Simultaneous Quantitation of Dicaffeoylquinic Acids in Rat Plasma After an Intravenous Administration of Mailuoning Injection Using Liquid Chromatography–Mass Spectrometry

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Abstract

A high-performance liquid chromatography electrospray ionization mass spectrometry method is developed and validated for the simultaneous quantitation of three major phenolic acids including 1,5-dicaffeoylquinic acid (1,5-DCQA), 3,4-dicaffeoylquinic acid (3,4-DCQA), and 3,5-dicaffeoylquinic acid (3,5-DCQA) in rat plasma. All analytes and internal standard (bergeninum) are extracted from plasma samples by liquid–liquid extraction with isopropanol. The chromatographic separation is accomplished on a stainless-steel column with a gradient 0.1% formic acid–acetonitrile solution as mobile phase at a flow rate of 0.2 mL/min with an operating temperature of 40°C. The selected ion monitoring is performed at *m/z* 515.2 for 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA, and *m/z* 327 for the internal standard bergeninum. Linear detection responses are obtained at a concentration range from 0.020 to 5.0 µg/mL for 1,5-DCQA, and 0.039 to 10.0 µg/mL for 3,4-DCQA and 3,5-DCQA. The lower limits of quantitation for 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA are 20, 39, and 39 ng/mL, respectively. The intra- and inter-day precisions (RSD%) are within 11.0%, and the deviations of the assay accuracies are within ± 12.0% for all analytes. The recoveries are greater than 84.0%. All analytes are proved to be stable during the sample preparation and analytic procedures. The method is successfully applied to the pharmacokinetic study of 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA following an intravenous dose of 10 mL/kg mailuoning injection to rats.

Introduction

Traditional Chinese medicine (TCM) is the natural therapeutic system used under the guidance of the theory of traditional Chinese medical sciences, which has played an indispensable role in the prevention and treatment of diseases in China and worldwide. In clinical application, most herbal medicines are prescribed in combination to obtain synergistic effects or to diminish possible adverse reactions. Mailuoning injection is made from the extracts of four medicinal materials, including *Flos lonicerae, Artemisiae scopariae, Scrophularia ningpoensis,* and *Achyranthes bientata* Bl. This injection has been clinically applied in China for more than 30 years to treat cerebral infarction, scleredema neonatorum, diabetes, vasculitis, and coronary artery disease (1).

As the major active components of mailuoning injection, chlorogenic acid (CGA), caffeic acid (CA), 1,5-dicaffeoylquinic acid (1,5-DCQA), 3,4-dicaffeoylquinic acid (3,4-DCQA), and 3,5-dicaffeoylquinic acid (3,5-DCQA) have been shown to have various biological activities such as antibacterial, antivirus, anti-inflammatory, and anti-oxidative stress effects (2–10). Although their pharmacological properties have been extensively studied, less is known about their pharmacokinetic behaviors. The main cause is possibly the lack of a high sensitive bioanalytical method for simultaneous quantitation of these phenolic acids.

There are many methods (11–25) for the single determination of CGA or CA that have been reported in the current literature, but few have contributed to the simultaneous determination of the five pharmacologically active phenolic acids in biological samples. For the therapeutic effect and clinical safety considerations, the pharmacokinetic behavior of TCM preparations, especially injections, should be clarified as clearly as possible for the active compounds contained. Developing a highly sensitive method for the simultaneous quantitation of as many of the compounds as possible is a prerequisite step for the pharmacokinetic study of TCM preparations. More recently, a method that could be reliably applied to the simultaneous analysis of 11 molecules (nine CGAs and their two metabolites) in human plasma (17) has been established. However, this method using no internal standard (IS) for mass detecting correction resulted in poor analytical accuracy for some of the molecules including 3-CQA, 3,5-DCQA, and 4,5-DCQA. Furthermore, this method has not been fully validated according to the current guidelines for

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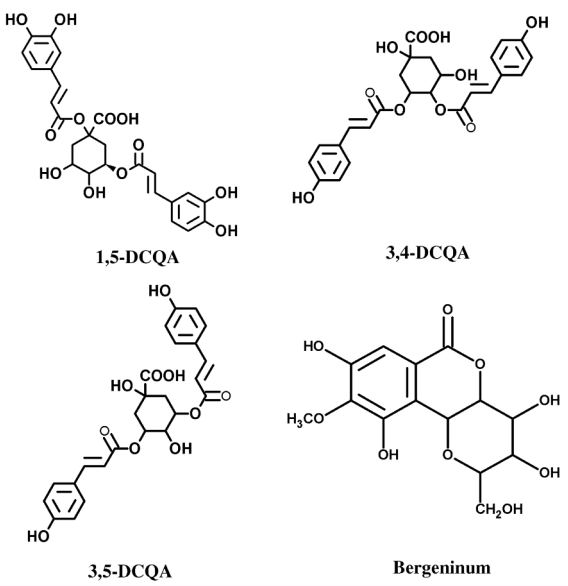

Figure 1. Chemical structures of 1,5-DCQA, 3,4-DCQA, 3,5-DCQA, and the IS bergeninum.

biosample analysis in pharmacokinetic study. Also, 2.5 mL plasma was needed for analysis, which is hardly suitable for pharmacokinetics study. Therefore, this method was not suitable for the pharmacokinetic study and might be only used for the rough or preliminary determination of phenolic acids' concentrations, as suggested by the authors themselves.

This study was thus designed to develop a simple, sensitive, and specific LC–MS method for the simultaneous determination of CA, CGA, 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA in rat plasma with bergeninum as the IS (see Figure 1). This method has been fully validated for its specificity, sensitivity, stability, accuracy, and precision, and has been successfully applied for the simultaneous pharmacokinetic studies of CA, CGA, 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA after a single intravenous administration of mailuoning injection to rats. Considering that the CA and CGA had been previously well studied, we only provided the data of three kinds of dicaffeoylquinic acids, while referencing and comparing the data obtained for CA and CGA with that previously reported.

Experimental

Materials and reagents

1,5-DCQA, 3,4-DCQA, and 3,5-DCQA (> 99% purity) were purchased from Tianjin YiFang S&T CO., Ltd. (Tianjin, China). Bergeninum (> 99% purity) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Mailuoning injection (10 mL/vial, containing 0.039 mg/mL of 1,5-DCQA, 1.0 mg/mL of 3,4-DCQA, and 0.5 mg/mL of 3,5-DCQA) was provided by Jinling Pharmaceutical Co. Ltd. (Nanjing, China). Acetonitrile was of HPLC grade (Merck, Whitehouse, NJ). All other chemicals were of analytical reagent grade. Ultrapure water, prepared using a Milli-Q Reagent water system (Millipore, MA), was used throughout the study.

Animals

Sprague-Dawley rats, weighing 180–220 g, were obtained from the experimental animal feeding center of China Pharmaceutical University. These animals were acclimatized for 1 week prior to beginning the study. The rats were housed in a windowless room, which was illuminated for 12 h each day at 22–18ºC. All animals were weighed daily and observed twice daily to assess their general health. Diet was prohibited for 12 h before the experiment, while water was taken freely.

LC–MS

The liquid chromatography–mass spectrometry (LC–MS) system consisted of a Shimadzu LC-10ADvp HPLC series liquid chromatograph and a Shimadzu LC–MS-2010A single quadrupole mass spectrometer equipped with an electrospray ionization (ESI) interface, and LC–MS solution (version 2.02) was used for data acquisition and processing (Japanese, Kyoto). LC separation was achieved using a Synergi Hydro-RP C_{18} (5 µm, 250 mm × 2.0 mm i.d.) column maintained at 40ºC. The mobile phase consisted of 0.1% formic acid (A) and acetonitrile (B) with a linear gradient elution. The gradient cycle consisted of an initial 0.7 min isocratic segment (89% A and 11% B). Solvent B was then increased to 23% within 1.0 min and maintained from 1.7 to 5.5 min. After increasing solvent B to 30% within 2.0 min, the mobile phase gradient was maintained at this composition from 7.5 to 10.5 min, then solvent B was returned to 11% within 3.5 min, which was maintained for 4 min (from 14.0 to 18.0 min) for column equilibration. The flow rate was 0.2 mL/min during the whole gradient cycle.

The effluent from the HPLC column was directed into the ESI probe. Mass spectrometer conditions were optimized to obtain maximal sensitivity. The curve dissolution line (CDL) temperature and the block temperature were maintained at 250ºC and 200ºC, respectively. The probe voltage (capillary voltage), CDL voltage, and detector voltage were fixed at –4.5 kV, –50 V, and 1.6 kV, respectively. Liquid nitrogen (99.995%, Nanjing University, China) was used as the source of nebulizer gas (1.5 L/min). Negative ion ESI was used to form deprotonated molecules at *m/z* 515.2 of 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA, and *m/z* 327 of the internal standard bergeninum. Selected ion monitoring (SIM) was used with a dwell time of 0.020 s.

Preparation of samples and method validation

Stock solutions of the analytes and IS were prepared by dissolving 10.0 mg of each authentic samples in 10 mL methanol, producing a concentration of 1.0 mg/mL, and were stored at 4ºC. The samples for standard calibration curves were prepared by spiking the blank rat plasma with the appropriate working solutions to yield the following concentrations: 0.020, 0.039, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, and 5 µg/mL (1,5-DCQA), and 0.039, 0.078, 0.156, 0.313, 0.625, 1.250, 2.5, 5, and 10 µg/mL (3,4-DCQA and 3,5-DCQA), respectively. Quality control (QC) samples were prepared from blank plasma at concentrations of 0.078, 0.625, and 5 µg/mL for 1,5-DCQA, 0.156, 1.250, and 10 µg/mL for 3,4-DCQA and 3,5-DCQA, respectively.

Plasma samples, calibration standards, and QC samples were extracted employing a liquid–liquid extraction technique. To each tube containing 50 µL plasma sample, 250 ng (10 µL × 25 µg/mL) of IS, 50 µL of 0.5 M acetic acid solution and 1.20 mL isopropanol (saturated with water) were added and then were vortexed for 3 min. Following centrifugation and separation, the organic layer (1 mL) was evaporated under a stream of nitrogen at 40ºC. The residue was reconstituted with 100 µL mobile phase. An aliquot of 5 µL was injected into the LC–MS system.

The recoveries of 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA from spiked rat plasma were determined by measuring an extracted sample against a post-extraction spiked sample and expressed as the ratio of the peak responses. Intra- and inter-day accuracy and precision for the assay were evaluated by analyzing rat plasma samples containing 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA at 5, 0.625, and 0.078 µg/mL, respectively. Intra-day accuracy and precision (each, n = 5) were evaluated by analysis of samples at different times during the same day. Inter-day accuracy and precision were determined by repeated analysis of samples over three consecutive days (n = 5 series per day). The stabilities of 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA in rat plasma during the sample storage were fully validated by analyzing quintupled QC samples for each analyte. The compound stability for 12 h after preparation in autosampler at 4ºC and for 6 h at room temperature in plasma was evaluated by repeated analysis (n = 5) of plasma samples. Long-term stability in plasma was also tested by assaying frozen plasma samples after storage at –80ºC for a month. The amount of the analytes in plasma samples was determined by using a newly prepared calibration curve. The obtained results were compared with the nominal concentration of the analytes. A compound was considered unstable if the calculated concentration was less than the nominal concentration by more than 15%. The matrix effect was measured by comparing the peak response of the post-extraction spiked sample with that of the standard solution.

Application to pharmacokinetic study

The studies were approved by the Animal Ethics Committee of China Pharmaceutical University. Ten Sprague-Dawley rats were fasted with free access to water for 12 h prior to experiment. Mailuoning was administered to the rats (10 mL/kg body weight, equivalent to 20.0 mg/kg of CGA, 0.39 mg/kg of 1,5-DCQA, 10.0 mg/kg of 3,4-DCQA, 5.0 mg/kg of 3,5-DCQA, and 9.0 mg/kg of CA) by intravenous injection. Blood samples (150 µL) were obtained from the oculi chorioideae vein before dosing and subsequently at 0.03, 0.08, 0.l6, 0.33, 0.5, 1, 2, 4, 6, and 8 h following administration, transferred to a heparinized eppendorf tube, and centrifuged at 4000 rpm for 10 min. A 50 µL plasma sample was subsequently collected and prepared for analysis immediately.

Data analysis

To determine the pharmacokinetic parameters of CGA, CA, 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA, the concentration-time data were estimated by compartmental methods using the DAS Ver1.0 (Drug and Statistics for Windows) program. All results were expressed as arithmetic mean ± standard deviation (SD). The compartment model could be a sum of the exponential of the following: $C = \sum Ci \times \exp(\lambda_i t)$, in which C is the predicted concentration, t is time, and Ci = (A, B, …) and λ_i (= α, β, …) are the pre-exponential and exponential coefficients; this was fitted to plasma concentration data using nonlinear regression analysis. The area under the concentration versus time curve (AUC_{0-t}) was calculated using the trapezoidal rule up to the last quantifiable time point and extrapolated to infinity ($AUC_{0-\infty}$) by using the terminal elimination; an analogous method was managed for the calculation of the area under the first moment curve ($AUMC_{0-\infty}$), but concentration vs. time data were used. Mean residence time ($MRT_{0-\infty}$) was calculated as $AUMC_{0-\infty}/AUC_{0-\infty}$. The elimination half-life ($t_{1/2}$) was determined as $0.693/k_{el}$, where k_{el}, the elimination rate constant, was determined by linear regression of the terminal log-linear phase of the plasma concentration-time curve (number of points = 5 in each animal). Volume of distribution (V_C) of the central compartment was determined as dose/C_0, and C_0 is the concentration measured right after the administration. Clearance (Cl) was determined as dose/AUC.

Results and Discussion

Optimization of LC–MS for quantitative analysis

The selection of mobile phase was a critical factor in achieving good chromatographic behavior (peak shape and resolution) and appropriate ionization. Modifiers such as formic acid and ammonium acetate alone or in combination in different concentrations were compared. The best peak shape and ionization were achieved using 0.1% formic acid solution. Linear gradient elution was used to make satisfactory separation and to elute endogenous substance residue from the column. In the negative ion scan mode,

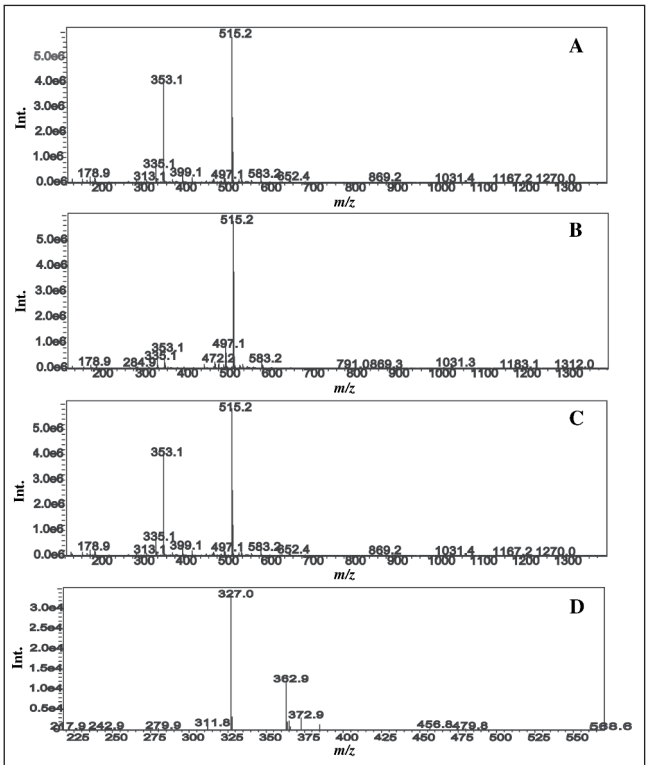

Figure 2. Mass spectra of 1,5-DCQA (10000 ng/mL) (A), 3,4-DCQA (10000 ng/mL) (B), 3,5-DCQA (10000 ng/mL) (C), and the IS bergeninum (2500 ng/mL) (D).

the molecular ions $[M-H]^-$ for 1,5-DCQA, 3,4-DCQA, 3,5-DCQA, and IS were the most abundant ions. Therefore, these were used in the SIM acquisition. Bergeninum was chosen as IS for its similarity with the analytes in structure (Figure 1), chromatographic behavior, and mass spectrographic behavior (Figure 2). The analytes and IS could be extracted from plasma sample under acid conditions. Ethyl ether, ethyl acetate, and isopropanol were preliminarily screened for their extraction power on the analytes from plasma. Isopropanol was finally selected for its satisfactory extraction power on all analytes and the IS.

Method validation

The method validation assays were carried out based strictly on the currently accepted Chinese State Food and Drug Administration bioanalytical method validation guidance (2005.3), and also in compliance with the FDA guidelines of the United States. The validation experiments and results obtained are described in the following.

Selectivity

Negative ion electrospray mass scan spectra of the analytes and IS were shown in Figure 2. The $[M-H]^-$ ions with *m/z* 515.2 for 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA and *m/z* 327 for IS (bergeninum) were chosen for SIM due to their high stability and intensity. Representative SIM chromatograms are shown in Figure 3. Assay selectivity was evaluated by analyzing blank plasma samples obtained from six rats. The retention times of 1,5-DCQA, 3,4-DCQA, 3,5-DCQA, and IS were 9.2, 14.1, 13.6, and 7.8 min, respectively. S/N of peaks of 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA at the lower limit of quantitation (LLOQ) were 21.38, 23.14, and 24.64, respectively. All samples were found to be free of interferences with the analytes of interest.

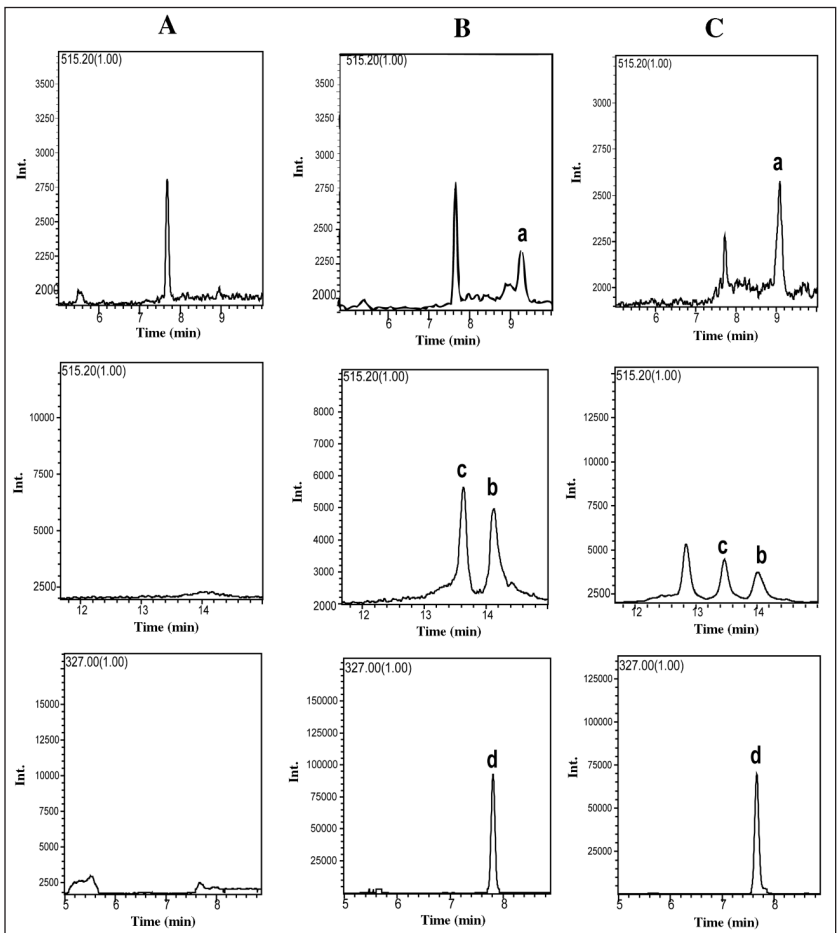

Figure 3. Representative chromatograms obtained from the selected ion monitoring of the deprotonated molecules of 1,5-DCQA, 3,4-DCQA, 3,5-DCQA (*m/z* 515.2) and the IS bergeninum (*m/z* 327) including: blank rat plasma (A); a blank plasma spiked at 20 ng/mL of 1,5-DCQA (peak a), 39 ng/mL of 3,4-DCQA (peak b), 3,5-DCQA (peak c), and 5000 ng/mL IS (bergeninum, peak d) (B); a rat plasma sample at 90 min after i.v. administration of mailuoning injection at 10 mL/kg (C).

Linearity and sensitivity

LLOQ of the assay, defined as the lowest concentration on the standard curve that can be quantitated with accuracy within ± 15% bias of nominal concentration and precision not exceeding 15% (RSD), was 20 ng/mL for 1,5-DCQA and 39 ng/mL for 3,4-DCQA and 3,5-DCQA, respectively. The calibration curves were constructed using linear regression of the peak area ratio of the analytes to IS (Y) against the corresponding spiked plasma concentrations of the analytes (X, ng/mL) over the range 0.020 to 5.0 µg/mL for 1,5-DCQA and 0.039 to 10.0 µg/mL for 3,4-DCQA and 3,5-DCQA, respectively. The regression equations were $R = 0.2175C + 0.0007$ ($r^2 = 1.0000$, $n = 5$) for 1,5-DCQA, $R = 0.2459C - 0.0003$ ($r^2 = 0.9999$, $n = 5$) for 3,4-DCQA, and $R = 0.1261C + 0.0007$ ($r^2 = 1.0000$, $n = 5$) for 3,5-DCQA, respectively. The correlation coefficient indicated a good linear detector response over the 200-fold dynamic range that was investigated. The concentrations in unknown samples were calculated using these calibration equations.

Assay precision and accuracy

Precision and accuracy of the assay were determined by replicate analyses ($n = 5$) of QC samples on the same day (intra-day) and also on 3 consecutive days (inter-day). The accuracy (% bias) was calculated from the nominal concentration (C_{nom}) and the mean value of the observed concentration (C_{obs}) as follows: bias (%) = $[(C_{obs} - C_{nom})/(C_{nom})] \times 100$. The intra- and inter-day precision and accuracy are summarized in Table I. The results demonstrate that the precision and accuracy of this assay were acceptable.

Recovery

The extraction recovery (absolute recovery) of analytes from rat plasma after the extraction procedure was assessed in quintuplicate by comparing the analyte/IS peak area ratio of extracted analytes ($R1$) with those of mobile phase spiked with standard solution ($R2$). IS was spiked before extraction in both cases. QC samples at three concentrations were evaluated. The extraction recovery was expressed as $(1.20R1/R2) \times 100\%$. The data are shown in Table II. The extraction recovery of the analytes was shown to be consistent and reproducible.

Matrix effect

The matrix effect (co-eluting, undetected

endogenous matrix compounds that may influence ionization of the analyte) was examined by comparing the peak areas of the analytes between two different sets of samples. In set 1, analyte standards were dissolved in the reconstitution solvent and analyzed at three concentrations of the analytes. These analyses were repeated five times at each concentration. In set 2, blank plasma samples obtained from five rats were extracted and then spiked with the same concentrations of analytes in the reconstitution solvent. Deviation of the mean peak areas of set 2 versus set 1 would indicate the possibility of ionization suppression or enhancement for analytes and IS. The results indicated that there was no significant difference between the signals of analytes extracted from rat plasma and the mobile phase, which proved that there was negligible matrix effect.

Stability

The stability of 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA was evaluated under the conditions mimicking situations likely to be encountered during the sample storage and analytical processes by analyzing five replicates of QC samples for all analytes, and the result is shown in Table III. QC samples were frozen and stored at –80ºC for a month. The determined concentration variations of 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA after one cycle of freezing and thawing were within n ± 20% (over ± 15%) of nominal concentrations, indicating that these compounds were somewhat unstable after a long-term storage, suggesting that the samples should be immediately analyzed to avoid the loss (26–28). Processed samples in the autosampler at 4ºC showed good stability for all analytes, as evidenced in that the responses varied within ± 15% at QC concentrations during a 12 h storing. After storage at ambient temperature for 6 h, the concentrations of 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA in plasma also deviated less than ± 15% from those in freshly spiked plasma.

Results of pharmacokinetic study

After i.v. administration of mailuoning injection (10 mL/kg) to ten rats, plasma concentrations of 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA were simultaneously determined by the described LC–ESI-MS (Figure 4). Considering that the concentrations of most analytes in the samples obtained at early phase after intravenous administration were expected to be near or over the upper limit of the calibration curves, the plasma samples obtained at 2 min and 5 min, and 10 min and 20 min post-administration were diluted 10 and 20-fold, respectively, using blank plasma prior to LC–MS analysis. It has been preliminarily validated that the dilution exerted no influence on the analytical accuracy and precision of all analytes. Although 1,5-DCQA failed to be detected at 4 h post administration, the present method was sensitive enough for the pharmacokinetic studies considering its short elimination half-life time (0.334 ± 0.050 h).

A two-compartment, first-order pharmacokinetic model appeared to fit the plasma concentration-time curves of all analytes following a 10 mL/kg mailuoning injection. The pharmacokinetic parameters calculated with the DAS Ver1.0 program are listed in Table IV. Interestingly, the pharmacokinetic parameter calculations revealed that the V of 1,5-DCQA was lower than that of 3,4-DCQA and 3,5-DCQA. This suggested 1,5-DCQA mainly existed in blood, while more 3,4-DCQA and 3,5-DCQA distributed to other tissues and organs in rats. The k_{10}, k_{12}, and k_{21} of 1,5-DCQA were much more than the other two, reveals faster elimination of 1,5-DCQA. $t_{1/2\beta}$ can also prove this, because $t_{1/2\beta}$ of 1,5-DCQA was 0.334 ± 0.050 h and $t_{1/2\beta}$ of 3,4-DCQA and 3,5-DCQA were 0.479 ± 0.155 h and 0.486 ± 0.125 h, respectively.

We also determined CGA and CA simultaneously by using this method. The results were similar to those reported previously (16). CGA and CA eliminated soon after i.v. of mailuoning injection to rats. $t_{1/2\beta}$ of CGA and CA was 0.649 ± 0.035 h and 0.330 ± 0.107 h, respectively.

Table I. Intra- and Inter-Day Accuracy and Precision of LC–MS Determination of 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA in Rat Plasma (n = 5)

Sample	Spiked conc. (µ/mL)	Measured conc. (µ/mL)	Accuracy (%)	Precision (%)
1,5-DCQA				
Intra-day	0.039	0.034 ± 0.003	87.20	9.15
	0.313	0.298 ± 0.027	95.23	9.24
	2.5	2.355 ± 0.171	94.19	7.25
Inter-day	0.039	0.032 ± 0.002	80.69	7.82
	0.313	0.329 ± 0.016	105.41	4.90
	2.5	2.506 ± 0.035	100.26	1.39
3,4-DCQA				
Intra-day	0.078	0.074 ± 0.007	95.01	8.99
	0.625	0.592 ± 0.044	94.74	7.39
	5	4.906 ± 0.290	98.12	5.9
Inter-day	0.078	0.081 ± 0.005	104.08	5.89
	0.625	0.636 ± 0.039	101.69	6.07
	5	4.978 ± 0.280	99.56	5.63
3,5-DCQA				
Intra-day	0.078	0.072 ± 0.003	92.78	3.57
	0.625	0.595 ± 0.044	95.15	7.37
	5	4.954 ± 0.236	99.07	4.77
Inter-day	0.078	0.071 ± 0.002	90.83	2.26
	0.625	0.634 ± 0.040	101.42	6.28
	5	4.994 ± 0.189	99.88	3.78

Table II. Recoveries of 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA in Rat Plasma (n = 5)

Sample	Spiked conc. (µg/mL)	Measured conc. (µg/mL)	Recovery (%)
1,5-DCQA	0.039	0.036 ± 0.002	92.85 ± 5.38
	0.313	0.290 ± 0.014	92.55 ± 4.39
	2.5	2.142 ± 0.030	85.69 ± 1.19
3,4-DCQA	0.078	0.070 ± 0.004	89.83 ± 5.35
	0.625	0.553 ± 0.034	88.40 ± 5.38
	5	4.332 ± 0.244	86.65 ± 4.88
3,5-DCQA	0.078	0.070 ± 0.001	90.18 ± 1.85
	0.625	0.577 ± 0.036	92.38 ± 5.74
	5	4.505 ± 0.170	90.09 ± 3.40

Table III. Stability of 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA in Rat Plasma (*n* = 5)

Sample	Nominal conc. (μg/mL)	Post-preparation in autosampler at 4°C (12 h)		Room temperature (6 h)		Stored at –80°C for a month	
		Measured conc. (μg/mL)	Accuracy (%)	Measured conc. (μg/mL)	Accuracy (%)	Measured conc. (μg/mL)	Accuracy (%)
1,5-DCQA							
	0.039	0.034 ± 0.002	86.59	0.033 ± 0.003	84.02	0.032 ± 0.002	81.65
	0.313	0.276 ± 0.012	88.23	0.258 ± 0.027	82.48	0.283 ± 0.012	90.55
	2.5	2.156 ± 0.066	86.22	2.099 ± 0.103	83.94	2.258 ± 0.066	90.30
3,4-DCQA							
	0.078	0.069 ± 0.003	87.88	0.067 ± 0.001	85.31	0.065 ± 0.005	83.92
	0.625	0.561 ± 0.025	89.79	0.544 ± 0.034	87.07	0.539 ± 0.017	86.17
	5	4.912 ± 0.321	98.25	4.901 ± 0.305	98.03	4.499 ± 0.465	89.99
3,5-DCQA							
	0.078	0.072 ± 0.002	92.35	0.068 ± 0.007	87.23	0.066 ± 0.003	84.79
	0.625	0.582 ± 0.027	93.11	0.528 ± 0.057	84.47	0.595 ± 0.077	82.26
	5	5.086 ± 0.207	101.71	4.936 ± 0.426	98.73	4.565 ± 0.415	91.30

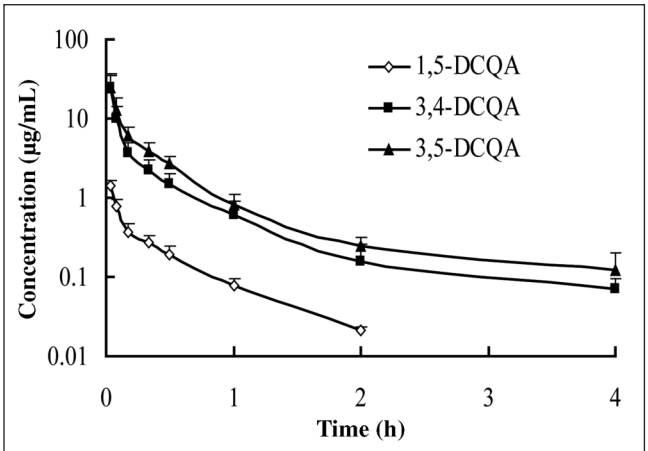

Figure 4. Plasma concentration-time curves of 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA after i.v. bolus administration of mailuoning injection at 10 mL/kg to rats (equivalent to 0.39 mg/kg of 1,5-DCQA, 10.0 mg/kg of 3,4-DCQA, and 5.0 mg/kg of 3,5-DCQA). Each point represents the mean ± SD of 10 rats.

Table IV. Mean Pharmacokinetic Parameters of 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA

Parameter	1,5-DCQA	3,4-DCQA	3,5-DCQA
k10(1/h)	17.238 ± 5.240	11.151 ± 5.872	8.273 ± 7.767
k12(1/h)	2.701 ± 0.966	0.838 ± 1.038	0.410 ± 0.497
k21(1/h)	1.865 ± 0.302	0.979 ± 0.681	1.041 ± 0.724
$T_{1/2\alpha}$ (h)	0.038 ± 0.018	0.100 ± 0.089	0.138 ± 0.081
$T_{1/2\beta}$ (h)	0.334 ± 0.050	0.479 ± 0.155	0.486 ± 0.125
CL(L/h/kg)	1.166 ± 0.218	3.157 ± 1.354	1.072 ± 0.534
$AUC_{(0-\infty)}$ (mg/L*h)	0.353 ± 0.062	3.620 ± 1.246	5.287 ± 1.627
V1(L/kg)	0.175 ± 0.050	0.740 ± 0.662	0.312 ± 0.218
V(L/kg)	1.818 ± 0.510	3.955 ± 0.949	2.775 ± 3.483
MRT (h)	0.323 ± 0.060	0.400 ± 0.128	0.444 ± 0.163

As presumed from a previous metabolism study of dicaffeoylquinic acids (10,26–28), the longer residence of CGA after i.v. administration of mailuoning injection might be the cause of 3,4-DCQA and 3,5-DCQA transformation into CGA, which led to the apparent longer residence of CGA in rat blood. This preliminary information should be useful for guiding further pharmacokinetic study, and contributed to the better understanding of the therapeutic efficacy or toxicity of mailuoning injection.

Conclusion

Simultaneous quantitation of the complicated components contained in the TCM preparations in the biological samples at low levels is still a great challenge and constitutes the main barrier for their pharmacokinetic study. In the present study, a highly selective and sensitive LC–ESI-MS method has been developed and validated for the simultaneous quantitation of 1,5-DCQA, 3,4-DCQA, and 3,5-DCQA in rat plasma after i.v. administration of mailuoning injection. Application with some modifications of the presently described methodology will probably be suitable for the biological determinations of other phenolic acids, which are widely distributed in many natural plants, herbal drugs, and their pharmaceutical preparations.

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