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Solid-phase extraction–liquid chromatographic method for the determination and pharmacokinetic studies of albiflorin and paeoniflorin in rat serum after oral administration of Si–Wu decoction

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Abstract

A sensitive and rapid high-performance liquid chromatography (HPLC) method with solid-phase extraction (SPE) to simultaneously determine albiflorin and paeoniflorin in rat serum was described. Serum samples were pretreated with solid-phase extraction using Extract-Clean™ cartridges, and the extracts were analyzed by HPLC on a reversed-phase C_{18} column and a mobile phase of acetonitrile-0.03% formic acid (17:83 (v/v)) with ultraviolet detection at 230 nm. Pentoxifylline was used as the internal standard (IS). The linear ranges of the calibration curves were 29–1450 ng/ml for albiflorin and 10–2000 ng/ml for paeoniflorin. The intra- and inter-day precisions (R.S.D.) were ≤10.49% for albiflorin and ≤11.29% for paeoniflorin, respectively. Mean recovery was determined to be 89.75% for albiflorin and 85.82% for paeoniflorin. The limit of quantification was 29 ng/ml for albiflorin and 10 ng/ml for paeoniflorin, respectively. The validated method was applicable to pharmacokinetic studies of albiflorin and paeoniflorin from rat serum after oral administration of Si–Wu decoction. The pharmacokinetic study indicated that albiflorin and paeoniflorin had poor absorption and rapid elimination. This assay result was necessary for the pharmacokinetic evaluation of Si–Wu decoction.

1. Introduction

Traditional Chinese medicine (TCM) is the natural therapeutic agent used under the guidance of the theory of traditional Chinese medical sciences. In clinical application, most herbal medicines are prescribed in combination to obtain the synergistic effects or to diminish the possible adverse reactions [1]. This medical approach has played an important role in the prevention and treatment of diseases.

Si–Wu decoction is one of the famous tonic Chinese prescriptions and widely used in China and Japan with the effect of improving a deficiency of blood, promoting blood circulation, regulating menstruation and relieving pains. Si–Wu decoction is composed of Baishao (*Radix paeoniae*), Dihuang (*Radix rehmanniae*), Danggui (*Radix angelicae*) and

Chuanxiong (*Rhizoma chuanxiong*), which were all recorded in Chinese Pharmacopoeia (2000 Edition).

R. paeoniae is the major component of Si–Wu decoction. Albiflorin and paeoniflorin (structures shown in Fig. 1), the major constituents in *R. paeoniae* and Si–Wu decoction, are water-soluble compounds isolated from the roots of *Paeonia lactiflora* [2,3]. Paeoniflorin has been reported to exhibit anti-coagulant [4], neuromuscular blocking [5–7], immunoregulating [8], cognition-enhancing [9–11] and anti-hyperglycemic effects [12]. Pharmacological investigation showed that paeoniflorin was one of the major active principles of Si–Wu decoction [13]. Albiflorin, as well as paeoniflorin, are the characteristic constituents of *R. paeoniae* and used as markers to evaluate the quality of *R. paeoniae* [14,15]. The metabolic process of albiflorin by human intestinal bacteria was similar to that of paeoniflorin [16]. In addition, it has been reported that albiflorin had relatively weak inhibitory effects on DNA cleavage [17] and completely inhibited the EEG power spectrum changes as well as the extracellular calcium and potassium

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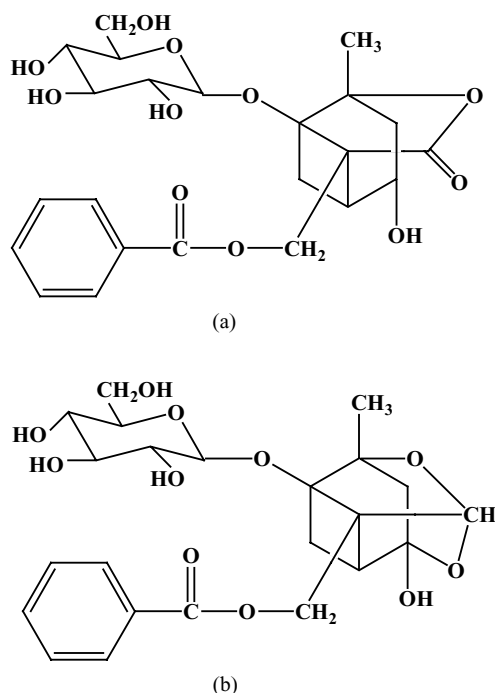*E-mail address:* gda@bjmu.edu.cn (D. Guo).

Fig. 1. Chemical structures of albiflorin (a) and paeoniflorin (b).

concentration changes related to seizure activity [18]. Thus, it is important to quantify albiflorin and paeoniflorin in rat serum and investigate their pharmcokinetics for evaluating the clinical applications of Si–Wu decoction. There are some reported quantitative methods and pharmacokinetic studies on paeoniflorin in biological samples [19–24]. But simultaneous determination of albiflorin and paeoniflorin in rat serum and their pharmacokinetic study were not previously reported. The present study described a sensitive and rapid solid-phase extraction– liquid chromatographic (SPE–HPLC) method to simultaneously determine albiflorin and paeoniflorin in rat serum after oral administration of Si–Wu decoction, so as to take a limited view of their pharmacokinetic profiles.

2. Experimental

2.1. Herbal materials

Baishao, Dihuang, Danggui and Chuanxiong, were purchased from Tong Ren Tang Pharmaceutical Group (Beijing, China). The sliced crude drugs were extracted twice by refluxing with boiling water (1:10, g/ml) for 1 h, and the solution obtained was concentrated and lyophilized. The dried powder was stored at 4 °C before use.

2.2. Chemicals and reagents

The reference standards of albiflorin and paeoniflorin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The internal standard (IS), pentoxifylline, was purchased from Sigma (St. Louis, MO, USA). Acetonitrile and methanol were of HPLC grade (J.T. Baker, Phillipsburg, NJ, USA). The deionized water was prepared from Millipore water purification system and was filtered with 0.45 μm membranes.

2.3. HPLC method

An Agilent 1100 liquid chromatograph system consisting of a quaternary pump, an autosampler and UV detector coupled with an analytical workstation was used. Separations were performed on an Inertsil ODS-3 C-18 reversed-phase column (5 μm, 250 mm × 4.6 mm) with a RP18 (5 μm) guard column (both from Dikma). The isocratic mobile phase was 0.03% formic acid–acetonitrile (83:17 (v/v)). The flow rate was 1 ml/min. Detector was set at 230 nm and all the measurements were performed at 25 °C.

2.4. Animals

Male Sprague–Dawley rats (200–220 g) were obtained from the Laboratory Animal Center of Peking University Health Science Center (Beijing, China). They were kept in environmentally controlled breeding room for 3 days before starting the experiments and fed with standard laboratory food and water ad libitum and fasted overnight before the test.

2.5. Content of albiflorin and paeoniflorin in Si–Wu decoction

To calculate the administered dose of albiflorin and paeoniflorin, their contents in Si–Wu decoction were quantitatively determined. The lyophilized extract of Si–Wu decoction was mixed with deionized water and diluted to the concentration of 4 mg/ml. The mixture was centrifuged at 3000 rpm for 10 min and the supernatant solution was obtained, and then 10 μl of this solution was injected into the HPLC system for analysis. The contents of albiflorin and paeoniflorin in the lyophilized extract of Si–Wu decoction were determined to be 0.38 and 0.61%, respectively, with external standard method.

2.6. Sample preparation-solid-phase extraction procedure

Extract-Clean™ cartridges (Alltech Associates Inc.) were applied for isolation of albiflorin and paeoniflorin from rat serum. The columns were conditioned and equilibrated with methanol followed by deionized water before use. Rat serum with albiflorin, paeoniflorin and IS (pentoxifylline) (4 ml) was transferred into a SPE column cartridge. After washing the cartridge with deionized water, the compounds were eluted using 60% methanol. The elute was evaporated to dryness at 30 °C in vacuo and dissolved in 240 μl of mobile

phase. Twenty microliters of the sample was injected into HPLC for analysis.

2.7. Calibration curve

Stock solutions of albiflorin, paeoniflorin and IS (pentoxifylline) were prepared with deionized water. All the standard solutions were diluted to give concentrations in the range of 1.16–58 μg/ml for albiflorin, 0.4–80 μg/ml for paeoniflorin and 15.36 μg/ml for pentoxifylline. Then, 100 μl of each solution was added together to blank serum (4 ml), the resulting serum contained 29–1450 ng/ml of albiflorin, 10–2000 ng/ml of paeoniflorin and 384 ng/ml of pentoxifylline, respectively. The serum was processed according to the procedure described above. The concentrations of albiflorin and paeoniflorin in serum samples were determined using internal standard method. Limit of detection was determined at a signal to noise of baseline in the ratio of 3:1. The limit of quantification in serum was defined as the lowest concentration on the calibration curve for which assay precision (coefficient of variation (CV)) was less than 20%.

2.8. Precision and accuracy

The precision and accuracy of this method were evaluated by analyzing serum samples spiked with four concentrations of albiflorin and paeoniflorin. The intra-day variance was determined by assaying the six replicates on the same day and inter-day variance was assayed on four consecutive days. Precision was expressed as the percent coefficient of variation. Accuracy was determined by comparing the calculated concentration using calibration curves to the known concentration.

2.9. Recovery

Recoveries for albiflorin and paeoniflorin from rat serum were assessed at four different concentrations (58, 145, 725, 1015 ng/ml for albiflorin and 80, 200, 400, 1000 ng/ml for paeoniflorin). The recovery of internal standard, pentoxifylline, was evaluated at the concentration used in sample analysis (384 ng/ml). Albiflorin, paeoniflorin and pentoxifylline were spiked into blank serum to give the above concentrations. The samples were treated as described in Section 2.6 and analyzed by HPLC. For albiflorin and paeoniflorin, the recoveries were calculated by comparing their peak area ratios to IS of the extracted serum samples with standard solutions at the same concentration level. For pentoxifylline, the extraction efficiency was determined by comparing the peak area of pentoxifylline to that of standard solution injected directly into the HPLC system without extraction.

2.10. Application of the method and pharmacokinetic study

The utility of the worked-out method was demonstrated in the in vivo conditions. Each rat was administered an oral dose of 24 g/kg of Si–Wu decoction. A blood sample was collected at times of 5, 10, 15, 20, 40, 60,120, 180, 240 min after dosing. Within 30 min after blood withdrawal, the samples were centrifuged and the separated serum samples were frozen in polypropylene tubes at −20 °C prior to analysis.

The rat serum samples (4 ml) with internal standard were performed as described under Section 2.6. Twenty microliters of this sample was analyzed. The serum concentrations of albiflorin and paeoniflorin at different time point were evaluated by means of linear regression analysis. Data were expressed as mean ± S.D. All statistical analysis was performed using Microsoft Excel 2000. The relevant pharmacokinetic parameters were calculated using the computer program 3p97 (the Chinese Society of Mathematical Pharmacology).

3. Result and discussion

3.1. Solid-phase extraction and recovery

In the present study, the method for estimation of albiflorin and paeoniflorin in rat serum was described. Due to the structural similarity, the chemical properties of albiflorin and paeoniflorin were very similar. Therefore, the method for isolation of paeoniflorin from rat serum was also applicable to the isolation of albiflorin. In the previous reports [22], the biological samples were obtained by precipitating protein with acetonitrile and extracted with ether to remove non-polar interfering impurities, which was cumbersome and involved in the use of toxic solvents. On the basis of our previous study [24], solid-phase extraction was performed to isolate the albiflorin and paeoniflorin from interfering compounds in rat serum. Proteins and interfering compounds can be removed by deionized water, and albiflorin and paeoniflorin retained on the Extract-Clean™ cartridge were completely eluted with 60% methanol. Most of the endogenous substances could be removed with SPE process. The mean extraction efficiency for albiflorin and paeoniflorin from rat serum were 89.75 and 85.82%, respectively (Table 1). In addition, the recovery of pentoxifylline was 92.93% at its concentration used in the assay, which suggested that the extraction for the analyzed compounds was quite efficient.

3.2. Selectivity

Chromatographic profiles of blank serum, blank serum spiked with albiflorin, paeoniflorin and IS, and serum obtained 15 min after oral administration of Si–Wu decoction were shown in Fig. 2. The retention times of albiflorin, paeoniflorin and IS were 10.30, 12.26 and 22.37 min, respectively. A baseline separation of these compounds was obtained under the specified chromatographic conditions. No interfering peaks were detected. This indicated that the selectivity of the elaborated procedure was appropriate.

Table 1
Recovery of the albiflorin and paeoniflorin assay ($n = 3$)

Compound	Spiked concentration (ng/ml)	Recovery (%)	R.S.D (%)	Average (%)
Albiflorin	58	90.52 ± 6.21	6.86	90.15
	145	95.43 ± 7.04	7.37	
	725	87.78 ± 0.59	0.67	
	1015	86.88 ± 1.05	1.21	
Paeoniflorin	80	81.69 ± 6.17	7.55	85.82
	200	85.27 ± 1.97	2.31	
	400	94.79 ± 2.23	2.35	
	1000	81.51 ± 0.52	0.64	

3.3. Standard curve

The standard curve for albiflorin in rat serum was linear over the range of 29–1450 ng/ml, producing a regression of $y = 0.001476x + 0.011465$ with a correlation coefficient r^2 of 0.9944 (where y is the peak area ratio and x the concentration of analyte), the limit of quantification was 29 ng/ml and the limit of detection was 9.5 ng/ml. While for paeoniflorin,
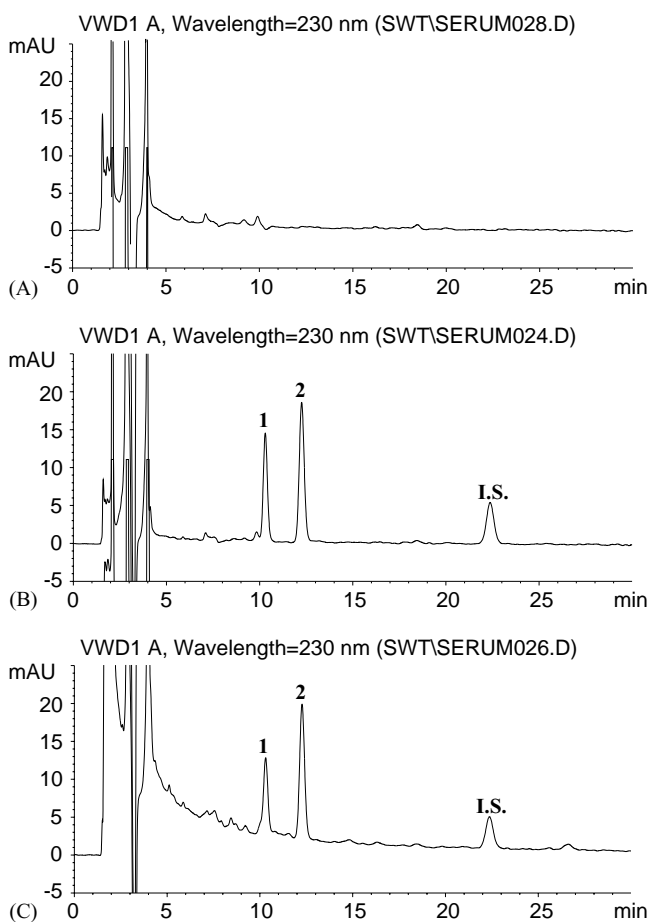

Fig. 2. Typical chromatograms for the determination of albiflorin and paeoniflorin in serum samples: (A) chromatogram of a blank serum sample; (B) chromatogram of a serum sample spiked with albiflorin (1) and paeoniflorin (2) and internal standard (IS); (C) chromatogram of the serum sample from a rat after 15 min of oral administration of Si–Wu decoction.

it was linear over the range of 10–2000 ng/ml, producing a regression of $y = 0.00133x + 0.065101$ with a correlation coefficient r^2 of 0.9961, the limit of quantification was 10 ng/ml and the limit of detection was 3.2 ng/ml.

3.4. Precision and accuracy

The intra- and inter-day precision and accuracy were evaluated in four concentration levels (albiflorin at 58, 145, 725 and 1450 ng/ml; paeoniflorin at 40, 200, 800 and 1400 ng/ml). As shown in Tables 2 and 3, the intra-day accuracy of albiflorin and paeoniflorin was 92.55–94.43 and 95.40–109.82% with the CV values less than 9.93 and 11.29%, respectively. The inter-day accuracy of albiflorin and paeoniflorin ranged from 90.45 to 92.89% and from 96.01 to 100.3% with the CV values less than 10.49 and 10.88%, respectively. The overall reproducibility of the method was acceptable.

3.5. Application of analytical method in pharmacokinetic studies

The described method was applied to analysis of serum samples after oral administration of Si–Wu decoction. Fig. 3 showed the mean serum concentration–time profile of albiflorin and paeoniflorin ($n = 5$). The absorption of albiflorin and paeoniflorin was rapid, with peak concentrations occurring at 15 min for albiflorin and 20 min for paeoniflorin after oral administration of Si–Wu decoction. The concentration was lower than limit of quantification after 3 h for albiflorin and 4 h for paeoniflorin.

As calculated from the serum concentrations of albiflorin and paeoniflorin following oral administration of Si–Wu decoction, the non-compartmental pharmacokinetic parameters were calculated and listed in Table 4. These parameters indicated that albiflorin and paeoniflorin were absorbed and cleared quickly from the body.

TCMs are widely used in China and Japan and in most cases they are prescribed in combination in clinics. The pharmacokinetic study is an important and useful method to approach the pharmacological actions of TCMs since knowledge on the pharmacokinetics might help to explain and predict a variety of events related to the efficacy and toxicity of herbal preparations [25,26].

Table 2
Validation of the intra-day assay ($n = 6$)

Compound	Spiked concentration (ng/ml)	Measured concentration (ng/ml)	Accuracy (%)	C.V. (%)
Albiflorin	58	53.68 ± 5.83	92.55	9.93
	145	136.62 ± 5.56	94.22	4.07
	725	684.62 ± 23.07	94.43	3.37
	1450	1341.96 ± 58.69	92.55	4.37
Paeoniflorin	40	43.93 ± 4.96	109.82	11.29
	200	194.36 ± 12.61	97.18	6.49
	800	763.20 ± 51.71	95.40	6.78
	1400	1389.46 ± 50.52	99.25	3.64

Table 3
Validation of the inter-day assay ($n = 4$)

Compound	Spiked concentration (ng/ml)	Measured concentration (ng/ml)	Accuracy (%)	C.V. (%)
Albiflorin	58	52.46 ± 5.50	90.45	10.49
	145	133.41 ± 9.90	92.01	7.42
	725	673.13 ± 21.83	92.85	3.24
	1450	1346.84 ± 49.30	92.89	3.66
Paeoniflorin	40	38.58 ± 4.20	96.45	10.88
	200	200.52 ± 14.28	100.3	7.12
	800	768.04 ± 72.51	96.01	9.44
	1400	1402.24 ± 75.93	100.2	5.41

TCM combination is used to cure diseases as a whole. Each herb that constitutes the formula is necessary to the integral effect. As the chemical constituents of the formula are complex, the pharmacokinetic study was usually focused on the main active constituents. Si–Wu decoction is the water extract of four herbal drugs in combination. According to the previous investigation, the chemical constituents of Si–Wu decoction were mainly monosaccharides, polysaccharides and water-soluble compounds [27,28]. Using HPLC with ultraviolet detection method, the fingerprint of Si–Wu decoction was analyzed and the marker substances of Si–Wu decoction were determined (data not shown). The results showed that albiflorin and paeoniflorin were two main constituents of aqueous solution of Si–Wu decoction. Therefore, the current investigation was designed to administer
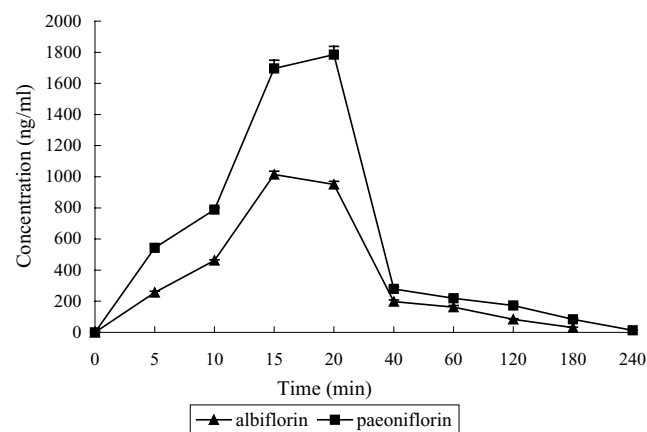

Fig. 3. Mean serum concentration–time profile for albiflorin and paeoniflorin in rat serum after oral administration of Si–Wu decoction.

aqueous Si–Wu decoction to animal subjects, and then to determine the serum profiles and pharmacokinetic parameters of albiflorin and paeoniflorin in Si–Wu decoction.

Previous studies indicated that paeoniflorin has a higher binding affinity to organs and a lower blood distribution and its low bioavailabity caused the low paeoniflorin concentration in plasma [22]. In order to study pharmacokinetics of albiflorin and paeoniflorin following oral administration of Si–Wu decoction, a large dose referred to clinical application was administered to rats. Although the oral administration dose of Si–Wu decoction contained 91.2 mg/kg albiflorin and 146.4 mg/kg paeoniflorin, their concentrations in rat serum were extremely low. The current investigation results were consistent with the above conclusion. And it was found that a relatively greater extent of albiflorin and paeoniflorin were absorbed after oral administration of Si–Wu decoction. Therefore, it is important to study their pharmcokinetics to explain the pharmacological synergistic

Table 4
Mean pharmacokinetic parameters of albiflorin and paeoniflorin in rat serum ($n = 5$) after oral administration Si–Wu decoction[a]

Parameter	Estimate (mean ± S.D.)	
	Albiflorin	Paeoniflorin
t_{max} (min)	13.77 ± 0.23	19.36 ± 10.34
C_{max} (ng/ml)	1014.36 ± 21.41	1784.74 ± 53.50
$AUC_{0\to\infty}$ (ng min/ml)	36915.01 ± 429.18	68423.41 ± 1333.29
$T_{1/2}$ (min)	43.71 ± 1.74	63.69 ± 10.26
MRT (min)	56.86 ± 1.72	93.32 ± 18.06

[a] $AUC_{0\to\infty}$: the area under curve concentration–time; C_{max}: maximum concentration at t_{max}; $T_{1/2}$: elimination half-life time; MRT: mean residence time.

effect of Si–Wu decoction in clinical use. In addition, if an ideal vehicle could be found to increase absorption of albiflorin and paeoniflorin, great benefit for improving clinical efficacy of albiflorin and paeoniflorin will be obtained.

4. Conclusions

A simple and reliable SPE-HPLC method has been developed for the determination of albiflorin and paeoniflorin in rat serum after oral administration of Si–Wu decoction. The assay provided adequate recovery with good precision and accuracy. The method was validated to meet the requirements of the pharmacokinetic investigation of the two compounds. This is the first report of pharmacokinetic studies of albiflorin and paeoniflorin in rat serum after oral administration of Si–Wu decoction. The pharmacokinetic results provide a firm basis for evaluating the clinical efficacy of Si–Wu decoction.

Acknowledgements

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