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Study on the pharmacokinetics and metabolism of paeonol in rats treated with pure paeonol and an herbal preparation containing paeonol by using HPLC–DAD-MS method

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Abstract

Paeonol, a principal bioactive component of the Chinese herb Moutan Cortex with anti-inflammatory and analgesic effects, was comparatively studied to determine its pharmacokinetic behavior and metabolic profile in rat following oral administration of the pure paeonol alone and an herbal preparation "Qingfu Guanjieshu" (QFGJS) containing paeonol. An HPLC–DAD method was developed and validated for determining the concentration of paeonol in rat plasma. The *in vivo* time curves and AUC of paeonol at three doses were increased in a dose-dependent manner, while the pharmacokinetic parameters of paeonol in QFGJS at a comparable dosage were significantly elevated in comparison with those of pure paeonol. By using LC-Q/TOF-MS technique, four metabolites of paeonol were identified in plasma at 5 min after dosing, with T_{max} around 20 min after treatment with the pure paeonol or QFGJS. Interestingly, relative concentrations of metabolites P2, P3 and P5 were markedly increased in plasma of rats treated with QFGJS compared with those of pure paeonol. These results indicate that other components in QFGJS could effectively influence the pharmacokinetic behavior and metabolic profile of paeonol in rats. The current studies emphasize the significance of the research toward an understanding of pharmacokinetic interactions of the co-existing components in the herbal preparations.

Keywords: Paeonol; Pharmacokinetic; Metabolite; LC–MS; QFGJS

1. Introduction

Paeonol (2′-hydroxy-4′-methoxyacetophenone, Fig. 1) is a major component in the medicinal herb Moutan Cortex, the root cortex of *Paeonia suffruticosa* A. (family Paeoniaceae), and is commonly prescribed for the treatment of pain and inflammatory ailments in Chinese medicine [1]. Paeonol has been demonstrated to inhibit carrageenan-evoked thermal hyperalgeisa in rats, to inhibit the pro-inflammatory cytokine cascade and overproduction of nitric oxide (NO) and prostaglandin E2 (PGE_2), to suppress ADP- or collagen-induced human blood platelet aggregation in a dose-dependent manner, and possess antipyretic and antibacterial properties [2,3].

Pharmacokinetic studies are useful to explain and predict a variety of events related to the efficacy and toxicity of drugs, thus it is very important to perform pharmacokinetic studies of the major bioactive components in the herb for evaluating their potentials in clinical usage. However, pharmacokinetics and metabolism of paeonol have not yet been systematically investigated although its anti-inflammation activity was extensively studied. The pharmacokinetics after intra-venous administration of paeonol were reported previously, but without satisfaction for the support of its clinical usage [4]. Riley and Ren developed an HPLC method for the determination of paeonol in rabbit plasma after oral administration of pure paeonol, but no paeonol was detected in the plasma at a dosage of 30 mg/kg body weight [5]. Failure to detect paeonol in plasma may be due to low sensitivity of the detection method and/or poor oral bioavailability.

Five metabolites of paeonol, *i.e.*, 2,4-dihydroxyacetophenone-5-*O*-sulfate (P1), resacetophenone-2-*O*-sulfate (P2), 2-hydroxy-4-methoxyacetophenone-5-*O*-sulfate (P3), paeonol-

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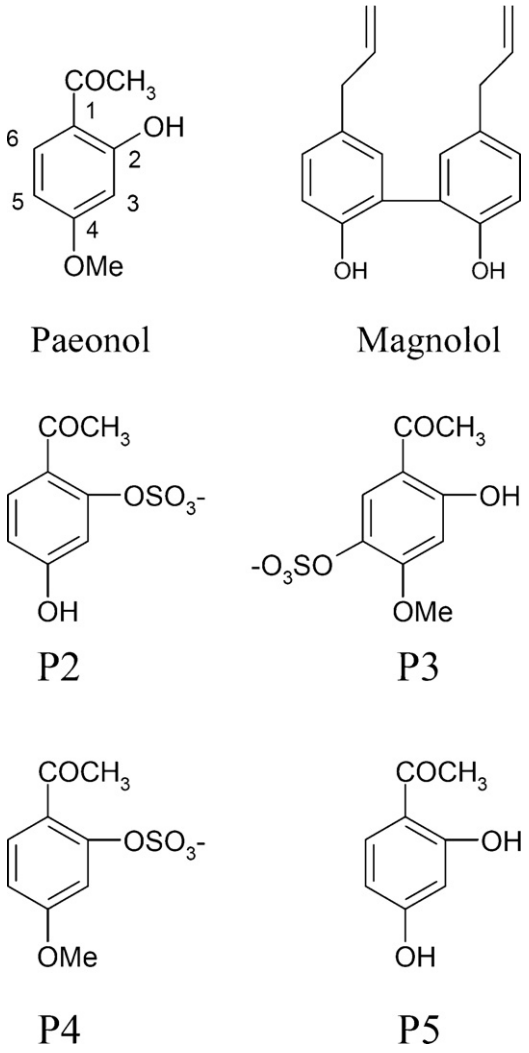

Fig. 1. Chemical structures of paeonol, magnolol (internal standard), and metabolites of paeonol including resacetophenone-2-*O*-sulfate (P2), 2-hydroxy-4-methoxyacetophenone-5-*O*-sulfate (P3), paeonol-2-*O*-sulfate (P4) and resacetophenone (P5).

2-*O*-sulfate (P4) and resacetophenone (P5), were first identified in rat urine by Yasuda et al. after oral administration of paeonol [6]. However, the need to develop more sensitive methods for the pharmacokinetic and metabolism studies of paeonol for the purpose of evaluating its clinical usage is apparent.

In clinics, Chinese medicine practitioners usually prescribe Moutan Cortex in collaboration with other herbs to treat diseases, rather than prescribe the single herb or purified compound alone. Thus, the herbal pharmacokinetic studies should focus on the herbal formula to maintain consistency with the mode of clinical usage of herbal medicines. This approach, however, presents some difficulties, as the potential interactions between the chemicals in the herbs or herbal formula must be taken into account. As a preliminary step, it is important to identify the pharmacokinetic behavior and metabolic profile of some representative compounds with confirmed bioactivities in the herb, as well as any variations due to the interactions with other components in the herbal preparation.

We hypothesized that the pharmacokinetic behavior and metabolism of the bioactive compounds in an herbal preparation could be altered through pharmacokinetic chemical–chemical interactions with other components in the herbal preparation. To study this hypothesis, the pharmacokinetic behavior of paeonol and its metabolic profile in rats were examined using two administrative protocols, either oral delivery of the pure paeonol alone, or an anti-arthritic herbal preparation, Qingfu Guanjieshu (QFGJS), containing paeonol. Being derived from a Chinese medicinal prescription, QFGJS is composed of five well-documented medicinal herbs (Caulis Sinomenii, Radix Paeoniae Alba, Moutan Cortex, Rhizoma Curcumae Longae, and Radix Aconiti Lateralis Preparata) for rheumatic and arthritic diseases. It has proven to be effective in suppression of experimental arthritis as well as various acute inflammatory and pain models in rodents in our previous studies [7–9]. As paeonol has been demonstrated to be an effective compound in anti-inflammation and analgesia, paeonol is likely to be one of the bioactive components of QFGJS responsible for treatment of inflammatory arthritic diseases. Therefore, in the current study, paeonol was also chosen as an indicative effective chemical for the pharmacokinetic study of QFGJS.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (HPLC grade), triethylamine, formic acid, trifluoroacetic acid (TFA) (all GR grade) were purchased from International Laboratory, USA. Hydrochloric acid (GR grade) was purchased from Merck, Germany. Deionized water was prepared using a Millipore water purification system (Billerica, MA, USA).

Reference chemical standards of paeonol and magnolol (internal standard) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. The identity and purity of these chemicals were further validated by LC–MS in our laboratory.

The QFGJS product was prepared from five herbs with modern pharmaceutical methods, according to our previous reports [7–10]. The concentration of paeonol in the QFGJS products was determined as 19.0 mg/g.

2.2. HPLC and LC–MS conditions

An Agilent 1100 series LC system (Agilent Technologies, CA, USA) consisting of a G1312A Binary Pumps, G1379A degasser, G1315B Diode-Array Detector and G1313A Autosampler was employed for this study.

The chromatographic analysis for determination of paeonol was carried out on a Phenomenex ODS (250 mm × 4.6 mm i.d.; particle size 5 μm; Phenomenex Inc., USA) protected by a Security Guard Cartridge (C18, 4 mm × 3.0 mm i.d.; Phenomenex Inc., USA). The mobile phase was acetonitrile (A) and aqueous solution (B) (containing 0.1% formic acid, adjusted with triethylamine to pH 3.5 ± 0.2). The conditions of the solvent gradient elution were 25–90% (A) in 0–23 min at a flow rate

of 1.0 ml/min. Detection was conducted with wavelength of 270 nm for paeonol at room temperature. All injection volumes were 50 μl.

The mobile phase for LC–DAD-MS was 0.05% TFA (A) and 50% acetonitrile with 0.05% TFA (B) with the following gradient system: 0–15% (B) in 0–20 min, 15–50% (B) in 20–60 min, 50–100% (B) in 60–70 min, and 100% (B) in 70–80 min at a flow-rate of 1.0 ml/min.

Electrospray ionization in negative and positive ion modes was used to analyze paeonol and its metabolites in rat plasma samples. All MS experiments were performed using a MicroTOF-Q mass spectrometer (Bruker Daltonics Inc., MA, USA). The following parameters were used for analysis: end plate offset voltage −500 V, capillary voltage 4500 V, capillary temperature 200 °C, dry gas (N_2) 4.0 l/min, dry gas temperature 200 °C, nebulizing gas (N_2) pressure 0.4 bar. Mass spectra were acquired in the 50–400 *m/z* range with resolution around 10,000.

2.3. Animals and surgery

Male Sprague–Dawley rats weighing 200–250 g were purchased from the Laboratory Animal Services Center of the Chinese University of Hong Kong. Animals were housed four per cage with food and water provided *ad libitum* and acclimated in the laboratory for at least 1 week prior to testing. After surgery, rats were housed individually in metabolite cages for 5 days' recovery and underwent pharmacokinetic treatment according to a jugular-catheterized rat model [11]. All procedures involving animals and their care were approved and under the regulations of the Committee on Use of Human & Animal Subjects in Teaching and Research (HASC) of Hong Kong Baptist University and the Department of the Health of Hong Kong Special Administration Region.

Rats were anesthetized by intra-peritoneal administration of 0.7% chloral hydrate saline solution at a dosage of 5 ml/kg of body weight as described previously [12]. A longitudinal skin incision was made over the area where the right external jugular vein passed dorsal to the pectoral major muscle. The catheter (Silastic cat. no. 602-155; Dow Corning, Midland, MI), filled with 20 units/ml heparinized saline solution, was inserted into the right jugular vein and then advanced into the sinus venosus. The catheter was inserted up to the first silicone stopper (Silastic cat. no. 801; Dow Corning), set at 26 mm, and anchored in place by suturing the stopper to muscle [13]. The free end of the catheter was passed under the skin of the dorsum of the neck just caudal to the ears and attached to the skin, together with a metal spring, which was covered with PVC tubing for protection of the outer part of the catheter. Finally, the catheter was filled with 500 units/ml heparinized saline solution, and a plug was inserted in the free end of the catheter.

2.4. Drug administration and blood sampling

Rats were randomly divided into four groups with eight rats per group. The oral dosage of QFGJS capsule was 3.89 g/kg body weight (a dose equivalent to 73.9 mg/kg of pure paeonol), prepared by dissolving the capsule content in 0.3% CMC-Na solution. This dosage (3.89 g/kg) is approximately 4.66 times of the equivalent dose in humans, previously demonstrated to significantly suppress arthritis and acute inflammation in animal models [7–9], and thus is a reasonable dose for animal studies. Pure paeonol was dissolved in propylene glycol and further diluted with 0.3% CMC-Na solution, and was given orally to three groups of animals at doses of 140, 70, and 35 mg/kg, respectively.

Prior to the experiments, the animals fasted for 24 h with water *ad libitum*, and maintained at 21 °C and 60% relative humidity, with a 12-h light/dark cycle. After administration, the jugular vein blood samples were collected (0.2 ml) in heparinized 1.5 ml micro-centrifuge tubes at the following time intervals: 0, 5, 15, 30, 45, 60, 90, 120, 240, 360, 540, and 720 min. After each blood sample, the catheter was gently flushed with the same volume of heparinized saline solution (20 units/ml) to replace the loss of volume of blood, and a plug was inserted at the end of the catheter. The blood samples were immediately centrifuged at 12,000 rpm for 5 min at room temperature, and the resulting plasma was collected and stored at −20 °C until analysis.

2.5. Preparation of sample solution

The rat plasma (100 μl) was mixed with 200 μl acetonitrile containing magnolol (internal standard, 26.23 μg/m) by vortexing for 30 s. The mixture was then centrifuged at 12,000 rpm for 10 min at room temperature to separate precipitated proteins. The supernatant was transferred into a 1.5-ml centrifuge tube containing 50 mg sodium chloride, mixed by vortexing, and incubated at room temperature for 20 min. After another round of vortexing and centrifugation (12,000 rpm for 5 min), 50 μl of the supernatant was directly injected into the HPLC system for analysis.

For preparation of the sample solution for LC–DAD-MS analysis, 100 μl of plasma was mixed with 300 μl methanol containing magnolol (internal standard, 26.23 μg/ml) by vortexing, and centrifuged at 12,000 rpm for 10 min at room temperature to separate precipitated proteins. The supernatant was evaporated to dryness below 40 °C under reduced pressure. The residue was dissolved in 150 μl methanol–water (1:1 v/v), the mixture was centrifuged twice (12,000 rpm, 15 min), and 40 μl of the supernatant was injected into the LC–DAD-MS system for analysis.

2.6. Calibration curve

Concentrated stock solutions of paeonol and magnolol were prepared separately in acetonitrile at final concentrations of 250 and 26.23 μg/ml, respectively, and stored at −20 °C. Spiked standard solutions for the calibration curve and quality control samples (QCs) were made by serial dilutions with acetonitrile. Calibration curves were prepared by spiking 100 μl of the corresponding spiked standard solutions to 0.9 ml blank plasma to reach the final concentrations, *i.e.*, 0.15, 0.30, 0.76, 1.51, 2.26, 3.02, and 4.53 μg/ml. The QCs were prepared in the same way as the calibration standards with three concentrations of paeonol and used for validation procedures and stability proposes. The

QCs were aliquoted (100 μl) into 1.5 ml micro-centrifuge tubes and stored at −20 °C until analysis. Extraction was performed in the same way as described in Section 2.4.

The linearity of the method was evaluated by the calibration curve of paeonol, including lower limit of quantification (LLOQ). A least-squares linear regression analysis was performed to determine slope, intercept and coefficient of correlation (r^2). The limit of detection (LOD) was determined as the concentration giving a signal-to-noise ratio of 3:1.

2.7. Method validation

The intra-day accuracy and precision of determination were evaluated by replicate analysis of six sets of samples at four concentration levels (LLOQ, low, middle, and upper concentrations) on the same day. For inter-day variation, six replicates of QC samples at three concentration levels were analyzed along with a standard curve on three different days. For acceptable intra- and inter-day values, accuracy should be within the range of 85–115% and the precision, *i.e.*, the coefficient of variation (CV%) which served as a measure of precision, should be below 15%, except for the LLOQ, where the accuracy should be between 80 and 120% and precision (CV%) should not exceed 20% [14].

The recovery of paeonol from plasma was evaluated using the QC samples at three different concentrations by comparing the peak areas of the prepared samples with those of standard solutions containing the corresponding concentrations that represent 100% of the recovery rate. The recovery rate of magnolol from plasma was determined using a concentration of 26.23 μg/ml by the same method.

2.8. Stability test on plasma samples

Stability of paeonol in plasma was tested using QC samples at three different concentrations by using four different sample preparatory methods, *i.e.*, freeze–thaw, long-term, short-term and post-preparative plasma samples. In each freeze–thaw cycle, the QC samples were frozen for about 24 h at −20 °C and thawed at room temperature (20 °C). The long-term stability was evaluated after keeping the QC samples frozen at −20 °C for 1 month. For short-term stability, frozen samples were kept at room temperature for 5 h before sample preparation. The post-preparative stability of the processed samples was tested after keeping the samples in HPLC autosampler vials at room temperature for 24 h. All the stability samples were analyzed and the results were compared to the mean of back-calculated values for the freshly prepared QC samples assayed at the first day of long-term stability testing.

2.9. Pharmacokinetic behavior and metabolites determination of paeonol in plasma

To quantify the paeonol in plasma samples, calibration curves and three QC samples were used with every set of 20 plasma-testing samples. The pharmacokinetic parameters for paeonol were evaluated by analyzing the data of plasma concentration–time profiles, which was calculated by the pharmacokinetic software, PK Solutions 2.0 (Summit Co., USA) with non-compartment analysis. The following non-compartmental pharmacokinetic parameters were calculated based on the moment method: half-life ($T_{1/2}$), mean residence time (MRT), volume of distribution (V_d), total clearance (CL/F), and area under the concentration–time curve (AUC).

For the determination of four metabolites of paeonol, the peak area ratio (R) of the peak area of the respective metabolite to that of internal standard was calculated, which allowed graphic representation of the time course in plasma. The value of R was calculated as follows: R (%) = [peak area of metabolite/peak area of internal standard] × 100. AUC and $T_{1/2}$ were calculated by analyzing the peak area ratio–time curve for each metabolite with the pharmacokinetic software, PK Solutions 2.0. The statistical significance of the difference among pharmacokinetic parameters was assessed with mean comparison Student's *t*-test. P values lower than 0.05 were considered to be significant.

3. Results

3.1. Chromatographic conditions and measurement of paeonol in rat plasma

For the quantification of paeonol concentration in rat plasma, an HPLC–DAD method was developed with method validation according to the guidance of the US Food and Drug Administration [14]. Another method of LC coupled with ESI (electrospray ionization)–Q-TOF (quadrupole/time-of-flight) MS was established for the structural identification of the metabolites of paeonol in rat plasma at different time intervals after oral administration of paeonol alone and QFGJS containing paeonol.

Due to the similarity of the chemical structure and anticipated resembleness in the extraction recovery, magnolol was chosen as an internal standard for the quantification. The HPLC chromatograms of blank plasma, plasma spiked with paeonol and magnolol, as well as plasma obtained 5 min after oral administration of paeonol (70 mg/kg dosage) and QFGJS (3.89 g/kg body weight dosage), are shown in Fig. 2. The gradient elution program was optimized in order to achieve a sufficient resolution of paeonol and magnolol in an acceptable run-time with sharp peaks and without interference (Fig. 2B). By comparing both the retention times and the UV spectra of the reference standards, peaks of paeonol and magnolol in rat plasma were unambiguously identified (Figs. 2C and D). The peak purity was confirmed by studying the DAD spectrum in which no indication of impurities of peaks was found. Furthermore, the peaks in the drug-free plasma samples failed to show any interfering influence (n = 6) (Fig. 2A).

3.2. Calibration curve

The linear regression analysis was constructed by plotting the peak area ratios versus concentrations of paeonol in a range of 0.15–4.53 μg/ml. The regression equation of these calibration curves and their correlation coefficients (r^2) were calculated as

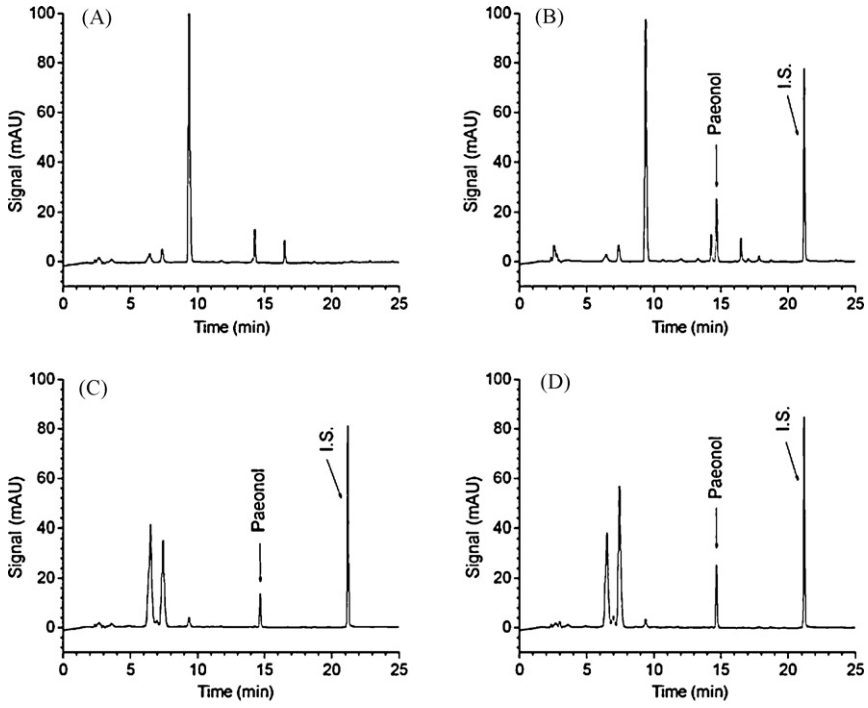

Fig. 2. Typical HPLC chromatograms of the measurements of paeonol in plasma samples: chromatogram of a blank plasma sample (A); chromatogram of a plasma sample spiked with paeonol (0.755 μg/ml) and magnolol (I.S., 26.23 μg/ml) (B); chromatogram of the plasma sample from a rat after 5 min of oral administration of paeonol alone at dosage of 140 mg/kg (C); QFGJS at dosage of 3.89 g/kg (D).

follows: $y = 0.4689x + 0.0024$, $r^2 = 0.9999$ (where y is the peak area ratio, x is the paeonol concentration).

The LLOQ in rat plasma was calculated as 0.15 μg/ml of paeonol, and it was the lowest concentration determined in which the accuracy and precision (CV%) of determinations were 100.5 and 3.38%, respectively. The LOD was 0.015 μg/ml at a signal-to-noise ratio of 3:1.

3.3. Accuracy, precision and recovery rate of the assays

The accuracy and precision of the intra- and inter-day assay variations in rat plasma are summarized in Table 1. The precision of the method was determined by calculating the percentage of deviation (CV%) observed in the analysis of QC samples. The intra-day accuracy varied between 100.5 and 102.7%, while the precision (CV%) was within 3.38%. The inter-day accuracy ranged from 99.8 to 101.2%; while the precision (CV%) was less than 3.50%. All the values of accuracy and precision including LLOQ fell within the limits stipulated by the FDA of USA, indicating the reproducibility and accuracy of the modified HPLC–DAD method to be highly acceptable [14].

Table 2 shows the absolute recovery rates of paeonol and magnolol in rat plasma. Regardless of the concentration of paeonol, the recovery rates ranged between 97.84 and 102.06%, with the CV ranging between 1.37 and 2.45%. A mean recovery rate of 96.06% was obtained for magnolol at 26.23 μg/ml. These results indicate that acetonitrile used in a 2:1 ratio (acetonitrile:plasma)

Table 2
Absolute recovery rates for determination of paeonol in rat plasma ($n = 6$)

Compounds	Concentrations (μg/ml)	Absolute recovery rates (%, $\bar{X} \pm$ S.D.)	CV (%)
Paeonol	0.30	102.1 ± 2.50	2.45
	1.51	98.50 ± 2.39	2.42
	3.02	97.84 ± 1.34	1.37
Magnolol	26.23	96.06 ± 2.50	2.45

Table 1
Accuracy and precision of the method for determination of paeonol in rat plasma ($n = 6$)

Assays	Spiked concentrations (μg/ml)	Measured concentrations (μg/ml, $\bar{X} \pm$ S.D.)	Accuracy (%)	Precision (CV%)
Intra-day	0.15	0.15 ± 0.01	100.5	3.38
	0.30	0.31 ± 0.01	102.7	1.71
	1.51	1.54 ± 0.01	101.8	0.82
	3.02	3.04 ± 0.03	101.2	1.12
Inter-day	0.30	0.31 ± 0.01	101.2	3.50
	1.51	1.51 ± 0.04	99.80	2.73
	3.02	3.02 ± 0.06	100.0	1.88

Table 3
Stability of paeonol in rat plasma

Stability conditions ($n=3$)	Concentrations (μg/ml, $\bar{X} \pm$ S.D.)		
	LQC	MQC	HQC
Initial	0.31 ± 0.01	1.53 ± 0.00	3.06 ± 0.05
Freeze–thaw	0.30 ± 0.02	1.50 ± 0.02	3.05 ± 0.08
Deviation	−1.82%	−2.12%	−0.33%
Short term	0.30 ± 0.00	1.51 ± 0.01	3.07 ± 0.05
Deviation	−2.52%	−1.56%	0.42%
Long term	0.29 ± 0.01	1.51 ± 0.00	3.14 ± 0.01
Deviation	−5.87%	−1.52%	2.42%
Post-preparative	0.30 ± 0.00	1.53 ± 0.01	3.07 ± 0.05
Deviation	−2.40%	−0.31%	0.17%

effectively removed proteins from blood plasma for the purpose of paeonol determination.

3.4. Stability test of paeonol in rat plasma samples

The QC samples at three concentrations of paeonol were used for stability experiments under a variety of storage and processing conditions. The results presented in Table 3 showed that the deviation of the mean test responses was within ±10% of the appropriate controls in all stability tests. Three freeze–thaw cycles of the QC samples appeared to have no influence on the quantification of paeonol. The results of long-term and short-term stability testing of QC samples indicated that the plasma samples remained stable for 1 month at −20 °C and for 5 h at room temperature. No influence on quantification was observed for all of the processed samples that were analyzed after storage at room temperature for 24 h. The conditions employed in the stability study reflected the logistics of pharmacokinetic studies of paeonol during sample handling, storage and preparation. These results also indicated that the rat plasma containing paeonol could be handled under normal laboratory conditions without significant loss of the amount of the drug.

3.5. Pharmacokinetic behavior of paeonol in rat plasma

The validated HPLC–DAD method discussed above was successfully applied to a pharmacokinetic study of paeonol in rats using two administrative protocols, *i.e.*, an oral administration of the pure paeonol alone, and oral dosing of QFGJS containing approximately equivalent paeonol. The pharmacokinetic behavior with mean plasma concentration–time curves of the three doses of pure paeonol, and of comparable paeonol doses in QFGJS, are presented in Fig. 3.

The pharmacokinetic parameters of paeonol obtained from the non-compartmental pharmacokinetic analysis are summarized in Table 4. As shown in Fig. 3, paeonol was absorbed very quickly into blood with the time to reach maximum plasma concentration (T_{max}) at 5.0 min. There were no significant differences in the apparent CL/F, T_{max}, half-time of absorption phase ($T_{1/2\ A\ phase}$), half-time of elimination phase ($T_{1/2\ E\ phase}$), half-time of distribution phase ($T_{1/2\ D/A\ phase}$), and V_d (area), when the paeonol dose was increased from 35 to 140 mg/kg

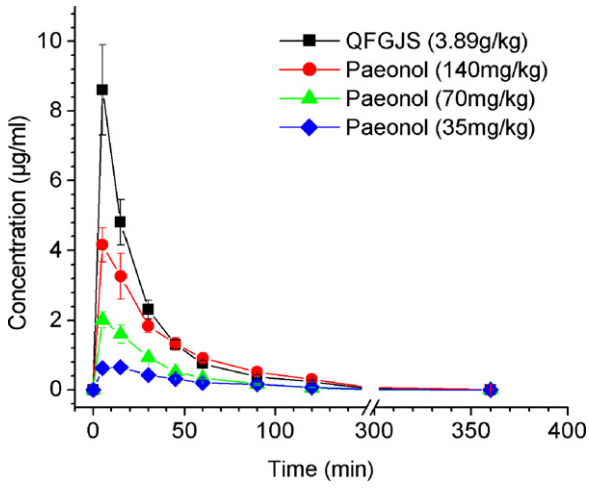

Fig. 3. Mean plasma concentration–time profiles of paeonol in rat plasma after oral administration of QFGJS capsule containing paeonol and paeonol alone at three doses ((■) QFGJS 3.89 g/kg, (●) 140 mg/kg, (▲) 70 mg/kg, (♦) 35 mg/kg); each point and bar represents the mean ± S.E.M. ($n=6$–8).

body weight. The AUC of paeonol increased proportionally from the dosage of 35 to 140 mg/kg body weight, which indicated that the pharmacokinetic behavior of paeonol is in a dose-dependent manner. Moreover, the results of the current study showed that the pharmacokinetic behavior of paeonol administered via the QFGJS preparation was significantly different from that of pure paeonol alone (Fig. 3). In the animals given QFGJS, the peak plasma concentration (C_{max}) of paeonol was markedly elevated ($P<0.01$), AUC was increased ($P<0.01$), and CL/F was decreased ($P<0.001$), indicating that some chemicals contained in QFGJS may influence the pharmacokinetic behavior of paeonol *in vivo*, particularly the intestinal absorption and transport of the drug. However, the T_{max}, $T_{1/2}$, and MRT were similar in comparison with animals given pure paeonol alone, at a comparable dose of 70 mg/kg body weight. In addition, quick intestinal absorption and blood distribution of paeonol were observed from the mean plasma concentration–time profiles of both administrative protocols.

3.6. Metabolic profile of paeonol in rat plasma

Although five metabolites of paeonol, P1–P5, were identified in rat urine, only P2–P4 were found in rat plasma after oral administration of paeonol [6]. In the present study, as shown in Fig. 4, we confirmed the presence of paeonol and P5 in rat plasma detected by the positive ion mode of ESI-MS, while P2–P4 were detected under the negative ion mode. Fig. 4 shows the extracted ion chromatograms (positive mode) for P5 at *m/z* 153.0609 and for paeonol at *m/z* 167.0701 (C), as well as the extracted ion chromatograms (negative mode) for P2 at *m/z* 230.9914, P3 at *m/z* 261.0102, and P4 at *m/z* 245.0114 (B). P5 was first detected in plasma of rats treated with purified paeonol alone, in a relatively abundant amount, compared with that of paeonol. Among the five metabolites of paeonol, P2, P3 and P5 are most prevalent, and they appeared quickly in rat plasma, just 5 min after administration of paeonol with both protocols. Unfortunately,

Table 4
Pharmacokinetic parameters of paeonol in rat plasma after single oral administration of paeonol alone and paeonol contained in QFGJS

Parameters	Paeonol			QFGJS
	35 mg/kg	70 mg/kg	140 mg/kg	3.89 g/kg
C_{max} (μg/ml)	0.73 ± 0.07	2.02 ± 0.23	4.16 ± 0.50	8.61 ± 1.30**
T_{max} (min)	9.00 ± 2.45	5.00 ± 0.00	7.00 ± 2.00	5.00 ± 0.00
$T_{1/2\ E\ phase}$ (min)	34.25 ± 3.91	34.18 ± 1.77	50.13 ± 4.11	38.25 ± 2.99
$T_{1/2\ D/A\ phase}$ (min)	12.02 ± 1.73	12.74 ± 1.69	12.79 ± 1.81	11.66 ± 0.62
$T_{1/2\ A\ phase}$ (min)	2.60 ± 0.53	1.92 ± 0.44	2.51 ± 0.38	1.58 ± 0.21
MRT (min)	51.62 ± 8.86	36.65 ± 2.18	56.14 ± 3.13	37.43 ± 2.58
V_d (ml/kg)	47,170 ± 5984	49,628 ± 6810	48,329 ± 6424	18,034 ± 1498*
CL/F (ml/(min kg))	1016.8 ± 167.7	1003.4 ± 110.5	761.0 ± 70.6	342.2 ± 39.8***
AUC_{0-t} (μg min/ml)	40.66 ± 9.99	74.13 ± 8.68	188.50 ± 16.35	229.64 ± 27.38**
$AUC_{0-\infty}$ (μg min/ml)	40.72 ± 10.01	74.38 ± 8.77	189.22 ± 16.47	229.88 ± 27.46**

Data are expressed as the mean ± S.E.M. of 6–8 rats. C_{max} (μg/ml), maximum plasma concentration; T_{max} (min), time to reach maximum plasma concentration; $T_{1/2\ E\ phase}$ (min), half-time of elimination phase; $T_{1/2\ D/A\ phase}$ (min), half-time of distribution phase; MRT, mean residence time; V_d (ml/kg), volume of distribution; CL/F (ml/(min kg)), total clearance; AUC_{0-t} (μg min/ml), area under the concentration–time curve from zero up to a definite time; $AUC_{0-\infty}$ (μg min/ml), area under the concentration–time curve from zero up to a infinite time. *, **, ***: P values are significantly different from that of the paeonol alone (at dosage of 70 mg/kg) group by Student's t-test at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively. Paeonol contained in QFGJS at dosage of 3.89 g/kg is equivalent to 70 mg/kg pure paeonol to rats.

P1 was not detected under either positive or negative ion modes in ESI-MS, indicating that P1 may exist in trace amounts in rat plasma.

The pharmacokinetic time course of the paeonol metabolites, P2–P5, in rat plasma was clearly identified through the curves of peak area ratios versus time (Fig. 5). The relative content of each metabolite in rat plasma was calculated from the UV chromatogram obtained under the conditions of LC–MS analysis, and expressed as the peak area ratio (R) by comparing the peak area of each metabolite with that of internal standard magnolol. All four metabolites were detected in rat plasma 5 min after dosing of paeonol with both administrative protocols (Fig. 5), and a maximum concentration was detected approximately 20 min after dosing. However, significantly higher plasma concentrations of P2, P3 and P5 were observed in animals administered with QFGJS than those treated with the pure paeonol alone. The differences of the pharmacokinetic parameters of the four metabolites in plasma of rats treated with paeonol alone or via QFGJS, along with statistical significance analysis, are presented in Table 5. The C_{max} and AUC of each metabolite in animals treated with QFGJS were significantly increased, while other parameters, such as T_{max}, $T_{1/2}$, and MTR, maintained similar levels between the two groups. These observations indicated that paeonol could be absorbed more effectively into blood circulation when administered via QFGJS, compared to the delivery of the pure paeonol alone.

4. Discussion

Paeonol, a major bioactive compound of Chinese herb Moutan Cortex, has been attributed with significant anti-inflammatory and analgesic effects, and potential for development as a novel anti-inflammatory agent [1–3]. In clinics, Chinese medicine practitioners usually prescribe an herbal prescription containing the herb Moutan Cortex rather than the single compound to treat various illnesses. Thus, in this work, we not only determined the pharmacokinetic behavior of paeonol itself, but also investigated the possible influences of co-existing chemicals in the QFGJS herbal preparation on the pharmacokinetic behavior and metabolic profile of paeonol in rats. Two administrative protocols, oral administration of pure paeonol alone and of the QFGJS preparation, were utilized for this study.

An HPLC–DAD method was developed and validated for the determination of paeonol concentrations in plasma of the jugular-catheterized freely moving rats. The method showed good reproducibility, accuracy, precision and recovery in assays

Table 5
Pharmacokinetic parameters of the paeonol metabolites P2–P5 after oral administration of QFGJS (3.89 g/kg) or paeonol alone at comparable dosage (70 mg/kg) ($n = 6$)

Name	Parameters	Paeonol metabolites			
		P2	P3	P4	P5
Paeonol (70 mg/kg)	C_{max}	154.6 ± 10.0	86.2 ± 14.7	14.0 ± 1.7	103.1 ± 11.9
	$T_{1/2\ E\ phase}$ (min)	60.48 ± 4.67	29.21 ± 8.15	23.71 ± 0.11	64.07 ± 5.84
	AUC_{0-t}	14,455 ± 1202	6825 ± 1241	1195 ± 105	8485 ± 778
QFGJS (3.89 g/kg)	C_{max}	285.8 ± 17.6***	152.9 ± 5.6**	19.7 ± 1.9	201.0 ± 12.9***
	$T_{1/2\ E\ phase}$ (min)	60.03 ± 5.58	22.71 ± 1.97	22.86 ± 0.17	65.91 ± 5.95
	AUC_{0-t}	24,956 ± 1459**	12,302 ± 958**	2009 ± 186*	17,414 ± 1861**

Data are expressed as the mean ± S.E.M. of six rats. Abbreviations are the same as those shown in Table 4.

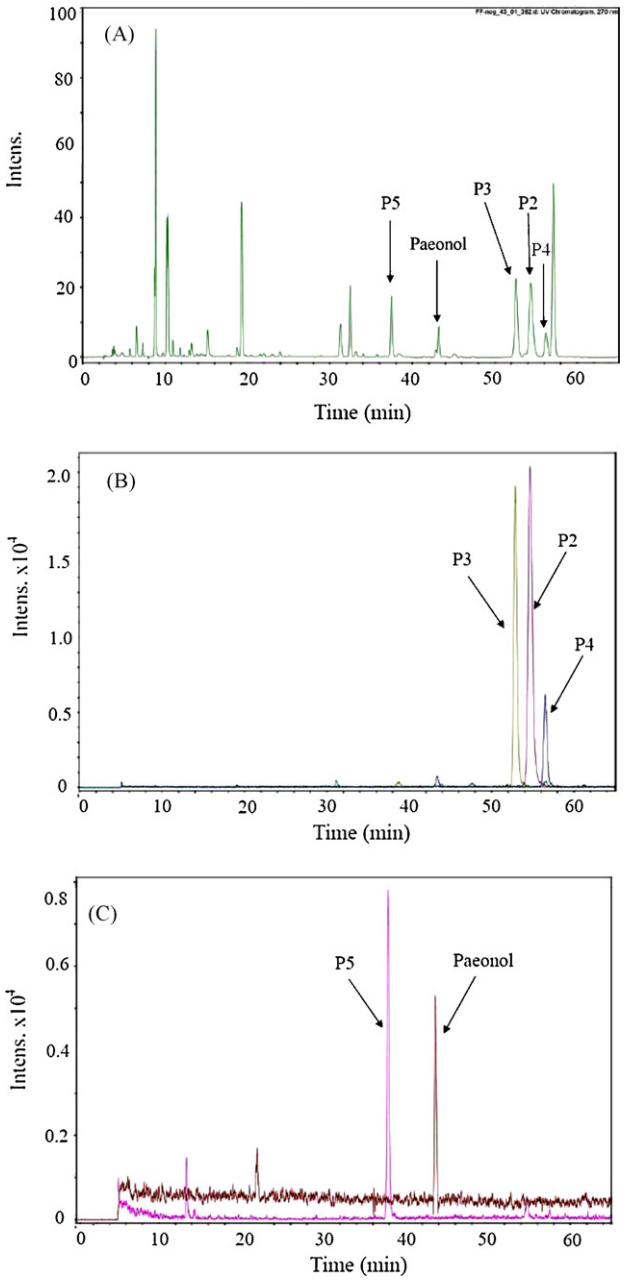

Fig. 4. Typical LC–UV and MS chromatograms for identification of paeonol and metabolites in plasma samples from a rat after 15 min of oral administration of QFGJS containing paeonol approximately equivalent to the dose of 70 mg/kg: (A) UV chromatogram with detection wavelength of 270 nm; (B) extract ion chromatograms under the positive ion mode (P2 at *m/z* 230.9914, P3 at *m/z* 261.0102, and P4 at *m/z* 245.0114); (C) extract ion chromatograms under the negative ion mode (P5 at *m/z* 153.0609 and paeonol at *m/z* 167.0701).

of paeonol in rat plasma. The paeonol contents in rat plasma after oral administration of the pure compound of paeonol and QFGJS at three comparable doses were successfully quantified with the modified HPLC method. The results showed the concentration–time curve and AUC of paeonol after oral administration alone, at doses of 35, 70 and 140 mg/kg body weight, to be in a dose-dependent manner. Furthermore, paeonol was observed in rat plasma as soon as 5 min after dosing, which is consistent with the previous report [15]. Although the mechanisms and sites for absorption and transport of paeonol in the gastrointestinal tract remain unclear, the rapid presence of paeonol and its conjugates suggest that absorption of paeonol may take place in the upper site of the gut.

Although the T_{max}, $T_{1/2}$, and MRT of paeonol were similar in both groups of animals, our results showed that the blood concentration and AUC of paeonol were significantly elevated in animals treated with QFGJS in comparison with animals treated with the pure paeonol alone at a comparable dose. These differences may result from the pharmacokinetic interaction between other co-existing chemicals in the QFGJS preparations, where the other components in QFGJS may function in improving the pharmacokinetic behavior of paeonol as by-players, or/and enhancer of the solubility and absorption of paeonol in the gastrointestinal tract.

Some literatures have suggested that the poor oral bioavailability of paeonol was due to the rapid and complete first-pass metabolism of the compound in blood [15]. In the current study, we determined the time course of paeonol metabolites in rat plasma following oral administration of the pure paeonol alone and QFGJS, using the LC-Q/TOF-MS analytical method, so as to further elucidate the metabolic profile of paeonol. This was the first time to employ this method with excellent high sensitivity for the analysis of the paeonol's metabolites *in vivo*. Four metabolites, P2–P5, were clearly identified in the plasma of the rats administrated with paeonol and QFGJS. The relative plasma concentration of these metabolites was found to be significantly higher in rats treated with QFGJS than those treated with pure paeonol at a comparable dose. This is in parallel with the results of the increase of blood concentration of paeonol. In addition, all four metabolites appeared as soon as 5 min after dosing and reached the maximum plasma concentrations approximately 20 min after oral administration of the drug, indicating that paeonol could quickly be metabolized into P2–P5 and circulate in blood, and/or tissues and organs. The detailed metabolic mechanisms warrant further investigation.

Together these results strongly suggest that the absorption and transport of paeonol in the gastrointestinal tract may be enhanced by other chemicals in the herbal preparation. Furthermore, the increased absorption of paeonol in plasma of rats orally treated with QFGJS might not be due to the inhibition of drug metabolism, but rather as a result from the enhancement of drug absorption in the gastrointestinal tract. Therefore, further examination of the effects of paeonol together with other chemicals in QFGJS on the intestinal P-glycoprotein-mediate efflux will be useful in elucidating the underlying mechanisms of the current results.

P5 was identified as a new metabolite of paeonol in rat plasma, while P1 was not detected in plasma in our current study. With respect to the possible metabolic route of paeonol *in vivo*, loss of the methoxyl group at the C-4 position, and hydroxylation and subsequently sulfating at C-5 position were identified as two major metabolic pathways of paeonol.

In conclusion, the current studies not only demonstrated the pharmacokinetic behavior and the metabolic profile of paeonol in jugular-catheterized moving rats, but also provided the first finding that the pharmacokinetic behavior and metabolism of

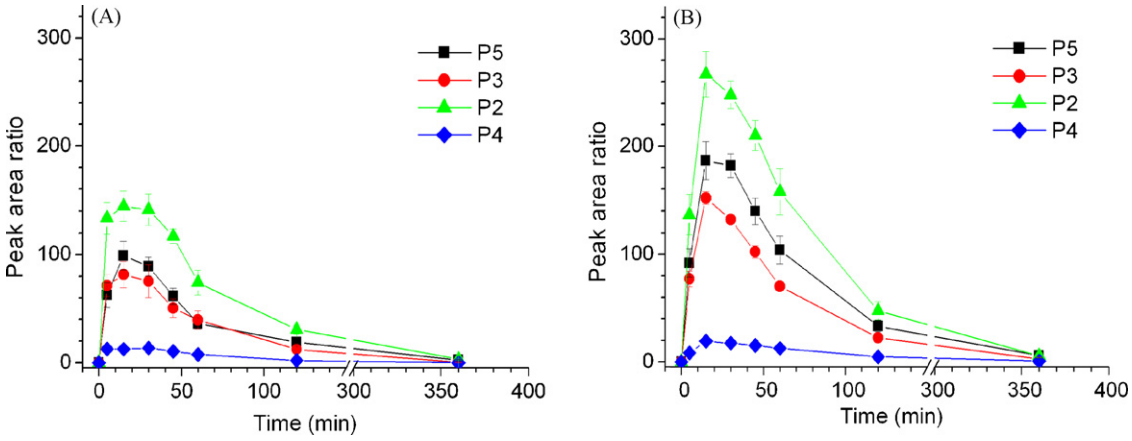

Fig. 5. Mean plasma concentration–time courses of the paeonol metabolites, P2–P5, in rat plasma after oral administration of the pure compound of paeonol alone (A) and QFGJS containing paeonol (B), in which the doses of paeonol administrated in two groups of animals were the same. Each point and bar represents the mean ± S.E.M. ($n = 6$).

paeonol could be altered by oral administration of QFGJS containing a number of small organic compounds. In addition, these results suggest that the influence of interactions between co-existing components in herbal preparations on the pharmacokinetic behavior and metabolic profile must be stressed in future investigations as well as the interaction of western drug and herb.

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