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Determination of linezolid in growth media by high-performance liquid chromatography with on-line extraction

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Abstract

An isocratic high-performance liquid chromatography (HPLC) method with on-line extraction has been developed to determine linezolid in Mueller-Hinton broth. The loading mobile phase consisting of water–acetonitrile 99:1 (v/v) allowed retention of the analyte on a LiChrocart 4-4 pre-column filled with a LiChrospher 100 RP-8, 5 μm. The transfer of the analyte by a backflush mode to a 150 mm × 4.6 mm I.D. Kromasil C8 5 μm column was performed using a mobile phase of water–acetonitrile 80:20 (v/v). UV detection at 254 nm allowed a quantification limit of 0.39 μg/mL with a 50-μL sample size. The method was successfully applied to in vitro pharmacokinetic–pharmacodynamic studies.

Keywords: Linezolid; Growth media; HPLC; On-line extraction

1. Introduction

Linezolid is the first of a new class of antimicrobial drugs, the oxazolidinones, that inhibit bacterial protein synthesis through a unique mechanism. In contrast to other inhibitors of protein synthesis, the oxazolidinones act early in translation by preventing the formation of a functional initiation complex [1]. Linezolid has inhibitory activity against a broad range of gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), glycopeptide-intermediate *S. aureus* (GISA), vancomycine-resistant enterococci (VRE) and penicillin-resistant *Streptococcus pneumoniae* [2]. In vitro staphylococci and enterococci resistant to linezolid can be selected only with difficulty, in association with mutations in genes encoding the central loop of domain V of 23S rRNA [3]. However, resistance to linezolid has been reported in clinical isolates of MRSA [3,4] and VRE [5]. These failures raise the question of whether appropriate serum levels of linezolid were achieved [4]. In vitro pharmacokinetic–pharmacodynamic (PK-PD) models that mimic serum concentration profiles could answer to this interrogation. Such in vitro investigations generate a large number of samples. Therefore, rapid and reliable analytical methods are required both for labor saving and accurate determination of linezolid in culture broths at variable concentration levels.

High-performance liquid chromatography (HPLC) methods involving different sample handling, i.e. deproteinization [6–11] and solid-phase extraction [12–14], were proposed for linezolid determination in plasma, serum, urine, brain heart infusion broth (BHIB) and microdialysis samples. An on-line extraction technique [15] was also applied to serum and urine samples. Taking into account the constraints of our investigations, an on-line extraction method was developed and validated for linezolid determination in Mueller-Hinton broth (M-HB). The on-line method involving a column-switching system consists of a first step of trapping the analyte in the pre-column (PC) and elution of biological matrix to the waste. In the second step, the

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analyte is transferred to the analytical column (AC) and separation occurs.

2. Experimental

2.1. Chemicals

Linezolid obtained as reference powder was provided by Pharmacia & Upjohn (Kalamazoo, USA). Acetonitrile was purchased from VWR (Fontenay-sous-Bois, France) and was of HPLC grade. Water was obtained with a Direct Q 5 purification system (Millipore, Saint-Quentin-Yvelynes, France).

2.2. Chromatographic system and conditions

The liquid chromatograph was composed of a Kontron 360 autosampler (Serlabo Technologies, Bonneuil-sur-marne, France) equipped with a 50-μL sample loop, a HP 1050 isocratic pump (pump 1) (Agilent Technologies, Massy, France) and a Kontron 420 pump (pump 2) delivering mobile phases 1 and 2, respectively. The electric-actuated switching system, depicted in Fig. 1, was equipped with a six-port Rheodyne valve and was controlled by the external time events of the HP 1050 pump. Sample injection starts its programmable time-relay.

The PC was a Merck LiChroCART® 4-4 filled with LiChrospher® 100 RP-8, 5 μm (VWR). The AC was a 150 mm × 4.6 mm I.D. Higgins Analytical stainless steel column filled with Kromasil C8, 5 μm (Bios Analytique, Labège, France). An Upchurch 0.5 μm pre-column filter (Cluzeau Info Labo, Sainte-Foy-La-Grande, France) was inserted between the PC and the AC.

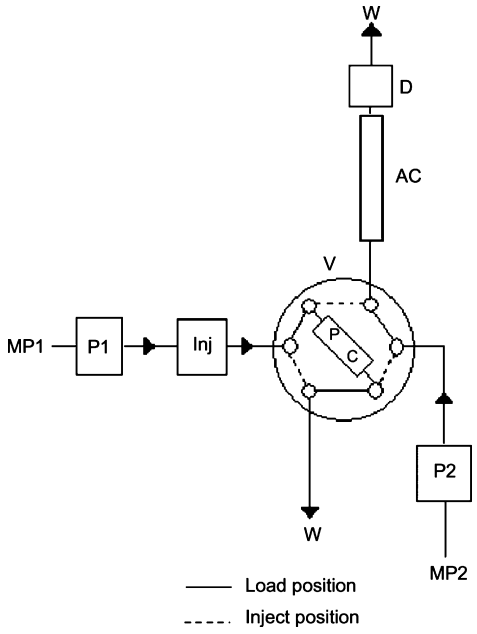

Fig. 1. Schematic diagram of the column switching system for the assay of linezolid. MP1 and MP2, mobile phase 1 and 2; P1 and P2, pumps 1 and 2; Inj, injector; PC, pre-column; AC, analytical column; V, six-port switching valve; D, UV detector; W, waste.

Detection was performed with a Kontron 332 detector (Serlabo Technologies) set at 254 nm. A recorder output range of 0.5 μA full scale was used. Data acquisition was performed with a HP 3396 A integrator (Agilent Technologies).

Mobile phase 1 used for the on-line extraction procedure consisted of water–acetonitrile (99:1, v/v) mixture. The analytical mobile phase (mobile phase 2) was composed of the same component as mobile phase 1, but at the ratio 80:20 (v/v). Flow rate was set at 1.0 mL/min for both.

Broth samples from PK-PD in vitro models, containing bacteria in suspension, underwent a 5-min centrifugation at 2000 × *g* and a 50-μL volume of the supernatant was injected.

The total sequence of automated sample analysis required 15 min and included the following three steps:

Step I (0–2 min, valve in load position): After injection of the sample, the mobile phase 1 with weak elution strength allowed the transfer of the analyte to the PC where it was retained, and elimination of unwanted components of the broth which were directly vented to waste.
Step II (2–7 min, valve in injection position): The mobile phase 2 with high elution strength allowed analyte transfer in backflush mode from PC to the AC where it was separated for quantification.
Step III (7–15 min, valve in load position): The PC was flushed again with mobile phase 1 and conditioned for the next injection, while the mobile phase 2 continuously went through the AC to complete analyte separation.

The PC and the AC were washed every week after a number of analyses with water–acetonitrile 70:30 (v/v) at 1.0 mL/min for at least 30 min.

2.3. Stock solutions and spiked broth samples

Linezolid was made up as 250 μg and 200 μg/mL stock solutions in water. The 250 μg/mL solution was diluted with blank M-HB to obtain calibration samples at 0.39, 1.56, 3.12, 6.25, 12.5 and 25 μg/mL. Quality control samples of 1, 10 and 20 μg/mL were prepared by successive dilution of the 200-μg/mL stock solution with the same blank matrix. Supplementary broth samples of 0.39 μg/mL were prepared for validation tests of the lowest limit of quantification (LLOQ), defined as the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision. Portions of 220 μL were transferred to Eppendorf tubes and stored at −20 °C.

2.4. Calibration and calculations

The concentration of unknown samples was calculated from a linear calibration curve. This curve was obtained daily by computing a nonweighted least-squares regression of the peak height *y* versus linezolid concentration *x* from six standard broth samples: 0.39, 1.56, 3.12, 6.25, 12.5 and 25 μg/mL.

Linearity was determined by assaying six standards in six separate assay runs within 2 weeks.

2.5. Recovery

The recovery was established for M-HB by six analyses of four linezolid broth concentrations: 0.39, 1, 10 and 20 μg/mL. The response of the worked-up sample was compared with that obtained by injection of linezolid aqueous solution at equivalent concentration directly in the AC.

3. Results and discussion

3.1. Sample clean-up procedure and HPLC system

Whilst little improvement can be made to the analytical speed of LC methods, the work capacity can be greatly increased if the system is completely automated to the extent of analyzing raw samples without operator intervention [16]. Biological samples usually require some form of preparation before injection onto the LC to remove compounds that would otherwise interfere with the separation or reduce the performance of the analytical column. A variety of methods have traditionally been found to be successful, but some are easier to automate than others. Although solid-phase extraction was being increasingly used for sample preparation, it proved impossible to fully automate without resorting to the use of laboratory robots. Column-switching techniques permitted the preparation of biological samples in an on-line manner using the benefits of solid-phase extraction [17].

The pre-column and the 0.5 μm filter were replaced after injection of about 100 samples to prevent high pressure in the chromatographic system and pre-column efficiency decrease. Memory effects from the pre-column (identified by injecting blank solutions after a run with definite amounts of drug) were not observed.

More than 1000 samples from method development and validation tests and from in vitro PK-PD model unknown samples were analyzed without appreciable decrease of the AC efficiency and, therefore, confirmed its stability under the chromatographic conditions retained as elution mobile phase containing 80% water. Kromasil belongs to the group of polar-embedded alkyl stationary phases. With the incorporation of a polar functional group in the alkyl ligand close to the surface of the silica, the phase remains solvated by water at low percentages of organic modifier and even 100% water. Under these conditions, the alkyl chains maintain their conformational freedom and can interact with polar analytes. This feature is especially useful for polar compound retention and leads to improved chromatographic performance [18], and therefore fits well with linezolid physical chemical characteristics. Indeed, linezolid is an amphophilic compound with an octanol–water partition coefficient of 0.55 [19].

3.2. Recovery

The recovery (Table 1) was 88.0 ± 5.33–93.9 ± 1.47% ($n = 6$) in the concentration range 0.39–20 μg/mL. If extraction recovery reached, ca. 100% after deproteinization of serum [6,9], plasma [8,11], urine [9], microdialysate [8], BHIB [10], it fluctuated between 92% [13] and 99.64–108.5% [12,14] after liquid–solid extraction from plasma, and fell to 95.5–98.8% and 89.2–95.2% for serum and urine, respectively, during on-line extraction [15]. These last results are close to that obtained for M-HB with our method.

Table 1
Extraction recovery of linezolid from Mueller-Hinton broth

Concentration (μg/mL)	Recovery (%) (mean ± S.D.) ($n = 6$)
0.39	88.0 ± 5.33
1	93.9 ± 1.47
10	93.5 ± 1.03
20	93.3 ± 1.12

3.3. Selectivity

Typical chromatogram of blank M-HB, M-HB spiked with linezolid at 0.39 and 1.56 μg/mL are shown in Fig. 2A–C, respectively. Linezolid retention time was approximately 10.40 min. No interference of broth components or culture by-products (data not shown) was noted.

In vitro activity of linezolid combined with other antibacterial agents against staphylococci, penicillin-susceptible and -resistant pneumococci, vancomycin-susceptible and -resistant enterococci has been investigated by Sweeny et al. [20,21]. Linezolid was associated with ampicillin, amoxicillin, oxacillin, penicillin, cefotaxim, cephalotin, cefotaxim, ceftazidim, cefpodoxim, cefdinir, aztreonam, imipenem, clindamycin, erithromycin, gentamicin, neomycin, tetracycline,

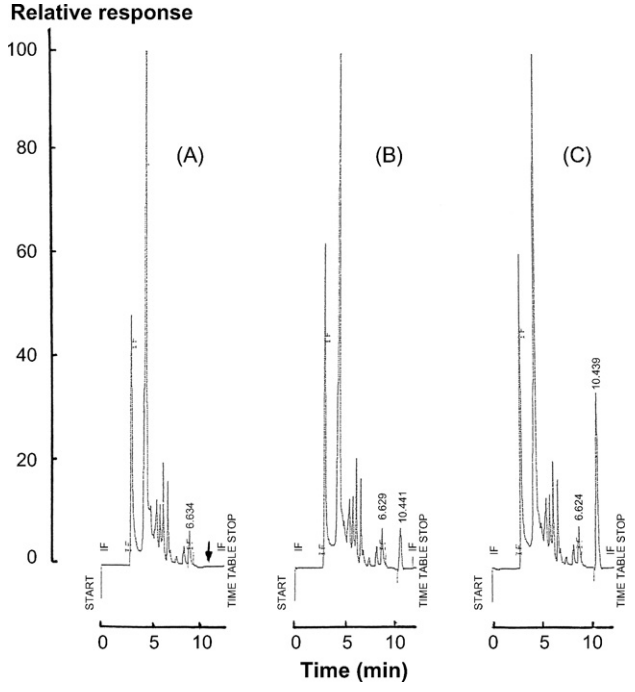

Fig. 2. Typical chromatograms from (A) blank Mueller-Hinton broth and (B, C) Mueller-Hinton broth spiked with linezolid at concentrations of 0.39 and 1.56 μg/mL, respectively.

Table 2
Intra- and inter-assay imprecision and inaccuracy of linezolid determination in Mueller-Hinton broth

Added concentration (μg/mL)	*n*	Concentration found (mean ± S.D.) (μg/mL)	R.S.D. (%)	Inaccuracy[a] (%)
Intra-assay				
0.39	6	0.43 ± 0.003	0.70	9.83
1.00	6	1.05 ± 0.01	1.05	5.10
10.0	6	10.2 ± 0.58	5.64	2.00
20.0	6	21.3 ± 1.44	6.77	6.70
Inter-assay				
0.39	12	0.44 ± 0.03	7.35	11.8
1.00	12	1.02 ± 0.01	0.83	2.11
10.0	12	10.4 ± 0.14	1.35	4.00
20.0	12	20.7 ± 0.73	3.53	3.35

[a] Mean relative error.

rifampicin, vancomycin, teicoplanin, fusidic acid, bacitracin, metronidazole and chloramphenicol. Overall, the in vitro data demonstrated the predominant additive/indifferent interaction of linezolid with the tested antibacterials. In contrast, in vitro synergy was found by Kato et al. [22] between linezolid and sulbactam/ampicillin against methicillin-resistant *S. aureus* clinical isolates. Therefore, analytical interference study was done on ampicillin and sulbactam. No overlapping peak was detected for these potential comedications under the selected chromatographic conditions.

3.4. Linearity

The correlation between linezolid concentration (x) and peak height (y) in the range 0.39–25 μg/mL led to a mean slope (±S.D.) of 162.7 (±1.55) and a mean y-intercept (±S.D.) of −5.64 (±4.17) from six separate assay runs. The mean correlation coefficient was 0.9999, indicating the linearity of standard curves. The use of internal standard was not necessary to generate valid results because of a good reproducibility of linezolid on-line extraction.

3.5. Imprecision and inaccuracy

The intra- and inter-assay imprecision (given by the relative standard deviation of replicate analyses) and the inaccuracy (given as mean error, i.e., the percentage deviation between found and added concentration) of the method were evaluated using low-, medium- and high-quality control broth samples (1, 10 and 20 μg/mL). To allow confirmation of the LLOQ, the same investigations were also done for a 0.39-μg/mL control sample. The intra-assay repeatability was determined by analyzing six specimens of spiked broth samples on the same day. The inter-assay repeatability was obtained by analyzing two specimens of broth samples on 6 days over a period of 2 weeks. The data in Table 2 demonstrate the good precision and accuracy for linezolid in M-HB over the concentration range investigated. Indeed, both imprecision and inaccuracy were <20% for the limit of quantification and <15% for the three quality control samples, according to FDA guidelines [23].

3.6. Stability

According to Li et al. [10], at 37 °C – temperature at which the pharmacodynamic experiments were conducted – linezolid was stable for up to 72 h in brain heart infusion broth. Boak et al. [11] found that linezolid concentrations remained stable following exposure of plasma to three freeze–thaw cycles, storage on the bench top at room temperature for up to 24 h and over a long-term period of 12 weeks at −20 °C; extracts were also stable in autosampler for 12 h. Similar results were obtained also by Toutain et al. [12] for plasma and bronchoalveolar lavage. Note that our experiments with Mueller-Hinton broth lasted 48 h and samples were analyzed within 1 h after sampling or within 1 week after storage at −20 °C. Therefore, classical stability tests were not investigated.

4. Application

The method described was successfully applied to the analysis of M-HB samples from an in vitro PK-PD model [24].

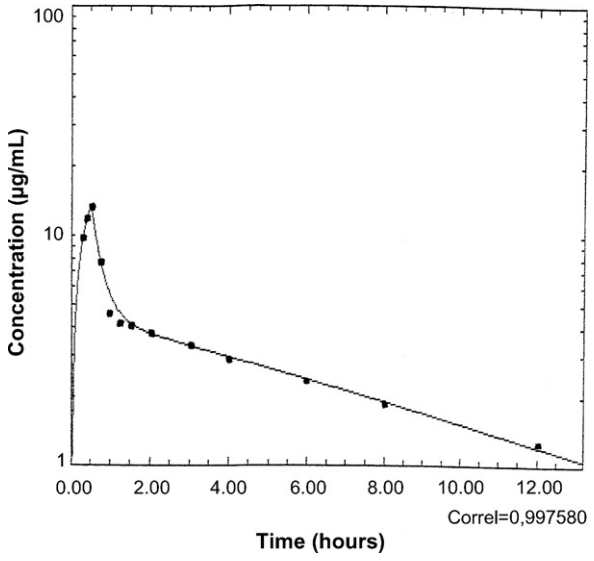

Fig. 3. Pharmacokinetic profile from simulation of a 0.5 h perfusion of a 600 mg dose of linezolid in the peripheral compartment of an in vitro pharmacokinetic–pharmacodynamic model using Mueller-Hinton broth.

Table 3
Linezolid pharmacokinetic parameters in the in vitro pharmacokinetic–pharmacodynamic model and in healthy volunteers

Parameter	In vitro model ($n = 12$)	Healthy volunteers [25] ($n = 10$)
C_{max} (μg/mL)	14.0 ± 0.88	14.1 ± 2.8
$T_{1/2}$ β (h)	6.05 ± 0.57	5.1 ± 2.6
$AUC_{0–24}$ (h × μg/mL)	72.7 ± 7.23	88.1 ± 34.0

The two-compartment kinetic model with a capillary unit was designed to expose bacteria to changing antibiotic concentration by simulating human plasma or tissue profile, without dilution of the bacterial inoculum together with the antibiotic. Fig. 3 shows the pharmacokinetic profile from simulation of a 0.5 h perfusion of 600 mg dose of linezolid in the peripheral compartment where the bacteria are confined. The derived pharmacokinetic parameters (Table 3) were close to that obtained in healthy volunteers [25].

5. Conclusion

Our method using column-switching technique provided a simple and fast analysis of linezolid in Mueller-Hinton broth, with a 15-min run time. The on-line process avoids time-consuming treatment of samples before injection. As a consequence, it allowed to perform consecutively in vitro pharmacokinetic–pharmacodynamic experiments and the determination of samples derived from such studies.

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