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Short communication

Simultaneous determination of catechin, epicatechin and epicatechin gallate in rat plasma by LC–ESI-MS/MS for pharmacokinetic studies after oral administration of *Cynomorium songaricum* extract

Qiu-Hong Zhang[a], Wen-Biao Wang[b], Jin Li[a], Yan-Xu Chang[a,*], Yue-Fei Wang[a], Jishu Zhang[b], Bo-Li Zhang[a], Xiu-Mei Gao[a,*]

[a] Tianjin State Key Laboratory of Modern Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin 300193, China
[b] Elion Resources Group Company, Beijing 100031, China

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ABSTRACT

A rapid and valid method was developed for simultaneous determination catechin, epicatechin and epicatechin gallate in rat plasmas using scopoletin (103 ng mL^{-1}) as an internal standard (IS). The separation was performed on Eclipse plus C18 column (100 mm × 4.6 mm, 1.8 μm) at a flow rate of 0.3 mL min^{-1}, and acetonitrile–0.1% formic acid was used as mobile phase. The recoveries of three analytes and IS were more than 78.9%. The lower limits of quantitation (LLOQ) in rat plasma were 2.14, 2.38 and 2.08 ng mL^{-1} respectively for catechin, epicatechin and epicatechin gallate. Intra-day and inter-day precisions were within 12%. The accuracies were more than 85%. After single oral administration of 15.25 g kg^{-1} *Cynomorium songaricum* extract, C_{max} of catechin, epicatechin and epicatechin gallate in rat plasma were respectively 86.69 ± 38.65, 32.57 ± 15.00 and 36.93 ± 12.62 ng mL^{-1} while T_{max} values were respectively 0.15 ± 0.09, 0.20 ± 0.10 and 0.20 ± 0.13 h. The results demonstrated that the present LC–MS/MS method was sensitive enough for pharmacokinetic study of catichins following oral administration of *C. songaricum* extract.

1. Introduction

Cynomorium songaricum Rupr. (Suoyang) which is a medicinal parasitic plant has been used as a Traditional Chinese Medicine to reinforce kidney, nourish Yang, strengthen essence and moisten the intestine (National Commission of Chinese Pharmacopoeia, 2010). The stem of *C. songaricum* could alleviate the symptoms of aging, improve male fertility, and had in vitro estrogenic, anti-dementia, anti-epilepsy, anti-stress, antioxidative, α-glucosidase and HIV-1 protease inhibitory activities [1–5], and so on.

The main catechins that have been isolated and identified from *C. songaricum* included catechin, epicatechin and epicatechin gallate [6,7]. Previous studies have reported that epicatechin might exert neuroprotective properties and prevent neuronal cell death [8,9]. Epicatechin gallate has anticancer effects [10], could inhibit β-catenin signaling and cyclin D1 expression [11]. Catechin could promote adipocyte differentiation [12] and reduce atherosclerotic lesion development [13]. The above studies suggested that catechins were major bioactive components of *C. songaricum* which may be responsible for therapeutic efficacy. Despite its various biological activities, the pharmacokinetic properties of these catechins of *C. songaricum* in animal or human have not been reported.

There were several articles published on the quantification of one or two catechins by HPLC-UV or LC–MS in *C. songaricum* [4,14] and green tea crude drug [15], and coconut water [16]. However, simultaneous pharmacokinetic studies of catechin, epicatechin and epicatechin gallate were scarcely reported after oral administration of *C. songaricum* extract in rats. In the present study, a sensitive LC–MS/MS method with a lower limit of quantification (LLOQ) of about 2 ng mL^{-1} was firstly developed and validated for the simultaneous determination of three catechins (catechin, epicatechin and epicatechin gallate) in rat plasma and their pharmacokinetics after oral administration of *C. songaricum* extract.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (Dikma Technologies Inc, USA) and methanol (Tianjin Concord Science Co. Ltd., Tianjin, China) were of HPLC grade. Catechin, epicatechin, epicatechin gallate and scopoletin (purity > 98%) were purchased from National Institute for the

* Corresponding author at: Tianjin State Key Laboratory of Modern Chinese Medicine, Tianjin University of Traditional Chinese Medicine, Tianjin 300193, China. Tel.: +86 22 59596163; fax: +86 22 59596163.
E-mail addresses: tcmcyx@126.com (Y.-X. Chang), gaoxiumei@tjutcm.edu.cn (X.-M. Gao).

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Control of Pharmaceutical and Biological Products (Beijing, China). Deionized water was purified with a Milli-Q Academic ultra-pure water system (Millipore, Milford, MA, USA).

2.2. LC/MS/MS instrument and analytical conditions

The LC–MS/MS system is composed of an Agilent 1200 series LC system (Agilent Technologies, USA),) consisting of a G1312A binary pump, a vacuum degasser unit (G1322A), a Hip-ALS autosampler (G13678) and an API 3200 triple quadrupole mass spectrometer with an ESI source (Concord, Ontario, Canada). Data acquisition was performed with Analyst 1.4.2 software (AB MDS Sciex).

The chromatographic separations were achieved on an Eclipse plus C18 (4.6 mm × 100 mm, 1.8 μm) column with a security guard C18 (2.1 mm × 12.5 mm, 5 μm) column (Agilent, USA). The mobile phase in HPLC determinations comprised (A) formic acid aqueous solution (0.1%) and (B) acetonitrile using a gradient elution of 20–44% B at 0–8 min, 44–80% B at 8–12 min, 80–20% B at 12–13 min, and the re-equilibration time of gradient elution was 5 min. The flow rate was set at 0.3 mL min^{-1}, the column oven temperature at 25 °C and an injection volume of 10 μL. Mass spectrometry was operated with an optimized spray voltage at −4500 V, temperature at 450 °C, and collision gas of 10 psi. The curtain gas, nebulizer and auxiliary gas were at 30, 50 and 60 psi, respectively. The detection was operated by multiple reaction monitoring (MRM) of the transitions of m/z 288.3 → 109.0 for catechin, 288.9 → 108.8 for epicatechin, 441 → 169.1 for epicatechin gallate and 191.2 → 175.9 for IS, respectively.

2.3. Preparation of herbal materials

1.0 kg *C. songaricum* was extracted with 10 L 60% alcohol under reflux two times each lasting for 2 h. Extract was filtered and concentrated to dryness under reduced pressure. The dried power (383 g) was dissolved with 800 mL water and then precipitated with alcohol, till solution strength of 70% alcohol was achieved. Precipitated polysaccharides were filtered out and the resultant solution was evaporated to dryness (199 g).

2.4. Plasma samples preparation

To a 100 μL aliquot of rat plasma, 10 μL IS (1030 ng mL^{-1} Scopoletin) solution and 1000 μL ethyl acetate were added to extract three catechins from the plasma. The sample was swirled for 1 min, and centrifuged for 10 min at 8000 rpm. Supernatant was transferred into another centrifuge tube and condensed to dryness by using nitrogen. 100 μL 70% methanol solutions were used to redissolve the dried residue. The solution was centrifuged at 14,000 rpm for 10 min, and a 10 μL aliquot of the solution was injected into the LC–MS/MS system for analysis.

2.5. Preparation of standard and quality control (QC) samples

The standard stock solution of catechin (1.07 mg mL^{-1}), epicatechin (1.19 mg mL^{-1}) and epicatechin gallate (1.04 mg mL^{-1}) and IS (1.03 mg mL^{-1}) was prepared separately in methanol and stored at 4 °C. An appropriate volume of each stock solution was mixed together. The mixture was subsequently diluted serially to prepare the reference working solutions.

Quality control (QC) samples of catechin, epicatechin and epicatechin gallate were respectively prepared at low (10.7, 11.9, 10.4 ng mL^{-1}), medium (107, 119, 104 ng mL^{-1}) and high levels (268, 298, 260 ng mL^{-1}) by spiking appropriate standard solutions to blank rat plasma with the required plasma concentrations.

2.6. Method validation

The method was validated in terms of specificity, lower limit of quantification (LLOQ), linearity, accuracy and precision, recovery, matrix effect and stability according to the USFDA guidelines [17] and literature [18]. The precisions were expressed as the relative standard deviations (RSDs). The stability was expressed as percentage of remains.

2.7. Pharmacokinetic study

Eight Sprague–Dawley rats (281 ± 20 g, n = 8) were housed to a cage with unlimited access to food and water except for 12 h before the experiment. Each rat was given *C. songaricum* extract at oral dose of 15.25 g kg^{-1}. Blood samples (about 250 μL) were collected in heparinized 1.5 mL polythene tubes before dosing and at 0.03, 0.08, 0.17, 0.33, 0.5, 0.75, 1, 2, 3, 7 and 12 h after dosing from their fossa orbitalis of rats. Then the samples were centrifugated at 4000 rpm for 10 min at 4 °C. A 100 μL volume of sample of plasma was finally obtained, and was stored at −20 °C until analysis.

2.8. Assay application

Pharmacokinetic parameters were calculated by using the computer program "Drug and Statistics 1.0" (DAS 1.0) (Medical College of Wannan, China) [18].

3. Results and discussion

3.1. Optimization of the chromatographic condition

The above described Eclipse plus C18 (4.6 mm × 100 mm, 1.8 μm) column was proved to be optimal under a gradient elution condition. Based on the fact that the three catechins were polyphenolic, formic acid was selected as mobile-phase modifier to achieve good resolution and sensitive signal of analytes. Meanwhile, a shorter chromatographic time can be obtained when acetonitrile was used as mobile-phase.

3.2. Assaying the dosage of oral administration of three catechins

The contents of catechin, epicatechin and epicatechin gallate were determined by HPLC–MS. The results showed that the dosage of catechin, epicatechin and epicatechin gallate was 106 mg kg^{-1}, 5 mg kg^{-1} and 38 mg kg^{-1}, respectively.

3.3. Method validation

3.3.1. Specificity, calibration curves, linearity and LLOQ

The specificity was assessed by analyzing six different blank plasma samples whether there were interferences at their retention time for analytes and IS. All samples showed that there were no interfering peaks in their peak region which was shown in Fig. 1. The method exhibited a good linearity of catechin, epicatechin and epicatechin gallate over the range of 2.14–428 ng mL^{-1}, 2.38–476 ng mL^{-1} and 2.08–416 ng mL^{-1}, respectively. The equations of the calibration curves were: $y = 0.00072x + 0.000232$ ($r = 0.9919$), $y = 0.00194x + 0.000689$ ($r = 0.9950$) and $y = 0.00237x + 0.00137$ ($r = 0.9936$). The lower limit of quantitations (LLOQ) of catechin, epicatechin and epicatechin gallate in rat plasma was 2.14, 2.38 and 2.08 ng mL^{-1}, respectively. The precision and accuracy of catechin at 2.14 ng mL^{-1} were 9.27% and 93.1%, epicatechin at 2.38 ng mL^{-1} were 11.7% and 104% and epicatechin gallate at 2.08 ng mL^{-1} were 1.48% and 103%, respectively.

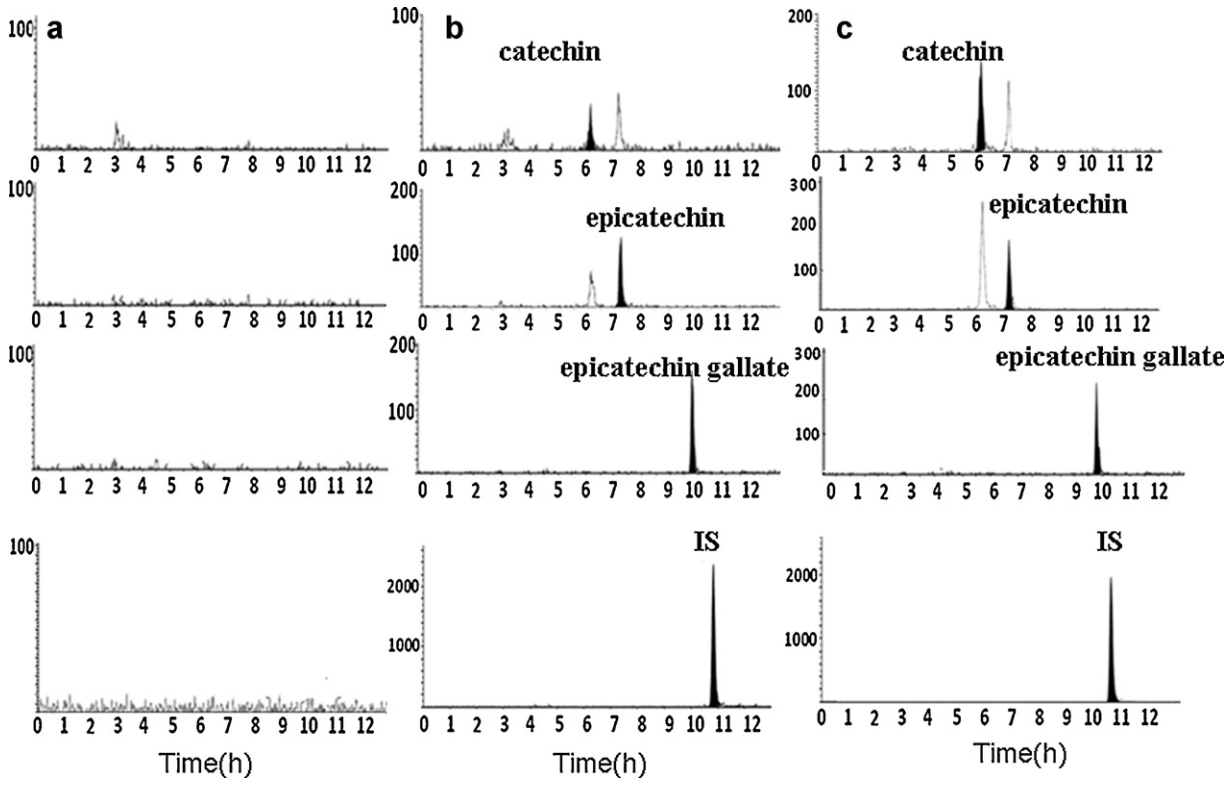

Fig. 1. MRM chromatograms of (a) blank rat plasma, (b) blank rat plasma spiked with catechin (2.14 ng mL^{-1}), and epicatechin (2.38 ng mL^{-1}), epicatechin gallate (2.08 ng mL^{-1}) and scopoletin (IS) at 103 ng mL^{-1}, and (c) real sample 30 min after oral administration of 15.25 g kg^{-1} *C. songaricum* extract.

3.3.2. Precision and accuracy

The values for inter-day and intra-day precision and accuracy are shown in Table 1. It can be seen that RSDs of inter-day and intra-day are less than 12% and accuracy is more than 85%. For the internal standard, the RSDs of inter-day and intra-day are 7.2% and 8.2%, respectively.

3.3.3. Stability

Autosampler, freeze/thaw and long-term stability data are summarized in Table 2. The data indicate that all analytes are stable in rat plasma at room temperature for 12 h, under freeze/thaw cycles and at −20 °C for one month. The effects of autosampler conditions on IS stability in plasma were evaluated. It was found that the RSD of IS was 7.3% for 24 h at room temperature.

3.3.4. Extraction recovery and matrix effect

The extraction recoveries of catechin, epicatechin and epicatechin gallate at different level of concentrations are shown in Table 2. The extraction recoveries of three level QC samples are more than 78.9%. The extraction recovery of IS was 94.6 ± 3.5%. The matrix effect of blank plasma of catechin, epicatechin and epicatechin gallate was found to be within the acceptable range; all values were more than 71.5% (Table 2). The matrix effect of IS was 108 ± 3%. Thus, it was demonstrated that the plasma matrix effect was negligible for the assay.

3.4. Pharmacokinetic study

The plasma drug–time curve of catechin, epicatechin and epicatechin gallate is presented in Fig. 2 and pharmacokinetic parameters are shown in Table 3. From this table, C_{max} of catechin, epicatechin and epicatechin gallate in rat plasma are 86.69 ± 38.65, 32.57 ± 15.00 and 36.93 ± 12.62 ng mL^{-1} while T_{max} values are 0.15 ± 0.09, 0.20 ± 0.10 and 0.20 ± 0.13 h, respectively. It was demonstrated that three catechins were rapidly absorbed in rat plasma after oral administration of *C. songaricum* extracts.

Table 1
Intra-day, inter-day accuracy and precision of catechin, epicatechin and epicatechin gallate (n = 6).

Compound	Concentration (ng L^{-1})	Intra-day		Inter-day	
		Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Catechin	10.7	103	7.4	85.9	5.0
	107	97.1	5.8	108	5.8
	268	104	3.9	106	2.5
Epicatechin	11.9	107	9.4	90.5	7.2
	119	101	9.8	102	5.9
	298	103	6.0	103	3.8
Epicatechin gallate	10.4	91.7	10	104	8.5
	104	94.7	12	102	4.1
	260	101	5.9	106	4.3

Table 2
Recoveries, matrix effects and stability of catechin, epicatechin and epicatechin gallate ($n = 6$).

Compound	Concentration ($ng\,mL^{-1}$)	Recovery		Matrix effect		Freeze–thaw cycles		At −20 °C for 1 month		Autosampler for 24 h	
		Average (%)	RSD (%)	Average (%)	RSD (%)	Remains (%)	RSD (%)	Remains (%)	RSD (%)	Remains (%)	RSD (%)
Catechin	10.7	102	2.8	101	3.1	95.3	6.5	88.8	14.7	115	8.9
	107	97.2	7.9	94.4	4.7	96.2	7.8	112	4.6	115	4.0
	268	98.3	3.1	88.5	5.2	96.6	4.2	101	11.3	109	5.1
Epicatechin	11.9	98.0	2.2	93.2	3.7	99.2	7.4	95.0	2.6	115	4.4
	119	95.7	8.1	96.1	8.6	97.4	11	112	4.2	112	3.3
	298	89.0	3.9	93.1	6.9	98.4	1.7	100	6.5	109	4.9
Epicatechin gallate	10.4	78.9	6.7	111	5.1	100	7.3	91.3	10.5	101	11
	104	82.0	3.3	79.6	1.1	103	5.7	106	2.4	115	4.7
	260	87.3	1.2	71.5	2.5	98.1	4.9	107	7.2	88.8	4.1

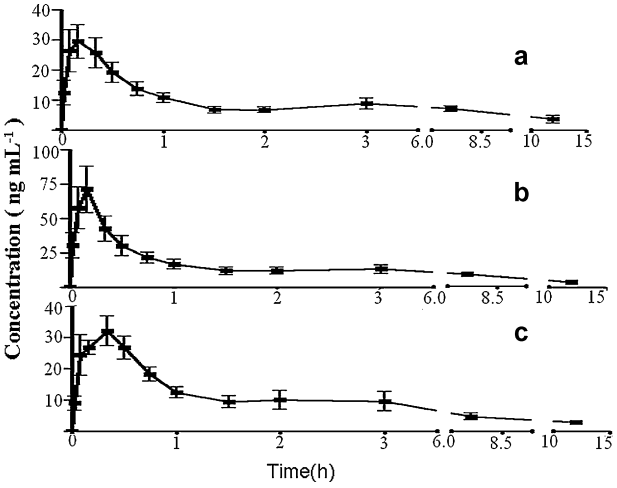

Fig. 2. Mean plasma concentration versus time profiles in rats after oral administration of 15.25 g kg^{-1} *C. songaricum* extract (a) catechin, (b) epicatechin, (c) epicatechin gallate.

Table 3
Pharmacokinetic parameters of catechin, epicatechin and epicatechin gallate after oral administration of 15.25 g kg^{-1} *C. songaricum* extract ($n = 8$, mean ± SD).

Parameters	Catechin	Epicatechin	Epicatechin gallate
C_{max} ($ng\,mL^{-1}$)	86.69 ± 38.65	32.57 ± 15.00	36.93 ± 12.62
T_{max} (h)	0.15 ± 0.09	0.20 ± 0.10	0.20 ± 0.13
$T_{1/2\alpha}$ (h)	0.33 ± 0.12	0.60 ± 0.34	0.51 ± 0.36
$T_{1/2\beta}$ (h)	6.38 ± 4.20	34.86 ± 25.30	4.70 ± 4.24
AUC_{0-12} (ng h/mL)	109.7 ± 57.0	67.66 ± 16.99	72.44 ± 49.93
$AUC_{0-\infty}$ (ng h/mL)	154.0 ± 97.2	214.0 ± 161.7	91.01 ± 67.99
MRT_{0-12} (h)	3.98 ± 0.33	4.54 ± 0.66	3.85 ± 0.78

4. Conclusion

In the present study, a convenient, rapid and reliable LC–MS/MS method was described for the determination of catechin, epicatechin and epicatechin gallate in rat plasma. The basic advantage of this method was that the lower limits of quantifications (LLOQs) of three analytes were about 2 ng mL^{-1}. The validated method was applied to evaluate the pharmacokinetics of catechin, epicatechin and epicatechin gallate following oral administration of *C. songaricum* extract for the first time.

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