SHORT COMMUNICATION

High Resolution UPLC-MS/MS Method for Simultaneous Separation and Determination of six Flavonoids from *Semen Cuscutae* Extract in Rat Plasma: Application to Comparative Pharmacokinetic Studies in Normal and Kidney-deficient Rats

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Abstract: *Semen Cuscutae*, which mainly consisted of flavonoids, is a traditional Chinese herbal medicine and used for nourishing the liver and kidneys. The aim of this study was to develop a sensitive and selective UPLC-MS/MS method for simultaneous separation and determination of six main active renoprotective components of Hyperoside, Astragalin, Isoquercitrin, Quercitrin, Quercetin, and Kaempferol from *Semen Cuscutae* in rat plasma, and to reveal the pharmacokinetic differences between normal and kidney deficient rats. The validated method has been successfully applied to comparing pharmacokinetic profiles of the six analytes in rat plasma. The results indicated that there was significant difference in pharmacokinetic parameters of the six analytes between two groups, while absorptions in kidney deficient group were significantly lower than those in normal group. This study would be helpful for evaluating the *Semen Cuscutae* as renoprotective drug candidates for pre-clinical and clinical research.

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Supplementary material

3. Experimental

3.1. Materials and reagents

The reference standards (purity > 98%) of hyperoside, astragalin, isoquercitrin, quercitrin, quercetin, kaempferol and baicalin (internal standard) were purchased from National Institute for Food and Drug Control (Beijing, China). *Semen Cuscutae* seed was purchased from Tianyitang TCM store (Shenyang, China). Hydrocortisone injection was purchased from Jinyao (Company Inc, Tianjin, China). Methanol and formic acid of HPLC grade were purchased from Sigma (Company Inc, USA). Distilled water prepared with Wahaha demineralized water was employed throughout the experiment.

3.2. Animals

12 adult female Wistar rats (age: 8-week old, weight: 200-300 g) used for this study were obtained from the Vital River Laboratories, Beijing, China. The animal study was carried out in accordance with the Guideline for Animal Experimentation of Shenyang Medical College and the protocol was approved by the Animal Ethics Committee of the institution.

Kidney deficient model rats were induced by hydrocortisone injection method (Gou et al. 2009), Kidney deficient group rats were intramuscularly injected with hydrocortisone injection 25 $\mu g \cdot kg^{-1}$ for 15 days. Rats presenting fear of cold, loose stools, spirit wilting, reduced independent activity and weight loss were proved as the success of the kidney deficient model.

3.3. Instruments and UPLC-MS/MS conditions

The UPLC-MS/MS system was performed on an ACQUITY UPLC system (Waters,

Milford, MA, USA) coupled with a Micromass Quattro Micro API mass pectrometer (Waters). Chromatographic separation was achieved using an ACQUITY UPLC BEH C_{18} column (50 mm × 2.1 mm, 1.7 μm; Waters) at 30 °C. The mobile phase consisted of methanol (solvent A) and aqueous 0.1% formic acid (solvent B), which was delivered at a flow rate of 0.3 mL·min^{-1}. The linear gradient elution program was decreased from 55 % B to 20 % B in 0.00-6.00 min, from 20% B to 55% B in 6.00-6.01 min, held at 55 % B for 1.5 min.

An ESI source interface operated in negative ionization mode in the multiple reaction monitoring mode (MRM) was used in this study. The capillary voltage was 3.00 kV. The flow rate of desolvation gas was 700 L/h. The source temperature and desolvation temperature were 120 and 400 °C, respectively. Data acquisition was acquired and processed using MassLynx 4.1 software (Waters Corp., Milford, MA, USA).

3.4. Preparation of Semen Cuscutae Decoction

The *Semen Cuscutae* seeds were pulverized to fine powder. And then powders were extracted three times by refluxing in water (1:10 w/v) for 2 h. The extracted solutions were concentrated under reduced pressure to 0.5 g·mL^{-1}.The decoction was stored in the refrigerator at 4 °C.

3.5. Preparation of Standard solution and quality control samples

The standard stock solution of the six analytes were prepared in methanol at the concentration of 10 μg·mL^{-1}. The stock solutions of the six analytes were further diluted with methanol-water (45:55, v/v) to obtain standard working solutions.

Calibration standard solutions were prepared by mixed these working solutions into blank rat plasma to set at concentrations of 1.0, 4.0, 20, 100, 400, and 800 ng·mL^{-1}.

Quality control samples were prepared separately in the same fashion. Internal standard working solution (200 ng·mL^{-1}) was prepared. All calibration standards and QC samples were stored at 4°C until analysis.

3.6. Sample preparation

100 μL plasma samples were spiked with 10 μL IS solution and 10 μL methanol-water (45:55, v/v). After vortexing for 30 s, the mixture was extracted with 1 mL ether. The analytes and IS were extracted from plasma by vortexing for 5 min and shaking for 5 min. Then the samples were centrifuged at 4000 × g for 5 min. The organic layer was quantitatively transferred to a 5 mL centrifuge tube and evaporated to dryness at 35 °C under a slight stream of nitrogen. Then, the dried extract was reconstituted in 100 μL methanol-water (45:55, v/v), and vortex-mixed for 1min,and then centrifuged at 12000 g for 3 min. Followed by injection of 5 μL aliquot into UPLC-MS/MS for analysis.

3.7. Method validation

The method was validated in accordance with US FDA guidelines (Food and Drug Administration 2004). The specificity was evaluated by comparing chromatograms of blank plasma from six different rats, blank plasma spiked with six analytes and IS, and plasma samples obtained after oral administration of *Semen Cuscutae* decoction.

The linearity of the assay was assessed by analyzing the calibration curves using least-squares linear regression of the peak area ratios of the analytes to the IS versus the nominal concentration of the calibration standard with a weighed factor ($1/C^2$). The lower limit of quantification (LLOQ) was defined as the lowest concentration on the calibration curve with an acceptable accuracy within ± 20% and the precision below 20%.

The accuracy and precision of intra-day and inter-day determinations were assessed

by analyzing QC samples at low, medium and high concentration in six replicates on the same day and on three consecutive validation days.

The recovery were determined at three QC levels with six replicates by comparing the peak areas from extracted samples with those in post-extracted samples spiked with the six analytes. The recovery of IS was at one concentration in the same way.

The matrix effect was evaluated by comparing the peak response of blank biosample extracts spiked with analytes with that of pure standard solution containing equivalent amounts of the analytes. The comparing two peak area ratios were used to evaluate the matrix effect.

Stability studies were investigated at three QC levels in different storage conditions: 8 h after prepared at 4 °C, at room temperature for 24 h, after three freeze-thaw cycles, and at -20 °C for at least 30 d.

3.8. Pharmacokinetic study application

12 male rats were divided randomly into two groups with 6 rats in each. The animals were fasted for 12 h with free access to water prior to the oral administration of *Semen Cuscutae decoction* with a dose of 7 $g\cdot kg^{-1}$ (equivalent to 8.8 $mg\cdot kg^{-1}$ of Hyperoside, 9.9 $mg\cdot kg^{-1}$ of Astragalin, 12.8 $mg\cdot kg^{-1}$ of Isoquercitrin, 10.2 $mg\cdot kg^{-1}$ of Quercitrin, 18.1 $mg\cdot kg^{-1}$ of Quercetin, and 7.9 $mg\cdot kg^{-1}$ of Kaempferol). Blood samples (about 250 μL) were collected in heparinized tubes via the postorbital venous plexus veins from each rat before administration and 0.08, 0.17, 0.33, 0.5, 1, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, 10, and 12 h after administration, and were immediately centrifuged and stored at -80 °C until analysis.

The pharmacokinetic parameters of the six analytes were calculated by the

non-compartmental analysis of plasma concentration versus time data using the DAS 2.2 software package (Chinese Pharmacological Society) to calculate AUC, C_{max}, $T_{1/2}$, T_{max}. Statistical analysis between two groups was possessed by SPSS 18.0 (Statistical Package for the Social Science) using independent samples T-test, while $p < 0.05$ was considered statistically significant for the test. All data were presented as means ± SD.

References

Food and Drug Administration. Guidance for Industry: Bioanalytical Method Validation. Available online: https://www.fda.gov/downloads/Drugs/Guidance/ucm070107.pdf (accessed on 18 June 2004).

Gou XJ, Han BX, Wang CT. 2009. The method study on the kidney deficiency syndrome. Jilin J TCM. 29: 814-815.

Table S1 Optimized multiple-reaction-monitoring (MRM) parameters for Hyperoside, Astragalin, Isoquercitrin, Quercitrin, Quercetin, Kaempferol, and baicalin (IS).

Components	m/z of precursor ion (Da)	m/z of prduct ion (Da)	Cone voitage (V)	Collision voltage (eV)	Acquisition time (min)
Hyperoside	447.1	283.9	-30	-45	1.96
Astragalin	463.0	300.0	-55	-40	2.12
Isoquercitrin	463.1	300.0	-40	-40	2.27
Quercitrin	447.0	300.0	-35	-40	3.05
Quercetin	301.0	151.0	-110	-70	3.60
Kaempferol	285.0	93.0	-45	-40	5.51
Baicalin (IS)	445.2	269.1	-70	-35	2.80

Table S2 Linear ranges, regression equations and correlation coefficients of the six components in rat plasma.

Components	Linear range ($ng \cdot mL^{-1}$)	Regression equation ($\times 10^{-3}$)	Correlation coefficient (*r*)
Hyperoside	1.0-1000	$y = 2.5\ x + 1.5$	0.9956
Astragalin	1.0-1000	$y = 1.5\ x + 2.1$	0.9978
Isoquercitrin	1.0-1000	$y = 2.1\ x + 2.0$	0.9932
Quercitrin	1.0-1000	$y = 4.1\ x + 2.2$	0.9919
Quercetin	1.0-1000	$y = 2.7\ x + 4.2$	0.9927
Kaempferol	1.0-1000	$y = 1.9\ x + 1.2$	0.9955

Table S3 Summary of accuracy, precision, recovery and matrix effect of the six components in rat plasma ($n = 6$).

Components	Concentration ($ng \cdot mL^{-1}$)	Recovery (%, mean ± SD)	R.S.D. (%)	Matrix effect (%,mean ± SD)	R.S.D. (%)
	4.0	91.2 ± 1.9	8.2	93.2 ± 2.4	8.5
Hyperoside	100	96.4 ± 4.8	7.2	96.2 ± 5.3	5.4
	640	88.5 ± 5.0	5.4	94.6 ± 2.8	4.1
	4.0	85.7 ± 9.3	9.2	89.3 ± 7.5	6.7
Astragalin	100	96.3 ± 4.7	5.9	91.8 ± 5.0	8.4
	640	85.5 ± 3.2	7.0	86.2 ± 9.1	4.4
	4.0	93.6 ± 3.0	8.7	92.3 ± 4.6	8.0
Isoquercitrin	100	91.5 ± 2.5	5.3	97.8 ± 0.5	2.2
	640	96.9 ± 3.9	6.7	89.4 ± 6.3	5.3
	4.0	87.1 ± 4.6	9.2	91.3 ± 2.3	9.8
Quercitrin	100	96.1 ± 8.1	6.3	88.3 ± 7.2	7.3
	640	93.2 ± 6.3	5.2	94.7 ± 6.1	5.8
	4.0	85.2 ± 4.1	8.8	89.1 ± 5.3	7.8
Quercetin	100	92.3 ± 4.4	5.1	91.3 ± 6.2	7.5
	640	90.3 ± 3.1	3.4	94.2 ± 4.3	8.8
	4.0	86.2 ± 6.7	9.0	92.8 ± 5.7	4.9
Kaempferol	100	94.1 ± 3.5	7.8	87.0 ± 9.5	5.2
	640	96.7 ± 2.7	6.1	94.2 ± 3.7	5.8

Table S4 Stability of the six components in rat plasma (n = 3)

Analytes	Concentration ($ng \cdot mL^{-1}$)	24 h, room temperature		8h, 4 °C		3 freeze-thaw cycles		30 days, −20°C	
		RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)
Hyperoside	4.0	8.8	2.9	1.8	4.9	6.1	5.0	-5.8	8.3
	100	9.4	8.3	-7.1	3.8	-9.0	7.1	5.9	8.0
	640	-11.9	9.3	8.0	9.2	7.1	5.2	3.2	4.8
Astragalin	4.0	-8.9	10.5	6.2	4.0	4.1	7.0	5.5	2.1
	100	4.8	1.9	7.8	2.2	-5.4	3.7	-9.7	8.3
	640	9.9	4.7	10.4	5.1	-2.7	2.2	-4.4	2.2
Isoquercitrin	4.0	-7.4	11.1	7.7	7.8	2.9	2.2	-2.4	4.2
	100	6.9	5.0	-4.9	3.1	8.8	2.9	1.8	4.9
	640	4.1	4.8	8.1	9.4	9.4	8.3	-7.1	3.8
Quercitrin	4.0	-6.7	6.3	8.0	9.1	-8.2	9.3	8.0	8.2
	100	3.4	4.9	-6.5	5.1	-8.9	13.2	6.2	4.0
	640	6.1	5.0	-5.8	8.3	4.8	1.9	7.8	2.2
Quercetin	4.0	-9.0	12.1	12.9	8.0	8.2	4.7	10.4	5.1
	100	7.1	10.1	3.2	4.8	4.1	4.8	8.1	1.2
	640	4.1	5.2	-3.7	9.2	-6.7	6.3	8.0	6.2
Kaempferol	4.0	-8.4	2.3	-9.3	8.7	3.4	4.9	-6.5	8.7
	100	-2.7	2.2	-4.7	3.4	6.1	5.0	-5.8	3.4
	640	2.9	2.2	5.0	2.2	2.5	5.2	4.2	2.7

Table S5 Main pharmacokinetic parameters of the six flavonoids after oral administration of *Cuscuta* decoction at 7 g·kg^{-1} to rats in kidney deficient group, *$p < 0.05$ compared with normal group. (mean ± SD; n = 6).

Parameters	Hyperoside	Astragalin	Isoquercitrin	Quercitrin	Quercetin	Kaempferol
			Normal group			
$AUC_{0\text{-}t}$(μgh/L)	640.8 ± 211.3	427.2 ± 140.9	3493 ± 927.3	1076 ± 313.2	3961 ± 1155	8450 ± 1377
$AUC_{0\text{-}\infty}$(μgh/L)	740.5 ± 226.4	493.7 ± 151.0	3957 ± 1057	1213 ± 360.2	4646 ± 1402	18341 ± 3612
$T_{1/2}$ (h)	2.39 ± 0.40	3.21 ± 0.40	1.52 ± 0.43	2.76 ± 0.31	1.71 ± 0.19	5.30 ± 1.60
T_{max} (h)	0.38 ± 0.20	0.32 ± 0.20	0.83 ± 0.41	0.31 ± 0.27	0.61 ± 0.20	2.70 ± 0.52
C_{max}(μg/L)	55.21 ± 13.62	35.02 ± 9.08	281.8 ± 41.80	142.1 ± 51.19	263.1 ± 82.49	400.2 ± 40.89
			Model group			
$AUC_{0\text{-}t}$(μgh/L)	322.4 ± 100.4*	215.1 ±67.15*	1926 ± 517.0*	806.4 ± 276.7*	3054 ± 842.1*	4658 ± 435.4*
$AUC_{0\text{-}\infty}$(μgh/L)	509.0 ± 38.24*	339.6 ±24.92*	2184 ± 588.8*	925.9 ± 318.3*	3598 ± 1041*	10382 ± 1744*
$T_{1/2}$ (h)	4.50 ± 1.72*	4.23 ± 1.56*	2.56 ± 1.03*	3.16 ± 0.78	2.55 ± 0.19*	6.77 ± 1.90*
T_{max} (h)	0.56 ± 0.07*	0.42 ± 0.09*	0.99 ± 0.26*	0.72 ± 0.09*	1.12 ± 0.32*	3.50 ± 0.55
C_{max}(μg/L)	31.18 ± 10.37*	17.12 ± 7.29*	165.7 ± 22.63*	91.67 ± 16.63*	180.2 ± 58.23*	246.2 ± 31.72*

$AUC_{0\text{-}t}$ $AUC_{0\text{-}\infty}$, area under the plasma concentration-time curve from time 0 to t, 0 to ∞; T_{max}, time to reach the maximum plasma concentration; C_{max}, peak plasma concentration; $T_{1/2}$, terminal elimination half life.

Figure S1. Chemical structures MS/MS spectrum of (A) Hyperoside, (B) Astragalin, (C) Isoquercitrin, (D) Quercitrin, (E) Quercetin, (F) Kaempferol, and (G; IS) baicalin.

Figure S2. Typical chromatograms of (A) blank rat plasma, (B) blank rat plasma spiked with six flavonoids and IS at LLOQ, (C) normal group rat plasma sample 2 h after administration of *Semen Cuscutae* decoction at a dose of 7.0 $g \cdot kg^{-1}$. Representative MRM chromatograms of (I) Hyperoside, (II) Astragalin, (III) Isoquercitrin, (IV) Quercitrin, (V) Quercetin, (VI) Kaempferol, and (VII; IS) baicalin.

Figure S3. Plasma concentration-time curves for (A) Hyperoside, (B) Astragalin, (C) Isoquercitrin, (D) Quercitrin, (E) Quercetin, (F) Kaempferol in rat plasma after oral administration of *Semen Cuscutae* decoction at 7.0 $g \cdot kg^{-1}$ to rats in normal and kidney deficient groups. Each point represents the mean ± S.D. (n = 6)

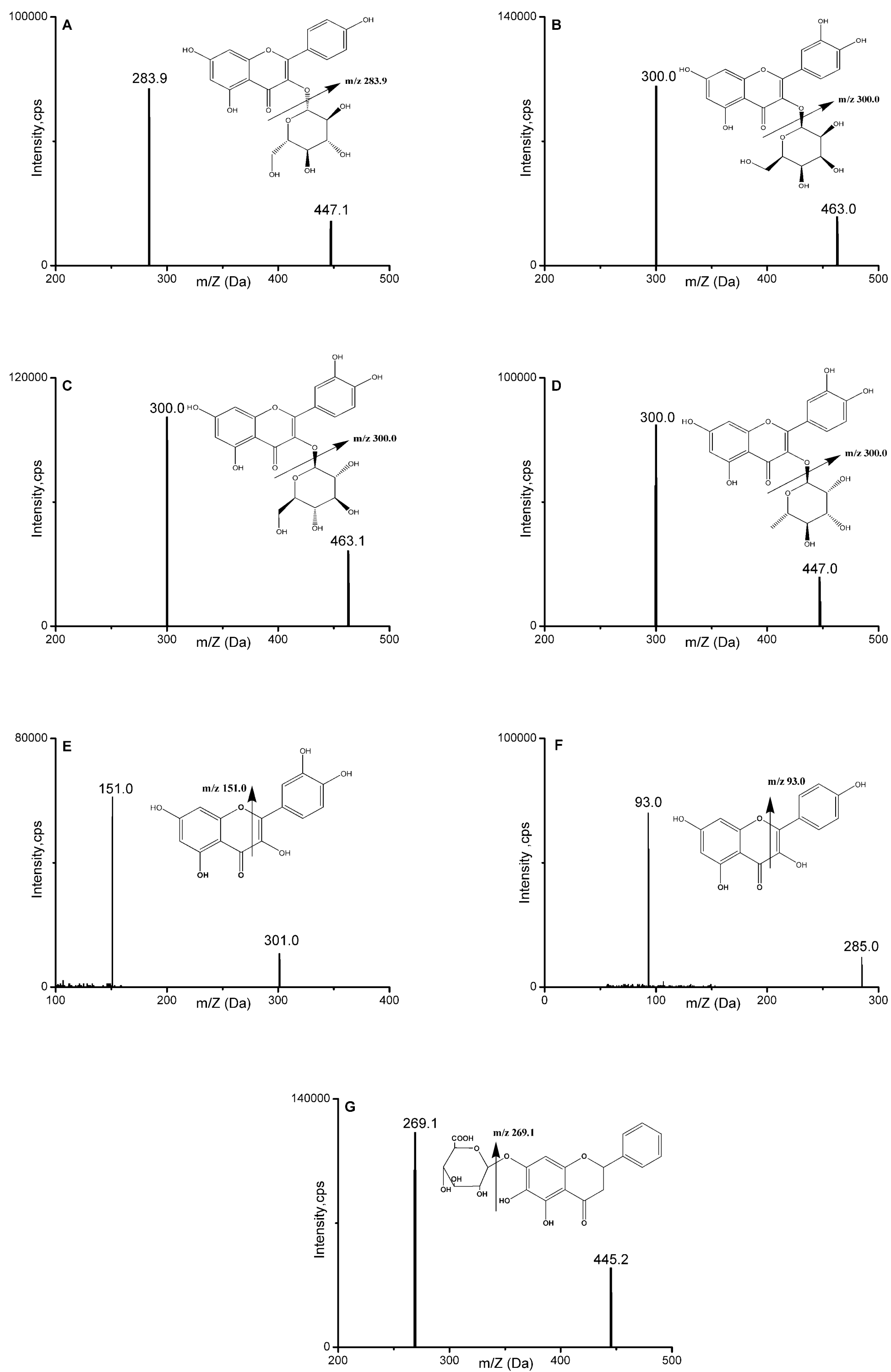
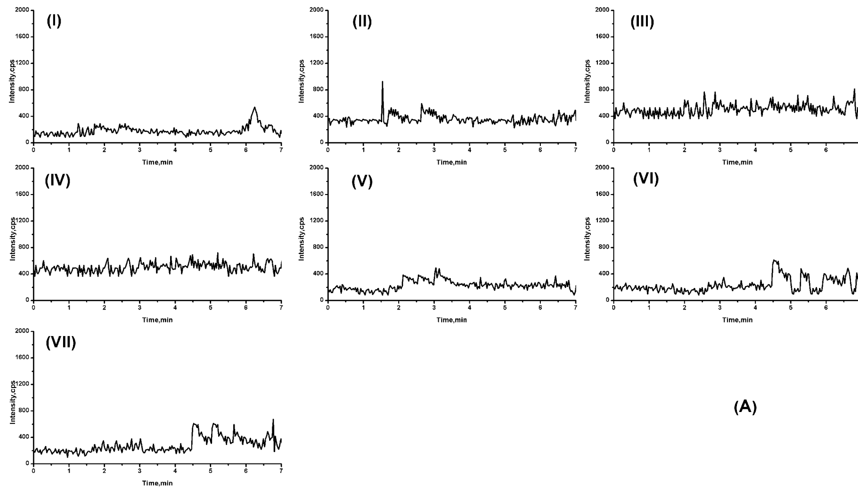
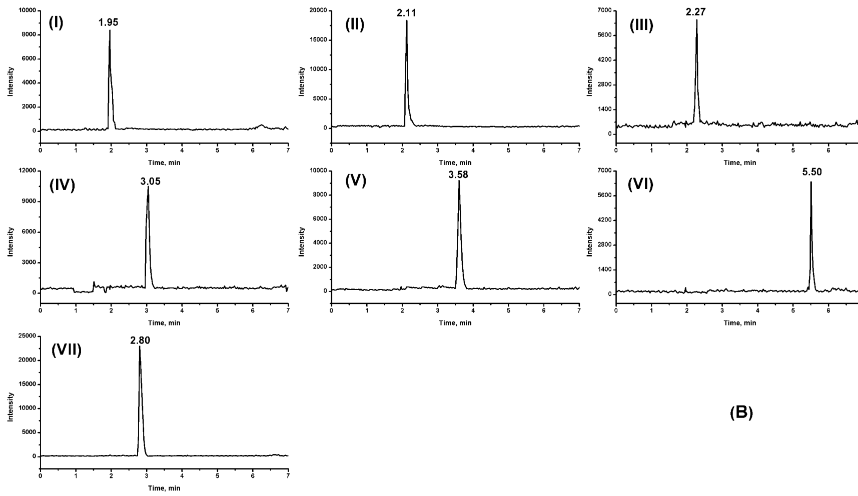


Fig. S1





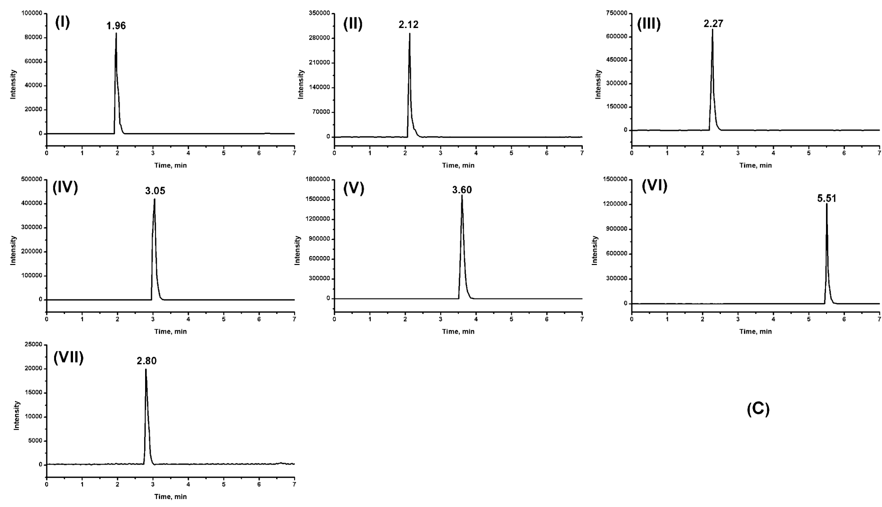


Fig. S2

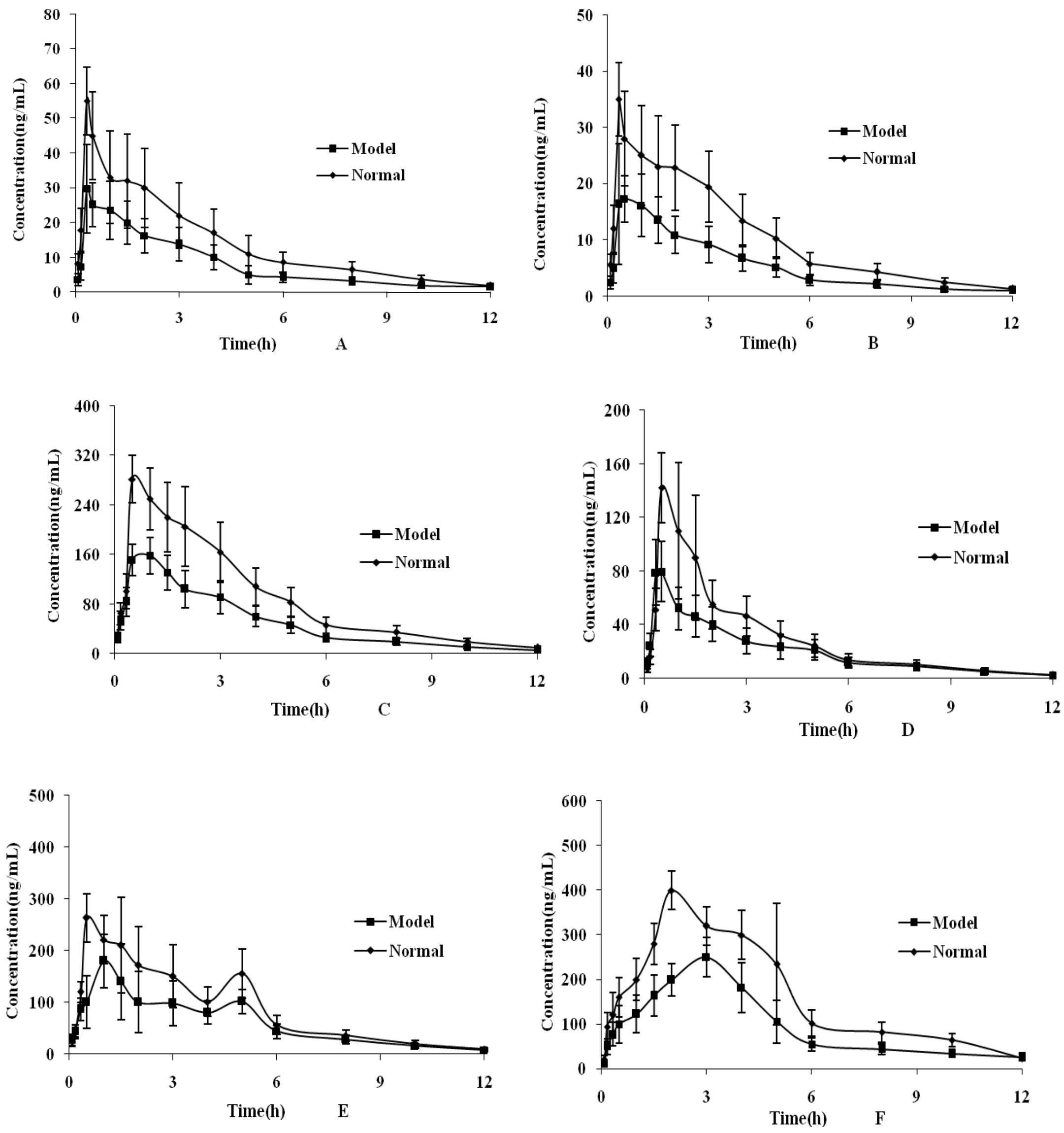


Fig. S3