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Journal of Pharmaceutical and Biomedical Analysis
35 (2004) 615–620
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Further method development for measurement of linezolid in human serum by MEKC

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Received 26 June 2003; received in revised form 10 January 2004; accepted 17 January 2004

Available online 20 March 2004

Abstract

A method for determining linezolid concentration in human serum using micellar electrokinetic capillary chromatography by direct injection of serum is described. A borate buffer (pH 8.0) containing sodium dodecyl sulfate was used as a run buffer and detection of linezolid was performed at 250 nm (its absorption maximum). The migration time of linezolid was 5.5 min and the detection limit was 0.5 mg/l (S/N = 3). The precision and accuracy of this method was good with no interference with the detection from bilirubin, hemoglobin and chyle of high concentrations. This provides a simple and easy method where samples of micro-quantity are used.

Keywords: Linezolid; Oxazolidinones; Micellar electrokinetic capillary chromatography; Serum direct injection

1. Introduction

Linezolid (PNU-100766) is an oxazolidinone anti-microbial drug, efficacious against multi-resistant Gram-positive infections (Fig. 1), which inhibits the initial stage of bacterial protein synthesis [1,2]. It exhibits an efficacy against infections caused by Gram-positive organisms that are resistant to vancomycin-resistant enterococci and methicillin-resistant *Staphylococcus aureus* [3,4]. A linezolid peak concentration of 18–20 mg/l (1–2 h post-dose) and a minimal serum concentration of 4 mg/l after a 625 mg dose can be expected [2]. Determination of the drug concentration in blood during treatment is essential for preventing side effects as well as identifying a trough. Traditionally, methods using high-performance liquid chromatography (HPLC) [5–8] or LC-MS [9] have been reported for determining linezolid in blood. LC-MS has disadvantages of its equipment being expensive and its sample preparation being complicated, which makes this method unsuitable for clinical laboratories. As a result, HPLC is generally used. Peng et al. [5] have extracted plasma using a solid phase cartridge, while Borner et al. [6] have de-proteinized serum with perchloric acid and Tobin et al. [7] by adding acetonitrile to serum, to use them as HPLC injection samples. All of these methods require complicated and time-consuming sample preparation which can cause measurement errors easily to occur. Ehrlich

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doi:10.1016/j.jpba.2004.01.020

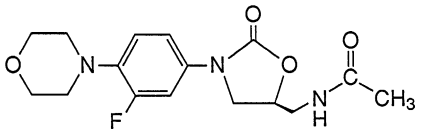

Fig. 1. The chemical structure of linezolid.

et al. [8] have reported a direct injection method of serum using 3 HPLC pumps and 1 (one) 6-port valve. Their method requires a large quantity of mobile phase solvent and injection samples (20 μl), resulting in higher running costs. Capillary electrophoresis (CE), compared with HPLC, requires extremely tiny volumes of solvent and samples and a hollow capillary is highly durable, which enables economic analysis. Furthermore, because its separation efficiency is high, recently, a purity determination of a linezolid preparation where CE is used instead of HPLC [10] has been reported [11]. A method for determining linezolid concentration in serum using CE has already been developed by us [12], but this method requires the pretreatment of serum, prolonging the entire analysis time and it also requires a comparatively large volume of serum. Therefore, this time, the purpose of the study is to develop a new linezolid determination method, which is simple, easy, fast as well as highly precise, using CE by direct serum injection. The assay validation of this method is also reported.

2. Experimental

2.1. Chemicals and reagents

Linezolid was obtained from Pharmacia (Kalamazoo, MI). Sodium dodecyl sulfate (SDS), boric acid, sodium tetraborate decahydrate and sodium hydroxide (0.1 M) were purchased from Wako Pure Chemicals (Osaka, Japan). “Interference check A plus”, a kit for evaluating interference from bilirubin, hemoglobin and chyle, was purchased from Sysmex (Kobe, Japan). The adjustment of these solutions was performed according to the instructions described in the handling manual attached to the kit. Various concentration series were prepared by adding drug-free human serum to undiluted solutions dissolved in albumin, each of which contains direct bilirubin (198 mg/dl), indirect bilirubin (204 mg/dl) and chyle (24700 formazin turbidity), and an undiluted solution of hemolytic hemoglobin (4570 mg/dl). Then, linezolid standard solution of the volume within 5 v/v% was added to them to prepare linezolid-added serum of 100 mg/l. All solvents and chemicals used were at least of an analytical grade.

2.2. Instrumentation and analytical conditions

A P/ACE^{TM} system MDQ by Beckman Coulter (Fullerton, CA, USA) equipped with a photodiode array detector was used as a CE system. An uncoated fused-silica tube (effective length 500 mm, bore 75 μm) (Beckman Coulter, Fullerton, CA, USA) was used as a capillary and was set for 25 °C and at 15 °C for the sample vial tray. A 50 mM boric acid/sodium tetraborate decahydrate buffer (pH 8.0) containing SDS (50 mM) was used as a run buffer. This run buffer was passed through a 0.45 μm filter (Millipore, Bedford, MA, USA) and then de-aired ultrasonically for 5 min before use. The samples introduced into the anodic site of the capillary at 0.5 psi for 8 s and micellar electrokinetic capillary chromatography (MEKC) was performed at 25 kV with normal polarity and detection at 250 nm (λ_{max}). At the beginning of the analysis, the capillary was rinsed with 0.1 M sodium hydroxide for 15 min (20 psi). After each sample analysis, it was rinsed with SDS solution (2 w/v%) for 5 min, with sodium hydroxide (0.1 M) for 3 min, with distilled water for 2 min and then with the run buffer for 5 min, all at a 20 psi pressure before serum samples were injected directly.

2.3. Sample preparation

Stock standard solution of linezolid was prepared and adjusted by dissolving 50 mg of linezolid in 50 ml of distilled water (1.0 mg/ml), which was further diluted with distilled water into working standard solutions of various concentrations. Serum standard solution was prepared by adding the linezolid standard solution of a volume within 5 v/v% to drug-free human serum.

2.4. Assay qualification

The identification of a linezolid peak was performed based on whether the migration times of the linezolid

peaks of linezolid-added serum and the standard solution coincided with each other. Calibration curves were drawn using linezolid-added serum samples of 0, 0.8, 1.6, 3.2, 6.3, 12.5, 25, 50 and 100 mg/l and a peak height was plotted for each linezolid concentration so that linear regression could be obtained. Limits of detection and limits of quantification were determined by using linezolid serum standard solutions and calculating an average of concentrations with which determination was possible when the signal-to-noise ratio of back ground noise was 3 or 10, as well as a coefficient of variation (C.V.). In order to obtain intra-day precision, linezolid-added serum samples of 5, 10, 20, 40 and 80 mg/l were prepared and each of them was measured 5 times in a consecutive manner. As for inter-day precision, linezolid-added serum samples of 7.5, 40 and 70 mg/l were prepared and each of them was measured 6 consecutive days. The samples were stored at −20 °C during this procedure. Accuracy (recovery rate) was calculated by comparing a linezolid-added serum sample of each concentration with a linezolid standard solution of the same concentration. The influence of endogenous substances contained in the serum of normal persons on the linezolid determination was evaluated by comparing chromatograms of drug-free human serum containing no linezolid and a linezolid-added serum sample. The influence of various interfering substances in serum on the determination was also evaluated in the following manner: The concentration series of direct bilirubin (0–39.6 mg/dl), indirect bilirubin (0–40.8 mg/dl), hemolytic hemoglobin (0–914 mg/dl) and chyle (0–4940 formazin turbidity) were prepared, the linezolid standard solution was added to them to make linezolid-added serum samples of 100 mg/l and their linezolid concentrations were measured, so that the influence of each interfering substance could be evaluated.

3. Results and discussion

3.1. Method development

In the previous method pH 10.0 was used for the run buffer [12]. In the present study, because serum samples were injected directly in this method, electrophoretic behaviors in the capillary were different from those described for the previous method and the effect of pH was re-investigated. Because interference with the linezolid peak from impurities contained in serum was observed for run buffers of pH equal to or less than 7.0 and equal to or more than 9.0, pH 8.0 was used for this method. The evaluation results of the borate buffer concentration ranging 25–150 mM showed that determination was not possible at 25 mM due to the interference from impurities in serum and that the migration time of the linezolid peak became longer as the molarity of the borate buffer increased. At 150 mM, the migration time became 1.17 times as long as that at 50 mM while the sensitivity (peak height) became lower by 24.6% than that at 50 mM. Thus, the 50 mM borate buffer was used for this method. The evaluation results of the SDS concentration ranging 10–100 mM showed that the interference from impurities in serum gradually increased at 50 mM or under and that determination was totally impossible at 20 mM or under. Therefore, determination at 50 mM or over was recommended. However, the linezolid peak migration time became longer as the SDS concentration increased, at 100 mM being 1.36 times as long as that at 50 mM, while the sensitivity increased by 18% at 100 mM compared to that at 50 mM. As for the injection time, 10 s. was adopted for the previous method, while 8 s. was adopted for this method because there was not a big difference in measurement sensitivity observed across the 8–10 s. range. In order to increase the sensitivity of CE determination, stacking method, sweeping method and extending the sampling time are the usual measures [13,14]. With this method, enough sensitivity was obtained because 50 mM SDS was used and the sampling time was set for 8 s. In our previous method, serum samples re-dissolved in distilled water following extraction with acetonitrile were used as CE samples. Here on the other hand, a rinsing process using SDS solution was added to the procedure so that it could be performed first to achieve analysis of high precision and accuracy even if serum was to be injected directly. As a result, analysis of high reproducibility could be carried out. Terabe et al. [15] has reported that, with MEKC, good separation of hydrophobic compounds was obtained when an excessive volume of urea was added to a run buffer. Thus, with this method, too, in order to shorten the migration time of linezolid, a high concentration of urea (7.0 M) was

added to the run buffer to increase the dissolution of linezolid in the run buffer. As a result, the migration time became shorter by about 1 min, but, because the dissolution of serum impurities also increased as a whole, the baseline became unstable. Therefore, under analytical conditions of this method, better separation was obtained when no urea was added. In the evaluation of electrophoretic load voltage, a material difference in measurement sensitivity was hardly observed in the 10–25 kV range, but migration time became longer by 10 min at 10 kV than at 25 kV. Thus, 25 kV was adopted so that rapid measurement could be performed (this was the identical load voltage that was adopted for the previous method [12]). As for the detection wavelength, 253 nm was adopted for the previous method, while 250 nm was adopted for this method. This is considered to be the result of a slight change of the λ_{max} value caused by a change in the composition of the run buffer. The maximum ultraviolet region absorption wave length of linezolid (λ_{max}) exhibited dual peaks, one in a region equal to or under 200 nm and another in a 250 nm region, but it became stable at 250 nm with enough measurement sensitivity. The data collection frequency was evaluated in the 4–8 Hz range and 4 Hz was the most suitable for this method. Therefore, after optimization the conditions used for further work were established.

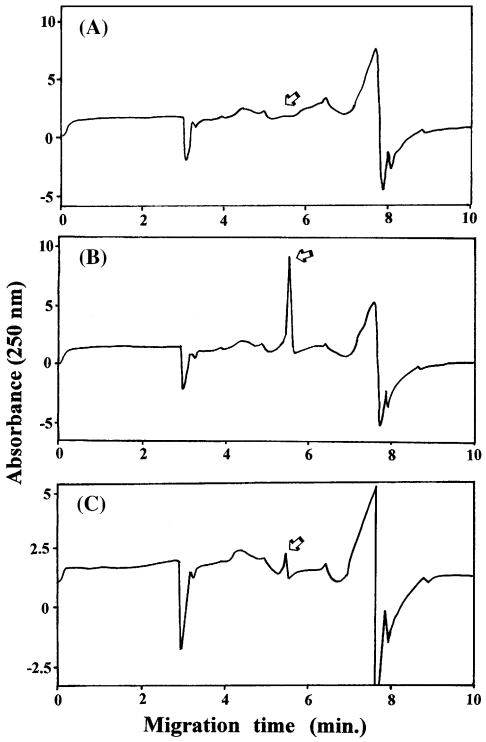

Fig. 2. Typical electropherograms of linezolid in (A) blank human serum, (B) human serum spiked with 40.0 mg/l linezolid, (C) human serum spiked with 1.0 mg/l linezolid.

3.2. Validation of the assays

Fig. 2(A) shows the electropherogram of the drug-free human serum and Fig. 2(B) and (C) that of the linezolid-added human serum. Good separation was obtained without interference from endogenous substances in serum with the determination. The apparent fronting is considered to have been unknown substances in the serum samples. There was no interference from them on the linezolid determination. In addition, the linezolid peak in the electropherogram had a shape good enough for quantification. The migration time of linezolid was 5.5 min and the total run time for each sample was 6.0 min. The linearity as 0–100 mg/l was good ($r = 0.9999$). The limit of detection was 0.5 mg/l at a signal-to-noise ratio (S/N) of 3 and the limit of quantification was 1.0 mg/l at a S/N of 10. Compared to the methods already reported, this method is characterized by the ability to allow us to determine linezolid by CE without the pretreatment of serum samples. The determination of real samples is considered to be possible using this method with almost the same precision and accuracy as shown in the assay validation results of this method. Table 1 shows the evaluation results of intra-day and inter-day precisions, and those of recovery rates. Those results indicated somewhat higher precision than those reported earlier by us [12]. For further comparison with HPLC methods, there are reports as follows; C.V. of intra-day assay as 1.8–2.5% and that of inter-day assay as 1.8–3.1% in the concentration range of 2.5–17.5 mg/l by Borner et al. [6]; C.V. of intra-day assay as 2.4–4.2% (0.63–15.3 mg/l) by Ehrlich et al. [8]; C.V. of intra-day assay as 3.4–5.6% and that of inter-day assay as 9.2–12.1% in the concentration range of 5–30 mg/dl by Tobin et al. [7]. For comparison with a LC-MS method, there is a report by Phillips et al. [9] where C.V. of intra- and inter-day assay precisions is 5.1–11.4%. Assay precision of this method was equal or superior to that of these meth-

Table 1
Intra-day and inter-day precision and accuracy of linezolid in serum

Concentration (mg/l)	Concentration found (mg/l)	S.D. (mg/l)	C.V. (%)	Accuracy (%)
Intra-day assay ($n = 5$)				
5.0	5.1	0.13	2.55	102
10.0	9.7	0.089	0.918	97
20.0	21.0	0.224	1.07	105
40.0	41.0	0.823	2.01	103
80.0	84.9	2.15	2.53	106
Inter-day assay (6 days)				
7.5	7.8	0.16	2.05	104
40.0	40.5	0.669	1.65	101
70.0	69.5	0.999	1.44	99

ods. No definitive report has been made regarding the fate of the drug, and metabolites are hard to obtain. Therefore, no evaluation was performed regarding the potential interference of the metabolites of linezolid for this method. With this method, serum is injected directly for measurement and therefore, the influence of bilirubin, hemolytic hemoglobin, lipid of high concentrations on the linezolid determination often poses a significant problem. Thus, the evaluation results of the influence of these interfering substances are shown in Fig. 3. From these results, the average was obtained as 98.6–102.8 mg/dl versus the linezolid target value of 100 mg/dl and C.V. as 1.19–2.47%, which proved that the determination was not influenced by these high concentration substances. Although the concentration ranges in Fig. 3 are extremely wide, the *M* (mean) value and the coefficient of variation (C.V.) value shown in the figure indicate that there was no effect of bilirubin, hemoglobin and chyle. Therefore, interference from these substances will not pose a problem in the clinical laboratory.

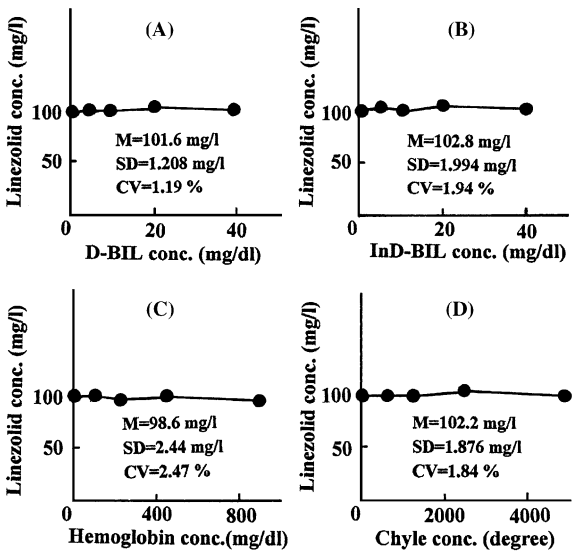

Fig. 3. Variations of serum linezolid concentration with (A) direct bilirubin (D-BIL), (B) indirect bilirubin (InD-BIL), (C) blood hemoglobin, and (D) chyle counted by formazin turbidity in human serum. *M*, S.D., and C.V. show the mean concentration of linezolid in serum, standard deviation, and coefficient of variation, respectively.

4. Conclusion

This method enjoys improvements over the linezolid determination method using CE reported earlier by us [12] such as (i) requiring no serum pretreatment, (ii) requiring substantially reduced volume of serum, (iii) requiring no use of internal standards, (iv) shortening the migration time of a linezolid peak, (v) having improvement in precision. Thus, this method enabled a simple, easy, fast, specific and highly precise analysis.

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