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Pharmacokinetics of paeoniflorin microemulsion after repeated dosing in rats with adjuvant arthritis

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An investigation was designed and conducted to detect pharmacokinetic differences between paeoniflorin (Pae) microemulsion and Pae saline. Pae microemulsion (25, 50,100 mg·kg^{-1}) was administered to three groups of rats with adjuvant arthritis (AA) while Pae (25, 50,100 mg·kg^{-1}) was given to another three groups of rats both for ten days. A HPLC assay was developed to determine the plasma concentrations of Pae. The plasma concentrations of Pae groups (25, 50 mg·kg^{-1}) were undetectable. Furthermore, compared with pharmacokinetic parameters of Pae group (100 mg·kg^{-1}), maximum concentration (C_{max}), the area under the plasma concentration–time curve ($AUC_{0\text{-}t}$), and mean retention time $MRT_{(0\text{-}\infty)}$(h) of Pae microemulsion (100 mg·kg^{-1}) increased apparently, while volume of distribution (Vd) and clearance rate (CL/F) decreased. These results indicate that a microemulsion significantly improves the absorption of Pae in AA rats.

1. Introduction

Rheumatoid arthritis (RA) is a chronic and disabling disease affecting approximately 1% of the population worldwide. The main clinical symptoms of RA are articular destruction and functional disability. So far, the precise etiology of RA is not known, but genetic and environmental influences clearly participate. Besides, the disease has attracted more and more attention as a result of significant morbidity and increased mortality (Firestein 2003).

Total glucosides of paeony (TGP), is an active compound extracted from the roots of *Paeonia lactiflora Pall* which has been recognized as a valuable herb used in the treatment of RA in traditional Chinese medicine (Zheng and Wei 2005). Previous research has demonstrated that TGP inhibits the chronic inflammation in adjuvant arthritis (AA) rats (Zheng and Wei 2005). As a disease-modifying drug, TGP has both anti-inflammatory and immune-regulatory effects on the treatment of RA (Zhang et al. 2008). Therefore, TGP was approved by the State Food and Drugs Administration in 1998. Paeoniflorin (Pae, Fig. 1), the main bioactive monoterpene glucoside in TGP (containing more than 90% Pae), has been proved effective in treating arthritis (Wu et al. 2007). In recent years, clinical practice of TGP has demonstrated its superiority in improving symptoms as well as fewer side effects (Min et al. 2005). However, TGP's slow effects seriously limit its application to RA patients (Liu et al. 2005). According to previous studies (Takeda et al. 1995, 1997), when administered orally, Pae was absorbed poorly in gastrointestinal tract, leading to a very low bioavailability (3–4%). Therefore, it is essential to design a novel formulation of Pae to improve its oral absorption and therapeutic effect.

Microemulsions are transparent or translucent preparations with low viscosity, good isotropic and thermodynamic stability. The advantages of microemulsions can be simply summarized as easy preparation and preservation, good solubilization for lipophilic and hydrophilic drugs, and favorable drug dispersion, all of which facilitate its application to pharmaceuticals (Zeng et al. 2010). Recently, numerous studies (Nornoo et al. 2009; Yin et al. 2009) of microemulsions were reported to have resolved the problem of poor absorption by oral administration, which may result from nano-particles, improvement of drug stability in gastrointestinal tract, and lymphatic absorption pathway (Wu et al. 2011, 2006; Lawrence and Rees 2000). As a result, Pae microemulsion has been prepared, characterized and optimized in our laboratory. We have previously confirmed that microemulsions improved the absorption of Pae in normal rats (data not shown), and the curative effect of Pae microemulsion on AA rats was superior to Pae (Lin et al. 2011). Furthermore, pharmacokinetic studies in the arthritic model, which can provide reference for clinical prescription, have scarcely been reported. Therefore, in the present study, we compared the pharmacokinetics parameters between Pae microemulsion and Pae (dissolved in physiological saline) in AA rats.

2. Investigations and results

2.1. Effects of Pae microemulsion and Pae on paw swelling of AA rats

As seen from Table 1, compared with normal group, chronic inflammation (paw swelling) was observed clearly in AA rats on day 17 after immunization ($P < 0.01$). Thus, AA model was established. Meanwhile, we also found that Pae microemulsion (25, 50, 100 mg·kg^{-1}) and Pae (50, 100 mg·kg^{-1}) inhibited paw swelling apparently after immunization. The inhibition rate of paw swelling by the Pae microemulsion (100 mg·kg^{-1}) was significantly higher than that of Pae ($P < 0.05$).

2.2. Pharmacokinetic study

Fig. 2 shows the HPLC chromatograms of blank plasma, blank plasma spiked with Pae and plasma sample from AA rats after

Table 1: Effects of Pae microemulsion and Pae saline on paw swelling of AA rats

Group	Dose ($mg \cdot kg^{-1}$)	Paw swelling (ml)		
		Day 17	Day 21	Day 24
Normal		0.079 ± 0.058	0.099 ± 0.059	0.121 ± 0.068
AA		$0.473 \pm 0.058^{\#\#}$	$0.587 \pm 0.050^{\#\#}$	$0.681 \pm 0.055^{\#\#}$
Pae saline	25	0.440 ± 0.077	0.503 ± 0.075*	0.584 ± 0.027**
	50	0.420 ± 0.079	0.475 ± 0.043**	0.506 ± 0.044**
	100	0.396 ± 0.041*	0.433 ± 0.065**	0.464 ± 0.038**
Pae microemulsion	25	0.418 ± 0.044	0.497 ± 0.048*	0.547 ± 0.062**
	50	0.402 ± 0.051	0.439 ± 0.039**	0.463 ± 0.036**
	100	$0.321 \pm 0.042^{**\Delta}$	$0.349 \pm 0.041^{**\Delta}$	$0.386 \pm 0.052^{**\Delta}$

Data represent the mean ± S.D. (n = 8). $^{\#\#}p < 0.01$ vs normal group, $^{*}p < 0.05$, $^{**}p < 0.01$ vs model group, $^{\Delta}p < 0.05$ vs Pae saline

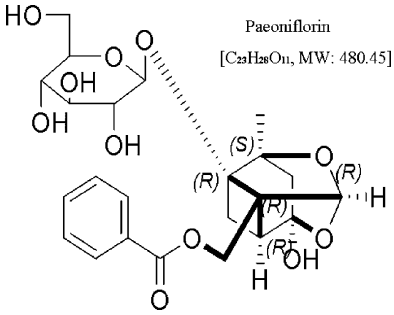

Fig. 1: Chemical structure of paeoniflorin

oral administration. The retention time of Pae was approximately 12 min. No interfering peaks were observed within the time frame in which Pae was detected.

The calibration curve of Pae was linear ($r^2 = 0.9996$) within the concentrations of 0.06–9.60 $\mu g \cdot mL^{-1}$. With the least-squares method, a regression equation of C ($ng \cdot mL^{-1}$) = 0.021X + 11.2 (C is for concentration of Pae in plasma; X is for the peak area of Pae) was obtained (Tables 2, 3). The recovery rates were higher than 98.10%. Intra-daily and inter-daily variations were lower than 6.83%. The Pae was stable in plasma under refrigeration. These results indicate that the method was suitable for present study.

The pharmacokinetic parameters of Pae microemulsion are listed in Table 2, and a two compartment open model was determined. There was no significant difference in $T_{1/2\alpha}$ under three different dosages, and likewise in $T_{1/2\beta}$. In addition, two linear equations of Pae microemulsion, including Y ($ng \cdot mL^{-1}$) = 15.699X + 94.947 (X: dosages, Y: C_{max}, r = 0.983) and Z ($ng \cdot h \cdot mL^{-1}$) = 63.212X + 5473.400 (X: dosages, Z: $AUC_{(0-\infty)}$, r = 0.998), were analyzed and then they revealed the process of first order kinetics in AA rats.

The plasma concentrations of Pae groups (25, 50 $mg \cdot kg^{-1}$) were not detected in the present study, which, on the other hand, has reflected the absorptive enhancement of microemulsion groups. As shown in Fig. 3, compared with those of Pae group (100 $mg \cdot kg^{-1}$), the concentrations of Pae in plasma can be obviously improved by microemulsion during 2.5 h to 24 h after oral administration. Moreover, in comparison with pharmacokinetic parameters of Pae saline (100 $mg \cdot kg^{-1}$) (Table 2), many parameters (C_{max}, $AUC_{(0-t)}$ and $MRT_{0-\infty}$) in group of Pae microemulsion (100 $mg \cdot kg^{-1}$) increased dramatically ($P < 0.01$), while CL/F and Vd decreased ($P < 0.01$).

3. Discussion

Recent studies have shown a low absorption (approximately 3-4% bioavailability) of Pae in rats, which limits the application of TGP in clinical settings (Liu et al. 2005). Microemulsion is considered to be a promising carrier for its superiority in enhancing absorption of both lipophilic and hydrophilic drugs. The most successful case was the wide application of ciclosporin microemulsion to clinic (Hirunpanich and Sato 2009). Moreover, our previous study confirmed that the absorption of Pae *in situ* intestines was improved by a microemulsion (Wang et al. 2009). Thus, in the present study, we have compared the pharmacokinetic process of Pae microemulsion with that of Pae in AA rats. As charted in Fig. 3 and listed in Table 3, Pae microemulsion and Pae saline significantly differ in both plasma concentrations and partial pharmacokinetic parameters. All these results indicate that the absorption of Pae can be improved by microemulsion.

Previous studies (Liu et al. 2006; He et al. 2007, 2003) concluded the causes of Pae's poor bioavailability as poor permeation, efflux via P-glycoprotein (p-gp), and hydrolytic degradation by esterase in the intestine. P-gp has been extensively studied for its inhibitive effect on absorption of drugs in the intestine, and p-gp's involvement in absorptive process of Pae has been demonstrated (Chan et al. 2006). Verapamil and quinidine (p-gp inhibitors) markedly enhanced the intestinal transportation and absorption of Pae (Chan et al. 2006). Thus, polysorbate-80, as one ingredient of the microemulsion in the present study, inhibited p-gp expression in the intestine (Nornoo et al. 2009; Wang et al. 2004; Shui et al. 2007), which may partly explain the absorption enhancement of Pae from the microemulsion. Furthermore, some other reasons may contribute to the absorption increase of Pae from the microemulsion. Firstly, partial Pae had a good stability in the gastrointestinal tract, as they were distributed in oleic acid (oil phase, interior phase). Secondly, as edible oil, oleic acid (one constituent of Pae microemulsion) was preferentially (Trevaskis et al. 2006a, b) assimilated by the lymphatic pathway in the intestine. However, the mechanism of absorption enhancement remains to be further studied.

4. Experimental

4.1. Animals

The present study was approved by the Ethical Committee on Animal Research at the Institute of Clinical Pharmacology, Anhui Medical University (approval number 038616, September 17, 2008). Sprague–Dawley (SD) rats (male, 160 ± 20 g, Grade II, Certificate No. 006) were purchased from the Animal Department of Anhui Medical University (Hefei, Anhui Province, China). All rats were housed under standard laboratory conditions at the temperature of 24 ± 1 °C, and given tap water and commercially available food *ad libitum*. The lighting duration in the breeding room was 12 h (7:00 a.m. to 7:00 p.m.).

4.2. Plant material

The roots of *Paeonia lactiflora* were collected from plants grown under controlled conditions in Bozhou (latitude 33°47'59.64 north and longitude 115°50'15.90 east), China. A voucher specimen was deposited at the Herbar-

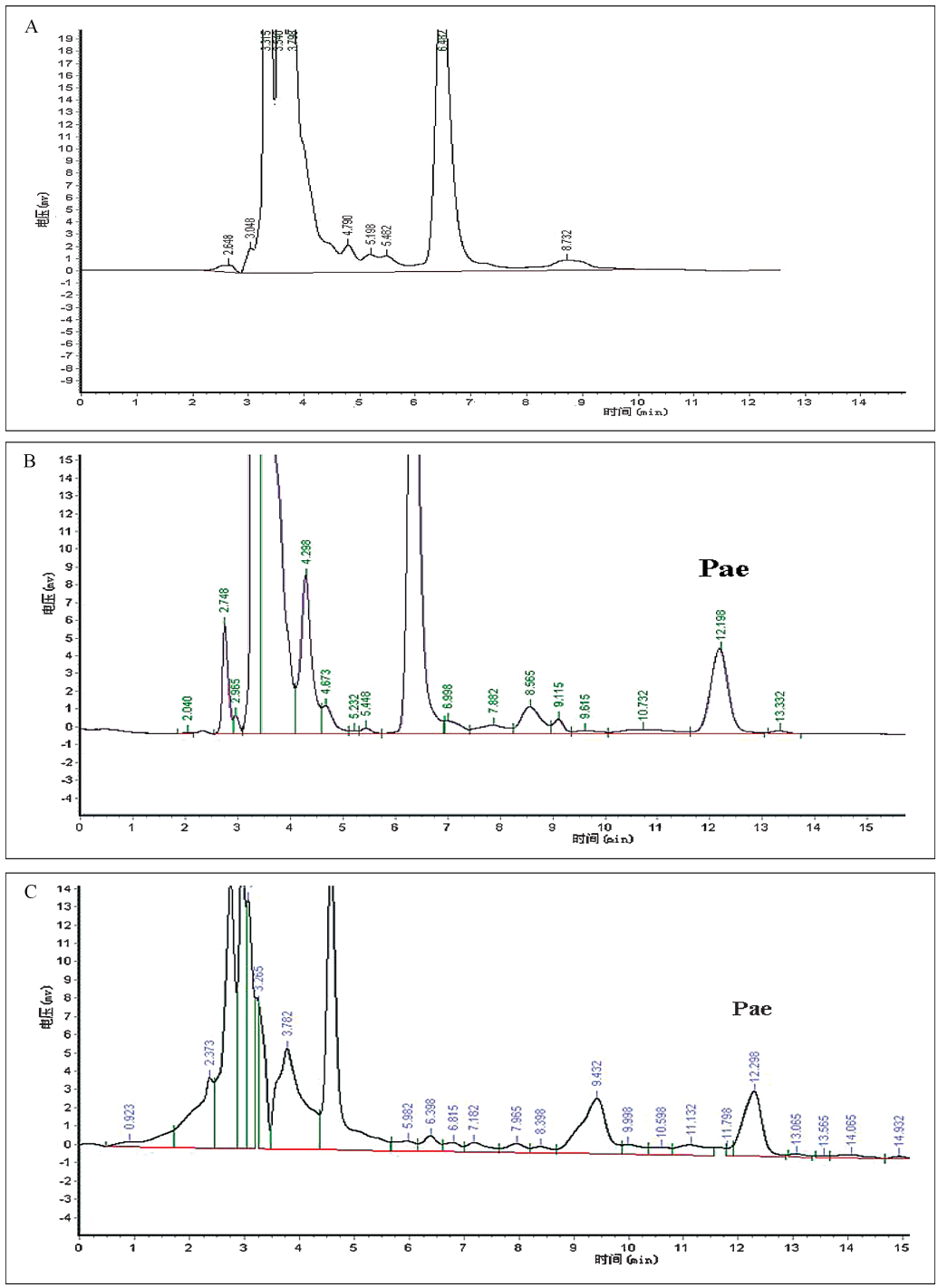

Fig. 2: HPLC chromatograms of Pae in rat plasma. **A**: blank plasma obtained from AA rats; **B**: blank plasma spiked with Pae; **C**: plasma sample obtained from AA rats

ium of Anhui Medical University (No.08274). The Voucher specimen was identified by Professor Lumin Pan in the Department of Materia Madica, School of Pharmacy, Anhui College of Traditional Chinese Medicine. Pae [$C_{23}H_{28}O_{11}$, MW: 480.45] was extracted from the roots of *Paeonia lactiflora* and purified with methods of solvent extraction and column chromatography in the Chemistry Lab of the Institute of Clinical Pharmacology of Anhui Medical University (Hefei, Anhui Province, China). The dried and powdered roots of *Paeonia lactiflora* were extracted with 70% ethanol under reflux. The concentrated extract was dissolved in water and tandem passed through a macroporous resin column. The column was washed with water first until the Molish reaction stopped, and was then repeated with 40% ethanol. Concentration of the 40% eluate under reduced pressure gave the TGP. The yellow powder was subjected to silica gel column chromatograph (column dimensions: 3 cm × 1.2 m; silica gel diameter: 74 μm; filling heights: 700 mm) and then eluated with ethyl acetate/methanol (20/1). Pae was yielded after the concentration of the collected eluate (Liu et al. 2005a). The purity of Pae (white crystal powder) was above 95% determined by HPLC assay (LC-10AD, Shimadzu Co., Japan).

4.3. Drugs and reagents

The following reagents were obtained commercially: methanol and acetonitrile (chromatography grade, Merck & Co., Darmstadt, Germany), Bacillus Calmette Guerin (BCG, Shanghai Biochemical Factory, Shanghai, China), ethyl acetate, oleic acid and polysorbate-80 (analytical reagent, Sinopharm Chemical Reagent Co, Ltd. Beijing, China), polyethylene glycol 400 (analytical reagent, Yasheng Chemical Co, Ltd, Wuxi, China). The reference standards of Pae (>98% purity) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Pae (>95% purity) was provided by Chemistry Lab of Institute of Clinical Pharmacology of Anhui Medical University (Hefei, Anhui Province, China). Pae saline and Pae microemulsion were obtained by dissolving Pae in physiological saline and microemulsion respectively in the same volume.

4.4. Preparation of paeoniflorin microemulsion

Microemulsion consisted of distilled water (51%, weight ratios), oleic acid (15%, weight ratios), polysorbate-80 (17%, weight ratios) and polyethy-

Table 2: Pharmacokinetic parameters of Pae saline and Pae microemulsion in AA rats

Parameters	Pae saline	Pae microemulsion		
	100 mg·kg^{-1}	25 mg·kg^{-1}	50 mg·kg^{-1}	100 mg·kg^{-1}
Vd (L·kg^{-1})	102.64 ± 58.41	25.71 ± 5.79	58.59 ± 25.32	51.66 ± 29.63**
$T_{1/2\alpha}$ (h)	1.31 ± 0.98	1.36 ± 0.87	1.58 ± 0.45	1.48 ± 0.47
$T_{1/2\beta}$ (h)	9.22 ± 4.13	12.01 ± 3.14	11.32 ± 2.69	11.55 ± 3.67
CL/F (L·h^{-1}·kg^{-1})	16.16 ± 0.61	3.62 ± 0.75	6.26 ± 1.50	8.55 ± 0.84**
T_{max} (h)	2.60 ± 0.65	2.50 ± 0.00	2.70 ± 0.27	3.20 ± 0.45
C_{max} (μg· L^{-1})	732.55 ± 172.19	572.12 ± 33.72	752.83 ± 83.14	1707.17 ± 337.11**
$AUC_{(0\text{-}t)}$ (μg·h·L^{-1})	5389.39 ± 264.89	5479.47 ± 883.22	6590.47 ± 1524.69	9544.07 ± 984.89**
$AUC_{(0\text{-}\infty)}$ (μg·h· L^{-1})	6194.80 ± 233.86	7145.37 ± 1440.36	8496.50 ± 2689.05	11840.41 ± 1158.68**
$MRT_{(0\text{-}t)}$ (h)	7.72 ± 0.56	8.84 ± 0.36	8.83 ± 0.70	8.10 ± 0.80
$MRT_{(0\text{-}\infty)}$ (h)	10.89 ± 0.41	15.29 ± 2.50	14.67 ± 3.15	13.22 ± 2.13*

Data represent the mean ± S.D. (n = 5), *$p < 0.05$, **$p < 0.01$ vs Pae saline group

lene glycol 400 (17%, weight ratios). Microemulsion was made by adding distilled water dropwise to the mixture of oleic acid and surfactant, under magnetic stirring at 37 °C. The particle diameter of microemulsion was determined by BT-9300H Laser particle size analyzer (Malvern Instruments, UK). The average particle diameter of Pae microemulsion was 110 nm.

4.5. Induction of AA rats and drug administration

Freund's complete adjuvant (FCA) was prepared by suspending heat-killed BCG in liquid paraffin at 10 mg·mL^{-1}. The model of AA was induced by a single intradermal injection of 0.1 mL FCA into the left hind metatarsal footpad of rat on day 0 (Zheng and Wei 2005; Wu et al. 2007).

Paw swelling (Zheng and Wei 2005; Wu et al. 2007): Basic value for right hind paw volume was determined with YLS-7A toe volume measurement instrument (Shandong academy of medical sciences, Jinan, China) before immunization in day 0(d0). The right hind paw volume measuring was repeated on d 14, d 17, d 24 after immunization, and the paw swelling (Δml) was calculated by subtracting the paw volume at d0.

After the onset of arthritis, animals were divided into six groups randomly. Rats with AA were given intragastrically Pae microemulsion (25, 50, 100 mg kg^{-1}) and Pae (25, 50, 100 mg kg^{-1}) once per day from d14 to d24 after immunization. Normal and AA model rats were given an equal volume of blank microemulsion at the same time (Wu et al. 2007). Furthermore, the remaining AA and normal rats were given an equal volume of blank microemulsion at the same time.

4.6. Blood collection and preparation of plasma samples

On day 24, at different time points (0.5,1, 1.5, 2, 2.5, 3, 4, 5, 7, 9, 12 and 24 h) after the last administration, approximately 0.2 mL blood samples were collected in heparinized tubes by retro-orbital sinus puncture from rats anesthetized by diethyl ether. The blood samples were centrifuged (8000 rpm, 10 min and 4 °C), and the plasma was separated and kept frozen at − 20 °C. The plasma (0.1 mL) was mixed with ethyl acetate (0.7 mL) by ultrasonic vortexing for 2 min. Then the ethyl acetate layer was separated and evaporated to dryness by nitrogen gas, and the residue in the tube was dissolved in 50 μL methanol. After centrifugation for 15 min (12000 rpm, 4 °C), 30 μl of methanol saline was injected into the HPLC system for analysis. The procedures mentioned above were also applied to the determination of calibration curve, recovery and precision test in plasma.

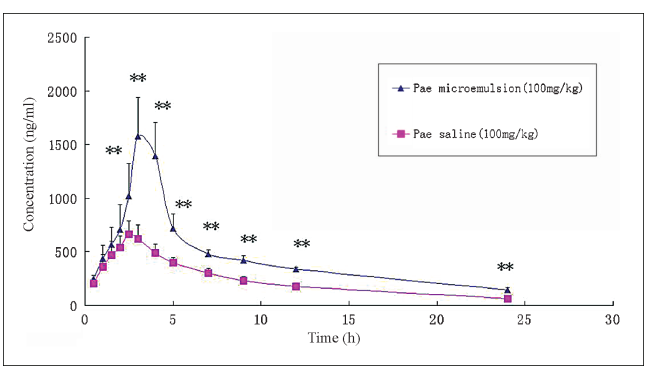

Fig. 3: Mean plasma concentration-time curve of Pae after oral administration Pae microemulsion and Pae in AA rats. Data represent the mean ± S.D. (n = 5). Triangles represent Pae microemulsion (100 mg·kg^{-1}), and squares represent Pae (100 mg·kg^{-1}). **$p < 0.01$ vs Pae group

4.7. High performance liquid chromatography analysis

HPLC included a pump (LC-10ATVP HPLC, Shimadzu, Japan), a UV detector (SPD-10AVP Spectrometer, Shimadzu, Japan), a Hypersil C_{18} column (4.6 mm × 250 mm, 5 μm, Elite analytical instrument Co., Ltd, Dalian, China) and a N2000 chromatographic work station (Intelligent Information Engineering of Zhejiang University, Hangzhou, China). Acetonitrile-distilled water- glacial acetic acid (18:82:0.4, V/V/V) was used as the mobile phase. The flow rate was set at 0.7 mL·min^{-1}. Detection was performed at a wavelength of 230 nm under a constant temperature of 30 °C.

4.8. Calibration curve, recovery, precision and stability tests

A calibration curve was constructed based on the HPLC analysis of blank rat plasma spiked with various concentrations (0.06, 0.12, 0.24, 0.48, 0.96, 1.92, 4.80, 9.60 μg·mL^{-1}) of Pae. Thus, the concentration of Pae in plasma was calculated from the peak area using the linear regression equation obtained from the calibration curve.

Three concentrations (0.24, 0.48, 0.96 μg·ml^{-1}) of Pae in blank plasma were prepared for recovery and precision. The peak area was recorded and compared with that of standard in methanol to provide recovery values. Intra-day variance was determined by assaying each sample for quintic on the same day, and inter-day variance was also assayed over five consecutive days.

The plasma samples of stability test were stored at −20 °C. The samples, which contained different concentrations of Pae (0.24, 0.48, 0.96 μg·ml^{-1}), were assayed at day0, day7 and day30. The changes in peak area of Pae were calculated.

4.9. Pharmacokinetic parameters and statistical analysis

The concentration–time data was subsequently processed by the Drug and Statistics for Windows ver 2.0 (Chinese Pharmacology Society).The pharmacokinetic parameters of t1/2, MRT, AUC, CL, Vd, C_{max} and T_{max} were calculated based on moment methods.

Data were expressed as mean ± standard deviation (S.D.). Significant differences between means were evaluated by one-way ANOVA and Dunnett's test (SPSS Software Products, USA). A difference was considered significant at $P < 0.05$.

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