Simultaneous Quantification of Three Pyranocoumarins of Peucedanum Praeruptorum in Rat Plasma by Liquid Chromatography–Tandem Mass Spectrometry: Application to Pharmacokinetic Study

Guangyu Zhou, Guozhen Chen and Hongbo Liu*

Department of Pediatrics, Yantai Yuhuangding Hospital, Yantai 264000, PR China

*Author to whom correspondence should be addressed. Email: hblin28@outlook.com

Received 12 February 2014; revised 27 May 2014

A simple, rapid and robust liquid chromatography–tandem mass spectrometry was established and validated for simultaneous quantifications of three pyranocoumarins (praeruptorin A–C) in rat plasma. Following a single-step liquid–liquid extraction, the analytes were separated on a reversed-phase C18 column with a mobile phase consisting of methanol and 10 mM ammonium acetate solution (70:30, v/v) at a constant flow rate of 0.3 mL/min. The linear calibration curves were obtained over the concentration ranges 2.93–1470 ng/mL for praeruptorin A, 1.47–734 ng/mL for praeruptorin B and 2.00–1000 ng/mL for praeruptorin C. The within-batch accuracy was −8.6 to 7.5% for praeruptorin A, −9.5 to 12.0% for praeruptorin B and −10.5 to 12.5% for praeruptorin C, respectively. The between-batch accuracy was −3.5 to 1.4% for praeruptorin A, −8.7 to 3.4% for praeruptorin B and −6.0 to 4.3% for praeruptorin C, respectively. The within-batch and between-batch precisions were ≤3.1 and ≤8.2%, respectively. This method is suitable to simultaneously determine the three pyranocoumarins in plasma and thus to investigate the pharmacokinetics of the pyranocoumarins of Peucedanum praeruptorum in rats.

Introduction

The dry roots of Peucedanum praeruptorum Dunn have long been used in traditional Chinese medicine for the treatment of certain respiratory diseases, such as asthma, chronic bronchitis and pulmonary hypertension (1). The major constituents of this herbal medicine are angular-type pyranocoumarins, specifically praeruptorins, including praeruptorin A (pA), praeruptorin B (pB), praeruptorin C (pC) and others that have shown certain effects on blood pressure, heart failure, ischemia of heart and brain and cancer (2, 3). pA (Figure 1) has inhibited the expression of apoptosis-related proteins and has reduced the level of proinflammatory factors in ischemia-reperfusion myocardioocytes (4). pA is a potentially novel drug for the treatment and prevention of cardiovascular diseases (5, 6). Recently, pB, as an analog of pA, has also drawn wide attention because of other specific pharmacological effects of pB such as inhibition of tumor promoter-induced phenomenon in vitro (7). pC has also inhibited the expression of apoptosis-related proteins and has reduced the level of proinflammatory factors in ischemia-reperfusion myocardioocytes (8).

To our knowledge, various analytical methods have been used in previous studies for determining the levels of pA, pB and pC in plant materials; these methods include high-performance liquid chromatography with ultraviolet (HPLC–UV) detection (9–11), gas chromatography (12, 13), 1H-nuclear magnetic resonance (14) and high-speed counter-current chromatography coupled with electrospray ionization (ESI) multistage mass spectrometry (3). Several studies have also reported on the pharmacokinetics of pA by liquid chromatography–tandem mass spectrometry (LC–MS/MS) (4, 15, 16). Zhu et al. (15) reported an LC–MS/MS method for pharmacokinetics, tissue distribution and excretion study of pA of P. praeruptorum in rats. However, their method was used only for the determination of pA and was not selective enough to determine other pyranocoumarins in biological samples after intragastric administration. Recently, an online solid phase extraction–chiral LC–MS/MS method has been developed for the simultaneous enantiospecific determination of (+)pA, (±)pB, (+)pE and other pyranocoumarins in rat plasma (17). However, this LC–MS/MS method was limited by a long chromatographic run time (25 min), time-consuming gradient elution program, complicated sample preparation process and sophisticated chromatographic column requirement. In this study, a simple, rapid and robust LC–MS/MS method was developed and validated to simultaneously determine the three pyranocoumarins (pA, pB and pC) in rat plasma. This method was suitable for the pharmacokinetics of pyranocoumarins after oral administration of P. praeruptorum extract.

Experimental

Materials and reagents

Reference standards of pA, pB and pC (>98% purity) and ginkgo-biloba A (internal standard, >98% purity) were purchased from Purechem-standard Co., Ltd. (Chengdu, China); the chemical structures are shown in Figure 1. HPLC-grade methanol and ammonium acetate were obtained from Tedia Co. (Fairfield, OH, USA). HPLC-grade water from Milli-Q system (Millipore, Bedford, MA, USA) was used. Blank plasma was provided by the Animal Center of Shandong Luye Pharmaceutical Co. (Yantai, China).

Preparation of Peucedanum praeruptorum extract

Peucedanum praeruptorum (80 g) was ground into suitable powder and extracted with boiling water decoction (1:20, w/v) for 1 h. The extraction solutions were combined for filtration, concentrated to 500 mL and then added with 200 mL of dehydrated ethanol for precipitation (18). The precipitation was filtered, and ethanol was removed under reduced pressure. The aqueous solutions were then concentrated to 100 mL to obtain the P. praeruptorum extract at 0.8 g/mL concentration. The contents of the three pyranocoumarins in the extract solution were quantitatively determined according to previously reported
methods (12, 13). The contents of pA, pB and pC in the solution were 1.05, 0.85 and 1.03 mg/mL, respectively.

**Chromatographic conditions**
A volume of 5 μL was injected into an Agilent 1200 HPLC (Agilent Technologies, Palo Alto, CA, USA) equipped with a Shim-pack Diol safeguard column (4.0 × 50 mm, 5 μm; Shimadzu, Kyoto, Japan). Separation was performed on a Venusil MP-C18 column (50 × 2.1 mm, 3 μm; Agela, DE, USA) with a mobile phase consisting of methanol and 10 mM ammonium acetate solution (70 : 30, v/v) at 30°C and a constant flow rate of 0.3 mL/min.

**Mass spectrometric conditions**
The mass spectrometer, an Agilent 6460 QQQ (Agilent Technologies) equipped with a jet stream ESI ion source, was operated in the positive ion mode. The flow of dry and sheath gas was 5 and 12 L/min, respectively. The temperature of dry and sheath gas was 325 and 350°C, respectively. The nebulizer gas pressure was 45 psi and the capillary voltage was 4.0 kV. The quantification was achieved by using the mass spectrometer in multiple reaction monitoring (MRM) mode. A single precursor ion-product ion transition was monitored for each analyte and the IS, internal standard. The transitions were m/z 83.1 → m/z 444.2 for pA, m/z 293.2 → m/z 83.1 for pB, m/z 446.2 → m/z 83.1 for pC, and m/z 426.3 → m/z 409.2 for IS, respectively (Figure 2). The collision energies were 10, 12, 18 and 20 eV for pA, pB, pC and IS, respectively.

**Preparation of analytical standards**
Mixed stock solutions that contained pA (2.2 mg/mL), pB (1.1 mg/mL) and pC (1.5 mg/mL) were prepared in methanol, kept at -20°C, and used within 1 week after preparation. The mixed stock solutions were successively diluted with methanol–water (50 : 50, v/v) to prepare working solutions in the concentration ranges of 0.293–147 μg/mL for pA, 0.147–73.4 μg/mL for pB and 0.200–100 μg/mL for pC. Up to 10 μL of standard working solution was added to 900 μL of blank plasma to prepare calibration standards. Plasma calibration concentrations of pA were 2.93, 7.33, 29.3, 73.3, 293.2, 733 and 1470 ng/mL; those of pB were 1.47, 3.67, 14.7, 36.7, 146.8, 367 and 734 ng/mL and those of pC were 2.00, 5.00, 20.0, 50.0, 200, 500 and 1,000 ng/mL. Quality control (QC) samples for pA, pB and pC were prepared at concentrations of 1323, 660.6 and 900 ng/mL (higher quality control, HQC); 88.0, 44.0 and 60.0 ng/mL (medium quality control, MQC) and 7.33, 3.67 and 5.00 ng/mL (lower quality control, LQC).

An internal standard working solution was prepared by diluting the internal standard stock solution (300 μg/mL with methanol to reach a final concentration of 800 ng/mL for ginkgolide A and was stored at -20°C.

**Sample preparation**
Plasma samples (50 μL) were mixed with 50 μL of IS solution (ginkgolide A, 800 ng/mL). The mixture was extracted with 3 mL of chloroform by vortexing for 5 min. After centrifugation at 5000 × g for 5 min, the supernatant was transferred into a clean glass tube and evaporated to dryness under a gentle stream of nitrogen at 40°C. The residue was reconstituted with 100 μL of mobile phase, and 5 μL of aliquot was injected into the LC–MS/MS system.

**Method validation**
A full method validation to ensure selectivity, sensitivity, linearity, precision, accuracy, recovery, matrix effect and stability was performed according to the US Food and Drug Administration guidelines (19). To evaluate selectivity, endogenous interference was determined by comparing the MRM chromatograms for blank plasma, blank plasma spiked with the three analytes and IS. For sensitivity, the lower limit of quantification (LLOQ) was determined at the concentration at which the accuracy was within ±20% of the bias, and the precision was <20% of the coefficient of variation (CV). Accuracy and precision were also evaluated by determining QC samples at low, medium and high concentration levels in three different validation days. The validation of % bias and % CV for QC samples were accepted within 15%. Recovery of pA, pB, pC and IS were determined by comparing the peak area of extracted analyte standards with that of nonextracted standards. Recovery of pA, pB and pC was determined at the corresponding concentrations of 7.33, 3.67 and 5.00 ng/mL (LQC), 88.0, 44.0 and 60.0 ng/mL (MQC) and 1323, 660.6 and 900 ng/mL (HQC), whereas that of IS was determined at the concentration of 800 ng/mL. The absolute matrix effect was determined by comparing the peak areas of analytes that were mixed postextraction with those of the neat standards at corresponding QC levels. Stability was evaluated by analyzing QC samples at three concentration levels (n = 6). Four types of stability were investigated, namely, freeze–thaw, short-term, long-term and postpreparative stability.
Applications in pharmacokinetics studies

The experimental protocol (no. 20130415) is approved by the Animal Ethics Committee of Yantai Yuhuangding Hospital. Six male Wistar rats (200–220 g) were kept in air-conditioned animal quarters at 22 ± 2°C and a relative humidity of 50 ± 10% with free access to food and water until 12 h prior to the experiment.

Figure 2. Product ion mass spectra of pA (A), pB (B), pC (C) and IS (D).

Simultaneous Quantification of Three Pyranocoumarins of *Peucedanum Praeruptorum* in Rat Plasma
The *P. praeruptorum* extract solution (0.8 g/mL) for intragastric administration was administered to the animals at a dose of 10 mL/kg. Briefly, the rats were orally given with the *P. praeruptorum* extract at a dose of 8 g/kg body weight, which is equal to each dose of pA, pB and pC at 10.5, 8.5 and 10.3 mg/kg body weight, respectively. Up to 200 µL of blood

**Figure 3.** Representative chromatograms for pA (MRM transition m/z 404.1 to m/z 83.1), pB (MRM transition m/z 444.2 to m/z 83.1), pC (MRM transition m/z 446.2 to m/z 83.1) and IS (MRM transition m/z 426.3 to m/z 409.2) from a drug-free blank plasma sample (A), a calibration standard plasma sample containing 73.3 ng/mL pA, 36.7 ng/mL pB and 50.0 ng/mL pC (B) and a plasma sample from a rat 3 h after oral administration of *P. praeruptorum* extract (C).
samples were collected in heparin-containing tubes via the orbital vein by capillary tube pre-dose (0 h) and at 0.083, 0.167, 0.333, 0.667, 1, 2, 3, 5, 8, 12 and 24 h post-dose, which were centrifuged at 6000 \( \times \) g for 10 min at 4°C. Plasma samples were collected and frozen at −20°C until LC–MS/MS analysis.

Results and Discussion

**Optimization of LC–MS/MS conditions**

The MS spectra of the three analytes and IS were recorded in positive ion mode. The negative ion mode was also tested, but the intensity obtained was very low for the three analytes and IS. To optimize the MS parameters, a neat standard solution containing individual analyte or IS was directly infused into the mass spectrometer. \([M + NH_4]^+\) was the predominant ion in the Q1 spectrum for each analyte and IS, which was used as the precursor ion to obtain the corresponding product ion spectra. The three analytes produced the most abundant characteristic product ions at \(m/z\) 83.1 each. The most abundant product ion for IS was \(m/z\) 409, which is similar to the previous report (20). The best MRM transitions were selected at \(m/z\) 404.1 → \(m/z\) 83.1, \(m/z\) 444.2 → \(m/z\) 83.1, \(m/z\) 446.2 → \(m/z\) 83.1 and \(m/z\) 426.3 → \(m/z\) 409.2 for pA, pB, pC and IS, respectively (Figure 2).

Chromatographic analysis of the analytes and IS was initiated under isocratic conditions to develop a simple separation process with a short run time. Feasibility of various mixture(s) of solvents, such as acetonitrile and methanol, using different buffers, such as formic acid, acetic acid and ammonium acetate, were evaluated to obtain the optimal mobile phase that produced the best sensitivity and separation efficiency. Electrolyte modification (ammonium acetate) of the mobile phase can significantly improve ESI efficiency and enhance coumarin responses (21). Thus, different concentrations of ammonium acetate (1.0, 2.0, 5.0 and 10 mM) were added into the aqueous solution to obtain the optimal mobile phase. A mixture that consisted of methanol and 10 mM ammonium acetate solution (70:30, v/v) could achieve this purpose and was finally used as the optimal mobile phase.

**Selection of IS**

The use of an optimal IS is necessary to obtain high accuracy when a mass spectrometer is used as a detector. Ginkgolide A (Figure 1) was chosen as the IS considering similarity of ionization mode with the analyte and reduced endogenous interferences at the product ion of \([M + NH_4]^+\) \(m/z\) 426.3.

**Method validation**

**Selectivity**

MRM scan mode can provide high selectivity in biological analysis. Figure 3 shows representative chromatograms obtained from blank plasma, blank plasma mixed with the analytes and IS and a rat plasma sample after oral administration of *P. praeruptorum* extract. No endogenous interference was observed during retention of the analytes and IS.

**Calibration curve, linearity and LLOQ**

The assay was linear over the concentration ranges of 2.93–1470 ng/mL for pA, 1.47–734 ng/mL for pB and 2.00–1000 ng/mL for pC. All calibration curves were weighted according to the
1/\(x^2\) weighting scheme. Typical equations of the calibration curves were as follows:

\[
\begin{align*}
\text{pA} : y &= 9.1 \times 10^{-4}x - 8.3 \times 10^{-4}, \quad r^2 = 0.9923 \\
\text{pB} : y &= 1.3 \times 10^{-3}x - 1.1 \times 10^{-3}, \quad r^2 = 0.9939 \\
\text{pC} : y &= 2.0 \times 10^{-3}x - 3.4 \times 10^{-3}, \quad r^2 = 0.9920
\end{align*}
\]

where \(y\) represents the ratio of the analyte peak area to that of the IS and \(x\) represents the plasma concentration of the analyte. Good linearity was shown in the stated concentration ranges. For pA, pB and pC, the present method offered an LLOQ of 2.93, 1.47 and 2.00 ng/mL, respectively. The matrix effect of all analytes ranged from 94.0 to 101.2%, and CV values were all below 6.9% (Table II).

Applications in pharmacokinetics studies

This validated LC–MS/MS method was successfully applied to simultaneously determine the plasma concentrations of pA, pB and pC in rats after a single intragastric administration of \(P. praeruptorum\) extract at a dose of 8 g/kg body weight. The mean plasma concentration–time curves are illustrated in Figure 4, and the main pharmacokinetic parameters are presented in Table IV. pA, pB and pC were detectable in rat plasma up to 24 h. The area under the plasma concentration–time curve from time zero to the last quantifiable time-point (AUC\(_{0\rightarrow t}\)) values for pA, pB and pC were 311.80 ± 42.38, 187.29 ± 15.02 and 91.64 ± 9.37 ng h/mL, respectively. The maximum plasma concentration (C\(_{\text{max}}\)) values for pA, pB and pC were 31.09 ± 4.84, 19.66 ± 4.25 and 7.59 ± 1.98 ng/mL, respectively. The obtained elimination half-life (t\(_{1/2}\)) values indicated that the three compounds were slowly eliminated with a long t\(_{1/2}\) of pA, 7.52 ± 1.00 h; t\(_{1/2}\) of pB, 8.20 ± 1.21 h and t\(_{1/2}\) of pC, 14.97 ± 3.66 h.
Praeruptorin A
Praeruptorin B
Praeruptorin C

have similar absorption and elimination behavior.

The results showed that the three pyranocoumarins were successfully applied in the determination of the three major pyranocoumarins of *P. praeruptorum* in rat plasma after intragastric administration. The method was simple and the analysis time was just 6.0 min. This method was developed and validated for simultaneous determination of pA, pB, and pC in rat plasma samples. The process of sample preparation was simple and the analysis time was just 6.0 min. This method was successfully applied in the determination of the three major pyranocoumarins of *P. praeruptorum* in rat plasma after intragastric administration. The results showed that the three pyranocoumarins have similar absorption and elimination behavior.

Table III

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<tr>
<th>Parameter</th>
<th>Mean found A (ng/mL)</th>
<th>Accuracy (% bias)</th>
<th>Precision (CV)</th>
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<td>F_max (ng/L)</td>
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Table IV

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<tr>
<td>C_max (ng/mL)</td>
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<td>19.66 ± 4.25</td>
<td>7.59 ± 1.98</td>
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<tr>
<td>T_max (h)</td>
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<td>8.20 ± 1.21</td>
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<td>MRT (h)</td>
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Conclusion

A simple, rapid and robust LC–MS/MS method had been developed and validated for simultaneous determination of pA, pB, and pC in rat plasma samples. The process of sample preparation was simple and the analysis time was just 6.0 min. This method was successfully applied in the determination of the three major pyranocoumarins of *P. praeruptorum* in rat plasma after intragastric administration. The results showed that the three pyranocoumarins have similar absorption and elimination behavior.

References


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