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Simple HPLC Method for the Determination of Caspofungin in Human Plasma

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Abstract

Antifungal caspofungin (CPFG) was approved for the treatment of febrile neutropenia (FN) as the empiric treatment. However, the relationship between pharmacokinetic properties of CPFG and its clinical effects in patients with FN has not been fully established yet. Thus, in the present study, we tried to establish the simple and quantitative HPLC method to measure CPFG in human plasma with liquid-liquid extraction. CPFG in human plasma was extracted by liquid-liquid extraction and was separated by 5C18 column with mobile phase containing 20 mM phosphate buffer (pH 2.5) and acetonitrile (65:35). CPFG and *p*-hydroxybenzoate ethyl ester, used as an internal standard (IS), were detected by a fluorescence detector (Ex: 224 nm, Em: 304 nm) and by UV-VIS (254 nm), respectively.

CPFG and IS were detected with retention times of 17.0 and 9.5 min, respectively, which were separated from matrix compounds. The calibration curves were linear from 1.0 to 20 μg/mL (R^2>0.99). The limit of detection, the limit of quantification and the lower limit of quantification were 0.53 μg/mL, 0.89 μg/mL and 1.0 μg/mL, respectively. The validation study revealed that the intra- and inter-day accuracy and precision were within the acceptable range and that CPFG was fairly stable after freezing and thawing, reconstitution, in autosampler and in stock solution at ambient temperature up to 8-24 h.

These results suggest that our established method to measure CPFG in human plasma would be applicable to measure plasma CPFG in patients with FN in order to establish the evidence for appropriate drug therapy.

Keywords: Caspofungin; HPLC; Human; Febrile neutropenia; Liquid-liquid extraction

Introduction

In patients who are receiving cancer chemotherapy, invasive fungal disease occurred frequently, subsequently causing febrile neutropenia (FN) [1]. Caspofungin (CPFG, Figure. 1), an antifungal agent of the echinocandin class [2], has antifungal activity against both Aspergillus and Candida species [3-6]. In Japan, on 2012, CPFG was approved for the treatment of FN, possibly caused by fungal infection, as the empiric treatment [7]. Global study employing white, black and other races including Asian except Japanese has already proved the relative efficacy and tolerability of CPFG than liposomal amphotericin B in patients with persistent fever and neutropenia [8]. However, there are, so far, no evidence related to the safety and efficacy of CPFG in Japanese patients with FN. Moreover, although several studies have already addressed the relationship between pharmacokinetics of CPFG and its clinical effects [5,9,10], the pharmacokinetic properties of CPFG in patients with FN have not been fully established yet.

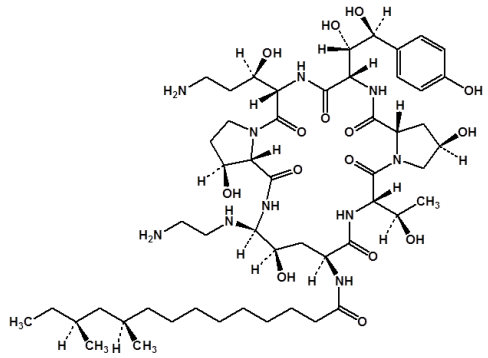

Figure 1: The chemical structure of CPFG.

For this purpose, it is deemed to establish to measure the concentration of CPFG in plasma. Several methods have already been reported by using liquid chromatography-tandem mass spectrometry (LC/MS/MS) [10-15] and high-performance liquid chromatography (HPLC) [9,16-22] in order to determine the levels of CPFG in human plasma. Regarding the extraction of CPFG from plasma sample, solid-phase extraction (SPE) [13,16,18-22] and liquid-liquid extraction

(LLE) [10,12,14,15,17] were employed. However, the required volumes of plasma samples were also varied (0.1-1 mL).

In this study, we selected HPLC system since 1) HPLC system can measure plasma CPFG at μg/mL order [9,16-22], 2) even trough level, plasma CPFG exceeds 1 μg/mL when administered 50-70 mg of CPFG [7,23] and 3) HPLC system is less expensive than LC/MS/MS. Moreover, although previous reports using HPLC employed SPE [18-22] according to the earlier report by Schwartz et al. [16], we selected LLE since LLE is a ready-to-use method that can be established easily without further equipment. Thus, in the present study, we tried to establish the simple and quantitative method to measure CPFG in plasma by using LLE and HPLC.

Materials and Methods

Chemicals

Caspofungin diacetate (CPFG) was purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade acetonitrile, p-hydroxybenzoate ethyl ester, used as an internal standard (IS), were purchased from Nacalai Tesque (Kyoto, Japan). Blank human plasma was purchased from Cosmo Bio (Tokyo, Japan). All other chemicals were of the highest grade that could be obtained commercially.

Standard solutions and sample preparation

Regarding IS, based on the preliminary experiment, among several ethylparabens, we selected p-hydroxybenzoate ethyl ester as IS, based on the retention time and the peak sharpness (see Figure. 2) although previous papers used a derivate of CPFG [9,13-16,19-22], roxithromycin [18] and clarithromycin [17] as IS.

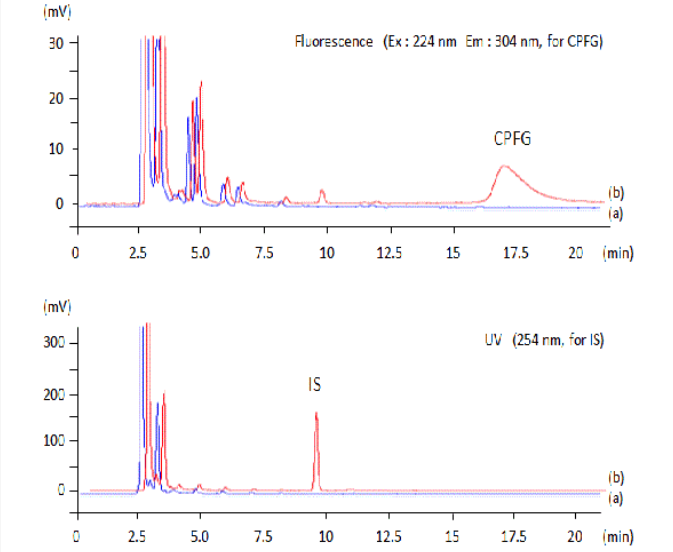

Figure 2: Typical HPLC chromatograms of CPFG and IS. (a) Blank plasma; (b) blank plasma spiked with 20 μg/mL CPFG and IS.

Standard stock solutions of CPFG and IS were solved in distilled water and methanol, respectively, at 1 mg/mL. Each aliquot was stored in polypropylene tubes at -30°C just before use.

The sample preparation method was followed by Uranishi et al. [24] with slight modification. Briefly, in polypropylene tube, 200 μL of plasma sample, 20 μL of distilled water and 600 μL of ice-cold acetonitrile containing IS (0.6 μg/mL) was added. The mixture was vigorously vortex-mixed for 30 sec and was centrifuged at 12,500 rpm for 10 min 4°C. The supernatant was collected to glass tube and was evaporated under nitrogen gas to dryness. The dried residue was re-dissolved in 80 μL of phosphate buffer (20 mM, pH 2.5) and 50 μL was injected into the HPLC. For a calibration curve, instead of plasma sample and water, blank human plasma and CPFG at designated concentrations were added.

HPLC instrumentation and conditions

Samples were analyzed using a Shimadzu HPLC system (LC-20AD Liquid Chromatography; SCL-10Avp System Control; SIL-10ADvp Autosampler; CT0-10Avp Column oven; SPD-10Av UV-VIS detector; RF-10A spectrofluorometric detector) (Kyoto, Japan). The column was heated to 50°C, and the autosampler temperature was set at room temperature (RT). Isocratic separation was carried out on a 5C18-MS-II COSMOSIL column (4.6 mm×250 mm, 5 mm particle size) which was protected by a 4.6 mm×10 mm guard column of the same stationary phase (Nacalai Tesque, Kyoto, Japan). The mobile phase was composed of a solution of 20 mM phosphate buffer (pH 2.5) and acetonitrile in a 65:35 volume ratio. The flow rate was 1.0 mL/min. The elution was monitored by a fluorescence detector for CPFG with excitation and emission at 224 nm and 304 nm, respectively, and by UV-VIS detector for IS at 254 nm.

Analytical method validation

According to Food and Drug Administration (FDA) guidelines [25], the recovery, the accuracy, the intra- and inter-assay precision, the selectivity and stability were validated. The linearity of each standard curve was confirmed by plotting the CPFG/IS standard peak area ratio versus the CPFG concentration. A calibration curve was generated using plasma spiked with 1.0, 2.5, 10 and 20 μg/mL of CPFG. Regarding the stability of CPFG, we confirmed the stabilities of 1) freeze and thaw cycles, 2) long-term (stored at -30°C) and 3) short-term; during the analytical process (after thawing, after drying and in autosampler). The extraction recovery was assessed by comparing the peak areas of the extracted samples with those of the unextracted standards.

Results and Discussion

As shown in the typical chromatograms (Figure 2), the peaks of CPFG and IS was detected with retention times of 17.0 and 9.5 min, respectively, which were separated from matrix compounds. Thus, it is likely that our HPLC setting would be suitable to measure CPFG in human plasma.

The calibration curves were linear from 1.0 to 20 μg/mL (Figure 3). A correlation coefficient, calculated with the peak area ratio of CPFG vs. IS, was over 0.99. The limit of detection (LOD), the limit of quantification (LOQ), and the lower limit of quantification (LLOQ) were 0.53 μg/mL, 0.89 μg/mL and 1.0 μg/mL, respectively. It has been reported that C24h (trough) of CPFG on day 1 and day 14 was 1.69 and 1.98 μg/mL, respectively in Japanese healthy male volunteers administered CPFG intravenously at dose of 70 mg on day 1 and 50mg daily up to day 14 [7]. Moreover, it has been reported that C24h of CPFG in Japanese patients with deep mycosis was exceeded these

values [7]. C24h of CPFG in patients with granulocytopenia and refractory fever was also above 1 μg/mL [9]. Considering the LLOQ of the presented result (1.0 μg/mL), we expect that our established method would be enough to measure the blood concentrations of CPFG for patients in clinical. A previous HPLC method for quantitation of CPFG showed the relatively lower LLOQ (10 ng/mL) using 1 mL of human plasma and a solid-phase extraction [16]. Thus, in order to measure CPFG below 1.0 μg/mL, the large volume of plasma sample and the alternative extraction method would be needed.

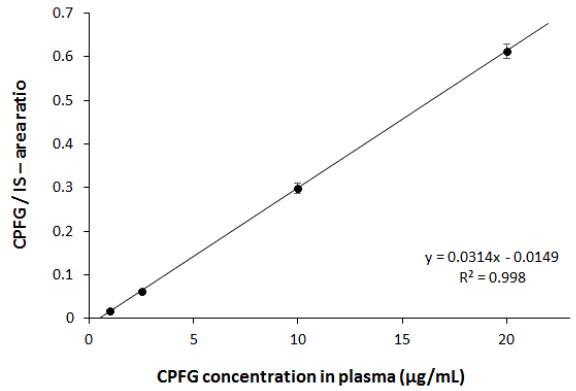

Figure3: The caliblation curve for CPFG in plasma (Each point represents mean ± SD of 5 samples.

As shown in Table, at four concentrations of CPFG (1.0, 2.5, 10 and 20 μg/mL), the intra-day accuracy and precision were ranged from -4.7 to 4.8% and 1.6-5.9%, respectively. Moreover, the inter-day accuracy and precision were ranged from -4.7 to 0.8% and 1.8-3.9%, respectively (Table 1). The extraction recovery of CPFG was within 70.1-83.4% (Table 1). This is comparable to the values for plasma of an earlier described methods using liquid-liquid extraction [12,15,17]. These results indicated that the proposed method was precise and accurate.

CPFG in human plasma (2.5 and 20 μg/mL) was stable up to three freeze-thaw cycles within 10% of the degradation rate, compared to day 0 (data was not shown). Our results were consistent with Rochat et al. [14] that three freeze-thaw cycles or storage of the plasma samples at -80°C for 6 months did not show significant degradation of CPFG.

CPFG stock solution diluted by water was stable for at least 30 days at -30°C, showing degradation rate was less than 1%. Furthermore, CPFG in plasma (2.5 and 20 μg/mL) was stable up to 30 days within the acceptable limit (less than 15%, data not shown).

As shown in Table 2, the degradation rate of CPFG at RT after thawing was also within the acceptable limit (less than 15 %) up to 24 h. The prepared samples containing CPFG, reconstituted in phosphate buffer, was also stable up to 8 h at RT in autosampler (Table 2). However, the degradation rate of extracted CPFG from plasma samples was exceeded 15% 4 h after drying (Table 2). CPFG stock solution was stable up to 24 h after both thawing and diluting by water at RT (Table 2).

Intra- and inter-day precision, accuracy and recovery						
Added C (μg/mL)	Found C (μg/mL)	Intra-run (n=5)		Inter-run (n=5)		Recovery (%) (n=3)
		RSD (%)	RE (%)	RSD (%)	RE (%)	
20	20.16 ± 0.54	1.58	-1.49	2.69	0.80	83.4
10	10.05 ± 0.39	3.44	-2.56	3.89	0.52	71.4
2.5	2.39 ± 0.08	5.86	-4.66	3.50	-4.56	70.1
1.0	0.98 ± 0.02	3.33	4.78	1.80	-2.29	81.4
RSD: precision, RE: accuracy.						

Table 1: Intra- and inter-day precision, accuracy and recovery for the determination or CPFG in plasma.

	Degradation rate (%)			
CPFG in plasma				
After thawing (n=3)	0 h	6 h	12 h	24 h
20 μg/mL	0	-1.4	-4.4	-13.1
2.5 μg/mL	0	-1.2	-0.9	-10.6
After drying (n=3)	0 h	2 h	4 h	6 h

20 μg/mL	0	-10.0	-18.0	-16.1
2.5 μg/mL	0	-17.0	-19.0	-23.1
In autosampler (n=3)	0 h	6 h	12 h	24 h
10 μg/mL	0	-4.5	-8.0	-17.2
CPFG in water				
After diluting (n=3)	0 h	6 h	12 h	24 h
20 μg/mL	0	1.7	-	-3.0
2.5 μg/mL	0	3.5	-	-5.7

Table 2: Short-term stability of CPFG.

In conclusion, present results suggest that we successfully establish the method to measure CPFG in plasma with HPLC and LLE. By using our methods, in order to establish the evidence for appropriate drug therapy, we will try to carry out clinical research to reveal the relationship between the blood concentration of CPFG and clinical efficacy and safety in Japanese patients with FN. We expected that our simple method to measure plasma CPFG would be easy for many laboratories to obtain the data in order to adjust the dose of CPFG in clinical.

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