Farm Hosp. 2010;**34(1):**27–31

Farmacia
HOSPITALARIA

www.elsevier.es/farmhosp

BRIEF REPORT

Measuring linezolid in biological fluids using high-efficiency liquid chromatography

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Received June 3, 2009; accepted July 28, 2009

KEYWORDS
Cerebrospinal fluid;
High-performance liquid chromatography;
Linezolid;
Pharmacokinetics;
Plasma;
Vitreous humour

Abstract
Objective: Evaluation of an analytic method for determining linezolid concentrations in biological fluids including plasma, vitreous humour and cerebrospinal fluid using high-efficiency liquid chromatography and subsequent ultraviolet detection.
Method: The method was validated by studying the following parameters: accuracy, precision, sensitivity, linearity and recovery. The drug was extracted from the biological matrix by means of a protein precipitation with perchloric acid. Chromatographic separation was performed by eluting linezolid with a mobile phase consisting of 80% K_2HPO_4 buffer solution (15 mM; pH=5) and 20% acetonitrile, and a stationary phase, NOVAPAK C18 150x3.9 mm with precolumn. The wavelength reading was 254 nm and the working flow rate was 1 ml/min.
Results: We obtained values with accuracies between 94.4% and 106.1%, and precisions between 0.88%–6% and 3.7%–5.6% for intra- and inter-day variability, respectively. Recovery obtained after analysing the plasma samples was at 93%. The method showed itself to be linear for the concentration levels under study.
Discussion: The method's behaviour can be described as linear, precise and accurate. Furthermore, the method is fast, sensitive, and inexpensive. It is useful for determining linezolid concentrations in multiple biological matrices. It can also be used as a basis for further clinical pharmacokinetic studies.

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PALABRAS CLAVE
Fluido cerebroespinal;
Cromatografía líquida de alta eficacia;
Linezolid;
Farmacocinética;
Plasma;
Humor vítreo

Determinación de linezolid en fluidos biológicos mediante cromatografía líquida de alta eficacia

Resumen
Objetivo: Validación de un método analítico para la determinación de concentraciones de linezolid (LNZ) en fluidos biológicos: plasma, humor vítreo y líquido cefalorraquídeo mediante cromatografía líquida de alta eficacia y posterior detección ultravioleta.
Método: El método se validó mediante el estudio de los siguientes parámetros: exactitud, precisión, sensibilidad, linealidad y recuperación. El fármaco se extrajo de la matriz biológica mediante una precipitación proteica con ácido perclórico. La separación cromatográfica se realizó mediante la elución de LNZ con una fase móvil compuesta por el 80%de un tampón de fosfato dipotásico-monohidrogenado (K_2HPO_4) (15 mM; pH = 5) con el 20%de acetonitrilo y una fase estacionaria NOVAPAK® C18 150 x 3,9 mm con precolumna. La longitud de onda de lectura fue de 254 nm y el flujo de trabajo fue de 1 ml/min.
Resultados: Se obtuvieron valores de exactitud entre el 94,4-106,1%y de precisión entre el 0,88-6%y el 3,7-5,6%para la variabilidad intradía e interdía, respectivamente. La recuperación obtenida tras el análisis de las muestras de plasma fue del 93%. El método mostró ser lineal para los intervalos de concentraciones estudiados.
Discusión: El método se comporta de forma lineal, precisa y exacta. Además, es rápido, sensible y de bajo coste económico. Es un método útil para la determinación de concentraciones de LNZ en múltiples matrices biológicas. Es posible su utilización como base para posteriores estudios de farmacocinética clínica.

Introduction

Linezolid (LNZ) is a synthetic oxazolidinone that acts by inhibiting initiation of protein synthesis in bacteria.[1] It has a wide spectrum of activity against gram-positive microorganisms, including methicillin-resistant Staphylococcus, penicillin-resistant Pneumococcus and vancomycin-resistant *Enterococcus faecalis*/*Enterococcus faecium*.[2]

On a pharmacokinetic level, LNZ presents total oral bioavailability (100%), an elimination half-life of five to six hours, and low plasma protein binding (31%). It also presents a distribution volume of 40 to 50 litres, which indicates good distribution to tissues. It reaches high concentrations in the skin, synovial fluid, aqueous humour, cerebrospinal fluid (CSF), pulmonary parenchyma, etc.[3] LNZ is a good treatment alternative for infections of ventriculoperitoneal origin and other foreign body infections in which gram-positive microorganisms predominate.[4] In these cases, it is crucial to know the degree of penetration of the antibiotic in order to ensure that it reaches therapeutic levels that permit its use for suspected or microbiologically confirmed infections.

Chromatography is an analytical separation technique which is highly sensitive, selective and exact. It is ideal for separating non-volatile compounds, thermally labile compounds, amino acids, proteins, hydrocarbons and drugs in general, and is considered a technique of reference in investigative studies. Various chromatography methods for determining LNZ levels in serum, urine and plasma have been described in medical literature.[5-7]

The main purpose of this study is the development, fine tuning and validation of a quick, precise and simple analytical method for determining LNZ levels in plasma, CSF and vitreous humour (VH) using high-performance liquid chromatography and ultraviolet detection (UV).

Material and methods

Reagents

LNZ was provided by Pharmacia & Upjohn (purity 100%). Acetonitrile for high-performance liquid chromatography, perchloric acid and dipotassium hydrogen orthophosphate (analysis grade, PA) were supplied by Merck-Farma y Química, S. L. (Barcelona) and the orthophosphoric acid by Panreac Química S. A. (Barcelona).

Equipment and chromatographic system

We have access to an analytical balance (Precisa-40S-M200A, Pacisa), a centrifuge for microtubes (Heraeus), a vortex shaker (REAX-2000, Heidolph) and a freezer (917, Forma-Scientific, Inc.).

The chromatography equipment (Hewlett Packard-1100) consists of a degasser, automatic injector, quaternary pump, UV detector and computer system (HP-Chemstation).

Calibrators and controls

To validate the technique in plasma, we used a stock solution of 1000 µg/ml to prepare calibration solutions, each at a different concentration: 5; 10; 50; 100; 150; 200; 500 and 1,000 gg/ml. These were subsequently diluted with plasma to a concentration ten times lower. For CSF and VH, we used

a stock solution of 10 µg/ml to prepare calibration solutions at concentrations of 0.1; 0.5; 1; 3; 5; 7.5 and 10 µg/ml.

The control samples to determine LNZ levels in plasma were prepared at concentrations of 1.5; 7.5 and 75 µg/ml in the same biological matrix. Control samples for VH and CSF were prepared at 0, 8, 4 and 8 µg/ml in water.

Preparing the samples

Linezolid was extracted from plasma using a protein precipitation process (1:1, v/v) with perchloric acid; 200 µl of a 3% $HClO_4$ were added to 200 µl of plasma. The mixture was shaken during 30 seconds and centrifuged at 10,900g during five minutes. Next, 100 gl of the supernatant was injected in the chromatography system.

The CSF and VH samples did not require this process, and were therefore injected directly: 100 gl of CSF and 50 gl of VH.

Chromatographic conditions

The mobile phase consisted of a mixture of acetonitrile and a 15 mM phosphate buffer adjusted to pH 5 with phosphoric acid (20:80, v/v). We used a reverse phase 150 mmx3.9 mm C18 NOVAPAK® analytical column with precolumn, (Waters, S. A.). Isocratic elution was performed at 1 ml/min and the UV wavelength was set at X=254 nm.

Validating the method

Complete validation was performed for the plasma samples only. It was not possible to carry out this step with CSF and VH since it was impossible to obtain blank biological matrix for preparing calibration samples and quality control samples.

Accuracy

We evaluated three concentration levels coinciding with control sample concentrations. We took five analytical measurements for each of the three levels. Accuracy was calculated according to:

%A=([LNZ Experimental]–[LNZ Theoretical]=[LNZ Theoretical])*100

Where:
A: accuracy.
LNZ Theoretical: theoretical LNZ concentration (µg/ml).
LNZ Experimental: resulting LNZ concentration (µg/ml).

Precision

We evaluated three concentration levels coinciding with control sample concentrations. We took five analytical measurements for each of the three levels once daily during three consecutive days. The intra-assay study was performed using the results obtained on one of the previous days. Precision was expressed as an inter-assay or intra-assay coefficient of variation (CV) (%):

%CV=(SD=X')*100

Where:
SD: standard deviation of the concentration.
X': mean value of the analytical measurements (µg/ml).

Sensitivity

Sensitivity was established using the quantification limit. This was defined as the smallest LNZ concentration which could actually be distinguished from baseline with acceptable levels of precision and accuracy (<20%), as determined by inter-day assays.

Recovery

LNZ recovery was calculated by determining the percentage of drug by extracting plasma samples with known LNZ concentrations and comparing them with injections of the same amount of the drug in methanol/water (1/1).

In a parallel manner we injected extracts of samples corresponding to three concentration levels that coincided with those in the quality control samples. We injected five extracts per concentration level and two model equivalent samples in methanol/water per concentration. We evaluated the areas of the resulting chromatograms:

$\%Rec = A_{probl} = A_{control} * 100$

Where:
Rec: recovery.
A_{probl}: chromatographic peak area in the problem sample.
$A_{control}$: chromatographic peak area in the control sample.

Results

Concentrations for the linearity study were as follows: 0.5 to 100 µg/ml for plasma and 0.1 to 10 µg/ml for cerebrospinal fluid and vitreous humour. The mean for plasma was R2=0.9996. Means for cerebrospinal fluid and vitreous humour were R2=0.9982 and R2=0.9998, respectively.

Figure 1 shows the chromatogram obtained following analysis of a plasma sample. Figures 2 and 3 show the chromatograms for CSF and VH samples, in that order. The LNZ retention time was approximately four minutes. The accuracy results for the study technique presented values between 94.4% and 106.1%. For precision, we obtained intervals of 0.88% to 6% and 3.7% to 5.6% for intra-day and inter-day variability, respectively. Recovery obtained after analysing the plasma samples was at 93%.

Discussion

This study's main point of interest is the possibility of determining and measuring LNZ concentrations in several different biological matrices, such as plasma, VH and CSF, in a reliable and precise way.

The main difference between the method we describe and that described by Tobin et al[5] is that they employ a complex mobile phase with mixed organic reagents for chromatographic separation. In contrast, our method uses a mobile phase composed of a simple mixture of a phosphate buffer with 20% acetonitrile. Furthermore, our study uses a simple precipitation with $HClO_4$ to eliminate proteins from the sample. To obtain the same result, Ehrlich et al[6] and Geoffrey et al[7] employ a solid phase extraction technique, which is more time-consuming and requires more monetary resources. Likewise, our method does not require adding an

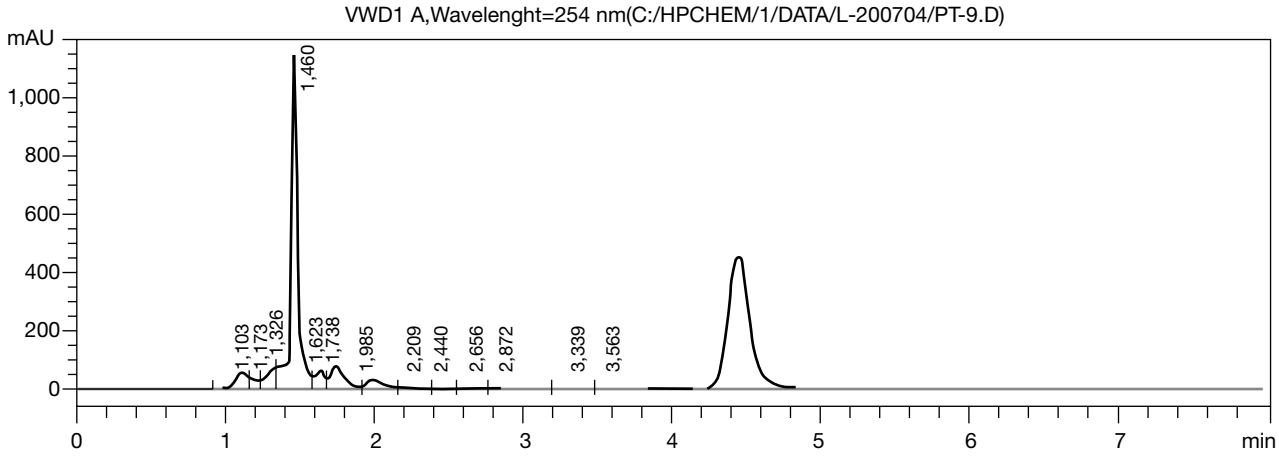

Figure 1 Chromatogram of a plasma sample from a patient treated with linezolid.

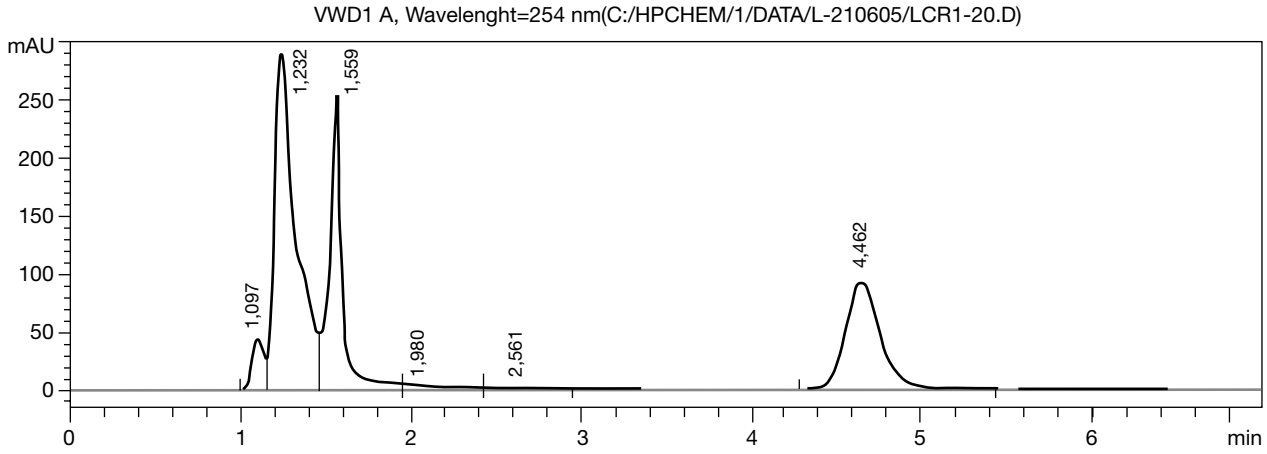

Figure 2 Chromatogram of a cerebrospinal fluid sample from a patient treated with linezolid.

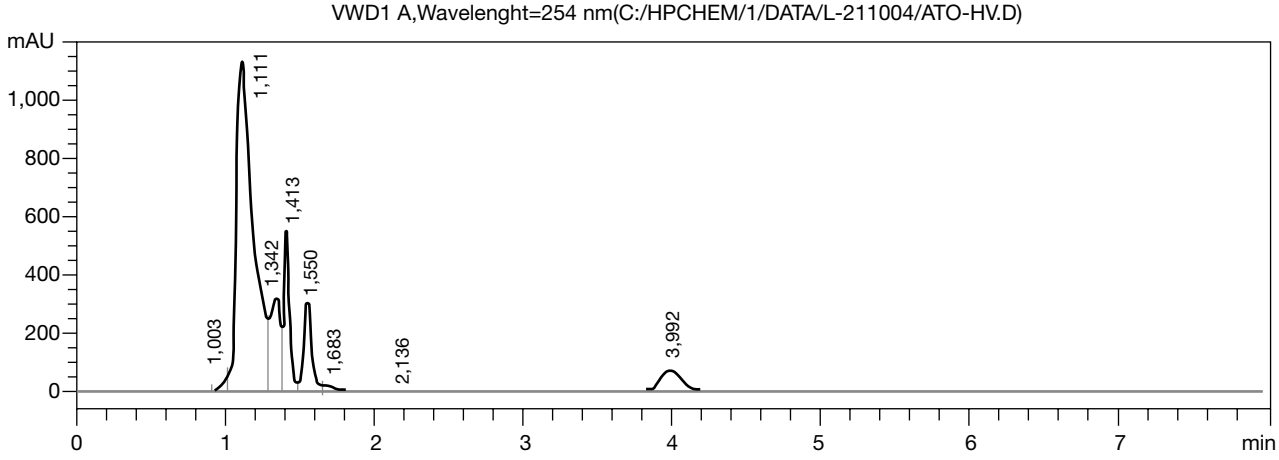

Figure 3 Chromatogram of a vitreous humour sample from a patient treated with linezolid.

internal model to quantify the samples, unlike those proposed by other authors.[7]

From what we have described above, we deduce that the main advantages of our method are its analytical simplicity, quickness, good analytical recovery and low monetary cost.

One of the study's limitations is that the method was not validated in VH and CSF. This was mainly due to the fact

that it was impossible to obtain blank biological matrix for purposes of preparing calibration and quality control solutions. CSF is a watery solution that is created by the two-way transport of substances between the plasma and the choroid plexus cells found in the interior of the central nervous system. CSF is produced and reabsorbed continuously to maintain a constant volume (150 ml) and composition. In a healthy individual, its composition on the ionic level is similar to that of plasma, but it differs from plasma in that it has a lower concentration of glucose and mainly proteins. It may therefore be considered a plasma ultrafiltrate.[8] On the other hand, VH is a gelatinous liquid mass with an approximate volume of 4 ml in an adult, and a 99%water content. The rest is made up of inorganic components, glucose and amino acids in quantities corresponding to 1/5 of those present in plasma.[9]

The absence of cells and the low protein concentration in both liquids permit them to be injected directly into the chromatographic system. These traits also allow extrapolation of the validation process performed in plasma without creating any analytical interference problems. The aqueous nature of these matrices means that they resemble the model samples in water that were analysed during method validation.

In conclusion, we may say that the method shows linear, precise and accurate behaviour. In addition, the method is fast, sensitive, and inexpensive. The methodology we describe enables LNZ concentrations to be measured in different biological matrices such as plasma, VH and CSF, and may be used as a base for additional studies in clinical pharmacokinetics.

Funding

Funding was provided by the *CIBER de Enfermedades Respiratorias* (Biomedical Research Networking Centre for Respiratory Diseases) on 06/06/08 and the *Fondo de Investigaciones Sanitarias* (FIS, Health Care Research Fund) as grant FIS PI070419.

Conflict of interest

The authors affirm that they have no conflicts of interest.

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