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Short communication

Method development for determining the antibacterial linezolid in human serum by micellar electrokinetic capillary chromatography

Toshihiro Kitahashi *, Itaru Furuta

Department of Laboratory Medicine, Kinki University School of Medicine, 377-2 Ohno-higashi, Osakasayama, Osaka, Japan

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Abstract

A precise method for determining linezolid concentration in human serum by micellar electrokinetic capillary chromatography has been developed and validated. Serum was deproteinized with acetonitrile and etofylline was used as an internal standard. A borate buffer (pH 10.0; 25 mM) containing sodium dodecyl sulfate (80 mM) was used as a running buffer. Detection was performed at UV253 nm by applying 25 kV voltage to a fused-silica capillary tube. Migration time of linezolid was approximately 14 min. Good linearity (0–100 mg/l) was obtained and the limit of detection was 0.5 mg/l (S/N = 3). This technique covered the clinical concentration (4 mg/l) measurement of this drug enough. The intra- and inter-day reproducibility was good. Serum recovery was 95–102%. No interference from other anti-microbial agents was observed. Linezolid after serum deproteinization showed high stability. This method was easy to operate as well as economical as a method for determining linezolid in serum.
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Keywords: Linezolid; Serum linezolid level; Capillary electrophoresis; Serum deproteinization; Etofylline

1. Introduction

Linezolid (PNU-100766) is an oxazolidinone anti-microbial agent, which acts on the initiation of bacterial protein synthesis (Fig. 1). It means that this drug exhibits anti-microbial properties by inhibiting the formation of peptide synthesis initiation complex. This action mechanism is different from that of traditional protein synthesis inhibiting agents, which suggests that this drug does not show cross-resistance with existing anti-microbial agents [1,2]. This drug has a wide spectrum of activity against gram-positive organisms including methicillin-resistant staphylococci and vancomycin-resistant *Enterococcus faecalis* and *E. faecium* [1]. Monitoring its concentration in blood is essential for the prevention of side effects as well as the validation of drug efficacy. High-performance liquid chromatography (HPLC) [3–6] and LC-MS-MS [7] have been reported as new methods for determining linezolid

* Corresponding author. Tel.: +81-72-366-0221; fax: +81-72-368-1141

E-mail address: kitahasi@med.kindai.ac.jp (T. Kitahashi).

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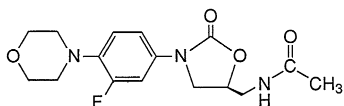

Fig. 1. Chemical structure of linezolid.

concentration in blood and urine. We paid attention to the fact that capillary electrophoresis (CE) used a hollow capillary tube of high durability and enabled economic determination requiring only tiny volumes of samples and electrophoresis buffer and offering higher theoretical plate number. Furthermore, linezolid itself showed strong UV absorption and its quantitative electrophoretic phenomenon in the capillary tube was observed. Our purpose of conducting this research is to develop a new method for determining linezolid concentration in serum by CE so that it can be introduced to clinical laboratories.

2. Materials and methods

2.1. Anti-microbial agents

Linezolid was provided by Pharmacia (Kalamazoo, MI). Amoxicillin, ampicillin, amikacin, amphotericin B, antipyrine, bacampicillin, cefalotin, cefazolin, cefotaxime, cefoxitin, cefuroxime, cefusulodin, carbenicillin, cefadroxil, cefatrizine, chloramphenicol, cimetidine, erythromycin, furosemide, 5-fluorocytosine, gentamycin, ketoconazole, methicillin, miconazole, minocycline, netilmycin, penicillin, piperacillin, sisomycin, streptomycin, tinidazole, tobramycin and vancomycin were purchased from Sigma (St. Louis, MO). All anti-microbial agents were at least analytical grade.

2.2. Reagents

Sodium dodecyl sulfate (SDS), sodium tetraborate decahydrate, sodium hydroxide (0.1 N) and HPLC-grade acetonitrile were purchased from Wako Pure Chemicals (Osaka, Japan). Etofylline purchased from Tokyo Kasei (Tokyo, Japan) was used as an internal standard (IS). All reagents were at least analytical grade.

2.3. Preparation of standard solutions

Linezolid stock standard solution was prepared with distilled water (50 mg/50 ml) and this was then diluted with appropriate volumes of distilled water to make working standard solutions of various concentrations. IS was dissolved in acetonitrile into a stock standard solution (50 mg/50 ml), which was further diluted with acetonitrile so that it could be used as a working standard solution of 18.75 mg/l.

2.4. Sample preparation

0.1 ml of serum sample and 0.4 ml of acetonitrile containing the IS were mixed up vigorously for 30 s and centrifuged at $12\,000 \times g$ for 2 min with a centrifuge (Kubota 1130, Tokyo, Japan). Then, its supernatant was poured into another sample tube. The supernatant was evaporated to dryness under reduced pressure at 30 °C using a centrifugal concentrator (VC-36, Taitec, Tokyo, Japan), and re-dissolved in distilled water of the same amount as the serum used for this preparation so that they could be used as CE samples.

2.5. CE instrumentation and running conditions

P/ACE^{TM} system MDQ as a CE system and an untreated fused-silica capillary tube (effective length 500 mm, 75 μm ID) both purchased from Beckman Coulter (Fullerton, CA) were used. A borate buffer (pH 10.0; 25 mM) containing SDS (80 mM) was used as a running buffer and its pH was adjusted with sodium hydroxide (0.1 N). This running buffer was passed through a 0.45 μm filter (Millipore, Bedford, MA) and then de-aired ultrasonically for 5 min before actual use. After each analytical run of 1 sample, the capillary tube was rinsed with sodium hydroxide (0.1 N) for 3 min, then with distilled water for 2 min and finally conditioned with the running buffer for 5 min (20 psi). Sampling time was 10 s (0.5 psi). Micellar electrokinetic capillary chromatography (MEKC) was performed with normal polarity under 25 kV and detection at λ_{max} 253 nm. The temperature was maintained at 25 °C for the capillary tube and at 15 °C for the sample vial chamber.

2.6. Validation of the assay

2.6.1. Linearity

Serum samples of 0, 3.13, 6.25, 12.5, 25, 50 and 100 mg/l prepared by adding the linezolid standard solution were measured. The results were plotted against linezolid peak height ratios versus IS and against linezolid concentrations in order to obtain linear regression.

2.6.2. The limit of quantification

Using serum samples added with the linezolid standard solutions, an average of concentrations measurable when the signal-to-noise ratio of back ground noise was 3 was obtained and R.S.D. was calculated from this so that the limit of detection could be determined.

2.6.3. Reproducibility

To obtain intra-day reproducibility, linezolid-added serum samples of five different concentrations were prepared, each of which were measured five times in a simultaneous and consecutive manner. To obtain inter-day reproducibility, serum samples of two different concentrations added with linezolid were prepared, each of which were measured for 5 consecutive days ($n = 2$). During this, the samples were stored at − 20 ° °C.

2.6.4. Recovery

Serum samples (5, 10, 20, 30, 40 mg/l) prepared by adding the linezolid standard solution to blank serum were measured ($n = 3$) and an average of the measurements, R.S.D. and recovery rate were calculated.

2.6.5. Stability

Linezolid-added serum samples of two different concentrations were pretreated and their re-dissolved solutions in distilled water were measured for 5 consecutive days ($n = 2$), so that fluctuations of these measurements could be observed. During this, the samples were stored at 4 ° °C.

2.6.6. Specificity

Influence of endogenous substances in serum on linezolid determination was evaluated by comparing the chromatogram of linezolid-added serum with that of normal pooled serum not containing linezolid. To evaluate interference from other antimicrobial agents with the determination, 33 drugs (the concentration of all these drugs was 2.0 g/l) were measured and their relative migration times (RMTs) versus linezolid were calculated.

3. Results

Fig. 2 shows the chromatogram of a serum blank sample and the separated linezolid peak. Good separation was obtained without interference with the determination from endogenous substances in serum. The linearity for 0–100 mg/l was indicated as $r = 0.999, y$ (linezolid peak height/IS peak height) $= 0.0317x$ (linezolid concentration) $+ 0.0773$. The limit of detection was 0.5 mg/l when the signal-to-noise ratio was 3. Reproducibility results are shown in Table 1. R.S.D.'s of intra-day reproducibility and inter-day reproducibility were equal or less than 3.82% and equal or less than 3.29%, respectively. Good results were obtained at recovery rate test (Table

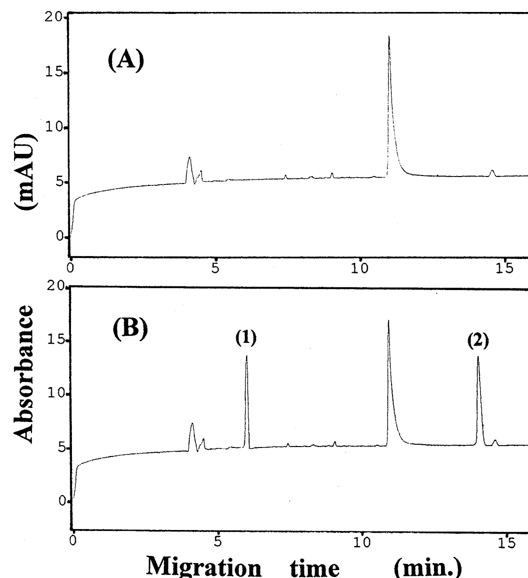

Fig. 2. Typical chromatograms of (A) a blank serum, (B) a serum standard spiked with linezolid (20.0 mg/l). (1) IS; (2) linezolid.

Table 1
Intra-day and inter-day precision of spiked serum samples for linezolid assay

Mean value (mg/l)	S.D. (mg/l)	R.S.D. (%)
Intra-day reproducibility ($n = 5$)		
4.7	0.172	3.66
9.9	0.337	3.40
16.8	0.641	3.82
23.5	0.649	2.76
38.3	0.388	1.01
Inter-day reproducibility (5 days, $n = 2$)		
15.7	0.516	3.29
30.0	0.279	0.930

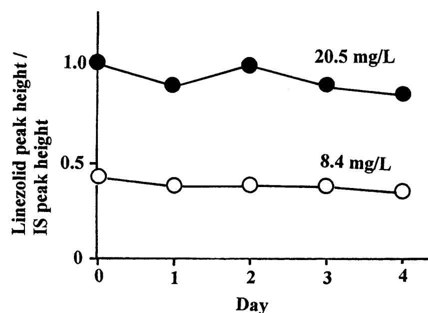

Fig. 3. Stability of 8.4 and 20.5 mg/l serum linezolid after preparation with acetonitrile.

2). Evaluation results of the stability of MEKC injected samples obtained from the serum pretreatment are shown in Fig. 3. It is presumed that samples can remain stable for 4 days or so as long as they are stored at 4 ° °C. Determination results of other anti-microbial agents under analytical conditions of this method were used to calculate ratios of their respective migration times versus the peak migration time of linezolid (RMT). MEKC determination of each agent took 16 min. These are the results: cefusulodin: 0.352, 5-fluorocytosine: 0.359, tinidazole: 0.421, cefotaxime: 0.449, cefuroxime: 0.476, cefazolin: 0.481, vancomycin: 0.484, piperacillin: 0.513, cefadroxil: 0.557, ampicillin: 0.614, furosemide: 0.662, antipyrine: 0.716, cimetidine: 0.720, amoxicillin: 0.757, penicillin: 0.842, cefatrizine: 0.884, cefoxitin: 0.896, cefalotin: 0.949, minocycline: 1.017. Drugs that remained undetected were amikacin, amphotericin B, bacampicillin, carbenicillin, chloramphenicol, erythromycin, gentamycin, ketoconazole, methicillin, miconazole, netilmycin, sisomycin, streptomycin and tobramycin. RMT of IS was 0.438, and therefore, no peaks of the anti-microbial agents evaluated overlapped with the peaks of linezolid and IS.

4. Discussion

Generally, when drugs are to be determined by CE, it is easier if serum samples are directly injected without pretreatment [8,9]. We have tried for the determination of linezolid, but the determination turned out to be impossible due to substantial interference from endogenous substances in serum, and therefore, deproteinization of serum was required [10]. Sádecká et al. [11] in determining vancomycin, have deproteinized serum by adding to serum ethanol of the equal amount of or one and half times as much as the serum and analyzed its supernatant by capillary isotachophoresis. Shihabi [12] recommends using

Table 2
Accuracy of spiked serum samples for linezolid assay ($n = 3$)

Theoretical concentration (mg/l)	Measured concentration (mg/l)	S.D. (mg/l)	R.S.D. (%)	Accuracy (%)
5.0	5.1	0.283	5.55	102.0
10.0	9.9	0.189	1.91	99.0
20.0	20.0	0.704	3.52	100.0
30.0	29.6	0.368	1.24	98.7
40.0	38.0	0.490	1.29	95.0

acetonitrile one and half times as much as serum for deproteinizing the serum. We also deproteinized using ethanol as well as acetonitrile one and half times as much as the serum, but linezolid peak determined by this method was overlapped with those of unremoved impurities in the serum. Brunner et al. [13] have deproteinized serum with acetonitrile, evaporated to dryness its supernatant by blowing air at normal temperature on it and then have re-dissolved the residue in distilled water. Although their method requires somewhat complicated sample preparation, it enables easier analysis because CE samples are of distilled water. Furthermore, with their method, CE samples can be virtually enriched by adjusting re-dissolution liquid volume and evaporation of samples hardly occurs. This method does not require immediate determination after sample preparation because re-dissolved liquid after deproteinization remains stable for a relatively long period of time. However, any of these pretreatment methods does not solve the problem of impurities remaining unremoved. Especially with high resolution factor analysis using CE, peaks of unremoved substances occasionally appear prominently [14]. Generally, when measurement sensitivity is not enough, a stacking method by CE is used [15]. As for linezolid, however, its therapeutic concentration in blood has been reported to be equal or more than 4 mg/l [1], and this method offered enough measurement sensitivity. Evaluation and comparison was performed between when SDS was added to the borate buffer to be used for this method (MEKC) and when SDS was not added (CZE). The results showed that the MEKC method enabled a separation without overlapping with the peaks of impurities in serum and improvement in the theoretical plate number, while these was not achieved by CZE [10]. We performed various evaluations regarding the composition of the running buffer used in this method and the results showed that, in the range of pH 8–11, the higher the pH value became, the shorter linezolid migration time was, though some interference from impurities was observed under pH 9.0. As measurement sensitivity being in maximum at pH 10.0 experimentally and electric current value for the loading voltage of 25 kV being approximately 130 μA both indicated stability, determination was performed at pH 10.0. As for measurement precision and accuracy, R.S.D.'s of intra-day reproducibility and inter-day reproducibility have been reported to be 1.8–4.2% for HPLC [4,5] and 5.1–11.4% for LC-MS-MS [7], and almost 100% of recovery rate has been reported for both. This method offered precision and accuracy of the same level as these methods. As for the limit of detection, 0.05–0.135 mg/l has been reported for other methods [3–5,7]. Although this figure indicates these methods are above this method, it is presumed this method can still enables satisfactory determination of linezolid over the whole range of its therapeutic concentration in blood. While HPLC is an easy analytical method, a HPLC column is a little expensive and comparatively low in durability. On the other hand, a capillary tube used for this method rarely has problems such as blinding or deterioration and is highly durable, requiring extremely tiny volumes of a running buffer and samples. These advantages enabled an economical analysis. As for LC-MS-MS, it has disadvantages of the system itself being expensive and its operation being neither simple nor easy. Thus, this method is believed to be a simple and easy method most suitable for the determination of linezolid in serum.

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