Brief Report

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Determination of anamorelin concentration in human plasma using a simple high-performance liquid chromatography-ultraviolet detection method

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SUMMARY Anamorelin, a non-peptide ghrelin analog and growth hormone secretagogue, is a novel oral drug used to treat cancer cachexia. Patients with cancer cachexia frequently use several drugs and anamorelin is a substrate of cytochrome P450 (CYP) 3A4; therefore, drug-drug interactions with CYP3A4 inhibitors and inducers pose a clinical problem. In this study, we aimed to determine the concentration of anamorelin in human plasma using a simple high-performance liquid chromatography-ultraviolet (HPLC-UV)-based method. The analysis involved extracting a 200-μL plasma sample and protein precipitation using solid-phase extraction. Anamorelin was isocratically separated using a mobile phase consisting of 0.5% potassium dihydrogen phosphate (pH 4.5) and acetonitrile (61:39, v/v) on a Capcell Pack C18 MG II column (250 mm × 4.6 mm) at a flow rate of 1.0 mL/min and monitored at a detection wavelength of 220 nm. The calibration curve was linear within a plasma concentration range of 12.5-1,500 ng/mL, with a coefficient of determination of 0.9999. The intra- and inter-day coefficients of variation were 0.37-6.71% and 2.05-4.77%, respectively. The accuracy of the assay and recovery were 85.25-112.94% and > 86.58%, respectively. This proposed HPLC-UV method is simple and can be applied in clinical settings.

Keywords cancer cachexia, drug-drug interactions, clinical settings

1. Introduction

Anamorelin, a non-peptide ghrelin analog and growth hormone secretagogue, is a novel oral drug used to treat cancer cachexia (*1*). Approximately 80% of patients with advanced cancer develop cancer cachexia, which is characterized by involuntary weight loss frequently accompanied by anorexia and altered metabolism (*2*). Anamorelin has been reported to increase appetite, overall body weight, lean body mass, and muscle strength in clinical studies involving patients with cancer experiencing cancer cachexia (*3,4*). However, this therapy for cancer cachexia is approved and marketed only in Japan. Anamorelin is a substrate of cytochrome P450 (CYP) 3A4; therefore, drug-drug interactions with CYP3A4 inhibitors and inducers pose a clinical problem. A three-fold increase in the plasma values for the area under the plasma concentration-time curve over a 24-h dosing interval was observed with ketoconazole, a CYP3A4 inhibitor (*5*). Patients with cancer cachexia use several medications and herbs more frequently than the general population to improve cancer-associated symptoms and quality of life, decrease cancer progression, and reduce the side effects of chemotherapy (*6*). However, such concomitant use and its effects on the pharmacokinetics of anamorelin have not been fully studied. Hyperglycemia, hepatotoxicity, and depression of the stimulant conduction system have been reported as common adverse events of anamorelin (*7*). Furthermore, the exposure-response and exposure-safety relationships of anamorelin levels in plasma have not been reported. We hypothesize that monitoring anamorelin levels in the blood will aid in determining the relationship between its blood levels and efficacy

or safety. Following this, anamorelin treatment can be effectively optimized in patients with cancer cachexia in clinical settings. To date, liquid chromatography coupled with tandem mass spectrometry and high-performance liquid chromatography-ultraviolet (HPLC-UV) have not been used to determine anamorelin concentration in human plasma. HPLC-UV offers many advantages over other detection systems, such as low operational cost, versatility, and simple operation. In this study, we aimed to measure anamorelin levels in human plasma using a HPLC-UV-based method that can be applied in clinical practice.

2. Materials and Methods

2.1. Standards and reagents

Anamorelin and ketoconazole were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Tokyo Chemical Industry Co. (Tokyo, Japan), respectively. HPLC-grade acetonitrile, methanol, and water were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan) and potassium dihydrogen phosphate (KH_2PO_4) was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). An Oasis hydrophilic-lipophilic balance (HLB) extraction cartridge was purchased from Waters Corp. (Milford, MA, USA). Human plasma and EDTA-2Na were purchased from Cosmo Bio Co., Ltd. (Tokyo, Japan).

2.2. HPLC apparatus and analytical conditions

The HPLC apparatus consisted of a pump (PU-4180; Jasco, Tokyo, Japan), UV detector (UV-4075; Jasco, Tokyo, Japan), and autosampler (AS-4550, Jasco, Tokyo, Japan). Separation was performed on a octadecylsilyl analytical column (Capcell Pack C18 MG II, 250 mm × 4.6 mm i.d., 5 µm, Osaka Soda, Tokyo, Japan). The detection wavelength was 220 nm and the mobile phase consisted of 0.5% KH_2PO_4 (pH 4.5) and acetonitrile (61:39, v/v) with a flow rate of 1.0 mL/min.

2.3. Preparation of stock and working solutions

Anamorelin and ketoconazole stock or working solutions were diluted in methanol and then stored at −60°C in the dark until use.

2.4. Sample preparation

A 10-µL anamorelin working solution was vortexed with 200 µL plasma for 1 min. Anamorelin-spiked plasma (200 µL) was mixed with 10 µL of the internal standard (IS; 10 µg/mL ketoconazole) and 800 µL water, vortexed for 30 s, and applied to the pre-conditioned Oasis HLB cartridges. The cartridges were washed with 1 mL of water and 60% methanol in water (v/v). The analytes were eluted with 1 mL of 100% methanol and vacuum-evaporated to dryness at 80°C using a rotary evaporator. The dried residues were reconstituted with 50 µL methanol, and a 20-µL aliquot was injected into the HPLC system.

2.5. Calibration and Method validation

The calibration concentrations of anamorelin were 12.5, 25, 100, 500, 1,000, and 1,500 ng/mL. Method validation was based on the Guidelines for the Validation of Methods for the Quantitative Analysis of Biological Samples by the US Food and Drug Administration (FDA) (*8*). The recovery and accuracy of the assay were determined at the calibration concentrations of anamorelin (12.5–1,500 ng/mL). The assay precision was evaluated by analyzing five sets of control samples on the same day (intra-day) and five different days (inter-day) at concentrations of 12.5, 25, 100, 500, 1,000, and 1,500 ng/mL.

2.6. Sample stability

Anamorelin stability in plasma samples was evaluated at three different concentrations (12.5, 100, and 1,500 ng/mL). Bench-top stability was evaluated using samples stored at 20°C for 6 h (n = 5). The stability of the processed samples was evaluated after storage at 4°C for 24 h (n = 5). Long-term stability was evaluated using samples stored at −60°C for 4 weeks (n = 5). The freeze and thaw stability were evaluated using samples thawed after storage at −60°C after three freeze-thaw cycles (n = 5).

2.7. Clinical application

Blood samples were collected after obtaining written informed consent from a patient with non-small-cell lung cancer (NSCLC) receiving anamorelin. Plasma samples were obtained by centrifuging the blood samples at 3,000× g for 5.5 min. Plasma and serum were stored at −80°C until analysis. This study was approved by the Institutional Review Board of Tokyo Metropolitan Bokutoh Hospital (approval number: #05–101) and conducted in accordance with the Declaration of Helsinki. Concomitant medications used by the patient were acetaminophen, loxoprofen, rabeprazole, naldemedine, magnesium oxide, oxycodone, prochlorperazine, trichlormethiazide, amlodipine, and insulin.

3. Results and Discussion

In the present study, we developed a sensitive HPLC-UV-based method to determine anamorelin concentrations in human plasma in a clinical setting.

The chromatograms of blank plasma and anamorelin in plasma (100 ng/mL) are provided in Figure 1A and 1B, respectively. The retention times for anamorelin and the IS were 10.2 and 17.4 min, respectively, and there were no interfering peaks (Figure 1B). The six-point standard calibration curve for anamorelin was linear over the 12.5–1500 ng/mL concentration range. The equation for the calibration curve was y = 0.0041x + 0.0026 (r^2 = 0.9999), where y and x are the peak height ratio and anamorelin concentration in plasma (ng/mL), respectively. The lower limit of quantification for anamorelin and the recovery were 6.25 ng/mL and > 86.58%, respectively. At these concentrations, the ranges of intra- and inter-day coefficients of variation were 0.37–6.71% and 2.05–4.77%, respectively (Table 1). The stability of anamorelin in plasma was assessed under various conditions (Table 2). Anamorelin was not significantly degraded, and the final concentration was within 85.25–112.94% of the theoretical values. The matrix effects were assessed by comparing the peaks of the quality control sample in the mobile phase with those of the supernatant from the extracted blank plasma. No significant peak suppression or enhancement was observed. Therefore, the intra- and inter-assay variations, accuracy, recovery, and stability under various conditions complied with the FDA guideline recommendations (*8*). In Japanese patients with NSCLC, the median maximum concentration (C_{max}) and trough concentration (C_{trough}) of 100 mg anamorelin on days 7, 21, and 42 at multiple doses were 1,120 ± 922 and 26.1 ± 25.2 ng/mL, respectively (*9*). In the present study, a concentration range of 12.5–

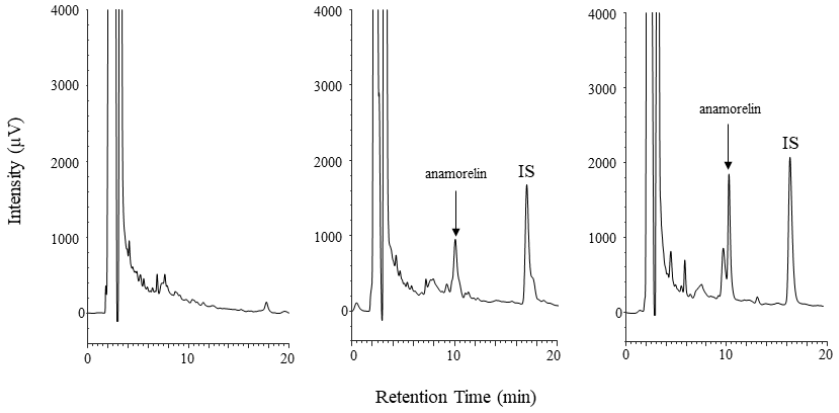

Figure 1. Chromatograms of the (A) blank plasma sample, (B) plasma sample containing 100 ng/mL anamorelin, and (C) plasma sample from a patient treated with 100 mg anamorelin (294.9 ng/mL).

Table 1. Intra- and inter-day accuracy and precision results for anamorelin samples

Anamorelin added (ng/mL)	Intra-day (n = 5)			Inter-day (n = 5)			Recovery (%)
	Mean ± SD (ng/mL)	CV (%)	Accuracy (%)	Mean ± SD (ng/mL)	CV (%)	Accuracy (%)	
12.5	12.51 ± 0.37	0.37	100.11	13.34 ± 0.64	4.77	106.73	89.49
25	26.75 ± 1.61	6.02	107.00	27.13 ± 0.87	3.20	108.51	86.58
100	96.51 ± 5.31	5.50	96.51	100.68 ± 3.54	3.51	100.68	98.14
500	474.05 ± 22.65	4.78	94.81	493.29 ± 10.10	2.05	98.66	91.46
1000	975.71 ± 65.45	6.71	97.57	991.27 ± 38.61	3.90	99.13	94.31
1500	1395.42 ± 58.66	4.20	93.03	1468.47 ± 35.57	2.42	97.90	98.62

CV, coefficient of variation; SD, standard deviation.

Table 2. Stability analyses under various conditions (n = 5)

Anamorelin added (ng/mL)	Stability condition (%)			
	Benchtop mean ± SD	Processed sample mean ± SD	4 weeks mean ± SD	Freeze-and-thaw mean ± SD
12.5	97.69 ± 7.95	112.94 ± 4.90	99.26 ± 5.88	108.55 ± 5.09
100	101.69 ± 3.44	95.40 ± 2.58	87.32 ± 3.07	98.93 ± 1.90
1500	108.28 ± 6.36	104.19 ± 6.03	85.25 ± 1.06	103.92 ± 1.29

SD, standard deviation.

1,500 ng/mL was covered using our method, indicating its suitability for the evaluation of C_{max} and C_{trough} of anamorelin in a clinical setting.

A 76-year-old man with NSCLC who received carboplatin + nab-paclitaxel + atezolizumab was started on anamorelin (100 mg/day) for anorexia. The loss of appetite improved after 5 days of anamorelin treatment, and body weight increased from 60.8 kg to 62.1 kg after 7 days of anamorelin treatment, indicating the effectiveness of anamorelin. At the initiation of anamorelin administration, the patient's aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were 28 and 34 IU/L, respectively. However, blood tests showed AST and ALT levels of 98 IU/L (grade 2 based on the Common Terminology Criteria for Adverse Events [ver.5.0]) and 187 IU/L (grade 3), respectively, on day 28 (Figure 2). The patient was admitted to the hospital for close examination of liver damage. Anamorelin-induced hepatotoxicity was suspected; therefore, anamorelin was discontinued after admission. Seven days after anamorelin discontinuation, the patient's AST and ALT levels improved to normal values, and the patient was diagnosed with anamorelin-induced hepatotoxicity. A plasma sample of anamorelin obtained on day 28 showed a value of 294.9 ng/mL 4 h after administration. The time for anamorelin to reach the maximum serum concentration ranges from 0.5 to 2.0 h (*10*), indicating its presence (half-life of 1–2 h) in the blood. In the ONO-7643-04 study by Ono Pharmaceutical, the mean level of anamorelin in the blood 4–6 h after multiple 100 mg doses was 385 ± 324 ng/mL in Japanese patients with NSCLC (*9*). Compared with the patient in the present study, those in the ONO-7643-04 study had higher blood anamorelin levels 4–6 hours after administration. There are no reports on the relationship between anamorelin levels in the blood and hepatotoxicity; however, there have been reports of moderately elevated but normalized AST and ALT levels in patients after anamorelin discontinuation (*11*), as in the patient in this study. Notably, only one case was studied; therefore, there may be no relationship between anamorelin blood levels and hepatotoxicity. In two phase III trials, the most frequent drug-related adverse events were hyperglycemia (4.2–5.3%). Only a few drug-related Grade ≥ 3 AEs were observed in both studies (0.9% and 2.7%, respectively) (*3,4*). In a phase 1 study, nine healthy male volunteers received 10, 25, and 50 mg oral doses of anamorelin, and serum growth hormone levels increased significantly in a dose-dependent manner (*12*). Therefore, hyperglycemia may be associated with anamorelin levels in the blood. In the future, we aim to evaluate the exposure-response and exposure-safety relationships in cases of hyperglycemia, a characteristic adverse event of anamorelin, in a large sample size using our assay. Nevertheless, the therapeutic range of anamorelin remains unclear. The number of older patients with cancer cachexia has increased with population aging, resulting in increased anamorelin use. Our proposed method can be applied to patients receiving anamorelin and can be used to explore the therapeutic target range.

Our reported method has a limitation, as it was applied to samples from only one patient. During full validation in blank human plasma, amamorelin and biogenic components were completely separated. Furthermore, no interferential peak near anamorelin was observed in six different lots of blank human plasma. However, in this one patient's plasma, with separation between anamorelin and the biogenic component being 1.0 (Figure 1C). Therefore, it may be necessary to reconsider the measurement conditions. Nevertheless, an interference peak near anamorelin in this case did not affect the quantification of anamorelin. It is therefore necessary to measure samples from a large number of patients in the future and assess foreign substances, such as concomitant medications and individual patient biogenic components.

In conclusion, we developed a simple HPLC-UV-based method to determine anamorelin concentration in human plasma. This method is simple and can be used to evaluate interactions between anamorelin and concomitant drugs in clinical settings. Further

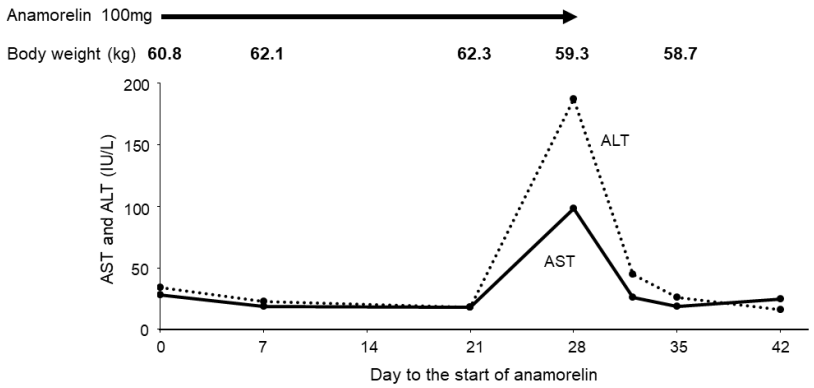

Figure 2. Clinical course of anamorelin for cancer cachexia in a patient with non-small cell lung cancer. The straight line indicates aspartate transaminase (AST), whereas the dashed line indicates alanine transaminase (ALT).

studies using this assay are required to elucidate the relationship between anamorelin blood levels and its clinical efficacy and safety.

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