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Original Research Article

A UHPLC-Q-Exactive-Orbitrap-MS method for simultaneous determination of three flavonoids from Parasitic loranthus and their pharmacokinetics in rat plasma

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Abstract

Purpose: *To develop and validate a chromatographic method for the simultaneous determination of plasma levels of rutin, avicularin and quercitrin using UHPLC-Q-Exactive-Orbitrap-MS.*
Methods: *A sensitive, selective, and reliable UHPLC-Q-Exactive-Orbitrap-MS method was developed and validated for simultaneous determination of the three flavonoids, with puerarin as internal standard (IS). Plasma samples were first treated with methanol, and then acidified using hydrochloric acid (HCl) prior to liquid-liquid extraction with ethyl acetate. The flavonoids were separated on a Syncronis C18 column (100×2.1mm, 1.7 µm) using an elution gradient of acetonitrile and 0.1 % formic acid at a flow rate of 0.3 mL/min.*
Results: *A linear correlation was obtained for the three flavonoids over the investigated concentration range, with correlation coefficients > 0.9954. The values of validated lower limit of quantification (LLOQ) were 0.68, 1.42 and 2.54 ng/mL for rutin, avicularin and quercitrin, respectively. Intra- and inter-day precision (RSD) were < 10 %, while accuracy (RE) ranged from −3.76 to 4.04 %.*
Conclusion: *The proposed method has been successfully validated and is suitable for studying the pharmacokinetics of the three analytes in rats treated with parasitic loranthus extract (PLE).*

Keywords: *Parasitic loranthus, Rutin, Avicularin, Quercitrin, UHPLC-Q-Exactive-Orbitrap-MS, Pharmacokinetics*

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INTRODUCTION

Sang ji sheng, the branches and leaves of parasitic loranthus, is a traditional Chinese medicine which was known in “Shennong's Herbal” eighteen hundreds years ago [1]. As far as is known, loranthus is a semi-parasitic plant, but the growth environment of the host trees varies widely [2]. The quality of medicinal parasitic loranthus is affected by the nature of the

host tree. These qualities include content of bioactive components and the efficacy of the drug. Therefore, studies on the parasitic nature of the relationship between loranthaceae and its host have continued to engage the attention of researchers [3-7].

In China, parasitic loranthus is widely used in many types of traditional Chinese medicinal prescriptions (TCMPs) for the treatment of soreness, threatened abortion, rheumatism and intractable dizziness [1]. Pharmacological studies demonstrate that parasitic loranthus exerts several pharmacological effects such as inhibition of fatty acid synthase and enhancement of weight loss [8]; diuretic and hypotensive effects [9,10], antidiabetic effects [11,12], and protective effects on learning and memory abilities [13]. Some reports show that the parasitic loranthus which is also named Taxillus, is rich in flavonoids [14,15], as well as hemiterpenoid derivatives [16].

Although the pharmacological and chemical properties of parasitic loranthus have attracted much research attention, not much is known about its pharmacokinetics. This study attempted to provide a new approach for determination of the bioactive components of parasitic loranthus in plasma, in order to characterize its pharmacokinetics. In previous studies, researchers found that the anti-rheumatic and anti-osteoporotic substances in loranthus were mainly flavonoids such as rutin, avicularin and quercitrin [17,18]. Rutin is one of the most active flavonoids, and it has been reported that it relieved pain and intra-ocular pressure when combined with vitamin C [19]. Avicularin and quercetin are potent inhibitors of fatty acid synthase [20]. Thus, it would be helpful to develop an approach which can be used for simultaneous determination of these three flavonoids. The UHPLC-MS/MS method provides high sensitivity and selectivity, and can be used for microanalysis of multi-components [21]. In recent years, it has been rapidly developed into an important method for measuring the composition of trace components in food and drugs [22-26]. The present study was carried out to determine the pharmacokinetics of rutin, avicularin and quercitrin in rats.

EXPERIMENTAL

Materials and reagents

Dried parasitic loranthus was purchased from Zhang Zhongjing Pharmacy, Henan, China. It was authenticated by Professor Suiqing Chen and Chengming Dong at Henan University of Chinese Medicine. Rutin, avicularin, and quercitrin were purchased from Sichuang Vicket Company (Chengdu, China). Puerarin was supplied by China Research Institute of Food and Drug Verification (Beijing, China). The structures of the three flavonoids and puerarin are shown in Figure 2. Heparin sodium, sodium carboxymethylcellulose (CMC-Na) and formic acid were supplied by Sigma–Aldrich (St. Louis, USA). Acetonitrile and methanol were purchased from Thermo Fisher. Hydrochloric acid and ethyl acetate obtained from Tianjin Dasen Chemical Products Sales Company, Tianjin, China.

A pressurized fluid extract (PLE) was prepared. The dried parasitic loranthus (500 g) was extracted twice with 70 % ethanol. The extract was concentrated to 0.08 g/mL, and subjected to separation and purification using elution through AB-8 macroporous adsorption resin. First, distilled water was used to elute the column, followed by elution with 50 % ethanol. The 50 % ethanol eluate was finally vacuum-dried to obtain a flavonoid-enriched extract (27.5 g). The flavonoid-enriched extract was subjected to HPLC analysis for determination of the contents of the three flavonoids. The results showed that the levels of rutin, avicularin and quercitrin in the PLE were 0.87, 1.10 and 3.74 %, respectively.

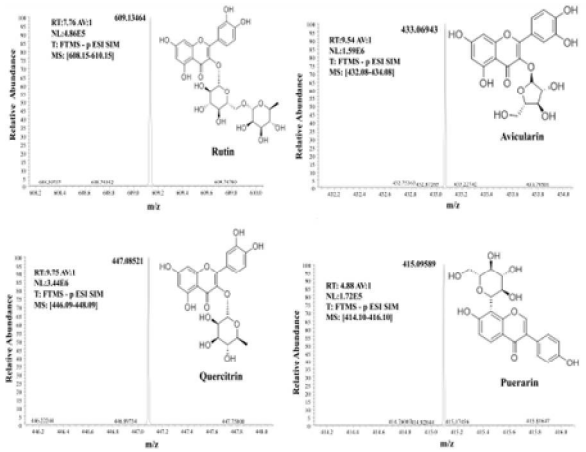

Figure 1: Molecular mass and structures of the analytes and IS in negative electrospray ionization mode

UHPLC-Q-Exactive-Orbitrap-MS instrument settings

Chromatographic analysis was performed using Thermo UPLC System. Syncronis C_{18} column was used at 30 ℃, and a gradient elution with water containing 0.1 % formic acid (A) and acetonitrile (B) was used at a flow rate of 0.30 mL/min as follows: 0 - 11 min with 14 - 42 % B; 11 - 11.2 min with 42 - 14 % B, and 11.2 - 14.2 min with 14 % B.

Mass spectrometric analysis was performed with Thermo Q-Exactive -Qibitrap-MS under the following conditions: voltage of 2.8 kV, 40 units of sheath gas flow, 10 units of auxiliary gas flow, nitrogen flow rate of 10 mL/min, and temperature of 350 ℃.

Calibration standards and quality control samples

Rutin, avicularin and quercitrin standards were dissolved in 10 mL of methanol to obtain standard solutions of concentrations 43.2, 91.2 and 162.4 µg/mL. The internal standard (IS) solution and working standard solution of IS were prepared in methanol. Calibration standard samples and quality control (QC) samples were prepared by spiking blank rat plasma with moderate working solutions. They were extracted using the same procedures for plasma samples.

Sample preparation

A liquid–liquid extraction (LLE) method was used to extract the three analytes and IS from rat plasma. All protocols in animal experiments were approved by the Ethics Committee on Animal Research of Henan University of Chinese Medicine (approval ref. No: HNUTCMEKY-201800361Z), and were carried out in line with the guidelines of the Committee on the Care and Use of Laboratory Animals in China [27]. The plasma was spiked with 100 µL of IS and 50 µL of 0.25 M HCl, and vortexed for 1 min. The proteins were precipitated by addition of 200 µL methanol. Thereafter, the mixture was extracted with 1 mL ethyl acetate through oscillation for 5 min at room temperature, followed by centrifugation at 12000 rpm for 5 min. The extract was then transferred to a clean tube and evaporated to dryness under mild nitrogen at 40 ℃. The resultant residue was dissolved in methanol.

Validation of method

The method was validated with respect to selectivity, matrix effect, recovery, linearity, precision, accuracy and stability, in line with the FDA guidelines for validation of bioanalytical methods.

Linearity and quantification

Linear interpolation from the calibration curves was used for calculating the concentrations of the flavonoids in all samples, while LLOQ was used for determination of the lowest concentrations.

Selectivity, matrix effect and recovery

The selectivity was assessed by analyzing 6 blank plasma samples (from different rats) which were spiked with rutin, avicularin, quercitrin and IS, and rat plasma sample treated with PLE (400 mg/kg) for 60 min.

The extraction recoveries and matrix effects of the analytes were performed as described by Sun [28].When the ratios *[(A/B × 100) %]* of the analytes and IS solution ranged from 85 to 115 %, the matrix effect was negligible.

Precision, accuracy and stability

The precision, accuracy and stability were determined according to the method described by Sun [28].

Pharmacokinetic studies

Six Sprague-Dawley rats (weighing 200 ± 20 g) were obtained from Henan Experimental Animal Center (Zhengzhou, China; certificate No: SCXK 2015-0004). All protocols of animal experiments received approval from the Ethics Committee of Animal Research, Henan University of Chinese Medicine (approval ref. No: HNUTCMEKY-201800361Z). The rats were fed in an environment with temperature of 25 ^{o}C, relative humidity of 60 %, under 12-h light/12-h dark cycle for 7 days. Prior to PLE administration, the rats were fasted for 12 h, but were allowed free access to clean drinking water. They were then given single oral administration of PLE (400 mg/kg) dissolved in 0.5 % CMC-Na. Blood samples were collected in heparinized hemostasia tubes 5, 10, 15, 30, 45, 60, 90, 120, 180, 240, 360, and 540 min after oral administration of PLE. The blood samples were centrifuged, and the plasma samples were kept frozen at −20 ^{o}C prior to analysis.

Chromatography and mass spectrometry

Various mobile phase conditions were tried in this study. Methanol, acetonitrile, 0.1% formic acid and water, and their combinations were tested as mobile phases. Finally, a gradient elution with acetonitrile and water containing 0.1% formic acid was selected for optimal peak symmetry, good resolution, and significantly enhanced sensitivity. Total chromatographic analysis was conducted in 11 min, with retention times of 7.76 min for rutin, 9.54 min for avicularin, 9.75 min for quercitrin, and 4.90 min for the IS.

In mass spectrometry (MS), it was found that

Target-SIM mode was best for collecting the molecular ion peaks.

Statistical analysis

All data are presented as mean ± standard deviation (SD). Relevant pharmacokinetic parameters were calculated using DAS3.0 software.

RESULTS

Selectivity, matrix effect, recovery

Selectivity was calculated through comparison of typical chromatograms of blank plasma (A), blank plasma spiked with rutin (10.8 ng/mL), avicularin (45.6 ng/mL), quercitrin (81.2 ng/mL), puerarin (25.0 ng/mL) (B), and plasma sample after oral administration of the PLE for 60 min (C), as shown in Figure 2. The retention times for rutin, avicularin, quercitrin and puerarin (internal standard) were 7.76, 9.54, 9.75 and 4.90 min, respectively. No endogenous interference was observed in full scan mode for each analyte.

In order to effectively remove the interference of the matrix from the analytical results, matrix effect and extraction recovery were determined, and data for all analytes are shown in Table 1. The ratios ranged from 87.79 - 101.61 %, and for IS, the ratio was 94.23 %. These results suggest that absence of endogenous substances and interference in the co-eluting matrix. The extraction recoveries ranged from 86.08 - 106.64 %, which were within acceptable range.

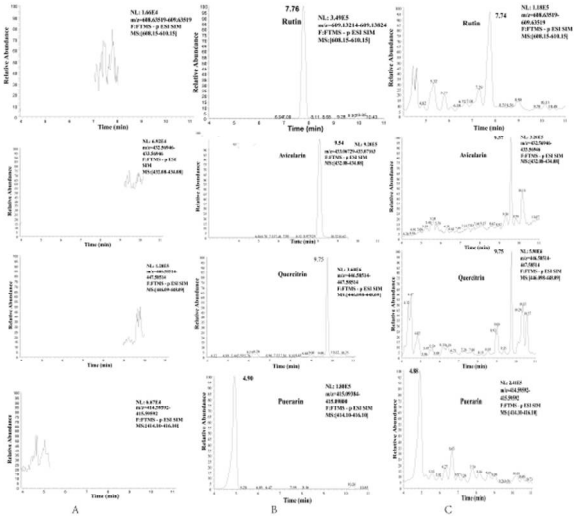

Figure 2: Representative chromatograms of the three analytes and IS in rat plasma. (A) Blank plasma, (B) blank plasma spiked with rutin, avicularin, quercitrin and the IS, and (C) rat plasma sample after oral administration of PLE (400 mg/kg) for 60 min

Linearity and the lower limits of quantitation (LLOQ)

Linear correlation was seen in the concentration ranges of 0.68 - 43.20 ng/mL for rutin, 1.42 - 91.20 ng/mL for avicularin, and 2.54 - 162.40 ng/mL for quercitrin. Linear regressions of the peak area ratios versus concentrations for these analytes in rat plasma are shown in Table 2. The LLOQ for rutin, avicularin and quercitrin were 0.68, 1.42, and 2.54 ng/mL, respectively.

Table 1: Matrix effects and extraction recovery of analytes from rat plasma (n = 6)

Analyte	Spiked concentration (ng/mL)	Matrix effect (mean±SD, %)	Extraction recovery (mean±SD, %)
Rutin	2.70	96.82 ± 3.87	101.91 ± 3.71
	10.80	90.39 ± 6.87	93.40 ± 2.36
	21.60	91.85 ± 3.10	106.64 ± 5.63
Avicularin	5.70	97.08 ± 4.25	101.78 ± 3.30
	22.80	96.91 ± 4.21	87.28 ± 6.28
	45.60	92.43 ± 1.66	94.97 ± 4.83
Quercitrin	10.15	87.79 ± 8.56	86.08 ± 7.87
	40.60	101.61 ± 2.65	99.31 ± 2.48
	81.20	93.96 ± 2.64	99.98 ± 1.90

Table 2: Regression equations for calibration curves and LLOQ

Analyte	y = ax+ b	Linear range (ng/mL)	R2	LLOQ (ng/mL)
Rutin	y=0.0659x+0.0564	0.68-43.20	0.9986	0.68
Avicularin	y=0.0592x+0.0648	1.42-91.20	0.9954	1.42
Quercitrin	y=0.2180x+0.2015	2.54-162.40	0.9989	2.54

Table 3: Precision and accuracy for analytes in rat plasma (n = 6)

Analyte	Spiked concentration (ng/mL)	Intra-day			Inter-day		
		Measured Concentration (ng/mL)	Precision (% RSD)	Accuracy (% RE)	Measured Concentration (ng/mL)	Precision (% RSD)	Accuracy (% RE)
Rutin	2.70	2.76 ± 0.05	1.82	2.35	2.75 ± 0.04	1.61	1.98
	10.80	10.58 ± 0.18	1.68	-2.04	10.58 ± 0.14	1.33	-2.01
	21.60	22.13 ± 0.67	3.01	2.47	22.33 ± 0.63	2.84	3.40
Avicularin	5.70	5.78± 0.37	6.32	1.40	5.79 ± 0.33	5.69	1.54
	22.80	22.08 ± 1.05	4.77	-3.17	22.06 ± 0.91	4.11	-3.24
	45.60	44.38 ±1.96	4.41	-2.67	44.76 ± 1.31	2.93	-1.84
Quercitrin	10.15	10.56 ± 0.96	9.10	4.04	10.51 ± 0.87	8.29	3.57
	40.60	39.42 ±1.02	2.59	-2.91	39.21 ± 1.11	2.84	-3.42
	81.20	78.15 ± 4.29	5.49	-3.76	78.48 ± 3.89	4.96	-3.36

Table 4: Stability of the analytes in rat plasma under different storage conditions (n = 6)

Analyte	Spiked concentration (ng/mL)	Room temperature for 8 h		4 °C temperature for 24 h		Three freeze-thaw cycles at −20 ◦C		Frozen at −20 °C for 30 days	
		Mean ± SD	RE (%)	Mean ± SD	RE (%)	Mean ± SD	RE (%)	Mean ± SD	RE (%)
Rutin	2.70	2.74 ± 0.10	1.4	2.67 ± 0.08	-1.1	2.73 ± 0.13	1.2	2.75 ± 0.15	1.8
	10.80	10.59 ± 0.18	-2.0	10.39 ± 0.12	-2.7	10.33 ± 0.47	-1.4	10.34 ± 0.58	-4.2
	21.60	23.08 ± 1.58	6.8	22.91 ± 1.42	6.1	22.58 ± 1.69	4.5	22.76 ± 1.49	5.4
Avicularin	5.70	5.77 ± 0.05	1.1	5.65 ± 0.27	-1.0	5.60 ± 0.23	-1.8	5.81 ± 0.12	1.9
	22.80	22.24 ± 2.70	-2.5	21.15± 1.77	-7.2	21.37 ± 2.28	-6.3	20.79 ± 0.96	-8.8
	45.60	44.25 ± 2.36	-3.0	44.08 ± 2.73	-3.3	44.88 ± 4.38	-1.6	45.72 ± 4.61	0.3
Quercitrin	10.15	9.92 ± 0.86	-2.3	10.10 ± 1.24	-0.5	9.94 ± 1.38	-2.1	10.01 ± 1.31	-1.4
	40.60	41.63 ± 1.38	2.5	41.44 ± 2.38	2.1	41.92 ± 3.32	3.3	42.22± 3.26	4.0
	81.20	80.20 ± 3.75	-1.2	78.87 ± 5.46	-2.9	79.03 ± 5.63	-2.7	79.24 ± 5.92	-2.4

Precision and accuracy

Data associated with precision and accuracy of the three analytes in QC samples are presented in Table 3. The results indicate that the intra- and inter-day precision (RSD) of these analytes ranged from 1.61 to 9.10 %, while the corresponding REs ranged from −3.76 to 4.04 %. These data demonstrate that the precision and accuracy achieved with this method were acceptable.

Stability

The stability of the three analytes in different conditions are presented in Table 4. The extracted samples were stable under all test conditions, which demonstrates that the samples were stable in typical conditions of conventional pharmacokinetic analysis of the three flavonoids.

Plasma concentration-time profiles for the three flavonoids

The average plasma concentration-time curves of the 3 flavonoids are shown in Figure 3. The area under curve (AUC), mean resident time (MRT), half-life ($t_{1/2}$), peak time (T_{max}), and maximum plasma concentration (C_{max}) were estimated using DAS3.0 software.

Pharmacokinetics of the three flavonoids

The main pharmacokinetic parameters of the three flavonoids are shown in Table 5.

DISCUSSION

Mulberry parasitic is the dry stalk of the mulberry parasitic plant which is used for treatment of rheumatism, weak bones and threatened miscarriage. It is clinically used for rheumatic pain, weak waist and knees, weak bones, and fetal dysphoria. Modern research has found that the chemical components contained in mulberry parasitic are mainly flavonoids, alkaloids, anthraquinones, organic acids, polysaccharides, proteins and lectins, with quercetin, rutin and avicularin as the major flavonoids.

The new method developed in this study was successfully applied in the determination of the concentrations of the three flavonoids after rats

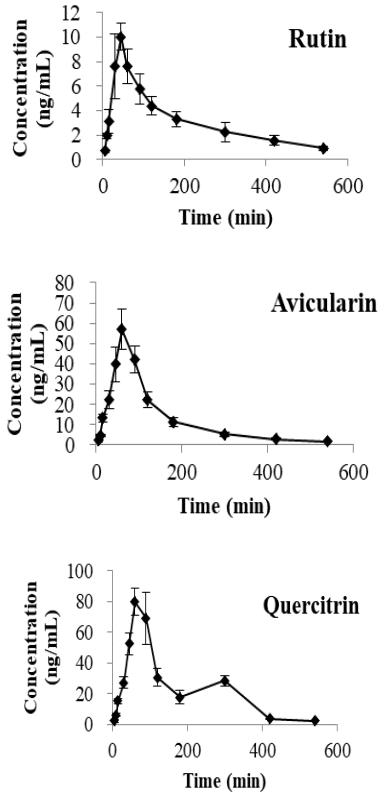

Figure 3: Plasma concentration-time curves of rutin, avicularin, quercitrin after oral administration of parasitic loranthus extract (n = 6)

were treated with PLE. The results indicated that rutin was quickly absorbed within 42 min and had minimum $AUC_{(0-t)}$ and C_{max}. This was mainly due to the fact that rutin was predominantly in its glycosidic form, so defective absorption and intestinal bacteria degradation occurred in the intestinal tract after oral intake [29,30]. The $t_{1/2}$ and T_{max} of avicularin and quercitrin were similar, due to their similar structural features. However, quercitrin had double absorption maxima at about 300 min which was distinctly observed in the concentration-time curve, possibly due to enterohepatic circulation [31]. Another reason might be that quercitrin undergoes metabolism through phase I hydrolysis to quercetin, and quercetin can be converted to quercetin glucuronide [32]. Furthermore, the possible explanation of the double-site absorption of the drug might be that the first peak was for absorption of the active ingredients in stomach, while the second peak was probably due to intestinal absorption.

This study is the first to report pharmacokinetics of rutin, avicularin and quercitrin after oral administration of PLE *in vivo*. The pharmacokinetic differences might be due to the complex constituents of plant extracts, which probably led to drug-drug interactions, with impacts on the pharmacokinetic behavior of each component.

CONCLUSION

A sensitive and selective UHPLC-Q-Exactive-Orbitrap-MS method for the simultaneous determination of rutin, avicularin and quercitrin from parasitic loranthus extract in rat plasma has been developed and validated in this study. These results might be useful in demonstrating possible mechanisms of action of parasitic loranthus. Moreover, the results provide basic understanding of the use of parasitic loranthus in clinical practice.

DECLARATIONS

Acknowledgement

This study was supported by National Natural Science Foundation of China (no. 81473368), the Collaborative Innovation Center for Respiratory Disease Diagnosis and Treatment, and the Chinese Medicine Development of Henan Province financial support. We are grateful to Professor Kai Ma for his routine management and careful maintenance of the UHPLC-Q-Exactive-Orbitrap-MS instrument.

Conflict of interest

No conflict of interest is associated with this work

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this

Table 5: Pharmacokinetic parameters of rutin, avicularin, quercitrin after oral administration of *parasitic loranthus* extract (n = 6).

Analyte	Dose (mg/kg)	$AUC_{(0-t)}$ (min, ng/mL)	$MRT_{(0-t)}$ (min)	$t_{1/2}$ (min)	Tmax (min)	Cmax (ng/mL)
Rutin	3.48	1657.00±200.81	181.64±10.38	201.88±41.03	42.50±6.12	10.35±1.49
Avicularin	4.40	6731.36±573.05	136.67±5.56	128.46±14.39	62.50±14.75	59.16±7.33
Quercitrin	14.96	12274.11±1427.83	171.26±5.61	128.64±17.74	65.00±12.25	80.64±10.61

article will be borne by the authors. Ying Cui supervised the experiments and acquired funding for the research. Ling-lng Li designed and performed the experiments, and wrote the manuscript. Shu Yang assisted in the experiments. Hong-li Wang and Jing Feng bred rats and collected blood. Kai Ma provided routine management and careful maintenance of the UHPLC Q-Exactive Orbitrap HRMS instrument. De-en Han contributed critical review of the manuscript.

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