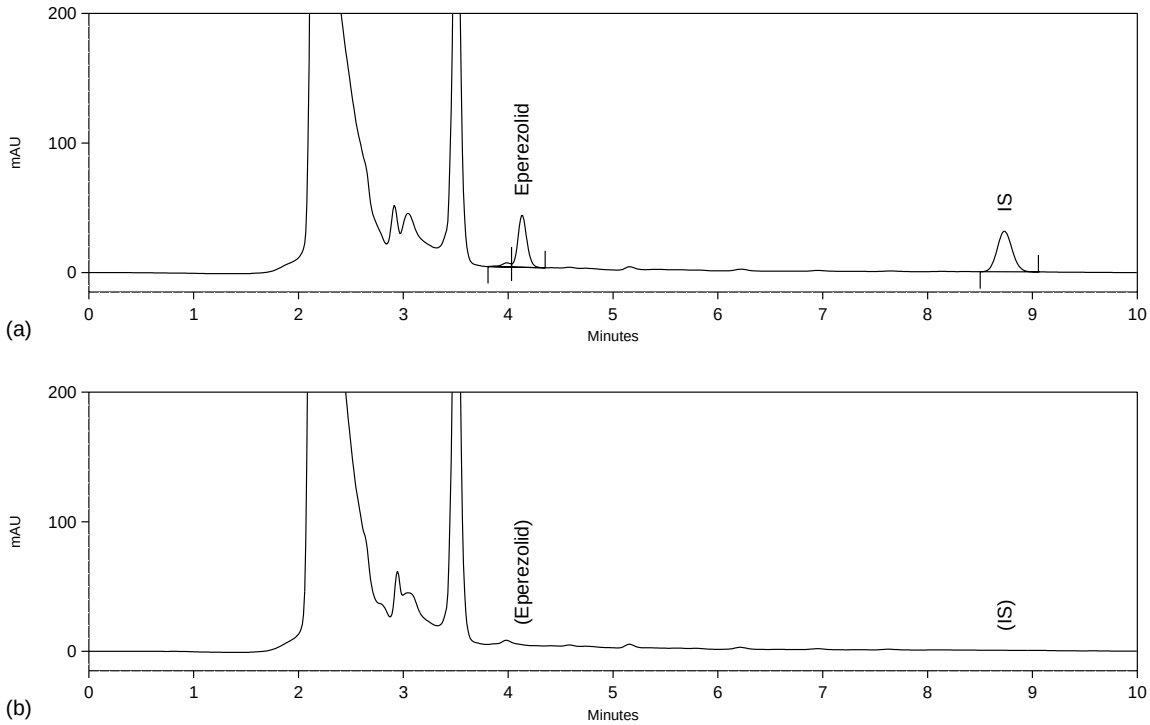

Fig. 2. Typical chromatograms obtained for a sample containing (a) 8.0 mg/l eperezolid and (b) blank BHI. For experimental details, see text.

Table 1
Accuracy and reproducibility for the assay of eperezolid in BHI broth

	Intra-day ($n = 6$)		Inter-day ($n = 3$)	
	0.30 mg/l	15 mg/l	0.30 mg/l	15 mg/l
Mean concentration (mg/l)	0.32	15.0	0.30	15.2
CV (%)	3.7	0.4	7.5	1.8

Table 2
Recoveries (±S.D., $n = 3$) of eperezolid

Concentration (mg/l)	Percentage recovery		
	Methanol	Acetonitrile	Trichloroacetic acid
1.0	104.7 ± 1.2	99.1 ± 4.8	104.2 ± 3.0
10	103.7 ± 1.0	107.0 ± 8.1	105.7 ± 1.0
20	103.8 ± 0.5	102.1 ± 4.5	102.6 ± 0.7

nation ($r^2 > 0.9987$). The linear regression equation for the relationship between ratios of peak area of eperezolid to that of the internal standard and concentrations of eperezolid had a mean (±S.D.) slope of 0.0928 ± 0.0008 and an intercept of 0.0055 ± 0.0008 ($n = 3$). The intra- and inter-day accuracy and reproducibility for the assay are presented in Table 1. The limit of quantification was 0.10 mg/l, at which concentration the mean value measured was 0.088 mg/l and the relative standard deviation (R.S.D.) 12.3% ($n = 6$). Sample pre-treatment with methanol, acetonitrile or 5% trichloroacetic acid gave approximately 100% recoveries (Table 2). After storage in BHI broth at 37 °C for 72 h, 105.7 and 110.8% of eperezolid remained in the broth at the concentrations of 1.0 and 10 mg/l, respectively. Stability of eperezolid was also studied in autosampler vials at ambient temperature. The samples at 1.0, 10 and 20 mg/l stored at ambient temperature after 72 h gave 96.9 ± 0.8, 100.2 ± 0.1 and 102.4 ± 0.1% ($n = 3$) of the UV responses at time zero, respectively.

4. Discussion

This is the first report documenting an HPLC assay for the quantification of eperezolid in a microbiological growth medium, BHI broth. BHI broth is one of the most common growth media used for in vitro pharmacodynamic modelling and other experiments which are important preclinical studies for development of novel antimicrobials targeting staphylococci, meningococci, pneumococci, streptococci and other fastidious organisms. Since the minimum inhibitory concentrations of eperezolid against methicillin-susceptible and resistant *S. aureus* range between 1.0 and 8.0 mg/l [17], concentrations of 0.10–20 mg/l were chosen for the calibration curves for analysis of samples from in vitro pharmacodynamic studies. The method for the quantification of eperezolid in BHI broth is simple, sensitive, reproducible and accurate. To obtain the highest sensitivity, 253 nm was chosen as the detection wavelength. A short run time of 10 min is ideal for the assay of large numbers of samples from in vitro models. There were no chromatographic interferences of eperezolid and internal standard in BHI samples after inoculation of a reference strain of *S. aureus* NCTC 6571 at 37 °C for 72 h (data not shown). Good linearity was achieved within the concentration range examined.

Sample pre-treatment was required prior to chromatographic analysis because BHI broth contains proteins. In preliminary assay development, the recoveries of eperezolid were evaluated by three different pre-treatment methods, i.e. using methanol, acetonitrile or 5% trichloroacetic acid for deproteinization. There were no differences among these three different pre-treatment procedures in the recovery of eperezolid (approximately 100% relative to corresponding water samples pre-treated in the same way). However, aqueous and BHI broth solutions of eperezolid treated with acetonitrile or trichloroacetic acid gave approximately 25 and 90%, respectively, of the UV response obtained by using methanol (data not shown), even though the recovery from BHI broth relative to the aqueous solution was essentially 100% with all three systems. The reason for the substantially lower UV response with acetonitrile treatment is unknown.

Because in vitro pharmacodynamic studies involve incubation of eperezolid in media for up to 72 h, it is essential to investigate its stability at 37 °C, the temperature used in the in vitro pharmacodynamic experiments. Eperezolid was demonstrated to be stable for up to 72 h under such conditions. The determination of concentrations slightly higher than 100% of starting concentration is most likely due to small evaporative

loss of broth liquid during the 72 h storage at 37 °C (mimicking the conditions in microbiological culture bottles during incubation in an actual experiment). The stability of eperezolid in the HPLC injector vials at ambient temperature suggested that assays can be run in batch mode over an HPLC run time of 72 h without errors from instability within the vials.

To conclude, a simple and sensitive HPLC method is described for the determination of eperezolid in BHI broth. Validation results proved that it was accurate and reproducible. The simple pre-treatment procedure and short run time facilitate analysis of large numbers of samples in in vitro pharmacodynamic studies of eperezolid.

Acknowledgements

We are grateful to Pharmacia Australia for supporting this study and the R&D Global Distribution Center of Pharmacia Corporation, MI, USA, for providing supplies of linezolid and eperezolid.

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