Analysis of Micafungin in Serum by Capillary Zone Electrophoresis

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Abstract

A method is developed for measuring the concentration of micafungin, an echinocandin antifungal agent, in serum, using capillary zone electrophoresis. With a 0.1M borate buffer (pH 10.0) as the run buffer, detection is carried out at 200 nm. Pretreatment is performed by adding acetonitrile to serum (1:2) for deproteinization, allowing the supernatant fluid to be taken for measurement. The detection limit of the assay is 0.5 mg/L at a signal-to-noise ratio of 3.0. Coefficients of variation for intra- and inter-assay precision are 3.45–4.47% and 2.61–7.15%, respectively, at a nominal concentration of 5.0–25.0 mg/L. Their recovery rates are 92–110%. It is convenient for the monitoring of micafungin therapy in patients contracting clinically important deep mycoses.

Introduction

Micafungin (MCFG) is a new echinocandin antifungal agent that has antifungal activity. MCFG exhibits antifungal activity against Candida species, Aspergillus species, etc., which are the major causal fungi of deep mycoses. They act by inhibiting the biosynthesis of 1,3-β-D-glucan, one of the major components of cell walls specific to fungi (1,2). The typical effective MCFG concentration range in the serum is not decided. It is necessary to measure the MCFG level in the serum of an individual patient. The kinetics of MCFG in the system needs to be monitored for the purpose of determining its doses for patients suffering its side-effects, such as anaphylactoid symptoms and decreased neutrophils, as well as for the elderly with lowered physiological functions (3,4). MCFG, when in plasma, is bonded with proteins at a rate of 99.8% or greater, most of which is excreted as feces (5). Therefore, measuring MCFG concentration in serum is useful as an indicator of its kinetics in the system.

Niwa et al. (6) quantitated MCFG with a fluorescence detector by separating it from the plasmas and tissues of the rat using high-performance liquid chromatography (HPLC). MCFG, having ionic functional groups as shown in its structural formula (Figure 1), can be analyzed electrophoretically. Capillary electrophoresis (CE) requires small samples and only small amounts of run buffer to be used for one run, and it employs a fused-silica capillary tube of high durability. These characteristics make analysis by CE more economical than HPLC. This study elaborates on a determination method of MCFG concentration in serum using capillary zone electrophoresis (CZE) and a serum sample preparation method. The analysis of this method, validation of the assay, and measurement of clinical samples are described in this paper.

Experimental

Reagents

Astellas Pharmaceuticals (Tokyo, Japan) kindly provided micafungin sodium. Sodium decahydrate tetrahydroborate, sodium hydroxide (0.1M), acetonitrile, and *L*-tryptophan (internal standard) were purchased from Wako Pure Chemicals (Osaka, Japan). All solvents and chemicals used were at least of an analytical grade.

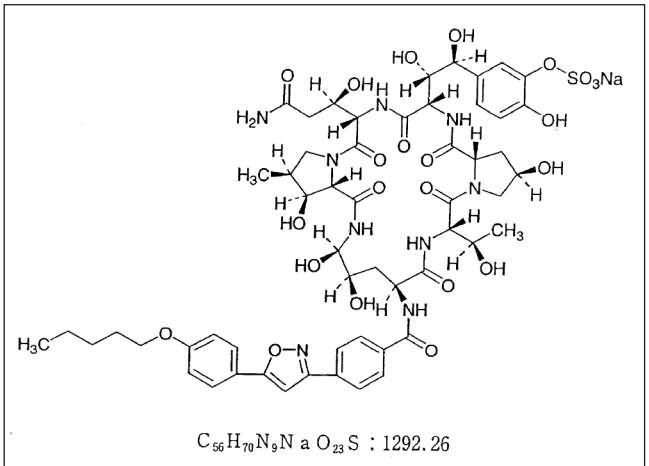

Figure 1. The chemical structure of micafungin.

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Electrophoresis

CZE was performed using the P/ACE system MDQ (Beckman Coulter, Fullerton, CA) equipped with a photodiode-array detector. A fused-silica capillary tube (total length, 70 cm; injector-detector length, 50 cm; internal diameter, 75 µm) was selected. A 0.1M borate buffer was prepared as the run buffer and adjusted to pH 10.0 with 0.1M sodium hydroxide. This run buffer was passed through a 0.45-µm filter (Millipore, Bedford, MA) and deaired ultrasonically for 5 min before use. The temperature of the capillary tube was set at 25°C. Injection time was 10 s under a pressure of 20 psi, the applied voltage was 25 kV, and detection was carried out at UV 200 nm. For each sample, the capillary tube was washed with 0.1M sodium hydroxide (3 min), followed by distilled water (2 min), and a final equilibrium with the CZE run buffer (5 min).

Preparation of calibration standards

MCFG stock solution (1.0 g/L) was prepared through dissolution with distilled water and stored at –20°C. Calibration standards were prepared by diluting the stock solution with distilled water. Serum calibration standards were prepared by adding the MCFG calibration standards to drug-free human serum so that the concentration of the standards became 5% (v/v) (5, 10, 15, 20, 25 mg/L).

Preparation of serum samples

A sample of 0.01 mL of *L*-tryptophan (0.2 g/L) was used as an internal standard (IS), and 0.1 mL of acetonitrile were added to 0.05 ml of serum for deproteinization. It was centrifuged at 12,000 × *g* for 2 min, and its supernatant fluid was used as CE samples.

Clinical application

The serum of patients contracting Aspergillosis or Candidiasis, taken just after the administration of 50 mg of MCFG (Funguard, Astellas Pharmaceuticals) intravenous drip, was used for the measurement. The patient's serums used by this method were remainders after an earlier diagnostic inspection. The data of the measured MCFG concentration was not used for a clinical diagnosis. The purpose of this research was to examine the possibility of measuring patient serum by this method. Therefore, this study did not generate the problem of an ethical issue.

Results and Discussion

Choice of analysis conditions

In evaluating the borate concentration of the run buffer, it was discovered that the higher its molarity became, the more the band diffusion of a peak was suppressed, resulting in better resolution. However, as the molarity increased, the electric current generated in the capillary tube also increased, causing instability of the baseline during analysis. Thus, 0.1M borate buffer was employed.

The evaluation of the optimal pH value of the run buffer over the range of 8.0–11.0 using a 0.1M borate buffer revealed that the highest sensitivity was obtained at pH 10.0. The migration time of MCFG became longer as the pH increased (Figure 2). The results of the capillary tube temperature (15–30°C) showed that, as the temperature became higher and sensitivity increased, the MCFG migration time became shorter and peak shape became sharper. Considering generated electric current, etc., 25°C was deemed to be optimal (Figure 3).

From the evaluation of applied voltage (15–30 kV), as the voltage decreased, sensitivity and the migration time tended to increase, with the peak shape becoming broader (Figure 4). For fast determination, 25 kV was deemed to be optimal. As sampling time became longer, sensitivity increased linearly. Considering

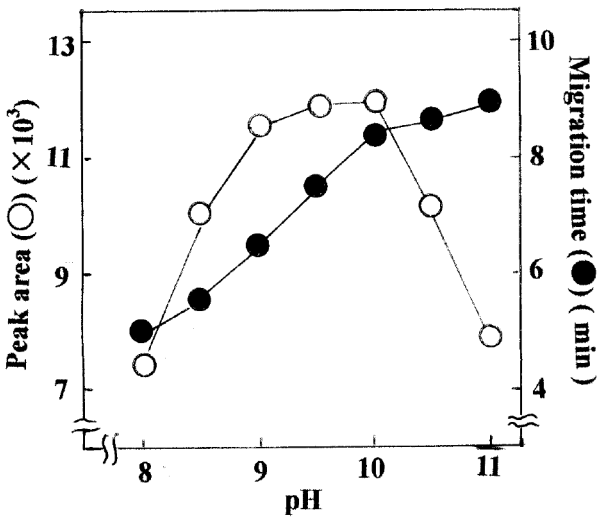

Figure 2. The effect of pH in borate buffer (0.1M) on peak area and the migration times of micafungin. The other conditions were: applied voltage, 25 kV; capillary temperature, 25°C; pressure injection time, 10 s; UV detection wavelength, 200 nm; and concentration of standard serum, 50.0 mg/L.

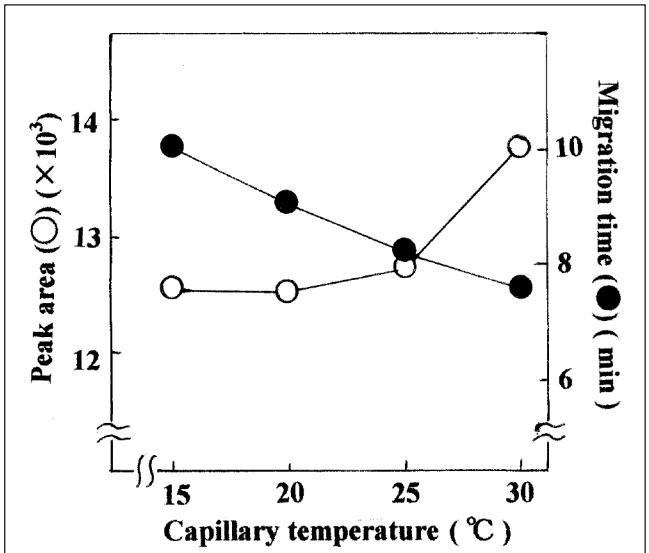

Figure 3. The effect of capillary temperature on peak area and the migration times of micafungin. The other conditions were: run buffer, borate buffer (0.1M, pH 10.0); applied voltage, 25 kV; pressure injection time, 10 s; UV detection wavelength, 200 nm; and concentration of standard serum, 50.0 mg/L.

the peak shape, etc., 10 s seemed to be the limit. As for the detection wavelength, MCFG had the maximum absorption in the range of 200 nm and under, as well as at 270 nm. However, sensitivity at 200 nm was several times higher, and therefore, detection was carried out at 200 nm, which was the measurement limit in terms of hardware.

Choice of serum sample preparation method

When serum was used as the sample, the protein it contained was the biggest interfering substance (7). The direct injection of serum without pretreatment was simple and convenient and offered excellence in precision, accuracy, and speed (8,9). However, this procedure caused difficulty in separating MCFG from endogenous substances, such as proteins, which accounted for the majority of serum. Extraction and deproteinization were two possibilities for MCFG pretreatment. Extraction with diethyl ether and ethyl acetate was attempted to no avail. The reason for this failure was possibly the inability of extracting MCFG with these organic solvents because of their high polarity. Either acetonitrile or methanol could be used for deproteinization, of which, acetonitrile offered a higher deproteinization ratio of serum and was used for analyzing various drugs (10). An evaluation on the mixing ratio between serum and acetonitrile (serum 1:acetonitrile 0.67–3.0) showed that baseline noise increased as the mixing ratio of acetonitrile became higher. The increase in the mixing ratio of acetonitrile also meant an increase in the dilution ratio with acetonitrile, which in turn caused a decrease in quantitation sensitivity. On the other hand, a decrease in the mixing ratio of acetonitrile caused an unsatisfactory removal of serum proteins, resulting in higher interference from them. Shihabi et al. (11–13) reported that, in analyzing drugs in serum by CE, an acetonitrile mixing ratio to serum at 1.5–2.0 offered the optimal analysis. From this evaluation, an acetonitrile mixing ratio to serum at 2 allowed good separation.

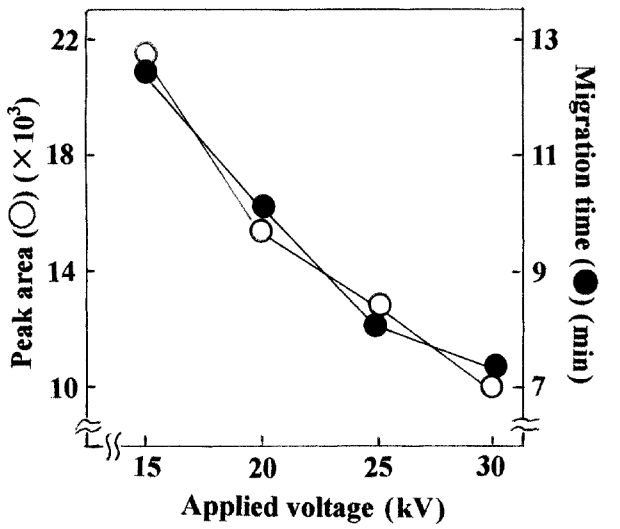

Figure 4. The effect of applied voltage on peak area and the migration times of micafungin. The other conditions were: run buffer, borate buffer (0.1M, pH 10.0); capillary temperature, 25°C; pressure injection time, 10 s; UV detection wavelength, 200 nm; and concentration of standard serum, 50.0 mg/L.

Choice of internal standard

In the pretreatment of serum by deproteinization, internal standard (IS) must be employed to ensure precision and accuracy. The IS should be a substance whose peak, together with a MCFG peak, can be separated well without overlapping and without impurity peaks appearing in the electropherogram. From the evaluation of several ISs, acetaminophen, procainamide, caffeine, tyramine, and *L*-tryptophan were eluted earlier than the MCFG peak, and ceftriazone and theophylline were eluted later than the MCFG peak. Of these, only *L*-tryptophan was separated well without overlapping with either MCFG or impurities, which was the reason why this substance was employed as the IS for this method.

Specificity

The electropherograms of blank serum and standard solution serum, measured using drug-free human serum, are shown in Figure 5. Both MCFG and IS were separated without interference from endogenous substances in the serum.

Linearity

The linearity of this method (corrected peak area ratio of MCFG–IS versus MCFG concentration) was evaluated over a concentration range of 0–25 mg/L (calibration standards: 0, 5, 10, 15, 20, and 25 mg/L). Regression analysis, performed by the least-squares method, gave $r^2 = 0.998$.

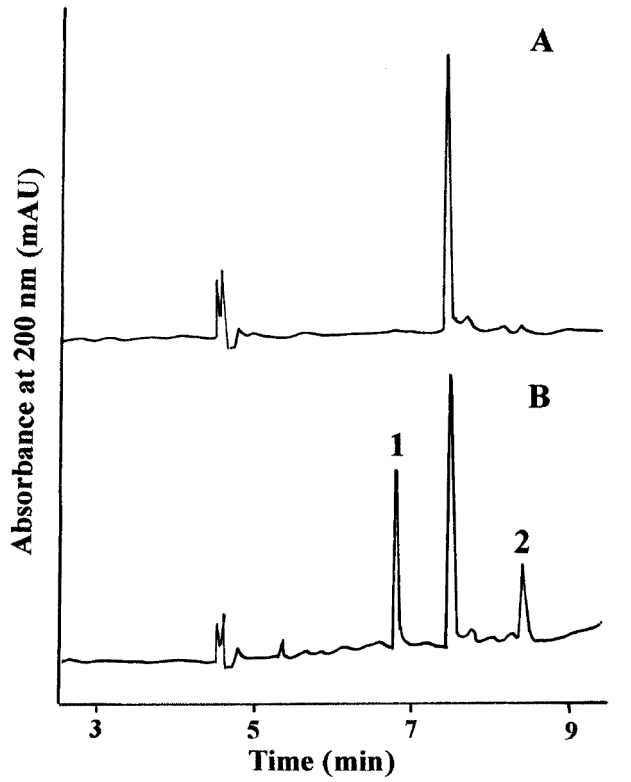

Figure 5. Typical electropherograms of a blank human serum (A) and serum standard (28.6 mg/L) with internal standard (peak 1) and micafungin (peak 2) (B). The other conditions were: run buffer, borate buffer (0.1M, pH 10.0); applied voltage, 25 kV; capillary temperature; 25°C; pressure injection time, 10 s; and UV detection wavelength, 200 nm.

Table I. Statistics of Intra-assay and Recovery (n = 5) to Determine Micafungin in Serum Samples

Nominal concentration (mg/L)	Measured concentration (mg/L)	CV (%)	Recovery (%)
5.0	5.5	3.45	110
10.0	9.5	3.47	95
15.0	14.0	4.20	93
20.0	18.2	4.47	92
25.0	26.2	4.47	104

Table II. Statistics of Inter-assay (Six Days) to Determine Micafungin in Serum Samples

Measured concentration (mg/L)	CV %
7.60	6.45
11.7	7.15
15.4	2.61
20.0	6.50
22.7	3.51

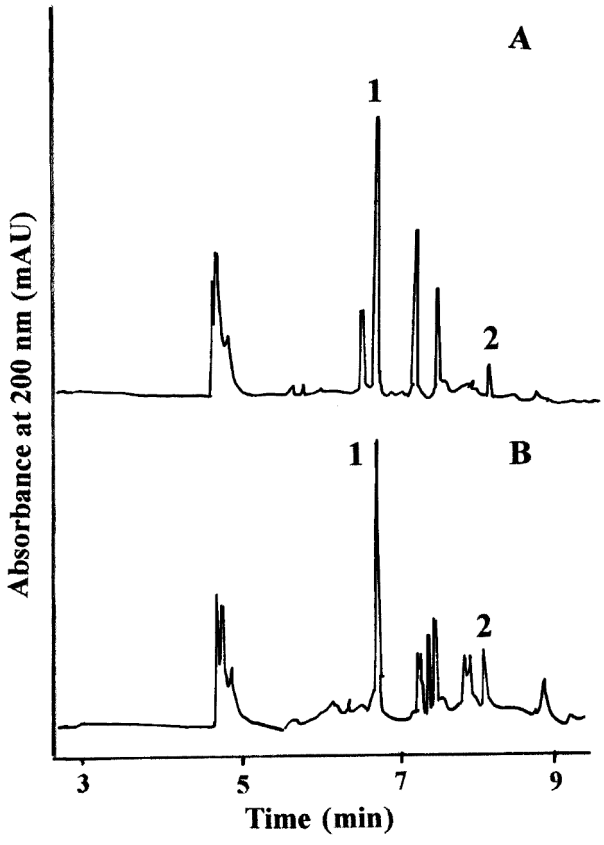

Figure 6. Electropherograms of serum samples from patients on micafungin [6.7 mg/L (A); 12.3 mg/L (B)] with internal standard (peak 1) and micafungin (peak 2). The other conditions were: run buffer, borate buffer (0.1M, pH 10.0); applied voltage, 25 kV; capillary temperature, 25°C; pressure injection time, 10 s; and UV detection wavelength, 200 nm.

Detection and quantitation limits

The detection limit, defined as the MCFG concentration, yielded a peak three times the noise level. In this method, the detection limit of MCFG was 0.5 mg/L at a signal-to-noise ratio of 3, and its quantitation limit was 2.0 mg/L. Niwa et al. (6) reported the quantitation limit of MCFG in rat plasma to be 0.05 mg/L (0.1 mL plasma sample) using HPLC and a fluorescence detection method. The cell capacity of a detector ordinarily used for CE was so minute compared with a HPLC detector, that it unavoidably caused a decline in sensitivity. Mrestani et al. (14), by using a high-sensitivity cell for CZE determination, increased detection sensitivity to 10 times higher than an ordinary cell; however, it was difficult to apply this cell to the CE system used in this method because of a technical reason. However, the quantitation limit of this method could, satisfactorily, be applied to clinical samples.

Repeatability and recovery

Intra-assay reproducibility, measured five consecutive times using the serum standard solution and recovery ratio test results obtained by adding the MCFG calibration standard to the serum calibration standards, are given in Table I. The coefficient of variation (CV) was 4.5% or less for both. Inter-assay reproducibility was evaluated based on six days, and the results are given in Table II. The CV was 7.2% or less.

Clinical application

MCFG concentration in the serum of five patients after intravenous drip infusion was measured, and the representative electropherograms of two of these five patients are shown in Figure 6. Variations in MCFG concentration in serum are observed among these three patients, which should be attributed to variations in their body weight and the severity of their ailments. The purpose of this study was to determine the MCFG concentration in patients' samples using a simple and easy method by CE. The possibility of making the determination using this method was confirmed from just a few examples of the patients evaluated in this study.

Conclusion

The proposed CZE method allowed fast determination with excellent specificity and excellent precision and accuracy only through the deproteinization of serum with acetonitrile. Therefore, this method is convenient and can be applied to monitoring the treatment of patients administered with MCFG as antifungal therapy.

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