Center for Clinical Pharmacology[1], University of Pittsburgh, USA, West China School of Pharmacy[2], Sichuan University, Chengdu, China

Pharmacokinetics and excretion of chlorogenic acid in beagle dogs

Shilong Zhong[1], Jing Liu[2], Xia Ren[2], Jie Zhang[2], Sha Zhou[2], Xiao-Ping Xu[2]

Received January 7, 2008, accepted February 11, 2008

Xiao-Ping Xu, Associate Professor, West China School of Pharmacy Sichuan University Chengdu 610041, China
xu106@sina.com

Pharmazie 63: 520–524 (2008) *doi: 10.1691/ph.2008.8506*

In order to characterize the pharmacokinetics and excretion of chlorogenic acid (ChA) in beagle dogs, a gradient high performance liquid chromatographic method has been developed and validated for determining ChA concentration in dog serum, urine and feces. The pharmacokinetic profile of ChA in dog after an intravenous injection was best described by a two-compartment model. Terminal half-lifes were similar at dosages of 5, 20 and 50 mg/kg, ranging from 41–45 min, while AUC and C_{max} values of ChA were dose proportional. There are no differences in Vd and CL at three doses of ChA, ranging from 133–252 mL/kg and from 4.4–6.3 mL/min/kg respectively. Excretion studies showed that more than 67% ChA was excreted into urine and less than 3% ChA was eliminated into feces.

1. Introduction

Chlorogenic acid (ChA) (3-*O*-caffeoyl-D-quinic acid) is found widespread in fruits and vegetables, particularly abundant in coffee, and can be isolated from the leaves and fruit (Clifford et al. 2007; Monteiro et al. 2007). It has been shown to possess a wide spectrum of biological and pharmacological effects, including antiviral (Chiang et al. 2002), antioxidant (Bouayed et al. 2007), anti-inflammatory (dos Santos et al. 2006; Miceli et al. 2005), anti-carcinogenic activities (Belkaid et al. 2006; Lee and Zhu 2006) and inhibits hepatic glucose-6-phosphatase (Arion et al. 1997; Belkaid et al. 2006). It has relatively low toxicity and side effects (dos Santos et al. 2006).

In view of its potential application in medicine, it is worthwhile to characterize the disposition of ChA. Although the pharmacokinetics of ChA in small animals have been investigated in several studies (Gonthier et al. 2003; Li et al. 2006; Ren et al. 2007; Yang et al. 2004), little is known of the pharmacokinetics of ChA in large animals such as dogs. Much less is known about excretion in large animals. In the present study, we developed a precise, accurate and sensitive HPLC method for the determination of ChA in beagle dog serum and excreta and studied the pharmacokinetics and excretion of ChA after intravenously administered doses. We also determined the protein binding extent to dog plasma.

2. Investigations and results

2.1. Selectivity

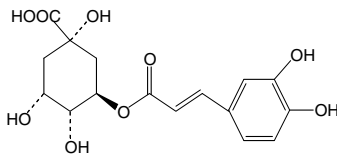
Typical chromatograms of blank serum, urine and feces, spiked serum, urine and feces and serum, urine and feces samples after administration of ChA injection are presented in Fig. 1. HPLC peaks of ChA and IS were identified on the basis of their retention times. No interfering endogenous compound peak was observed at the retention time of analytes. Under current chromatographic conditions, the retention times of ChA and IS1 were 19 and 23 min respectively in serum and those of ChA and IS2 were 19 and 10 min respectively in urine and feces.

2.2 Limit of quantitation (LOQ)

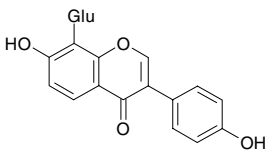
The LOQ of ChA in dog serum, feces and urine was calculated as the minimum concentration that could be quantified with no more than 15% relative standard deviation (RSD) and 15% absolute values of relative error (RE). LOQ of ChA in present study is 9.84 ng/mL.

2.3. Recovery and stability

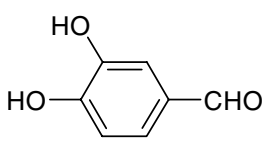
Recovery or extraction efficiency of ChA from dog serum, feces and urine were determined by comparison of peak areas of extracted QC samples and those of corresponding



Chlorogenic acid



Puerarin



Protocatechualdehyde

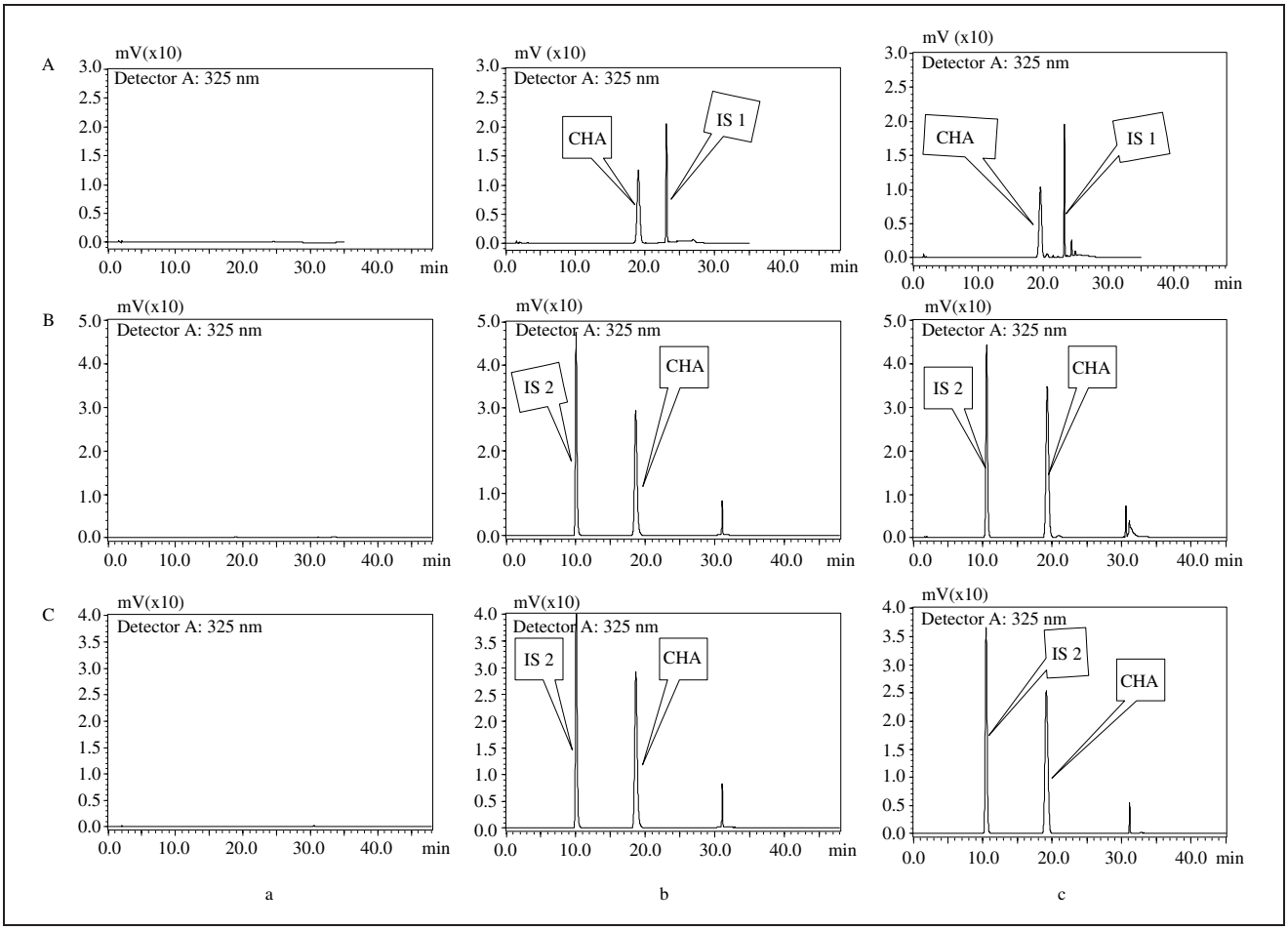


Fig. 1: Chromatograms of A) ChA and puerarin (IS1) in beagle dog serum; B) chlorogenic and protocatechualdehyde (IS2) in urine; C) chlorogenic and protocatechualdehyde (IS2) in feces. a) Blank samples; b) Samples spiked ChA with 25.6 mg/L IS1 for serum, 409.6 mg/L IS2for urine and feces; c) Serum sample 30 min, urine sample 100 min and feces sample 120 min after iv administration of ChA 20 mg/kg to a beagle dog, respectively

standard solutions (n = 6). The recoveries of ChA from serum, urine and feces ranged from 94.9 to 100.2% and were similar at all analyte concentrations. This indicated that the extraction efficiency for ChA using methanol was not concentration-dependent and was acceptable.

The stability of ChA was investigated under various storage and process conditions. ChA was stable (relative errors within ±15%) in dog serum, feces and urine at room temperature for 24 h, under −20 °C storage conditions for 15 days, and after four freezing and thawing cycles.

2.4. Linearity, precision and accuracy

Calibration standards exhibited excellent linearity over a range of 0.1–819.2 mg/L in serum and 0.0656–820.0 mg/L in urine and feces. Table 1 shows the standard curves, correlation coeffient values and linear rages of ChA in dog serum, feces and urine.

Intra- and inter-day precision and accuracy were based on analysis of six replicates of the low, medium and high QC samples. Intra-day precision ranged between 0.13–1.38% and the inter-day precision ranged between 0.52–0.99%. The mean intra-day error was between 0.10–4.71% and the mean inter-day error was between 0.10–3.85%.

2.5. Pharmacokinetics in dogs

Figure 2 illustrates serum ChA concentration-time courses after intravenous injection of ChA of 5, 20 and 50 mg/kg to dogs. Table 2 shows pharmacokinetic parameters calculated from the serum data by two-compartmental analysis. ANOVA showed that terminal half-lifes were similar at doses of 5, 20 and 50 mg/kg, ranging from 41–45 min, while both AUC_{0-180} and C_{max} values of ChA after intravenous injection were linearly dependent on doses of the drug ($r = 0.9856$ for AUC_{0-180}, $r = 1.000$ for C_{max}). The AUC_{0-180} were 733.96, 2681.37, and 9504.13 mg · min/L and C_{max} were 18.89, 68.14, 243.61 μg/mL for 5, 20 and 50 mg/kg respectively. There are no differences in Vd and

Table 1: Standard curves, correlation coeffients and linear rages of ChA in dog serum, feces and urine

	Standard curve	Range (mg/L)	Correlation coefficients	IS, concentration (mg/L)
Serum	$Y = 0.17X - 0.0001$	0.1–819.2	0.9999	Puerarin, 84
Urine	$Y = 0.0079X - 6 \times 10^{-6}$	0.0656–820.0	0.9997	Protocatechualdehyde, 402
Feces	$Y = 0.0079X + 8 \times 10^{-6}$	0.0656–820.0	0.9998	Protocatechualdehyde, 402

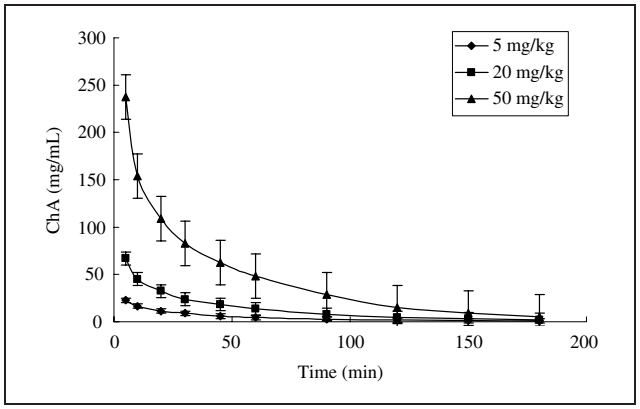


Fig. 2: Serum ChA concentration-time courses after intravenous injection of ChA of 5, 20 and 50 mg/kg to dogs. Each point represents mean ± SD (n = 6)

CL at three doses of ChA, ranging from 133–252 mL/kg and from 4.4–6.3 mL/min/kg respectively.

2.6. Chlorogenic acid excretion

The excretion of unchanged ChA in urine and feces after intravenous injection of ChA of 5, 20 and 50 mg/kg to dogs was summarized in Table 3. These data indicated that ChA was predominantly and rapidly excreted in urine and to a lesser extent in feces. The total recovery of unchanged ChA after intravenous injection of 5, 20 and 50 mg/kg to dogs was 67.0%, 94.6% and 81.5% respectively in urine and 2.85%, 0.83% and 1.39% at feces during 0–48 h after dosing. The extent of ChA excreted into urine is highest during 0–1 h and more than 90% unchanged ChA excreted in urine were excreted during this period.

3. Discussion

According to the proposal of the study, puerarin was used as internal standard to determine ChA concentration in serum. Because of interfering from the chromatographic peak of endogenous compound in urine and feces, we changed puerarin to protocatechualdehyde as internal standard to determine ChA concentration in urine and feces. Both protocatechualdehyde and puerarin share similar physicochemical properties of phenol to ChA. The gradient HPLC method based on the two internal standards showed acceptable precision, accuracy, linearity, stability and specificity. The method was further applied to the study of pharmacokinetics and excretion of ChA in beagle dogs.

The ChA concentration-time course in beagle dog serum showed bi-phase for three doses and the pharmacokinetic characteristics of ChA after an intravenous injection were best described by a two-compartment model, which is similar to that in rat (Wang et al. 2006). There were signs of apparent dose-dependent pharmacokinetics. AUC_{0-180} and C_{max} increased linearly with increasing intravenous administration doses to dog. ChA was eliminated quickly with $t_{1/2\beta}$ of 41–45 min, longer than that in rat with $t_{1/2\beta}$ of 28 min (Wang et al. 2006).

The present study showed that more than 67% ChA was excreted into urine and less than 3% ChA was eliminated into feces. Therefore, we expect that metabolised ChA will be less than 30% after an intravenous injection, but it need further studies to characherise the metabolism of

Table 2: Pharmacokinetic parameters of ChA after intravenous infusion of 5, 20 and 50 mg/kg doses to dogs

Parameters	5 mg/kg	20 mg/kg	50 mg/kg
A	20.598 ± 11.16	47.405 ± 16.40	244.72 ± 22.09
B	8.86 ± 3.28	32.03 ± 14.99	130.395 ± 26.75
$t_{1/2\alpha}$ (min)	4.942 ± 2.66	8.195 ± 6.27	4.431 ± 8.90
$t_{1/2\beta}$ (min)	45.437 ± 25.34	44.47 ± 15.32	41.235 ± 15.05
k_{10} (1/min)	0.034 ± 0.010	0.025 ± 0.008	0.034 ± 0.014
k_{12} (1/min)	0.069 ± 0.053	0.032 ± 0.041	0.074 ± 0.041
k_{21} (1/min)	0.053 ± 0.010	0.043 ± 0.033	0.065 ± 0.023
C_{max} (mg/L)*	18.89 ± 6.40	68.14 ± 15.24	243.61 ± 52.61
AUC_{0-180} (mg/L · min)*	733.96 ± 198.2	2681.37 ± 633.0	9504.13 ± 216.95
Vd (mL/kg)	170.0 ± 60.0	252.0 ± 74.2	133.2 ± 97.4
CL (L/min/kg)	6.1 ± 2.3	6.3 ± 2.5	4.4 ± 2.4

* Statistical differences were detected by ANOVA
Values are expressed as mean ± SD (n = 6)

Table 3: Excretion of ChA in dog urine and feces after intravenous injection of ChA to dogs at 5, 20 and 50 mg/kg (n = 6, mean ± SD)

Time (h)	5 mg/kg		20 mg/kg		50 mg/kg	
	Urine (mg)	Feces (mg)	Urine (mg)	Feces (mg)	Urine (mg)	Feces (mg)
0–1	31.40 ± 1.70	–	221.50 ± 69.73*	0.70 ± 0.86	364.8 ± 158.1*	1.02 ± 1.24
1–4	0.83 ± 0.10	0.23 ± 0.16	2.35 ± 1.76	0.44 ± 0.26	172.60 ± 259.60	1.24 ± 1.48
4–8	0.22 ± 0.16	0.22 ± 0.12	0.12 ± 0.10	0.82 ± 0.44	0.41 ± 0.10	0.31 ± 0.29
8–24	0.78 ± 0.68	0.49 ± 0.34	2.20 ± 3.63	0.73 ± 0.61	0.54 ± 0.40	4.05 ± 4.86
24–30	0.18 ± 0.17	0.19 ± 0.07	0.15 ± 0.12	0.20 ± 0.06	0.31 ± 0.40	0.71 ± 0.85
30–36	0.13 ± 0.10	0.33 ± 0.16	0.52 ± 0.55	0.35 ± 0.21	0.16 ± 0.13	1.00 ± 1.23
36–48	–	–	0.30 ± 0.23	0.23 ± 0.09	0.15 ± 0.24	0.44 ± 0.20
0–48 h (%)	67.0 ± 5.48	2.85 ± 1.40	94.6 ± 28.65	0.83 ± 0.33	81.5 ± 27.31	1.39 ± 0.56

* determined after dilution; – undetectable

ChA. In contrast to the excretion profile of ChA after intravenous injection, the recovery of chlorogenic acid in urine after oral administration was low, with recovery yields varying from 0.3 to 2.3% (Gonthier et al. 2003; Olthof et al. 2003). It has been reported that the gut absorption of ChA is poor and only a small part of intact ChA is absorbed (Dupas et al. 2006). The bioavailability of ChA largely depends on its metabolism by the gut microflora (Gonthier et al. 2003). Thus, it will be difficult to achieve effective concentration of ChA after oral administration. As shown in the present study, intravenous injection can reduce ChA metabolism and keep a majority of intact ChA in circulation. It is an acceptable way to take advantages of biological and pharmacological effects of ChA.

4. Experimental

4.1. Chemicals

Chlorogenic acid (MW = 354, purity = 99.56%) was provided by Jiuzhang Biochemical Engineering Science and Technology Development Co (Sichuan, China). The internal standard (IS) 1 puerarin (MW = 416.38, purity >98%) and IS2 protocatechualdehyde (MW = 138.12, purity > 98%) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Chemical structures of ChA, puerarin and protocatechualdehyde were depicted in Fig. 1. HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific Inc (Fair Lawn, NJ, USA). All other chemicals were of analytical grade and used without further purification. Distilled water, prepared from demineralized water, was used throughout the study.

4.2. Chromatography

The HPLC system is consisted of a Shimadzu LC-6A high pressure pump, a spd-10Avp UV detector (detection wave length: 323 nm), Sepu3000 ChemStation. A Gemini C18 ODS column (150 mm × 4.6 mm i.d, 5 μm particle size, phenomenex Inc.) was used throughout all the chromatographic experiments. The flow-rate was 1.0 mL/min and chromatography was performed at 40 °C. Mobile phases A and B were methanol and 2 mM phosphoric acid, respectively. Solvent gradients were used to determine ChA concentration in serum, urinary, and fecal samples. For serum samples, the elution gradient was from A/B 9/91 to 18.5/81.5 during 17 min, to 39/61 during 5 min and maintained for 2 min, then from 39/61 to 9/91 within 1 min and maintained for 10 min. For urinary and fecal samples, the elution gradient was from A/B 8/92 to 15/85 during 10 min, to 19/81 during 16 min, to 40/60 during 2 min and maintained for 4 min, then from 40/60 to 8/92 within 2 min and maintained for 8 min.

4.3. Standard solutions and quality control samples

A stock solution of standard ChA was prepared in MeOH−0.2% H_3PO_4 (20 : 80; v/v) to give a final concentration of 8.19 mg/mL for serum measurements and in MeOH−0.2% H_3PO_4 (35 : 65; v/v) to give a final concentration of 8.20 mg/mL for urine and feces measurements. The solution was then serially diluted with MeOH−0.2% H_3PO_4 to obtain standad working solutions over a concentration range of 0.1–819.2 mg/L for serum measurements and 0.0656–820 mg/L for urine and feces measurements. Quality-control (QC) solution (0.4, 25.6, 409.6 mg/L) for serum measurements, and (0.4, 6.56, 164 mg/L) for urine and feces measurements were diluted in the same manner as the standard calibration. The solution of puerarin (IS1 for serum measurements, 84 mg/L) was prepared in methanol, while the solution of protocatechualdehyde (IS2 for urine and feces measurements, 402 mg/L) was prepared in MeOH−0.2% H_3PO_4 (35 : 65; v/v). All solutions were stored at 4 °C and brought to room temperature before use.

4.4. Sample preparation

Serum: 0.1 mL of the standard solutions were added to 0.1 mL of drug-free serum to prepare 0.1, 0.4, 1.6, 6.4, 25.6, 51.2, 102.4, 204.8, 409.6, 819.2 mg/L calibration solutions of ChA. Low, medium and high QC samples were similarly prepared at concentrations of 0.4, 25.6 and 409.6 mg/L in serum respectively. To 0.1 mL of serum samples, 0.1 mL of MeOH−0.2%H_3PO_4 (20 : 80; v/v) was added. Serum samples, calibration or QC solutions were then mixed with 0.1 mL of the IS1 solution (puerarin 84 mg/L). After 0.1 mL methanol being added into each tube, the tube was then vortex-mixed for 3 min on a vortexer and centrifuged at 12 000 × g for 10 min, and a volume of 20 μL of the supernatant was injected into the HPLC system.

Urine: 0.1 mL of the standard solutions were added to 0.1 mL of drug-free urine to prepare 0.0656, 0.2624, 1.312, 6.56, 32.8, 82.0, 164.0, 328.0, 820.0 mg/L calibration solutions of ChA. Low, medium and high QC samples were similarly prepared at concentrations of 0.4, 6.56 and 164.0 mg/L in urine, respectively. To 0.1 mL of urine samples, 0.1 mL of MeOH−0.2%H_3PO_4 (35 : 65; v/v) was added. Urine samples, calibration or QC solutions were then mixed with 0.1 mL of the IS2 solution (protocatechualdehyde 402 mg/L).The tube was then vortex-mixed for 3 min on a vortexer and centrifuged at 12 000 × g for 10 min, and the supernatant was filtered through 0.45 μm filter and injected into the HPLC system.

Feces: To prepare feces solution, 1 mL methanol was added to 0.02 g homogenised feces and mixed for 3 min. Standard solutions (0.1 mL) were added to 0.1 mL of drug-free feces solution to prepare 0.0656, 0.2624, 1.312, 6.56, 32.8, 82.0, 164.0, 328.0, 820.0 mg/L calibration solutions of ChA. Low, medium and high QC samples were similarly prepared at concentrations of 0.4, 6.56 and 164.0 mg/L in feces, respectively. To 0.1 mL of feces samples, 0.1 mL of MeOH−0.2%H_3PO_4 (35 : 65; v/v) was added. Feces samples, calibration or QC solutions were then mixed with 0.1 mL of the IS2 solution (protocatechualdehyde 402 mg/L). The tube was then vortex-mixed for 3 min on a vortexer and centrifuged at 12 000 × g for 10 min, and the supernatant was filtered through 0.45 um filter and injected into the HPLC system.

4.5. Animals

Six purebred beagle dogs (three males and three females, aged 8 ∼ 2 years, weighing 10 ± 0.4 kg) were purchased from the Experimental Animal Center of Sichuan Academy of Medical Sciences (Chengdu, China). The dogs were housed under 12 h light/dark cycles, with a room temperature of 20 ± 1 °C, relative humidity of 55 ± 15% and with MIL Morini diet and tap water *ad libitum*.

4.6. Biological specimen collection

Dosing dogs were fasted for 12 h before study and fed 48 h after the dose. All procedures were in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of People's Republic of China. Six dogs received an intravenous bolus injection of 5, 20 and 50 mg/kg of ChA every one week. Blood samples (5 mL) were drawn from the right front leg vein before and at 5, 10, 20, 30, 45, 60, 90, 120, 150 and 180 min after drug administration began. After stored at 4 °C for 2 h, blood was processed for serum by centrifugation at 3000 × g for 15 min. Pooled urine and feces from each dog was collected in metabolism cages before and at 1, 4, 8, 24, 30, 36, 48 h after the administration. All biological samples were frozen and maintained at −20 °C until analysis.

4.7. Pharmacokinetic analysis

Pharmacokinetic parameters were estimated using Drug and Statistics version 2.0 program (Anhui Provincial Center for Drug Clinical Evaluation, China) with a two-compartment model and a weighing function of $1/C^2$ for data fitting and parameter estimation. All data were expressed as mean ±SD. Statistical analysis was performed using statistical program SPSS (SPSS, Chicago, IL). To evaluate dose proportionality, the relationships of AUC_{0-180} and C_{max} with doses were analyzed by linear regression. Statistical differences of the pharmacokinetic parameters were determined by analysis of variance (ANOVA).

Acknowledgements: Torch High Technology Industrial Development Center of The Ministry of Science and Technology of the People Republic of China (Project Ref. 2005EB021419) supported this work.

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