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Development of Sensitive Analytical Method of Rhodanthpyrone A by a LC-MS/MS and its Application to Bioavailability Study in Rats

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Abstract : A sensitive analytical method of rhodanthpyrone A in rat plasma was developed using a liquid chromatography-tandem mass spectrometry (LC-MS/MS). Rhodanthpyrone A and rhodanthpyrone B (internal standard) in rat plasma were extracted by a liquid-liquid extraction method with ethyl acetate. This extraction method gave results in high and reproducible extraction recovery in the range of 73.75-79.90% with no interfering peaks around the peak elution time of rhodanthpyrone A and B. The standard calibration curves for rhodanthpyrone A ranged from 0.5 to 2000 ng/mL were linear with $r^2 > 0.994$ and the inter- and intra-day accuracy and precision and the stability were within acceptance criteria. Using this validated analytical method, pharmacokinetics of rhodanthpyrone A following intravenous and oral administration of rhodanthpyrone A at doses of 2 mg/kg and 30 mg/kg, respectively, were investigated. Rhodanthpyrone A in rat plasma showed multi-exponential elimination pattern with high clearance and volume of distribution values. The absolute oral bioavailability of this compound was calculated as 3.7%. Collectively, the newly developed sensitive LC-MS/MS analytical method of rhodanthpyrone A could be successfully applied to investigate the pharmacokinetic properties of this compound and would be useful for the further studies on the efficacy, toxicity, and biopharmaceutics of rhodanthpyrone A.

Keywords : Rhodanthpyrone A, LC-MS/MS, bioavailability, rat plasma

Introduction

Gentiana rhodantha has been employed as anti-inflammatory, hepatoprotective, and anti-microbial activities because of higher content of iridoids and polyphenols.[1] In addition to these iridoids and polyphenols components, Yao et al recently reported two a-pyrone derivatives from Gentiana rhodantha and names as rhodanthpyrone A and B.[2] a-Pyrone derivative is a six membered cyclic unsaturated ester and showed antimicrobial and anticancer effect.[2-4] Moreover, the pyrone moiety and its fused derivatives has been recognized as a privileged scaffold.[5] Since rhodanthpyrone A and B are rare component of Gentiana rhodantha, our group developed a concise synthetic method for these compound.[3] For the further experiments for investigating its activity and toxicity, development of analytical method for monitoring rhodanthpyrone A concentration in biological matrix is an inevitable process. Therefore, the aim of this study was to develop and validate a sensitive and reproducible liquid chromatography tandem mass spectrometry (LC-MS/MS) method for measuring the concentrations of rhodanthpyrone A and to apply this analytical method to the pharmacokinetic study of rhodanthpyrone A in small animals.

To achieve the substantial extraction recovery and negligible matrix effect from biological matrix such as plasma, the most widely used sample preparation methods are protein precipitation (PPT) and liquid-liquid extraction (LLE).[6] Since LLE has the advantage in lowering interferences from the sample matrix and increasing the sensitivity of the analyte, our approach for developing an assay of rhodanthpyrone A in plasma samples was based on LLE. Moreover, our method was fully validated according to the U.S. Food and Drug Administration Guideline for Bioanalytical Method for its linearity, selectivity, accuracy, precision, stability, recovery, and matrix effects.[7]

Experimental

Chemicals and reagents

Rhodanthpyrone A and rhodanthpyrone B (Figure 1) were synthesized with a purity of > 99.0% and confirmed

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by nuclear magnetic resonance spectroscopy and mass spectroscopy.[3] Rhodanthpyrone B was used as the internal standard (IS). The ethyl acetate was obtained from 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, MA, USA). All solvents and chemicals were of HPLC and reagent grade.

Preparation of stock and working solutions

The stock solutions were prepared individually by dissolving the rhodanthpyrone A and rhodanthpyrone B in methanol at concentrations of 1 mg/mL. The rhodanthpyrone A working solutions were prepared by diluting the stock solution serially with acetonitrile and made their final concentrations 5, 10, 50, 200, 500, 2000, 5000, 10000, and 20000 ng/mL. The rhodanthpyrone B solution was prepared at a concentration of 25 ng/mL by diluting the stock solution with water.

Preparation of calibration curve and QC samples

The calibration curve and quality control (QC) samples were prepared by spiking a 5 μL aliquot of the working solution with 45 μL aliquot of blank rat plasma. The final concentrations of calibration standards and QC samples were 0.5, 1, 5, 20, 50, 200, 500, 1000, 2000 ng/mL and 1.5 (low QC), 150 (middle QC), 1500 (high QC) ng/mL, respectively.

Sample preparation

The calibration curve and QC samples were added with 50 μL of rhodanthpyrone B solution (25 ng/mL in water) and 800 μL of ethyl acetate. The mixture was vigorously vortexed for 15 min then centrifuged at 16,100 × g for 5 min. The supernatant was transferred to a clean tube and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 200 μL of mobile phase and 6 μL aliquot of the solution was injected into the LC-MS/MS system.

Instrument conditions

The LC system was an Agilent Infinity 1260 Infinite II HPLC system (Agilent Technologies, Santa Clara, CA, USA), and chromatographic separation was carried out using a Luna C18 (150 × 2.0 mm, 5 μm; Phenomenex, Torrance, CA, USA). The mobile phase was pumped with isocratic elution of water containing 0.1% formic acid : acetonitrile containing 0.1% formic acid (50:50, v/v). The flow rate was 0.2 mL/min with column temperature maintained at 30°C. Total run time for each injection was 3.7 min. The Agilent 6470 triple quadrupole MS equipped with an electrospray ionization source was used for mass spectrometric detection and quantitative analysis. The mass spectrometer was operated in the positive ion mode with multiple reaction monitoring (MRM) transitions at *m/z* 263.2→247.1 for rhodanthpyrone A and at *m/z* 233.1→218.0 for rhodanthpyrone B with optimized fragmentor of 135 V and collision energy (CE) of 20 or 25 eV, respectively.

Method validation

The developed method was validated according to the U.S. FDA guideline for bioanalytical method for its specificity, linearity, accuracy, precision, extraction recovery, matrix effect, and stability.[7] Blank plasma samples from six different rat were used for assessing the specificity. Signals of six blank plasma samples were compared with those of corresponding lower limit of quantification (LLOQ) samples and IS. By plotting the ratio of the peak areas of the analyte and IS versus the concentrations of rhodanthpyrone A, a nine point calibration curve (0.5-2000 ng/mL) was generated by a least square linear regression using $1/x^2$ as weighting factors. The extraction recovery and matrix effect was determined using three QC samples (low-, middle-, and high QC) of rhodanthpyrone A and IS solution (25 ng/mL). The extraction recovery was calculated by comparing the mean peak areas of extracted samples with mean peak areas of post-extracted plasma reconstituted with rhodanthpyrone A and IS. The matrix effect was determined by comparing the mean peak areas of post-extracted sample with the peak areas of neat samples spiked with corresponding concentrations. The intra-day precision and accuracy were analyzed the six replicates at three QC levels on the same day. The inter-day precision and accuracy were determined for five consecutive days. The bench-top stability was assessed by placing QC samples at 25°C for 6 h. The stability of three freeze-thaw cycles was analyzed by comparing QC samples that underwent three freeze-thaw cycle (from -80°C to 25°C for 6 h as one cycle) with those of control group. Autosampler stability was evaluated by placing processed QC samples in the autosampler at 6°C for 26 h.

Pharmacokinetic study

All animal procedures were approved by the Animal Care and Use Committee of the Kyungpook National University (Permission no. 2016-0019). The male Sprague-Dawley rats (7-8 weeks old, 250-280 g) were purchased from the SAMTAKO (Osan, Korea). Rats 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 before the pharmacokinetic experiments. On the day of study, the femoral arteries and femoral veins of rats were cannulated with PE50 polyethylene tubing (Jungdo, Seoul, Korea) under anesthesia with isoflurane (30 mmol/kg). Blood samples (0.20 mL) were collected via the femoral artery using heparinized collection tube at 0.083, 0.25, 0.5, 1, 1.5, 2, 6, and 8 h following the intravenous administration of rhodanthpyrone A (2 mg/kg dissolved in 1 mL mixture of DMSO : saline = 20:80 (v/v)) via the femoral vein. Blood samples (0.20 mL) were collected via

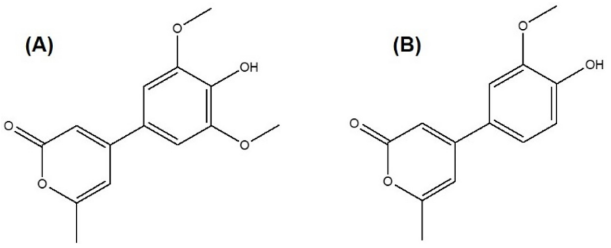

Figure 1. Structures of (A) rhodanthpyrone A, and (B) rhodanthpyrone B.

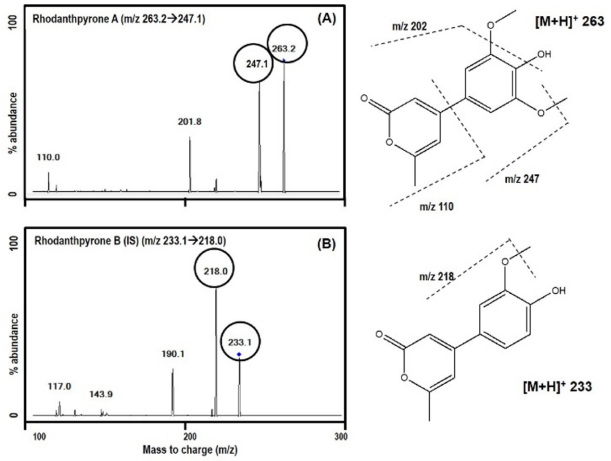

Figure 2. Product ion spectra of (A) rhodanthpyrone A, and (B) rhodanthpyrone B (IS).

the femoral artery using heparinized collection tube at 0.083, 0.25, 0.5, 1, 1.5, 2, 6, and 8 h following oral administration (30 mg/kg suspended in 2 mL of 0.5% carboxymethyl cellulose suspension) using oral gavage. The blood was centrifuged at 16,100 × g to separate the plasma for 10 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) and oral bioavailability (BA) was calculated by dividing AUC_{PO}, which was normalized with rhodanthpyrone A dose (30 mg/kg) by AUC_{IV}, which was also normalized by the IV dose of rhodanthpyrone A (2 mg/kg).

Results and Discussion

Optimization of MS conditions

In order to optimize ESI conditions for rhodanthpyrone A and IS, each compound was injected directly into the mass spectrometer ionization source. The rhodanthpyrone A and IS showed optimal ionization in positive mode. MRM transition of rhodanthpyrone A was selected from the precursor ion ($[M+H]^+$, *m/z* 263.2) and the most frequent product ion (*m/z* 247.1). MRM transition of IS was selected from the precursor ion ($[M+H]^+$, *m/z* 233.1) and the most frequent product ion (*m/z* 218.0). Figure 2 shows the structure and product ion scan spectra of rhodanthpyrone A and IS. Therefore, the optimal MRM transition for rhodanthpyrone A and IS were in the condition at *m/z* 263.2→247.1 for rhodanthpyrone A, and at *m/z* 233.1→218.0 for IS.

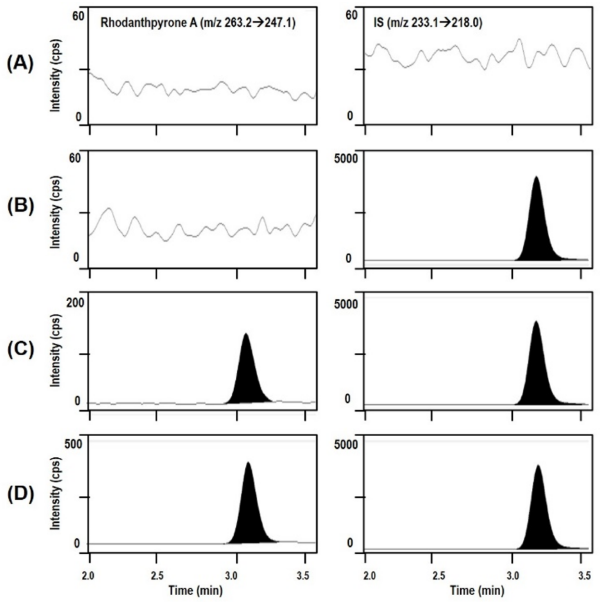

Figure 3. Representative MRM chromatograms of rhodanthpyrone A and IS in rat (A) double blank sample, (B) zero blank sample, (C) LLOQ sample (0.5 ng/mL), and (D) plasma sample at 2 h following oral administration of rhodanthpyrone A.

Analytical method validation

Figure 3 shows the typical chromatograms of double blank sample, zero blank sample, LLOQ sample (0.5 ng/mL), and plasma sample after oral administraion of rhodanthpyrone A. The retention times for rhodanthpyrone A and IS were 3.1 min and 3.2 min, respectively. The signal-to-noise (S/N) ratio of rhodanthpyrone A was more than 10.0 in the LLOQ samples and there was no significant matrix interference at the retention times of rhodanthpyrone A and IS in the blank samples compared with the LLOQ samples when judged from the chromatograms shown in Figure 3.

The results of extraction recoveries and matrix effects

Table 1. Extraction recoveries and matrix effects of rhodanthpyrone A and IS.

Analyte	Nominal concentration (ng/mL)	Extraction recovery (%)	CV (%)	Matrix effects (%)	CV (%)
Rhodanthpyrone A	1.5	79.90 ± 6.31	7.90	103.91 ± 2.45	2.36
	150	74.95 ± 2.52	3.36	98.14 ± 2.38	2.43
	1500	73.75 ± 4.68	6.35	96.86 ± 1.92	1.98
IS	25	86.49 ± 2.15	2.49	100.69 ± 1.07	1.06

Data represented as mean ± SD from six independent experiments.

Table 2. Intra- and inter- day precision and accuracy of rhodanthpyrone A in rat plasma.

	Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Precision (%)	Accuracy (%)
Intra-day (n=6)	1.5	1.40 ± 0.03	2.14	93.33
	150	156.39 ± 0.72	0.46	104.26
	1500	1503.40 ± 11.21	0.75	100.23
Inter-day (n=5)	1.5	1.58 ± 0.15	9.49	105.33
	150	155.82 ± 4.95	3.18	103.88
	1500	1486.20 ±14.44	0.97	99.08

Data represented as mean ± SD from five or six independent experiments.

Table 3. Stability of rhodanthpyrone A in rat plasma.

Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Precision (%)	Accuracy (%)
Bench-top stability			
Low QC (1.5)	1.64 ± 0.13	7.93	109.33
High QC (1500)	1536.69 ± 10.64	0.69	102.45
Freeze-thaw stability			
Low QC (1.5)	1.49 ± 0.03	2.01	99.33
High QC (1500)	1485.80 ± 21.83	1.47	99.05
Autosampler stability			
Low QC (1.5)	1.52 ± 0.04	2.63	101.33
High QC (1500)	1503.78 ± 13.59	0.90	100.30

Data represented as mean ± SD from three independent experiments.

are summarized in Table 1. The extraction recoveries for rhodanthpyrone A were calculated at three levels of QC samples and were found to be high and reproducible in the range of extraction recovery 73.75-79.90% and coefficient of variation (CV) 3.36-7.90%. This demonstrated that the LLE method employed in this study was capable of efficiently extracting rhodanthpyrone A from the rat plasma. The matrix effects were between 96.86-103.91%, indicating that co-eluting substances did not interfere with the ionization of the rhodanthpyrone A. The low CV in the matrix effect indicated that there were no significant differences for the peak areas of rhodanthpyrone A at three concentrations in the 6 batches of rat plasma matrix so it was possible to exclude any matrix effect of ion suppression or enhancement.[6] The extraction recovery and matrix effects of IS was also high and reproducible.

The calibration curves showed good linearity over the concentration range of 0.5-2000 ng/mL ($r^2 > 0.994$). Table 2 summarizes the intra- and inter-day precision and accuracy for rhodanthpyrone A from three levels of QC samples. The intra- and inter-day precision was from 0.46 to 9.49% for rhodanthpyrone A and the intra- and inter-day accuracy was from 93.33 to 105.33%, which in the acceptable criteria (less than 15%).[7]

The results of stability experiments are presented in Table 3. The accuracy of rhodanthpyrone A was within 99.05-109.33% and the precision was within 7.93%. As there was no significant difference observed for each concentration compared with nominal concentration under various experimental conditions, these results confirmed that the rhodanthpyrone A is stable up to 6 h on bench top at 25°C for bench-top stability, and 26 h in autosampler at 6°C for autosampler stability, and over three freeze-thaw cycles.

Pharmacokinetic study

Temporal profiles for the plasma concentration of rhodanthpyrone A after intravenous and oral administration are shown in Figure 4, and the relevant pharmacokinetic parameters are listed in Table 4. The plasma concentration of rhodanthpyrone A after intravenous injection declined sharply in early phase (up to 1 h) and declined gradually in

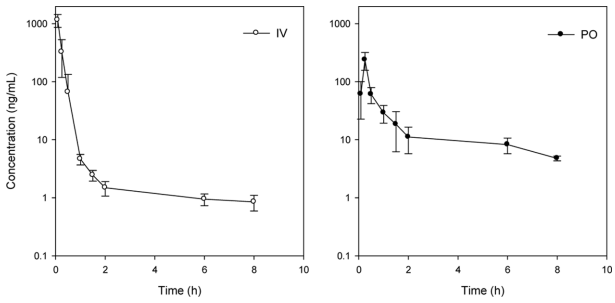

Figure 4. Plasma concentration-time profile of rhodanthpyrone A in rats following an intravenous (IV, 2 mg/kg, $n = 5$), and an oral (PO, 30 mg/kg, $n = 5$) administration of rhodanthpyrone A.

Table 4. Pharmacokinetic parameters of rhodanthpyrone A following IV and PO administration in rats.

Parameters	IV (2 mg/kg)	PO (30 mg/kg)
Half-life (h)	4.5 ± 1.6	3.1 ± 1.5
C_{max} (ng/mL)	-	204.6 ± 96.1
T_{max} (h)	-	0.2 ± 0.08
AUC_{last} (ng·h/mL)	347.7 ± 105.3	175.7 ± 26.8
AUC_{∞} (ng·h/mL)	353.1 ± 105.7	196.7 ± 21.4
CL(mL/h/kg)	6108.1 ± 1868.9	-
$V_{d,ss}$ (mL/kg)	2645.2 ± 1177.9	-
BA (%)	-	3.7

Each data represents the mean ± SD from five different rats per group.

late phase (up to 8 h), which implied rhodanthpyrone A showed the multi-exponential elimination pattern with the fast distribution phase and gradual elimination phase. As this consequence, the clearance and volume of distribution of this compound was very high (Table 4), suggesting that this compound may undergo substantial metabolism or distribution although the underlying mechanism need to be further investigated. The plasma concentration of rhodanthpyrone A following oral administration reached peak values at the early sampling time (i.e. 5~15 min), indicating that the gastrointestinal absorption of rhodanthpyrone A is rapid. The AUC of intravenous and oral administration was calculated as 353.1 ± 105.7 and 196.7 ± 21.4 ng·h/mL, respectively, yielding a 3.7% of absolute bioavailability (BA).

Conclusions

A sensitive LC-MS/MS method has been developed and validated for the quantification of the rhodanthpyrone A in rat plasma and this analytical method was successfully applied to evaluate the pharmacokinetic properties of the rhodanthpyrone A in rats. To the best of our knowledge, this is the first report of an LC-MS/MS method for the determination of rhodanthpyrone A from rat plasma and, consequently, the analytical method and the pharmacokinetic features obtained from this study will facilitate further preclinical investigation on the rhodanthpyrone A.

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