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Report

Development and Validation of an HPLC-UV Method for Capivasertib Quantification in Human Plasma

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Capivasertib is an oral molecular targeted drug for treating hormone receptor-positive, human epidermal growth factor receptor 2-negative inoperable or recurrent breast cancer with *phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha, AKT serine/threonine kinase 1,* and *phosphatase and tensin homolog* mutations. Moderate intra- and inter-individual variabilities have been reported in the pharmacokinetic study of capivasertib. Furthermore, because capivasertib is a substrate and weak inhibitor of CYP3A4, drug–drug interactions are expected in elderly patients with breast cancer due to the concomitant use of multiple drugs for comorbidities. These factors suggest that the therapeutic drug monitoring (TDM) of patients receiving capivasertib may improve therapeutic efficacy while decreasing the onset of adverse events. However, to the best of our knowledge, there are no reports on methods to measure the capivasertib concentration in human plasma. Therefore, this study proposes a method for determining the capivasertib concentration in human plasma using HPLC-UV. Following protein precipitation with methanol, capivasertib and pirfenidone (internal standard) were separated using isocratic elution on a C18 column using a mobile phase of 0.5% KH_2PO_4 (pH 4.5)/acetonitrile in a 73:27 (vol/vol) ratio, pumped at a constant flow rate of 1.0 mL/min. Quantification was performed at 219 nm. The calibration curves were linear over the range of 50–1000 ng/mL. Intra- and inter-day coefficients of variation were less than 10.2%. The assay accuracy ranged from -7.2–2.9%, and the recovery was >93.8%. This simple and cost-effective method may contribute to the TDM of capivasertib in clinical practice.

Key words high-performance liquid chromatography-ultraviolet, human plasma concentration, capivasertib, breast cancer

INTRODUCTION

Breast cancer is the most diagnosed cancer in women, with approximately 43,780 deaths reported in 2022.[1] Approximately 70% of breast cancer patients are hormone receptor (HR)-positive,[2,3] and among them, mutations in phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), AKT serine/threonine kinase 1 (*AKT1*), and phosphatase and tensin homolog (*PTEN*) mutations are frequent, occurring in up to 50% of breast cancer patients.[4-6] Therapy for metastatic or recurrent breast cancer is aimed at maintaining or improving the quality of life and prolonging survival, as achieving a radical cure is challenging. The recommended first-line therapy for HR-positive human epidermal growth factor receptor 2 (HER2)-negative metastatic or recurrent breast cancer is the combination of endocrine therapy and cyclin-dependent kinase (CDK) 4/6 inhibitors.[7] However, most patients eventually develop resistance to the CDK4/6 inhibitor, necessitating a change in therapy.[8] Therefore, capivasertib was developed as the first oral AKT inhibitor that blocks the *PIK3CA*/*AKT1*/*PTEN* signaling pathway.[9] In combination with fulvestrant, capivasertib is a new treatment option for second-line therapy of HR-positive, HER2-negative metastatic or recurrent breast cancer with one or more *PIK3CA*, *AKT1*, or *PTEN* gene mutations.

In the Phase III study, diarrhea (72.4%), nausea (34.6%), rash (38.0%), and hyperglycemia (16.3%) were observed as adverse events, with 19.7% and 13.0% of patients experiencing dose reduction and discontinuation of capivasertib, respectively.[9] Capivasertib is also a substrate drug for CYP3A4[10] and simultaneously a weak inhibitor of CYP3A4.[11] Thus, the drug–drug interactions caused by multiple drug combinations in elderly breast cancer patients with increasing comorbidities are a concern. Moderate intra- and inter-individual variabilities in the pharmacokinetics study of capivasertib have been reported.[10,12] These factors suggest that dose adjustment while monitoring blood levels in patients receiving capivasertib may improve the therapeutic efficacy while minimizing the adverse events. However, to the best of our knowledge, there are no reports on methods to measure the capivasertib concentration in human plasma. Therefore, we propose a novel method to measure the capivasertib concentration in human plasma

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according to the Food and Drug Administration (FDA) analytical validation guidelines.[13)]

MATERIALS AND METHODS

Reagents and Chemicals Capivasertib and pirfenidone (internal standard, IS) were obtained from Toronto Research Chemicals Inc. (Ontario, Canada) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), respectively (Fig. 1). Fulvestrant, loperamide, and metformin were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). HPLC-grade acetonitrile, methanol, distilled water (Kanto Chemical, Co., Inc., Tokyo, Japan), and KH_2PO_4 (Wako, Osaka, Japan) were used in the HPLC mobile phase. Human plasma (pool) and EDTA-2Na were purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan).

Equipment and Chromatographic Conditions A Jasco HPLC system (Tokyo, Japan), comprising a pump (PU-4180), a UV detector (UV-4075), and an auto-sampler (AS-4550), was utilized. Analyses were performed on a Capcell Pak C18 MG II (Osaka Soda, Tokyo, Japan) reversed-phase column (250 mm × 4.6 mm i.d.) and Capcell Pak C18 MG II guard column (10 mm × 4.0 mm; Osaka Soda) at ambient temperature. The mobile phase was composed of 0.5% KH_2PO_4 (pH 4.5) and acetonitrile (73:27, vol/vol). The flow rate was 1.0 mL/min, and the total run time was 14 min. UV detection was performed at 219 nm.

Preparation of Stock and Working Solutions Stock solutions of capivasertib and IS were prepared in methanol at a concentration of 1 mg/mL. The capivasertib stock solution was diluted further with methanol to obtain working solutions with concentrations of 0.25, 0.5, 1.25, 2.5, 4.0, and 5.0 µg/mL. IS was diluted with methanol to obtain a working solution of 50 µg/mL. These stock and working solutions were aliquoted and stored at −60°C in the dark.

Sample Preparation Before analysis, human plasma and working solutions were thawed and vortexed. A protein precipitation procedure was used to extract capivasertib and IS. Blank plasma (50 µL) was spiked with 10 µL capivasertib, vortex-mixed for 5 s, followed by the addition of 10 µL of IS and 130 µL of methanol chilled to −60°C, and vortex-mixed for 1 min. The sample was centrifuged at 15,000 × *g* for 10 min at 4°C, and 50 µL of the supernatant was directly injected into the HPLC system for analysis (Fig. 2).

Specificity and Selectivity Samples were collected from six different lots of human plasmas and analyzed to confirm if the endogenous matrix of these six plasmas eluted near the retention time of capivasertib or the IS. Capivasertib is used in combination with fulvestrant.[9)] In the Phase III study, diarrhea (72.4%) and hyperglycemia (16.3%) were observed as adverse events with capivasertib, and were treated with loperamide and metformin, respectively.[9)] Therefore, fulvestrant, loperamide, and metformin are likely to be used in combination with capivasertib, and considering the possibility of adulteration with capivasertib or IS in the present method, the retention times of loperamide and metformin were checked.

Calibration Curve Accuracy and linearity were evaluated by analyzing a set of standards ranging from 50–1000 ng/mL. The precision and accuracy of this method were evaluated using spiked samples with six different concentrations of

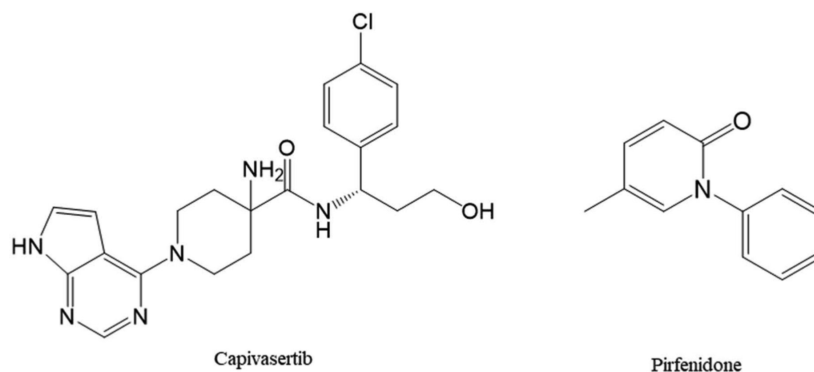

Fig. 1. Chemical Structures of Capivasertib (Left) and Pirfenidone (Right)

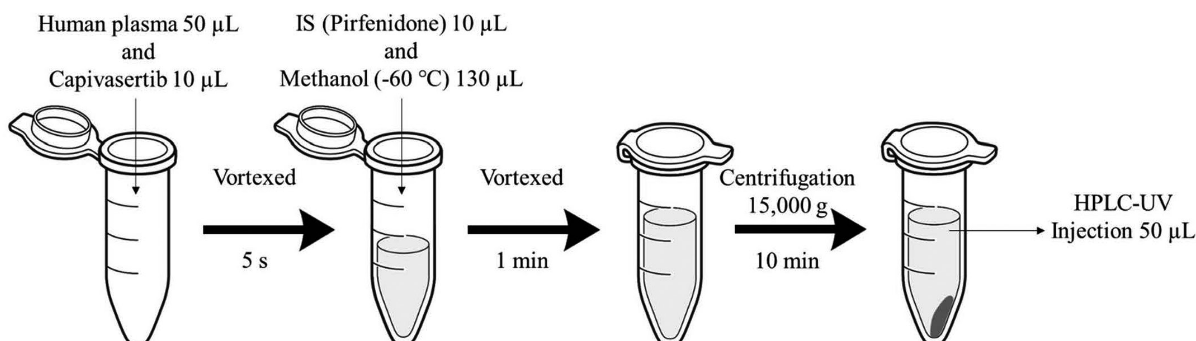

Fig. 2. Sample Preparation

capivasertib (50, 100, 250, 500, 800, and 1000 ng/mL). A calibration graph was constructed by plotting the known concentration (X) against the ratio of the capivasertib height to that of IS. This data was analyzed using least squares linear regression analysis (Y) to establish the relationship.

Recovery Recovery was evaluated using control plasma samples at the six concentrations indicated above. It was calculated by comparing the capivasertib concentration extracted from these control plasma samples to that extracted from saline.

Method Validation For intra-day assay precision and accuracy, five replicates of quality control samples at each concentration were assayed simultaneously within a day. The inter-day assay precision and accuracy were determined by analyzing the quality control samples on five consecutive days. Method validation was based on the Bioanalytical Method Validation guidelines published by the FDA[13], with a precision of ≤ 15.0% and an accuracy within ± 15.0%. The precision at each concentration was calculated from the coefficient of variation (CV). Accuracy was calculated by comparing the ratio of the theoretical concentration to the difference between the mean measured and theoretical concentrations.

Stability Analyte stability in human plasma was tested using three concentrations (50, 500, and 1000 ng/mL) for bench-top, short-term, long-term, and freeze-thaw stability. The stability of capivasertib was assessed using three concentrations (50, 500, and 1000 ng/mL) for bench-top, short-term, long-term, and freeze-thaw stabilities. The bench-top stability samples were kept at room temperature (22°C) for 6 h, the short-term stability samples were stored at 4°C for 24 h, the long-term stability samples were stored at −60°C for one month, and the freeze-thaw samples underwent three cycles of freezing at −60°C or below in a freezer and thawed at room temperature. The stability samples were analyzed against a calibration curve prepared using the freshly spiked analyte, and the obtained concentrations were then compared to the nominal values.

RESULTS AND DISCUSSION

We developed an HPLC-UV method to quantify the concentration of capivasertib in human plasma according to FDA analytical validation guidelines. Although LC-MS has been used to measure human plasma capivasertib concentrations in Phase I clinical trials, detailed LC-MS conditions have not been described, making it unsuitable for clinical applications.[12] In addition, LC-MS is susceptible to ion suppression and can misinterpret samples containing multiple drugs of the same mass.[14] Moreover, the high cost of LC-MS and its limited availability in general hospitals prompted us to establish an HPLC-UV method.

In the proposed method, calibration curves for capivasertib were linear over a range of 50–1000 ng/mL. The six-point capivasertib standard calibration curve was expressed as $y = 0.0013x + 0.0099$ ($r^2 = 0.9999$). Capivasertib was administered orally at a dose of 400 mg/day for four days with a 3-day rest period. The mean pre-dose concentration of capivasertib on Day 25 was 304 ng/mL, with a CV of 73% and a range of 104–795 ng/mL.[15] The LC-MS method used in the Phase I study to measure human plasma capivasertib concentrations can measure a range of 1–1000 ng/mL.[12] Conversely, our method could not quantify plasma capivasertib concentrations below 1 ng/mL, but it adequately covered the blood concentrations in clinical practice. Therefore, assessing plasma concentrations in patients receiving capivasertib in the clinical setting is feasible. Representative chromatograms of blank human plasma samples are depicted in Fig. 3A. Capivasertib and IS were well separated from the co-extracted materials under the described chromatographic conditions at retention times of 7.4 and 11.8 min, respectively. No interfering peaks from endogenous human plasma components were observed at the retention times of capivasertib and IS (Fig. 3B and C).

We considered pirfenidone, gefitinib, birgatinib, niraparib, and olaparib as IS candidates, and their retention times were 11.8, 20.6, 7.2, 5.2, and 15.6 min, respectively. Pirfenidone was selected as the IS because it had the least interference effect with the matrix, a high separation from capivasertib (7.79), and the shortest measurement time per sample. In addition, six different lots of human plasma samples were used to confirm that the endogenous matrix did not cause an effect near the retention time of capivasertib or IS. No fulvestrant peaks were detected during the analysis time (14 min). In addition, loperamide and metformin, which are more likely to be used as a therapy for adverse events caused by capivasertib, had a retention time of less than 3 min, and did not elute near the retention time of capivasertib or the IS. The present

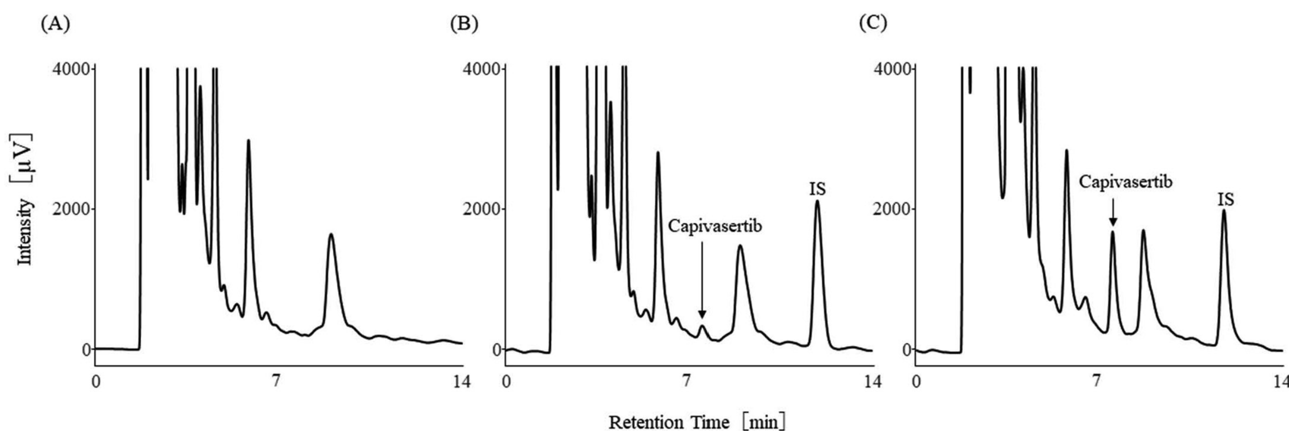

Fig. 3. Chromatograms of the (A) Blank Plasma Sample, (B) Plasma Sample Containing 50 ng/mL Capivasertib, and (C) Plasma Sample Containing 500 ng/mL Capivasertib

Table 1. Intra-day and Inter-day Accuracy and Precision

Theoretical capivasertib concentration (ng/mL)	Intra-day (n = 5)			Inter-day (n = 5)			
	Detected (ng/mL) mean ± SD	CV(%)	Accuracy (%)	Detected (ng/mL) mean ± SD	CV (%)	Accuracy (%)	Recovery (%)
50	50.6 ± 5.2	10.2	1.2	49.1 ± 3.0	6.2	-1.8	93.8
100	102.9 ± 4.4	4.2	2.9	98.6 ± 5.2	5.2	-1.4	96.4
250	254.9 ± 13.4	5.3	2.0	238.0 ± 15.3	6.4	-4.8	94.7
500	503.2 ± 38.1	7.6	0.6	464.1 ± 22.4	4.8	-7.2	94.3
800	811.4 ± 49.3	6.1	1.4	744.7 ± 34.2	4.6	-6.9	97.6
1000	1017.3 ± 72.9	7.2	1.7	959.7 ± 49.8	5.2	-4.0	96.4

Table 2. Stability Analysis

Stability test conditions	Ratio of plasma concentration to the spiked value (%)		
	50 ng/mL (mean ± S.D.)	500 ng/mL (mean ± S.D.)	1000 ng/mL (mean ± S.D.)
Benchtop storage (22°C, 6 h)	94.5 ± 9.0	96.0 ± 2.6	98.3. ± 2.4
Short-term storage (4°C, 24 h)	106.7 ± 6.6	109.8 ± 3.4	108.1 ± 1.9
Long-term storage (−60°C, 1 month)	96.2 ± 5.6	93.5 ± 4.5	105.2 ± 3.2
Freeze–thaw, three cycles (−60°C to room temperature)	98.6 ± 5.5	107.3 ± 6.6	108.4 ± 5.4

method has an analysis time of 14 min and a plasma requirement of 50 µL. Zhang *et al*. compared liquid–liquid extraction and protein precipitation methods as sample deproteinization methods and chose the acetonitrile-based protein precipitation method for extraction recovery.[16] However, in our study, we used methanol for the protein precipitation method because it showed sharp chromatographic peaks and was more sensitive than acetonitrile.

The intra-day and inter-day CVs and accuracies are shown in Table 1. Our method was comparable in accuracy to that of Zhang *et al*.[16] and adhered to the FDA analytical validation guidelines.[13] Notably, our method demonstrated better recovery, exceeding 85.8%, compared to the method of Zhang *et al.*[16] This may be due to the differences in the organic solvent used for extraction (methanol or acetonitrile) and the measuring instrument. Previous reports have shown that acetonitrile exhibits strong ionization suppression,[17] and acetonitrile may be a suitable organic solvent for protein precipitation in the case of LC-MS, where ionization effects are considered. However, the present method with UV detection, which does not consider ionization effects, used methanol, which has the highest precipitation activity among organic solvents.[18] In addition, solid phase extraction (SPE) was used to quantify human plasma capivasertib concentrations in the method of Dean *et al.*[12] While SPE provides a clean sample that minimizes the effects of blood components, it poses challenges in terms of cost, extraction time, and reproducibility.[19,20] Therefore, the present method using protein precipitation with methanol is rapid and cost-effective and is expected to be helpful for the TDM of capivasertib in routine practice. The results of the stability studies are shown in Table 2. Under all conditions and at each capivasertib concentration, the stability rates were > 93.5 ± 4.5%. These results confirmed the short- and long-term stability of capivasertib.

However, there are limitations to this study. Measuring capivasertib concentrations in patients receiving the drug in Japan was not possible, as capivasertib has not been marketed there for some time. Therefore, it is unclear whether the present method can accurately measure capivasertib without the influence of metabolites in patients receiving loperamide or metformin, which are used for symptomatic therapy. In addition, patients with recurrent breast cancer are often older and take multiple concomitant medications to manage comorbidities and adverse events. Therefore, it was impossible to evaluate the selectivity of the method concerning concomitant medications or their metabolites. In the future, selectivity should be confirmed in clinical samples from patients receiving capivasertib.

In conclusion, we have developed a novel method for determining capivasertib concentration in human plasma using HPLC-UV. Future studies will involve measuring plasma samples from patients treated with capivasertib to investigate the relationship between plasma levels, efficacy, and adverse events.

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Conflict of interest The authors declare no conflict of interest.

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