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Development of a Sensitive Analytical Method of Polynemoraline C Using LC-MS/MS and Its Application to a Pharmacokinetic Study in Mice

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Abstract : Polynemoraline C, a pyridocoumarin alkaloid, exhibits anticholinergic, anti-inflammatory, antitumor, and antimicrobial activities. A sensitive analytical method of polynemoraline C in mouse plasma was developed and validated using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Polynemoraline C and ^{13}C-caffeine (internal standard) in mouse plasma were extracted using a liquid-liquid extraction method coupled with ethyl acetate. This extraction method resulted in high and reproducible extraction recovery in the range of 73.49%-77.31% with no interfering peaks around the peak retention time of polynemoraline C and ^{13}C-caffeine. The standard calibration curves for polynemoraline C were linear over the range of 0.5-200 ng/mL with $r^2 > 0.985$. The accuracy, precision, and the stability of the data were within acceptable limits on the FDA guideline. After intravenous and oral administration of polynemoraline C at doses of 5 and 30 mg/kg, respectively, the present method was successfully applied to the pharmacokinetic study of polynemoraline C. Polynemoraline C in mouse plasma showed a multi-exponential elimination pattern with a high volume of distribution values. This compound's absolute oral bioavailability was found to be 17.0%. Polynemoraline C's newly developed LC-MS/MS method can be used for further studies on the efficacy, toxicity, and biopharmaceutics of polynemoraline C, as well as its pharmacokinetic studies.

Keywords : Polynemoraline C, LC-MS/MS analysis, Pharmacokinetics, Bioavailability

Introduction

Polyalthia nemoralis A. DC., a shrub distributed in southern China and Vietnam,[1,2] has been used for the treatment of infectious diseases such as malaria, hepatitis, pneumonia, and syphilis. The roots of this plant, called Radix *Polyalthia nemoralis*, have been used for the treatment of chronic gastritis and indigestion.[3,4] Various chemicals, such as terpenoids and alkaloids, were isolated from the roots, branches, and leaves of Polyalthia nemoralis A. DC.. The strong antimalarial activity was found in zincpolyanemine and cupric bis(pyridineN-oxide-2-thiolate).[5] Several types of alkaloids such as aporphines, pyridocoumarins, benzylisoquinolines, and azafluorene alkaloids extracted from this plant exhibited cytotoxical,[2,6] antimicrobial,[2,7] antimalarial,[2,8] and antiHIV activities.[2,9]

Polynemoraline C (Figure 1), a pyridocoumarin alkaloid, also exhibits various pharmacological activities such as anticholinergic,[10,11] anti-inflammatory,[10,12] antitumor,[10,13] and antimicrobial activities.[10,14] Recently, Lu et al. reported method for the extraction of polynemoraline A, B, C, and D from the bark of Polyalthia nemoralis using ethanol.[15] Yoon et al. reported a silver nitrate catalyzed cycloisomerization method for the synthesis of polynemoraline C.[10]

To examine polynemoraline C's pharmacological efficacy, we aimed to develop and validate a sensitive and reproducible LC-MS/MS method for measuring the concentration of polynemoraline C and to apply this analytical method to a pharmacokinetic study of polynemoraline C in mice.

Protein precipitation (PPT) and liquid-liquid extraction (LLE) are the most widely used sample preparation methods for achieving a significant extraction recovery and negligible matrix effect from a biological matrix such as plasma.[16] LLE has an advantage in lowering interferences from the sample matrix and increasing the sensitivity of the analyte,[16,17] thus LLE method was utilized in this study for the sample preparation method for the extraction of polynemoraline C from 30 μL aliquot of mouse plasma samples. In addition, we examined our newly developed

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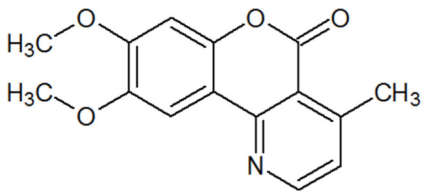

Figure 1. The structure of polynemoraline C.

analytical method in terms of its linearity, selectivity, accuracy, precision, stability, recovery, and matrix effects according to the U.S. Food and Drug Administration Guideline for Bioanalytical Method.[18]

Experimental

Chemicals and reagents

Polynemoraline C (Figure 1) was synthesized with a purity of > 99.0%, as confirmed by nuclear magnetic resonance and mass spectroscopy by Yoon et al.[10] ^{13}C-Caffeine was used as the internal standard (IS), which was purchased from Sigma-Aldrich (St. Louis, MO, USA). J.T. Baker provided us with ethyl acetate (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). HPLC or reagent grade solvents and chemicals were used throughout.

Preparation of calibration standards and quality control (QC) samples

Using acetonitrile, the polynemoraline C working solutions were serially diluted polynemoraline C stock solution (0.25 mg/mL in acetonitrile). The final concentrations of polynemoraline C working solutions were 0.5, 1, 2, 5, 20, 50, 100, and 200 ng/mL for calibration standards and 1.5, 30, and 150 ng/mL for QC samples. The IS solution was prepared at a concentration of 20 ng/mL of ^{13}C-caffeine by diluting the stock solution with water. Spiking a 30 μL aliquot of the polynemoraline C working solution, evaporating under a gentle stream of nitrogen gas, and reconstituting with a 30 μL aliquot of mouse blank plasma were used to make the calibration standards and QC samples. The final concentrations of polynemoraline C calibration standard and QC samples were 0.5, 1, 2, 5, 20, 50, 100, and 200 ng/mL and 1.5, 30, and 150 ng/mL, respectively.

Sample preparation

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

Instrument conditions

The LC system was an Agilent Infinity 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA), and chromatographic separation was carried out using a Luna C18 (150 mm × 2.0 mm, 5 μm; Phenomenex, Torrance, CA, USA) system. The mobile phase was pumped with an isocratic elution of water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid (20:80, v/v). The column temperature was kept at 30°C and the flow rate was 0.2 mL/min. Each injection took 4 min to run in total. For mass spectrometric detection and quantitative analysis, an Agilent 6430 triple quadrupole MS with an electrospray ionization (ESI) source was used. Gas flow was 10 L/min with a gas temperature of 300°C. Nebulizer and capillary conditions were set at 35 psi and 4000 V, respectively. The mass spectrometer was operated in the positive ion mode with multiple reaction monitoring (MRM) transitions at *m/z* 272.1 → 228.1 for polynemoraline C and *m/z* 198.0 → 140.0 for ^{13}C-caffeine.

Method validation

Blank plasma samples from six different mice were used for assessing selectivity. Signals of six blank plasma samples were compared to those of the corresponding lower limit of quantification (LLOQ) samples (0.5 ng/mL of polynemoraline C) and IS. The linearity of the calibration curve with eight non-zero standards (0.5-200 ng/mL) was generated using a least-square linear regression with $1/x^2$ as weighting factors by plotting the ratio of the analyte and IS peak areas versus the concentrations of polynemoraline C. The extraction recovery and matrix effect were determined using three levels of QCs (1.5, 30, and 150 ng/mL). By comparing the mean peak areas of extracted and post-extracted spiked samples, the extraction recovery was calculated. By comparing the mean peak areas of the post-extracted spiked sample to those of standard solutions with the same concentrations, the matrix effect was determined. The intraday precision and accuracy were analyzed for the six replicates at LLOQ and three levels of QCs (0.5, 1.5, 30, and 150 ng/mL) on the same day. The interday precision and accuracy were determined by measuring the LLOQ and three levels of QCs for five consecutive days. The bench-top stability was assessed by placing three levels of QCs at 25°C for 5 h. Freeze-thaw stability was assessed with three levels of QCs 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 three levels of QCs in the autosampler at 6°C for 24 h.

Pharmacokinetic study

The Animal Care and Use Committee at Kyungpook National University gave its approval to all animal procedures (Permission no. 2019-0126). The Samtako company (Osan, Korea) provided the male ICR mice (7-8 weeks old, 30-35 g). Mice were acclimated to the Kyungpook National University's

animal facility for a week with free access to food and water and fasted for 12 h before performing the pharmacokinetic experiments. Polynemoraline C was dissolved in DMSO :saline=20:80 (*v/v*) to make final concentration of 2.5 mg/kg/mL and 2 mL/kg solution was injected via tail vein. It was also suspended in 0.5% carboxymethyl cellulose suspension to make final concentration of 7.5 mg/kg/mL and 4 mL/kg suspension was administered orally using oral gavage. Blood samples were collected with sparse sampling method 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. The plasma sample (30 μL) was separated from the blood by centrifuging it at 10,000 × g for 1 min and storing at −80 °C until the analysis.

Protein binding

A rapid equilibrium dialysis kit (ThermoFisher Scientific Korea, Seoul, Korea) was used to determine plasma protein binding according to the manufacturer's instructions. In the inner sample chamber surrounded by a semipermeable membrane (molecular weight cut-off 8,000 Da), 50 μL of mouse plasma with a polynemoraline C concentration of 150 ng/mL were added. To the outer chamber, 300 μL of phosphate-buffered saline (PBS) was added. The samples were then incubated for 4 h at 37°C. After collecting 25 μL aliquots of each chamber, 25 μL of fresh PBS or blank mouse plasma was added to match the matrices. The samples were mixed with 20 μL of ^{13}C-caffeine solution (20 ng/mL in water) and 400 μL of ethyl acetate and vortexed vigorously for 10 min before centrifugation at 16,000 × g for 5 min. The supernatant was transferred to a clean tube and dried under a gentle stream of nitrogen gas. The residue was reconstituted in 150 μL of a mobile phase and 5 μL aliquot of the solution and was injected into the LC-MS/MS system. Protein binding fraction was calculated by subtracting a free fraction from unity and free fraction was calculated by dividing drug concentration in PBS chamber by drug concentration in a plasma chamber.[16,19] As a positive control, propranolol showed 93.0% protein binding, which was in line with the 85%−96% reference value.[19,20]

Solubility

Polynemoraline C (3 mg) was weighed and added to 1 mL of water. The tube was incubated for 24 h on a Multi Reax shaker (Heidolph, Schwabach, Germany) and filtered through a 0.45 μm membrane filter. The filtrate was 10-fold diluted with acetonitrile, followed by 10-fold diluted with the mobile phase. Equal volume of ^{13}C-caffeine solution (20 ng/mL in water) was added to the solution. The mixture was vigorously vortexed for 10 min and then centrifuged at 16,000 × g for 5 min. The 5 μL aliquot of the solution was injected into the LC-MS/MS system.

Data analysis

Non-compartmental analysis (WinNonlin® 5.1; Pharsight, Mountain View, CA, USA) was used to determine the pharmacokinetic parameters, and oral bioavailability (BA) was calculated by dividing $AUC_{24h,PO}$, which was normalized with polynemoraline C oral dose (30 mg/kg), by $AUC_{24h,IV}$, which was also normalized with polynemoraline C IV dose (5 mg/kg).

Results and Discussion

Optimization of MS conditions

A Luna C18 (150 mm × 2.0 mm, 5 μm) column showed good and stable peak shape, selectivity, and sensitivity for polynemoraline C and ^{13}C-caffeine (IS) with an isocratic elution of water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid (20:80, *v/v*) compared with Synergi polar RP (150 mm × 2.0 mm, 4.0 μm, Phenomenex), Luna CN (150 mm × 2.0 mm, 5 μm, Phenomenex), and Omega Polar C18 (50 mm × 2.1 mm, 1.6 μm, Phenomenex) column.

Each compound was injected directly into the mass spectrometer ionization source to optimize ESI conditions for polynemoraline C and ^{13}C-caffeine (IS). In positive mode, polynemoraline C and ^{13}C-caffeine showed optimal ionization. The MRM transition of polynemoraline C was selected from the precursor ion ($[M+H]^+$, *m/z* 272.1) and the most frequent product ion (*m/z* 228.1), as shown in Figure 2. Similarly, the MRM transition of ^{13}C-caffeine was selected from the precursor ion ($[M+H]^+$, *m/z* 198.0) and the most frequent product ion (*m/z* 140.0). The fragmentor voltage of polynemoraline C and ^{13}C-caffeine was set at 135 V and 120 V, respectively. The collision energy of polynemoraline C and ^{13}C-caffeine was optimized as 35 V and 20 V, respectively.

Figure 3A shows the representative MRM chromatograms of a double blank sample, zero blank sample, LLOQ sample

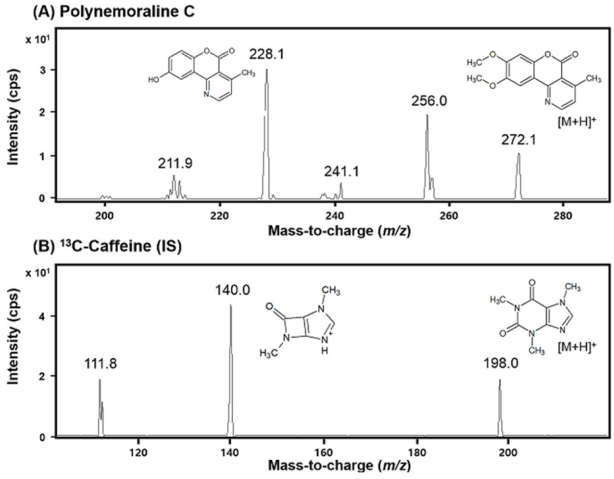

Figure 2. Product ion spectra of (A) polynemoraline C and (B) ^{13}C-caffeine (IS).

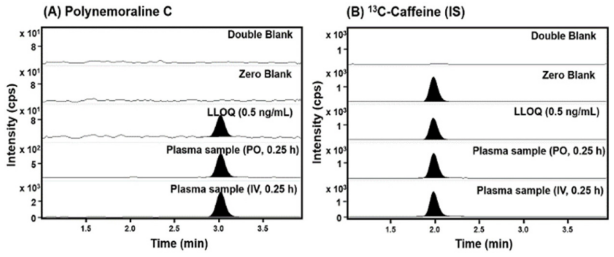

Figure 3. Representative MRM chromatograms of (A) polynemoraline C and (B) ^{13}C-caffeine (IS) in mouse plasma of double blank, zero blank, LLOQ sample (0.5 ng/mL), and plasma sample following an oral (PO) and intravenous (IV) administration of polynemoraline C.

(0.5 ng/mL), and plasma sample after PO and IV administration of polynemoraline C. Polynemoraline C had a retention time of 3.1 min, and IS had a retention time of 2.0 min. In the LLOQ samples (0.5 ng/mL), polynemoraline C had a signal-to-noise (S/N) ratio of 11.8. The results revealed no significant interference peaks in the retention times of the analytes, indicating that the current method has good selectivity (Figure 3A). Figure 3B shows a typical MRM chromatogram of a mouse plasma sample spiked with ^{13}C-caffeine at 20 ng/mL.

Validation of the analytical method

The extraction recoveries for polynemoraline C were found to be high and reproducible, with the range of extraction recoveries between 73.49%-77.31% and a coefficient of variation (CV) 5.58%-12.73% (Table 1), suggesting that the LLE method with ethyl acetate was capable of efficiently extracting polynemoraline C from mouse plasma. Ethyl acetate was selected based on its high and reproducible exraction compared to methyl-tertiary butyl ether.

The matrix effects ranged from 74.53% to 93.57% (Table 1), indicating that co-eluting substances did not interfere with the polynemoraline C ionization, and the sample preparation method of polynemoraline C could exclude any matrix effect by ion suppression or enhancement.

The calibration curves showed good linearity over the concentration range of 0.5-200 ng/mL ($r^2 > 0.985$). Table 2 summarizes the precision and the accuracy of polynemoraline C from four levels of QC samples intraday and interday. The intraday and interday precision and accuracy were found to range from 2.86% to 13.55% and from 86.82% to 102.7%, respectively, meeting the acceptability criteria.[18]

The results of the stability test are presented in Table 3. The precision of QC samples for polynemoraline C was found to be within 8.44% for bench-top stability, 11.25% for freeze-thaw stability, and 12.01% for autosampler stability. For the three types of stability evaluations, the accuracy ranged from 86.33% to 112.2%. As a result of these three types of stability tests performed during the bioanalytical procedure, polynemoraline C in mouse plasma samples was found to be stable.

Table 1. Extraction recoveries and matrix effects of polynemoraline C in mouse plasma.

Nominal concentration (ng/mL)	Extraction recovery (%)	CV (%)	Matrix effects (%)	CV (%)
1.5	75.39 ± 9.60	12.73	76.54 ± 9.05	11.82
30	73.49 ± 6.11	8.31	74.53 ± 6.25	8.38
150	77.31 ± 4.31	5.58	93.57 ± 8.31	8.88

Data represented as mean ± SD (n = 6)

Table 2. Intraday and interday precision and accuracy of polynemoraline C in mouse plasma.

Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Precision (%)	Accuracy (%)
Intraday (n = 6)			
0.5	0.48 ± 0.03	6.63	94.86
1.5	1.40 ± 0.06	4.25	93.52
30	25.99 ± 0.74	2.86	86.82
150	158.9 ± 8.73	5.50	105.9
Interday (n = 5)			
0.5	0.51 ± 0.02	4.12	102.7
1.5	1.53 ± 0.20	12.88	102.5
30	26.92 ± 3.65	13.55	89.87
150	152.6 ± 11.89	7.79	101.9

Data represented as mean ± SD

Table 3. Stability of Polynemoraline C in mouse plasma.

Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Precision (%)	Accuracy (%)
Bench-top stability (25°C, 5 h)			
1.5	1.63 ± 0.14	8.44	108.4
30	25.90 ± 0.59	2.26	86.33
150	154.6 ± 10.12	6.54	103.1
Freeze-thaw stability (3 cycles)			
1.5	1.68 ± 0.15	9.04	112.2
30	28.23 ± 3.18	11.25	94.11
150	161.2 ± 2.81	1.74	107.5
Autosampler stability (6°C, 24 h)			
1.5	1.52 ± 0.14	8.97	101.3
30	28.35 ± 3.40	12.01	94.49
150	152.7 ± 13.30	8.71	101.8

Data represented as mean ± SD (n = 3)

Pharmacokinetic study

The plasma concentrations of polynemoraline C after intravenous and oral administration in mice are shown in Figure 4, and the relevant pharmacokinetic parameters are listed in Table 4.

After IV injection, the plasma concentrations of polynemoraline C showed a multi-exponential decay. It declined sharply for 1 h but slowly declined for 4-24 h, and the initial concentration (C_o) of polynemoraline C was 41.82 ± 7.34 ng/mL. Volume of distribution at central compartment (V_1) was caculated as 122.2 ± 19.92 L/kg and volume of distribution at steady state ($V_{d,ss}$) was calculated as 797.1 ± 211.3 L/kg (Table 4). The results (i.e., the large V_1 and $V_{d,ss}$ value and much higher $V_{d,ss}$ than V_1) suggested that polynemoraline C may have highly distributed characteristics into central compartment as well as peripheral compartment.[21] The elimination half-life ($T_{1/2}$) was calculated as 13.84 ± 7.46 h when mice received 5 mg/kg of polynemoraline C intravenously.

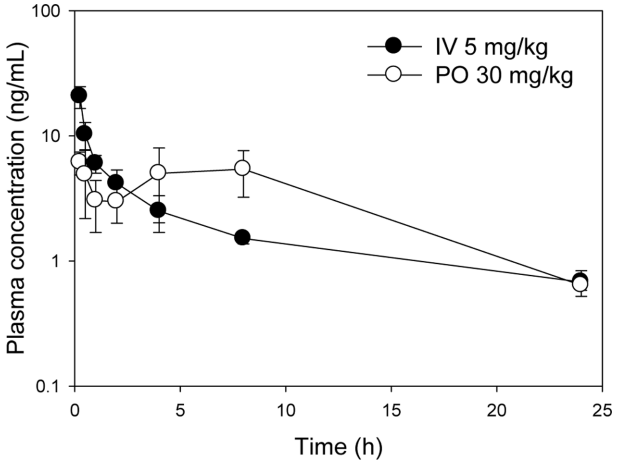

Figure 4. Plasma concentration-time profile of polynemoraline C in mice following an intravenous (IV, 5 mg/kg) and oral (PO, 30 mg/kg) administration of polynemoraline C. Each data point represents the mean ± SD (n = 4).

Table 4. Pharmacokinetic parameters of polynemoraline C in mice.

Parameters	IV (5 mg/kg)	PO (30 mg/kg)
$T_{1/2}$ (h)	13.84 ± 7.46	11.45 ± 4.70
C_0 (ng/mL)	41.82 ± 7.34	-
C_{max} (ng/mL)	-	7.66 ± 1.43
T_{max} (h)	-	3.13 ± 3.70
AUC_{24h} (ng·h/mL)	48.69 ± 12.35	49.71 ± 24.01
CL (mL/h/kg)	89.97 ± 28.97	-
V_1 (L/kg)	122.2 ± 19.92	-
$V_{d,ss}$ (L/kg)	797.1 ± 211.3	-
BA (%)	-	17.0

Each data represents the mean ± SD (n = 4)
V_1, Volume of distribution at central compartment calculated by dividing C_0 with dose; $V_{d,ss}$, Volume of distribution at steady state calcaulted by multiplying mean residence time with CL

Table 5. Physicochemical properties of polynemoraline C.

Parameters	Mean ± SD (n = 3)
Log P [a]	2.23
Solubility (μg/mL)	5.46 ± 1.92
Protein binding (% Bound)	96.19 ± 0.83

[a] Log P value was obtained from Molinspiration Cheminformatics

Orally administered polynemoraline C showed multiple peaks; the first peak appeared in 0.25-0.5 h and the second peak appeared in 4-8 h (Figure 4), which resulted in a variable T_{max} value (CV of 118%). The polynemoraline C's lipophilic nature (i.e., high log P value of 2.23 and high protein binding properties, Table 5) and small molecular weight could contribute to polynemoraline C's rapid gastrointestinal absorption. In addition, the low aqueous solubility (5.46 ± 1.92 mg/mL, Table 5) of this compound after oral administration of high dose (30 mg/kg/2 mL) may contribute to delayed and steady absorption. The multiple peak phenomenon may also be influenced by the enterohepatic circulaiton. Polynemoraline C's absorption properties need to be investigated further. The AUC_{24h} of intravenous and oral administration were calculated as 48.69 ± 12.35 and 49.71 ± 24.01 ng·h/mL, respectively, yielding a 17.0% of oral BA. Because of the slow elimination and the appearance of second peak of polynemoraline C, the percentage of extrapolated part in AUC_{IV} and AUC_{PO} (i.e. AUC calculated from 24 h to infinity) was 17.7% and 48.8%, respectively. Therefore, we provided $AUC_{24h,IV}$ and $AUC_{24h,PO}$ values in Table 4 and used for BA calculation.

Conclusions

For the quantification of polynemoraline C in mouse plasma, a sensitive analytical method of polynemoraline C using an LC-MS/MS system was developed and validated. The pharmacokinetic parameters in mice and the physicochemical properties of polynemoraline C were successfully evaluated using this analytical method. To the best of our knowledge, this is the first report of using the LC-MS/MS method for the determination of polynemoraline C from mouse plasma and, consequently, the analytical method and pharmacokinetic features obtained from this study will facilitate the further preclinical investigation of polynemoraline C.

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