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Liquid Chromatography-Tandem Mass Spectrometric Analysis of Nannozinone A and Its Application to Pharmacokinetic Study in Mice

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Abstract : We aimed to develop and validate a sensitive analytical method of nannozinone A, active metabolite of Nannochelins A extracted from the Myxobacterium Nannocytis pusilla, in mouse plasma using a liquid chromatography-tandem mass spectrometry (LC-MS/MS). Mouse plasma samples containing nannozinone A and ^{13}C-caffeine (internal standard) were extracted using a liquid-liquid extraction (LLE) method with methyl *tert*-butyl ether. Standard calibration curves were linear in the concentration range of 1 - 1000 ng/mL ($r^2 > 0.998$) with the inter- and intra-day accuracy and precision results less than 15%. LLE method gave results in the high and reproducible extraction recovery in the range of 78.00–81.08% with limited matrix effect in the range of 70.56-96.49%. The pharmacokinetics of nannozinone A after intravenous injection (5 mg/kg) and oral administration (30 mg/kg) of nannozinone A were investigated using the validated LC-MS/MS analysis of nannozinone A. The absolute oral bioavailability of nannozinone A was 8.82%. Plasma concentration of nannozinone A after the intravenous injection sharply decreased for 4 h but plasma concentration of orally administered nannozinone A showed fast distribution and slow elimination for 24 h. In conclusion, we successfully applied this newly developed sensitive LC-MS/MS analytical method of nannozinone A to the pharmacokinetic evaluation of this compound. This method can be useful for further studies on the pharmacokinetic optimization and evaluating the druggability of nannozinone A including its efficacy and toxicity.

Keywords : Nannozinone A, LC-MS/MS analysis, pharmacokinetics

Introduction

Nannochelins A (Figure 1), a siderophores extracted from the Myxobacterium Nannocytis pusilla, strain MNa109131, was discovered to have cytotoxic activity as an iron complex.[1] Nannozinone A, a metabolite of nannochelins A, posseses a dihydropyrrolopyrazinone structure, and has antibacterial activity against some gram-positive bacteria, fungi, and viruses and has also shown to possess anti-cancer activity.[1-3] However, these biological activities of nannozinone A were previously only investigated in cell systems. The bioanalysis and pharmacokinetic properties of nanozinones A should be conducted during the early stages for investigating its in vivo activity and toxicity as well as its its potential as therapetic agent. Therefore, we aimed to develop and validate the bioanalysis of nanozinones A in mouse plasma samples and to investigate its pharmcokinetic properties when administered to ICR mice using our developed analytical method.

We used the liquid-liquid extraction (LLE) method for sample preparation since LLE has the advantage of lowering interferences from the sample matrix and increasing analyte sensitivity.[4] Moreover, our method was fully validated by observing the the U.S. Food and Drug Administration Guideline for Bioanalytical Method with regard to its linearity, selectivity, accuracy, precision, stability, recovery, and matrix effects.[5]

Experimental

Chemicals and reagents

Nannozinone A (Figure 1) were synthesized, with a purity of > 99.0%, and purtiy was confirmed by nuclear magnetic resonance spectroscopy and mass spectroscopy.[3] ^{13}C-Caffeine was used as the internal standard (IS), which was purchased from Sigma-Aldrich (St. Louis, MO, USA). The methyl *tert*-butyl ether (MTBE) was obtained from

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Mallinckrodt Baker (Phillipsburg, NJ, USA). Acetonitrile, water, and methanol were purchased from Tedia (Fairfield, CT, USA). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). All solvents and chemicals were of HPLC or reagent grade.

Preparation of stock and working solutions

Stock solutions were prepared by dissolving the nannozinone A in acetonitrile at a concentrations of 2 mg/mL. The nannozinone A working solutions were prepared by diluting the stock solution serially with acetonitrile and to achieve final concentrations of 10, 20, 50, 200, 500, 2000, 5000, and 10000 ng/mL. The ^{13}C-caffeine solution was prepared at a concentration of 20 ng/mL in water.

Preparation of standard calibration curve and quality control (QC) samples

The standard calibration curve and quality control (QC) samples were prepared by spiking 5 μL aliquot of the working solution with 45 μL aliquot of blank mouse plasma. The final concentrations of standard calibration curve and QC samples were 1, 2, 5, 20, 50, 200, 500, 1000 ng/mL and 1 (QC for lower limit of quantification; LLOQ QC), 3 (low QC), 100 (middle QC), 750 (high QC) ng/mL, respectively.

Sample preparation

The standard calibration curve and QC samples were added to 20 μL of ^{13}C-caffeine solution (20 ng/mL in water) and 400 μL of methyl *tert*-butyl ether. The mixture was vigorously vortexed for 10 min then centrifuged at 16,000 × *g* for 10 min. The supernatant was transferred to a clean tube and dried under a gentle stream of nitrogen. The residue was reconstituted in 150 μL of mobile phase and 5 μL aliquot of the solution and was injected into the LC-MS/MS system.

Instrument conditions

Nannozinone A in mouse plasma samples were analyzed using an Agilent 6430 triple quadrupole liquid chromatography-tandem mass spectrometry (LC-MS/MS) system (Agilent Technologies, Wilmington, DE, USA) equipped with an Agilent Infinity 1260 Infinite II HPLC system. Chromatographic separation was performed on a Luna C18 column (150 × 2.0 mm, 5 μm; Phenomenex, Torrance, CA, USA). Isocratic mobile phase consisting of mixture of water and acetonitrile (20:80, *v/v*) containing 0.1% formic acid was used at a flow rate of 0.2 mL/min with a column temperature maintained at 30 °C. The total run time for each injection was 4 min. The mass spectrometer was operated in the positive ion mode with multiple reaction monitoring (MRM) transitions at *m/z* 241.1→150.1 for nannozinone A and at *m/z* 198.2→140.0 for ^{13}C-caffeine with optimized fragmentor of 115 V and collision energy of 25 eV, respectively.

Method validation

Blank plasma samples from six different mouse were used for assessing selectivity. Signals of six blank plasma samples were compared to those of the corresponding LLOQ samples and IS. By plotting the ratio of the peak areas of the analyte and IS versus the concentrations of nannozinone A, the linearity of an eight-point standard calibration curve (1–1000 ng/mL) was generated using a least square linear regression utilizing $1/x^2$ as weighting factors. The extraction recovery and matrix effect was determined using three levels of QC samples (low-, middle-, and high QC) of nannozinone A and IS solution (20 ng/mL). The extraction recovery was calculated by comparing the peak areas of nannozinone A in QC samples through the extraction process with those in blank plasma extracts spiked with correstponding concentrations. The matrix effect was determined by dividing the peak areas in blank plasma extracts spiked with QC concentrations by those in neat solutions of the corresponding concentrations. The intra-day precision and accuracy were analyzed for the six replicates at four levels of QC samples (LLOQ-, low-, middle-, and high QC) on the same day. The inter-day precision and accuracy were determined by measuring the four levels of QC samples for six consecutive days. The bench-top stability was assessed by placing QC samples at 25°C for 5 h. The freeze-thaw stability was analyzed by comparing QC samples that underwent three freeze-thaw cycles (from -80°C to 25°C for 5 h as one cycle). Autosampler stability was evaluated by placing processed QC samples in the autosampler at 6°C for 24 h.

Pharmacokinetic study

All animal procedures were approved by the Animal Care and Use Committee of the Kyungpook National University (Permission no. 2019-0126). The male ICR mice (7-8 weeks old, 30-35 g) were purchased from the Samtako (Osan, Korea). Mice were acclimated to the animal facility of Kyungpook National University for a week with free access to food and water and fasted for 12 h prior to performing the pharmacokinetic experiments. Blood samples were collected via the Retro-Orbital plexus using heparinized collection tube at 0, 0.25, 0.5, 1, 2, 4, 8, and 24 h under anesthesia with isoflurane following the intravenous administration of nannozinone A (5 mg/kg dissolved in 1 mL mixture of DMSO : saline = 20:80 (*v/v*)) via the tail vein or following the oral administration of nannozinone A (30 mg/kg suspended in 2 mL of 0.5% carboxymethyl cellulose suspension) using oral gavage. The blood was centrifuged to separate the plasma at 16,000 × *g* for 1 min, and the plasma sample was stored at -80°C until analysis.

Data analysis

The pharmacokinetic parameters were determined by the non-compartmental analysis (WinNonlin® 2.0; Pharsight, Mountain View, CA, USA).[6] The area under the plasma

concentration-time curve from time 0 to the last measurement (AUC_{last}) was calculated using the linear trapezoidal method. The area under the plasma concentration-time curve from zero to infinity (AUC_{∞}) was calculated by the trapezoidal extrapolation method. Elimination rate constant (k) was the slope obtained from the plasma concentration-time curve. Half-life ($T_{1/2}$) was calculated as 0.693/k. Absolute oral bioavailability (BA) was calculated by dividing dose normalized AUC after intravenous injection (AUC_{IV}/$Dose_{IV}$) by dose normalized AUC after oral administration (AUC_{PO}/$Dose_{PO}$). Mean residence time (MRT), the average time a molecule stays in the body, was calculated by summing the total time in the body and dividing by the number of molecules. Maximum plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) was read from the pharmacokinetic data. All data are expressed as the mean ± standard deviation (SD)

Results and Discussion

MS conditions

Nannozinone A and ^{13}C-caffeine (IS) showed optimal ionization in positive mode when monioted from the direct injection of nannozinone A and ^{13}C-caffeine into the mass spectrometer ionization source. MRM transition of nannozinone A was selected from the precursor ion ($[M+H]^+$, *m/z* 241.1) and the most frequent product ion (*m/z* 150.1), as shown in Figure 1. Similarly, MRM transition of ^{13}C-caffeine was selected from the precursor ion ($[M+H]^+$, *m/z* 198.2) and the most frequent product ion (*m/z* 140.0).[7]

Analytical method validation

Figure 2 shows the representative MRM chromatograms of double blank sample, zero blank sample, LLOQ sample (1 ng/mL), and plasma sample after oral administraion of nannozinone A. The retention times for nannozinone A and IS were 2.35 min and 1.99 min, respectively. The signal-to-noise (S/N) ratio of nannozinone A was more than 10.0 in the LLOQ samples and there was no significant matrix interference for the retention times of nannozinone A and IS in the blank samples compared with the LLOQ samples.

The extraction recoveries for nannozinone A were calculated at three levels of QC samples and were found to be high and reproducible, with a the range of extraction recoveries between 78.00-81.08% and a coefficient of variation (CV) 3.22-5.18% (Table 1), suggesting that the sample preparation method developed in this study was capable of efficiently extracting nannozinone A from mouse plasma. The matrix effects were between 70.56-96.49% with a CV of lower than 13.6%, indicating that co-eluting substances did not interfere with the ionization of the nannozinone A (Table 1).[5]

The standard calibration curves showed good linearity over the concentration range of 1–1000 ng/mL ($r^2 > 0.998$). Table 2 summarizes the intra- and inter-day precision and accuracy for nannozinone A from four levels of QC samples. The intra- and inter-day precision was found to

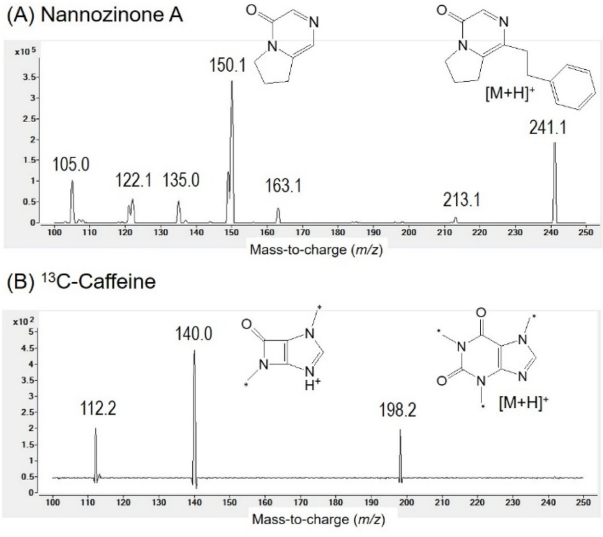

Figure 1. Product ion mass spectra of (A) nannozinone A and (B) ^{13}C-caffeine (IS).

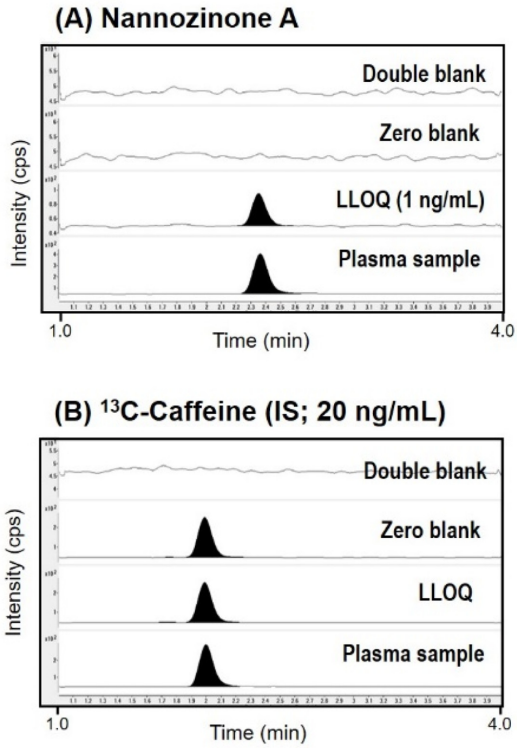

Figure 2. Representative MRM chromatograms of (A) nannozinone A and (B) ^{13}C-caffeine (IS) in mouse plasma of double blank, zero blank, LLOQ sample (1 ng/mL), and plasma sample at 2 h following oral administration of nannozinone A.

Table 1. Extraction recoveries and matrix effects of nannozinone A.

Analyte	Nominal concentration (ng/mL)	Extraction recovery (%)	CV (%)	Matrix effects (%)	CV (%)
Nannozinone A	3	81.08 ± 2.81	3.47	72.81 ± 1.70	2.33
	100	78.00 ± 2.51	3.22	70.56 ± 1.42	2.01
	750	79.18 ± 4.10	5.18	96.49 ± 13.1	13.6

Data represented as mean ± SD from six independent experiments.

Table 2. Intra- and inter-day precision and accuracy of nannozinone A in mouse plasma.

	Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Precision (%)	Accuracy (%)
Intra-day	1	1.01 ± 0.09	9.31	101.04
	3	2.64 ± 0.13	4.91	87.87
	100	93.07 ± 12.61	13.55	93.07
	750	794.76 ± 63.86	8.04	105.97
Inter-day	1	1.02 ± 0.09	9.07	99.98
	3	3.00 ± 0.21	7.02	99.91
	100	97.39 ± 7.49	7.69	97.39
	750	756.22 ± 72.81	9.63	100.83

Data represented as mean ± SD from six independent experiments.

Table 3. Stability of nannozinone A in mouse plasma.

Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Precision (%)	Accuracy (%)
Bench-top stability			
Low QC (3)	2.77 ± 0.18	6.34	92.23
High QC (750)	861.21 ± 21.14	2.45	114.83
Freeze-thaw stability			
Low QC (3)	2.94 ± 0.22	7.38	98.08
High QC (750)	788.34 ± 50.59	6.42	105.11
Autosampler stability			
Low QC (3)	3.17 ± 0.03	0.82	105.53
High QC (750)	747.25 ± 29.17	3.90	99.63

Data represented as mean ± SD from three independent experiments

range between 4.91 to 13.55% for nannozinone A and the intra- and inter-day accuracy was from 87.87 to 105.97%, which statisfies the acceptability criteria (less than 15%).[5] The results of the stability experiments are presented in Table 3. It was found that the accuracy of QC samples was within 114.83% for bench-top stability, within 105.11% for freeze-thaw stability, and within 105.53% autosampler stability. These results confirmed that nannozinone A is stable for up to 5 h on the bench-top at 25°C, and for over three freeze-thaw cycles, and for 24 h in an autosampler at 6°C.

Pharmacokinetic study

The plasma concentrations of nannozinone A after intravenous and oral administration in ICR mouse are shown in Figure 3, and the relevant pharmacokinetic parameters are listed in Table 4. The plasma concentrations of nannozinone A after intravenous injection declined sharply for 4 h, but the plasma concentrations of nannozinone A following oral administration showed a sharp decrease for 4 h and gradual decrease for 4-24 h, suggesting a high distribution kinetics. Consisently with this phenomenone, the clearance and volume of distribution of this compound were high (Table 4). It suggests that nannozinone A may undergo substantial metabolism or distribution, although the underlying mechanisms need to be further investigated.

The terminal half-life after oral administration of nannozinoe A was calculated as 8.0 ± 4.7 h, which was significantly longer compared to that of the intravenous injection (0.29 ± 0.12 h). Moreover, the mean absorption time, calculated by subtracting the mean residence time from the intravenous injection (MRT_{IV}) from the MRT_{PO} form the oral administration,[6,8] was calculated as 3.66 h, suggesting a long absorption time. The T_{max} value of nannozinone A was 15 min, indicating the rapid gastrointestinal

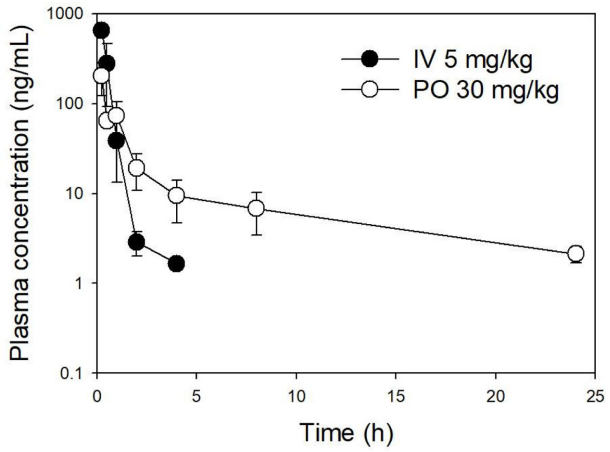

Figure 3. Plasma concentration-time profile of nannozinone A in mouse following an intravenous (IV, 5 mg/kg), and an oral (PO, 30 mg/kg) administration. Each data point represents the mean ± SD from four independent experiments.

Table 4. Pharmacokinetic parameters of nannozinone A following an intravenous (IV) and an oral (PO) administration in mouse.

Parameters	IV (5 mg/kg)	PO (30 mg/kg)
C_{max} (ng/mL)	-	205.37 ± 82.91
T_{max} (h)	-	0.25 ± 0.00
AUC_{last} (ng·h/mL)	535.67 ± 100.73	258.32 ± 83.93
AUC_{∞} (ng·h/mL)	536.73 ± 100.89	284.14 ± 87.86
$T_{1/2}$ (h)	0.29 ± 0.12	8.0 ± 4.7
MRT (h)	0.24 ± 0.08	3.9 ± 1.6
CL (mL/h/kg)	9561.30 ± 1762.99	-
$V_{d,ss}$ (mL/kg)	2269.34 ± 819.15	-
BA (%)	-	8.82

Data represents the mean ± SD from four independent experiments

absorption of nannozinone A.The AUC of intravenous and oral administration were calculated as 536.73 ± 100.89 and 284.14 ± 87.86 ng·h/mL, respectively, yielding a 8.82% of absolute oral bioavailability (BA). Taken together, once nannozinone A was given orally to mouse, nannozinone A was absorbed rapidly and stayed for long time but the extent absorbed was not great considering the low oral bioavailability. We should note the distinctive different half-life ($T_{1/2}$) after an intravenous and oral administration. The limited aqueous solubility of nannozinone A (4.44 ± 0.06 mg/mL), which was lower than oral dose (30 mg/kg/ 2 mL) and the long absorption time (3.66 h) could be attributed to the higher $T_{1/2,\ PO}$ than $T_{1/2,\ IV}$ since $T_{1/2,\ PO}$ could reflect the elimination and the delayed absorption.

Conclusions

In this study, we developed and validated a sensitive LC-MS/MS analytical method for nannozinone A in mouse plasma and we successfully applied this newly developed sensitive LC-MS/MS analytical method of nannozinone A to the pharmacokinetic evaluation of this compound. Consequently, the analytical method, and the pharmacokinetic features obtained from this study will facilitate the further preclinical investigation of nannozinone A.

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