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Short communication

Determination of pinostrobin in rat plasma by LC–MS/MS: Application to pharmacokinetics

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ABSTRACT

A rapid and sensitive method for the determination of pinostrobin in rat plasma was developed using liquid chromatography tandem mass spectrometry (LC–MS/MS) for the first time. Isoliquiritigenin was used as an internal standard in rat plasma. Chromatographic separation was performed on an HiQ Sil C_{18} column with isocratic elution at a flow rate of 1 mL/min. The mobile phase consisted of water and methanol (9:91, v/v) containing 0.1% formic acid. The quantification limit was 10 ng/mL within a linear range of 10–1000 ng/mL ($R = 0.9984$). The intra- and inter-day assay precision ranged from 3.8–5.3% to 3.2–5.2%, respectively, and the intra- and inter-day assay accuracy was between 93.2–95.1% and 95.5–104.3%, respectively. Our results indicated that the LC–MS/MS method is effective for pharmacokinetic study of pinostrobin in rat plasma.

1. Introduction

Flavonoids are a large group of diphenolic compounds that are present in fruit, vegetables, grains, tea, and wine, etc. These compounds have received a great deal of attention due to their wide range of biological activities, such as antiviral, antioxidant, anti-inflammatory, antimutagenic, and anticarcinogenic activities [1–7]. Pinostrobin (5-hydroxy-7-methoxyflavanone, PI) is a natural flavonoid. This compound is known to be a very effective antispasmodic agent [8]. It has also been reported to possess anti-ulcer, anti-inflammatory, antinociceptive, antifungal, and flatulence eliminating activities [9,10]. Furthermore, it can intensively induce mammalian phase 2 detoxication enzymes and antioxidant enzymes [11]. It can also inhibit the human placental aromatase, and decrease the proliferation of MCF-7 cells induced by dehydroepiandrosterone sulphate and 17β-estradiol [12]. The chemical structures of PI and the chemically related compound isoliquiritigenin, which served as internal standard, are shown in Fig. 1.

Determination of PI using HPLC methods applicable to pharmacokinetic study has been reported [9,13–15]. However, these methods need long chromatographic run time and large volumes of organic solvent, while having low sensitivity. To investigate pharmacokinetic characteristics, more sensitive methods like liquid chromatography–tandem mass spectrometry (LC–MS/MS) are preferred. To our best knowledge, using LC–MS/MS to determine and quantify PI in rat plasma has not been reported.

The purpose of this study is to develop a sensitive and highly selective method based on liquid chromatography–tandem mass spectrometry (LC–MS) to determine PI in rat plasma.

2. Experimental

2.1. Chemicals and reagents

Pinostrobin (98%, HPLC grade) was separated and purified in our laboratory. The structure was confirmed by comparing the IR, 1H- and 13C-nuclear magnetic resonance (NMR) and MS data with reported data [16]. Isoliquiritigenin (ISL, ≥98%) was purchased from Sigma–Aldrich Inc. (Taufkirchen, Germany). Other chemicals used in the present investigation were nitrogen gas (purity 99.99%, Liming Gas Corp., China), methanol, ethyl acetate (J & K Chemical Ltd., China) and formic acid (96%, DIMA Technology Inc., USA). Deionized water used for all experiments was of Milli-Q quality (Millipore Corp., Bedford, MA, USA).

2.2. Animals

Female Kunming rats, weighing 220–250 g, were obtained from Harbin Veterinary Research Institute Animal Co. Ltd. (Harbin, Hei-

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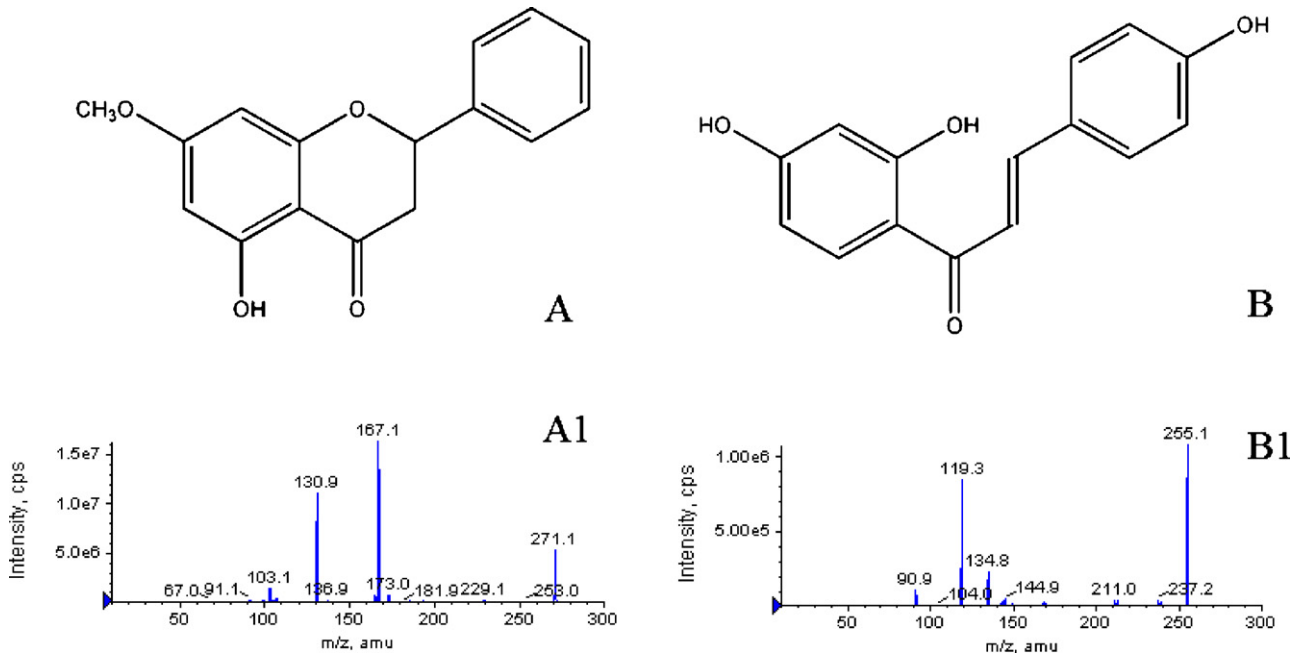

Fig. 1. Chemical structures and MS/MS spectra of (A) pinostrobin (PI) and (B) isoliquiritigenin (ISL).

longjiang, China). After a single dose of orally applied PI (0.5 mg/kg) to healthy rats ($n = 6$), blood samples were collected in heparinized tubes at 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12 and 24 h post-dose, immediately centrifuged at 3000 × *g* for 15 min, and then stored at −70 °C until analysis.

The protocol of the study was approved by the Ethics Committee of the Chengdu University of Traditional Chinese Medicine. The investigation was conducted in accordance with the ethical principles of animal use and care.

2.3. Instrumentations and chromatographic conditions

Chromatographic separation was performed with an Agilent 1100 series HPLC system (Agilent Technologies, San Jose, CA, USA) equipped with a G1312A binary pump, a 7725i manual injector, and a G1379A degasser. Column effluent was monitored with an API3000 triple-stage quadrupole mass spectrometer (Applied Biosystems, Concord, Canada) equipped with an electrospray ionization (ESI) source. The chromatographic separation was performed on an HiQ Sil C_{18} column (4.6 mm × 250 mm, KYA TEACH, Japan) at 40 °C. The mobile phase for PI was water and methanol (9:91, v/v) containing 0.1% formic acid. The flow rate was 1.0 mL/min, and the sample injection volume was 10 μL. The ion spray voltage was set at 5500 V. Compounds parameters, i.e., declustering potential (DP), collision energy (CE), entrance potential (EP), and collision exit potential (CXP), were 55, 30, 10, and 8 V for PI, and 80, 35, 10, and 8 V for ISL, respectively. The mass spectrometer was operated in positive ESI mode, and the detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring the transition of *m*/*z* 271.1 precursor ion $[M+H]^+$ to the *m*/*z* 167.0 product ion for PI, and *m*/*z* 255.1 precursor ion $[M+H]^+$ to the *m*/*z* 119.3 product ion for ISL.

2.4. Standard and quality control sample preparation

Standard stock solutions of 1 mg/mL of PI and internal standard were both prepared in methanol. Standards were prepared by spiking blank rat plasma at seven concentrations ranging from 10 to 1000 ng/mL. For validation, QC samples were prepared by spiking rat plasma at three concentration levels (10, 100 and 500 ng/mL). The standards and QC were stored at −20 °C until analysis. The internal standard solution was prepared by dissolving ISL in methanol to produce the final concentration of 1 mg/mL and stored at 4 °C.

2.5. Sample preparation

The ISL solution (20 μL, 400 ng/mL in methanol) was added to 100 μL of rat plasma sample. A 200 μL formic acid (10%) was added to the sample to allow the analyte to release sufficiently. The mixture was then precipitated and extracted with 500 μL ethyl acetate. After vortexing for 2 min, the samples were centrifuged at 13,200 × *g* for 10 min. The supernatant fluid was transferred to a glass insert and evaporated to dryness under vacuum by a speed-Vac concentrator. The residue was reconstituted in 100 μL of the mobile phase and 10 μL were injected to the column.

2.6. Method validation

The method validation assays were carried out according to the currently accepted U.S. Food and Drug Administration (FDA) bioanalytical method validation guidance [17].

2.6.1. Specificity

Specificities were investigated by comparing chromatograms of plasma samples after an oral dose with those of blank plasma samples and samples spiked with PI and ISL. Each blank sample was tested for interference using the proposed extraction procedure and chromatographic-mass conditions. The apparent responses at the retention time of PI and ISL were compared with the response at a concentration near the lower limit of quantification (LLOQ).

2.6.2. Calibration curve

Calibration curves of seven concentrations of PI (10, 20, 50, 100, 200, 500 and 1000 ng/mL) were extracted and assayed. Peak-area ratios of the PI to ISL were calculated and the calibration curve was established by fitting these ratios to the corresponding concentrations using linear regression.

2.6.3. Accuracy and precision

Precision and accuracy of the method were evaluated at concentrations of 10, 100 and 500 ng/mL plasma. For the evaluation of intra-day precision and accuracy, five aliquots of each sample were analyzed in the same day. For inter-day precision and accuracy, six aliquots of each sample were analyzed on six consecutive days. The criteria for acceptability of the data included accuracy within ±15% standard deviation (S.D.) from the nominal values and a precision within ±15% relative standard deviation (R.S.D.), except for LLOQ, of which neither accuracy nor precision should exceed ±20%.

2.6.4. Recovery

The absolute recovery of the extraction was determined by comparing the peak area obtained from the plasma sample with those obtained by direct injection of pure PI standard solutions in the mobile phase at three different concentration levels. Quantification of chromatogram was performed by using peak area ratios of PI to ISL.

2.6.5. Stability

Stability was assessed at three concentration levels (10, 100 and 500 ng/mL). Freeze and thaw stability was assessed by using stability QC samples prepared at three concentrations stored at −20 °C and subjected to three freeze–thaw cycles. Short-term temperature stability was assessed at the same three concentrations, which were thawed at room temperature and kept at this temperature from 4 h to 24 h and then analyzed. The long-term stability was assessed at three concentration levels after storage at −20 °C for 2 weeks. Post-preparative stability was assessed during storage in the auto sampler at 4 °C and performed by repeated injection every 4 h for a period of 24 h.

2.7. Application to pharmacokinetic study

Pharmacokinetic parameters (observed maximum concentration, $C_{max.}$, and time taken to reach the maximum drug concentration, T_{max}) were calculated using the Bioavailability Program Package (BAPP, Version 2.0, Center of Drug Metabolism and Pharmacokinetics, China Pharmaceutical University). The area under the plasma PI concentration versus time curve (AUC) and the area under the first moment curve (AUMC) were calculated by the linear trapezoidal rule. Mean residence time (MRT) was calculated as AUMC/AUC. Terminal half-life ($t_{1/2}$) was calculated as 0.693 × MRT.

3. Results and discussion

3.1. Chromatographic separation and LC–MS/MS conditions

In order to optimize ESI conditions for PI and ISL, quadrupole full scans were carried out in positive ion detection mode. The most abundant ions were *m/z* 271.0 precursor ion $[M+H]^+$ to the *m/z* 137.0 fragment ion for PI, and *m/z* 257.3 precursor ion $[M+H]^+$ to the *m/z* 167.0 fragment ion for ISL in full scan mass spectra, respectively. The positive mass spectra of PI and ISL are shown in Fig. 1.

Extracted ion chromatograms are shown in Fig. 2. The retention times of PI and ISL were about 5.33 and 3.46 min, respectively. The two compounds were subjected to separation by reverse-phase HPLC on an HiQ Sil C_{18} column using water and methanol (9:91, v/v) containing 0.1% formic acid as mobile phase at 20 °C. The addition of 0.1% formic acid to the mobile phase increased the sensitivity of the analytes.

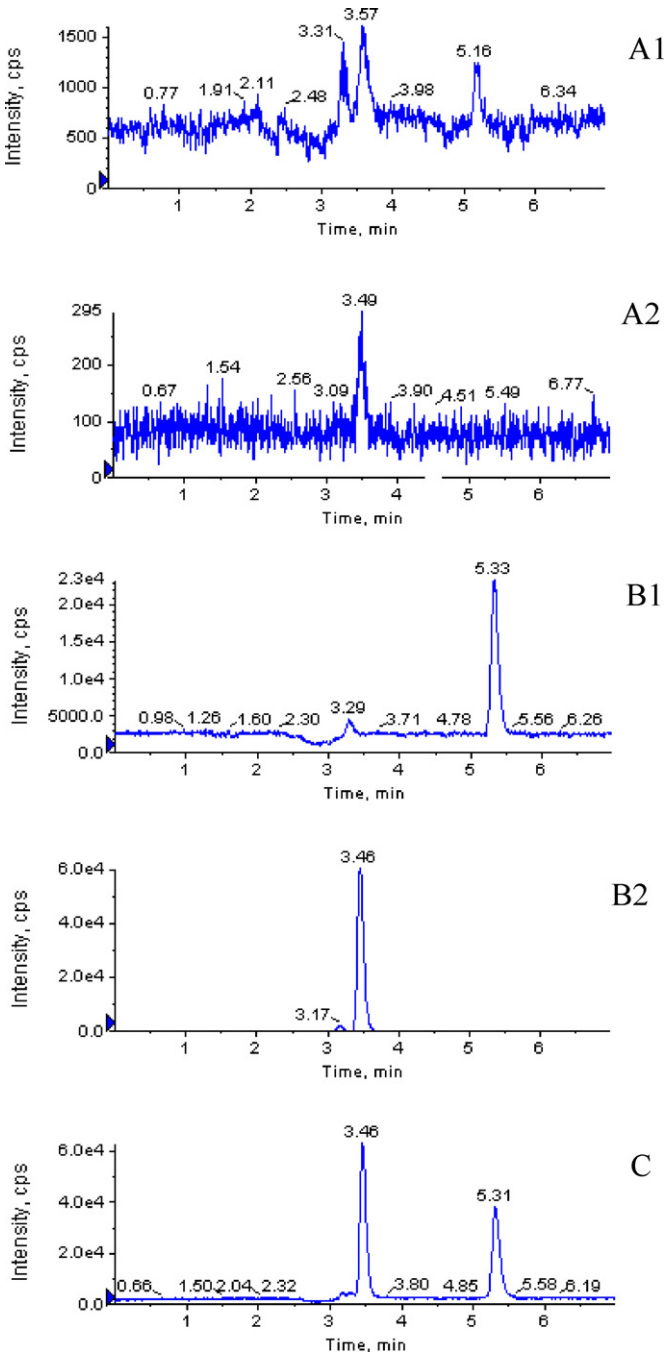

Fig. 2. Representative chromatograms of plasma samples with LC–MS/MS (A) blank rat plasma for PI (A1) and ISL (A2), (B) plasma spiked with PI (10 ng/mL, LLOQ, B1) and ISL (400 ng/mL, B2), and (C) plasma obtained 4 h after intragastric administration of PI (0.5 mg/kg) to rats.

3.2. Method validation

3.2.1. Chromatography and specificity

Selectivity was assessed by comparing the chromatograms of six different batches of blank rat plasma and PI in rat plasma after single oral administration. PI and ISL eluted at approximately 5.33 and 3.46 min, respectively. Endogenous peaks at the retention time of the analytes were not observed for any of the blank rat plasma batches, indicating no significant endogenous interference during the detections.

Table 1
Accuracy, precision and stability for the analysis of PI ($n = 5$).

Concentration (ng/mL)	Intra-day ($n = 5$)		Inter-day ($n = 5$)		Freeze and thaw stability	Short-term stability	Long-term stability	Post-preparative stability
	Accuracy (mean %)	Precision (RSD, %)	Accuracy (mean %)	Precision (RSD, %)	Accuracy (mean ± SD, %)			
10	94.6	4.7	104.3	3.2	103.2 ± 5.7	96.3 ± 3.0	93.6 ± 2.8	95.7 ± 2.7
100	95.1	3.8	96.1	4.6	97.7 ± 0.7	104.2 ± 4.3	98.6 ± 2.5	104.4 ± 2.7
500	93.2	5.3	95.5	5.2	94.3 ± 4.7	93.8 ± 6.4	91.6 ± 5.6	103.4 ± 2.9

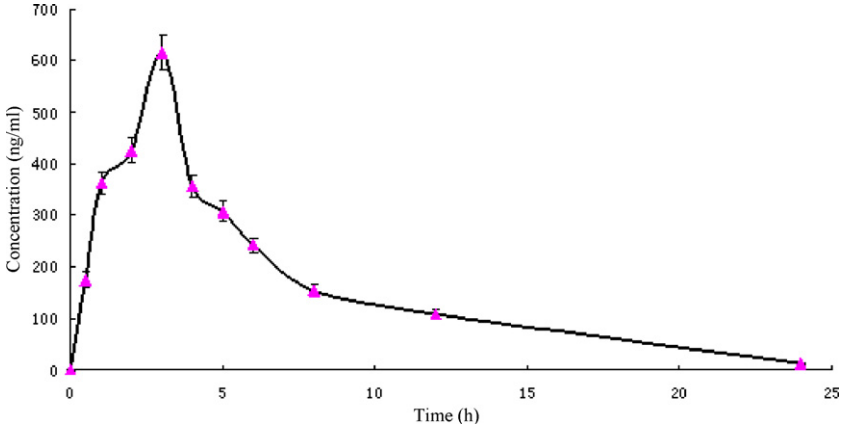

Fig. 3. Mean plasma concentration-time curve of PI in rats ($n = 5$) obtained after intragastric administration of PI (0.5 mg/kg).

3.2.2. Linearity

The peak area of calibration curve was proportional to the concentration of analyte in each assay over the nominal concentration range of 10–1000 ng/mL. The mean regression equation from 5 replicate calibration curves on different days was: $R_p = 0.00254C + 0.00171$ ($R = 0.9997$), where R_p corresponds to the peak area ratio of PI to the ISL, and C (ng/mL) refers to the concentration of PI added to plasma. The lowest limit of qualification (LLOQ) for PI was 10 ng/mL. The method was found to be sufficiently sensitive for the determination of pharmacokinetic analysis of PI in rats.

3.2.3. Accuracy and precision

Intra- and inter-day precision and accuracy are summarized in Table 1. The accuracy and precision of the developed method were evaluated by analyzing 5 replicates at three concentrations of QC samples. Intra-day variation was assessed by injecting one batch containing these samples on the same day. Inter-day variation was assessed by injecting one batch on six different days. The intra-day and inter-day precision of PI ranged from 3.8% to 5.3% and from 3.2% to 5.2%, respectively. Likewise, the intra-day and inter-day accuracy of PI were found to range from 93.2% to 95.1% and from 95.5% to 104.3%, respectively. The results demonstrated that the assay method was reliable and reproducible.

3.2.4. Recovery

The recovery of PI from rat plasma is shown in Table 2. The mean recoveries of PI were higher than 90% for all three concentration levels.

3.2.5. Stability

QC samples of PI at three concentrations (10, 100, 500 ng/mL) were used for stability experiments. Results of short-term stability, freeze/thaw stability, autosampler stability, and long-term stability are shown in Table 1. The results showed a very good stability under these conditions and were found to be within the assay variability limits during the entire process. The remaining percentages of PI for short-term temperature stability, long-term stability and post-preparative stability test were determined as 94.3% to 103.2%, 93.8% to 104.2%, 91.6% to 98.6% and 95.7% to 104.4%, respectively, after freeze and thaw stability.

Table 2
Recovery of PI and ISL from plasma ($n = 5$).

Compound	Spiked concentration (ng/mL)	Recovery (mean ± SD%)	RSD (%)
PI	10	104.6 ± 3.6	4.3
	100	96.4 ± 3.0	3.6
	500	95.3 ± 1.9	1.9
ISL	400	98.6 ± 2.2	2.6

3.3. Application to pharmacokinetic studies

The method described above was successfully applied to a pharmacokinetic study in the plasma of rats after an oral dose of 0.5 mg/kg PI. The plasma concentration–time profile of CSA in rats is shown in Fig. 3. The pharmacokinetic parameters of PI are listed in Table 3. As for intragastric administration, the terminal half-life ($t_{1/2}$) was 6.26 ± 0.31 h. The area under the plasma concentration curve ($AUC_{0\to\infty}$) of PI after intragastric administration was 3817.80 ± 352.89 ng × min/mL.

Table 3
Relevant pharmacokinetic parameters of PI in rats ($n = 5$) obtained after intragastric administration of PI (0.5 mg/kg).

Parameter	Unit	Administration
$AUC_{0\to t}$	ng min/mL	3817.80 ± 352.89
MRT	h	6.26 ± 0.31
C_{max}	ng/mL	615.35 ± 32.89
T_{max}	h	4 ± 0.18
$t_{1/2}$	min	4.34 ± 0.24

4. Conclusion

An accurate, precise, and highly sensitive LC–MS/MS method for the determination of PI in rat plasma has been developed and validated. The method is highly sensitive with a lower quantification limit of 10 ng/mL, shows wide linearity and high specificity, and is free from interferences of endogenous substances. It has been successfully applied to a pharmacokinetic study following oral administration of PI (0.5 mg/kg) in rats. The method is also time saving and economical, and therefore suitable for pharmacokinetic applications.

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