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Short communication

High-performance liquid chromatography/tandem mass spectrometry for simultaneous determination of four dicaffeoylquinic acids in rat plasma

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ABSTRACT

A specific and reliable HPLC–MS/MS method was developed and validated for the simultaneous determination of four dicaffeoylquinic acids (DCQA): 3,4-DCQA, 1,5-DCQA, 3,5-DCQA and 4,5-DCQA. The analytes were separated on a C_{18} column (150 mm × 2.1 mm, 1.8 μm) and a triple-quadrupole mass spectrometry equipped with an electrospray ionization (ESI) source was applied for detection. The plasma sample was prepared by a liquid–liquid extraction method and the recovery for the four analytes was around 80%. The calibration curves were linear over a concentration range of 10.6–1060.0 ng/mL for 3,4-DCQA, 19.2–1920.0 ng/mL for 1,5-DCQA, 14.0–2900.0 ng/mL for 3,5-DCQA, 9.7–970.0 ng/mL for 4,5-DCQA. The intra-day and inter-day precision was less than 15% and the relative error (RE) were all within ±15%. The validated method was successfully applied to a pharmacokinetics study in rats after oral administration of the extracts of Ainsliaea fragrans cham (a traditional Chinese herb).

1. Introduction

Dicaffeoylquinic acids (DCQAs) are a class of natural polyphenolic compounds widely distributed in many herbs. 3,4-DCQA, 1,5-DCQA, 3,5-DCQA and 4,5-DCQA (Fig. 1) were the typical analogs of DCQAs. They possessed various biological activities, such as antibacterial [1], antivirus [2–4], anti-inflammatory [5–7] and antioxidative stress effects [8,9]. In the present study, a large amount of polyphenolic compounds have been extracted from Ainsliaea fragrans cham, a traditional Chinese herb, and they were applied for the treatment of some gynecological diseases. Therefore, to support the pharmacokinetic (PK) study of the extracts of Ainsliaea fragrans cham, it was desired to develop a simple and sensitive method for the simultaneous determination of the four DCQAs mentioned above in the biological fluids.

Several HPLC and HPLC–MS/MS methods have been reported for the determination of DCQAs in biological samples [10–12]. Wang et al. [10] and Matsui et al. [11] developed LC–MS methods to determine three DCQAs in human and rat plasma, respectively. However, to the best of our knowledge, no published reports are available with regard to the simultaneous quantification of the four DCQAs in plasma.

In the present study, we attempted to develop a facile HPLC–MS/MS method to determine the four DCQAs simultaneously in rat plasma. The validated results showed this method was selective, sensitive and accurate to measure 3,4-DCQA, 1,5-DCQA, 3,5-DCQA and 4,5-DCQA in plasma. The lower limit of quantification (LLOQ) was as follows: 10.6 ng/mL for 3,4-DCQA, 19.2 ng/mL for 1,5-DCQA, 14 ng/mL for 3,5-DCQA and 9.7 ng/mL for 4,5-DCQA, which was sufficiently sensitive to measure relatively low concentrations of DCQA in plasma with a simple liquid–liquid extraction procedure using as little as 50 μL plasma. Finally, it was successfully applied to assess the PK characteristics after the extracts of Ainsliaea fragrans cham were orally administrated to the Sprague-Dawley rat.

2. Experimental

2.1. Chemicals and reagents

3,4-DCQA, 1,5-DCQA, 3,5-DCQA, 4,5-DCQA (purity >98%) and extracts of Ainsliaea fragrans cham were provided by Jiangxi herbfine Co. Ltd. (Nanchang, China).

The content of 3,4-DCQA 3,5-DCQA 4,5-DCQA in the extracts of Ainsliaea fragrans cham was 7.2%, 12.5% and 8.0%, respectively. The

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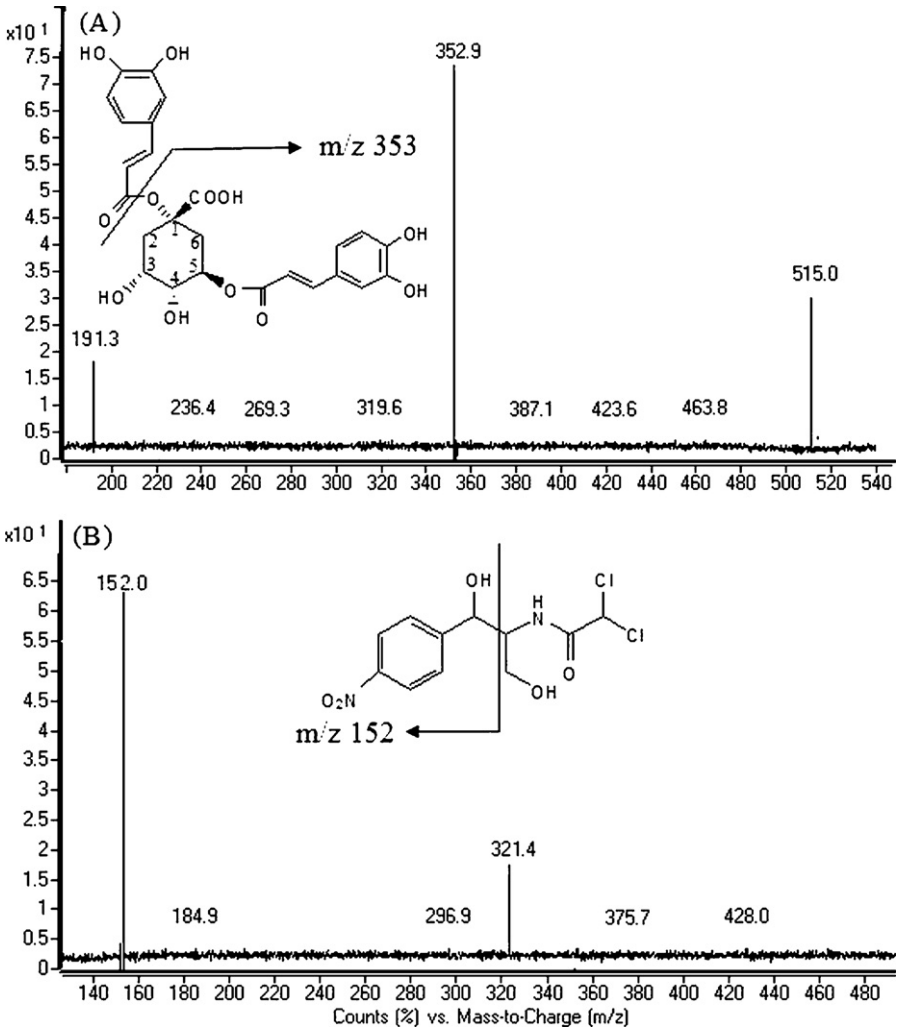

Fig. 1. Product ion mass spectra of $[M-H]^-$ ion of 1,5-DCQA (A) and chloromycetin (B).

content of 1,5-DCQA was less than 0.5%. Chloromycetin (Internal standard, purity >99%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Isopropanol was purchased from Shanghai Lab Reagents Co. Ltd. (Shanghai, China). Methanol and acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA, USA). Formic acid (FA) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Water was prepared with a Mill-Q Simplicity® system (Millipore Corp., MA, USA). All other chemicals are of analytical grade.

2.2. Instrumentation

An Agilent 1290 ultra-performance liquid chromatography and an Agilent 6460 triple-quadrupole tandem mass spectrometer (Agilent Technologies, Santa Clara, CA) were used for the determination of the analytes. An Agilent ZORBAX SB-C_{18} column (150 mm × 2.1 mm, 1.8 μm) was used to separate the analytes. All data were acquired and processed using Masshunter™ 2.0 software.

2.3. HPLC/MS/MS conditions

A gradient elution program was conducted for chromatographic separation with the mobile phase A (acetonitrile), and the mobile phase B (water containing 0.1% formic acid) as follows: 0 min (23%, A), 2 min (23%, A), 3.5 min (10%, A), 5 min (20%, A), 10.5 min (20%, A), 14 min (70%, A), 14.01 min (23%, A), 18 min (23%, A). The flow rate was 0.2 mL/min and the column temperature was 30 °C.

The mass spectrometry was operated in the negative mode. Quantification was performed by multiple reaction monitoring (MRM). N_2 (purity of 99.9%) was used as drying gas (10 L/min) and nebulizing gas (40 L/min). Gas temperature was 300 °C. Capillary voltage was 4000 V. The MRM transition 515 → 353 was used for the detection of 3,4-DCQA, 1,5-DCQA, 3,5-DCQA and 4,5-DCQA with fragmentor of 125 V. The transition 321 → 152 was for chloromycetin with fragmentor of 100 V. N_2 (purity of 99.999%) was used as collision gas and the collision voltage was 20 V. The dwell was 200 ms.

2.4. Preparation of standard and quality control samples

The stock mixing standard solutions of 3,4-DCQA, 1,5-DCQA, 3,5-DCQA and 4,5-DCQA was prepared in methanol at the concentration of 1.06 μg/mL, 1.92 μg/mL, 1.4 μg/mL and 0.97 μg/mL, respectively. Then, the solution was serially diluted with methanol to provide working standard solutions of the desired concentrations. The internal standard solution was prepared with methanol to 3.62 μg/mL. The calibration curves with seven non-zero standard levels contained 3,4-DCQA/1,5-DCQA/3,5-DCQA/4,5-DCQA in the concentration of 10.6–1060.0 ng/mL for 3,4-DCQA, 19.2–1920.0 ng/mL for 1,5-DCQA, 14.0–2900.0 ng/mL for 3,5-DCQA, 9.7–970.0 ng/mL for 4,5-DCQA. The calibration curves

were prepared by spiking 50 μL of blank rat plasma with 50 μL of the mixing standard solution of DCQA, 20 μL of internal standard solution. The quality control (QCs) samples at low, medium, high concentration levels were prepared in the same way as the calibration curves. The nominal plasma concentrations of QC samples were 3,4-DCQA (21.2, 106, 530 ng/mL), 1,5-DCQA (38.4, 192.0, 960.0 ng/mL), 3,5-DCQA (58.0, 290.0, 1450.0 ng/mL), 4,5-DCQA (19.4, 97.0, 485.0 ng/mL). All solutions were stored at −40 °C.

2.5. Sample preparation

To a 50-μL aliquot of plasma sample, 20 μL of internal standard solution (3.62 μg/mL) and 50 μL of methanol were added. The sample was briefly vortex-mixed following the addition of 100 μL of acetic acid (1 mM) and 25 μL of water containing 30% acetonitrile. Then 2 mL isopropanol was added and mixed for 5 min. The sample was centrifuged at 3000 × *g* and 4 °C for 10 min. The supernatant was transferred to 10 mL clean glass tube and evaporated to dryness at 37 °C under a gentle stream of N_2. The residue was reconstituted in 300 μL of water containing 30% acetonitrile by vortex mixing for 1 min and transferred to an autosampler vial at 4 °C. Then, a 5-μL aliquot was injected into the HPLC–MS/MS system for analysis. All operations were conducted in darkness and on ice.

2.6. Method validation

Selectivity was assessed by comparing chromatograms of six different batches of blank rat plasma with the corresponding spiked rat plasma. Linearity was assessed by weighted ($1/x^2$) least-squares analysis of six different calibration curves. Intra- and inter-day precision (the relative standard deviation, RSD) and accuracy (the relative error, RE) were determined by analysis of low, medium, and high QC samples ($n = 6$) on 3 different days. The matrix effect was investigated by comparing the peak areas of analytes in the post-extraction spiked blank plasma at low and high concentrations with those of the corresponding standard solutions. The extraction recovery was determined by comparing the mean peak areas of six extracted samples at low, medium, and high QC concentrations with the mean peak areas of spike-after-extraction samples. The stability of low and high QC samples ($n = 3$) in three complete freeze/thaw cycles (−80 to 23 °C), long-term sample storage (−80 °C for 30 days), and bench-top (23 °C for 2 h) was assessed. The ready to-injection stability of extracted samples in the autosampler rack at 4 °C for 6 h was also evaluated.

2.7. Pharmacokinetic study

Male Sprague-Dawley rats weighing from 210 to 240 g were used for PK study. All animal experiments were performed in accordance with institutional guidelines and were approved by the University Committee on Use and Care of Animals, Jiangxi University of Traditional Chinese Medicine. The suspension of the extracts of Ainsliaea fragrans cham were administrated to rats by gavage at 180 mg/kg (calculated as the total amount of extracts). Serial blood samples (0.2 mL) were obtained at 5, 10, 15, 30 min and 1, 2, 4, 6, 10 h after oral administration separately. During sampling, rats were anesthetized with ether. All samples were placed into heparinized tubes. After centrifugation at 800 × *g* and 4 °C for 10 min, plasma was collected and frozen at −80 °C until analysis.

3. Results and discussion

3.1. Method development

1,5-DCQA had six hydroxyl groups and a carboxyl group in the molecular structure. It has a stronger mass response under the negative ionization mode than the positive mode. In addition, ESI source provided a better response than APCI source for 1,5-DCQA. The product ions of the $[M-H]^-$ of 1,5-DCQA was dependent on the collision voltage. The major fragment ion m/z 191 was formed at higher collision voltage, and the most abundant ion m/z 353 was formed at lower collision voltage. The transition of m/z 515 → 353 gave a higher signal-to-noise (S/N) ratio and better response than that of m/z 515 → 191 during the analysis of the spiked plasma samples. As a result, the transition of m/z 515 → 353 was selected for MRM analysis of 1,5-DCQA (Fig. 1.). Because 1,5-DCQA, 3,4-DCQA, 3,5-DCQA and 4,5-DCQA were isomers and they displayed the similar mass response and fragment ion mode, m/z 515 → 353 at the collision voltage of 20 V was also chosen for the analysis of 3,4-DCQA, 3,5-DCQA and 4,5-DCQA in the same optimization way (Fig. 1.). The transition m/z 321 → 152 was selected for the analysis of chloromycetin with collision voltage of 20 V.

The four DCQAs have the same precursor and product ions in mass spectrometry. Therefore, it was indispensable to separate the four isomers with HPLC. Two C_{18} columns (50 mm × 2.1 mm, 1.8 μm and 100 mm × 2.1 mm, 1.8 μm) did not separate the four DCQAs from each other even under the highly aqueous mobile phase because of the high similarity in the physiochemical properties of the four analytes. A C_{18} column (150 mm × 2.1 mm, 1.8 μm) elicited a suitable retention and a base-line separation between the analytes. To equilibrate the column, the run time was extended to 18 min. The presence of a small amount of formic acid was able to improve the peak shape of the analytes.

3.2. Method validation

Fig. 2 shows the typical chromatograms of a blank, a spiked plasma sample with 3,4-DCQA (10.6 ng/mL), 1,5-DCQA (19.2 ng/mL), 3,5-DCQA (14.0 ng/mL) and 4,5-DCQA (9.7 ng/mL) and the internal standard, a plasma sample from a rat after an oral administration of the extracts of Ainsliaea fragrans cham. No interference from the endogenous compound with the analytes and the internal standard was found.

The linear regressions of the peak area ratios versus concentration were fitted over the concentration range of 10.6–1060.0 ng/mL for 3,4-DCQA, 19.2–1920.0 ng/mL for 1,5-DCQA, 14.0–2900.0 ng/mL for 3,5-DCQA, and 9.7–970.0 ng/mL for 4,5-DCQA in rat plasma. The typical equation of the calibration curves was as follows: 3,4-DCQA: $y = 0.0032x - 0.0132$, $r = 0.9990$; 1,5-DCQA: $y = 0.0021x - 0.008$, $r = 0.9990$; 3,5-DCQA: $y = 0.0462x - 0.0687$, $r = 0.9980$; 4,5-DCQA: $y = 0.0069x - 0.0226$, $r = 0.9990$, where y represents the peak area ratio of analytes to IS and x represents the concentration of the analytes in plasma. The correlation coefficient (r) exceeded 0.99, showing a good linearity among the concentration range.

The lower limit of quantification (LLOQ) was 10.6 ng/mL for 3,4-DCQA, 19.2 ng/mL for 1,5-DCQA, 14.0 ng/mL for 3,5-DCQA and 9.7 ng/mL for 4,5-DCQA, which were sensitive enough to determine the low concentration of the analytes of interest in rat plasma. The intra- and inter-RSD were both less than 15% and the RE were within ±8.2% at LLOQ level, which were within the accepted limits.

The matrix effects calculated were in the range of 93.1–108.4%. Therefore, ion suppression or enhancement from rat plasma was negligible under the current conditions.

Table 1 summarizes the intra- and inter-day precision for the analytes in QC samples. The intra- and inter-day RSD were below 9.1%, and the relative errors were from −10.1% to 8.5%. All the values were within the accepted range and the method was accurate and precise.

The mean extraction recoveries were 80.3 ± 5.3%, 77.0 ± 6.9%, 78.9 ± 4.6% and 78.5 ± 4.6% for 3,4-DCQA, 1,5-DCQA, 3,5-DCQA and 4,5-DCQA, respectively. The mean recovery of the internal standard was 85.3 ± 4.1% (Table 1).

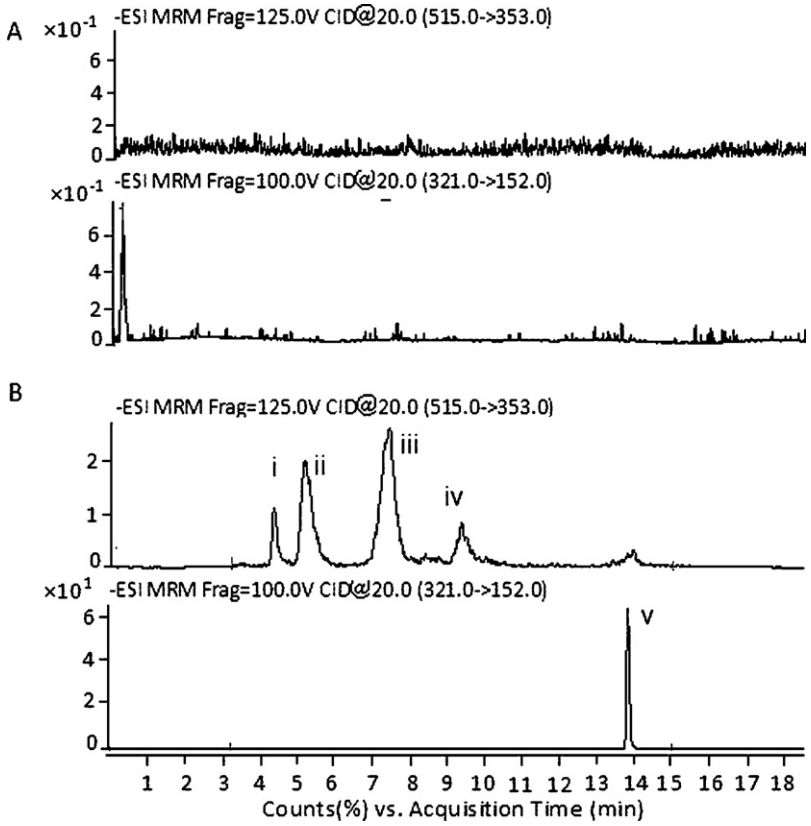

Fig. 2. Representative MRM chromatograms of 3,4-DCQA (i), 1,5-DCQA (ii), 3,5-DCQA (iii) and 4,5-DCQA (iv) and chloromycetin (IS) (v) in rat plasmas: (A) a blank rat plasma sample; (B) a rat plasma sample following an oral dose of the extracts of Ainsliaea fragrans cham at 180 mg/kg (calculated as the total amount of extracts) to a Sprague-Dawley rat.

The four DCQAs were not stable after three complete freeze/thaw cycles (−80 °C to 23 °C). But they were stable after long-term sample storage (−80 °C for 30 days) and bench-top (23 °C for 2 h). The extracted samples on the autosampler rack at 4 °C were stable for 6 h. In the preliminary study (unpublished data), the transition between the four DCQAs happened under in vitro incubation and light. The stability results were consistent with our previous study (Table 2), and we should pay attention to the operation circumstance during the PK study.

Table 1
Precision of four DCQAs in rat plasma (in three validation days, six replicates at different concentration level per day) and the mean recovery.

Concentration (ng/mL)		RSD (%)		Mean recovery (%)
Added	Found (mean)	Intra-day	Inter-day	
3,4-DCQA				
21.2	19.9	8.7	4.3	
106.0	97.7	8.0	5.3	80.3 ± 5.3
530.0	494.4	7.3	6.6	
1,5-DCQA				
38.4	34.7	9.1	7.3	
192.0	206.8	7.5	6.2	77.0 ± 6.9
960.0	933.2	7.0	5.6	
3,5-DCQA				
58.0	57.1	6.2	5.1	
290.0	308.5	5.2	5.5	78.9 ± 4.6
1450.0	1573.5	3.6	6.3	
4,5-DCQA				
19.4	17.4	8.0	7.4	
97.0	91.9	7.0	5.3	78.5 ± 4.6
485.0	504.4	6.3	7.9	

3.3. Application to PK study in the Sprague-Dawley rats

This validated method was successfully applied to the PK studies after the oral administration of the extracts of Ainsliaea fragrans cham to the Sprague-Dawley rat. The typical plasma concentration–time profiles of 3,4-DCQA, 1,5-DCQA, 3,5-DCQA and 4,5-DCQA were shown in Fig. 3. It was clear that 3,4-DCQA, 3,5-DCQA and 4,5-DCQA may transform to 1,5-DCQA in vivo because the content of 1,5-DCQA in the extract was very low.

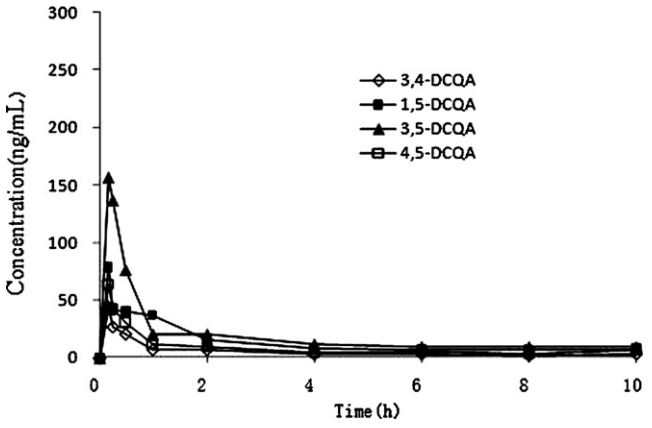

Fig. 3. Plasma concentration–time profiles of the four DCQAs in the Sprague-Dawley rat. (■) 1,5-DCQA, (◊) 1,5-DCQA, (▲) 3,5-DCQA, and (□) 4,5-DCQA after oral administration of the extracts of Ainsliaea fragrans cham to a Sprague-Dawley rat at 180 mg/kg (calculated as the total amount of extracts).

Table 2
Stability data of four DCQAs in rat plasma under different conditions.

	Concentration (ng/mL)		RSD (%)	RE (%)
	Added	Found (mean)		
Bench-top (23 °C for 2 h)				
3,4-DCQA	21.2	22.3	7.5	5.3
	530.0	587.1	8.9	10.8
1,5-DCQA	38.4	43.2	10.3	12.5
	960.0	1045.2	6.3	8.9
3,5-DCQA	58.0	63.2	8.1	8.9
	1450.0	1543.3	9.3	6.4
4,5-DCQA	19.4	21.3	12.0	10.0
	485.0	465.2	7.5	−4.1
Three freeze/thaw cycles (−80 to 23 °C)				
3,4-DCQA	21.2	18.3	12.0	−13.6
	530.0	430.5	8.6	−18.8
1,5-DCQA	38.4	30.7	8.4	−20.2
	960.0	804.2	7.3	−16.2
3,5-DCQA	58.0	34.8	6.2	−40.1
	1450.0	1231.3	9.0	−15.1
4,5-DCQA	19.4	13.2	7.5	−31.9
	485.0	376.2	7.8	−22.4
Autosampler rack at 4 °C for 6 h				
3,4-DCQA	21.2	23.7	9.0	11.6
	530.0	523.2	5.3	−1.3
1,5-DCQA	38.4	43.2	4.6	12.6
	960.0	1004.3	7.0	4.6
3,5-DCQA	58.0	63.21	6.5	9.0
	1450.0	1509.6	7.3	4.1
4,5-DCQA	19.4	18.3	5.9	−5.6
	485.0	509.3	9.0	5.0
Freezing at −80 °C for 30 days				
3,4-DCQA	21.2	19.1	7.6	−10.3
	530.0	587.3	6.9	10.8
1,5-DCQA	38.4	42.1	9.0	9.7
	960.0	998.8	7.5	4.0
3,5-DCQA	58.0	63.2	8.0	9.0
	1450.0	1537.5	10.0	6.0
4,5-DCQA	19.4	21.3	5.4	10.0
	485.0	509.5	6.6	5.1

4. Conclusion

The present study developed and validated a facile, specific, and sensitive HPLC–MS/MS method for the simultaneous determination of 3,4-DCQA, 1,5-DCQA, 3,5-DCQA and 4,5-DCQA in rat plasma. It was very convenient to use a C_{18} column for chromatographic separation under the reversed-phase conditions. The simple liquid–liquid extraction pretreatment provided high recovery (about 80%) and clean sample. This method has been successfully applied to PK studies after oral administration of the extracts of Ainsliaea fragrans cham to the Sprague-Dawley rat.

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References

[1] E. Scholz, M. Heinrich, D. Hunkler, Planta Med. 60 (1994) 360.
[2] W.E.J. Robison, M. Cordeiro, S. Abdel-Malek, Q. Jia, S.A. Chow, M.G. einecke, W.M. Mitchell, Mol. Pharmacol. 50 (1996) 846.
[3] W.E.J. Robison, M. Cordeiro, S. Abdel-Malek, Q. Jia, S.A. Chow, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 6326.
[4] P.J. King, G. Ma, W. Miao, J. Med. Chem. 42 (1999) 497.
[5] L. Gongora, R.M. Giner, S. Manez, Life Sci. 71 (2002) 2995.
[6] A.M. Abeysekera, U.K. De Silva, S.R. De Silva, Fitoterapia 70 (1999) 317.
[7] J. Heilmann, I. Merfort, M. Weiss, Planta Med. 61 (1995) 435.
[8] Y. Chuda, H. Ono, M. Ohnishi-Kameyama, T. Nagata, T. Tsushida, J. Agric. Food Chem. 44 (1996) 2037.
[9] K. Azuma, M. Nakayama, M. Koshioka, K. Ippoushi, Y. Yamaguchi, K. Kohata, Y. Yamauchi, H. Ito, H. Higashi, J. Agric. Food Chem. 47 (1999) 3963.
[10] W. Wang, H.P. Hao, G.J. Wang, N. Cui, C.N. Zheng, Y.X. Wang, J. Chromatogr. Sci. 47 (2009) 216.
[11] Y.J. Matsui, S. Nakamura, N. Kondou, Y. Takasu, R. Ochiai, Y. Masukawa, J. Chromatogr. B 858 (2007) 96.
[12] R.L. Gu, G.F. Dou, J. Wang, J.X. Dong, Z.Y. Meng, J. Chromatogr. B 852 (2007) 85.