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Determination of the Plasma Concentrations of Amygdalin and Paeoniflorin in Rat Serum Using Liquid Chromatography-tandem Mass Spectrometry

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Abstract: *Objective*: To establish a liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method to determine amygdalin and paeoniflorin in rat blood serum. *Method*: After protein precipitation by acetonitrile, a gradient elution procedure was performed to separate the samples by using acetonitrile and ammonium acetate buffer as the mobile phase. The quantitative determination was based on an HPLC-MS/MS in negative electron spray ionization mode and in multiple reaction monitoring (MRM) mode. The precursor to product ion transitions for amygdalin and paeoniflorin were 456.2 to 323.1 and 479.2 to 121.0, respectively. *Results*: The linear ranges of amygdalin and paeoniflorin were 1–300 ng/mL (rAmy = 0.9994，rPae = 0.9993) with the lowest limits of quantification were 0.3 ng/ml. The extract recovery of amygdalin and paeoniflorin were 91.1%, 94.2%, 92.0% and 90.3%, 93.7%, 94.1% at concentrations of 3, 20, 100 ng/mL, respectively. All the intra and inter day precisions of analysis were less than 15%, and the RE were in the range of ± 15%. *Conclusion*: The method is specific, sensitive, accurate, and suitable for determination of amygdalin and paeoniflorin in rat blood serum.

Keywords: Fei Xin Decoction; Chinese medicinal preparation; Amygdalin; Paeoniflorin; Rat blood serum; HPLC-MS/MS

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1. Introduction

The material basis for the efficacy of traditional Chinese medicine (TCM) lies in the chemical constituents within the herbs or compound formulas, which are crucial for elucidating the mechanisms underlying their therapeutic effects. TCM and its compound formulas contain numerous components, but only those absorbed into the bloodstream can exert pharmacological effects *in vivo*. The TCM compound formula “Feixin Decoction”, primarily composed of over ten herbs including almonds, red peony root, astragalus, and safflower, is used for the prevention and treatment of pulmonary hypertension, with almonds and red peony root playing the most critical roles. Its main functions are promoting blood circulation and removing blood stasis. The primary active ingredients in

almonds and red peony root are amygdalin and paeoniflorin, respectively. Previous studies have reported the use of high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) to detect paeoniflorin concentrations in serum or plasma samples [1–5]. However, no method has been reported for measuring amygdalin concentrations *in vivo*. Therefore, this study established a specific, sensitive, accurate, and rapid LC-MS/MS method to detect amygdalin and paeoniflorin in rat serum after intragastric administration of "Feixin Decoction". This lays the foundation for elucidating the in vivo metabolism and pharmacological mechanisms of the TCM compound formula "Feixin Decoction" and provides support for subsequent serum pharmacology experiments in this study.

2. Materials and methods

2.1. Instruments

A 4000 QTRAP LC-MS/MS system, including an electrospray ionization (ESI) source and data processing system (AB, USA); an Agilent 1200 high-performance liquid chromatograph equipped with an online degasser, quaternary pump, DAD UV detector, autosampler, and column oven (Agilent, USA); an M37610-33 vortex mixer (Thermo Scientific, USA); a Sigma 2-16K tabletop high-speed refrigerated centrifuge (Sigma, Germany); and a Mettler XP26 electronic balance (Mettler, Switzerland).

2.2. Drugs and reagents

Amygdalin reference standard (batch number: MUST-13011801, HPLC purity: ≥ 98%) and paeoniflorin reference standard (batch number: MUST-12113009, HPLC purity: ≥ 98%) were purchased from Chengdu Mansite Biotechnology Co., Ltd. Methanol and acetonitrile were of chromatographic grade (Merck, Germany). Ultrapure water was prepared using a Millipore ultrapure water system. All other reagents were of analytical grade.

2.3. Animals

Healthy adult male SPF-grade SD rats weighing approximately 300 g were provided by Changsha Tianqin Biotechnology Co., Ltd. (license number: SCXK (Xiang) 2009-0012).

2.4. Chromatographic and mass spectrometric conditions

(1) Pre-column

Phenomenex C18 guard column (4 mm × 2.0 mm, 5 μm); analytical column: XBridge BEH C18 column (2.1 × 50 mm, 2.5 μm); mobile phase: acetonitrile-20 mmol/L ammonium acetate + 0.1% ammonia water (gradient elution, specific program: 0–0.9 min 5% acetonitrile, 0.9–1.0 min 5–95% acetonitrile, 1.0–2.5 min 95% acetonitrile, 2.5–2.6 min 95–5% acetonitrile, 2.6–4.0 min 5% acetonitrile); flow rate: 0.4 mL/min; column temperature: 40 ℃; injection volume: 20 μL.

(2) Ion source

Electrospray ionization (ESI) source; ionization mode: negative mode; electrospray voltage: -4500 V; temperature: 550 ℃; in-source gas (GS1, N2) pressure: 40 psi; heating gas (GS2, N2) pressure: 60 psi; curtain gas (CUR, N2) pressure: 20 psi; collision gas (CAD, N2) pressure: medium; scanning mode: multiple reaction monitoring (MRM); ion reactions used for quantitative analysis were m/z 456.2 to 323.1 (amygdalin) and 479.2 to 121.0 (paeoniflorin); declustering potential (DP) values were -105 and -103 V, respectively; collision energy (CE) values were -18 and -32 V, respectively; detector voltage: 2200 V.

2.5. Preparation of reference standard stock solutions

Ten milligrams each of amygdalin and paeoniflorin reference standards were accurately weighed and placed in separate 10 mL volumetric flasks. Five milliliters of methanol were added to dissolve the standards, which were then diluted to 10 mL with methanol to obtain 1 mg/mL stock solutions of amygdalin and paeoniflorin. Subsequent reference solutions were prepared by diluting these stock solutions with 50% methanol-water to the desired concentrations.

2.6. Collection and processing of serum samples

(1) Animal grouping

Twelve healthy male SD rats were randomly divided into a TCM group (n = 6) and a saline group (n = 6).

(2) Dosage administration

The dosage for the TCM group was calculated using the formula proposed by Wang Liqian [6,7]: dosage = Commonly used clinical doses × animal equivalent area coefficient × serum dilution in culture medium. Using this formula, the equivalent dose for rats was theoretically 6.25 times the human dose per unit mass. Since the drug serum needed to be diluted to 20% (dilution factor of 5), the intragastric dosage per unit mass for rats was 31.25 times the human dose per unit mass, resulting in an approximate dosage of 25 mL/kg (containing 28 g/kg of "Feixin Decoction") per rat per administration. The saline group received the same feeding regimen as the TCM group but was administered an equivalent volume of saline.

(3) Preparation of TCM Decoction

Ten grams of peach kernels, 10 g of safflower, 20 g of red peony root, 30 g of *salvia miltiorrhiza*, 30 g of *lepidium* seeds, 10 g of white ginseng, 10 g of prepared aconite, 30 g of raw *astragalus*, 20 g of *plantago* seeds, 15 g of *poria*, and 12 g of *atractylodes* were added to 1500 mL of re-distilled water and boiled for 20 minutes. The filtrates from two boilings were combined and centrifuged at 14000 r/min for 15 minutes to obtain the supernatant.

(4) Collection of drug-containing serum

Rats were fasted for 12 hours before the first intragastric administration but had free access to water. A specific amount of the decoction was administered to the experimental group rats using a dedicated intragastric needle, while the saline group received an equivalent volume of saline. Administrations were performed twice daily at 8 AM and 8 PM. On the 10th day, serum samples were collected from the inferior vena cava under anesthesia 1 hour after administration. Serum samples from rats in the same group were pooled, inactivated in a 56 ℃ constant-temperature water bath for 30 minutes, filtered through a 22 μm membrane for sterilization, and stored in an ultra-low temperature freezer for subsequent in vitro culture of pulmonary artery smooth muscle cells.

(5) Processing of serum samples

A 0.4 mL aliquot of serum was transferred to a 2 mL centrifuge tube, followed by the addition of 0.8 mL of acetonitrile. The mixture was vortexed for 2 minutes and centrifuged at 12000 r/min for 10 minutes. The supernatant was directly injected into the LC-MS/MS system for analysis.

3. Results and discussion

3.1. Liquid chromatography and mass spectrometry investigation and optimization

The structures of amygdalin and paeoniflorin are shown in **Figure 1**. As polyhydroxy compounds, they exhibit

strong water solubility and lack acidic or basic functional groups, which is not conducive to electrospray ionization mass spectrometry response. In the experiments, positive ESI mode was initially selected for condition optimization. However, under various conditions, both amygdalin and paeoniflorin primarily responded as $[M + Na]^+$ ions, with weak $[M + H]^+$ ion responses. When selecting $[M + Na]^+$ ions for MS2 fragmentation experiments, stable secondary fragmentation ions could not be obtained, making MRM experiments difficult. Therefore, this method opted for further investigation and optimization under negative ESI mode, examining the response intensity of $[M–H]^-$ ions of amygdalin and paeoniflorin under acidic (0.1% formic acid), neutral (without any mobile phase modifier), and alkaline (20 mmol/L ammonium acetate + 0.1% ammonia water) mobile phase conditions. The experimental results indicated that stable $[M–H]^-$ ions could be generated for both amygdalin and paeoniflorin under all conditions. However, under alkaline conditions, the $[M–H]^-$ ion intensity of amygdalin was three times and two times that under neutral and acidic conditions, respectively, while that of paeoniflorin was 2.5 times and two times, respectively. Therefore, this method ultimately selected negative ESI mode and an alkaline mobile phase.

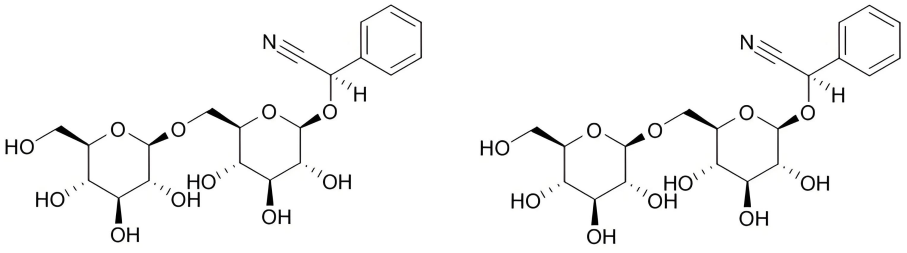

Figure 1. Chemical structural formulas of amygdalin and paeoniflorin.

To enhance method sensitivity, MRM monitoring mode was proposed for quantification. **Figure 2** shows the primary MS scan spectra and MS2 daughter ion scan spectra of $[M–H]^-$ ions for amygdalin and paeoniflorin under negative ESI mode. The $[M–H]^-$ ion of amygdalin was m/z 456.2, with the highest abundance of m/z 323.1 in its MS2 daughter ions; the $[M–H]^-$ ion of paeoniflorin was m/z 479.2, with the highest abundance of m/z 121.0 in its MS2 daughter ions. Therefore, the final selected MRM quantification channels were m/z 456.2 to 323.1 (amygdalin) and m/z 479.2 to 121.0 (paeoniflorin).

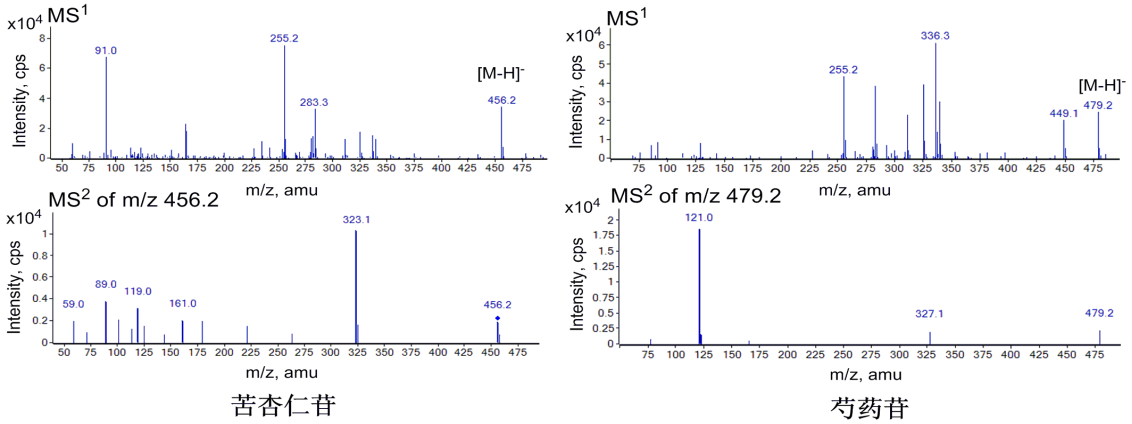

Figure 2. Primary MS scan spectra of amygdalin and paeoniflorin, along with their MS2 daughter ion scan spectra of $[M–H]^-$ ions.

Regarding the liquid chromatography elution method, isocratic elution was initially selected in the experiments. Although it could achieve separation of amygdalin and paeoniflorin, the peak shapes were poor, and there were concerns that some strongly retained components in the serum matrix might not be eluted under this condition. Therefore, this method ultimately adopted a large gradient elution method. Although amygdalin and paeoniflorin co-eluted under this method, subsequent specificity investigations proved that they did not interfere with each other's determination. This gradient method could obtain sharp peak shapes and ensure that components in the serum matrix could be eluted in a single injection without interfering with subsequent determinations.

3.2. Method specificity investigation

Blank serum, blank serum spiked with amygdalin and paeoniflorin (both at 10 ng/mL), blank serum spiked only with amygdalin (10 ng/mL), blank serum spiked only with paeoniflorin (10 ng/mL), and serum samples were processed and injected for determination according to Section 1.6. The results showed (see **Figure 3**) that the sample spiked with amygdalin and paeoniflorin in blank serum exhibited peaks at 2.01 min in the m/z 456.2 to 323.1 channel and at 2.02 min in the m/z 479.2 to 121.0 channel; the blank serum sample showed no significant peaks in either the m/z 456.2 to 323.1 or m/z 479.2 to 121.0 channels; the sample spiked only with amygdalin in blank serum showed no significant peak in the m/z 479.2 to 121.0 channel; and the sample spiked only with paeoniflorin in blank serum showed no significant peak in the m/z 456.2 to 323.1 channel. The specificity experiments proved that there were no endogenous matrix interferences in the plasma affecting the determination of amygdalin and paeoniflorin, and that amygdalin and paeoniflorin did not interfere with each other.

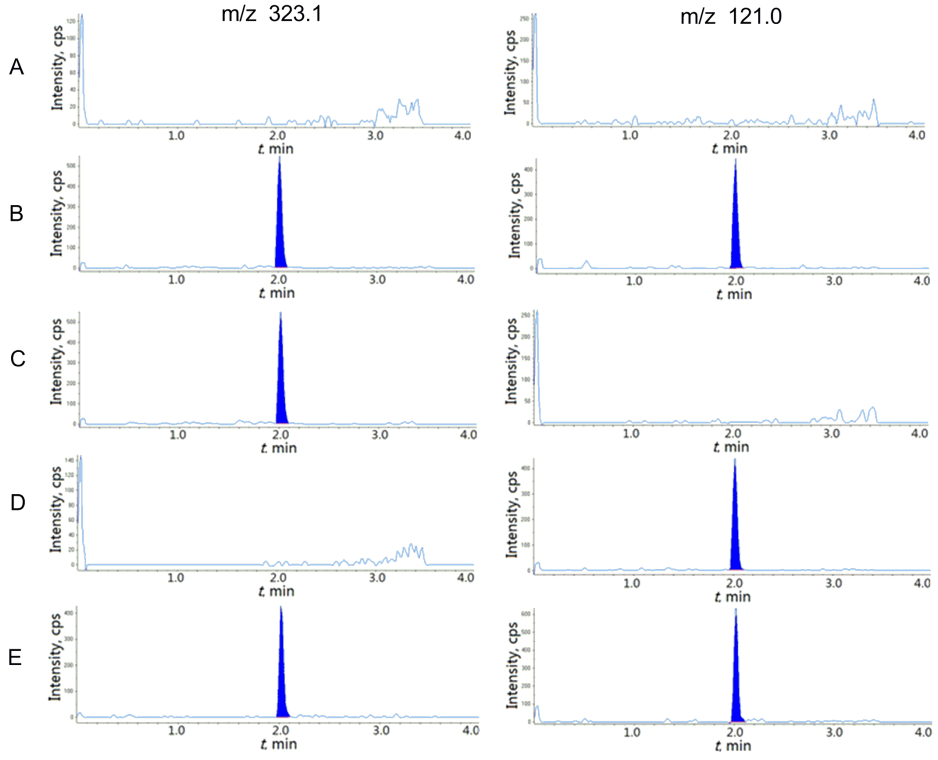

Figure 3. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) chromatograms: A: Blank serum; B: Blank serum + Amygdalin + Paeoniflorin; C: Blank serum + Amygdalin; D: Blank serum + Paeoniflorin; E: Serum sample.

3.3. Matrix effect investigation

Multiple portions of blank serum were processed according to Section 1.6. Processed blank serum extracts were used to prepare high, medium, and low (amygdalin and paeoniflorin at 100, 20, and 3 ng/mL, respectively) concentration spiked blank samples, with three parallel preparations for each concentration. Simultaneously, neat solvents (water/acetonitrile = 1/2) were used to prepare spiked solutions with the same high, medium, and low concentrations, with three parallel preparations for each concentration. Injection analysis was performed according to the method in Section 1.4. The matrix effect was defined as the ratio of the peak areas of the target analytes in the processed spiked blank samples to those in the neat solvent spiked samples. The results showed that under this method, the matrix effects for amygdalin at high, medium, and low concentrations were 94.2% (RSD = 5.2%, n = 3), 95.7% (RSD = 2.2%, n = 3), and 99.1% (RSD = 3.4%, n = 3), respectively, while those for paeoniflorin were 97.2% (RSD = 3.7%, n = 3), 95.5% (RSD = 4.1%, n = 3), and 94.3% (RSD = 2.6%, n = 3), respectively. The results indicated that this method had no significant matrix effects.

3.4. Extraction recovery

Blank serum was taken, and pre-extraction spiking and post-extraction spiking were performed with consistent absolute amounts added. High, medium, and low concentrations (relative to pre-extraction serum, amygdalin and paeoniflorin at 100, 20, and 3 ng/mL, respectively) were also examined. The extraction method was according to Section 1.6, with three parallel preparations for each condition. The extraction recovery was defined as the ratio of the peak areas in the pre-extraction spiked blank samples to those in the post-extraction spiked blank samples. The results showed that under this method, the extraction recoveries for amygdalin at high, medium, and low concentrations were 91.1% (RSD = 7.0%, n = 3), 94.2% (RSD = 4.1%, n = 3), and 92.0% (RSD = 10.2%, n = 3), respectively, while those for paeoniflorin were 90.3% (RSD = 5.8%, n = 3), 93.7% (RSD = 8.2%, n = 3), and 94.1% (RSD = 12.5%, n = 3), respectively. The results indicated that this method had good extraction recovery with minimal losses during sample processing.

3.5. Linear range and lower limit of quantification investigation

Blank serum was taken and spiked to prepare blank serum spiked samples with amygdalin and paeoniflorin concentrations of 1, 2, 5, 10, 20, 50, 100, and 300 ng/ml. Three portions were prepared for each concentration, processed according to the method in Section 1.6, and injected for analysis according to the method in Section 1.4. Linear regression was performed with the mass concentrations (c) of amygdalin and paeoniflorin as the abscissa and their peak areas (Y) as the ordinate. The regression equations for amygdalin and paeoniflorin were Y = 455.0558c + 114.7196, r = 0.9994, and Y = 370.8633c + 38.5879, r = 0.9993, respectively. The linear ranges for both analytes were 1–300 ng/mL, and the lower limits of quantification for amygdalin and paeoniflorin were both 0.3 ng/mL (signal-to-noise ratio (S/N) > 10, relative standard deviations (RSDs) were 9.1% and 11%, respectively).

3.6. Precision and accuracy

Blank serum spiked samples with amygdalin and paeoniflorin concentrations of 3, 20, and 100 ng/mL were prepared, with six parallel preparations for each concentration. The preparations and determinations were performed continuously for 3 days. The measured concentrations of each sample were calculated based on the daily working curves, and the experimental results are shown in **Table 1**. The results showed that the intra-day and inter-day pre-

cision RSDs were both < 15%, and the relative errors (REs) were within ± 15%, indicating that this method had good precision and accuracy, meeting the determination requirements for biological samples.

Table 1. Results of precision and accuracy tests

Drug	Added Concentration (ng/mL)	Measured Concentration (ng/mL)	Precision RSD/%		Accuracy/%	RE/%
			Intra-day (n = 6)	Inter-day (n = 18)		
Amygdalin	3	3.231	3.4	5.2	107.7	7.7
	20	21.951	2.9	5.4	109.8	9.8
	100	97.999	5.7	6.9	98.0	-2.0
Paeoniflorin	3	3.355	3.9	4.5	111.8	11.8
	20	19.357	2.7	3.8	96.8	-3.2
	100	102.619	4.1	6.3	98.1	-1.9

3.7. Stability investigation

Blank serum was taken and spiked to prepare blank serum spiked samples with amygdalin and paeoniflorin mass concentrations of 3, 20, and 100 ng/mL. Three parallel preparations were made for each concentration. The drug concentrations were examined after placing at room temperature for 24 h, undergoing three freeze-thaw cycles at -40 ℃, and long-term freezing at -40 ℃ for 20 days. The results showed that the REs between all measured concentrations and theoretical concentrations were within ± 15%, indicating that amygdalin and paeoniflorin were stable during room temperature placement, repeated freeze-thaw cycles, and long-term freezing of serum samples, and that these storage conditions did not affect the accurate determination of the samples.

3.8. Sample determination

Serum samples were processed according to the method in Section 1.6, and the blood drug concentrations of amygdalin and paeoniflorin in various serum samples obtained from three dosages (high, medium, and low) were determined by recording the peak areas under the chromatographic-mass spectrometric conditions in Section 1.4. The results are shown in **Table 2**. The determination results indicated that amygdalin and paeoniflorin could be detected in the serum of rats after intragastric administration, and the higher the dosage, the greater the detected amount in the serum, proving the presence of traditional Chinese medicine ingredient groups represented by amygdalin and paeoniflorin in the drug-containing serum of rats after oral administration of “Feixin Decoction”.

Table 2. Test results of test samples

Dosage level	Sample name	Test Concentration (ng/mL)	
		Amygdalin	**Paeoniflorin**
Low	L_1	2.41	2.89
	L_2	2.12	4.85
	L_3	2.43	2.33
	L_4	2.74	3.53
	L_5	0.86	1.77
	L_6	1.38	2.41
	Mean	**1.99**	**2.96**
Medium	M_1	5.07	5.91
	M_2	2.99	9.23
	M_3	6.59	11.10
	M_4	3.08	6.58
	M_5	4.54	18.40
	M_6	2.48	6.85
	Mean	**4.12**	**9.67**
High	H_1	2.65	8.75
	H_2	8.25	22.85
	H_3	7.525	28.75
	H_4	6.625	10.05
	H_5	9.75	30.00
	H_6	8.20	18.22
	Mean	**7.17**	**19.77**

4. Conclusion

In this study, a high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was established to determine the serum concentrations of amygdalin and paeoniflorin in serum samples. A specific, sensitive, accurate, and rapid LC-MS/MS method was developed for research in fields such as cytology and pharmacology related to traditional Chinese medicine compounds. This approach inherits and advances the research direction of traditional Chinese medicine, laying a foundation for elucidating the in vivo metabolism and pharmacodynamic mechanisms of the traditional Chinese medicine compound "Feixin Decoction", and providing support for subsequent serum pharmacological experiments in related research.

Disclosure statement

The author declares no conflict of interest.

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