Journal of Ethnopharmacology 169 (2015) 145–155

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

UPLC-MS/MS determination and gender-related pharmacokinetic study of five active ingredients in rat plasma after oral administration of *Eucommia cortex* extract

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ARTICLE INFO

Article history:
Received 26 September 2014
Received in revised form
8 April 2015
Accepted 10 April 2015
Available online 22 April 2015

Chemical compounds studied in this article:
Pinoresinol di-O-β-d-glucopyranoside (PubChem CID:44202790)
Geniposide (PubChem CID: 107848)
Geniposidic acid (PubChem CID: 443354)
Aucubin (PubChem CID: 91458)
Chlorogenic acid (PubChem CID: 1794427)
Pinoresinol (PubChem CID: 73399)
Epipinoresinol (PubChem CID: 637584)
Syringaresinol (PubChem CID: 443023)
Medioresinol (PubChem CID: 181681)
Rutin (PubChem CID: 5280805)

Keywords:
Eucommia cortex
Pinoresinol di-O-β-d-glucopyranoside
Geniposide
Geniposidic acid
Aucubin
Chlorogenic acid
UPLC-MS/MS
Rat plasma
Pharmacokinetics

ABSTRACT

Ethnopharmacological relevance: *Eucommiae cortex* (EC), the bark of *Eucommia ulmoides* Oliv., has been traditionally used to treat many diseases in China for more than 2000 years. The pharmacological effects are primarily attributed to the presence of lignans, iridoids and phenolics, which are main active ingredients in EC.

Aim of the study: First, to investigate the active ingredients that can be absorbed into the rat plasma according to which ingredients exhibit significant correlation of drug concentration–time curve. Second, to establish an efficient ultra-performance liquid chromatography/tandem mass spectrometry (UPLC-MS/MS) method for simultaneous determination of ingredients absorbed in rat plasma. Finally, to investigate gender effect on the pharmacokinetics of the ingredients absorbed in male and female rats plasma after oral administration with EC extract.

Materials and methods: 18 ingredients from EC were detected by UPLC-MS/MS, 9 out of 18 ingredients were absorbed into rat plasma. And 5 ingredients exhibit significant correlation of drug concentration–time curve. They were pinoresinol di-O-β-d-glucopyranoside (PDG), geniposide (GE), geniposidic acid (GA), aucubin (AN) and chlorogenic acid (CA). The analytes were extracted from rat plasma via a simple protein precipitation procedure and osalmid was used as the internal standard. Chromatographic separation was achieved on a Waters ACQUITY HSS T3 column (2.1 mm × 100 mm, 1.8 μm) using a gradient elution program with acetonitrile and 0.1% formic acid water as the mobile phase, with a flow rate of 0.3 mL min^{-1}. The detection was performed on a triple-quadrupole tandem mass spectrometer by multiple reactions monitoring (MRM) mode in a positive ion mode via electrospray ionization (ESI). The transition monitored were /*z* 683.00$[M+H]^+ \rightarrow$235.10 for PDG, / *z* 389.00$[M+H]^+ \rightarrow$208.80 for GE, *m*/*z* 375.00$[M+H]^+ \rightarrow$194.79 for GA, *m*/*z* 364.00$[M+NH_4]^+ \rightarrow$148.81 for AN, *m*/*z* 355.10$[M+H]^+ \rightarrow$162.84 for CA and *m*/*z* 230.03$[M+H]^+ \rightarrow$120.77 for internal standard.

Results: The developed method showed good linearity over a wide concentration range, the lower limits of quantification and higher accuracy and precision for determination of the 5 analytes. Then the method was applied to study the pharmacokinetics in rats, and the results indicated that there were significant differences in pharmacokinetic parameters of the analytes between the male and female rats, and absorptions of these analytes in male group were all significantly higher than those in female group.

Conclusion: This study established an efficient, sensitive and selective UPLC-MS/MS method for simultaneous determination of the five ingredients in rat plasma, and it could be successfully applied to the comparative pharmacokinetic studies in male and female rats after oral administration with EC extract.

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1. Introduction

Eucommia ulmoides Oliv. (Chinese, Du-zhong; Korean, Tu-chung; Japanese, Tochu) is an elm-like deciduous, precious tree native to

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http://dx.doi.org/10.1016/j.jep.2015.04.007
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China, which is the sole member of the *Eucommiaceae* family (Du, 1996; Li and Du, 2001). It has been widely used in China, Japan, Korea and some other countries (Lee et al., 2005; Luo et al., 2010). *Eucommiae cortex* (EC), the bark of *Eucommia ulmoides* Oliv., is acted either as a single herb or in combination with some other herbs (Du et al., 2009). It is originaly recorded in the Chinese medical classic Shen Nong Ben Cao Jing and currently listed in Chinese Pharmacopoeia (2010). It has been tradionally used to nourish liver and kidney, strengthen muscles and bones, and sooth the fetus (Chinese Pharmacopoeia Commission, 2010; Chien, 1957). Modern pharmacological studies have shown that EC possesses various activities such as antihypertensive (Luo et al., 2010), antioxidant (Xu et al., 2010), antimicrobial (Tsai et al., 2010), anti-inflammatory (Tsai et al., 2010), neuroprotective (Kwon et al., 2011) activity and inhibitory effect on osteoclast and osteoblastic apoptosis (Zhang et al., 2009).

The extensive pharmacological activities can be attributed to the various active ingredients in EC. Lignans, iridoids, phenolics, steroid, terpenoid, flavonoids and others have been isolated from EC (He et al., 2014). Pinoresinol di-O-β-d-glucopyranoside (PDG) is one of lignans and has been proved to possess antihypertensive, anticomplement activity, and estrogenic property (Deyama et al., 1988; Oshima et al., 1988; Shi et al., 2013; Sih et al., 1976; Wang et al., 2011). Geniposide (GE), Geniposidic acid (GA) and Aucubin (AN) are three iridoid glycoside compounds and usually found in EC. GE has anti-inflammatory, antithrombotic and antitumor activitiy (Isiguro et al., 1986; Suzuki et al., 2001). GA has been reported to have antihypertensive, anti-aging and neuroprotective effect (Deyama et al., 2001; Li et al., 1998; Zhou et al., 2009). AN has been shown to possess anti-inflammatory, anti-aging, antibacterial and estrogenic property (Li et al., 1998; Wang et al., 2011; Zheng et al., 2012a, 2012b). Chlorogenic acid (CA) is a kind of phenolics, and has been demonstrated to exhibit neuroprotective, antibacterial, antioxidative and antimutagenic activity (Dai et al., 2013; Zhang et al., 2001; Zhou et al., 2009).

Previous studies suggest that lignans, iridoids and phenolics are the important constituents contributing to the pharmacological efficacies of EC. Therefore, to characterize the pharmacokinetics of them will be useful and informative for assessing fully the pharmacological efficacies of EC. Several LC–MS methods have been developed to separately determine PDG (Wang et al., 2012), GA (Zheng et al., 2012a, 2012b) and AN (Xu et al., 2012) in rat plasma. However, there is almost no report on LC-MS method for the pharmacokinetic study of simultaneous determination of lignans, iridoids and phenolics in rat plasma after administration of EC extract.

In this paper, based on analyzing the bioactive ingredients absorbed into the rat plasma following the method for determination of 18 ingredients from lignans, iridoids and phenolics in EC, we screened out five ingredients, PDG, GE, GA, AN and CA exhibited significant correlation of drug concentration–time curve in rat plasma. An efficient and sensitive UPLC-MS/MS method was developed by optimizing the extraction, separation and analytical conditions for simultaneous determination of the five ingredients in rat plasma after oral administration with EC extract. Furthermore, the established and optimized method was applied to investigate the pharmacokinetics in male and female rats, and to evaluate whether gender affects the absorption of the 5 ingredients from EC. It was the first study with detailed pharmacokinetic characterizations for lignans, iridoids and phenolics of EC extract. The results of this study would be helpful for improving clinical therapeutic efficacy and further pharmacological studies of EC.

2. Experimental section

2.1. Materials, reagents and animals

EC was purchased from the Sichuan Xinghehua TCM Electuary Co., Ltd. (Sichuan, China) and identified by Prof. Li-hong Wu, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine. Standards substances of pinoresinol-di-O-β-D-glucopyranoside (63902-38-5) and osalmide (OS, 20100609) (purity $>99\%$) were purchased from the National Institute for Food and Drug Control (Beijing, China). Geniposide (24512-63-8), geniposidic acid (27741-01-1), phenolics (327-97-9), rutin (153-18-4), quercetin (117-39-5), astragalin (480-10-4) and kaempferol (520-18-3) (purity $>99\%$) were purchased from the Standardization of Chinese Medicine Research Center in Shanghai. Pinoresinol-4-O-β-D-glucopyranoside (69251-96-3), pinoresinol (487-36-5) (purity $>98\%$) were purchased from Yunnan Xili Technology Co., Ltd. Aucubin (479-98-1) and 5-hydroxymethyl furfural (67-47-0) (purity $>98\%$) were purchased from Shanghai Yongheng Biological Technology Co., Ltd. Syringaresinol, medioresinol, epipinoresinol, lirioresinol-A, coniferyl aldehyde, sinspslfrhyde, vanillic acid and pyromucic acid were obtained from our laboratory preparation (purity $>98\%$).

HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). HPLC-grade water (>18 mΩ) was obtained from a Milli-Q water purification system (Bedford, MA, USA). HPLC-grade formic acid was purchased from Tedia Company Inc. (Fairfield, OH, USA). Ethanol absolute was purchased from Tianjin Kermel Chemical Reagent Co., Ltd (Kermel, AR).

Sprague–Dawley rats, weighing 250 ± 10 g (male) and 210 ± 10 g (female) (certificate no. SYXK (Shanghai) 2009-0069), were obtained from SLAC Lab Animal Ltd. (Shanghai, China) and housed by the Animal Center of Shanghai University of Traditional Chinese Medicine. The rats were housed in an air-conditioned room at temperature of 22 ± 2 °C and a relative humidity of $50\pm10\%$ with a 12 h dark–light cycle (light on from 7:00 to 19:00) and allowed food and water spontaneously. The animals were acclimatized to the facilities for 7 days and then were kept under fasting with free access to water 12 h before the experiments. The animal study was conducted in accordance with the Institute's Guide for the Care and Use of Laboratory Animals and was approved by the Ethical Committee of Shanghai University of Traditional Chinese Medicine (approval no.: ACSHU-2011-056, approved in 11 November, 2011).

2.2. Apparatus and operation conditions

Liquid chromatography condition: the separation was performed on an Acquity UPLC/TQ-2000 system (Waters Corp., Milford, MA, USA) using an Acquity UPLC HSS T3 column (100×2.1 mm^2, i.d., 1.8 μm, Waters, USA). After optimizing the chromatographic conditions, the column temperature was adjusted to 35 °C and the autosampler was conditioned at 4 °C. 5 μL sample or standard solution was injected into the system for analysis. The mobile phase were composed of 0.1% formic acid aqueous solution (A) and acetonitrile (B) at a flow rate of 0.3 mL min^{-1}. (1) The gradient elution program for 18 ingredients: to explore the active ingredients that could be absorbed into the rat plasma, using a simple linear gradient elution program that started at 10% B, then was increased to 90% B in 4 min. Next, the percentage of B was reversed to the original condition at the time point of 4 min and held at the level for 2 min. (2) The gradient elution program for 5 ingredients: PDG, GE, GA, AN and CA were proved to be absorbed into the rat plasma and screened out as the ingredients of EC for further pharmacokinetic studies, using a gradient started at 10–18% B at 0–2.0 min; 18–18% B at 2.0–3.0 min; 60–60% B at 3.0–5.0 min; 90–90% B at 5.0–6.0 min, and the re-equilibration time of gradient elution was 2 min.

Mass spectrometric condition: the mass spectrometric detection was carried out on an Acquity™ triple-quadrupole-2000 tandem mass spectrometer (Waters Corp., Milford, MA, USA) with an electrospray ionization (ESI) interface set in positive or negative ionization mode. ESI-MS/MS was operated in positive ion mode (ESI$^+$) for the

analyses of syringaresinol (SL), lirioresinol-A (LA), pinoresinol (PL), epipinoresinol (EPL), medioresinol (ML), geniposide (GE), aucubin (AN), chlorogenic acid (CA), sinspslfrhyde (SE), rutin (RN), quercetin (QN), astragalin (ASN), kaempferol (KL), 5-hydroxymethyl furfural (5-HF), in negative ion mode (ESI^-) for pinoresinol-4-O-β-D-glucopyranoside (PG) and coniferyl aldehyde (CE). The ESI-MS/MS of pinoresinol-di-O-β-D-glucopyranoside (PDG) and geniposidic acid (GA) could be operated both in ESI^+ and ESI^-. (1) The MS condition for 18 ingredients: qualitative analysis of the active ingredients that could be absorbed into rat plasma was performed by monitoring $[M+H]^+$ or $[M-H]^-$ for 18 ingredients in multiple reaction monitoring (MRM) mode, as shown in Table 1. (2) The MS condition for 5 ingredients: quantification analysis (pharmacokinetics) of the five ingredients was further optimized and finally performed in ESI^+ using MRM mode of precursor-product ion transitions at *m/z* 683.00 $[M+H]^+ \rightarrow$235.10 for PDG, *m/z* 389.00$[M+H]^+ \rightarrow$208.80 for GE, *m/z* 375.00$[M+H]^+ \rightarrow$194.79 for GA, *m/z* 364.00$[M+NH_4]^+ \rightarrow$148.81 for AN, *m/z* 355.10$[M+H]^+ \rightarrow$162.84 for CA and *m/z* 230.03$[M+H]^+ \rightarrow$120.77 for osalmide (OE) as internal standard. The optimized MS parameters were as follows: capillary voltage, 3.0 kV; cone voltage, 30 V; extracter voltage, 3 V; RF lens voltage, 0.1 V. The source temperature and desolvation temperature were set at 120 and 350 °C, while desolvation gas (nitrogen), cone gas (nitrogen) and collision gas (argon) flow were 500 L h^{-1}, 50 L h^{-1} and 0.15 mL min^{-1}, separately. The cone voltage and collision voltage varied with different analytes. All data were acquired in centroid mode and processed using Masslynx 4.1 software (Waters Corporation, Milford, MA, USA).

2.3. Preparation of EC extract

For preparation of the extract, the dried powder of EC (500 g) was extracted under reflux with 4000 mL ethanol–water (75:25, v/v) for three times, 3 h each time, and then filtrated. The combined filtrate was evaporated to dryness and the residue was reconstituted in 250 mL 5% ethanol–water to get a concentration equivalent to 2 g mL^{-1} of EC. In order to calculate the administered dose, the contents of the five ingredients in administration solution were quantitatively determined. The contents of PDG, GE, GA, AN and CA of EC extract were 6.61, 1.04, 4.74, 0.27 and 0.38 mg g^{-1}, respectively.

2.4. Preparation of standard and quality control samples

Stock solutions were prepared by dissolving PDG (4.84 mg), GE (5.40 mg), GA (5.70 mg), AN (5.12 mg), CA (5.00 mg) and OE (internal standard, 5.07 mg) into methanol to yield a concentration of about 0.5 mg mL^{-1}. A mixed stock solution containing 19.36 μg mL^{-1} of PDG, 20.16 μg mL^{-1} of GE, 22.80 μg mL^{-1} of GA, 20.48 μg mL^{-1} of AN and 20.00 μg mL^{-1} of CA was prepared in methanol. A series of working standard solutions were prepared by successive dilution of the mixed stock solution with methanol. OE was used as the internal standard (IS), and was dissolved in acetonitrile to produce a 150 ng mL^{-1} IS stock solution. Calibration standards were prepared by spiking 10 μL of the appropriate standard working solutions into 50 μL blank plasma to yield calibration concentrations of 4.84, 9.68, 24.20, 48.40, 72.60, 121.00, 242.00, 726.00 and 968.00 ng mL^{-1} for PDG; 5.40, 10.80, 27.00, 54.00, 135.00, 270.00, 810.00 and 1080.00 ng mL^{-1} for GE; 5.70, 11.40, 28.50, 57.00, 142.50, 285.00, 855.00 and 1140.00 ng mL^{-1} for GA; 5.12, 10.24, 25.60, 51.20, 128.00, 256.00, 768.00 and 1024.00 ng mL^{-1} for AN and 5.00, 10.00, 25.00, 50.00, 125.00, 250.00 750.00 and 1000.00 ng mL^{-1} for CA. All the stock and working solutions were stored at 4 °C and brought to room temperature before use. Quality control (QC) samples were prepared separately in the same process. The QC samples were prepared at 9.68, 242.00 and 968.00 ng mL^{-1} for PDG; 10.80, 270.00 and 1080.00 ng mL^{-1} for GE; 11.40, 285.00 and 1140.00 ng mL^{-1} for GA; 10.24, 256.00 and 1024.00 ng mL^{-1} for AN; and 10.00, 250.00 and 1000.00 ng mL^{-1} for CA.

2.5. Preparation of plasma samples

The plasma samples were frozen and stored at −20 °C in the refrigerator and were prepared to thaw at room temperature by vortex before use. Aliquots of 10 μL of IS was spiked into 50 μL of plasma sample, vortexed for 1 min and let it stand for 10 min. Then, 140 μL of acetonitrile was added to the mixture in order to precipitate protein, vortexed for approximately 1 min, and then allowed to stand for 30 min. Thus, the precipitate was removed by centrifugation at 13,000 rpm for 15 min. The supernatant was dried with a stream of nitrogen gas at room temperature. The residue was re-dissolved in 50 μL of acetonitrile/water (20:80, v/v) and vortexed again for 1 min. The solution was then centrifuged at 13,000 rpm at 4 °C for 15 min, and 5 μL supernatant was injected into the UPLC-MS/MS system for analysis. All samples were processed in different tunes rendered with equal skill correspondingly.

2.6. Method validation

The established method was validated according to the Food and Drug Administration (FDA) guidance for bioanalytical method validation, including linearity, matrix effect, selectivity, precision, accuracy, recovery and stability (US Food and Drug Administration, 2001).

Selectivity is the ability of an analytical method to differentiate and quantify the analytes in the presence of other components in the sample. In this paper, the selectivity was ascertained by comparatively analyzing blank plasma samples from six individual rats, corresponding blank plasma spiked with the five analytes and IS, and the plasma samples from the rats after oral administration of EC extract at a dose of 50 g kg^{-1} body weight.

The calibration curves were constructed by plotting the peak area ratio versus the concentration of the five analytes with linear regression using standard plasma samples at seven concentrations. Sensitivity was evaluated by determining limit of detection (LOD) and limit of quantification (LOQ). LOD and LOQ were determined using the signal-to-noise ratio (S/N) of 3:1 and 10:1, respectively. The LOQ was defined as the lowest analytical concentration of the calibration curve at which the measured precision expressed as relative standard deviation (RSD) was within 20% and the accuracy expressed as relative error (RE) was in the range of −20% to +20%.

The intra- and inter-day precision and accuracy were evaluated by the determination of QC samples at three concentration levels (LQC, MQC and HQC) of the five analytes in six replicates on the same day and on three different days, respectively. The precision was defined as relative standard deviation (RSD) of the measured concentration and the accuracy as the relative error (RE) of the measured mean value deviated from the nominal value.

The extraction efficiency of the five analytes was determined by analyzing six replicates of plasma samples at LQC, MQC and HQC levels. The recovery was evaluated by comparing the peak areas of the five analytes from the QC samples with those obtained from blank plasma samples with the five analytes spiked into the post-extraction supernatant. The matrix effect was due to a possible ionization competition between the analytes and the components in the sample matrix, and it was evaluated by comparing the peak areas obtained from samples with the five analytes spiked into the post-extraction supernatant with those from pure standard solutions containing equivalent concentrations of the five analytes at LQC, MQC and HQC levels. The extraction recovery and the matrix effect were similarly evaluated for IS at one concentration.

Table 1
The molecular weight, ionization mode, monitoring ion, parent ion, daughter ion, cone voltage, collision voltage, dwells, retention time and classification of 18 constituents of EC extract.

Analytes	Molecular weight	Ionization mode	Monitoring ion	Parent ion	Daughter ion	Cone (V)	Collision (eV)	Dwells	Retention time (min)	Classification
pinoresinol-di-O-β-D-glucopyranoside (PDG)	682	ESI^-	$[M-H]^-$	681.36	519.22	40	15	0.10	1.80	Lignans
pinoresinol-4-O-β-D-glucopyranoside (PG)	520	ESI^-	$[M-H]^-$	519.55	357.16	35	16	0.10	2.25	Lignans
Syringaresinol (SL)	418	ESI^+	$[M+H]^+$	419.16	265.02	15	7	0.05	2.89	Lignans
Lirioresinol-A (LA)	418	ESI^+	$[M+H]^+$	419.16	264.98	20	8	0.05	3.00	Lignans
Pinoresinol (PL)	358	ESI^+	$[M+H]^+$	359.33	341.21	15	5	0.10	3.14	Lignans
Epipinoresinol (EPL)	358	ESI^+	$[M+H]^+$	359.10	341.20	15	8	0.10	3.02	Lignans
Medioresinol (ML)	388	ESI^+	$[M+H]^+$	389.19	371.18	18	8	0.05	2.95	Lignans
Geniposide (GE)	388	ESI^+	$[M+H]^+$	388.93	208.85	12	10	0.10	1.88	Iridoids
Geniposidic acid (GA)	374	ESI^-	$[M+H]^+$	372.80	210.90	40	12	0.10	1.41	Iridoids
Aucubin (AN)	346	ESI^+	$[M+H]^+$	347.30	285.36	48	28	0.10	3.79	Iridoids
Chlorogenic acid (CA)	354	ESI^+	$[M+H]^+$	355.08	162.65	15	12	0.10	1.77	Phenolics
Sinspslfrhyde (SE)	208	ESI^+	$[M+H]^+$	208.82	176.72	35	18	0.05	2.70	Phenolics
Coniferl aldehyde (CE)	178	ESI^-	$[M-H]^-$	176.72	161.77	28	14	0.10	2.75	Phenolics
Rutin (RN)	610	ESI^+	$[M+H]^+$	610.89	302.99	28	25	0.10	2.04	Flavonoids
Quercetin (QN)	302	ESI^+	$[M+H]^+$	302.93	152.67	50	35	0.05	2.88	Flavonoids
Astragalin (ASN)	448	ESI^+	$[M+H]^+$	449.00	286.90	23	10	0.15	2.28	Flavonols
Kaempferol (KL)	286	ESI^+	$[M+H]^+$	286.90	152.60	55	33	0.05	3.19	Flavonols
5-hydroxymethyl furfural (5-HF)	126	ESI^+	$[M+H]^+$	126.70	108.60	30	10	0.15	1.61	Others

The stability of the five analytes in samples including freeze–thaw stability (three freeze at −20 °C and thaw cycles), long-term stability (storage for 2 weeks at −20 °C), room temperature stability (storage for 12 h at ambient temperature), and post-preparation stability (storage for 8 h after sample preparation at 4 °C) was tested at LQC, MQC, HQC levels with six replicates at each level. All stability testing QC samples were determined by using the calibration curves of freshly prepared standard samples.

2.7. Pharmacokinetic study

Six male Sprague–Dawley rats (250 g ± 10 g) and six female Sprague–Dawley rats (210 g ± 10 g) were divided into two groups according to sex before treatment. The animals were kept under fasting for 12 h and fed with water prior to the administration of the EC extract at a dose of 50 g kg^{-1} (equivalent to 330.4 mg kg^{-1} of PDG, 52.0 mg kg^{-1} of GE, 237.0 mg kg^{-1} of GA, 13.5 mg kg^{-1} of AN and 18.8 mg kg^{-1} of CA). The animals had free access to water during the experiment. Blood samples (about 300 μL) were collected in heparinized tubes via the postorbital venous plexus veins from each rat before administration and 0.03, 0.08, 0.17, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 12, 24, 36 h after administration. After centrifugation at 13,000 rpm for 15 min, plasma samples were separated and frozen at −20 °C until analysis. All experimental procedures were approved by the Ethics Committee of Shanghai University of Traditional Chinese Medicine.

Plasma concentrations of the five ingredients were measured using accompanying calibration curves run with each batch of samples. The pharmacokinetic parameters of the five analytes were calculated by the non-compartmental analysis of plasma concentration versus time data using the "PKSOLN 2.0" software package (Montrose, USA). The maximum plasma concentration (C_{max}) and the time of maximum plasma concentration (T_{max}) were observed directly from the measured data. The elimination rate constant (K_e) was calculated by linear regression of the terminal points in a semilog plot of the plasma concentration against time and the elimination half-life ($t_{1/2}$) was calculated using the formula: $t_{1/2}=0.693/K_e$. The area under plasma concentration–time curve to the last measurable plasma concentration (AUC_{0-t}) was estimated by using the linear trapezoidal rule. The area under the plasma concentration–time curve to time infinity ($AUC_{0-\infty}$) was calculated as $AUC_{0-\infty}=AUC_{0-t}+C_t/K_e$. Clearance (CL) was calculated by dividing dose by AUC_{0-t}. Value of mean resident time (MRT) stood for the time of 63.2% drug eliminated from body and was calculated by $AUMC_{0-\infty}/AUC_{0-\infty}$. Volume of distribution (V_d) was theoretical volume that drug would occupy and was calculated to divide total amount of drug in the body by drug plasma concentration. All results were expressed as arithmetic mean ± standard deviation (SD).

3. Results and discussion

3.1. Screening of the ingredients absorbed into rat plasma

To explore which active ingredients of EC could be absorbed into the rat plasma, the UPLC-MS/MS method with electro-spray ionization (ESI) mode for simultaneous determination of 18 kinds of active ingredients of EC extract has been established (Table 1). Typical MRM chromatograms of blank plasma spiked with the mix standard (500 ng mL^{-1}) that contained 18 ingredients of EC and the blank rat plasma are shown in Fig. 1. As shown in Fig. 1, the analysis using MRM function was highly selective with no interfering endogenous substances. Since PL and EPL, SL and LA were two pairs of optical isomers, double peaks appeared in the channels of PL, EPL, SL and LA. The retention time of PL, EPL, SL and LA were 3.16, 3.02, 2.89 and 3.00 min, respectively. MRM chromatograms of the active ingredients that could be detected in the plasma samples at 0.083 h, 0.5 h and 4 h after single oral administration of EC extract in rats are shown in Fig. 2(A)–(C), respectively. As shown in Fig. 2(A), there were 9 ingredients, namely AN, EPL (RT: 3.00 min), SL (RT: 2.89 min), GE, 5-HF, CA, PG, PDG and GA, could be detected in the rat plasma at 0.083 h after administration of EC extract. However, the contents of EPL and PG were below the LOQ in rat plasma till 0.5 h after administration. With the passage of time, the content of SL dropped to the LOQ at 4 h after oral administration. The ingredients that always could be detected in rat plasma till 8 h after administration were AN, GE, 5-HF, CA, PDG and GA. In summary, considering the integrity of the concentration–time curves of the detected ingredients in rat plasma, AN, GE, CA, PDG and GA were selected as the ingredients for pharmacokinetic studies (5-HF is unrepresentative for EC).

3.2. Mass spectra

The first step in developing the method was to select precursor ions and product ions of the analytes and IS for MRM mode analysis

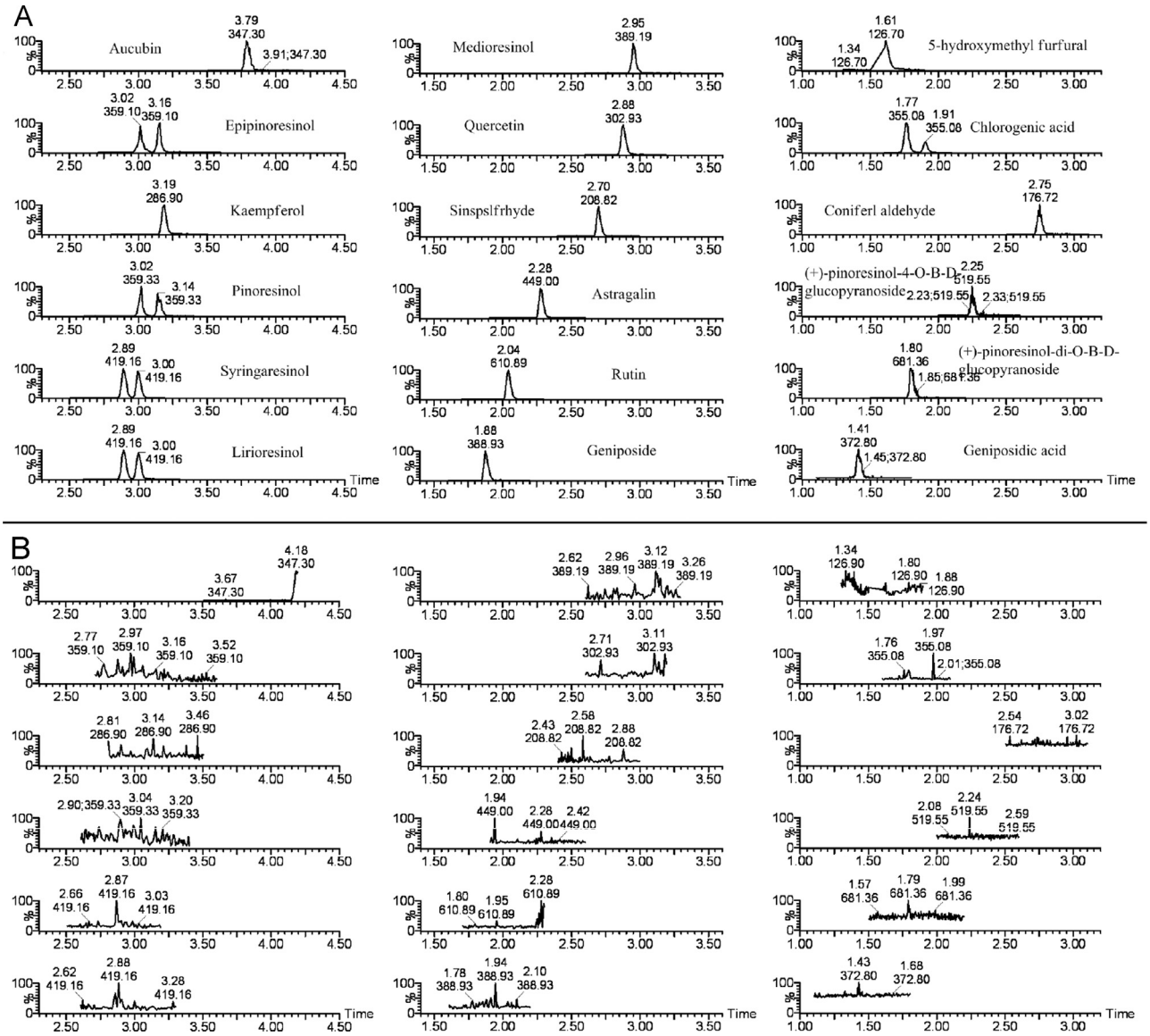

Fig. 1. Typical MRM chromatograms of AN, EPL, KL, PL, SL, LA, ML, QN, SE, ASN, RN, GE, 5-HF, CA, CE, PG, PDG and GA in (B) blank plasma and (A) blank plasma spiked with the 18 analytes at 500 ng mL^{-1}.

from the characteristic mass spectra. Meanwhile, their precursor ions and product ions were ascertained for use in MRM. Different ionization methods (including positive and negative modes) were tested and compared to obtain good specificity and sensitivity for PDG, GE, GA, AN and CA. In our experiment, all the analytes and IS could respond in the positive ion mode. And it was discovered that the response of PDG, GA and CA observed in positive ion mode was higher than that in negative ion mode. Moreover, GE and AN had no response in negative ion mode. Thus, positive ion mode was finally employed. The parenion of AN was selected as $[M+NH_4]^+$ because of its better response than $[M+H]^+$, and its response was enough for the mass analysis. Fig. 3 shows the product ions scan spectra of the analytes and IS. To get the richest relative abundance of precursor and product ions, the parameters of cone energy and collision energy were optimized. Table 2 shows the MS/MS transitions and energy parameters of the five compounds.

3.3. Chromatography

The chromatographic conditions were optimized to improve peak shape, increase signal intensity of the analytes and shorten run time. The mobile phase systems of acetonitrile–water and methanol–water at different proportions were tested. The response of analytes was obviously greater in acetonitrile–water mobile phase than that in methanol–water. The ionization in ESI mode occurs in the solution state, and the additives to the mobile phase may have a significant influence on the ionization of the analytes. With addition of formic acid (0.1%) to the mobile phase and gradient elution, the peak intensity and peak symmetry of the five analytes and IS were all dramatically improved. It was reported (Deng et al., 2008) that cross-talk may exist and affect the accuracy of MS quantification while some of the analytes have very similar structures and same fragmentation mechanisms. To eliminate the undesirable cross-talk effects, it seemed advisable to achieve a complete chromatographic resolution for this method. It is worth noting that the high chromatographic resolution of the UPLC system enabled to increase both analysis speed and peak capacity. Furthermore, the use of Acquity UPLC HSS T3 column (100×2.1 mm^2, i.d., 1.8 μm, Waters, USA) helped in the separation and elution of all the components in a very short time. Meanwhile, the mobile phase composition, flow rate and column temperature had been adjusted to obtain an acceptable resolution with no

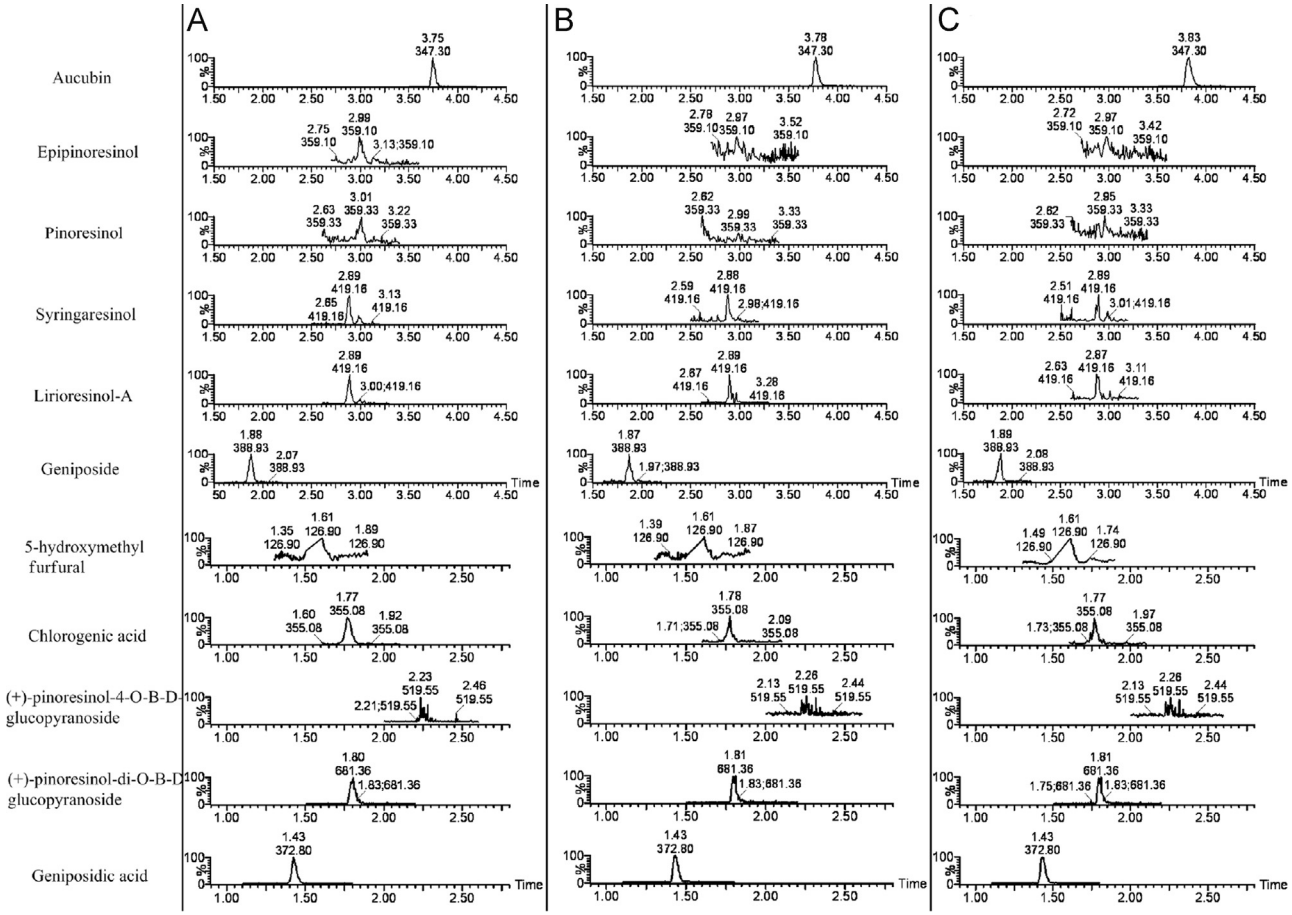

Fig. 2. Representative MRM chromatograms of AN, EPL, SL, GE, 5-HF, CA, PG, PDG and GA in rat plasma samples obtained at (A) 0.083 h (B) 0.5 h and (C) 4 h after single oral administration of EC extract.

cross-talk observed. Best chromatographic separations were achieved at 35 °C with a gradient elution program consisting of acetonitrile – 0.1% formic acid water at a flow rate of 0.3 mL min^{-1} with an acceptable run time of 7.0 min.

3.4. Extraction method

To obtain better extraction efficacy and less endogenous interference, various sample extraction approaches including protein precipitation and liquid–liquid extraction with different solvents were investigated. Above all, several solvent combinations were tested for liquid–liquid extraction of the analytes and IS. However, liquid–liquid extraction was not a viable option that could not provide satisfactory extraction efficiency due to the highly hydrophilic nature of the analytes. Subsequently, we used the protein precipitation reagents including acetonitrile and methanol, which provided satisfactory extraction efficiency. In the end, acetonitrile was chosen as the extraction solvent because of its higher extraction efficiency than methanol.

3.5. Method validation

3.5.1. Selectivity

The selectivity of the method towards endogenous plasma matrix was evaluated with plasma from six rats. Six channels were used for recording and the retention times of PDG, GE, GA, AN, CA and IS were 3.20, 2.99, 1.43, 1.03, 2.51 and 4.44 min, respectively. The typical chromatograms of blank plasma, plasma sample spiked with the analytes and IS, and the plasma sample from a rat after oral administration of EC extract are shown in Fig. 4. All the peaks of the analytes and IS were detected with excellent resolution as well as peak shapes, and no interference from the endogenous substances was observed at the retention time of the analytes and IS. The analytes could be easily differentiated from the rat plasma matrix and quantitatively determined at the LOQ level.

3.5.2. Linearity and lower limit of quantification

The typical equation of calibration curves and linearity ranges for the five analytes are shown in Table 3. The results showed that there was excellent correlation between the ratio of peak area and concentration for each compound within the selected concentration ranges with correlation coefficients (r) between 0.9967 and 0.9998. The LOQ of PDG, GE, GA, AN and CA in plasma were 9.68, 4.85, 10.00, 10.24 and 10.00 ng mL^{-1}, respectively.

3.5.3. Precision and accuracy

The intra-day precision, inter-day precision and accuracy of the five analytes in rat plasma had been validated. All the results of the tested samples were within the acceptable criteria (RSD%: $< 15\%$; RE%: $\pm 15\%$). The RSD values of intra-day precision and inter-day precision for 5 analytes were within 8.46% for HQC and MQC ($n=6$), while within 12.04% for LQC ($n=6$). Accuracy at the three QC levels ranged from -8.68% to 9.36% for HQC and MQC ($n=3$), and ranged from -13.32% to 13.04% for LQC ($n=3$). The results are shown in Table 4, which indicate that the present method has good precision and accuracy.

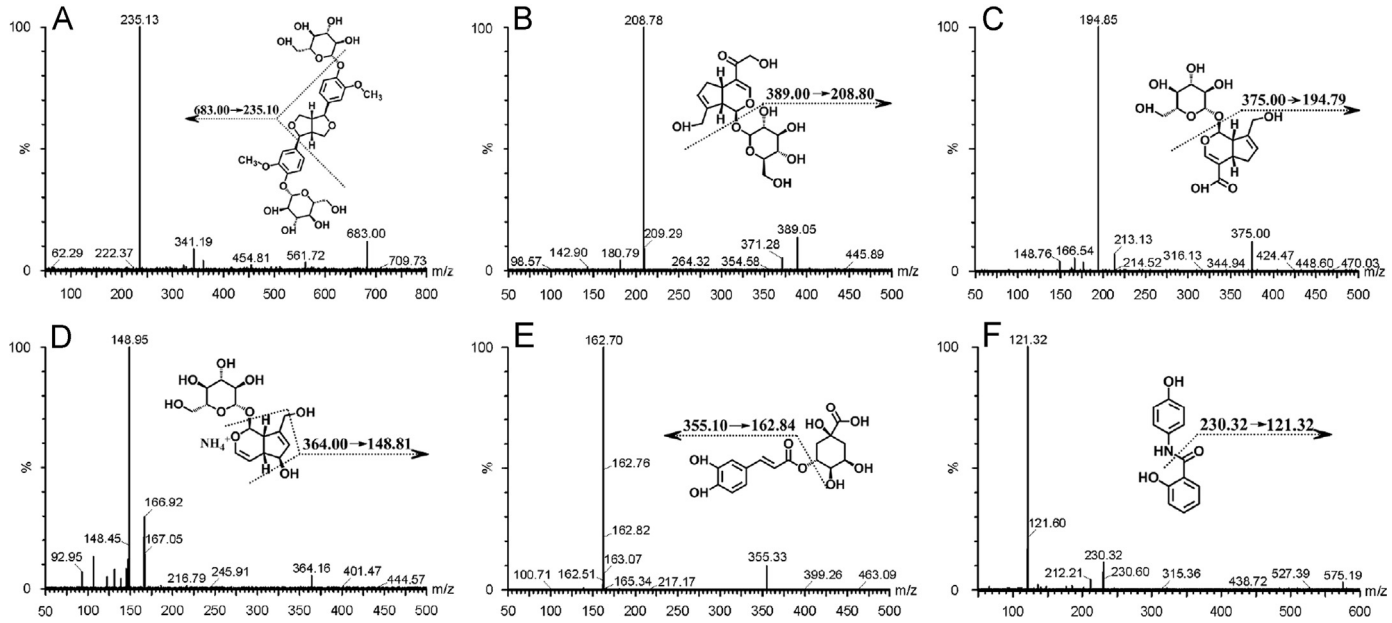

Fig. 3. Product ion mass spectra of PDG (A), GE (B), GA (C), AN (D), CA (E) and IS (F).

Table 2
The molecular weight, ionization mode, monitoring ion, parent ion, daughter ion, cone voltage, collision voltage, dwells and retention time of 5 ingredients of EC extract and IS for pharmacokinetic studies.

Analytes	Molecular weight	Ionization mode	Monitoring ion	Parent ion	Daughter ion	Cone (V)	Collision (eV)	Dwells	Retention time (min)
PDG	682.67	ESI^+	$[M+H]^+$	683.00	235.10	12	10	0.25	3.34
AN	346.33	ESI^+	$[M+NH_4]^+$	364.00	148.81	15	12	0.10	1.21
GE	388.37	ESI^+	$[M+H]^+$	389.00	208.80	12	10	0.10	3.13
GA	374.34	ESI^+	$[M+H]^+$	375.00	194.79	12	10	0.30	1.57
CA	354.31	ESI^+	$[M+H]^+$	355.10	162.84	18	12	0.35	2.64
IS: OE	229.23	ESI^+	$[M+H]^+$	230.03	120.77	30	22	0.20	4.44

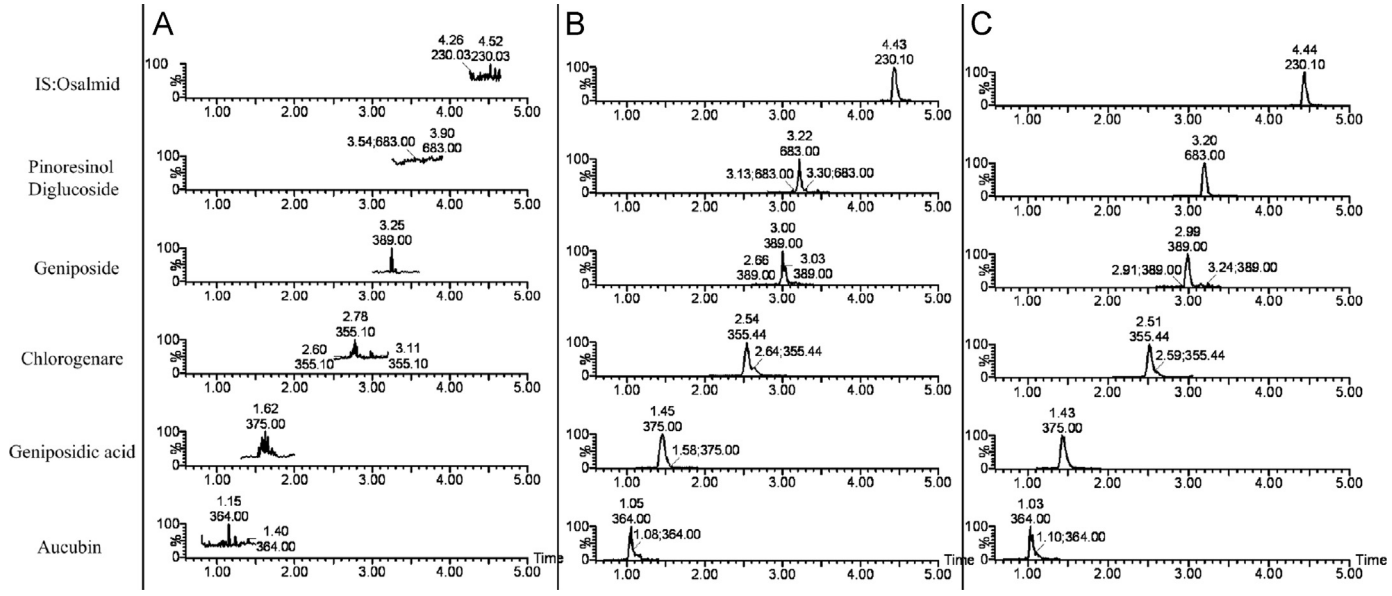

Fig. 4. Typical chromatograms of (A) blank plasma, (B) plasma sample spiked with the analytes (500 ng mL^{-1}) and IS (30 ng mL^{-1}), and (C) the plasma sample from a rat 0.083 h after oral administration of EC extract.

3.5.4. Extraction recovery and matrix effect

The mean extraction recoveries of the five analytes from rats plasma were all more than 86.82% at different concentration levels (Table 4), and the average extraction recovery of IS was 92.13%, which indicated the recoveries of the five analytes and IS were consistent, precise and reproducible at different concentration levels in various plasma biosamples. The matrix effect of the analytes ranged from 88.89% to 105.42% at the three QC levels

Table 3
The regression equations, correlation coefficients, linear ranges, LOQs and LODs for the determination of the analytes in rat plasma.

Analytes	Calibration curves	Correlation coefficients (r)	Linearity ranges (ng mL^{-1})	LOQ (ng mL^{-1})	LOD (ng mL^{-1})
PDG	$y=0.0001x-0.0013$	0.9967	9.68–968.00	9.68	4.84
GA	$y=0.0002x-0.0005$	0.9994	10.00–1000.00	10.00	5.00
CA	$y=0.0023x-0.0878$	0.9968	10.00–1000.00	10.00	5.00
GE	$y=0.0004x-0.0015$	0.9984	4.85–970.00	4.85	2.43
AN	$y=0.00004x-0.0016$	0.9998	10.24–1024.00	10.24	5.12

Table 4
Summary of accuracy, precision, recovery and matrix effect of the five analytes in rat plasma ($n=6$).

Analytes	Concentration (ng mL^{-1})	Intra-day precision RSD (%)	Inter-day precision RSD (%)	Accuracy RE[a] (%)	Matrix effect (%)	Recovery (%)
PDG	9.68	10.96	10.59	13.04	105.42	86.82
	242.00	4.65	8.02	−4.68	98.55	95.78
	968.00	3.63	6.16	3.22	94.93	92.33
GA	10.00	9.20	12.04	−7.05	94.74	89.89
	250.00	5.17	7.15	−2.07	95.51	92.94
	1000.00	1.83	4.87	6.15	98.11	94.65
CA	10.00	6.75	6.79	9.46	88.89	97.50
	250.00	6.31	5.27	9.36	93.67	93.24
	1000.00	4.03	3.81	−8.68	91.85	94.08
GE	9.70	8.12	5.49	−13.32	93.33	92.86
	242.50	3.71	1.84	4.93	104.37	87.70
	970.00	8.24	1.38	−2.45	96.25	98.28
AN	10.24	9.44	7.43	8.93	93.32	92.73
	256.00	2.70	2.76	5.92	99.65	92.16
	1024.00	8.46	5.33	−6.21	91.74	92.98

[a] RE is expressed as (measured concentration/freshly prepared concentration − 1) × 100%.

(Table 4), and the matrix effect of IS was 96.41%, indicated that no significant matrix effect was observed for the five analytes and IS.

3.5.5. Stability

The stability of all the analytes was assessed under various conditions. The measured concentration for five analytes at each QC levels deviated within 14.74%, which demonstrated that they were stable in biosamples at room temperature for 12 h, at −20 °C for at least 2 weeks, after three freeze and thaw cycles, and at 4 °C in autosampler for 8 h after prepared. The results are presented in Table 5, which are well within the acceptable limit.

3.6. Pharmacokinetic study

The validated method was successfully applied to pharmacokinetic studies of five ingredients in rat plasma after a single oral administration of 50 g kg^{-1} EC extract The mean plasma concentration–time profiles of the analytes in female and male groups are illustrated in Fig. 5. The pharmacokinetic parameters including half-time ($t_{1/2}$), maximum plasma concentration (C_{max}), time to reach the maximum concentrations (T_{max}), elimination rate constants (K_e), area under concentration–time curve ($AUC_{0\to t}$ and $AUC_{0\to\infty}$), mean resident time (MRT), clearance (CL) and volume of distribution (V_d) calculated by non-compartment model are presented in Table 6.

It can be seen that five ingredients exhibited good absorption, distribution and elimination process after oral administration of EC extract both in male and female rats. The profiles of concentration–time curve of each ingredient in males were similar to females. The concentration–time curves of PDG, GE and CA exhibited a relatively rapid absorption process, of which the plasma concentration achieved the peak from 10 to 20 min and showed a relatively sharp peak shape. In addition, GA and AN exhibited double peaks after administration of EC extract. The first peak sharply appeared at 10–25 min after treatment. However, the second peak occurred at 6–10 h post-dose, and maintained at a quite high level for several hours. Bimodal phenomenon of GA and AN might be due to multiple-sites absorption or enterohepatic circulation. The plasma concentration of PDG and CA declined to the detection limits around 8 h after oral administration, which exhibited a faster elimination process. By contrast, GA and AN experienced a slower elimination process, of which the plasma concentration was lower than the detection limits at 36 h after oral administration.

The pharmacokinetics of the five analytes is closely related to their chemical structures and metabolism mechanisms. The molecular structures of lignans (PDG) and iridoids (GE, GA and AN) contain glucose, which might be easily hydrolyzed. Han et al. (2011) study suggested that GE was metabolized in rat through deglycosylation of the iridoid glycoside, and followed by glucuronidation and the pyran-ring cleavages. Jin et al. (2007) study showed that the metabolic processes of PDG include deglycosylation, ring cleavage, demethylation, dehydroxylation and oxidation . CA is the ester formed by caffeic acid and quinic acid. It was reported that CA and its metabolites were estimated in the stomach, small intestine and cecal contents as well as in bladder urine and plasma by HPLC with coulometric detection at several time points (1.5, 3, 4.5, and 7 h) after the beginning of the meal. And it could be quickly absorbed in the rat stomach in its intact form (Lafay et al., 2006).

Through comparing C_{max} and AUC, the absorption of GA was higher than the other four analytes while CA was the poorest one. As shown in Fig. 5 and Table 6, the results demonstrated that there were significant differences ($p<0.05$) in pharmacokinetic parameters including AUC_{0-t}, $AUC_{0-\infty}$, C_{max}, $T_{1/2}$, K_e, MRT, V_d and CL for the five ingredients between the male and female group. A remarkable increase ($p<0.05$) in the value of AUC_{0-t}, $AUC_{0-\infty}$, C_{max}, and decrease ($p<0.05$) of V_d and CL were observed in male group after oral administration of EC extract compared to female

Table 5
Stability of the five analytes in rat plasma ($n=3$).

Analytes	Concentration (ng mL^{-1})	Short-term stability RSD (%) ($n=3$)	Long-term stability RSD (%) ($n=3$)	Freeze–thaw stability RSD (%) ($n=3$)	4 °C, 8 h after prepared RSD (%) ($n=3$)
PDG	9.68	6.64	5.96	9.01	8.63
	242.00	9.21	14.74	7.02	6.29
	968.00	9.83	7.56	12.32	10.06
GA	10.00	3.04	11.34	6.25	5.22
	250.00	8.21	4.78	8.76	7.08
	1000.00	12.89	11.75	14.00	12.87
CA	10.00	8.66	4.18	12.06	9.39
	250.00	3.29	10.59	12.65	2.93
	1000.00	4.03	5.97	4.54	8.29
GE	9.70	7.15	6.97	5.82	3.65
	242.50	2.19	3.54	13.45	3.82
	970.00	12.75	8.96	9.54	6.64
AN	10.24	4.59	6.82	6.10	10.02
	256.00	9.13	12.53	10.48	5.39
	1024.00	11.60	10.16	12.91	9.48

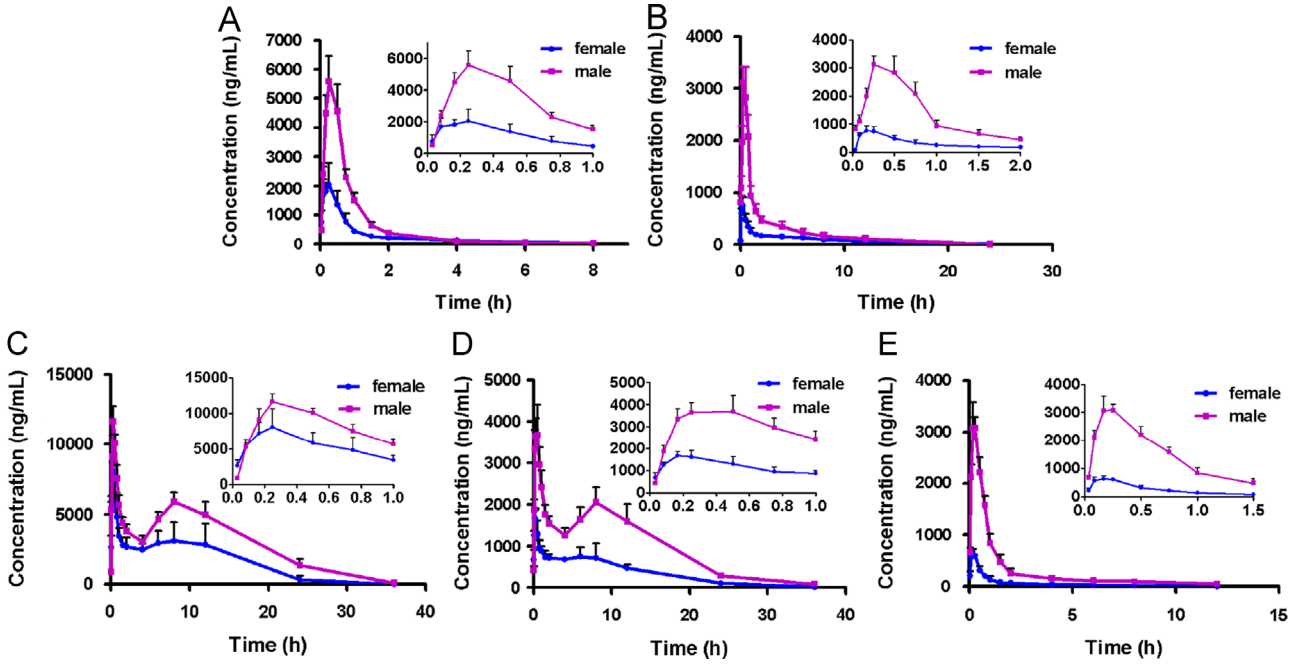

Fig. 5. Plasma concentration–time curves for PDG (A), GE (B), GA (C), AN (D) and CA (E) in rat plasma after oral administration of EC extract at 50 g kg^{-1} to rats in male and female groups. Each point represents the mean ± SD ($n=6$).

group. The results pointed out that the absorptions of the five analytes were increased while the distribution and elimination processes were decreased in male group in contrast with female group. The results might be attributed to the different mechanisms of absorption and metabolism between the different body status of males and females.

Gender-related differences in pharmacokinetics of the five active principles could be explained by many factors. One of the factors is anatomical and physiological characteristics, such as percentage of body fat, plasma volume, organ blood flow, proteins responsible for drug binding, microflora and endogenous substances (Carrasco-Portugal and Flores-Murrieta, 2007). Sex specific activity and expression of drug metabolism enzymes is another factor that must be considered. Gender differences in pharmacokinetics in rats also have been found in many compounds such as schisandrin (Cao et al., 2010), lumefantrine (Wahajuddin et al., 2012), celastrol (Zhang et al., 2012), peiminine (Chen et al., 2013) and emodin (Liu et al., 2011), mainly due to sex specific expression of metabolism enzymes. Take cytochrome P450s as an example, the cytochrome P450 isoforms 2C11, 2C13, and 3A2 are expressed in male rats whereas 2C12 and 3A18 are female dominant forms (Czerniak, 2001; Martignoni et al., 2006). UDP-Glucuronosyltransferase, Glu-tathione and S-transferase enzymes also express in a sex-dependent manner in rats (Srivastava and Waxman, 1993; Zhu et al., 1996). Drug transport systems also must be the third reason, such as intestinal first-pass elimination and efflux mediated by P-glycoprotein. Further investigations are needed to clarify the metabolic mechanisms of the five ingredients of EU underlying the differences between the male and female group. The Bile excretion study, the transport mechanism study in Caco-2 cells and the metabolism study of the active principles will be more persuasive and important topics for our further studies. This paper maybe

Table 6
Main pharmacokinetic parameters of the five ingredients after oral administration of EC extract at 50 g kg^{-1} to rats in male and female groups.

Parameters	PDG	GA	CA	GE	AN
The male rats group ($n=6$)					
$T_{1/2}$ (hr)	3.29 ± 0.79	3.21 ± 0.51	1.97 ± 1.53	3.29 ± 0.98	2.92 ± 1.09
K_e(1/hr)	0.21 ± 0.03	0.22 ± 0.02	0.35 ± 0.04	0.21 ± 0.03	0.24 ± 0.04
C_{max} (μg/L)	5637.00 ± 773.52	11,658.55 ± 1092.75	3349.82 ± 363.58	3142.80 ± 349.69	3901.29 ± 634.27
T_{max} (hr)	0.29 ± 0.04	0.30 ± 0	0.24 ± 0.05	0.34 ± 0.09	0.41 ± 0.11
AUC_{0-t} (μgh/L)	4850.24 ± 663.15	106,451.50 ± 7310.01	3812.08 ± 169.39	5831.02 ± 1055.39	35,177.77 ± 5689.34
$AUC_{0-\infty}$(μgh/L)	4994.00 ± 688.82	106,955.07 ± 7261.13	4296.76 ± 149.29	5876.12 ± 1054.33	36,172.73 ± 5627.37
MRT (hr)	1.35 ± 0.18	10.80 ± 0.62	2.36 ± 0.99	4.22 ± 0.74	8.80 ± 0.26
V_d/kg (L/kg)	314.81 ± 5.16	10.37 ± 0.46	15.00 ± 3.47	42.16 ± 4.56	1.80 ± 0.08
CL/kg (L/kg)	66.49 ± 0.64	2.26 ± 0.04	5.29 ± 0.06	8.89 ± 0.34	0.42 ± 0
The female rats group ($n=6$)					
$T_{1/2}$ (hr)	1.25 ± 1.06	3.80 ± 1.45	0.71 ± 0.24	4.25 ± 1.62	5.76 ± 1.28
K_e(1/hr)	0.56 ± 0.09	0.18 ± 0.03	0.98 ± 0.15	0.16 ± 0.02	0.12 ± 0.03
C_{max} (μg/L)	2278.00 ± 633.84	8359.83 ± 2360.59	673.59 ± 43.13	798.15 ± 122.56	1768.67 ± 155.94
T_{max} (hr)	0.22 ± 0.10	0.27 ± 0.05	0.19 ± 0.07	0.18 ± 0.07	0.25 ± 0.05
AUC_{0-t} (μgh/L)	2114.35 ± 494.79	58,242.82 ± 14,021.06	699.76 ± 65.66	2379.65 ± 533.18	12873.17 ± 1669.39
$AUC_{0-\infty}$(μgh/L)	2181.93 ± 585.67	58,608.20 ± 16,076.52	674.16 ± 22.69	2441.12 ± 716.97	13,030.28 ± 1652.83
MRT (hr)	1.73 ± 0.48	9.47 ± 2.03	1.80 ± 6.72	6.67 ± 9.56	9.42 ± 0.55
V_d/kg (L/kg)	376.90 ± 15.11	22.52 ± 9.21	28.60 ± 32.36	130.63 ± 36.24	8.65 ± 0.14
CL/kg (L/kg)	153.62 ± 2.37	4.06 ± 0.25	27.63 ± 1.12	21.73 ± 0.99	1.07 ± 0.01

useful for deeply studies on the metabolic pathways of EC in vivo for better understanding its pharmacokinetic profiles.

4. Conclusion

9 ingredients, namely AN, EPL, SL, GE, 5-HF, CA, PG, PDG and GA could be detected out in rat plasma 0.083 h after oral administration with EC extract following the method for determination of 18 ingredients. Among them, PDG, GE, GA, AN and CA were selected as the ingredients for pharmacokinetic studies due to the significant correlation of drug concentration–time curve of them. A new efficient and sensitive UPLC-MS/MS method for simultaneous determination of the five ingredients of EC in rat plasma had been developed and completely validated. The method was successfully applied to compare the pharmacokinetic behaviors of the five ingredients in rat plasma after a single oral administration of EC extract between the male and female groups. The results indicated that the five ingredients exhibited a better absorption and slower elimination in male group than that in female group. This study provided the theoretical basis for better application of EC to clinical practice.

Acknowledgment

The authors acknowledge the support of the Opening Project of Shanghai Key Laboratory of Complex Prescription (Shanghai University of Traditional Chinese Medicine) (13DZ2272300). This work is also supported by the "Three-Year Action Plan Project" for Development of TCM from Shanghai Health Bureau (ZYSNXD-GH-FFYJ, 2011–2013) and Postdoctoral fund Projects of China (3013M531203) and Postdoctoral fund Projects of Shanghai(13R21415700).

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