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Short communication

Direct-injection HPLC method of measuring micafungin in human plasma using a novel hydrophobic/hydrophilic hybrid ODS column

Hiroaki Uranishi[a], Mitsuhiro Nakamura[a,*], Hiroki Nakamura[b], Yukari Ikeda[b], Mayuko Otsuka[a], Zenichiro Kato[c], Teruo Tsuchiya[d]

[a] Laboratory of Drug Informatics, Gifu Pharmaceutical University, 1-25-4, Daigaku-nishi, Gifu 501-1196, Japan
[b] Gifu Research Center for Public Health, 4-6 Akebono-cho, Gifu 500-8148, Japan
[c] Department of Pediatrics Graduate School of Medicine, Gifu University, Yanagido 1-1, Gifu 501-1194, Japan
[d] Laboratory of Clinical Pharmacy, Gifu Pharmaceutical University, 1-25-4, Daigaku-nishi, Gifu 501-1196, Japan

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ABSTRACT

A direct-injection HPLC-based method has been developed for determining amounts of micafungin in human plasma using a novel hydrophobic/hydrophilic hybrid ODS column. The method is easy to perform and requires only 10 μL of a filtered plasma sample. The chromatographic separations were carried out with a gradient mode. The fluorescence detection wavelengths of excitation and emission were set at 273 nm and 464 nm, respectively. Retention times for micafungin and IS were 22.4 and 23.7 min, respectively. Micafungin and FR195743 (IS) peaks were completely separated with little tailing, and no interference was observed. The calibration curve of micafungin showed good linearity in the range of 0.5–20.0 μg/mL (r^2 = 1.00). The intra-day accuracy ranged from −4.5 to 5.3%. The inter-day accuracy ranged from −9.8 to 1.5%. The precisions were less than 10%. This method is useful for the determination of micafungin in human plasma.

1. Introduction

Micafungin (MCFG), an antifungal agent, is a water soluble cyclic hexapeptide with a fatty acyl side chain, similar in structure to echinocandins (Fig. 1) [1]. MCFG has a unique mechanism of action that inhibits the synthesis of 1, 3-β-D glucans in the fungal cell wall [2,3]. MCFG has been approved for the treatment of esophageal candidiasis, and for the prophylaxis of candida infections in patients undergoing hematopoietic stem cell transplantation [1,4–6]. Since plasma concentrations of MCFG show substantial variability, appropriate blood concentrations for therapeutic drug monitoring of MCFG are yet to be established [7–10]. For these reasons, the analysis of MCFG in plasma is of great interest to clinicians.

There have been several reports of assays for MCFG in blood using reversed-phase (RP) high performance liquid chromatography (HPLC) with fluorescence detection [6,11–14]. These methods showed a lower limit of quantitation (LLOQ) of 50 ng/mL using 100 μL of sample [12,14]. On the other hand, Farowski et al. have reported using LC–tandem mass spectrometry for the determination of anidulafungin, caspofungin, isavuconazole, posaconazole, voriconazole, and MCFG [15]. Despite efforts to enhance the signal intensity of MCFG, the LLOQ was 160 ng/mL with their method.

The preparation of samples prior to chromatography is an essential part of the analytical process. Traditional techniques rely on extraction with solvents, including liquid–liquid extraction, solid-phase extraction, and protein precipitation. In some reports, a diluted serum sample was directly injected into an on-line solid-phase extraction column connected to a HPLC system to measure antifungals [16,17]. Protein precipitation is one of the simplest bioanalytical pre-treatments. It only involves the addition of a precipitating solvent, subsequent homogenization, and centrifugation. Protein precipitation with acetonitrile for the determination of MCFG is common [12,14]. These samples often require complex time-consuming multistep procedures and the consumption of large amounts of solvents.

Recently, a novel hydrophobic/hydrophilic hybrid ODS column (Cadenza HS-C18, Imtakt Corp. Kyoto, Japan) has been developed [18]. It possesses the structure of a hybrid ODS stationary phase combining hydrophobic and hydrophilic groups and enables the direct injection of plasma. RP-ODS columns typically have a high level of hydrophobicity. This hydrophobicity makes eluting a blood plasma protein via an aqueous mobile phase very difficult. Although a number of RP columns that allow blood serum samples to be injected directly are currently available, they (1) require pre-treatments such as column switching, (2) can only be used with isocratic elution, and (3) require non-volatile buffers (e.g. phos-

* Corresponding author. Tel.: +81 58 230 8100x3613; fax: +81 58 230 8105.
E-mail address: mnakamura@gifu-pu.ac.jp (M. Nakamura).

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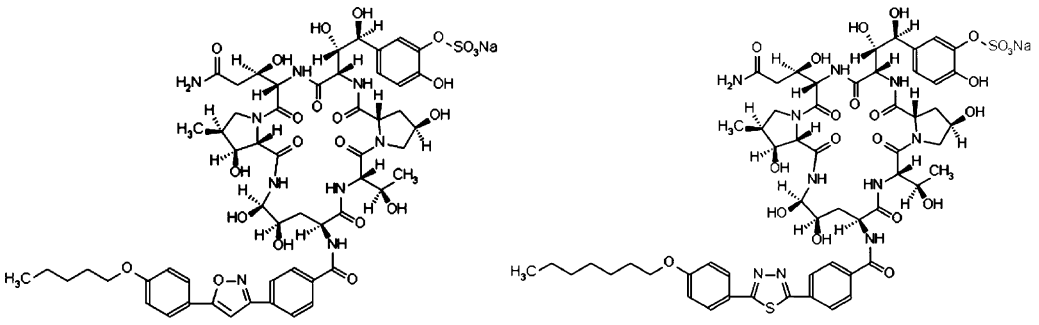

Fig. 1. Chemical structures of MCFG and FR195743.

phate buffers) to give clearer peaks for basic compounds. These limitations can be resolved with an ODS stationary phase that contains a hydrophilic group, such as Cadenza HS-C18. On the other hand, direct injection is generally messier than conventional methods of extraction. A major drawback with the direct injection of a biological matrix is susceptibility to endogenous compounds. In order to reduce the effects of endogenous compounds, we used selective fluorescence detection for MCFG.

The purpose of the present study was to establish a direct-injection HPLC-based method for determining amounts of MCFG using a novel hydrophobic/hydrophilic hybrid ODS column. This specific method was proved valid for the quantification of MCFG. It was also successfully used to determine amounts of injectable MCFG.

2. Methods

2.1. Chemicals and materials

A standard solution of MCFG, an internal standard (IS) solution of FR195743, and Funguard™ 25 mg for infusion (production lots: 3071 and 3380) were kindly supplied by Astellas Pharma Inc. (Tokyo, Japan). HPLC-grade acetonitrile was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). Ammonium acetate was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Heparinized human plasma was purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan). All reagents were of analytical reagent grade.

2.2. Standard solutions

The stock solution of MCFG was dissolved in ethanol at a concentration of 100 μg/mL. The stock solution of FR195743 was dissolved in 0.02 mol/L potassium phosphate/acetonitrile (50/50, v/v) at a concentration of 100 μg/mL. MCFG-spiked plasma was prepared as follows: the MCFG stock solution was evaporated under nitrogen gas with 43 °C. The residue was dissolved in human plasma, to which was added phosphoric acid (diluted three-fold with water) at a ratio of 10 μL to 1 mL of the plasma in order to stabilize the MCFG, to final concentrations of 0.5, 1.0, 5.0, 10.0, and 20.0 μg/mL. The IS solution was prepared as follows: the FR195743 stock solution was diluted with 0.05 mol/L ammonium acetate at a concentration of 4.0 μg/mL.

2.3. Sample preparation

MCFG-spiked plasma (100 μL) was transferred to a 1.5-mL Eppendorf tube, 100 μL of the IS solution was added, and the mixture was vortex-mixed for 10 s. The mixture was filtered with Millex®-HV (0.45 μm) (Millipore Corp., Billerica, MA), and 10 μL of filtrate was injected into the HPLC system.

2.4. HPLC

The HPLC system consisted of a system controller (SCL-10AVP), pump (LC20-AD), fluorometric detector (RF-10AXL), and electronic integrator (Chromatopac C-R8A) (all from Shimadzu, Kyoto, Japan). Samples were introduced with a syringe into a Rheodyne Model 7725(i) injector (Rheodyne, Rohnert Park, CA). The separation of MCFG was achieved using a hydrophobic/hydrophilic hybrid ODS column (Cadenza HS-C18, 3.0 mm × 150 mm, particle size 3 μm) as an analytical column and Cadenza HS-C18 (2.0 mm × 5.0 mm) as a guard column (these from Imtakt Corp., Kyoto, Japan).

The chromatographic separations were carried out with a gradient. The mobile phase consisted of solvent A (0.05 mol/L ammonium acetate) and solvent B (acetonitrile). The gradient was as follows: from 0 to 1 min, 0% B (isocratic); from 1 to 6 min, 0 to 22% B (linear gradient); from 6 to 16 min, 22 to 32% B (linear gradient); from 16 to 21 min, 32 to 80% B (linear gradient); from 21 to 26 min, 80% B (isocratic); from 26 to 31 min, 0% B (isocratic). The wavelengths of excitation and emission were set at 273 nm and 464 nm, respectively. The flow rate was set at 0.4 mL/min. The column temperature was 50 °C.

2.5. Method of validation

A calibration curve was generated using plasma spiked with 0.5, 1.0, 5.0, 10.0, and 20.0 μg/mL of MCFG. A correlation coefficient was calculated with the peak area ratio of MCFG vs. IS. The limit of detection (LOD) and the limit of quantification (LOQ) were determined as the concentration with a signal-to-noise (S/N) ratio of 3 and 10, respectively. The methods of intra-day and inter-day precision and accuracy were estimated by assaying replicates of the 1.0, 10.0, and 20.0 μg/mL MCFG-spiked plasma. The intra-day precision and accuracy were determined by analyzing 6 replicates of each of the concentrations on the same day. As for the inter-day precision and accuracy, 6 replicates of each plasma concentration were analyzed along with one set of standard samples on each of 6 days using the same instruments. Recovery of MCFG was determined by comparing the peak areas of the MCFG-spiked plasma to the peak areas of the MCFG standard solution diluted with 0.05 mol/L ammonium acetate.

2.6. Application

We estimated the amount of MCFG in Funguard™ 25 mg (production lots: 3071 and 3380). Each lot was measured 5 times and MCFG content, precision, and accuracy were calculated.

3. Results and discussion

Cadenza HS-C18 has the structure of a hybrid ODS stationary phase combining hydrophobic and hydrophilic groups and differs

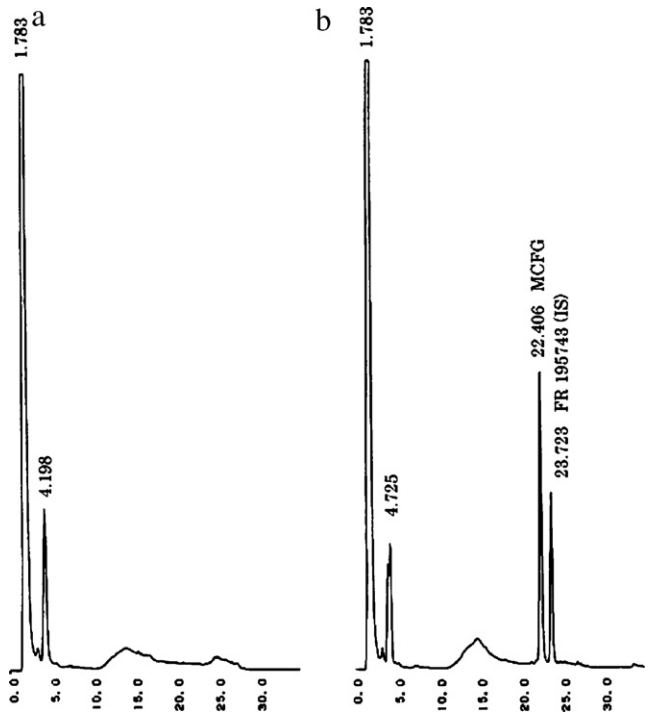

Fig. 2. Typical HPLC chromatograms of MCFG and FR195743. (a) Blank plasma, (b) blank plasma spiked with 20.0 μg/mL MCFG and 4.0 μg/mL IS.

from conventional ODS in that it has a lower density and more hydrophilic groups [18]. Therefore, Cadenza HS-C18 exhibits a different separation characteristics from a conventional ODS column and enables the direct injection of blood plasma. Fig. 2 shows representative chromatograms obtained from blank plasma, and blank plasma spiked with MCFG and IS. Table 1 summarizes the chromatographic data for Cadenza HS-C18. Retention times for MCFG and IS were 22.4 and 23.7 min, respectively. In this assay, no interference due to the direct injection of blood plasma was observed, and MCFG and IS peaks were well separated (resolution: 2.26) with little tailing. The use of a LC–mass spectrometry-compatible mobile phase (e.g. ammonium acetate) is unique compared to other direct injection columns, which require non-volatile buffers (e.g. phosphate buffers). With our method, a gradient system using ammonium acetate and acetonitrile showed the successful separation of highly polar materials, MCFG and IS.

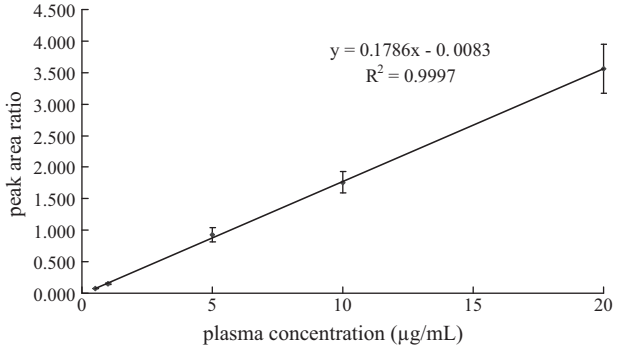

Fig. 3. Calibration curve for MCFG in plasma.

The linear regression of peak–area ratio versus concentration was fitted over the concentration range of 0.5–20.0 μg/mL in human plasma (Fig. 3). The calibration curve was fitted with the following equation:

$$y = 0.1786x + 0.008$$

where y is the peak–area ratio of MCFG to IS, and x is the concentration (μg/mL) of MCFG. The correlation coefficient (r) was 0.999 ($n = 3$). The LOD (0.11 μg/mL) and LOQ (0.33 μg/mL) using our method were comparable with other reports [12,14,15]. In a clinical study in Japan, the C_{max} values of MCFG were 14.30 μg/mL (single administration of 150 mg) and 2.52 μg/mL (single administration of 25 mg) [19]. Shimoeda et al. recommended 5.0 μg/mL or higher as a target blood trough level of MCFG in establishing a dose for treating aspergillosis in patients with blood diseases [20]. The sensitivity of our method was considered satisfactory for clinical application. On the other hand, M1 (catechol form of MCFG) and M2 (methoxy form of MCFG), active metabolites of MCFG, were detected in patient plasma after treatment [21,22]. Since the pharmacological effects of these metabolites were reported to be weak [23,24], we did not target them in our study.

Table 2 summarizes the intra- and inter-day precision, accuracy, and recovery at the three concentrations of MCFG (1.0, 10.0 and 20.0 μg/mL). The intra-day accuracy ranged from −4.5 to 5.3% and the precision (coefficient value, C.V.) was 2.7–9.8%. The inter-day accuracy ranged from −9.8 to 1.5% and the precision was 6.3–9.9%. The mean recoveries of MCFG-spiked plasma at the three levels were in the range of 99.8–115.9% (Table 3). These results indicated that the proposed method was precise and accurate.

Table 1
Chromatographic data of Cadenza HS-C18.

Compounds	t_R (min)	k'	α	Rs	N	H (μm)	Tf	LOD (μg/mL)	LOQ (μg/mL)
MCFG	22.4	11.68	–	–	29,709	5.05	1.28	0.098	0.328
FR1957463	23.7	12.44	1.07	2.26	38,238	3.92	1.17	–	–

t_R, retention time; k', capacity factor [$k' = (t_R - t_0)/t_0$].
α, coefficient of separation [$\alpha = k'_2/k'_1$]; Rs, resolution [$Rs = 1.18(t_{R2} - t_{R1})/(W_{0.5h2} + W_{0.5h1})$].
N, number of theoretical plates [$N = 5.55(t_{R2}/W_{0.5})^2$].
H, height equivalent of a theoretical plate [H = length of column/N].
Tf, tailing factor [$Tf = W_{0.05h}/2a_{0.05}$]

Table 2
Intra-day and inter-day precision, accuracy for the determination of micafungin in plasma.

Nominal concentrations (μg/mL)	Intra-day ($n = 6$)			Inter-day ($n = 6$)		
	Mean ± S.D. (μg/mL)	C.V. (%)	Accuracy (%)	Mean ± S.D. (μg/mL)	C.V. (%)	Accuracy (%)
1	0.95 ± 0.03	2.7	−4.5	0.90 ± 0.06	6.3	−9.8
10	9.97 ± 0.66	6.6	−0.3	10.15 ± 0.84	8.3	1.5
20	21.06 ± 2.06	9.8	5.3	19.47 ± 1.92	9.9	−2.7

C.V., coefficient of validation.

Table 3
Mean recovery of micafungin.

Nominal concentrations (μg/mL)	Intra-day ($n = 6$)		Inter-day ($n = 6$)	
	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
1	103.7	6.3	115.9	21.2
10	99.8	3.9	110.6	13.6
20	107.9	6.5	115.2	9.3

C.V., coefficient of validation.

The amounts of injectable MCFG (Funguard™, production lots: 3071 and 3380) were 24.5 ± 1.2 and 25.6 ± 1.5 mg (mean ± S.D., $n = 5$), respectively. This result indicated that there was no significant lot-to-lot variation. Accuracies of injectable MCFG (lots 3071 and 3380) were −8.1 and 8.3%, respectively. Precisions were 4.9 and 5.8%. These results meet the standard of content uniformity test in the Japanese Pharmacopoeia, fifteenth edition.

In conclusion, we developed a direct-injection HPLC-based method for the analysis of MCFG in human plasma. This method does not require solid-phase extraction, column switching, or other complicated processes. We consider that our method has significant advantages with respect to the reduction of sample preparation; thus it may be useful for therapeutic drug monitoring of MCFG. We achieved good performance with a hydrophobic/hydrophilic hybrid ODS column and found it to be a valuable addition to the various columns available commercially.

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