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Short Communication

Simultaneous determination of 3,3′,4′,5,7-pentamethylquercetin and its possible metabolite 3,3′,4′,7-tetramethylquercetin in dog plasma by liquid chromatography–tandem mass spectrometry and its application to preclinical pharmacokinetic study

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ABSTRACT

A sensitive, simple and rapid ultra fast liquid chromatography (UFLC)–ESI-MS/MS method was established for the simultaneous determination of 3,3′,4′,5,7-pentamethylquercetin (PMQ) and its possible metabolite 3,3′,4′,7-tetramethylquercetin (TMQ) in dog plasma using 4′,5,7-trimethylapigenin (TMA) as the internal standard. The plasma sample was pretreated with acetonitrile for protein precipitation and the analytes were separated on an Ultimate XB-CN column (5 μm, 2.1 mm × 150 mm) with the mobile phase consisting of acetonitrile and water (2:1, v/v). Detection was performed on a triple-quadrupole tandem mass spectrometer under a positive multiple reaction-monitoring mode (MRM). The mass transition ion-pair was followed as *m/z* 373.1–312.1 for PMQ, 359.1–344.0 for TMQ and 313.1–298.1 for TMA. The validated concentration ranged from 1.272 to 3060 ng/mL for PMQ and from 10.35 to 1725 ng/mL for TMQ. The lower limit of quantifications for PMQ and TMQ were 1.272 ng/mL and 10.35 ng/mL, respectively. The developed-method was successfully applied for the pharmacokinetic study of PMQ and its metabolite TMQ in dogs following a single oral dose.

1. Introduction

3,3′,4′,5,7-pentamethylquercetin (PMQ) and 3,3′,4′,7-tetramethylquercetin (TMQ) are the methylated derivatives of quercetin. As we know, quercetin (QUE) is a typical flavonol-type flavonoid which is ubiquitously present in fruits and vegetables [1]. It exhibits antioxidative, anti-inflammatory, vasodilating and potential anti-cancer effects, and it was also speculated to be a multidrug resistance (MDR) modulator and thus a potential chemosensitizer [2–5]. Recently, it has been proved that quercetin has the potential to protect DNA [6,7]. However, as a drug, there are still many defects in the application of quercetin, such as low oral bioavailability, a short elimination half-life and low titer [8]. These shortcomings restrict its further development and utilization. Fortunately, it was demonstrated that full methylation of dietary flavonoids can result not only in a dramatic increase in their hepatic metabolic stability but also in great improvement of their intestinal absorption, both of which should greatly increase their oral bioavailability [9]. PMQ is just the fully methylated derivative of quercetin. Moreover, a number of studies have demonstrated that PMQ have important therapeutic potential in cardiovascular disease and cancer [10–12].

In order to investigate the metabolism of PMQ, the dog plasma before and after medication were compared for PMQ, TMQ and QUE contents. The results showed that even though PMQ and TMQ could be seen only after drug administration, QUE could not be detected in both of the plasma samples. The PMQ preparation given to the dogs was also analyzed, and did not show TMQ and QUE. It indicated that TMQ is probably a metabolite of PMQ in dog plasma. According to reports in the literature, TMQ also have some important pharmacologic effects, such as inhibiting infectivity of virus and inducing quinone reductase to prevent cancer [13,14]. Determination of TMQ will help us to research the mechanism of PMQ better.

To the best of our knowledge, up to now there is no method reported for the determination of PMQ or TMQ in dog plasma. The goal of this paper was to develop a rapid, sensitive and simple high performance liquid chromatography–tandem mass spectrometry

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(HPLC–MS/MS) method for the pharmacokinetic study of PMQ and its possible metabolite TMQ in dogs.

2. Experimental

2.1. Chemicals and reagents

PMQ (99.17% purity), TMQ (98.77% purity) reference standards, 4′,5,7-trimethylapigenin (TMA, IS, 100.00% purity) and PMQ preparation were provided by Department of Pharmacology, Tongji Medical College, Huazhong University of Science and Technology (Wuhan, PR China). HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Emerson, IA, USA). Water was distilled and purified using a Milli-Q Water Purification System (Millipore, Bedford, MA, USA) and used throughout the study.

2.2. Liquid chromatography

The chromatography was carried out on Shimadzu ultra fast liquid chromatography (UFLC) system (Chiyoda-Ku, Kyoto, Japan) equipped with two LC-20AD pumps, DGU-20A3 on-line degasser, SIL-20ACHT autosampler, CTO-20AC column thermostat.

Chromatographic separation was performed on an Ultimate XB-CN column (2.1 mm × 150 mm, 5 μm, MD, USA) protected by a Phenomenex ODS guard column (4.0 mm × 3.0 mm i.d., 5 μm, Torrance, CA, USA). The mobile phase consisted of acetonitrile and water (2:1, v/v), while the flow rate was 0.3 mL/min. The temperature of column and autosampler were maintained at 35 and 15 °C, respectively. The chromatographic run time of each sample was 6.0 min.

2.3. Mass spectrometry

Mass spectrometric analysis was performed on an API 3200 LC/MS/MS System (Applied Biosystems, Foster City, CA, USA) equipped with an electrosprayion source in the positive ionization mode. The curtain gas (CUR) and collision activated dissociation (CAD) were 27.00 psi and 4.00 psi, respectively. The other working parameters were set as followings: spray voltage 5500.00 V, source temperature 550.00 °C, GAS1 45.00 psi, GAS2 40.00 psi. The declustering potential (DP), entrance potential (EP), collision energy (CE), collision cell exit potential (CXP) were optimized as 50.00 V, 8.00 V, 35.00 V, 4.50 V for PMQ; 62.00 V, 8.00 V, 30.00 V, 4.00 V for TMQ; 58.00 V, 6.00 V, 33.00 V, 4.00 V for TMA. Quantification was done using multiple reaction monitoring (MRM) to monitor the transitions m/z 373.1–312.1 for PMQ, 359.1–344.0 for TMQ and 313.1–298.1 for TMA, with a dwell time of 200 ms per transition. Data acquisition and analysis were achieved by using the Analyst 1.5 software (Applied Biosystems, Foster City, CA, USA).

2.4. Analytical procedure

2.4.1. Preparation of standard and quality control samples

Primary stock solutions of PMQ, TMQ and IS were prepared in methanol to a final concentration of 1.02 mg/mL, 1.38 mg/mL and 1.50 mg/mL, respectively. Working standard solutions of PMQ and TMQ were prepared by taking appropriate amount of each primary stock solution and diluting to the scale of a volumetric flask with methanol to obtain the desired concentrations of 12.72, 63.60, 318.0, 1272, 6360, 12,240, 21,420, 30,600 ng/mL for PMQ, and 103.5, 414.0, 1035, 2070, 5175, 8625, 12,075, 17,250 ng/mL for TMQ. IS working solution was prepared at 1500 ng/mL by dilution of the stock standard in methanol. All solutions were stored at −20 °C for further use.

Calibration standard samples were prepared by adding 20 μL of working standards of PMQ and TMQ at different concentrations in 200 μL of drug-free dog plasma. The final concentrations were 1.272, 6.360, 31.80, 127.2, 636.0, 1224, 2142, 3060 ng/mL for PMQ, and 10.35, 41.40, 103.5, 207.0, 517.5, 862.5, 1207.5, 1725 ng/mL for TMQ. The quality control (QC) samples containing 3.816 ng/mL (LQC), 63.60 ng/mL (M1QC), 510.0 ng/mL (M2QC) and 2448 ng/mL (HQC) of PMQ and 31.05 ng/mL (LQC), 172.5 ng/mL (M1QC), 517.5 ng/mL (M2QC), 1380 ng/mL (HQC) of TMQ were prepared in a manner similar to that used for preparation of the calibrator samples.

2.4.2. Preparation of samples

To an aliquot of 200 μL plasma, 20 μL of IS working solution and 40 μL methanol was added and vortex-mixed for 30 s. The mixture was precipitated with 500 μL acetonitrile, vortex-mixed for 1 min, and then centrifuged at 16,600 rpm for 10 min. Then, 200 μL of supernatant was transferred into autosampler vials and 5 μL was injected into the LC–MS/MS system.

2.5. Method validation

2.5.1. Linearity and lower limit of quantification (LLOQ)

The linearity of the method for the determination of PMQ and TMQ was assessed by weighted (1/X2) least squares regression of analyte-IS peak area ratios. It based on calibration curves containing eight points in the range of 1.272–3060 ng/mL for PMQ and 10.35–1725 ng/mL for TMQ. The calibration curve required a correlation coefficient (r) of 0.99 or better.

The sensitivity of the method was expressed as the lower limit of quantification (LLOQ), and it was defined as the lowest concentration on the calibration curve. Each LLOQ sample should be obtained with an acceptable accuracy (RE) within ±20% and a precision (RSD) not greater than 20% to validate the method.

2.5.2. Precision, accuracy and recovery

The intra-day accuracy and precision of the assay were assessed by analyzing five replicates of QC samples at four concentrations on the same day. The inter-day accuracy and precision were determined by analyzing the QC samples at four levels on three consecutive days. Accuracy and precision were expressed as relative error (RE) and relative standard deviation (RSD), respectively.

The recoveries of PMQ and TMQ from plasma were determined by comparing the peak area of samples spiked before being deproteinized with those spiked after being deproteinized. Recoveries were performed at four QC levels (n = 5).

2.5.3. Matrix effect

The matrix effect was defined as the ion suppression/enhancement on the ionization of analytes. It was evaluated by comparing peak areas of PMQ, TMQ spiked in protein precipitated samples (A) with those of analytes in neat solution at equivalent concentration (B). The ratio (A/B × 100)% was used to evaluate the matrix effect [15]. The same treatment was performed for the IS.

2.5.4. Stability studies

Short-term, long-term and three freeze–thaw stabilities of PMQ and TMQ were evaluated using QC samples containing known concentrations of 3.816, 63.60, 510.0 and 2448 ng/mL of PMQ and 31.05, 172.5, 517.5 and 1380 ng/mL of TMQ. Short-term stabilities were assessed by analyzing the fresh QC samples kept at ambient temperature for 12 h before sample preparation. To evaluate long-term stabilities of PMQ and TMQ, the plasma samples were stored at −80 °C for 30 days. The freeze–thaw stabilities of the analytes were determined over three freeze–thaw cycles. In each freeze–thaw cycle, the samples were frozen and stored at −20 °C for 24 h, then

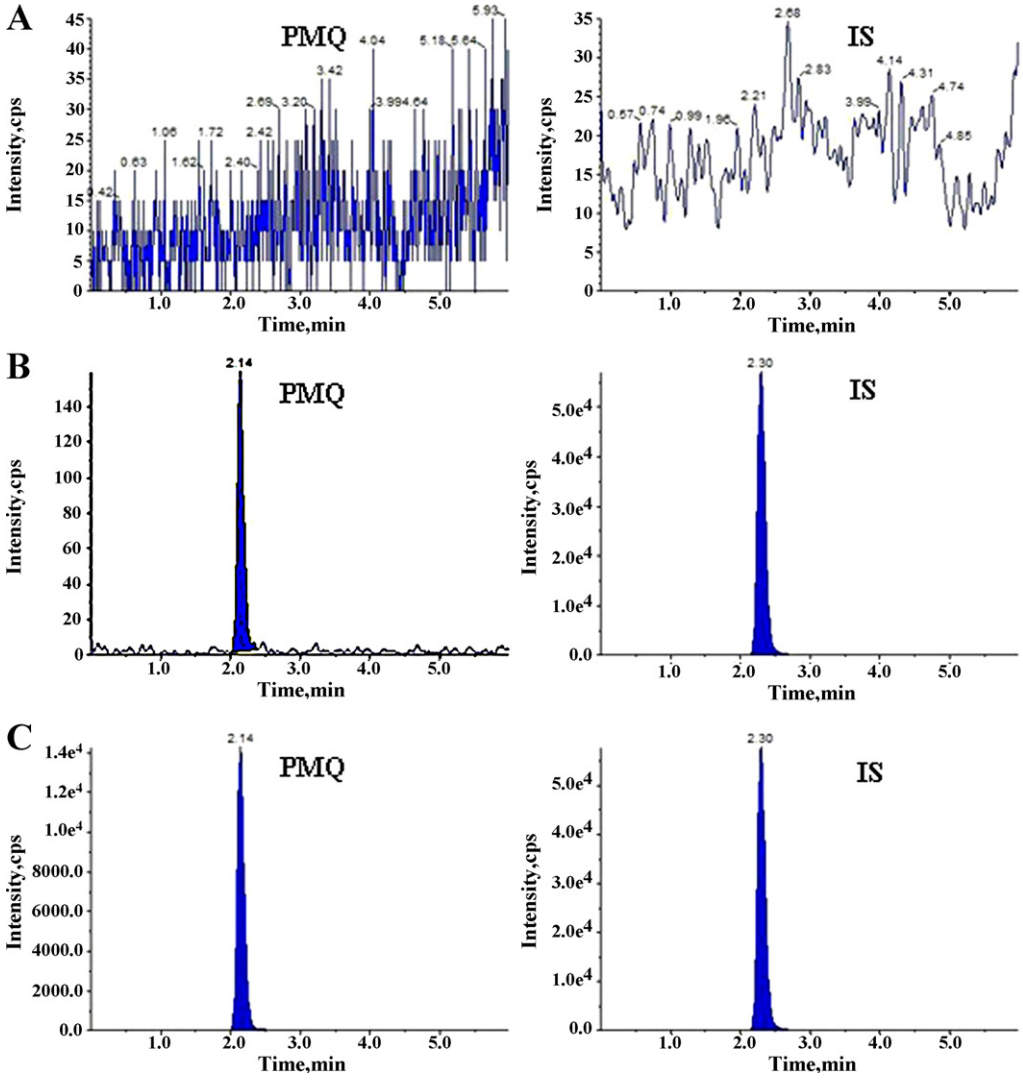

Fig. 1. Typical MRM chromatograms of (A) blank dog plasma; (B) blank plasma spiked with PMQ at LLOQ (1.272 ng/mL) and IS (150 ng/mL); (C) dog plasma after 0.25 h oral administration.

thawed at room temperature. Samples were considered to be stable if their assay values were within 15% error of the nominal values.

2.6. Pharmacokinetic experiments in dogs

The method was successfully applied to the simultaneous determination of PMQ and TMQ in plasma obtained from four healthy adult male Beagle dogs following a single intragastric administration of PMQ. The oral solution was a mixture of DMSO containing fully dissolved PMQ and 5% polyvinyl pyrrolidone (1:9, v/v). Dogs were fasted overnight but were allowed water ad libitum and received a single intragastric administration of 20 mg/kg. Blood samples were drawn from the forelimb cubital vein pre-dose, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8.0, 12.0 and 24.0 h post-dosing. The blood samples were centrifuged immediately at 13,000 × *g* for 10 min to obtain plasma which was transferred to EP tubes. The separated plasma was immediately frozen and stored at −80 °C until analysis. Pharmacokinetic analyses of PMQ and TMQ concentrations in plasma were performed using compartmental model via the proprietary DAS (Drug and Statistics) computer software package (Chinese Pharmacology Society).

3. Results and discussion

3.1. Method development

To the best of our knowledge, there is no report yet on the simultaneous determination of PMQ and TMQ in biological fluids by LC–MS/MS. Thus, the aim of the study was to establish a sensitive and specific LC–MS/MS method with a short overall analysis time for the simultaneous quantification of PMQ and TMQ in dog plasma and to make a preliminary investigation on the pharmacokinetics of PMQ in Beagle dogs.

In order to optimize mass spectrometric conditions, the solutions containing PMQ, TMQ and IS were directly infused into the mass spectrometer at a flow rate of 10 μL/min via a syringe pump, respectively. MS scans were carried out in positive and negative ion mode, and the results both showed that positive ion mode produced higher sensitivity and fewer fragments for PMQ and TMQ. From the product ion scan (MS2), it could be identified that the fragment ions with *m*/*z* 312.1 for PMQ, 344.0 for TMQ and 298.1 for TMA were most abundant. Thus, we selected the transitions m/z 373.1–312.1 for PMQ, 359.1–344.0 for TMQ and 313.1–298.1 for TMA for determination.

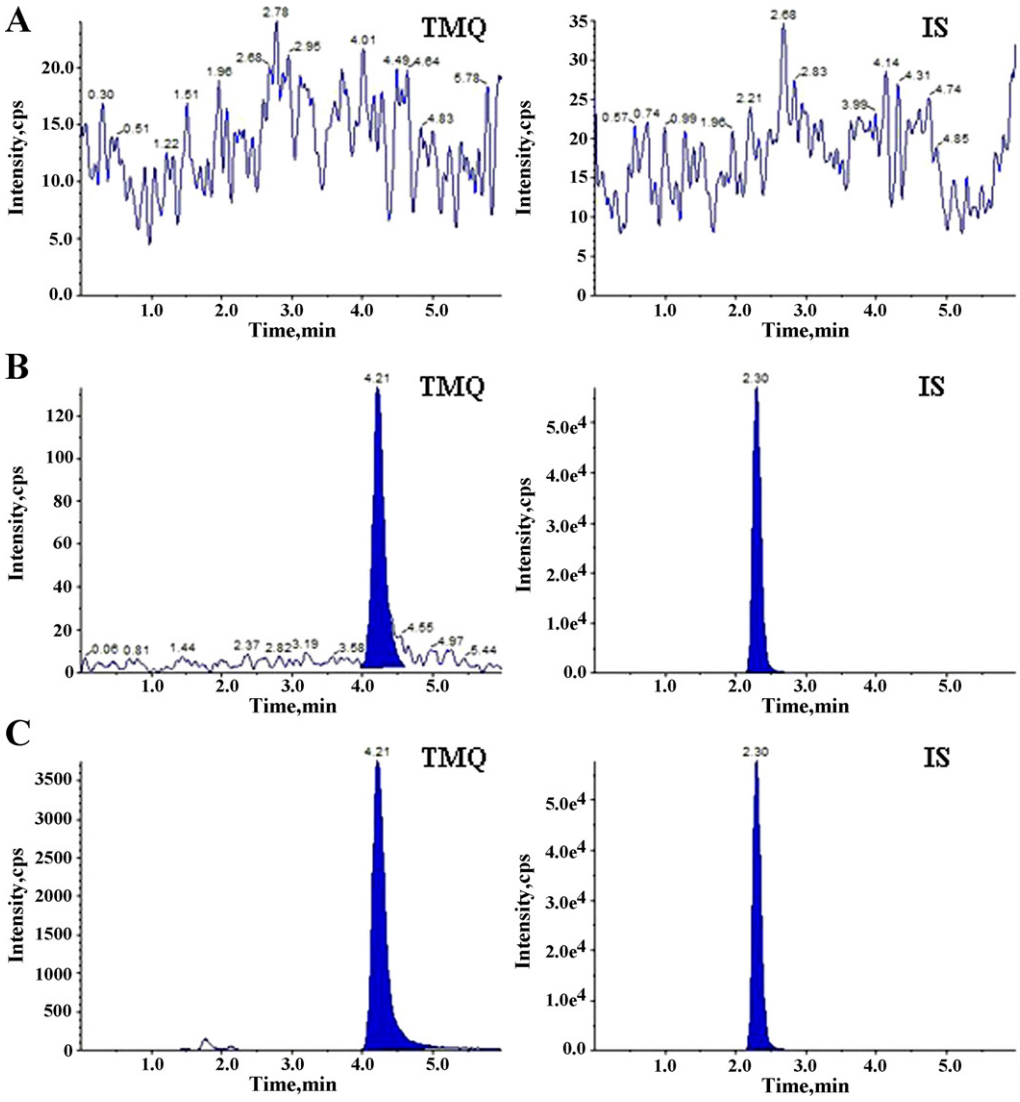

Fig. 2. Typical MRM chromatograms of (A) blank dog plasma; (B) blank plasma spiked with TMQ at LLOQ (10.35 ng/mL) and IS (150 ng/mL); (C) dog plasma after 0.25 h oral administration.

To get the most suitable mobile phase, methanol, acetonitrile, water and typical buffers such as formic acid and ammonium acetate were mixed at differentratios. The results revealed that the addition of formic acid or ammonium acetate could not enhance the MS ionization efficiency, neither could it improve the peak shapes of PMQ, TMQ or IS. Finally, a mixture of acetonitrile and water at ratio of 2:1 (v/v) was adopted in this study, which provided good peak shapes of PMQ, TMQ and IS as well as high sensitivity. Different types of column (Ultimate C18, Ultimate XB-CN, Ultimate AQ-C18) were evaluated and the Ultimate XB-CN column provided the best chromatographic separation. TMA, as a homologue of PMQ and TMQ, was selected as the internal standard. The retention times of PMQ, TMQ and IS were 2.14, 4.21 and 2.30 min, while no interference peak was detected at related retention time in blank samples (Figs. 1 and 2).

3.2. Method validation

3.2.1. Linearity and range

A linear relationship was found between peak area ratio and PMQ or TMQ concentration within the range of 1.272–3060 ng/mL or 10.35–1725 ng/mL, respectively. Typical equations for the calibration curves were as follows: $Y = 0.000855X + 0.00136$ ($r = 0.9958$) for PMQ and $Y = 0.000313X + 0.0000647$ ($r = 0.9911$) for TMQ, where Y was peak-area ratio of analyte to IS and X was the plasma concentration of analyte. The LLOQ of the analytes were 1.272 ng/mL for PMQ and 10.35 ng/mL for TMQ in dog plasma.

3.2.2. Precision and accuracy

The intra- and inter-day precisions were evaluated in five replicates and presented in Table 1. The results indicated that intra-day RSDs were between 6.2 and 10.9% for PMQ and lower than 8.7% for TMQ. The relative error ranged from −8.8 to 3.8% for PMQ and from −3.1 to 6.4% for TMQ. Moreover, the inter-day RSDs were between 7.0 and 9.1% for PMQ while 4.4 and 6.2% for TMQ. The relative error ranged from −5.4 to 0.4% for PMQ and from −3.3 to 7.1% for TMQ. The values are well within the acceptable criteria where the precision should not exceeding 15% and accuracy within ±15% of the actual value. It indicated that the present LC/MS/MS method was accurate, reliable and reproducible.

3.2.3. Recovery and matrix effect

The mean recoveries for PMQ at LQC, M1QC, M2QC and HQC levels were 89.9, 94.5, 86.7 and 88.2% and that for TMQ were 84.2, 80.6, 87.3 and 81.8%, respectively, with RSD% between them of 3.8% for PMQ and 3.5% for TMQ. Thus, the consistency in recoveries of PMQ

Table 1
Precision and accuracy for PMQ and TMQ in dogs plasma ($n = 3$ days, five replicates per day).

Analytes	Nominal con. (ng/mL)	Intra-day (RSD%)	Inter-day (RSD%)	Intra-day (RE%)	Inter-day (RE%)
PMQ	3.816	10.9	9.1	−2.2	−1.6
	63.60	9.9	7.4	−8.8	−5.4
	510.0	8.0	8.6	3.8	0.4
	2448	6.2	7.0	2.4	−0.8
TMQ	31.05	8.7	6.2	−3.1	−1.9
	172.5	7.4	5.9	6.4	7.1
	517.5	4.7	5.6	−2.5	−3.3
	1380	5.4	4.4	4.7	2.9

and TMQ supported the extraction procedure for its application to routine sample analysis.

The absolute matrix effects for PMQ and TMQ at LQC, M1QC, M2QC and HQC levels were all within 85–115%, respectively (Table 2). These results showed that ion suppression or enhancement from plasma matrix was negligible in the present condition.

3.2.4. Stability

The stability of PMQ and TMQ under different storage and handling conditions were fully evaluated by analyzing QC samples. In the short-term stabilities test, the measured concentration (mean ± SD, ng/mL) at LQC, M1QC, M2QC and HQC levels were 3.829 ± 0.233, 62.03 ± 2.24, 520.4 ± 21.4, 2430 ± 207 for PMQ and 32.42 ± 0.96, 171.3 ± 7.7, 526.3 ± 8.8, 1363 ± 79 for TMQ, respectively. The data for the long-term stabilities were 3.838 ± 0.319, 64.78 ± 2.86, 501.0 ± 13.1, 2340 ± 108 for PMQ and 30.43 ± 3.01, 174.7 ± 6.25, 492.7 ± 31.7, 1359 ± 72 for TMQ. For the test of the three freeze–thaw stabilities, the data were 3.763 ± 0.417, 64.49 ± 2.46, 507.9 ± 28.3, 2365 ± 248 for PMQ and 29.91 ± 1.36, 173.9 ± 9.1, 516.2 ± 18.3, 1300 ± 42 for TMQ. The relative errors of all samples were within 9.1%, which indicated that analytes exhibited no significant degradation under the above conditions. The result meant a good stability of the analytes over all steps of the determination.

3.3. Application to preclinical pharmacokinetic study

The validated method was successfully employed to determine the plasma concentration of PMQ and TMQ to support preclinical pharmacokinetic studies in dogs following intragastric administrations (20 mg/kg). The statistical software DAS was used to analyze the plasma concentration–time data by means of the least-square method. With comparing AIC-value, a single compartment model was available. The mean plasma concentration–time profiles of PMQ and TMQ were presented in Fig. 3. OBS is the measured plasma concentrations of four Beagle dogs while PRED is the fitted value. The figure showed that there exists a good agreement between them. It was also found that PMQ was rapidly absorbed into the circulation system and reached its peak concentration at around 1.0 h after oral administration, and rapidly metabolized to the metabolite TMQ.

Table 2
Matrix effect for PMQ and TMQ in dogs plasma ($n = 5$).

Analytes	Concentration (ng/mL)	(A/B × 100)% (mean ± SD)	RSD (%)
PMQ	3.816	97.2 ± 6.1	6.3
	63.60	100.9 ± 4.3	4.3
	510.0	102.6 ± 3.7	3.6
	2448	98.6 ± 5.2	5.3
TMQ	31.05	94.8 ± 2.0	2.1
	172.5	96.1 ± 1.5	1.6
	517.5	107.5 ± 3.4	3.2
	1380	95.4 ± 4.1	4.3
IS	150.0	105.7 ± 5.5	5.2

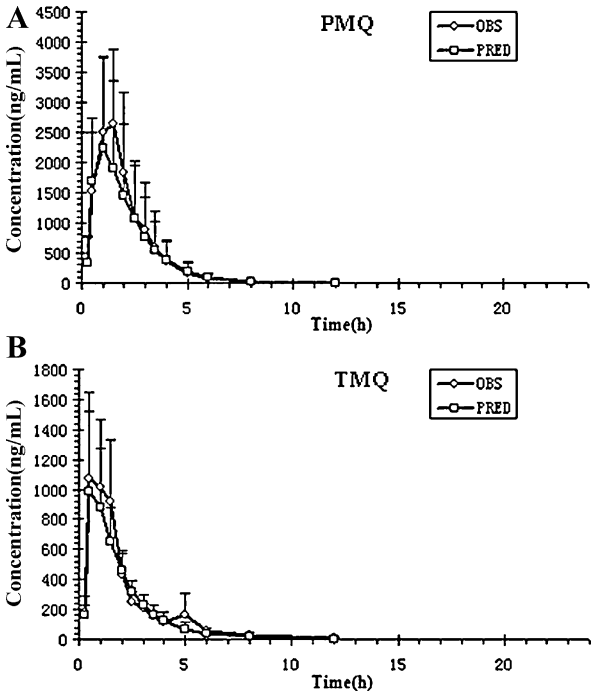

Fig. 3. Mean plasma concentration–time profiles of PMQ(A) and TMQ(B) in four Beagle dogs after oral administration.

4. Conclusion

A sensitive, simple and rapid UFLC–ESI-MS/MS assay for simultaneous determination of PMQ and TMQ in dog plasma was developed and validated with respect to sensitivity, accuracy, precision and reproducibility. Calibration curves were linear over a wide range of 1.272–3060 ng/mL for PMQ and 10.35–1725 ng/mL for TMQ. This method provided a sample preparation by protein precipitation with acetonitrile which could save considerable time and simplify the operating process. The results also indicated that the method could be applied to preclinical pharmacokinetic studies of PMQ and its possible metabolite TMQ with acceptable precision and accuracy in dogs following oral administration.

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