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Simultaneous determination of the 10 major components of Da-Cheng-Qi decoction in dog plasma by liquid chromatography tandem mass spectrometry

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ABSTRACT

A liquid chromatography tandem mass spectrometry (LC–MS/MS) method was developed for the simultaneous determination of the 10 major components of Da-Cheng-Qi decoction (rhein, emodin, aloe-emodin, chrysophanol, rheochrysidin, naringin, naringenin, hesperidin, magnolol and honokiol) in dog plasma. Plasma samples were spiked with internal standard (ibuprofen), acidified with HCl and extracted twice by liquid–liquid extraction using ethyl acetate. Separation was performed on a YMC-Pack ODS-A C_{18} column (5 μm, 150 mm × 4.6 mm) and a C_{18} guard column (5 μm, 4.0 mm × 2.0 mm) with methanol–water (92:8, v/v) at a flow rate of 0.3 mL/min. The LC/MS system was operated under the multiple reaction monitoring mode using electrospray ionization in the negative ion mode. All analytes showed good linearity over a wide concentration range ($r > 0.99$). The linear range of the calibration curves was 5000–19.53 ng/mL for rhein; 400–3.13 ng/mL for emodin; 800–3.13 ng/mL for aloe-emodin, chrysophanol, naringin, naringenin, hesperidin, magnolol and honokiol; 160–0.63 ng/mL for rheochrysidin. The lower limit of quantification was: 19.53 ng/mL for rhein; 3.13 ng/mL for emodin, aloe-emodin, chrysophanol, naringin, naringenin, hesperidin, magnolol and honokiol; 0.63 ng/mL for rheochrysidin. The overall mean accuracy for the 10 major components of Da-Cheng-Qi decoction was 90.40–108.60%. Intra-day and inter-day precision was ≤12.43% and ≤11.32%, respectively. We conclude that this method is appropriate for simultaneous determination of the 10 major components of Da-Cheng-Qi decoction in dog plasma and the investigation of the pharmacokinetics of Da-Cheng-Qi decoction in dog.

1. Introduction

Determination of the pharmacokinetic properties of traditional Chinese medicines (TCMs) is necessary to promote the efficacy and safety of these medicines and to avoid side adverse effects. Pharmacokinetic study of TCMs can be a challenge owing to the complex compositions, uncharacterized active constituents, and low plasma concentrations of TCMs. Therefore, a sensitive and selective method is needed for simultaneous determination of the multiple components of TCMs in a biological matrix [1].

Da-Cheng-Qi decoction (DCQT) is a widely used traditional Chinese medicine first described in the Shang-Han-Lun. Da-Cheng-Qi decoction has been used as a purgative for the treatment of constipation and for clearing of internal heat in the stomach and intestine. Clinically, DCQT is effective in the treatment of acute diseases of the abdomen such as intestinal obstructions and adhesions. The decoction consists of four crude ingredients: rhubarb, immature orange fruit, magnolia bark and Mirabilitum (Na_2SO_4) [2]. Rhubarb possesses a wide spectrum of bioactivities including inhibition of the production of nitric oxide (NO) and malondialdehyde (MDA) induced by lipopolysaccharide, anti-bacterial activity, and hepatoprotection. Rhubarb is the principal ingredient in DCQT, exerting the major therapeutic activity of the decoction. Anthraquinone compounds of rhubarb, such as rhein, emodin, aloe-emodin, chrysophanol and rheochrysidin, are effective in the induction of diarrhea [2,3]. Naringin, naringenin and hesperidin are bioactive flavonoids present in immature orange fruit. Like most flavonoids, naringin, naringenin and hesperidin possess antioxidant, anti-inflammatory and anti-ulcer properties [4,5]. Honokiol and magnolol are isomers of neolignans that inhibit intracellular calcium mobilization in platelets as well as relax vascular smooth muscle. They also exert antihemostatic and antithrombotic effects [6,7].

At present, no analytical methods have been reported for the simultaneous determination of rhein, emodin, aloe-emodin, chrysophanol, rheochrysidin, naringin, naringenin, hesperidin, honokiol and magnolol in DCQT in a biological matrix. However, several analytical methods have been reported for the quantification of anthraquinones [8,9], flavonoids [10,11] and neolignans [12] as separated components and combined components [13].

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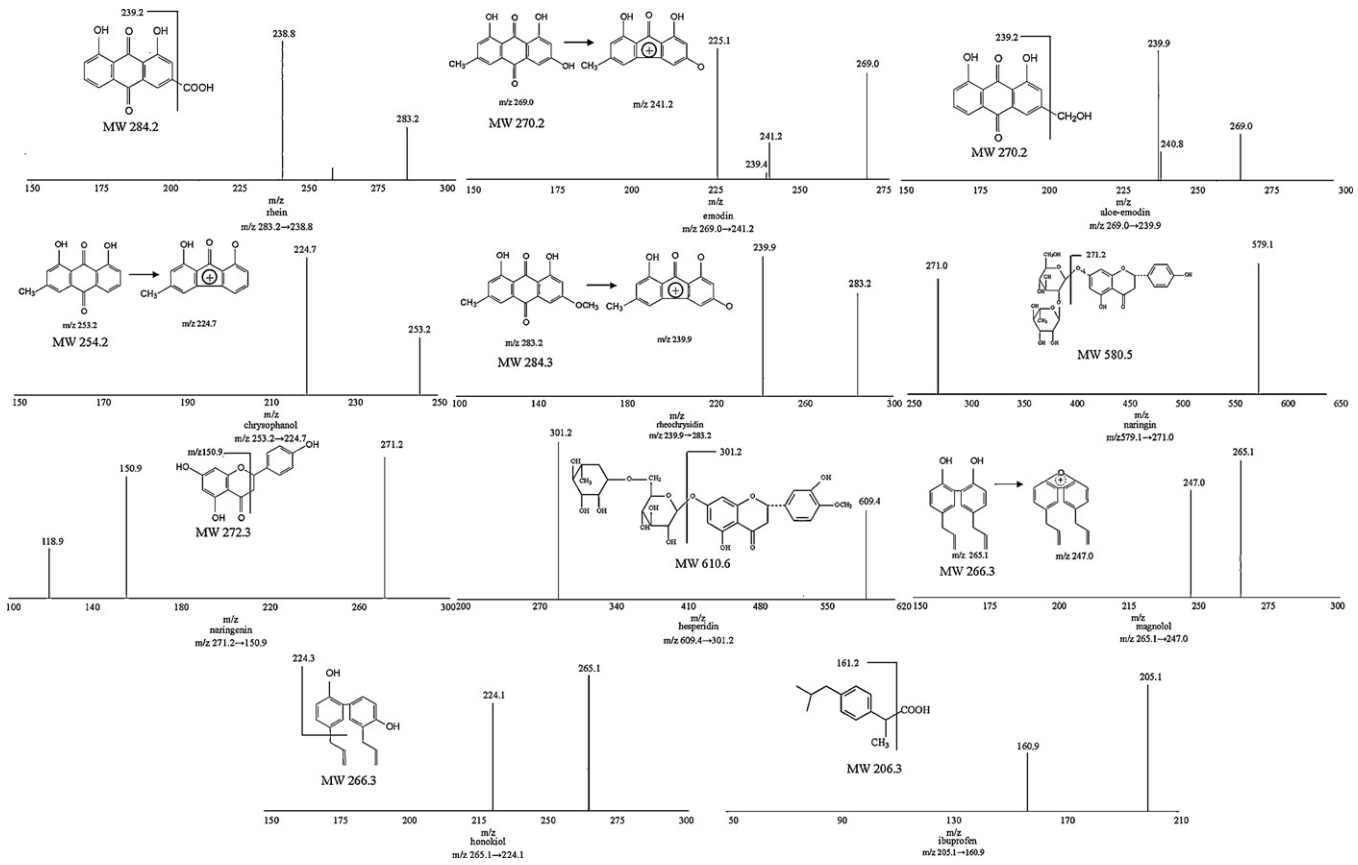

Fig. 1. Product ion mass spectra (ESI^-) and ion structures of the 10 major components of DCQT and IS.

The purpose of this study was to develop and validate a high-performance liquid chromatography–mass spectrometry (LC–MS/MS) method for the simultaneous determination of 10 major active components of DCQT in dog plasma.

2. Experimental

2.1. Chemicals and materials

Dahuang, Houpu, Zhishi and Mangxiao were purchased from Chengdu Green Herbal Pharmaceutical Co. Ltd (Chengdu, China). The crude components of the formula were extracted twice by refluxing with boiling distilled water (1:12, g/mL) for 1 h, and the solution obtained was concentrated and spray-dried. The dry powder was stored at 4 °C until use.

Reference standards of rhein, emodin, aloe-emodin, chrysophanol, naringin, hesperidin, magnolol and honokiol were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), that for rheochrysidin was purchased from Nanjing Chongyuan Biotechnology Co. Ltd (Nanjing, China) and that for naringenin was purchased from Nanjing TCM Institute of Chinese Materia Medical (Nanjing, China). Internal standard (ibuprofen) was kindly supplied by Glaxo Smith Kline Pharmaceutical Co. Ltd (Tianjin, China). Methanol, ethyl acetate and formic acid (HPLC grade) were obtained from Tedia Co. Ltd (Fairfield, OH, USA), and ammonium acetate, sodium hydroxide and hydrochloric acid (analysis grade) were obtained from Chengdu Kelong Chemical Reagent Factory (Sichuan, China). All aqueous solutions and buffers were prepared with de-ionized water from a Millipore Rios^{TM}-16 water purifier (Millipore, Billerica, MA, USA).

2.2. Apparatus

The LC–MS/MS system consisted of a SIL-HT*c* autosampler (Shimadzu, Kyoto, Japan), an LC-10AD*vp* pump (Shimadzu), an API3000 triple-quadrupole LC–MS system (Applied Biosystems, Foster City, CA, USA). The system was controlled with Analyst 1.4.2 software.

2.3. Chromatographic conditions

Separation was performed on a YMC-Pack ODS-A C_{18} column (5 μm, 150 mm × 4.6 mm, YMC, Kyoto, Japan) and a C_{18} guard column (5 μm, 4.0 mm × 2.0 mm, Phenomenex Inc., Torrance, CA, USA). The mobile phase consisted of methanol–water (92:8, v/v) at a flow rate of 0.3 mL/min. The column was maintained at ambient temperature and the injection volume was 80 μL.

2.4. Mass spectrometry conditions

The mass spectrometer was operated using an electrospray source configured to negative ion mode and quantification was performed using multiple reaction monitoring (MRM). Product ion mass spectra of the analytes are shown in Fig. 1 where $[M-H]^-$ of each analyte was selected as the precursor ion, and the most abundant or specific fragment ion was selected as the product ion in MRM acquisition. Instrumental parameters were optimized for each analyte by infusion of the corresponding standard solution at a flow rate of 5 μL/min, using a syringe pump integrated into the API 3000 mass spectrometer. Nitrogen was used as the curtain and auxiliary gas and air was used as the nebulizer gas. Electrospray conditions for the 10 major components and I.S. were curtain gas, 6.0 L/min; ion-spray voltage, −4500 V; nebulizer gas, 6.0 L/min;

Table 1
MS parameters of the 10 main compositions of DCQT with LC–MS/MS.

Optimized parameters	MRM	DP	FP	EP	CE	CXP
Rhein	283.2/238.8	−27	−150	−10	−12	−6
Emodin	269.0/241.2	−52	−200	−10	−37	−6
Aloe-emodin	269.0/239.9	−64	−260	−10	−36	−6
Chrysophanol	253.2/224.7	−55	−210	−10	−35	−6
Rheochrysidin	283.2/239.9	−86	−180	−12	−34	−8
Naringin	579.1/271.0	−140	−375	−11	−51	−7
Naringenin	271.2/150.9	−79	−180	−12	−26	−12
Hesperidin	609.4/301.2	−45	−240	−10	−17	−6
Magnolol	265.1/247.0	−61	−240	−10	−32	−6
Honokiol	265.1/224.1	−77	−315	−12	−35	−12
Ibuprofen	205.1/160.9	−19	−90	−5	−10	−6

auxiliary gas, 7.0 L/min; turbo temperature, 450 °C, respectively. Optimized mass spectrometry parameters for each compound and the internal standard are listed in Table 1.

2.5. Preparation of stock and working solutions

Standard stock solutions were prepared by dissolving the reference standards (100 μg/mL for emodin, aloe-emodin, chrysophanol, naringin, naringenin, hesperidin, magnolol and honokiol; 20 μg/mL for rhein and rheochrysidin) and internal standard (40 μg/mL for ibuprofen) in methanol. Stock solutions were stored at −20 °C.

Working standard solutions were prepared freshly by diluting stock solutions in sodium hydroxide solution (0.1 mol/L). Internal standard working solution (200 ng/mL) was prepared by diluting the stock solution in methanol–water (1:1, v/v).

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

Ten calibration standards were prepared by spiking 200 μL of blank plasma with 100 μL of each working solution to obtain plasma concentration of 5000, 3750, 2500, 1250, 625, 312.5, 156.25, 78.13, 39.06, 19.53 ng/mL for rhein; 400, 200, 100, 50, 25, 12.5, 6.25, 3.13 ng/mL for emodin; 800, 600, 400, 200, 100, 50, 25, 12.5, 6.25, 3.13 ng/mL for aloe-emodin, chrysophanol, naringin, naringenin, hesperidin, magnolol and honokiol; 160, 120, 80, 40, 20, 10, 5, 2.5, 1.25, 0.63 ng/mL for rheochrysidin. Quality control (QC) samples were prepared to obtain plasma concentrations of 3750, 625, 156.25 and 39.06 ng/mL for rhein; 100, 25 and 6.25 ng/mL for emodin; 600, 100, 25 and 6.25 ng/mL for aloe-emodin, chrysophanol, naringin, naringenin, hesperidin, magnolol and honokiol; 120, 20, 5 and 1.25 ng/mL for rheochrysidin. The spiked plasma samples (standard and QC samples) were pretreated and detected in each analytical batch along with the unknown samples.

2.7. Extraction procedure for plasma samples

To a 200 μL plasma sample, 100 μL of internal standard working solution (20 ng/mL) and 100 μL sodium hydroxide solution (0.1 mol/L) were added, followed by the addition of 200 μL hydrochloric acid (0.1 mol/L) to neutralize sodium hydroxide and acidify the sample. A volume of 3.5 mL ethyl acetate was then added and the samples were extracted by vortex-mixing for 7 min. Aqueous and the organic layers were separated by centrifugation at 3000 rpm for 7 min, and the organic layer was transferred to another tube and evaporated to dryness at 50 °C. The remaining aqueous layer was extracted again with additional 3.5 mL acetic ether. After centrifugation for 7 min (3000 rpm), the upper layer was collected along with the previously acquired organic layer. The mixture was then evaporated to dryness under nitrogen at 50 °C. The residue was reconstituted with 100 μL mobile phase and vortex-mixed. A volume of 80 μL was injected automatically into the LC–MS/MS system.

2.8. Assay validation

Chromatogram comparisons of blank and spiked dog plasma were used to evaluate the selectivity of the method. Calibration curves were constructed from the peak area ratios of each analyte to internal standard. versus plasma concentrations using a $1/c^2$ weighted linear least-squares regression model. The lower limit of quantification (LLOQ) was set at the concentration of the lowest non-zero calibration standard (S/N ≥ 10:1) that can be measured with an acceptable accuracy and precision (≤20% for both parameters). Intra- and inter-day precision were determined by assessing measured results of QC samples at low, medium and high concentrations. Accuracy was determined as the percentage difference between the mean detected concentrations and the nominal concentrations. Extraction recoveries of the 10 analytes were determined by comparing peak areas obtained from dog plasma samples with those of unextracted standard solutions at the same concentration. Bench-top stability of the 10 analytes in dog plasma was determined by assessing QC samples after 4 h at room temperature. Freeze–thaw stability was checked after two cycles and long-term stability was determined by assessing QC samples stored at −30 °C for 14 d. QC samples were prepared and injected, and reinjected after the samples of that batch were maintained in the autosampler at 8 °C for 12. Stability of the analytes was checked by comparing measured results with those of freshly prepared samples of the same concentration.

2.9. Data acquisition and analysis

Data acquisition, peak integration and calibration were all calculated with Analyst 1.4.2 software. Calibration curves were constructed with peak area ratios of the analytes to internal standards, and the linear regression between plasma concentration and peak area ratios was weighted by $1/x^2$. Concentrations of QC and unknown samples were calculated by interpolation from the calibration curves.

2.10. Pharmacokinetic study

Six male mongrel dogs (weight 11–14.6 kg) were used in the pharmacokinetics study. Da-Cheng-Qi decoction was orally administered at a dose of 6 g/kg. Blood samples (1.5 mL) were collected at 0 (prior to administration), 5 min, 10 min, 15 min, 20 min, 30 min, 45 min, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, and 10 h after a single dose. The dogs were given free access to water during the experiment. Blood samples were immediately heparinized and centrifuged at 2500 rpm for 10 min, and the supernatants were placed into 1-mL polypropylene tubes and stored at −30 °C until analysis. The following pharmacokinetic parameters were calculated: maximum plasma concentration (C_{max}) and the time to reach maximum (T_{max}) were determined directly from the maximum measured data; the terminal elimination rate constant (k_e) was determined by linear least-squares regression of the terminal portion of the plasma concentration–time curve, and the elimination half-life ($t_{1/2}$) was calculated as $0.693/k_e$; the area under the plasma concentration–time curve from time 0 to the time of the last measurable concentration ($AUC_{0\rightarrow t}$) was calculated by the trapezoidal rule.

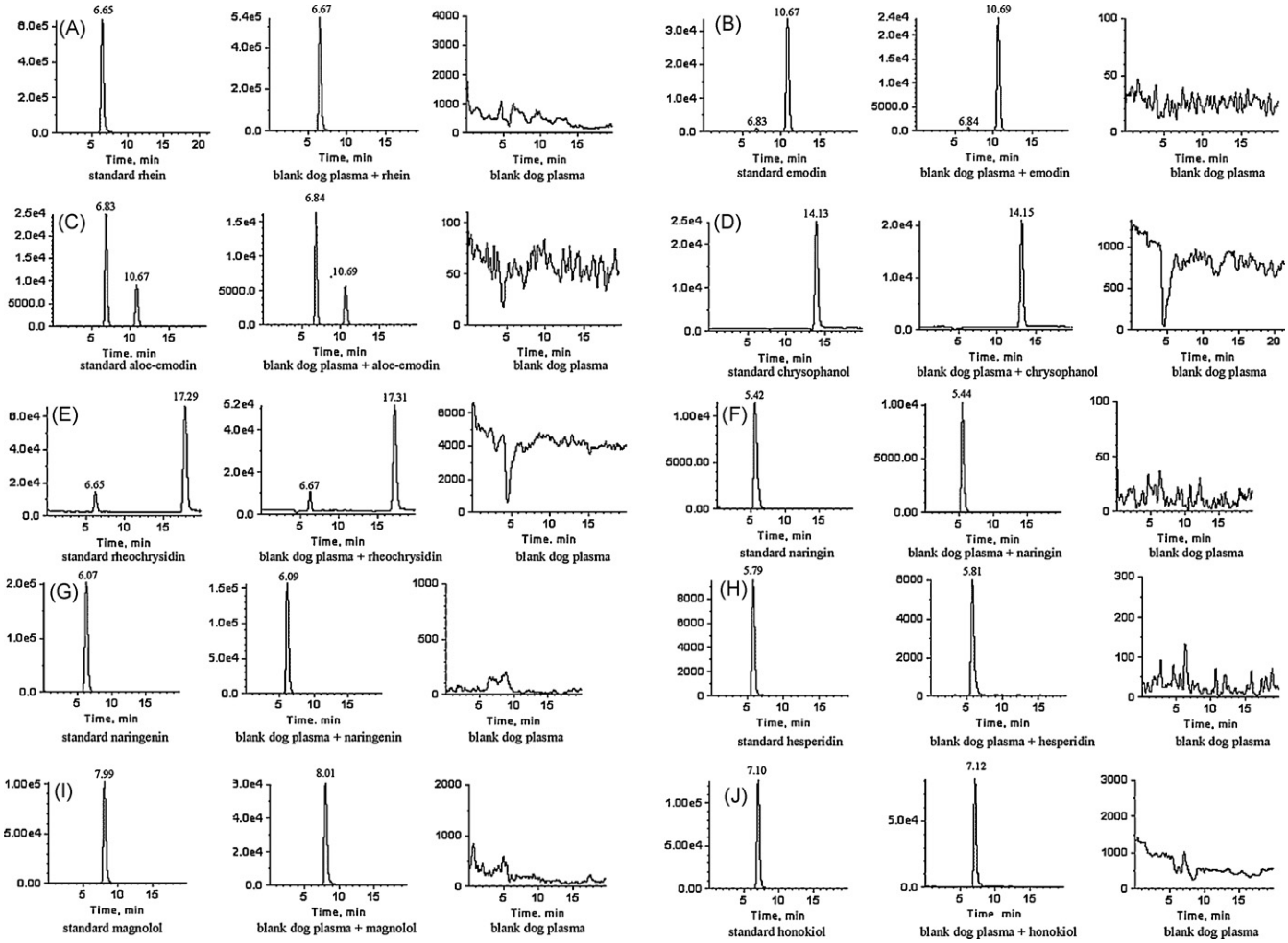

Fig. 2. Chromatography of the 10 major components of DCQT in dog plasma with LC–MS/MS.

3. Results and discussion

3.1. Analytical characteristics

3.1.1. Mass spectrometry

Product ion mass spectra of the 10 major components of DCQT and the internal standard are shown in Fig. 1. For rhein, the product ion mass spectrum was recorded in negative ion mode from the precursor ion *m*/*z* 283.2 $(M-H)^-$ and the most abundant fragment was monitored at *m*/*z* 238.8 $(M-H-COOH)^-$. For emodin, chrysophanol and rheochrysidin, the signals were monitored by loss of CO and molecular transposition from the deprotonated molecular ion. For aloe-emodin, the most abundant fragment obtained from the precursor ion *m*/*z* 269.0 was detected at *m*/*z* 239.9. Emodin and aloe-emodin are isomers; they have two identical product ion though their intensions are different (for example, they both have daughter ions of 239 and 241). The same phenomenon was observed from rhein–rheochrysidin and magnolol–honokiol. The chemical structure of magnolol makes it easy to lose a molecule of H_2O. For honokiol, the most abundant fragment was detected by the loss of $(C_3H_5)^-$ because of the stereospecific blockade of two hydroxyls groups. Naringenin and hesperidin are glycosides

Table 2
Recovery of the 10 main components of DCQT with different extract solvents (%).

	Rhein	Emodin	Aloe-emodin	Chrysophanol	Rheochrysidin	Naringin	Naringenin	Hesperidin	Magnolol	Honokiol
Hexane	0.0	0.5	8.1	69.2	62.5	0.0	0.0	0.0	38.7	36.1
Hexane:isopropanol (1:1)	0.0	46.3	45.6	72.7	80.9	0.0	2.6	0.0	87.8	76.3
Dichloromethane	20.6	76.4	52.0	64.2	53.1	0.0	63.1	0.0	78.6	70.0
Ether	31.5	18.6	58.4	65.5	65.8	0.0	75.6	0.0	79.3	81.3
Ethyl acetate	66.2	77.2	72.0	67.1	74.0	75.3	76.5	67.6	49.0	59.5
Petroleum:ether (7:3)	3.5	61.6	58.5	72.4	71.8	0.0	51.0	0.0	91.1	83.7
Ethyl acetate:dichloromethane (1:1)	34.8	74.5	57.4	75.6	87.2	0.0	74.6	3.0	66.5	79.2
Ether:ethyl acetate:dichloromethane (1:1:1)	43.2	81.1	69.4	70.8	66.1	14.1	73.5	11.5	67.5	56.2

and their fragments were the result of the deprotonated ion losing its glycons. In addition, for naringin, its fragment was the result of losing of $(C_8H_6O)^-$. For the internal standard (ibuprofen), the product ion was *m*/*z* 160.9 $(M–COOH)^-$. Our results on the negative ionization of the 10 major components and internal standard were rhein *m*/*z* 283.2 → 238.8, emodin 269.0 → 241.2, aloe-emodin 269.0 → 239.9, chrysophanol 253.2 → 224.7, rheochrysidin 283.2 → 239.9, naringin 579.1 → 271.0, naringenin 271.2 → 150.9, hesperidin 609.4 → 301.2, magnolol 265.1 → 247.0, honokiol 265.1 → 224.1 and internal standard 205.1 → 160.9, respectively.

3.1.2. Liquid chromatography

Different mobile phases were investigated to optimize analytical performance. We found that the response of the analytes decreased with increased formic acid concentration in the mobile phase. When the mobile phase consisted of methanol and water, the response was higher and peaks were of better shape. A long analytical column (YMC-Pack ODS-A C_{18} column; 5 μm, 150 mm × 4.6 mm), methanol–water mobile phase of 92:8 (v/v), and a flow rate of 0.3 mL/min were determined to be the best chromatographic conditions. Under these conditions, analytes and internal standard were eluted within 20 min. The MRM chromatograms for each analyte are shown in Fig. 2. As mentioned, emodin and aloe-emodin have two identical product ions with different intensions. Therefore, emodin showed a response at the retention time for aloe-emodin and vice-versa (Fig. 2B and C). The retention time for emodin was 6.83 min, and that for aloe-emodin was 10.67 min. Similar results were observed for rhein–rheochrysidin and magnolol–honokiol.

Table 3
LLOQ for the 10 major components of DCQT in dog plasma QC samples (*n* = 5).

Components	Spiked amount (ng/mL)	RSD (%)	RE (%)
Rhein	19.53	6.73	101.27
Emodin	3.13	6.40	99.75
Aloe-emodin	3.13	5.25	97.78
Chrysophanol	3.13	5.45	97.33
Rheochrysidin	0.63	7.53	104.14
Naringin	3.13	7.27	101.85
Naringenin	3.13	5.90	104.73
Hesperidin	3.13	6.93	99.52
Honokiol	3.13	5.92	99.38
Magnolol	3.13	7.76	102.45

3.1.3. Extraction procedure development

Obstacles in sample preparation of the 10 major components of DCQT in dog plasma included differences in properties of

Table 4
Precision, accuracy and extraction recovery for the 10 major components of DCQT in dog plasma QC samples.

Components	Spiked amount (ng/mL)	Intra-day (*n* = 5)		Inter-day (*n* = 15)		Extract recovery (%) (*n* = 3)
		RSD (%)	RE (%)	RSD (%)	RE (%)	
Rhein	3750	4.17	91.79	10.26	96.87	85.83
	625	8.71	103.04	7.05	104.76	80.81
	156.25	12.43	105.22	9.12	105.34	77.57
	39.06	10.94	101.48	7.70	107.38	84.78
Emodin	100	4.42	109.00	5.90	107.03	77.32
	25	11.70	90.40	8.64	104.77	78.53
	6.25	10.34	106.72	9.59	104.13	75.54
Aloe-emodin	600	5.21	108.60	11.32	95.98	61.76
	100	4.05	106.80	9.38	96.63	60.70
	25	3.95	102.24	9.27	99.47	62.84
	6.25	3.53	90.66	3.52	89.77	64.10
Chrysophanol	600	6.95	98.43	6.68	97.26	80.50
	100	3.20	96.62	5.58	100.22	81.99
	25	2.37	92.88	6.03	96.53	83.30
	6.25	8.47	97.28	7.08	102.43	84.46
Rheochrysidin	120	2.97	95.00	5.82	99.61	82.32
	20	3.63	96.60	6.89	101.00	80.75
	5	5.51	100.12	6.07	102.37	83.65
	1.25	8.38	101.12	6.22	102.83	83.01
Naringin	600	4.49	101.93	4.77	107.18	51.96
	100	3.85	106.20	8.63	101.28	50.24
	25	8.87	102.08	8.88	97.71	54.09
	6.25	7.43	98.66	6.96	98.20	55.63
Naringenin	600	9.19	101.67	7.61	103.69	51.27
	100	8.05	103.22	7.54	103.61	50.35
	25	5.96	91.92	8.68	100.35	53.58
	6.25	5.48	95.01	6.26	95.56	53.49
Hesperidin	600	9.56	99.53	7.06	106.07	52.36
	100	5.43	105.48	7.98	102.53	63.09
	25	9.40	100.72	7.30	98.29	51.09
	6.25	6.05	100.00	6.27	97.34	57.59
Honokiol	600	10.00	99.43	7.08	97.80	63.61
	100	2.80	98.18	6.19	100.46	61.32
	25	9.47	99.04	8.31	95.76	64.59
	6.25	7.57	95.81	8.29	99.94	60.85
Magnolol	600	4.12	92.63	9.54	99.46	61.89
	100	4.20	91.62	8.44	95.16	63.58
	25	4.54	95.44	5.45	92.43	62.13
	6.25	3.99	91.49	3.59	90.60	66.76

Table 5
Stability for the 10 major components of DCQT in dog plasma QC samples (n = 3).

	Spiked amount (ng/mL)	Bench-top bias (%)		Autosampler bias (%)		Freeze–thaw bias (%)		Long-term bias (%)	
		2 h	4 h	6 h	12 h	1	2	7 d	14 d
Rhein	3750	1.10	0.27	−2.93	−3.93	4.94	2.83	4.94	3.56
	625	0.15	−1.96	−5.21	−4.75	−5.73	−1.14	−5.73	−3.77
	156.25	−0.21	−1.46	−2.93	2.51	−3.35	−4.18	−3.35	−2.72
	39.06	1.75	0.75	−0.08	3.91	−5.08	−2.66	−5.08	0.75
Emodin	100	−3.23	−3.78	3.44	−0.83	−4.97	−3.09	−4.97	−2.64
	25	1.23	−4.50	1.91	5.45	−0.27	3.13	−0.27	−1.77
	6.25	−5.34	4.18	−1.71	−3.93	−3.43	−2.02	−3.43	−3.13
Aloe-emodin	600	−4.70	−5.92	5.02	2.85	−2.75	−4.33	−2.75	−6.02
	100	−4.01	−0.94	−1.17	2.37	1.27	6.35	1.27	−1.27
	25	−5.64	−1.79	4.62	1.41	−4.87	−3.97	−4.87	−4.62
	6.25	3.67	6.23	−3.06	3.89	1.39	5.95	1.39	−0.44
Chrysophanol	600	−3.00	−5.50	0.00	1.36	3.92	−2.23	3.92	6.21
	100	−0.39	−4.39	0.59	−2.72	−2.79	2.95	−2.79	4.59
	25	5.91	4.36	−3.59	−4.88	−3.59	3.21	−3.59	−2.31
	6.25	5.60	0.10	1.87	1.76	−6.38	−0.78	−6.38	−1.45
Rheochrysidin	120	−2.60	−5.97	−4.94	−3.64	2.34	−5.19	2.34	−2.34
	20	2.45	1.96	−4.08	−4.89	3.92	6.36	3.92	−0.33
	5	−4.25	0.26	−1.42	2.58	4.06	6.44	4.06	−0.64
	1.25	−4.35	0.26	4.60	−1.79	−4.86	−1.02	−4.86	−3.07
Naringin	600	3.51	−1.48	−6.53	−3.19	−3.84	−5.99	−3.84	−0.71
	100	3.30	5.93	0.63	1.40	5.02	1.40	5.02	2.74
	25	−2.20	−0.14	−1.10	2.89	−2.48	−1.24	−2.48	1.10
	6.25	−4.52	−1.66	−2.08	−3.48	−4.42	1.56	−4.42	−4.94
Naringenin	600	2.78	2.11	5.33	−3.56	0.72	−3.61	0.72	5.83
	100	2.71	3.41	4.51	4.88	1.72	1.76	1.72	3.63
	25	−0.29	−1.46	−1.89	2.04	2.62	−1.89	2.62	1.31
	6.25	−2.34	2.40	4.68	3.51	0.58	0.94	0.58	1.11
Hesperidin	600	−4.30	−4.74	2.18	−3.79	−2.85	3.24	−2.85	−1.23
	100	4.17	2.76	0.65	5.38	−0.69	0.86	−0.69	−0.07
	25	−2.63	4.71	0.55	2.35	4.99	1.11	4.99	1.11
	6.25	−1.39	−4.58	−3.50	1.60	−0.82	−3.76	−0.82	−1.44
Honokiol	600	1.56	3.42	6.71	3.90	5.94	1.86	5.94	4.80
	100	−0.07	−1.71	2.18	1.78	−5.09	2.68	−5.09	−3.69
	25	−2.62	−5.24	−4.98	6.42	1.83	−2.62	1.83	−1.44
	6.25	−4.64	−3.29	−3.59	−2.00	−3.09	−2.39	−3.09	−2.39
Magnolol	600	−4.63	−6.51	3.77	1.48	−6.23	−4.91	−6.23	−3.83
	100	−4.17	−0.99	−2.08	4.58	−3.28	−4.58	−3.28	−4.41
	25	−4.27	−1.25	−3.14	−2.38	−4.89	−4.39	−4.89	−4.39
	6.25	−4.51	−4.14	−5.35	−2.78	−5.50	−4.56	−5.50	−3.51

dissolution, pK_a, stability, and concentration within the biological matrix. We therefore investigated three methods of plasma sample preparation: liquid–liquid extraction, solid-phase extraction and deproteination. We found no endogenous interference at the retention time of the analytes and internal standard by liquid–liquid extraction. We also investigated different extraction solvents (acetic ether, dichloromethane, petroleum ether, mixed solvent, etc.; see Table 2). Only ethyl acetate was able to extract each analyte. Rhein is acidic because of its carboxy group and it was much easier to extract from plasma samples under acidic conditions. Narignin and hesperidin are glycoside; their recoveries were higher under neutral condition. When 0.1 mol/L HCl and two-step extraction were applied to the sample preparation procedure, extract recoveries of all analytes were greater than 50%.

3.2. Method validation

3.2.1. Specificity

Six blank samples of dog plasma from different sources were analyzed. There was no interference observed at the selected mass transitions for all analytes and the internal standard. As a result, the absence of endogenous interference at the retention time of the compounds clearly demonstrated the high selectivity of the method.

3.2.2. LLOQ, calibration and linearity

The LLOQ was 19.53 ng/mL for rhein; 3.13 ng/mL for emodin, aloe-emodin, chrysophanol, naringin, naringenin, hesperidin, magnolol and honokiol; 0.63 ng/mL for rheochrysidin. The accuracy of the five different dog plasma samples spiked at LLOQ were within 97.33% and 104.73%, the precision was within 5.25% and 7.76% (Table 3). The linearity of the calibration curves was determined as detailed in Section 2.6. Regression analysis over the validation days ($k = 7$) showed that the determination coefficients (r) were always higher than 0.99 and for each point on the calibration curve, the concentration back-calculated from the regression equation was constantly below 15% of the nominal value, below 20% of the LLOQ.

3.2.3. Accuracy and precision

A summary of inter- and intra-day precision and accuracy at QC concentrations is shown in Table 4. The precision (RSD, %) for all was less than 15% and the accuracy (RE, %) was within ±15%. The results indicated that the assay had remarkable reproducibility with acceptable accuracy and precision.

Table 6
Pharmacokinetics parameters for the 10 major components after administration of DCQT to dog ($n = 6$).

Parameters	T_{max} (h)	C_{max} (ng h/mL)	$AUC_{0\rightarrow t}$ (ng h/mL)	k_e (h^{-1})	$t_{1/2}$ (h)
Rhein	1.9 ± 1.2	343.0 ± 117.5	1124.4 ± 552.0	0.33 ± 0.14	2.4 ± 1.2
Emodin	1.5 ± 1.0	8.5 ± 3.9	30.2 ± 20.7	0.54 ± 0.38	2.8 ± 1.7
Aloe-emodin	1.7 ± 1.1	23.7 ± 14.3	95.3 ± 72.7	0.34 ± 0.15	2.7 ± 1.1
Chrysophanol	2.5 ± 1.4	33.4 ± 15.5	175.8 ± 86.8	0.18 ± 0.09	4.3 ± 2.0
Rheochrysidin	11.6 ± 0.6	2.1 ± 0.5	9.3 ± 1.4	0.20 ± 0.06	3.8 ± 1.2
Naringin	1.6 ± 0.9	91.0 ± 32.8	361.9 ± 132.5	0.31 ± 0.16	3.1 ± 1.3
Naringenin	3.2 ± 3.9	22.2 ± 8.5	99.5 ± 39.8	0.20 ± 0.08	3.9 ± 1.4
Hesperidin	1.6 ± 0.9	152.5 ± 37.6	710.2 ± 311.5	0.36 ± 0.18	3.9 ± 0.7
Honokiol	2.2 ± 1.2	32.5 ± 13.5	163.8 ± 76.3	0.36 ± 0.09	2.1 ± 0.6
Magnolol	0.9 ± 0.2	14.1 ± 8.5	53.3 ± 29.9	0.36 ± 0.18	3.9 ± 0.7

3.2.4. Extraction recovery

Table 4 also summarises the extraction recoveries for all analytes. The mean extraction efficiencies were all over 50%. Thus, extraction efficiency was within the acceptance criteria.

3.2.5. Stability

The 10 major components of DCQT were found to be stable under following conditions: in dog plasma at room temperature for 4 h, in the autosampler at 8 °C for 12 h, two freeze–thaw cycles, or in plasma at −30 °C for 14 d. All RE values between post-storage and initial QC samples were within ±15%, which is shown in Table 5.

3.3. Application of the method

The method was applied to study the pharmacokinetics of oral administration of DCQT in dogs. The pharmacokinetic parameters are presented in Table 6.

4. Conclusion

To our knowledge, this is the first fully validated LC–MS/MS method for the simultaneous quantification of the 10 active components of DCQT (including rhein, emodin, aloe-emodin, chrysophanol, rheochrysidin, naringin, naringenin, hesperidin, honokiol and magnolol) in dog plasma. The significance of this study is in the innovative analytic method used. We successfully combined a two-step liquid–liquid extraction method with a highly selective analytical process. A long analytical column and negative MRM mode were applied to optimize selectivity and sensitivity. The method was specific, sensitive, accurate and reproducible and was successfully applied in the determination of the 10 major components of DCQT in dog plasma after oral administration.

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