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Original article

Development of a high sensitivity UHPLC-MS/MS method to determine the twelve compounds of Physochlainae Radix extract and application to a pharmacokinetic study in rats

Zhenguo Lv [a], Caixia Li [a], Tiantian Wu [a], Peng Zhao [a], Yang Liu [a], Huizi Ouyang [a], Jihong Feng [b,*], Jun He [a,*]

[a] State Key Laboratory of Component-based Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin 301617, China
[b] The Second Affiliated Hospital of Tianjin University of Traditional Chinese Medicine, 300250 Tianjin, China

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ABSTRACT

Physochlainae Radix (PR) is generally applied for treating cough and asthma. In this study, a sensitive ultra-high-performance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC-MS/MS) method was established and validated for the simultaneous determination of twelve components (scopolamine, anisodamine, hyoscyamine, protocatechuic acid, fabiatrin, scopolin, 4-hydroxybenzoic acid, caffeic acid, tropic acid, scopoletin, isoquercitrin, and scoparone) of PR extract in rat plasma. ACQUITY UPLC CSH C18 column was employed for the chromatographic separation with the mobile phase system consisting of acetonitrile and 0.1 % (v/v) formic acid aqueous solution. The intra-day and inter-day precisions of twelve analytes was less than 9.33 %, and the accuracy ranged from −11.08 to 11.89 %. The extraction recoveries of the analytes ranged from 76.42 to 93.17 % and the matrix effects varied from 76.02 to 90.56 %. The results of stability tests demonstrated that the analytes were stable under the different conditions and their relative standard deviation were less than 13.06 %. The developed method was firstly successfully applied to the pharmacokinetic study of the twelve analytes of PR extract in rats, and the pharmacokinetic results showed that the maximum concentration (C_{max}, 1553.51 ± 736.52 ng/mL) of tropic acid were relatively large, which was related to the fact that scopolamine and anisodamine were metabolized to tropic acid *in vivo*. The elimination half-life ($T_{1/2}$) of fabiatrin, scopolin, 4-hydroxybenzoic acid, and tropic acid were 6.45, 6.16, 9.87, and 7.12 h, suggesting that these analytes were kept for a longer duration *in vivo*. This research would provide meaningful reference for PR in clinical use.

1. Introduction

Medicinal plants have shown promising efficacy and accessibility, alleviating the harm of diseases to human health, which has led to an increased attention in recent years (Wheatley, 2005; Ardalani et al., 2020; Gregory et al., 2021; Ben-Shabat et al., 2020). Physochlainae Radix (PR) is derived from the dried roots of *Physochlaina infundibularis* Kuang in the family Solanaceae (State Pharmacopoeia Commission, 2020). Due to its remarkable clinical efficacy, PR is widely used in medications. Traditionally, PR is employed to relieve cough and phlegm, warm the lung, and calm the mind. Currently, more and more pharmacological studies have demonstrated that PR has been found to possess a variety of properties, such as antitussive, expectorant, antioxidant and analgesic (Yohannes et al., 2019; Wang et al., 2023; Tang et al., 2019). Meanwhile, clinical studies have indicated that PR is effective in the treatment of chronic bronchitis, asthmatic bronchitis, allergic purpura (Yohannes et al., 2019; Zhou et al., 2017), etc.

Medicinal plants have these properties depending on the bioactive metabolites they contain. In phytochemical studies, various components have been identified in PR, mainly including alkaloids, coumarins, and organic acids (Lou et al., 2011; Dai and Song, 2012; Zhao et al., 2013), etc. Among them, tropane alkaloids are considered both as the main

Abbreviations: **PR**, Physochlainae Radix; **UHPLC-MS/MS**, ultra-high-performance liquid chromatography coupled with triple quadrupole mass spectrometry; **IS**, internal standard; **MRM**, Multiple reaction monitoring; **QC**, quality control; **S/N**, signal-to-noise ratio; **RE**, relative error; **RSD**, relative standard deviation; **LLOQ**, lower limits of quantification; $\mathbf{T_{1/2}}$, elimination half-life; $\mathbf{T_{max}}$, time to reach maximum concentration; $\mathbf{C_{max}}$, maximum concentration; **AUC**, area under the curve.

* Corresponding authors.
E-mail addresses: 493507647@qq.com (J. Feng), hejun673@163.com (J. He).

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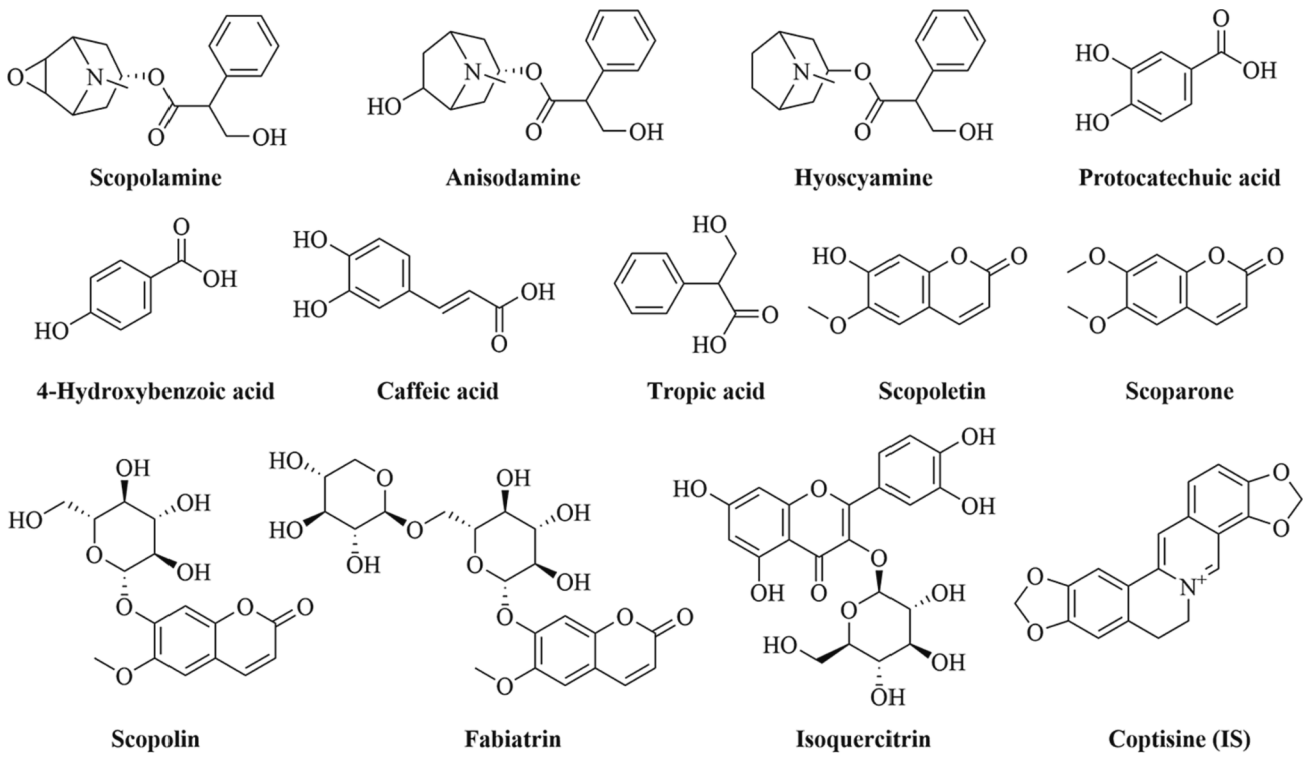

Fig. 1. Chemical structures of twelve compounds and IS.

Table 1
Mass spectra properties of twelve analytes and IS.

Compounds	Rt (min)	Precursor ion (*m/z*)	Product ion (*m/z*)	Fragmentor (V)	Collision energy (V)	Ion mode
Scopolamine	1.541	304.1	138.1	80	24	Positive
Anisodamine	1.643	306.1	140.1	80	28	Positive
Hyoscyamine	2.237	290.1	124.1	80	10	Positive
Protocatechuic acid	2.455	153.0	109.0	99	16	Negative
Fabiatrin	2.744	531.1	191.0	99	8	Negative
Scopolin	2.911	399.1	191.0	84	8	Negative
4-Hydroxybenzoic acid	3.474	137.0	93.0	84	16	Negative
Caffeic acid	3.704	179.0	135.0	84	16	Negative
Tropic acid	4.053	165.0	103.0	69	4	Negative
Scopoletin	4.545	191.0	176.0	84	12	Negative
Isoquercitrin	4.593	463.0	300.0	135	29	Negative
Scoparone	5.469	207.0	107.0	108	40	Positive
Coptisine (IS)	4.206	321.0	321.0	130	6	Positive

active ingredients for the treatment of various airway diseases and as the cause of toxicity in PR (Flynn et al., 2009; Wigenstam et al., 2021; Gadzikowska and Grynkiewicz, 2002; Kohnen-Johannsen and Kayser, 2019). Hence, it is necessary to figure out how these ingredients are metabolized to minimize the occurrence of side effects *in vivo*.

Pharmacokinetics of components in herbal medicine is mainly used for investigating absorption, distribution, metabolism, and excretion of drugs *in vivo* (He et al., 2011), which is an essential bridge connecting the herb compositions and active components (Laddha and Kulkarni, 2023; Huang et al., 2022). Meanwhile, the pharmacokinetic characteristics can illustrate the dynamically changing process of compounds *in vivo*. These are extremely valuable for improving the safety and efficacy in clinical use of drugs and helping to determine the suitable dosing and administration time (Hanley et al., 2023; Ma et al., 2023). Although PR is widely used clinically, its pharmacokinetic properties *in vivo* have not been reported.

In this study, a rapid and sensitive ultra-high-performance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC-MS/MS) method was established and validated for the simultaneous determination of twelve components (scopolamine, anisodamine, hyoscyamine, protocatechuic acid, fabiatrin, scopolin, 4-hydroxybenzoic acid, caffeic acid, tropic acid, scopoletin, isoquercitrin, and scoparone) after oral administration of the PR extract in rat plasma. This research is the first pharmacokinetic study of both PR extract and fabiatrin, which can provide some valuable references for further pharmacological studies and applications.

2. Materials and methods

2.1. Chemicals, reagents, and plant materials

Scopolamine, anisodamine hydrobromide, hyoscyamine, protocatechuic acid, fabiatrin, scopolin, 4-hydroxybenzoic acid, caffeic acid, tropic acid, scopoletin, isoquercitrin, scoparone and coptisine (internal standard [IS], purity $\geq$ 98 %) were purchased from Chengdu Desite Biotechnology Co., Ltd. (Chengdu, China). Fig. 1 shows the chemical

Table 2
The content of twelve analytes in PR extract ($n = 3$).

Compounds	Content (μg/g)
Scopolamine	1537.52 ± 20.80
Anisodamine	4811.42 ± 30.59
Hyoscyamine	4238.32 ± 64.62
Protocatechuic acid	121.50 ± 0.76
Fabiatrin	22213.64 ± 199.22
Scopolin	4488.79 ± 67.24
4-Hydroxybenzoic acid	260.42 ± 3.87
Caffeic acid	164.00 ± 7.33
Tropic acid	1457.80 ± 18.99
Scopoletin	6053.56 ± 72.47
Isoquercitrin	0.41 ± 0.05
Scoparone	38.21 ± 2.77

structures of the twelve analytes and IS. Fisher Scientific (Fair Lawn, NJ, USA) provided the methanol and acetonitrile (chromatographic purity) used in this study. Chromatographic purity formic acid was prepared from ROE (St. Louis, MO, USA). The demineralized water was obtained from Millipore's Milli-Q water purification system (Milford, MA, USA). PR was collected from Shaanxi province (China), and preserved at Tianjin University of Traditional Chinese Medicine, China.

2.2. Instruments and conditions

In UHPLC-MS/MS, an Agilent 1290 ultra-high performance liquid chromatography system along with an Agilent 6470 series triple quadrupole mass spectrometer were used. ACQUITY UPLC CSH C18 column was employed for the chromatographic separation, and the column temperature of 30 °C was maintained. As mobile phases, 0.1 % formic acid in water (A) and acetonitrile (B) were used with the following gradient elution method: 0 – 7 min, 10 – 55 % B; 7 – 8 min, 55 – 61 % B, post run time of 4 min. The flow rate and injection volume were 0.3 mL/min and 2 μL, respectively. Multiple reaction monitoring (MRM) mode was applied for analysis in both positive and negative ionization mode simultaneously. The instrumental parameters were set as follows: gas temperature at 350 °C, gas flow rate at 11 L/min, and nebulizer pressure at 30 psig. The quantitative parameters of twelve components and IS were listed in Table 1. The mass spectroscopy characterization of twelve compounds were shown in Figure S1.

2.3. PR extract preparation

PR (700.0 g) was weighed accurately, and ten times 85 % ethanol (v/v) was extracted under hot reflux for two times, each time for two hours. Extract was mixed and concentrated by evaporation at reduced pressure. The dried PR extract was crushed and stored. The contents of scopolamine, anisodamine, hyoscyamine, protocatechuic acid, fabiatrin, scopolin, 4-hydroxybenzoic acid, caffeic acid, tropic acid, scopoletin, isoquercitrin, and scoparone in PR extract were listed in Table 2.

2.4. Preparation of standard solutions, calibration standards and quality control samples

Scopolamine, anisodamine, hyoscyamine, protocatechuic acid, fabiatrin, scopolin, 4-hydroxybenzoic acid, caffeic acid, tropic acid, scopoletin, isoquercitrin, scoparone and coptisine (IS) were separately weighed and dissolved with methanol at a concentration of 1.0 mg/mL.

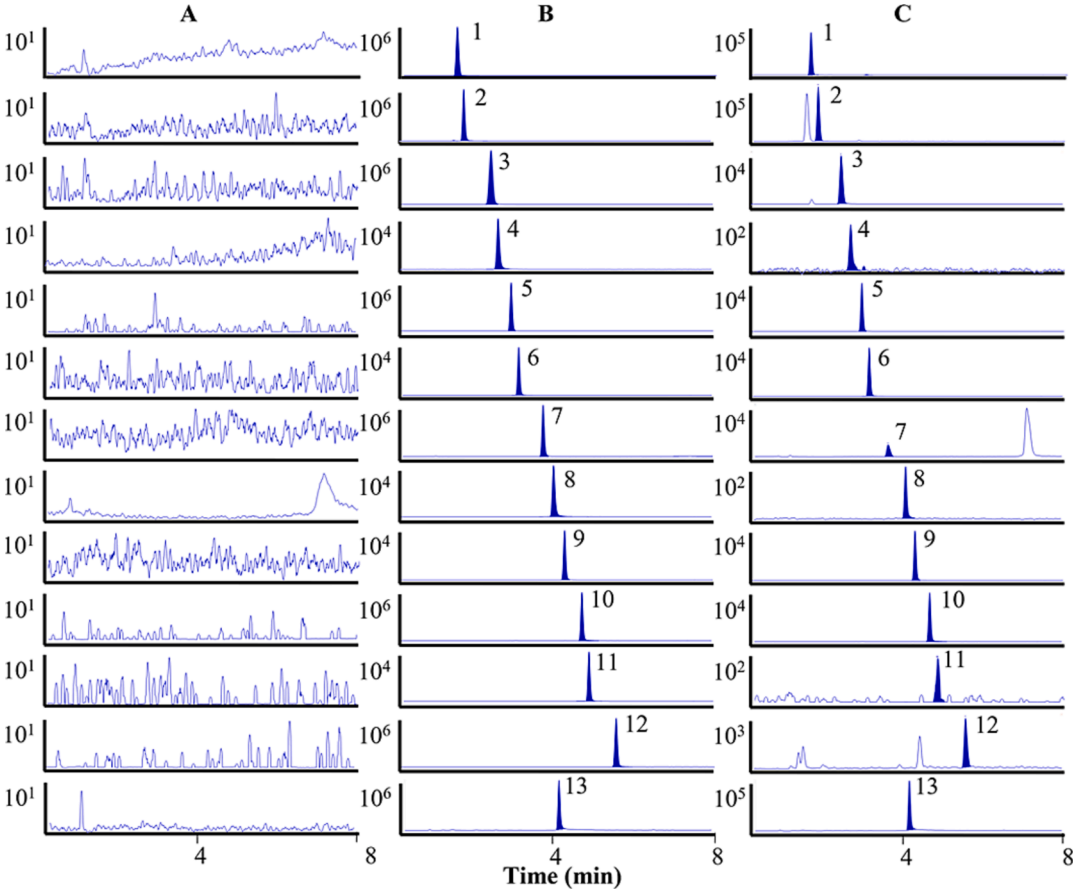

Fig. 2. MRM chromatograms of twelve analytes and IS. Blank plasma sample (A); Blank plasma spiked with twelve analytes and IS (B); Plasma samples after oral administration of PR extract (C). 1. scopolamine, 2. anisodamine, 3. hyoscyamine, 4. protocatechuic acid, 5. fabiatrin, 6. scopolin, 7. 4-hydroxybenzoic acid, 8. caffeic acid, 9. tropic acid, 10. scopoletin, 11. isoquercitrin, 12. scoparone, and 13. coptisine (IS).

Table 3
Calibration curves, linear range, and correlation coefficients (r), and LLOQ of twelve analytes.

Compounds	Calibration curves	r	Linear range (ng/mL)	LLOQ (ng/mL)
Scopolamine	$Y = 1.6869X + 3.0132E^{-004}$	0.9960	0.3–96	0.3
Anisodamine	$Y = 1.6171X + 0.0031$	0.9962	2–640	0.4
Hyoscyamine	$Y = 0.2129X + 2.7028E^{-004}$	0.9950	2–640	1.0
Protocatechuic acid	$Y = 0.0271X + 1.1599E^{-005}$	0.9952	1–320	1.0
Fabiatrin	$Y = 0.1597X + 8.1309E^{-005}$	0.9966	2–640	2.0
Scopolin	$Y = 0.0172X + 1.0846E^{-005}$	0.9953	1–320	1.0
4-Hydroxybenzoic acid	$Y = 0.0286X + 0.0011$	0.9953	1–320	1.0
Caffeic acid	$Y = 0.0098X + 2.3091E^{-004}$	0.9955	1–320	1.0
Tropic acid	$Y = 0.0097X-1.7889E^{-004}$	0.9961	10–3200	2.0
Scopoletin	$Y = 0.0299X-2.5028E^{-006}$	0.9954	2–640	1.0
Isoquercitrin	$Y = 0.0216X + 4.5810E^{-005}$	0.9983	1–320	1.0
Scoparone	$Y = 0.2856X + 7.1036E^{-007}$	0.9951	0.3–96	0.3

The calibration solutions were obtained by adding appropriate volumes of mixture working solution and 20 μL of IS into 100 μL blank rat plasma, resulting in concentrations: 1, 2, 4, 10, 20, 40, 80, 160, and 320 ng/mL for scopolin, 4-hydroxybenzoic acid, isoquercitrin, protocatechuic acid, and caffeic acid; 2, 4, 8, 20, 40, 80, 160, 320, and 640 ng/mL for scopoletin, fabiatrin, anisodamine, and hyoscyamine; 0.3, 0.6, 1.2, 3, 6, 12, 24, 48, and 96 ng/mL for scopolamine and scoparone; 10, 20, 40, 100, 200, 400, 800, 1600, and 3200 ng/mL for tropic acid. Three levels (low, medium, and high concentrations) of quality control (QC) samples were prepared in the same way.

2.5. Plasma sample preparation

20 μL of methanol, 20 μL of IS (coptisine, 1 μg/mL) were added to 100 μL of plasma sample and then vortex-mixed. The mixture was extracted with 600 μL methanol for 5 min. After centrifuging at 14,000 g for 10 min, the upper purified liquid was collected and evaporated under a stream of nitrogen until dry. The residue was redissolved in 100 μL of 50 % methanol. Finally, 2 μL of upper purifying solution was injected into the UHPLC-MS/MS system for analysis.

2.6. Method validation

The method was evaluated for specificity, linearity, sensitivity, precision and accuracy, recovery, matrix effect, and stability to ensure that it meets the U.S. Food and Drug Administration (FDA) guideline for accurate quantitation. (Tang et al., 2021; Xu et al., 2019; Zhang et al., 2022).

Table 4
Precision and accuracy of twelve analytes in rat plasma ($n = 6$).

Compounds	Spiked concentration (ng/mL)	Intra-day			Inter-day		
		Measured (ng/mL)	RE (%)	RSD (%)	Measured (ng/mL)	RE (%)	RSD (%)
Scopolamine	0.6	0.54 ± 0.05	−10.03	8.37	0.64 ± 0.05	6.37	7.17
	6	5.67 ± 0.24	−5.42	4.20	5.86 ± 0.16	−2.29	2.70
	76.8	80.24 ± 4.42	4.48	5.51	75.30 ± 3.53	−1.95	4.68
Anisodamine	4	4.38 ± 0.23	9.48	5.32	4.29 ± 0.27	7.33	6.40
	40	41.42 ± 3.36	3.56	8.10	38.73 ± 1.62	−3.17	4.19
	512	493.08 ± 25.40	−3.70	5.15	524.34 ± 24.29	2.41	4.63
Hyoscyamine	4	4.23 ± 0.20	5.80	4.78	3.83 ± 0.18	−4.34	4.78
	40	41.47 ± 3.43	3.68	8.28	39.07 ± 1.95	−2.34	5.00
	512	553.48 ± 21.39	8.10	3.87	520.63 ± 24.60	1.68	4.73
Protocatechuic acid	2	2.06 ± 0.10	3.08	5.01	1.89 ± 0.14	−5.49	7.45
	20	20.67 ± 1.29	3.35	6.25	19.19 ± 1.13	−4.06	5.88
	256	272.56 ± 15.45	6.47	5.67	259.73 ± 19.79	1.46	7.62
Fabiatrin	4	4.11 ± 0.22	2.84	5.31	3.83 ± 0.20	−4.17	5.20
	40	41.33 ± 1.71	3.33	4.14	38.72 ± 2.29	−3.19	5.93
	512	534.45 ± 12.05	4.38	2.25	526.02 ± 24.87	2.74	4.73
Scopolin	2	2.24 ± 0.09	11.89	3.82	2.15 ± 0.14	7.50	6.64
	20	18.22 ± 0.65	−8.88	3.57	18.85 ± 0.72	−5.75	3.83
	256	250.30 ± 8.98	−2.23	3.59	264.55 ± 22.99	3.34	8.69
4-Hydroxybenzoic acid	2	2.06 ± 0.10	2.77	4.84	2.02 ± 0.13	1.22	6.65
	20	20.31 ± 1.89	1.54	9.33	20.61 ± 0.78	3.04	3.80
	256	260.44 ± 6.87	1.74	2.64	264.06 ± 14.51	3.15	5.50
Caffeic acid	2	2.07 ± 0.15	3.71	7.40	2.12 ± 0.18	5.86	8.43
	20	21.13 ± 0.69	5.63	3.27	20.07 ± 1.07	0.36	5.32
	256	253.06 ± 12.38	−1.15	4.89	260.00 ± 6.64	1.56	2.55
Tropic acid	20	20.98 ± 0.75	4.91	3.58	20.54 ± 0.85	2.69	4.13
	200	179.75 ± 9.21	−10.12	5.12	202.67 ± 13.20	1.34	6.51
	2560	2487.37 ± 100.74	−2.84	4.05	2548.56 ± 57.95	−0.45	2.27
Scopoletin	4	4.24 ± 0.09	6.06	2.24	4.07 ± 0.23	1.67	5.63
	40	41.19 ± 2.62	2.98	6.36	40.15 ± 1.73	0.39	4.30
	512	538.84 ± 27.26	5.24	5.06	521.40 ± 20.08	1.84	3.85
Isoquercitrin	2	1.78 ± 0.02	−11.08	0.97	1.95 ± 0.10	−2.73	4.89
	20	21.23 ± 1.10	6.17	5.17	19.07 ± 1.32	−4.64	6.92
	256	265.11 ± 15.49	3.56	5.84	260.44 ± 15.58	1.74	5.98
Scoparone	0.6	0.59 ± 0.05	−2.27	8.08	0.63 ± 0.03	5.02	4.65
	6	6.09 ± 0.19	1.55	3.07	6.12 ± 0.28	1.99	4.52
	76.8	74.81 ± 5.72	−2.59	7.65	78.21 ± 2.58	1.84	3.30

Table 5
Extraction recoveries and matrix effects of twelve analytes in rat plasma ($n = 6$).

Compounds	Spiked concentration (ng/mL)	Extraction recovery (%)	RSD (%)	Matrix effect (%)	RSD (%)
Scopolamine	0.6	81.55 ± 8.05	9.87	87.97 ± 11.49	13.06
	6	84.94 ± 2.11	2.49	81.48 ± 2.80	3.44
	76.8	84.35 ± 4.81	5.70	82.69 ± 5.50	6.66
Anisodamine	4	84.51 ± 5.67	6.71	84.60 ± 4.48	5.29
	40	78.33 ± 3.52	4.49	86.63 ± 3.34	3.85
	512	76.42 ± 2.79	3.65	83.04 ± 4.84	5.83
Hyoscyamine	4	83.82 ± 3.64	4.35	84.50 ± 3.76	4.45
	40	79.28 ± 8.00	10.09	82.99 ± 3.39	4.08
	512	80.48 ± 4.23	5.25	82.04 ± 2.04	2.48
Protocatechuic acid	2	84.83 ± 4.02	4.74	76.02 ± 3.64	4.79
	20	82.97 ± 7.95	9.58	79.41 ± 10.88	13.70
	256	80.52 ± 2.33	2.90	78.86 ± 5.36	6.80
Fabiatrin	4	81.25 ± 4.04	4.97	78.03 ± 9.87	12.66
	40	78.75 ± 4.16	5.29	78.67 ± 3.88	4.93
	512	82.44 ± 7.38	8.95	86.67 ± 5.70	6.58
Scopolin	2	86.85 ± 5.72	6.58	81.86 ± 10.67	13.03
	20	80.49 ± 5.49	6.82	78.48 ± 5.43	6.92
	256	80.88 ± 4.92	6.08	90.56 ± 5.77	6.37
4-Hydroxybenzoic acid	2	83.14 ± 9.12	10.97	81.45 ± 6.35	7.79
	20	83.77 ± 4.12	4.92	80.07 ± 2.56	3.19
	256	80.10 ± 11.31	14.12	80.69 ± 4.05	5.02
Caffeic acid	2	79.51 ± 8.09	10.18	89.29 ± 9.10	10.19
	20	85.72 ± 11.18	13.04	80.31 ± 7.80	9.71
	256	86.26 ± 4.97	5.76	84.50 ± 5.26	6.22
Tropic acid	20	93.17 ± 9.84	10.56	78.89 ± 4.50	5.70
	200	91.54 ± 7.74	8.45	78.93 ± 2.93	3.72
	2560	86.15 ± 5.79	6.72	80.48 ± 4.23	5.25
Scopoletin	4	86.39 ± 9.25	10.70	83.79 ± 5.84	6.98
	40	81.35 ± 6.42	7.90	86.87 ± 2.91	3.35
	512	83.11 ± 10.82	13.01	84.76 ± 2.22	2.61
Isoquercitrin	2	81.35 ± 4.46	5.48	80.33 ± 5.67	7.06
	20	81.82 ± 5.68	6.94	86.40 ± 10.46	12.10
	256	81.27 ± 5.81	7.15	81.83 ± 7.06	8.63
Scoparone	0.6	83.20 ± 11.24	13.51	80.57 ± 9.67	12.00
	6	80.86 ± 8.98	11.10	85.05 ± 4.43	5.21
	76.8	82.41 ± 10.19	12.36	84.23 ± 5.33	6.33

2.6.1. Specificity

Specificity was assayed by comparing chromatograms of blank plasma samples, blank plasma samples spiked with twelve ingredients and IS, and plasma samples collected after oral administration of PR extract.

2.6.2. Linearity and lower limits of quantification

Calibration curves were prepared by plotting the relationship between the peak area ratios of each analyte to IS versus the concentration of the corresponding analyte, and $1/x^2$ was used as weighting coefficient. The LLOQ was the lowest concentration that could be measured with a signal-to-noise ratio (S/N) of 10.

2.6.3. Precision and accuracy

Precision and accuracy were assessed by analyzing six replicates of QC samples at low, medium, and high concentrations levels on the same day and on three consecutive days. Accuracy was evaluated by relative error (RE), while precisions were assessed by relative standard deviation (RSD).

2.6.4. Extraction recovery and matrix effect

Extraction recoveries were measured by comparing the peak response of the analytes in extracted samples with those in post-extraction spiked samples. Matrix effects were evaluated by calculating the ratio of the peak response of the analytes in the post-extracted spiked samples to those of the unextracted samples.

2.6.5. Stability

By analyzing the QC samples under various conditions: stored in auto-sampler for 12 h, at room temperature for 4 h, under three freeze–thaw cycles, and stored at −80 °C for 7 days, the stability of analytes in plasma samples was evaluated.

2.7. Pharmacokinetic study

Six SD rats (SPF, 220 ± 10 g, Male) were prepared from HuaFuKang Bioscience Co., Inc. (Beijing, China). Rats were allowed to drink freely and fasted for 12 h prior to the study. The PR extract was dissolved to a concentration of 62 mg/mL with 0.5 % CMC-Na aqueous solution. A suspension of 0.62 g/kg was administered orally to rats and roughly 300 μL of blood were collected before and at 0, 0.03, 0.08, 0.17, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 10, 12, 24, and 36 h after oral administration. After centrifugation at 7000 rpm for 10 min, the plasma is frozen at −80 °C until analysis. Pharmacokinetic parameters were calculated using "Drug and Statistics 3.0" (DAS 3.0) (Medical College of Wannan, China).

3. Result and discussion

3.1. Optimization of LC and MS/MS conditions

In the paper, the analytes were separated by UHPLC and characterized by mass spectrometry (QQQ-MS/MS). The UHPLC conditions, including column stationary phase type, mobile phase and additives, were optimized to obtain better separation. The stationary phase is crucial for obtaining satisfactory separation results. Main chemical components of most traditional Chinese medicines are readily absorbed by reversed-phase stationary phases, and three commonly used reversed-phase stationary phases were employed in this study: ACQUITY UPLC BEH C18 (2.1 × 100 mm, 1.7 μm), CORTECS UPLC C18 (2.1 mm × 100 mm, 1.6 μm), and ACQUITY UPLC CSH C18 (2.1 × 100 mm,

Table 6
Stability of twelve analytes in rat plasma ($n = 6$).

Compounds	Spiked concentration (ng/mL)	Room temperature for 4 h		Autosampler for 12 h		Three freeze–thaw cycles		−80 °C for 7 days	
		Measured (ng/mL)	RSD (%)	Measured (ng/mL)	RSD (%)	Measured (ng/mL)	RSD (%)	Measured (ng/mL)	RSD (%)
Scopolamine	0.6	0.59 ± 0.02	4.19	0.64 ± 0.05	7.17	0.59 ± 0.06	10.30	0.62 ± 0.05	8.26
	6	5.77 ± 0.31	5.29	5.86 ± 0.16	2.70	5.96 ± 0.46	7.79	6.28 ± 0.25	4.05
	76.8	75.84 ± 7.72	10.18	75.30 ± 3.53	4.68	75.69 ± 2.79	3.68	71.66 ± 5.49	7.65
Anisodamine	4	4.05 ± 0.21	5.10	4.29 ± 0.27	6.40	4.15 ± 0.19	4.55	4.13 ± 0.33	8.02
	40	40.92 ± 2.30	5.62	38.73 ± 1.62	4.19	41.44 ± 2.06	4.96	42.46 ± 1.60	3.77
	512	504.68 ± 24.24	4.80	524.34 ± 24.29	4.63	516.33 ± 22.52	4.36	519.63 ± 38.80	7.47
Hyoscyamine	4	3.95 ± 0.22	5.45	3.83 ± 0.18	4.78	3.92 ± 0.26	6.60	4.12 ± 0.23	5.53
	40	39.82 ± 1.70	4.27	39.07 ± 1.95	5.00	41.82 ± 1.48	3.54	41.03 ± 1.61	3.93
	512	520.46 ± 24.21	4.65	520.63 ± 24.60	4.73	507.81 ± 29.07	5.73	513.61 ± 17.64	3.44
Protocatechuic acid	2	2.05 ± 0.15	7.40	1.89 ± 0.14	7.45	1.93 ± 0.21	10.85	2.07 ± 0.16	7.67
	20	21.10 ± 1.53	7.24	19.19 ± 1.13	5.88	20.43 ± 1.34	6.54	20.73 ± 0.61	2.95
	256	258.98 ± 6.08	2.35	259.73 ± 19.79	7.62	263.92 ± 15.00	5.68	262.52 ± 7.51	2.86
Fabiatrin	4	3.97 ± 0.11	2.78	3.83 ± 0.20	5.20	3.81 ± 0.17	4.53	4.08 ± 0.13	3.22
	40	39.98 ± 1.00	2.49	38.72 ± 2.29	5.93	40.12 ± 1.91	4.76	41.02 ± 3.62	8.84
	512	503.62 ± 26.48	5.26	526.02 ± 24.87	4.73	506.80 ± 20.88	4.12	523.43 ± 18.32	3.50
Scopolin	2	2.05 ± 0.11	5.50	2.15 ± 0.14	6.64	2.03 ± 0.12	6.13	2.10 ± 0.12	5.84
	20	19.67 ± 0.77	3.90	18.85 ± 0.72	3.83	19.94 ± 0.68	3.40	20.80 ± 1.01	4.86
	256	253.94 ± 6.14	2.42	264.55 ± 22.99	8.69	266.25 ± 6.83	2.56	266.84 ± 12.34	4.63
4-Hydroxybenzoic acid	2	2.07 ± 0.13	6.42	2.02 ± 0.13	6.65	1.97 ± 0.06	3.04	1.95 ± 0.10	5.18
	20	19.70 ± 0.81	4.13	20.61 ± 0.78	3.80	20.40 ± 1.04	5.11	20.84 ± 0.86	4.12
	256	266.00 ± 9.59	3.61	264.06 ± 14.51	5.50	263.45 ± 17.39	6.60	261.22 ± 14.73	5.64
Caffeic acid	2	1.94 ± 0.21	10.88	2.12 ± 0.18	8.43	2.05 ± 0.07	3.30	2.02 ± 0.22	11.02
	20	19.88 ± 0.47	2.39	20.07 ± 1.07	5.32	19.82 ± 0.77	3.87	20.88 ± 1.36	6.50
	256	258.28 ± 16.63	6.44	260.00 ± 6.64	2.55	265.10 ± 16.59	6.26	264.15 ± 9.29	3.52
Tropic acid	20	21.02 ± 0.66	3.15	20.54 ± 0.85	4.13	19.77 ± 2.58	13.06	19.58 ± 0.77	3.91
	200	198.65 ± 14.51	7.30	202.67 ± 13.20	6.51	206.11 ± 9.54	4.63	200.49 ± 10.89	5.43
	2560	2627.14 ± 76.20	2.90	2548.56 ± 57.95	2.27	2515.33 ± 75.77	3.01	2615.37 ± 54.20	2.07
Scopoletin	4	4.03 ± 0.11	2.70	4.07 ± 0.23	5.63	4.00 ± 0.12	3.09	3.96 ± 0.24	6.11
	40	39.80 ± 0.94	2.37	40.15 ± 1.73	4.30	39.92 ± 1.38	3.46	40.76 ± 1.97	4.82
	512	507.99 ± 23.78	4.68	521.40 ± 20.08	3.85	512.75 ± 32.15	6.27	512.64 ± 25.98	5.07
Isoquercitrin	2	2.06 ± 0.07	3.56	1.95 ± 0.10	4.89	2.04 ± 0.14	6.68	2.01 ± 0.06	2.91
	20	19.83 ± 0.52	2.63	19.07 ± 1.32	6.92	20.44 ± 1.26	6.16	20.15 ± 1.54	7.64
	256	251.41 ± 13.80	5.49	260.44 ± 15.58	5.98	257.45 ± 9.47	3.68	260.76 ± 9.26	3.55
Scoparone	0.6	0.59 ± 0.02	4.03	0.63 ± 0.03	4.65	0.58 ± 0.03	5.97	0.61 ± 0.07	11.38
	6	6.10 ± 0.26	4.31	6.12 ± 0.28	4.52	6.11 ± 0.24	3.94	6.18 ± 0.32	5.14
	76.8	79.27 ± 5.54	6.98	78.21 ± 2.58	3.30	78.25 ± 2.75	3.52	80.35 ± 2.73	3.40

1.7 μm) to optimize the separation of twelve analytes. In comparison, the ACQUITY UPLC CSH C18 (2.1 × 100 mm, 1.7 μm) column provided better retention and shorter analysis time. Moreover, different mobile phases (ACN/H2O, ACN/0.1 %FA-H2O, ME/H2O, and ME/0.1 %FA-H2O) were compared and the results showed that ACN/0.1 %FA-H2O had higher sensitivity for analytes and IS. The twelve analytes and IS were eluted within eight min without any interference peaks (Fig. 2).

For QQQ-MS/MS conditions, key parameters of the mass spectrometry that may affect the ion response were optimized. Three main ion source parameters, including gas flow rate (5–11 L/min), gas temperature (350–380 °C), and nebulizer pressure (20–50 psig), were optimized using a single factor experiment. Figure S2 shows the histogram of the trend of ion response for these twelve compositions at different parameter levels. The final optimized MS/MS conditions were as follows: gas temperature of 350 °C, gas flow rate of 11 L/min and nebulizer pressure of 30 psig.

3.2. Sample preparation

Sample preparation by appropriate methods is a key step in pharmacokinetic study. Three methods, ethyl acetate liquid–liquid extraction, acetonitrile precipitated protein, and methanol precipitated protein were compared for plasma sample preparation. Results revealed that methanol precipitated protein method has higher extraction efficiency, lower matrix effect, and simpler operation procedure. In order to meet the demands of this experiment for biological sample determination, methanol precipitated protein method was adopted for sample preparation in this study.

3.3. Method validation

3.3.1. Specificity

The MRM chromatograms of blank plasma sample (A), blank plasma spiked with twelve analytes and IS (B), and plasma samples collected in rats (C) are displayed in Fig. 2. No endogenous interference was found in the samples.

3.3.2. Linearity and LLOQ

The regression equations, linear ranges, correlation coefficients, and LLOQs for the twelve analytes are presented in Table 3. The results indicated that the calibration curves for the twelve analytes showed excellent linearity over the corresponding concentration range. ($r >$ 0.9950). The LLOQs of scopolamine, anisodamine, hyoscyamine, protocatechuic acid, fabiatrin, scopolin, 4-hydroxybenzoic acid, caffeic acid, tropic acid, scopoletin, isoquercitrin, and scoparone were 0.3, 0.4, 1.0, 1.0, 2.0, 1.0, 1.0, 1.0, 2.0, 1.0, 1.0, and 0.3 ng/mL, respectively.

3.3.3. Precision and accuracy

The accuracy and intra- and inter-day precision were evaluated based on the RE and RSD values. As shown in Table 4, the intra- and inter-day RSD values were less than 9.33 %, the intra-day RE ranged from −11.08 to 11.89 %, while the inter-day RE ranged from −5.75 to 7.50 %. The results suggested that this method has acceptable limits of precision and accuracy.

3.3.4. Extraction recovery and matrix effect

The results of extraction recovery and matrix effect are provided in

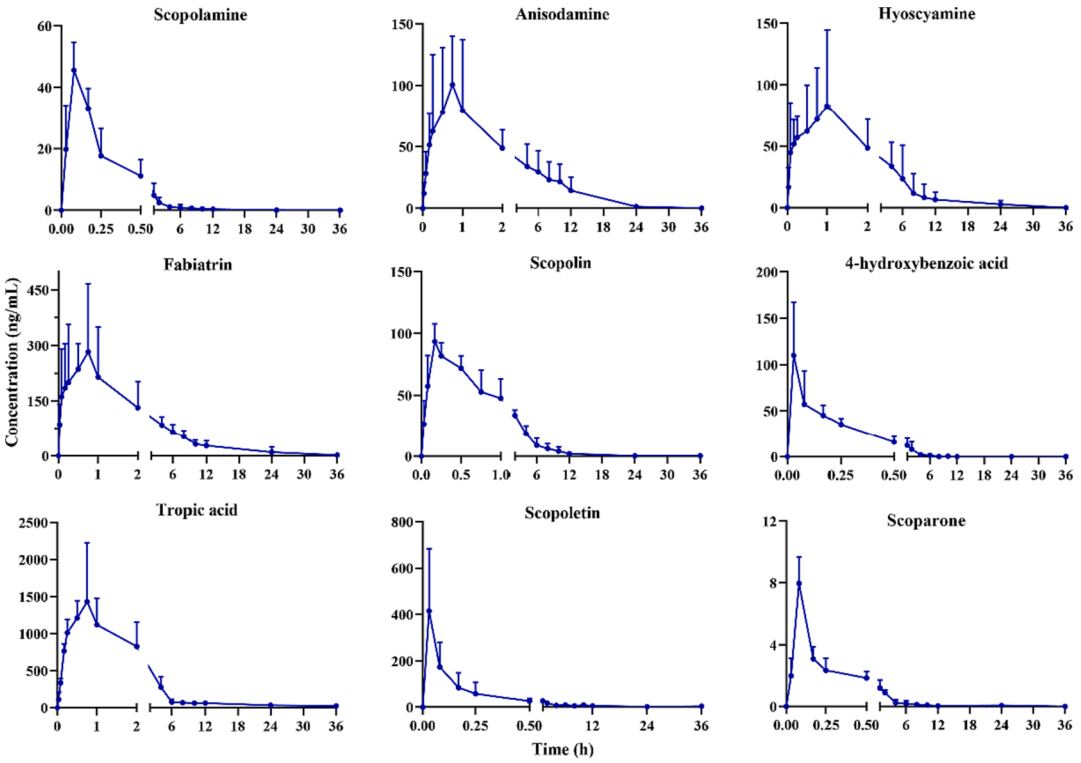

Fig. 3. Mean plasma concentration–time curves of scopolamine, anisodamine, hyoscyamine, fabiatrin, scopolin, 4-hydroxybenzoic acid, tropic acid, scopoletin, and scoparone after oral administration of PR extract (mean ± SD, $n = 6$).

Table 7
The main pharmacokinetic parameters of nine analytes in rat plasma ($n = 6$).

Compounds	T_{max} (h)	C_{max} (ng/mL)	$T_{1/2}$ (h)	$AUC_{(0-t)}$ (h·ng/mL)	$AUC_{(0-\infty)}$ (h·ng/mL)	CLz/F (L/h/kg)
Scopolamine	0.08 ± 0.00	45.60 ± 9.04	2.99 ± 0.79	30.36 ± 13.00	30.37 ± 13.31	23.48 ± 6.37
Anisodamine	0.75 ± 0.27	142.35 ± 43.30	3.15 ± 1.68	570.90 ± 272.72	571.90 ± 272.04	1.36 ± 0.52
Hyoscyamine	0.88 ± 0.14	124.86 ± 30.02	2.08 ± 0.21	409.23 ± 254.71	409.24 ± 254.71	1.88 ± 0.48
Fabiatrin	0.81 ± 0.24	360.72 ± 172.63	6.45 ± 0.74	1317.65 ± 521.24	1333.83 ± 521.43	0.52 ± 0.17
Scopolin	0.21 ± 0.04	96.02 ± 13.15	6.16 ± 1.89	261.99 ± 60.90	264.93 ± 60.78	2.44 ± 0.56
4-Hydroxybenzoic acid	0.05 ± 0.06	113.91 ± 51.22	0.41 ± 0.07	53.94 ± 22.72	53.94 ± 22.72	10.16 ± 2.48
Tropic acid	0.70 ± 0.21	1553.51 ± 736.52	9.87 ± 1.77	4793.51 ± 986.20	5222.61 ± 1336.85	0.11 ± 0.03
Scopoletin	0.08 ± 0.07	432.44 ± 245.83	7.12 ± 1.65	260.58 ± 58.49	273.54 ± 59.68	2.03 ± 0.86
Scoparone	0.08 ± 0.00	7.98 ± 1.73	4.22 ± 0.16	5.88 ± 1.16	5.88 ± 1.16	85.01 ± 14.44

Table 5. The extraction recoveries for the three concentration levels of analytes in rat plasma samples ranged from 76.42 % to 93.17 %, and the matrix effects ranged from 76.02 to 90.56 %. These results indicated that both the matrix effects and extraction recoveries were within acceptable limits.

3.3.5. *Stability*

The stability results are shown in Table 6. The analytes were stable at room temperature for 4 h, in the auto-sampler for 12 h, under three freeze–thaw cycles, and at −80 °C for 7 days. The RSD values were less than 13.06 %, indicating that these analytes were stable under the above conditions.

3.4. *Pharmacokinetic study*

In this study, a high sensitivity UHPLC-MS/MS method was developed to determine twelve compounds in rat plasma after administration of PR extract. However, the plasma concentrations of some compounds were too low to attain a complete pharmacokinetic curve, such as protocatechuic acid, caffeic acid, and isoquercitrin. This may be caused by their low content in PR extract. Eventually, a total of nine detectable compounds were fitted with pharmacokinetic parameters. The mean plasma concentration–time curves of the other nine components are shown in Fig. 3. The main pharmacokinetic parameters, elimination half-life ($T_{1/2}$), time to reach maximum concentration (T_{max}), maximum concentration (C_{max}), and area under the curve (AUC), are summarized in Table 7.

As shown in Table 7, the $AUC_{(0-t)}$ and $AUC_{(0-\infty)}$ values of all nine compounds were close, indicating that the monitoring time of this study was appropriate. The $AUC_{(0-t)}$ value of tropic acid was larger than the other analytes, indicating that this component had a higher level of plasma exposure. The C_{max} value of tropic acid was 1553.51 ± 736.52 ng/mL, and the blood concentration was higher than other compounds, which was related to the fact that scopolamine and anisodamine were metabolized to tropic acid *in vivo* (Chen et al., 2008; Chen et al., 2005a, Chen et al., 2005b). Meanwhile, fabiatrin also had a higher level of plasma exposure, which might be attributed to its higher content in PR extract.

The T_{max} values of scopolamine, anisodamine, hyoscyamine, fabiatrin, scopolin, 4-hydroxybenzoic acid, tropic acid, scopoletin, and scoparone were 0.08, 0.75, 0.88, 0.81, 0.21, 0.05, 0.70, 0.08, and 0.08 h. These results showed that these nine components were absorbed quickly

in vivo. The T_{max} values of scopolamine, scopolin, scopoletin, and scoparone were similar to those previously reported (Zhang et al., 2014; Zhang et al., 2020; Li et al., 2019; Yin et al., 2012). The $T_{1/2}$ values of scopolamine, anisodamine, hyoscyamine, 4-hydroxybenzoic acid, and scoparone were 2.99, 3.15, 2.08, 0.41 and 4.22 h, respectively, indicating that these five analytes are eliminated shortly after oral administration of PR extract. The $T_{1/2}$ values of fabiatrin, scopolin, 4-hydroxybenzoic acid, and tropic acid were 6.45, 6.16, 9.87, and 7.12 h, respectively, which revealed that these four analytes are present *in vivo* for a relatively longer period of time and may result in a more durable effect.

4. Conclusions

An UHPLC-MS/MS method was developed and validated for the simultaneous determination of the twelve components (scopolamine, anisodamine, hyoscyamine, fabiatrin, scopolin, 4-hydroxybenzoic acid, tropic acid, scopoletin, and scoparone) in rat plasma. The results indicated that fabiatrin, scopolin, 4-hydroxybenzoic acid, and tropic acid have longer elimination half-life compared to other compounds. Additionally, the blood concentration and plasma exposure of tropic acid were higher than other compounds ware related to the fact that scopolamine and anisodamine were metabolized to tropic acid *in vivo*. More importantly, this research was the first pharmacokinetic study of both PR extract and fabiatrin, which provided a reference for the clinical applications and further development of PR.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics statement

The animal protocol was approved by the Animal Ethics Committee of Tianjin University of Traditional Chinese Medicine (TCM-LAEC2023059).

Authorship contribution statement

Conceptualization, Jun He and Jihong Feng; **formal analysis**, Zhenguo Lv; **data curation**, Tiantian Wu; **writing—original draft preparation**, Zhenguo Lv; **writing—review and editing**, Peng Zhao, Yang Liu, Caixia Li and Huizi Ouyang; **supervision**, Jun He and Jihong Feng. All authors have read and agreed to the published version of the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.arabjc.2024.105664.

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