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Rapid simultaneous quantification of five active constituents in rat plasma by high-performance liquid chromatography/tandem mass spectrometry after oral administration of *Da-Cheng-Qi* decoction

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Abstract

A rapid, sensitive and specific liquid chromatography-electrospray ionization/tandem mass spectrometry (LC-ESI/MS/MS) method has been developed and validated for simultaneous determination of five active constituents (including magnolol, honokiol, rhein, emodin and aloe-emodin) from *Da-Cheng-Qi* decoction (DCQD) in rat plasma. After the addition of gliquidone as the internal standard (IS), plasma samples were prepared by one-step protein precipitation using methanol and separated by HPLC on a short reversed phase C_{18} column packed with smaller particles (100 mm × 3.0 mm, 3.5 μm) using a mobile phase of methanol–0.1% formic acid aqueous solution (70:30, v/v). Analytes were determined in a triple-quadrupole mass spectrometer in the selected reaction-monitoring (SRM) mode using electrospray source with negative mode. The method was proved to be rapid, sensitive, specific, accurate and reproducible and has been successfully applied to the determination of the five compounds in rat plasma after oral administration of low dose DCQD for pharmacokinetic study.

Keywords: *Da-Cheng-Qi* decoction; Multiple constituents; High-performance liquid chromatography/tandem mass spectrometry; Rat plasma; Pharmacokinetics

1. Introduction

Da-Cheng-Qi decoction (DCQD and *Ta-Cheng-Chi-Tang* (TCCT)) is a famous purgative formula consisting of four crude drugs: the root and bark of *Rheum palmatum* L. (Polygonaceae), the bark of *Magnolia officinalis* Rehd. et Wils. (Magnoliaceae), the immature fruit of *Citrus aurantium* L. (Rutaceae) and *Mirabilitum* (mirabilite, crystals of sodium sulfate, and Na_2SO_4) which was described in *Shang-Han-Lun*, a classical piece of Traditional Chinese Medicines (TCMs) literature of the Han dynasty [1]. Nowadays, DCQD is indicated in diseases like acute intestinal obstruction without complications, acute cholecystitis and acute appendicitis [2]. Other researchers have shown that DCQD was also effective in treating posttraumatic respiratory distress syndrome [3]; reducing acute-phase protein levels in patients with multiple organ failure syndromes [4], increasing plasma motilin, enhancing gastrointestinal motility, improving gastric dysrythmia, and reducing gastroparesis after abdominal surgery [5] and inflammatory mediators in patients after a tumor operation [6]. Recent research findings indicate that DCQD has anti-inflammatory effects in addition to its traditionally known purgative activities [7]. But up to now the mechanism of its action especially the relationship between clinical effects and chemical components of DCQD is still controversial.

It was well accepted that TCMs expressed its effects through multi-components and multi-targets. The multiple constituents were usually responsible for its therapeutic effects by synergistic and/or antagonistic interaction. The pharmacokinetic profile of multiple active constituents of TCMs might be the

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bridge connecting chemical components and clinical effects, and furthermore facilitate the curative mechanism investigation of them. So, rapid, sensitive and selective analytical method is needed to simultaneous determine of multiple components of TCMs in biological matrix with low concentrations.

Anthraquinones and lignans were two types of active components existing in DCQD. Up to now, no analytical method has been reported for the simultaneous determination of these two types of constituents. But several analytical methods have been reported to quantification anthraquinones alone or in combination. Tang *et al.* [8,9] developed an HPLC method to determine rhein, aloe-emodin and chrysophanol in rat plasma after oral administration of DCQD using ultra-violet detection with the LLOQ of 30, 28 and 25.6 ng mL^{-1}, respectively. Besides, Yan *et al.* [10] reported an HPLC method with fluorescence detection for quantification of five anthraquinones (aloe-emodin, rhein, emodin, chrysophanol and physcion) in rat plasma after oral administration of *Xie-Xin* decoction with the LLOQ >6 ng mL^{-1} for all the five anthraquinones. The above-mentioned methods were either time consuming or not sufficiently sensitive to properly evaluate the pharmacokinetics of these compounds in rat after oral administration of lower dose of DCQD.

So the aim of this work was to develop and validate a novel approach for simultaneous quantification of five active constituents, i.e. magnolol, honokiol, rhein, emodin and aloe-emodin in rat plasma rapidly for multiple constituents pharmacokinetic investigation of DCQD with low dose. Recently, LC/MS has shown its wide applications in many areas of research and played an important role in pharmacokinetics. So, our interest was to utilize a simple single step sample protein precipitation procedure and employ HPLC–MS/MS in order to improve method selectivity and sensitivity with less analysis time. The method was validated sensitive enough and was applied successfully to profile the pharmacokinetics of these compounds in rat after oral administration of DCQD.

2. Experimental

2.1. Chemical and reagents

Magnolol (Batch No.: 110729-200309), Honokiol (Batch No.: 110730-200307), Rhein (Batch No.: 0757-200206) and Emodin (Batch No.: 110756-200110) reference standard were purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Aloe-emodin (Batch No.: 1174-060526) was purchased from Jiangxi Herbfine Hi-tech Co. Ltd. (National Pharmaceutical Engineering Center for Solid Preparation in Chinese Herbal Medicine). Gliquidone (99.7% purity) was identified and supplied by Chengdu Hengrui pharmaceutical Co. Ltd. (Chengdu, China).

Methanol (HPLC grade) was purchased from VWR International Company (Darmstadt, Germany). Formic acid (analytical reagent) was purchased from Nanjing Chemical Reagent No. 1 Factory (Nanjing, China). Water was distillated twice before use.

The medicinal plants and materials used in the experiment including the root and bark of *Rheum palmatum* L. (Polygonaceae), the bark of *Magnolia officinalis* Rehd. et Wils. (Magnoliaceae), the immature fruit of *Citrus aurantium* L. (Rutaceae) and *Mirabilitum* (mirabilite, crystals of sodium sulfate, and Na_2SO_4) were purchased from a traditional Chinese medicinal store in Nanjing, China and authenticated by Prof. Ping Li (Key Laboratory of Modern Chinese Medicines, Ministry of Education, China Pharmaceutical University, Nanjing, China).

2.2. Preparation of stock solutions of standard reference

The stock solutions of all the above-mentioned standard reference were prepared after the correcting for purity and were stored at 4 °C.

Stock solutions of IS was prepared at 1.0 mg mL^{-1} in methanol and was further diluted with methanol (0.1% formic acid) to give the final solution containing 20 ng mL^{-1} of gliquidone.

The stock solution of magnolol, honokiol, emodin and aloe-emodin were prepared at 0.5 mg mL^{-1} in acetonitrile and were further diluted with methanol to prepare the working solutions at 5.0 ng mL^{-1}, 50 ng mL^{-1}, 500 ng mL^{-1} and 5.0 μg mL^{-1}.

The stock solution of rhein was prepared at 0.2 mg mL^{-1} in methanol and was further diluted with methanol to prepare the working solutions at 20 ng mL^{-1}, 200 ng mL^{-1}, 2.0 μg mL^{-1} and 20 μg mL^{-1}.

2.3. Preparation of Da-Cheng-Qi decoction

The DCQD was prepared according to the method and procedure described in *Shang-Han-Lun* and was optimized [11]: the bark of *M. officinalis* Rehd. et Wils. (Magnoliaceae, 24 g) and the immature fruit of *C. aurantium* L. (Rutaceae, 15 g) were immersed in 300 mL distilled water and boiled until half of the original amount was left. This procedure was repeated. The two water extracts were combined. The root and bark of *R. palmatum* L. (Polygonaceae, 12 g) were then immersed in the above mentioned combined water extracts and boiled until half of the original amount was left and then *Mirabilitum* (mirabilite, crystals of sodium sulfate, Na_2SO_4, 6 g) was dissolved in the water extract. The extract was then filtered and dilution to 250 mL with distilled water and stored at 4 °C until use.

2.4. Sample preparations

For quantitative analysis, an aliquot (0.2 mL) of rat plasma was pipetted into 1.5 mL plastics centrifuge tubes with addition of 500 μL methanol (containing 0.1% formic acid and 20 ng mL^{-1} IS) to precipitate protein. Then the samples were vortex mixed for 3 min and centrifuged at 13,800 × *g* for 8 min. The upper layer was transferred into another 1.5 mL plastics centrifuge tubes and centrifuged at 13,800 × g for 8 min again. Only 10 μL aliquots of the supernatant were injected into the LC–MS/MS system.

2.5. Instrumentation and operation conditions

Liquid chromatographic separation and mass spectrometric detection were achieved by employing the Finnigan™ TSQ Quantum Discovery MAXTM LC–MS/MS system consisted of a Finnigan Surveyor LC pump, a Finnigan Surveyor auto-sampler and combined with a triple quadrupole TSQ Quantum mass spectrometer (Thermo Electron Corporation). The chromatography was on a Zorbax SB-C_{18} (100 mm × 3.0 mm, 3.5 μm) analytical column at 45 °C. The isocratic mobile phase composition was a mixture of 0.1% formic acid/methanol (30/70, v/v), which was pumped at a flow rate of 0.4 mL min^{-1}.

The tandem MS system is equipped with an ESI source, and run with the Xcalibur 2.0 software (Thermo Electron Corporation). The mass spectrometer was operated in negative ion and SRM mode with precursor to product qualifier transition *m/z* 265.0 → 247.0 for magnolol, *m/z* 265.0 → 223.9 for honokiol, *m/z* 283.0 → 238.9 for rhein, *m/z* 268.9 → 224.9 for emodin, *m/z* 269.0 → 239.9 for aloe-emodin and *m/z* 526.0 → 400.9 for IS. The mass spectrometric conditions were optimized in order to achieve maximum sensitivity and as followed: spray voltage at −4000 V, heated capillary temperature at 350 °C, sheath gas and auxiliary gas (nitrogen) pressure at 45 and 25 arbitrary units (set by the LCQ software, Thermo Electron Corporation), respectively. Argon was used as collision gas at a pressure of 1.5 mTorr and collision energy was 23 V for magnolol, 26 V for honokiol, 15 V for rhein, 30 V for emodin and 25 V for aloe-emodin. The scan width for SRM was 0.01 *m/z* and scan time was 0.3 s. The peak width settings (FWHM) for both Q_1 and Q_3 were 0.7 *m/z*.

2.6. Method validation

The method validation assays were carried out according to the currently accepted US Food and Drug Administration (FDA) bioanalytical method validation guidance [12] on specificity, linearity, sensitivity, accuracy, precision, recovery and stability. Three different concentration levels of each analyte were selected to experience the method validation, i.e. 5, 50 and 2000 ng mL^{-1} for rhein, 0.5, 5 and 200 ng mL^{-1} for emodin and magnolol, 1.25, 12.5 and 500 ng mL^{-1} for honokiol and aloe-emodin, respectively.

The method's specificity was tested by screening six different batches of drug-free rat plasma for the exclusion of any endogenous co-eluting interference at the peak region of each analyte and IS. Each blank sample was tested for interference using the proposed extraction procedure and chromatographic/spectroscopic conditions and was compared with those obtained with an aqueous solution of the analyte at a concentration near to the LLOQ.

The matrix effect on the ionization of analytes was evaluated by comparing the peak area of analytes resolved in blank sample (the final solution of blank plasma after protein precipitation) with that resolved in mobile phase. Three different concentration levels of the five analytes (including magnolol, honokiol, rhein, emodin and aloe-emodin) were evaluated by analyzing six samples at each level. The blank plasma used in this study was six different batches of healthy rat blank plasma. If the ratio <85% or >115%, an exogenous matrix effect was implied.

The effects of suppression from compounds existing in DCQD on the five analyzed markers were evaluated using a post-column infusion system. In this system, continuous post-column infusion of the DCQD or water is performed at 0.2 mL min^{-1}, while standard solutions of the five analyzed compound are injected onto the LC column. Three concentration levels for the five compounds were involved, i.e. 1.5, 15 and 550 ng mL^{-1} for rhein, 0.15, 1.5 and 55 ng mL^{-1} for emodin and magnolol, 0.5, 3.5 and 140 ng mL^{-1} for honokiol and aloe-emodin, respectively. The effect of suppression from compounds existing in DCQD on the five analyzed compounds was calculated by comparing the peak area of the analyte obtain with the infusion of DCQD to that obtained with infusion of water. If the ratio <85% or >115%, a suppression from compounds existing in DCQD was implied.

Calibration curves were prepared by spiking different samples of 0.2 mL blank plasma each with proper volume of one of the above-mentioned working solutions to produce the calibration curve points equivalent to 2, 5, 10, 50, 200, 500 and 2000 ng mL^{-1} for rhein, 0.2, 0.5, 2, 5., 20, 50 and 200 ng mL^{-1} for magnolol and emodin, 0.5, 1.25, 5, 12.5, 50, 125 and 500 ng mL^{-1} for honokiol and aloe-emodin. A blank plasma sample was also analyzed to confirm absence of interferences and was not used to construct the calibration function. Calibration function was constructed by determining the best-fit of peak area ratios (peak area of analyte/peak area of IS) vs. concentration, and fitted to the equation $C = bR + a$ by least-squares linear regression with weighting and using $1/x$ (x, concentration) as weighting factors, where R corresponds to the peak area ratio and C refers to the concentration added to plasma. Four out of seven none zero standards including LLOQ and ULOQ were to meet the following acceptance criteria: no more than 20% deviation at LLOQ and no more than 15% deviation for standards above the LLOQ. The acceptance criterion for correlation coefficient was 0.99 or more, otherwise the calibration curve should be rejected. Five replicate analyses were done.

For sensitivity determination, the lowest standard concentration in the calibration curve was considered as the lower limit of quantification, and was to meet the following criteria: LLOQ response should be ten times the response of the blank and the LLOQ response should be identifiable, discrete and reproducible with a precision correspondence to max 20% R.S.D. Reproducibility and precision were also determined.

The precision of the assay was determined from the QC plasma samples by replicate analyses of three concentration levels of the five analytes (including magnolol, honokiol, rhein, emodin and aloe-emodin). Within-batch precision and accuracy were determined by repeated analysis of the group of standards on 1 day ($n = 5$). Between-batch precision and accuracy were determined by repeated analysis on 3 consecutive days ($n = 5$ series per day).

The absolute recovery of each analyte through the extraction procedures was determined at three concentrations. A known amount of analyte was added to blank rat plasma prior to extraction as described in Section 3.1, and then the IS was added after

protein precipitation to eliminate bias introduced by sample processing. As standards, the same amounts of analyte and IS were evaporated to dryness, then the residue was dissolved in the upper layer of blank rat plasma after protein precipitation. The extraction recovery was calculated by comparing the peak area ratio of analyte/IS of extracted samples to the peak area ratio of analyte/IS of standards.

Short-term temperature stability: Stored plasma aliquots were thawed and kept at room temperature for a period of time exceeded that expected to be encountered during the routine sample preparation (around 6 h). Samples were analyzed as mentioned above.

Post-preparative stability: The auto-sampler stability was conducted reanalyzing extracted QC samples kept under the auto-sampler conditions (15 °C) for 12 h.

Freeze and thaw stability: QC plasma samples containing analytes were tested after three freeze (−20 °C) and thaw (room temperature) cycles.

Long-term stability of each analyte in rat plasma was studied for a period of 8 weeks employing QC samples at three different levels. If after the stability study the analyte was found to be unstable at −20 °C, then it should be stored at −70 °C.

The stability of each analyte and internal standard working solutions were evaluated by testing their validity for 6 h at room

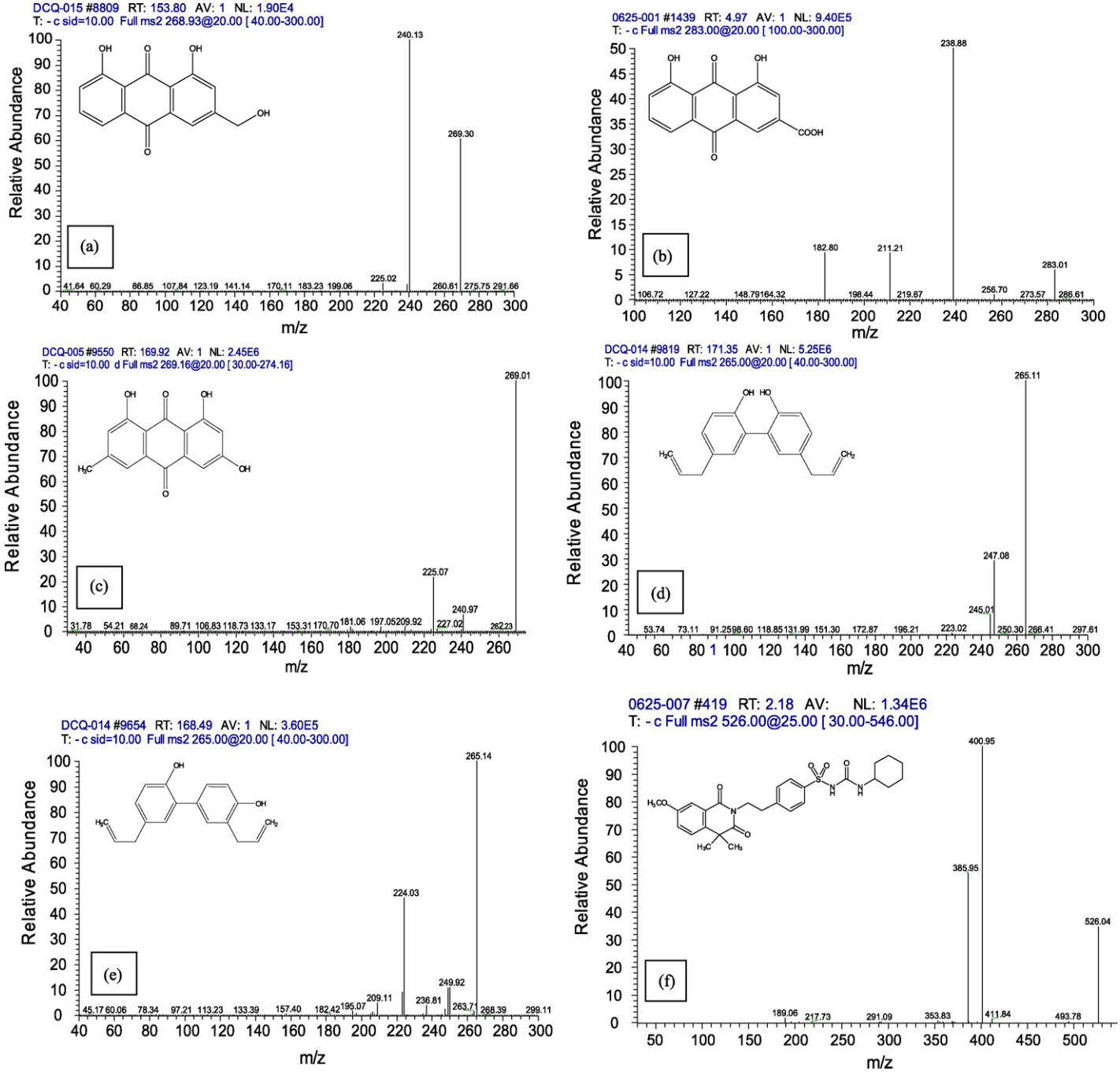

Fig. 1. Chemical structure and MS/MS spectra of aloe-emodin (a), rhein (b), emodin (c), magnolol (d), honokiol (e) and IS (f).

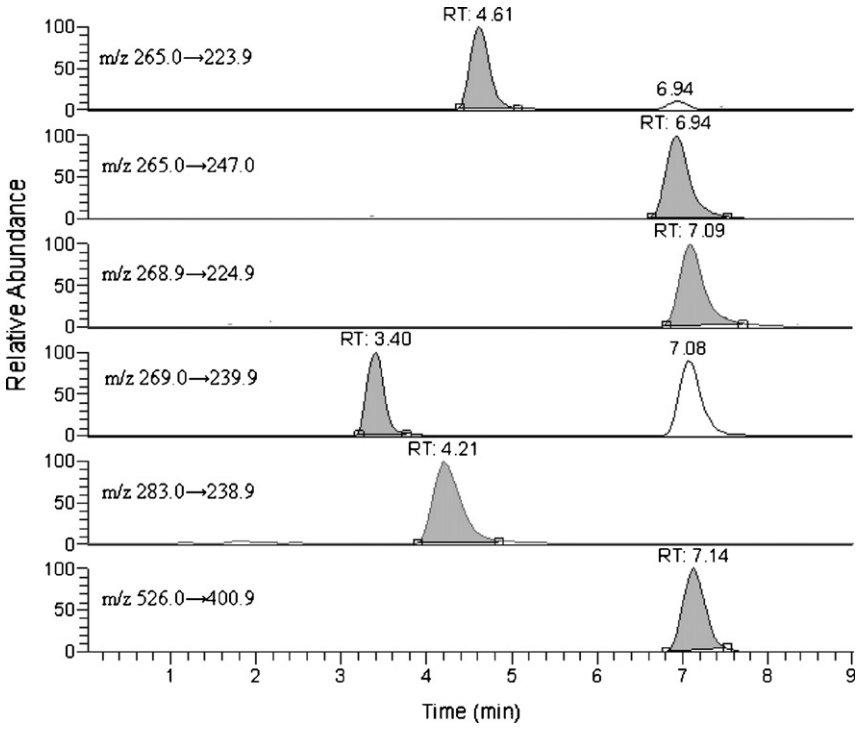

Fig. 2. The SRM chromatograms extracted from supplemented plasma. The retention times of aloe-emodin (125 ng mL^{-1}), rhein (500 ng mL^{-1}), honokiol (125 ng mL^{-1} L), magnolol (50 ng mL^{-1}), emodin (50 ng mL^{-1}) and IS (50 ng mL^{-1}) were 3.4, 4.2, 4.6, 6.9, 7.1 and 7.2 min, respectively.

temperature. Stability of working solutions was expressed as percentage recovery.

A calibration curve was generated to assay samples in each analytical run and was used to calculate the concentration of each analyte in the unknown samples in the run. The calibration was analyzed in the middle of each run. In order to monitor the accuracy and precision of the analytical method a number of QC samples were prepared to ensure that method continues

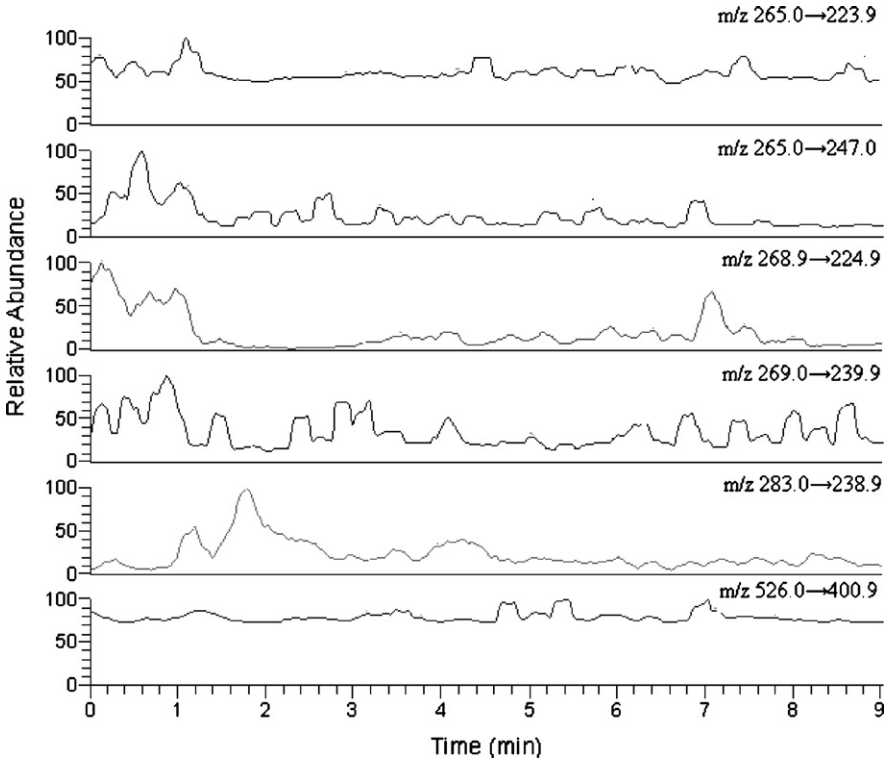

Fig. 3. The SRM chromatogram for a blank plasma sample. The retention times of aloe-emodin, rhein, honokiol, magnolol, emodin and IS were 3.4, 4.2, 4.6, 6.9, 7.1 and 7.2 min, respectively.

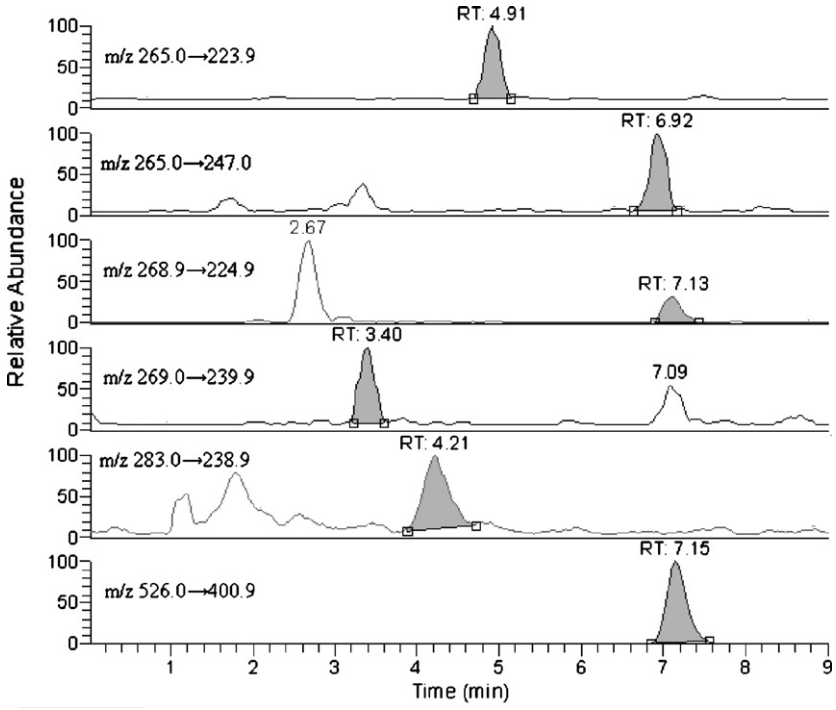

Fig. 4. The SRM chromatogram of LLOQ. The retention times of aloe-emodin (0.5 ng mL^{-1}), rhein (2.0 ng mL^{-1}), honokiol (0.5 ng mL^{-1}), magnolol (0.2 ng mL^{-1}), emodin (0.2 ng mL^{-1}) and IS (50 ng mL^{-1}) were 3.4, 4.2, 4.6, 6.9, 7.1 and 7.2 min, respectively.

to perform satisfactorily. The QC samples in duplicate at three concentrations were prepared and were analyzed with processed test samples at intervals based on the total number of samples per batch.

2.7. Method application: animals, drug administration and plasma collection

Six Sprague–Dawley rats (three male and three female; body weight, 190 ± 10 g) were supplied by the Animal Multiplication Center of Qinglong mountain (SCXK 2007-0007). The experimental protocol was approved by the University Ethics Committee for the use of experimental animals and conformed to the Guide for Care and Use of Laboratory Animals. The rats were maintained in air-conditioned animal quarters under the following conditions: temperature 22 ± 2 °C, relative humidity 55 ± 10%, free access to water, and feeding with laboratory rodent chow (Nanjing, China). The animals were acclimatized to the facilities for 10 days and were then fasted with free access to water for 12 h prior to the experiment.

It was orally administered to rats at a dosage of 15 mL kg^{-1} (3.42 g kg^{-1} for raw medicinal materials) for DCQD. Blood samples (0.5 mL) were collected at time points of 0 (prior to administration), 0.083, 0.166, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12, 16, 24, 36 and 48 h after a single dose. The rats were intragastric administrated with 2 mL of water at the time points of 0.5, 4 and 12 h, respectively. The rats had free access to water during the experiment. The blood samples were immediately heparinized and centrifuged at 7885 × *g* for 5 min, and the super-

Table 1
The within- and between-batch precision, accuracy of the method for determination of rhein, emodin, magnolol, honokiol and aloe-emodin (within-batch: $n = 5$; between-batch: $n = 15$ series per day; the three QC concentration levels were 5, 50 and 2000 ng/mL for rhein, 0.5, 5 and 200 ng mL^{-1} for emodin and magnolol, 1.25, 12.5 and 500 ng mL^{-1} for honokiol and aloe-emodin, respectively)

Conc. levels	Rhein		Emodin		Magnolol		Honokiol		Aloe-emodin	
	Accuracy (%)	R.S.D. (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)	R.S.D. (%)	Accuracy (%)	R.S.D. (%)
Within-batch										
Low	109.3	9.7	111.3	8.4	100.2	10.6	100.5	10.6	112.9	9.8
Middle	100.4	5.3	99.5	4.1	107.4	7.2	100.2	5.2	100.5	4.6
High	98.6	1.6	100.6	3.9	99.7	4.9	99.4	1.4	98.9	1.7
Between-batch										
Low	112.4	10.7	106.1	9.8	105.5	14.6	106.2	10.1	111.3	12.9
Middle	109.0	4.9	102.0	7.2	100.3	5.4	98.3	4.4	103.7	7.4
High	99.7	2.2	100.3	1.6	98.8	6.3	102.4	3.5	100.1	3.8

Table 2
Recovery of rhein, emodin, magnolol, honokiol and aloe-emodin from plasma ($n = 5$, the three QC concentration levels were 5, 50 and 2000 ng/mL for rhein, 0.5, 5 and 200 ng mL^{-1} for emodin and magnolol, 1.25, 12.5 and 500 ng mL^{-1} for honokiol and aloe-emodin, respectively)

Conc. levels	Rhein		Emodin		Magnolol		Honokiol		Aloe-emodin	
	Recovery (mean ± S.D., %)	R.S.D. (%)	Recovery (mean ± S.D., %)	R.S.D. (%)	Recovery (mean ± S.D., %)	R.S.D. (%)	Recovery (mean ± S.D., %)	R.S.D. (%)	Recovery (mean ± S.D., %)	R.S.D. (%)
Low	91.2 ± 9.8	10.7	88.5 ± 7.2	8.1	92.5 ± 9.2	9.9	88.1 ± 7.2	8.2	86.5 ± 5.7	6.7
Middle	89.8 ± 6.6	7.3	91.6 ± 4.8	5.2	94.7 ± 3.8	4.0	84.6 ± 4.8	5.7	90.1 ± 5.4	6.0
High	90.4 ± 3.5	3.9	90.7 ± 2.9	3.2	92.7 ± 3.6	3.9	87.7 ± 2.4	2.7	90.7 ± 3.1	3.4

natant was harvested into 0.2 mL aliquots and stored in 1.5 mL polypropylene tubes at −4 °C prior to analysis.

3. Results and discussion

3.1. Sample preparation procedure

One obstacle for multiple constituent analyses in biological matrix was the sample preparation owing to their different property of dissolution, pK_a, stability as well as the concentrations in biological matrix. So the ideal sample preparation procedure should possess the following prosperities: extract the entire compound with high recovery and no endogenous interference at the retention time, avoid the degradation of all the compounds, and concentrate samples. Owing to utilizing the inherent selective and sensitive LC–MS/MS technique, the first two prosperities were more important for us to seek an ideal sample preparation procedure. At the same time, in order to make the procedure simple and time saving, protein precipitation (PPT) becomes our priority. So, three types of precipitation reagents (methanol, acetonitrile, and perchloric acid) were investigated during the experiment. Methanol was eventually proved to be the best among the three reagents in terms of the higher extraction recovery and absences of endogenous interference at the retention time of analytes and the IS in the chromatogram.

3.2. Mass spectrometry conditions

Negative ion electrospray mode was selected owing to that the five constituents we analyzed were all phenolic acid compounds. Fig. 1 shows the chemical structures and MS/MS spectra of each compound, from which we could find that honokiol vs. magnolol, emodin vs. aloe-emodin were two pairs of isomers but the MS/MS fragmentation behaviors of them were very different.

For honokiol (Fig. 1e), *m/z* at 250, 247, 237, 224 and 209 were the major fragment ions of its $[M-H]^-$ *m/z* 265 in MS/MS spectra, among which ions at *m/z* 250, 247, 237 and 224 was proposed -as yield through the loss of $CH_3\cdot$, H_2O, CO and $CH_2{=}CHCH_2\cdot$, respectively from *m/z* 265; magnolol (Fig. 1d) gave rise the

Table 3
Data showing stability of rhein, emodin, magnolol, honokiol and aloe-emodin in plasma at different QC levels ($n = 5$, the three QC concentration levels were 5, 50 and 2000 ng mL^{-1} for rhein, 0.5, 5 and 200 ng mL^{-1} for emodin and magnolol, 1.25, 12.5 and 500 ng mL^{-1} for honokiol and aloe-emodin, respectively)

Conc. levels	Accuracy (mean ± S.D, %)			
	Freeze and thaw stability	Short-term stability	Long-term stability	Post-preparative stability
Rhein				
Low	111.4 ± 13.2	102.8 ± 9.2	103.7 ± 8.8	98.7 ± 10.0
Middle	100.5 ± 6.4	105.7 ± 5.1	100.2 ± 3.6	94.1 ± 5.2
High	99.7 ± 1.3	103.1 ± 3.2	97.8 ± 5.2	93.6 ± 3.9
Emodin				
Low	94.6 ± 10.4	87.3 ± 9.2	101.9 ± 8.4	91.1 ± 9.1
Middle	91.4 ± 3.3	98.6 ± 4.1	93.7 ± 4.2	95.2 ± 7.9
High	105.5 ± 1.7	92.3 ± 3.5	107.1 ± 2.9	88.4 ± 4.3
Magnolol				
Low	101.4 ± 10.1	96.4 ± 13.2	104.8 ± 9.9	95.0 ± 10.2
Middle	98.5 ± 6.7	93.8 ± 6.6	101.2 ± 5.0	100.5 ± 5.8
High	99.3 ± 3.2	103.5 ± 5.2	98.7 ± 3.1	99.5 ± 2.3
Honokiol				
Low	101.0 ± 9.2	97.3 ± 8.4	100.3 ± 11.2	96.5 ± 7.4
Middle	97.4 ± 3.7	101.9 ± 6.9	91.4 ± 8.5	104.4 ± 6.7
High	100.9 ± 5.8	95.8 ± 4.3	104.5 ± 5.4	100.0 ± 4.2
Aloe-emodin				
Low	96.5 ± 9.3	111.7 ± 12.5	98.4 ± 10.1	99.7 ± 9.5
Middle	100.1 ± 8.0	94.5 ± 6.9	103.5 ± 8.2	97.8 ± 5.6
High	98.4 ± 5.2	100.3 ± 4.7	95.5 ± 5.7	102.8 ± 4.5

MS/MS ion of *m/z* at 247 and 245 from its $[M-H]^-$ *m/z* 265, among which *m/z* at 247 was base peak and was proposed generated through the loss of H_2O from *m/z* 265.

Aloe-emodin and emodin are isomers with the same $[M-H]^-$ ions at *m/z* 269, while their MS/MS spectra were different. For emodin (Fig. 1c), the $[M-H]^-$ ions produce two major fragments at *m/z* 241 and 225 by loss of CO and CO_2, respectively. While for aloe-emodin (Fig. 1a), only one fragment was generated through the elimination of CHO.

Rhein (Fig. 1b) give the $[M-H]^-$ ions at *m/z* 283, after a loss of CO_2 leading to the fragment at *m/z* 239. A further loss of CO gives rise to two types of fragment at *m/z* 211. These ions could further lose one molecule of CO to produce *m/z* 183.

In order to obtain the highest sensitivity, the MS parameters, SRM ions and collision energies were optimized by auto-tuning. Finally, the precursor to product ion transitions *m/z* 265.0 → 247.0 for magnolol, *m/z* 265.0 → 223.9 for honokiol, *m/z* 283.0 → 238.9 for rhein, *m/z* 268.9 → 224.9 for emodin, *m/z* 269.0 → 239.9 for aloe-emodin and *m/z* 526.0 → 400.9 for IS were achieved.

3.3. Chromatography conditions and sample separation

To achieve symmetric peak shape as well as a short run time for the simultaneous analysis the five compounds, the chromatographic conditions were optimized through trials. Formic acid with the concentration of 0.1% was accepted as mobile phase to balance the peak shape, sensitivity and retention time of each analyte. At the same time Zorbax SB-C18 (100 mm × 3.0 mm, 3.5 μm) column was adopted instead of Zorbax SB-C18 (250 mm × 4.6 mm, 5.0 μm) column in order to decrease the analysis time.

Under the developed chromatographic conditions for the simultaneous determination of the five compounds of DCQD, all analytes were eluted rapidly within 9.0 min (Fig. 2). The retention times of aloe-emodin, rhein, honokiol, magnolol, emodin and IS were 3.4, 4.2, 4.6, 6.9, 7.1 and 7.2 min, respectively.

3.4. Method validation

No interferences of the analytes were observed. Fig. 3 shows an HPLC chromatogram for a blank plasma sample indicating no endogenous peaks at the retention positions of aloe-emodin, rhein, honokiol, magnolol, emodin and IS. All the ratios of the peak area resolved in blank sample compared with that resolved in mobile phase are between 85% and 115%, which means no matrix effect for aloe-emodin, rhein, honokiol, magnolol, emodin and IS in this method. No suppression from compounds existing in DCQD was found for the five analyzed compounds.

The calibration curves of each analyte showed good linearity in the ranges of 2.0–2000.0 ng mL^{-1} for rhein, 0.2–200.0 ng mL^{-1} for magnolol and emodin, 0.5–500.0 ng mL^{-1} for honokiol and aloe-emodin, respectively. The mean regression equations from five replicate calibration curves on different days: $C = 4.89 \times R - 0.0396$ ($r = 0.9990$) for the rhein, $C = 7.10 \times R + 0.00473$ ($r = 0.9966$) for the magnolol, $C = 1.76 \times R - 0.00819$ ($r = 0.9951$) for the emodin, $C = 21.6 \times R + 0.00806$ ($r = 0.993$) for the honokiol,

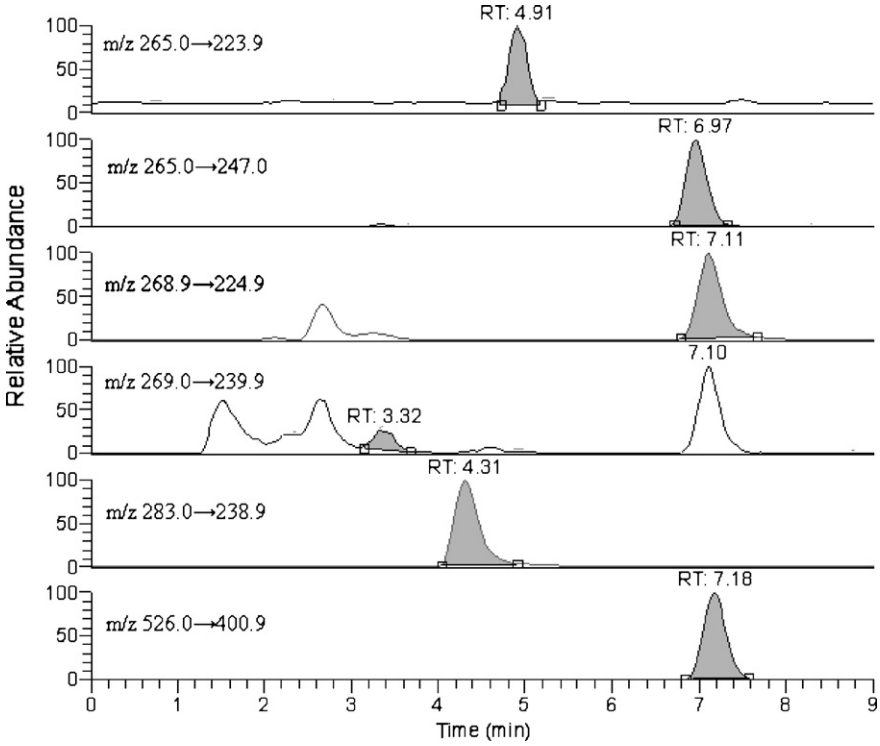

Fig. 5. The SRM chromatogram for plasma sample obtained at 0.5 h after oral administration of *Da-Cheng-Qi* decoction. The retention times of aloe-emodin (0.8 ng mL^{-1}), rhein (386.7 ng mL^{-1}), honokiol (0.7 ng mL^{-1}), magnolol (1.6 ng mL^{-1}), emodin (1.2 ng mL^{-1}) and IS (50 ng mL^{-1}) were 3.4, 4.2, 4.6, 6.9, 7.1 and 7.2 min, respectively.

$C = 9.36 \times R - 0.0538$ ($r = 0.9985$) for the aloe-emodin, respectively. All the above-mentioned calibration curves met the acceptance criteria with good back-calculated accuracy and precision.

Fig. 4 shows the SRM chromatogram of LLOQ for each compound. And the lower limits of detection (LLOD) for rhein, magnolol, emodin, honokiol and aloe-emodin were 1.0 ng mL^{-1}, 0.1 ng mL^{-1} L, 0.1 ng mL^{-1}, 0.2 and 0.2 ng mL^{-1}, respectively.

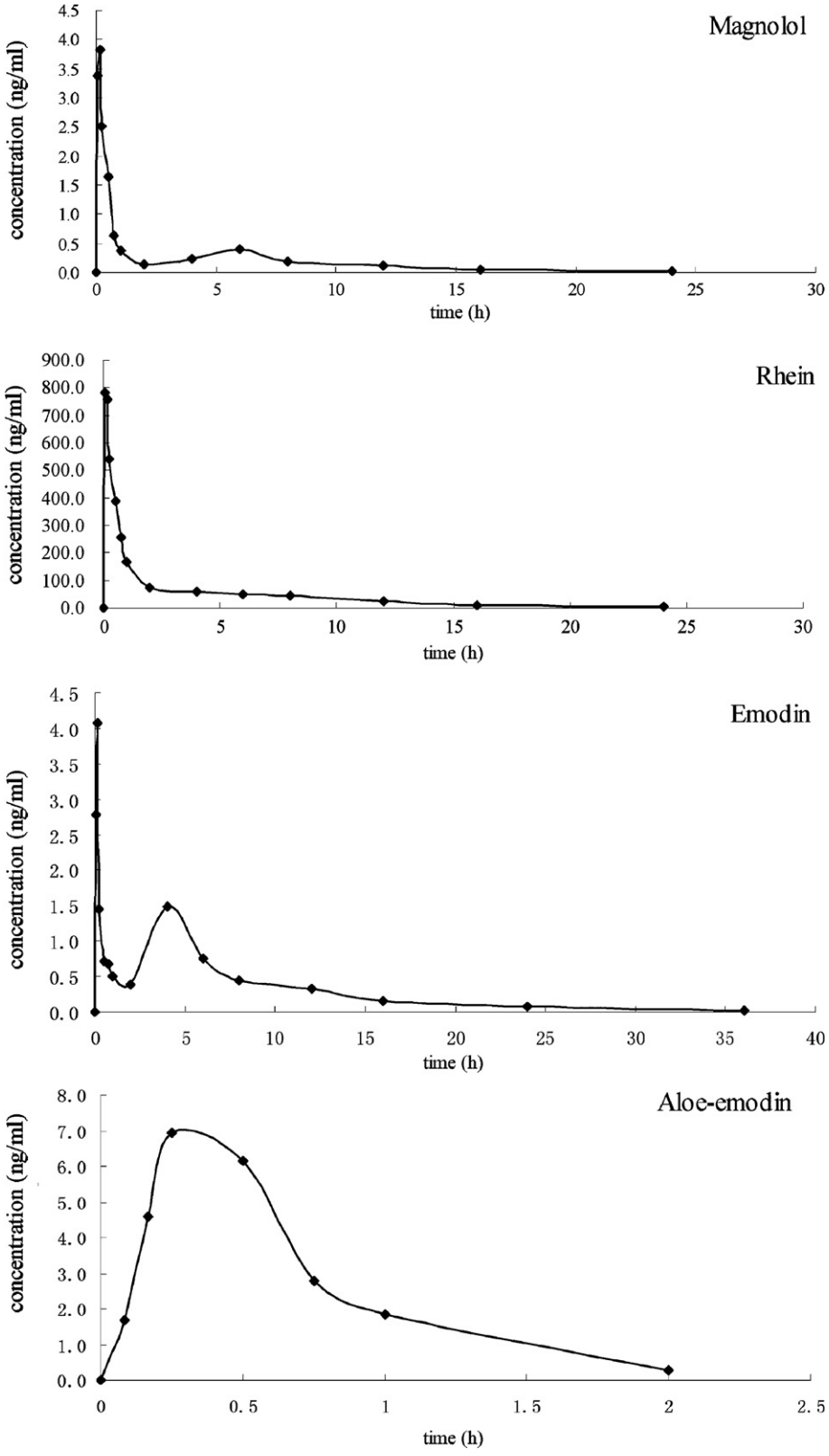

Fig. 6. Mean pharmacokinetic profiles of magnolol, rhein, emodin and aloe-emodin after oral administration of DCQD.

Data for within-batch and between-batch precision and accuracy of the method for rhein, magnolol, emodin, honokiol and aloe-emodin are presented in Table 1. The accuracy deviation values are within 15% of the actual values. The precision determined at each concentration level does not exceed 15% of the relative standard deviation (R.S.D.). The results revealed good precision and accuracy.

The extraction recovery determined for rhein, magnolol, emodin, honokiol and aloe-emodin were shown to be consistent, precise and reproducible. Data was shown below in Table 2.The extraction recovery of IS was more than 85%.

Table 3 summarizes the freeze and thaw stability, short-term stability, long-term stability and post-preparative stability data of rhein, magnolol, emodin, honokiol and aloe-emodin. All the results showed the stability behavior during these tests and there were no stability-related problems during the samples routine analysis for the pharmacokinetic studies.

The stability of working solutions was tested at room temperature for 6 h. Based on the results obtained, these working solutions were stable within 6 h.

3.5. Application

The method described above was successfully applied in the multiple constituents pharmacokinetic study. Fig. 5 shows the representative chromatogram for plasma sample obtained after oral administration of DCQD. The mean pharmacokinetic profiles of magnolol, rhein, emodin and aloe-emodin after oral administration of DCQD were shown in Fig. 6. The pharmacokinetic profile of honokiol was not got for its too low concentration at many time points.

4. Conclusion

An LC–MS/MS assay for the rapid simultaneous quantification of five active constituents (including magnolol, honokiol, rhein, emodin and aloe-emodin) in rat plasma has been developed and fully validated for the first time. The significant feature of this paper was the combination of one-step protein precipitation, short analytical column packed with smaller particles and high selective and sensitive SRM mode of MS/MS to decrease analysis time as well as increase selectivity and sensitivity. The method was proved to be rapid, sensitive, specific, accurate and reproducible and has been successfully applied to the determination of the five compounds in rat plasma after oral administration of DCQD for pharmacokinetic study. This paper provides an example for multiple components analysis of TCMs in biological matrix of low concentrations.

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