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Short communication

A new and rapid method for monitoring the new oxazolidinone antibiotic linezolid in serum and urine by high performance liquid chromatography-integrated sample preparation

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Abstract

A sensitive and rapid HPLC-assay for determining the new oxazolidinone antibiotic linezolid in serum and urine is described. HPLC-integrated sample preparation permits the direct injection of serum and urine samples without any pre-treatment. The in-line extraction technique is realized by switching automatically from the extraction column to the analytical column. After the matrix has passed the extraction column the retained analyte will be quantitatively transferred to the analytical column where separation by isocratic HPLC will be performed. Linezolid is detected according to its absorption maximum at 260 nm. The quantification limits are estimated to be 0.3 and 0.5 μg/ml in serum and urine samples, respectively. The described procedure allows sample clean-up and determination of the antibiotic within 20 min, thereby facilitating drug-monitoring in clinical routine.

Keywords: Oxazolidinones; Linezolid

1. Introduction

Linezolid (Zyvox®) is a member of a new class of synthetic antibacterial drugs called oxazolidinone derivatives that are chemically unrelated to currently used antibiotics. It is active against gram-positive bacteria by inhibition of formation of ribosomal initiation complex [1], including methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE) [2]. The chemical structure of linezolid is shown in Fig. 1.

As a new antimicrobial drug, licensed by the FDA since April 1st, 2000, only few pharmacokinetic data are available. The elimination half-life of linezolid is ~5 h. Most of an oral dose is excreted in the urine, 30% unchanged [3].

The pharmacokinetic behaviour in particular diseases has not yet been investigated in detail. Drug-

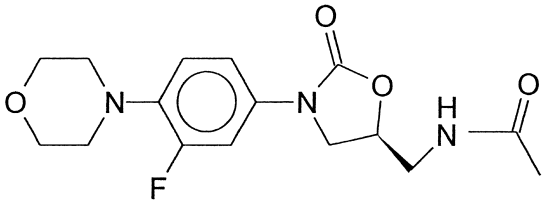

Fig. 1. Chemical structure of linezolid.

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monitoring could help to find the best dose regime, therefore the aim of the present study was to provide a rapid, accurate and sensitive analytical method to determine linezolid in serum and urine. HPLC-integrated extraction overcomes time-consuming sample pre-treatment, such as liquid–liquid or solid-phase extraction prior to HPLC-determination [4].

HPLC-integrated sample preparation described here uses special column packing materials which allow direct and repetitive injection of untreated biofluids. LiChrospher® RP-ADS belongs to the unique family of restricted access material. It possesses two chemically different surfaces. Hydrophilic, electroneutral diol-groups are bound at the outer surface of the spherical particles with a diameter of 25 μm. This chemically inert layer protects the column from any unwanted contamination caused by interaction with the protein matrix, even if used repetitively. The inner surface of the porous particles is covered with a hydrophobic dispersion phase (C-4, C-8, C-18 alkyl-chains). These adsorption centres are accessible for low molecular analytes [5–7].

2. Material and methods

2.1. Reagents and chemicals

Linezolid was kindly supplied by Pharmacia and Upjohn (Erlangen, Germany). Acetonitrile (LiChrosolv®) and sodium dihydrogenphosphate monohydrate were purchased from Merck (Darmstadt, Germany).

All reagents were at least analytical grade, except for acetonitrile which was gradient grade. HPLC-grade water was generated by using a Milli-Q water-purification system from Millipore (Molsheim, France). Pooled blank sera were purchased from Plasmadienst (Offenbach, Germany). Blank urine samples were available from healthy volunteers.

2.2. Apparatus

The in-line extraction system was constructed with LiChroCART® cartridge (25×4 mm), packed with LiChrospher®ADS C_8, cartridge holder manuCART® and in-line filter (2-μm sieve; Merck, Darmstadt, Germany). The HPLC-system consisted of two LC-10AT HPLC-pumps, CTO-10AC column oven, SPD-10A UV detector and FCV-12AH 6-Port Valve, controlled by a CBM-10A Module (all items obtained from Shimadzu, Duisburg, Germany). Samples were injected via Rheodyne 7125 manual injection valve equipped with a 20-μl sample loop. Chromatography was performed on a Nucleosil C_{18} 250-mm×4.6-mm, 5-μm analytical column preceded by a guard column CC 8/4 Nucleosil 100-5 C_{18} (Macherey and Nagel, Düren, Germany).

2.3. Analytical procedure

The direct injection of serum or urine samples was the only manual step in the analytical procedure. Extraction and chromatography was carried out automatically by the HPLC-integrated-extraction system. A block diagram of the HPLC-integrated sample preparation is shown in Fig. 2.

2.3.1. HPLC-integrated extraction

The operation of the HPLC-integrated extraction consists of three main components: sample application and fractionation, transfer of the analyte fraction and chromatographical separation.

The mobile phase used for the extraction procedure contained 50 m*M* sodium dihydrogenphosphate buffer and was adjusted to pH 5.0 (reservoir of pump A).

An aliquot (20 μl) of the biological fluid was injected into the manual injector. The mobile phase (pump A set to 0.8 ml/min) carried the sample to the extraction column. The antibiotic analyte was retained on this column, while higher molecular mass matrix compounds were discharged to waste together with the eluent (Fig. 2A).

After 8 min, the matrix had been washed out of the extraction column. The software time-schedule automatically switched the high-pressure valve into transfer position (Fig. 2B) thereby linking the extraction column with the HPLC-circulation.

Reservoir of pump B contained acetonitrile and reservoir of pump C consisted of 50 m*M* sodium dihydrogenphosphate buffer (pH 5.0). In this valve position the analytical mobile phase delivered from pump B and C in a ratio of 25:75, v/v, passed the extraction column in inverse direction at a flow of 1.0 ml/min. Compared to the mobile phase of the

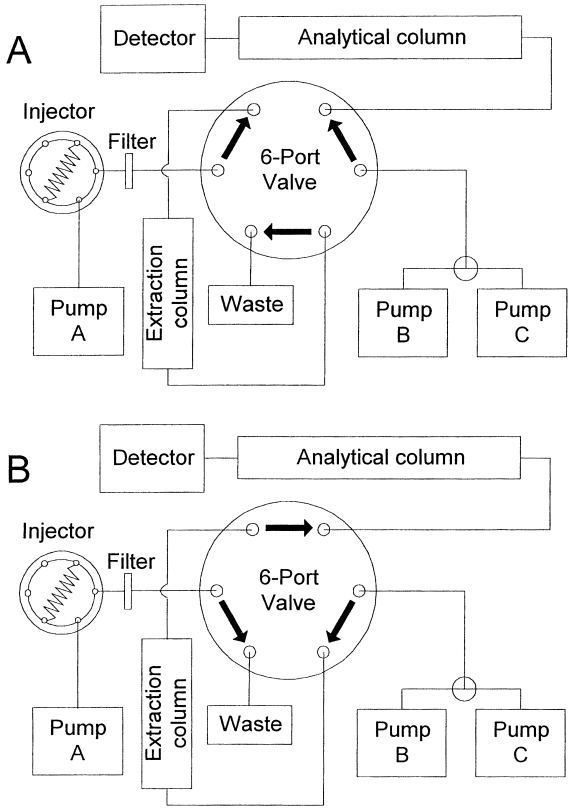

Fig. 2. Scheme of the HPLC-integrated sample preparation. (A) The system in initial position, ready for sample injection. HPLC circulation is isolated from extraction side. (B) The transfer step. The extraction column is connected with the analytical column. See Section 2.3 for details.

extraction procedure, the higher elution power of the analytical mobile phase desorbed the analyte from the extraction column and transferred it to the analytical column. After 12 min the transfer was completed and the valve was switched to initial position (Fig. 2A).

The extraction column was re-equilibrated by pump A while simultaneously the disconnected HPLC-circulation performed a conventional chromatography. Pump B and C settings remained as described above and the column oven was set to 35°C. The eluent was monitored at 260 nm.

The extraction column was washed every day after a number of analyses with methanol–water (50:50, v/v) at 1.0 ml/min for at least 15 min.

2.4. Quantification and statistics

2.4.1. Quantification

Standards for the linearity study were made by diluting an aqueous stock solution of linezolid in a range of 0.64–32.0 μg/ml.

All standards were injected into the in-line extraction system. The calibration curve was based on the peak area of each standard plotted versus the nominal antibiotic concentration using least-squares linear regression.

2.4.2. Recovery, accuracy and precision

Pooled blank serum and urine samples were spiked with standard solution of linezolid to yield concentrations between 0.64 and 16.0 μg/ml.

The chromatogram peak for linezolid was identified by the retention time and quantified by peak area. Accuracy of the linezolid urine and serum assay was determined by calculating the mean percentage differences between nominal and measured concentrations. The assay precision was characterized by mean value and coefficient of variation (C.V.).

The average quotient of measured concentrations and nominal concentrations indicated the ratios of the recovered antibiotic. The detection and quantification limits were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively.

3. Results and discussion

For the new antibiotic linezolid a reliable and cost effective method for determination in serum and urine is necessary for monitoring studies. Such a method should be also suitable for monitoring linezolid in routine clinical use. For this purpose speed and reliability in combination with cost effectiveness are extremely important. As linezolid is not volatile HPLC is the method of choice. In combination with the in-line extraction procedure presented here the requirements as described above are met.

Best separation results with regard to extraction columns were achieved by using ADS-RP C_8. Injection of 60 μg/ml aqueous linezolid standard

solution showed no breakthrough of the antibiotic during the matrix elution period of 8 min.

A typical chromatogram of linezolid in pooled serum compared with a drug free serum sample is shown in Fig. 3(A). Chromatograms of blank and spiked urine samples are shown in Fig. 3(B). Linezolid was well separated from other detectable components in serum and urine at the selected wavelength. The equation of the calibration curve of aqueous linezolid standards was $y=66351x+1846.2$ ($r^2=0.9999$), where x represents the analyte concentration in μg/ml and y the corresponding peak area.

Recovery, precision and accuracy of the linezolid assay for urine and serum samples are summarized in Table 1. The assays detection and quantification limits are shown in Table 2. The serum assay was slightly better because of the superior matrix washout during the matrix elution step (see matrix peaks in Fig. 3(A) vs. those in (B)).

The serum and urine assay described for linezolid overcomes time-consuming procedures of sample preparation and is therefore cost-effective. Time is saved because of fast extraction and enrichment of the analyte by the ADS-system. It allows determination of antibiotic levels within 20 min after sampling. The relatively high price of a single column (about US$250) is unimportant when the saved working time due to the complete automation and the requirement for fast results in clinical routine

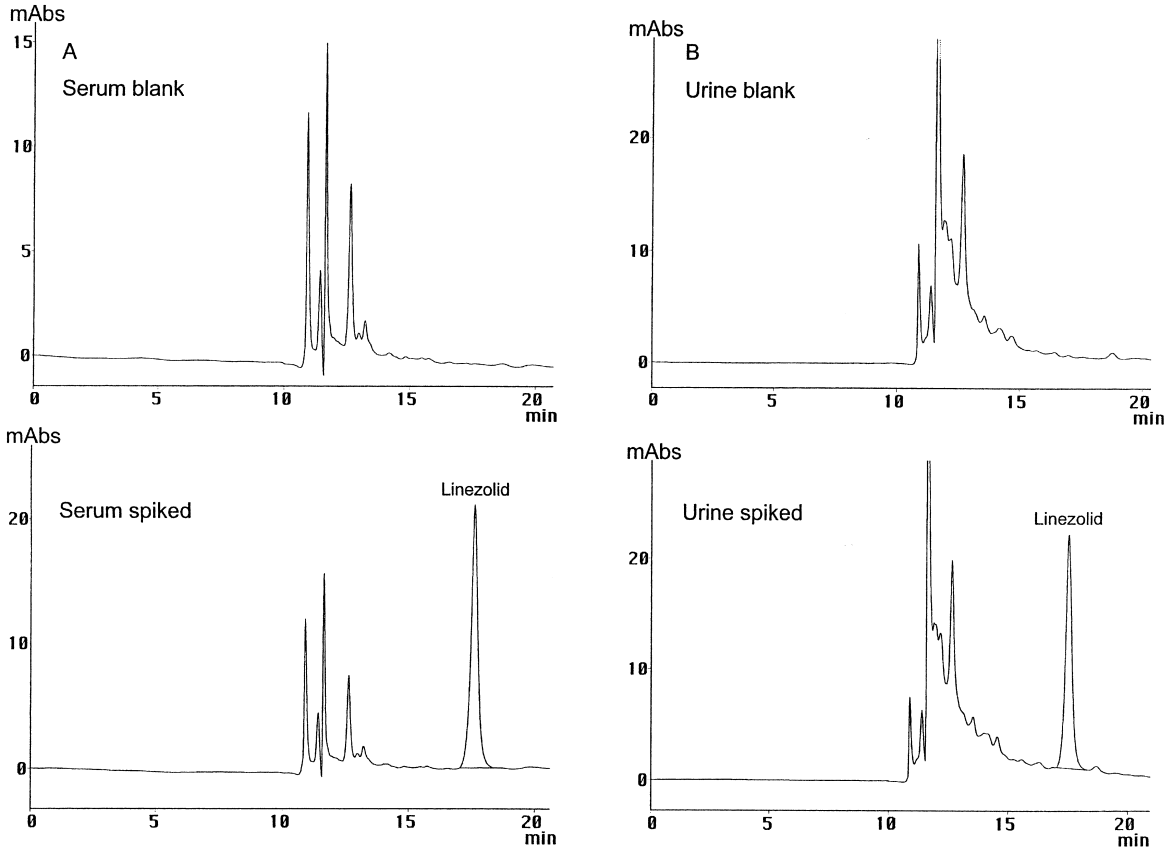

Fig. 3. Chromatograms of spiked matrix versus blank matrix. (A) Blank serum versus spiked serum (6.5 μg/ml linezolid). (B) Blank urine versus spiked urine (6.5 μg/ml linezolid).

Table 1
Statistics of spiked serum and urine samples for linezolid assay

Nominal conc. (μg/ml)	*n*	Precision		Accuracy		Recovery	
		Mean (μg/ml)	C.V (%)	Mean difference from nominal (μg/ml)	Mean difference from nominal (%)	Mean recovery from nominal (%)	SD
Serum							
16.00	6	15.28	2.4	0.72	4.5	95.5	0.02
6.40	6	6.32	2.8	0.08	1.2	98.8	0.03
0.64	6	0.63	4.2	0.01	1.6	98.4	0.04
Urine							
16.00	6	14.68	4.0	1.32	8.3	91.7	0.04
6.40	6	6.09	1.6	0.31	4.8	95.2	0.02
0.64	6	0.57	2.3	0.07	10.8	89.2	0.02

or drug monitoring is considered. The reuse of the extraction column was tested more than 50 times. No change in the performance was observed.

4. Conclusion

The presented assay for the determination of linezolid in serum and urine samples is sufficiently fast, sensitive and reliable for drug-monitoring in clinical routine. It is also suitable for pharmacokinetic studies in these biological fluids. The detection limits of the assay allow determination of antibiotic levels down to the MIC_{90} of the most gram-positive pathogens including methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococci* (VRE) [2,8].

Table 2
Detection and quantification limits of the linezolid assay

Sample matrix	Detection limit, *S/N* 3:1 (μg/ml)	Quantification limit, *S/N* 10:1 (μg/ml)
Serum	0.10	0.3
Urine	0.15	0.5
Millipore water	0.08	0.2

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