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Simultaneous determination of danshensu, ferulic acid, cryptotanshinone and tanshinone IIA in rabbit plasma by HPLC and their pharmacokinetic application in danxiongfang

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Abstract

A selective and sensitive reversed-phase high performance liquid chromatography method was developed and validated for the simultaneous determination of danshensu, ferulic acid, cryptotanshinone, and tanshinone IIA in rabbit plasma using *p*-hydroxybenzoic acid as internal standard. Liquid–liquid extraction was used for sample preparation. Chromatographic separation was successfully achieved on an Agilent HC-C_{18} column using a mobile phase composed of methanol–water (from 20:80 to 80:20, v/v) containing 0.5% (v/v) glacial acetic acid. The mobile phase was employing gradient elution at a flow rate of 1.0 ml/min. The method showed good linearity and no endogenous material interfered with the marked compounds and I.S. peaks. The limit of quantification of danshensu, ferulic acid, cryptotanshinone, and tanshinone IIA were 0.1, 0.03, 0.05, and 0.05 μg/ml, respectively. The average extract recoveries of the four compounds from rabbit plasma were all over 60%. The precisions determined from 5 days were all within 10%. The established method has been successfully applied in the pharmacokinetic study and drug interaction of danshensu, ferulic acid, cryptotanshinone, and tanshinone IIA in rabbits after intravenous administration of danxiongfang, a useful compound preparation of traditional Chinese medicine.

Keywords: Danshensu; Ferulic acid; Cryptotanshinone; Tanshinone IIA; Quantification; Pharmacokinetics; HPLC

1. Introduction

Traditional Chinese medicine (TCM) prescriptions are playing an indispensable role in the prevention and treatment of diseases due to their particular effectiveness in the orient public life for more than 2000 years. The remarkable efficacies of many TCM preparations are increasingly being recognized and accepted by more and more people in the world. In clinical practice, most traditional Chinese herbals are prescribed in combination to obtain the synergistic effects or diminish the adverse reactions.

Salvia miltiorrhiza Bunge, which is a well-known traditional Chinese medicine named "Danshen", has been widely adopted in TCM compound preparations and used for treating coronary heart diseases such as angina pectoris, myocardial infarction, anticoagulant and atherosclerosis [1–4], hepatitis and liver fibrosis, anti-inflammatory, antibacterial and antineoplastic action [5–8] in clinical practice. *Ligusticum chuanxiong* Hort [9–11], another famous TCM named "Chuanxiong", is similar in the therapeutic effects to *S. miltiorrhiza* Bunge. For gaining more satisfyingly therapeutic efficacy, *S. miltiorrhiza* Bunge and *L. chuanxiong* Hort were commonly combined to form the compound preparations. At present, there have already been some component mixture preparations containing the two herbs, such as guanxinning tablet [12] and danxiong tongmai pellet [13] in the Orient Market.

According to the chemical structures, the major bioactive constituents in *S. miltiorrhiza* Bunge can be classified into two groups: the phenolic compounds such as danshensu (3,4-dihydroxyphenyl lactic acid), and the tanshinone compounds (abietane-type diterpenes) such as cryptotanshinone and tanshinone IIA. From *L. chuanxiong* Hort, the high content and bioactive component is ferulic acid (3-methoxy-4-hydroxy cinnamic acid). On the basis of these investigations above, danshensu, the major water-soluble component, and tanshinones, the main lipophilic component extracted from *S. miltiorrhiza*

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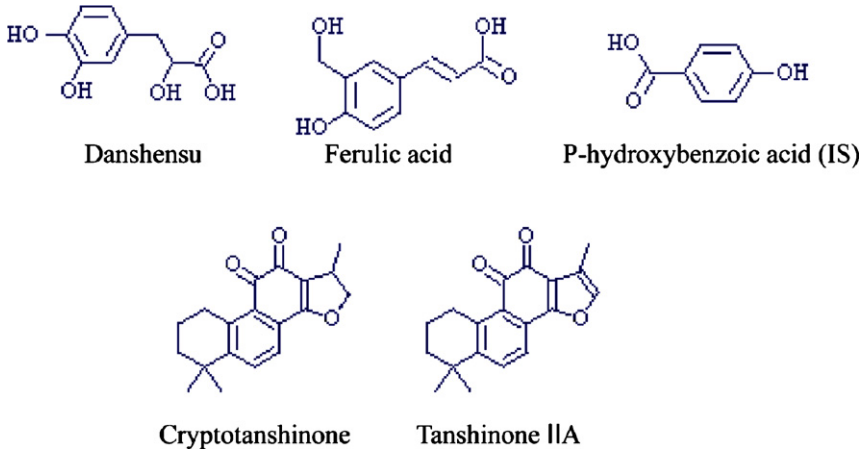

Fig. 1. Chemical structure of danshensu, ferulic acid, cryptotanshinone, tanshinone IIA, and *p*-hydroxybenzoic acid (I.S.).

Bunge, and ferulic acid, the major component from *L. chuanxiong* Hort were combined to form the danxiongfang, a new compound preparation manufactured by our laboratory. The constituents of compound preparation of the two herbs were more complex than that of the single herb preparation. So it is very necessary to develop a more efficient method for simultaneous determination of the four active components, danshensu, ferulic acid, cryptotanshinone and tanshinone IIA which were as the markers in the compound preparation for the quality control of the manufacturing process, the pharmacokinetics and drug interaction *in vivo* and the therapeutic monitoring of danxiongfang.

Each quantification method of HPLC equipped with UV [14–17] or MS, MS/MS detection [18–20] about danshensu, ferulic acid, cryptotanshinone and tanshinone IIA *in vivo* has been reported. However, those methods can only determine the water-soluble or the lipophilic component, respectively, but could not simultaneously determine the water-soluble and the lipophilic component in *S. miltiorrhiza* Bunge, especially not including ferulic acid. In our paper, a gradient HPLC–UV method has been developed for simultaneous determination of danshensu, ferulic acid, cryptotanshinone and tanshinone IIA in biological samples. This method was specific, linear, precise and accurate for the four compounds and successfully applied to the pharmacokinetic study and the drug interaction *in vivo* of danxiongfang in rabbits after an intravenous administration of the component preparations with a single dose.

2. Experimental

2.1. Chemicals and reagents

Danshensu (DS), cryptotanshinone (CT), tanshinone IIA (TS) and ferulic acid (FA) were purchased from National Institute for Control of Pharmaceuticals and Biological Products (Beijing, China). *p*-Hydroxybenzoic acid (internal standard, I.S.) was purchased from Beijing Reagent Chemical Company (Beijing, China). The chemical structures of these compounds were shown in Fig. 1. The raw materials of tanshinones, danshensu and ferulic acid were purchased from Xi'an Honson Biotechnology Co. Ltd. (Xi'an, China). Danxiongfang, which was composed of danshensu, cryptotanshinone, tanshinone IIA and ferulic acid, was prepared with distilled water containing 3% tween-80 and 0.5% 1,2-propanediol by our laboratory. Methanol used was of HPLC grade and obtained from Fisher Scientific Products (Fair Lawn, NJ, USA). Water was triply distilled. The other chemicals, reagents and solvents used were all of analytical grade.

2.2. Instrument and chromatography conditions

All analysis were performed on an Agilent high performance liquid chromatography system (Series 1100, Agilent technology, Palo Alto, CA, USA) which consisted of a G1310A quaternary pump, G1322A vacuum degasser, G1316A column thermostat, G1314A VWD and 7725I manual sample injector. The chromatography data were recorded and processed with HP chemstation software. The analytical column was an Agilent HC-C_{18} (150 mm × 4.6 mm, i.d. 5 μm) column coupled with a C_{18} guard column. All chromatography was performed at 25 °C.

The mobile phase was a mixture of methanol–water containing 0.5% (v/v) glacial acetic acid employing gradient elution (from 20:80 to 80:20, v/v) at a flow rate of 1 ml/min. The gradient elution was shown in Table 1. The solvent was filtered through a 0.45 μm filter and degassed. Danshensu and ferulic acid were determined at 281 nm. Cryptotanshinone and tanshinone IIA were detected at 254 nm. The sample injection volume was 20 μl.

Table 1
Gradient elution program using mobile phase containing A and B

Time (min)	Flow rate (ml/min)	Mobile phase A (%)	Mobile phase B (%)
0	1.0	20	80
4	1.0	45	55
9	1.0	45	55
12	1.0	80	20
25	1.0	80	20

A: methanol; B: water containing 0.5% acetic acid.

The chromatographic data were processed using the I.S. method of plotting peak area ratios of analyte/I.S. versus the relative concentration followed by least square regression of these data.

2.3. Preparation of calibration samples and quality control samples

The mixture of stock standard solution containing danshensu (0.4 mg/ml), ferulic acid (1 mg/ml), cryptotanshinone (0.2 mg/ml), and tanshinone IIA (0.2 mg/ml) was prepared in methanol. Working standard solution was prepared by serial diluting of the stock solution with methanol. The internal standard stock solution (*p*-hydroxybenzoic acid) of 1 mg/ml was also prepared in methanol. Internal standard working solution (50 μg/ml) was prepared by diluting the stock solution with methanol. Calibration samples in plasma were prepared by mixing solutions of standard mixture and I.S. with rabbit blank plasma at a volume ratio of 100 μl:100 μl:400 μl to form a concentration series of 20, 10, 2, 1, 0.2, 0.1 μg/ml for danshensu; 50, 25, 5, 0.5, 0.25, 0.05, 0.01 μg/ml for ferulic acid; 10, 5, 1, 0.5, 0.1, 0.05 μg/ml for cryptotanshinone and tanshinone IIA and 12.5 μg/ml for I.S. Quality control (QC) samples (20, 2, 0.2 μg/ml for danshensu; 50, 5, 0.5 μg/ml for ferulic acid and 10, 1, 0.1 μg/ml for cryptotanshinone and tanshinone IIA) were also prepared in the same way. All solutions were stored at 4 °C before use.

2.4. Sample preparation

Blood samples were collected in heparinzed tubes and centrifuged at 3500 rpm for 15 min immediately. The plasma was separated and stored at −40 °C until further analysis. The extraction procedure of samples was adopted from Xue and Zhuang's published work [21,22]. Plasma samples (0.4 ml) were spiked with 100 μl each of working solution containing danshensu, cryptotanshinone, tanshinone IIA and ferulic acid and 100 μl of I.S. stock solution. Then 100 μl of 1 mol/l HCl and 1 ml of ethyl acetate were added. The combined samples were adjusted to pH 3, vortex-mixed for 1 min and centrifuged at 3000 rpm for 10 min. Each sample was extracted two times and the upper organic potions were combined and evaporated to dryness under a stream of nitrogen flow at 40 °C. The residue was reconstituted in 100 μl of HPLC mobile phase before HPLC analysis and then an aliquot (20 μl) was injected into the LC system.

2.5. Method validation

The method was validated for its selectivity, linearity, limits of detection (LOD), limit of quantification (LOQ), accuracy, precision, recovery and stability. To evaluate the selectivity, five independent rabbit blank plasma were analyzed by comparing with the plasma-spiked analytes for excluding endogenous material interference. Quantification was based on the I.S. method of plotting peak areas ratios of the analyte/I.S. versus the concentration of the samples with a weighting factor 1, the calibration curves were reduplicated five times. The LOD was considered as the final concentration that produced a signal-to-noise (S/N) ratio of 3 and the LOQ as the final concentration that produced a signal-to-noise (S/N) ratio of 10. The precision and accuracy of method were assessed by performing replicate analyses of QC samples against calibration standards. The precision was determined from inter-day and intra-day using five determinations of low, middle, and high concentration and expressed as relative standard deviation (R.S.D.%). The extraction recovery was determined by calculating the ratio of the amount of the extracted compounds from drug-free plasma spiked with known amounts of danshensu, cryptotanshinone, tanshinone IIA, and ferulic acid to the amount of these compounds added at the same concentrations to mobile phase solution. The stability of the sample was assessed by measuring the analysis data of QC samples under ambient, frozen and froze-thaw storage conditions with fresh prepared QC samples.

2.6. Animal pharmacokinetic study and data analysis

Male New Zealand rabbits, weighing 2.2 ± 0.5 kg, were obtained from Laboratory Animals Center of Capital Medical University (LAC, Beijing, China). The rabbits were housed with unlimited access to food and water except for fasting 12 h before experiment, with water available ad libitum. Pooled drug-free plasma was obtained from the healthy rabbits, after aliquoting, plasma controls were stored at −40 °C and then thawed at room temperature for use in calibration curves and QC samples.

Rabbits were administrated danxiongfang via an ear vein. The blood samples were collected from the other ear vein pre-dose and at 1, 2, 5, 10, 30, 45, 60, 90, 120, 240, and 360 min after receiving a single intravenous dose of danxiongfang (containing danshensu 5 mg/kg, ferulic acid 5 mg/kg, cryptotanshinone 4.5 mg/kg, and tanshinone IIA 2.5 mg/kg). Four hundred microlitres of plasma was separated by centrifuging at 3500 rpm for 15 min, collected and stored at −40 °C until analysis.

The RP-HPLC procedure was successfully applied to investigate the plasma concentration–time profiles of danshensu, ferulic acid, cryptotanshinone and tanshinone IIA in rabbits, respectively. The pharmacokinetic model and the parameters were calculated by the practical pharmacokinetic program-version 87 (3P87), edited by the Committee of the Mathematic Pharmacology, the Chinese Society of Pharmacology. The compartment model was established by the methods of the survival square sum (SUM), the Akaike's information criterion (AIC) and the fitted degree (r^2).

3. Results and discussion

3.1. Selectivity

The results for selectivity are shown in Fig. 2. The RP-HPLC method described was selective and specific. The analysis from the plasma samples showed that there were no endogenous substance peaks and drug metabolite peaks interfered with analytes

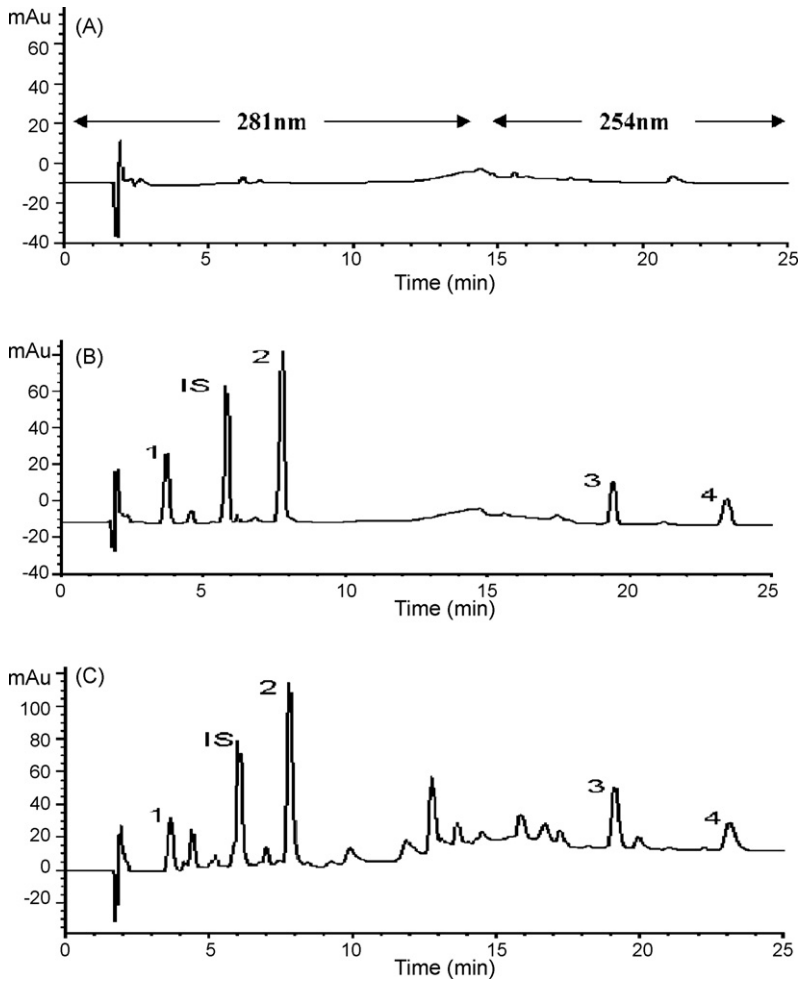

Fig. 2. Representative HPLC chromatography of a blank rabbit plasma (A), a plasma sample spiked with danshensu, ferulic acid, cryptotanshinone, tanshinone IIA, and I.S. (B), and a plasma sample obtained from a rabbit containing 9 μg/ml of danshensu, 12 μg/ml of ferulic acid, 2 μg/ml of cryptotanshinone and 1 μg/ml of tanshinone IIA after i.v. administration of danxiongfang (C). Peak: 1, danshensu; 2, ferulic acid; 3, cryptotanshinone; 4, tanshinone IIA. Wavelength detection: 0–14 min for 281 nm; 15–25 min for 254 nm.

and I.S. at the retention times. The retention times were 3.76, 5.85, 7.78, 19.35, and 23.34 min for danshensu, I.S., ferulic acid, cryptotanshinone and tanshinone IIA, respectively. Fig. 2 shows the representative chromatograms of blank rabbit plasma, a plasma sample spiked with both the four analytes and I.S. and a plasma sample obtained from a rabbit after i.v. administration of danxiongfang.

3.2. Linearity and sensitivity

All the linear regression of danshensu, ferulic acid, cryptotanshinone, and tanshinone IIA in rabbit plasma displayed good linear relationships between the ratios of the peak areas of the analytes to the internal standard over the range of concentrations studied. For a standard curve the ratio of the chromatographic peaks area (analytes/I.S.) as ordinate variables were plotted versus the concentration of these drugs as abscissa. The standard calibration for danshensu, ferulic acid, cryptotanshinone, and tanshinone IIA were linear over the range of 0.1–20 μg/ml, 0.03–50 μg/ml, 0.05–10 μg/ml, and 0.05–10 μg/ml in rabbit plasma, respectively. The mean values of regression equation of the analytes in rabbit plasma were: $y = 0.3834x + 0.0008$ ($\gamma = 0.9998$, danshensu), $y = 2.9388x + 0.0117$ ($\gamma = 0.9996$, ferulic acid), $y = 4.1767x + 0.0011$ ($\gamma = 0.9991$, cryptotanshinone) and $y = 3.8836x + 0.0047$ ($\gamma = 0.9995$, tanshinone IIA).

Sensitivity was evaluated by determining the LOD and the LOQ, which are defined as the lowest concentration that can be reliably and reproducibly measured at least five replicates. To determine the LOD, pooled plasma samples were spiked to contain 0.05 μg/ml danshensu, 0.01 μg/ml ferulic acid, 0.02 μg/ml cryptotanshinone, and 0.02 μg/ml tanshinone IIA, respectively and were analyzed on five different days. The peak area in chromatograms for the spiked plasma samples containing the above lowest concentrations was compared with the noise signal. The LOD had to have precision of ≤10% and a signal/noise ratio ≥ 3. To determine the LOQ, pooled plasma samples were spiked to contain 0.1 μg/ml danshensu, 0.03 μg/ml ferulic acid, 0.05 μg/ml cryptotanshinone, and 0.05 μg/ml tanshinone IIA, respectively and were analyzed

on five different days. The analyte peaks had to be distinct from noise peaks and for verification of LOQ; the peak area in chromatograms for the spiked plasma samples containing the above lowest concentrations was compared with the noise signal. The LOQ had to have precision of ≤10% and a signal/noise ratio ≥ 10.

3.3. Precision and accuracy

The precision and accuracy of the method were assessed in plasma by performing replicate analyses of spiked samples against calibration standards. The procedure was repeated on the same day and between five different days on the same spiked standard series. The within-day and between-day precision and accuracy of the method are shown in Table 2. The precisions (R.S.D.%) were all less than 10%. The data indicated that the precision and accuracy of the method are acceptable.

3.4. Extraction recovery

The extraction recoveries of danshensu, ferulic acid, cryptotanshinone and tanshinone IIA from rabbit plasma are shown in Table 3. The extraction recoveries were determined for five replicates of rabbit plasma spiked with low, medium and high concentrations of the four analytes. The mean recoveries of the samples were more than 60% and the average extraction recovery of internal standard was 77.31%. The data indicated that the extraction recoveries of danshensu, ferulic acid, cryptotanshinone, tanshinone IIA and I.S. from the plasma was

Table 2
Precision and accuracy of HPLC analysis of danshensu, ferulic acid, cryptotanshinone and tanshinone IIA

Theoretical concentration (μg/ml)	*n*	Measured concentration (μg/ml) (mean ± S.D.)	Precision (R.S.D.%)	Accuracy percent error (%)
Danshensu[a]				
Within-day				
0.2	5	0.198 ± 0.012	6.15	−1.0
2	5	2.002 ± 0.038	1.89	0.10
20	5	19.517 ± 0.329	6.15	−2.42
Between-day				
0.2	15	0.184 ± 0.017	9.45	−8.0
2	15	2.035 ± 0.056	2.73	1.75
20	15	19.327 ± 1.216	6.29	−3.37
Ferulic acid[a]				
Within-day				
0.5	5	0.504 ± 0.006	1.22	0.80
5	5	4.959 ± 0.104	2.09	−0.82
50	5	53.437 ± 0.770	1.43	6.87
Between-day				
0.5	15	0.486 ± 0.02	4.08	−2.80
5	15	4.970 ± 0.146	2.93	−0.60
50	15	53.746 ± 1.16	2.15	7.49
Cryptotanshinone[a]				
Within-day				
0.1	5	0.099 ± 0.006	5.78	−1.0
1	5	1.079 ± 0.019	1.8	7.90
10	5	10.163 ± 0.12	1.17	1.63
Between-day				
0.1	15	0.0953 ± 0.006	6.13	−4.70
1	15	1.020 ± 0.039	3.78	2.0
10	15	10.291 ± 0.155	1.5	2.91
Tanshinone IIA[a]				
Within-day				
0.1	5	0.0981 ± 0.005	5.25	−1.90
1	5	1.023 ± 0.04	3.9	2.30
10	5	10.062 ± 0.067	0.66	0.62
Between-day				
0.1	15	0.101 ± 0.006	5.89	1.0
1	15	0.999 ± 0.043	4.37	−0.02
10	15	10.074 ± 0.183	1.83	0.74

[a] Analyte.

Table 3
Extraction recovery of danshensu, ferulic acid, cryptotanshinone and tanshinone IIA in rabbit plasma

Analyte	Added (μg/ml)	Recovery (%) (mean ± S.D.)	R.S.D. (%)
Danshensu	0.2	64.05 ± 8.31	12.97
	2	59.97 ± 4.70	7.85
	20	72.70 ± 1.91	2.63
Ferulic acid	0.5	65.25 ± 1.57	2.41
	5	90.58 ± 3.55	3.92
	50	91.07 ± 1.91	2.10
Cryptotanshinone	0.1	59.10 ± 3.38	5.71
	1	82.72 ± 2.35	2.84
	10	88.10 ± 2.57	2.92
Tanshinone IIA	0.1	79.05 ± 2.49	3.14
	1	84.39 ± 1.77	2.10
	10	85.97 ± 2.81	3.27

concentration-independent in the concentration range evaluated and was acceptable.

3.5. Stability

The stability of the solution kept at 20 °C and frozen plasma samples (−40 °C), as well as frozen plasma extracts, was checked. Plasma QC samples were: (1) allowed to stand at ambient temperature for at least 48 h before extraction and (2) subjected to two freeze-thaw cycles for at least 3 days. Analysis of these samples consistently afforded values that were nearly identical to those of freshly prepared QC samples, thus confirming the overall stability of danshensu, ferulic acid, cryptotanshinone and tanshinone IIA in plasma under frozen storage, assay processing and freeze-thaw conditions.

3.6. Pharmacokinetics of danxiongfang in rabbits

The RP-HPLC method showed satisfactory results for the simultaneous determination of danshensu, ferulic acid, cryptotanshinone, and tanshinone IIA in rabbit plasma and was successfully used for the pharmacokinetic study of danxiongfang following an intravenous administration in rabbits. The plasma concentration–time profiles for danshensu, ferulic acid, cryptotanshinone, and tanshinone IIA, the main active components of danxiongfang are shown in Fig. 3 and the main pharmacokinetic parameters of danshensu, ferulic acid, cryptotanshinone and tanshinone IIA in rabbits are presented in Table 4.

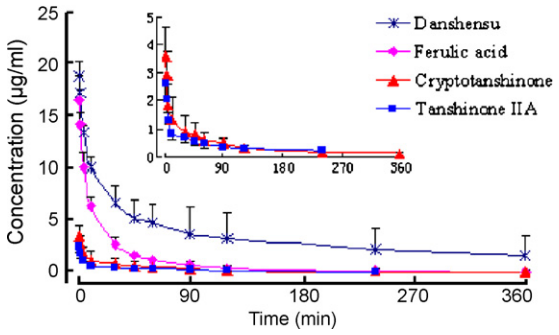

Fig. 3. Pharmacokinetic profiles of danshensu, ferulic acid, cryptotanshinone and tanshinone IIA in plasma following administration of a single intravenous dose of danxiongfang to rabbits.

From the results above, the plasma drug concentration–time data of danshensu, ferulic acid, cryptotanshinone, and tanshinone IIA in rabbits were all best fitted to a two-compartment open model and there were some differences in the distribution and elimination among danshensu, ferulic acid, cryptotanshinone, and tanshinone IIA. It showed that the distributions of the four drugs in rabbits were fast and the eliminations of ferulic acid and cryptotanshinone in rabbit blood were also fast and the eliminations of danshensu and tanshinone IIA were slow relatively. The results showed that danshensu, ferulic acid, cryptotanshinone, and tanshinone IIA in danxiongfang were transported quickly from blood into tissues and organs and eliminated from blood rapidly. It was found that cryptotanshinone could be biotransformed to tanshinone IIA in animals [21,23] and the concentration of tanshinone IIA from the animal plasma after administrating the danxiongfang was an accumulation of the parent drug and the metabolite of cryptotanshinone, thus

Table 4
The main pharmacokinetic parameters describing disposition danshensu, ferulic acid, cryptotanshinone and tanshinone IIA in rabbits after i.v. a single dose of danxiongfang

Parameter	Mean ± S.D.			
	Danshensu	Ferulic acid	Cryptotanshinone	Tanshinone IIA
A (μg/ml)	12.7 ± 1.60	13.58 ± 2.11	3.45 ± 1.87	2.54 ± 0.72
B (μg/ml)	6.41 ± 2.15	3.06 ± 1.67	1.02 ± 0.51	0.69 ± 0.20
α (min^{-1})	0.10 ± 0.06	0.12 ± 0.048	0.32 ± 0.10	0.29 ± 0.07
β (min^{-1})	0.007 ± 0.007	0.014 ± 0.008	0.01 ± 0.004	0.01 ± 0.002
$V(c)$ (l/kg)	0.32 ± 0.042	0.37 ± 0.056	1.08 ± 0.34	0.65 ± 0.20
$t_{1/2\alpha}$ (min)	8.60 ± 4.25	6.99 ± 2.84	2.33 ± 0.67	2.47 ± 0.50
$t_{1/2\beta}$ (min)	202.9 ± 152.1	65.06 ± 35.99	85.01 ± 42.46	135.1 ± 36.3
K_{21} (min^{-1})	0.042 ± 0.03	0.035 ± 0.024	0.09 ± 0.04	0.07 ± 0.02
K_{10} (min^{-1})	0.018 ± 0.015	0.049 ± 0.016	0.04 ± 0.03	0.02 ± 0.01
K_{12} (min^{-1})	0.052 ± 0.032	0.046 ± 0.020	0.20 ± 0.09	0.20 ± 0.04
AUC (μg ml^{-1} min)	2163.1 ± 1711.9	362.1 ± 94.16	129.3 ± 44.74	137.6 ± 18.79
CL(s) (l/(kg min))	0.0054 ± 0.004	0.017 ± 0.004	0.04 ± 0.01	0.01 ± 0.002

the pharmacokinetics of tanshinone IIA in danxiongfang was complex and need to be investigated and described further. The results provided important information for studying the drug interaction *in vivo* and the therapeutic monitoring of danxiongfang and for developing a novel TCM drug and for obtaining a more effectual and safe remedy for clinical practice.

4. Conclusion

A reversed-phase HPLC method has been developed for the simultaneous determination of danshensu, ferulic acid, cryptotanshinone and tanshinone IIA in rabbit plasma from danxiongfang, an effectual TCM compound preparation. The performance criteria for specificity, precision, accuracy, recovery, sensitivity, linearity and stability have been assessed and were within the SFDA recommended guidelines. The results indicated that the method could be successfully used for simultaneous determination of the four main active components in danxiongfang from the blood of animals. The pharmacokinetic profile showed that distribution of danshensu, ferulic acid, cryptotanshinone and tanshinone IIA were all fast and the elimination of danshensu and tanshinone IIA were slow in rabbits. The results provided the important information for studying the drug interaction *in vivo* and for developing a novel TCM drug and for obtaining a more effectual and safe remedy for clinical practice.

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