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Journal of Pharmaceutical and Biomedical Analysis
33 (2003) 521–527
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

SPE-HPLC method for the determination and pharmacokinetic studies on paeoniflorin in rat serum after oral administration of traditional Chinese medicinal preparation Guan-Xin-Er-Hao decoction

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Received 8 January 2003; received in revised form 9 May 2003; accepted 10 May 2003

Abstract

A new HPLC method for the determination of paeoniflorin in rat serum with solid-phase extraction (SPE) for preconcentration is introduced. Paeoniflorin and an internal standard (pentoxifylline) were extracted from serum by means of SPE using cartridges with octadecyl chemically bound phase. The HPLC separation was then performed on a reversed-phase C_{18} column using acetonitrile–water (18:82, v/v) as eluting solvent system, and UV detection at 230 nm to measure the analyte with a limit of quantitation about 10 ng ml^{-1}. The calibration curve for paeoniflorin was linear ($r = 0.9938$) in the concentration range of 10–1200 ng ml^{-1}, both intra- and inter-day precision of the paeoniflorin were determined and their coefficience of variation did not exceed 10%. The validated method has been successfully applied for pharmacokinetic studies of paeoniflorin from rat serum after oral administration of Guan-Xin-Er-Hao decoction.

Keywords: SPE; HPLC; Paeoniflorin; Guan-Xin-Er-Hao decoction

1. Introduction

Traditional Chinese medicine (TCM) is the natural therapeutic agent used under the guidance of the theory of traditional Chinese medical science and has been applied by TCM practitioners for thousands of years. It is used mostly in combination, in which the composite formulae will produce a synergistic effect or antagonistic action. This medical approach has played an important role in the prevention and treatment of diseases.

Guan-Xin-Er-Hao (GXEH) has been used to treat coronary heart diseases in China and Japan and has produced a favorable effect [1]. The formula consists of five crude drugs including Radix Salviae Miltiorrhizae, Rhizoma Chuanxiong, Flos Carthami, Lignum Dalbergiae Odoriferae, and Radix Paeoniae Rubrae in a ratio of

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0731-7085/03/$ - see front matter
doi:10.1016/S0731-7085(03)00294-2

2:1:1:1:1 on the dry weight basis. Paeoniflorin (Fig. 1), a water-soluble compound first isolated from the root of *Paeonia lactiflora* in 1963 [2], has been reported to exhibit anticoagulant [3], neuromuscular blocking [4–6], immunoregulating [7], antinociceptive [8] and antihyperglycemic activities [9]. Thus paeoniflorin can be used as one of the marker compounds to characterize the GXEH. HPLC [10–17] and CE [18–23] are the two major methods that have been used for the determination of paeoniflorin contained in Chinese medicinal preparations. Owing to the complexity of composite formulae, there has been few report regarding their pharmacokinetic studies. Since most Chinese medicines are administered orally as extract powders or decoctions in clinics, the administration of purified paeoniflorin [24,25] and *Paeonia* extract reported in the literature [26] could not be used as suitable references for clinical application. In this paper, we have developed a simple and rapid solid-phase extraction (SPE)-HPLC method to determine paeoniflorin in rat serum after oral administration of GXEH decoction, so as to take a limited view of its pharmacokinetic profiles.

2. Experimental

2.1. Crude drugs

Danshen (*Salvia miltiorrhiza* Bge.) was purchased from Zhongjiang Danshen Cultivation Base (Sichuan province, PR China); Honghua (*Carthamus tinctorius* L.) was purchased from Hetian, (Xinjiang Uighur Autonomous Region, PR China); Chishao (*P. lactiflora* Pall.) was from Duolun Medicine Corporation Ltd. (Inner Mongholia, PR China); Chuanxiong (*Ligusticum chuanxiong* Hort.) was from Dujiangyan Medicine Corporation Ltd. (Sichuan province, PR China); Jiangxiang (*Dalbergia odorifera* T. Chen) was from Zhixin Medicine Corporation Ltd. (Guangzhou, PR China). The herbal materials were extracted twice by refluxing in water (1:8, g/ml) for 1 h and the water extract was concentrated and lyophilized. The dried powder was stored at 4 °C before use.

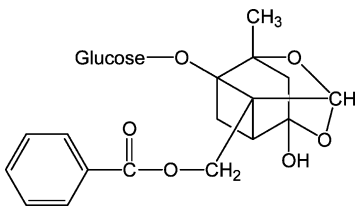

Fig. 1. The chemical structure of paeoniflorin.

2.2. Chemicals and reagents

Paeoniflorin was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). The internal standard pentoxifylline was purchased from Sigma (St. Louis, MO). Acetonitrile and methanol were of HPLC grade (Fisher, Leics, UK). House triple distilled water from silica glass equipment was always used.

2.3. Animals

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

2.4. HPLC conditions

The HPLC system consists of a Waters 600E pump, a Waters 2487 UV–Vis detector set at 230 nm, a 100-μl injection loop, a LC workstation for data collection and an inertsil ODS-3 C_{18} reversed-phase column (5 μm, 250 × 4.6 mm) which was protected by RP18 (5 μm) guard column (both from Dikma). The mobile phase was water–acetonitrile (82:18, v/v) filtered through a 0.45-μm millipore filter and degassed prior to use. The flow rate was set at 1 ml min^{-1}.

2.5. Content of paeoniflorin in GXEH

The content of paeoniflorin was calculated first, and then GXEH was orally administered by rats at

a dose containing 125 mg kg^{-1} paeoniflorin. To calculate the content of paeoniflorin, the lyophilized extract of GXEH was dissolved in distilled water and diluted to a concentration of 0.5 mg ml^{-1}. The mixture was centrifuged at 2500 rpm for 10 min and the supernatant solution was obtained, and then 20 μl of this solution was injected into the HPLC system for analysis. The content of paeoniflorin in the lyophilized extract of GXEH was determined to be 1.12%, from the peak area ratios by using equation for linear regression obtained from the calibration curve.

2.6. Calibration curve

Stock solutions of paeoniflorin and pentoxifylline were prepared with triple distilled water. Paeoniflorin was prepared at concentrations of 0.1, 0.4, 0.8, 1.2, 2.0, 4.0, 8.0 and 12.0 μg ml^{-1} and pentoxifylline at 4.3 μg ml^{-1}. Then 100 μl of each solution was added together to blank rat serum so that the resulting serum contained 10, 40, 80, 120, 200, 400, 800 and 1200 ng ml^{-1} paeoniflorin and 430 ng ml^{-1} pentoxifylline. Then the serum was processed according to the procedure SPE specified below. The limit of quantification (LOQ) in serum was defined as the lowest concentration on the calibration curve for which assay precision (coefficience of variation, CV) was lower than 10%.

2.7. Solid phase extraction procedure

Cartridges (3 ml capacity, Extract-Clean™, Alltech Associates, Inc.) with liquid phase C_{18} chemically bound to silica gel (500 mg) were applied to isolate paeoniflorin from rat serum. The columns were conditioned with 2×3 ml of methanol and 2×3 ml of triple distilled water before use. Rat serum with paeoniflorin and I.S. (1 ml) was transferred into a SPE column cartridge. Columns with absorbed paeoniflorin were purified by 2×3 ml distilled water. The compounds were washed with 4 ml of 60% methanol under low vacuum (flow rate at 30 drops per min) using a water pump. The resulting solution was evaporated to dryness at 40 °C in vacuo. The evaporated residue was dissolved in 100 μl of mobile phase and 20 μl of the solution was injected into HPLC for analysis.

2.8. Recovery

The recovery for paeoniflorin at concentrations of 40, 200 and 800 ng ml^{-1} was calculated. The first group consisted of five 1 ml serum samples and each sample was spiked with 100 μl of 0.4 μg ml^{-1} paeoniflorin standard solution and 100 μl of 4.3 μg ml^{-1} I.S. solution, which contained 40 ng ml^{-1} of paeoniflorin and 430 ng ml^{-1} of the I.S. in the end. The samples were processed according to the above mentioned SPE method. The second group consisted of five serum samples with I.S. only and paeoniflorin was added into the eluting solution after the above SPE procedure. Recoveries were calculated as the area ratio of paeoniflorin to I.S. from the spiked samples and unextracted standard solution. The recovery for 200 and 800 ng ml^{-1} of paeoniflorin were calculated in the same manner.

2.9. Application of the method for in vivo studies

Aqueous solution of GXEH were orally administrated to rats at a dose of 125 mg kg^{-1} of paeoniflroin, and blood samples were collected at times of 5, 10, 15, 20, 40, 60, 120, 180, 240 and 360 min after dosing. Within 30 min after blood withdrawal, the samples were centrifuged and the separated serum samples were frozen in plastic vials at 4 °C until analysis was carried out. Data from these samples were used to construct pharmcokinetic profiles by plotting drug concentration versus time. All data were subsequently processed by the computer program 3P87 (Chinese Pharmaceutical Association, 1987).

3. Results and discussion

3.1. Solid phase extraction and recovery

For the isolation of paeoniflorin from rat serum, solid phase extraction was employed in this study instead of classical liquid–liquid extraction. In the previous reports [26], the biosamples were ob-

tained 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. In addition, recoveries obtained by the method were rather low (74.49–80.56%), when compared with that of SPE method (87.3–93.7%) (Table 1).

To establish a simple and accurate HPLC method for in vivo analysis of paeoniflorin, SPE procedure was considered. The eluting distribution of paeoniflorin in the SPE column cartridge was investigated. One milliliter of the stock solution (containing 1.2 μg ml^{-1} paeoniflorin and 0.43 μg ml^{-1} I.S.) was transferred into the SPE column, and 5 ml of water was used as cleaning solution, then 4 ml of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% methanol were used successively to elute the column. The eluting solution (20 μl) was injected into the HPLC column to determine the content of paeoniflorin and to calculate the accumulative recovery on the basis of the chromatographic conditions described above. Analysis of eluting distribution plot (Fig. 2) showed that paeoniflorin and pentoxifylline could not be eluted out by water while 60% methanol could elute them completely. To remove the non-polar interfering impurities, the best SPE procedure was formulated: water as the cleaning solution, and 60% methanol as the eluting solution.

3.2. Selectivity

The separated peaks of paeoniflorin and I.S. (Fig. 3) revealed that the retention times of paeoniflorin and I.S. were 10.4 and 17.3 min, respectively, and no interfering peaks were detected. This indicated that the selectivity of the elaborated procedure was satisfactory.

Table 1
Recovery of paeoniflorin from rat serum [a]

Spiked concentration (ng ml^{-1})	% Recovery ± S.E.M.	Average (%)
40	87.3 ± 1.6	
200	93.5 ± 1.8	91.5
800	93.7 ± 2.1	

[a] $n = 5$.

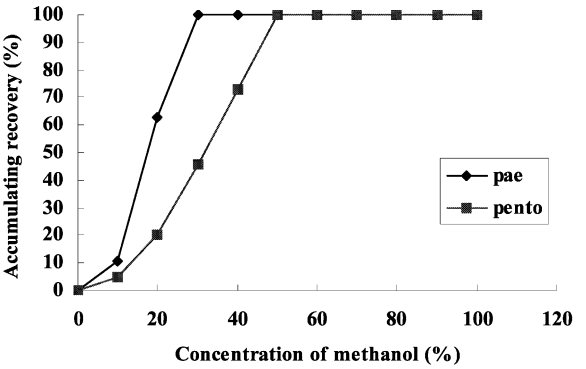

Fig. 2. Profile of eluting distribution.

3.3. Standard curve

The standard curve was prepared for paeoniflorin in the range of 10–1200 ng ml^{-1}, which covered the levels following the administration of a single dose of 125 mg kg^{-1} paeoniflorin. The standard curve was described by equations $y = 0.0025x + 0.0179$, $r = 0.9938$, where y is the peak area, x the concentration, and r the correlation coefficient. The LOQ in serum was defined as the lowest concentration on the standard curve for which the assay precision was reflected by CV $\leq$ 10%, and it amounted to 10 ng ml^{-1}.

3.4. Precision and accuracy

The intra-day and inter-day accuracies were estimated and the studied concentrations (10, 80, 200, 800 and 1200 ng ml^{-1}) were lower than 10%, as indicated by the respective values of CV (Table 2). This showed that the method is quite precise. Moreover, the small difference ($\leq 10\%$) noted between added levels and the estimated concentrations have documented an appropriate accuracy of the elaborated method.

3.5. In vivo application of the method

In this study, the established method successfully quantified paeoniflorin after oral administration of GXEH decoction, and the pharmacokinetic parameters of paeoniflorin are listed in Table 3. Although the oral administration dose of GXEH

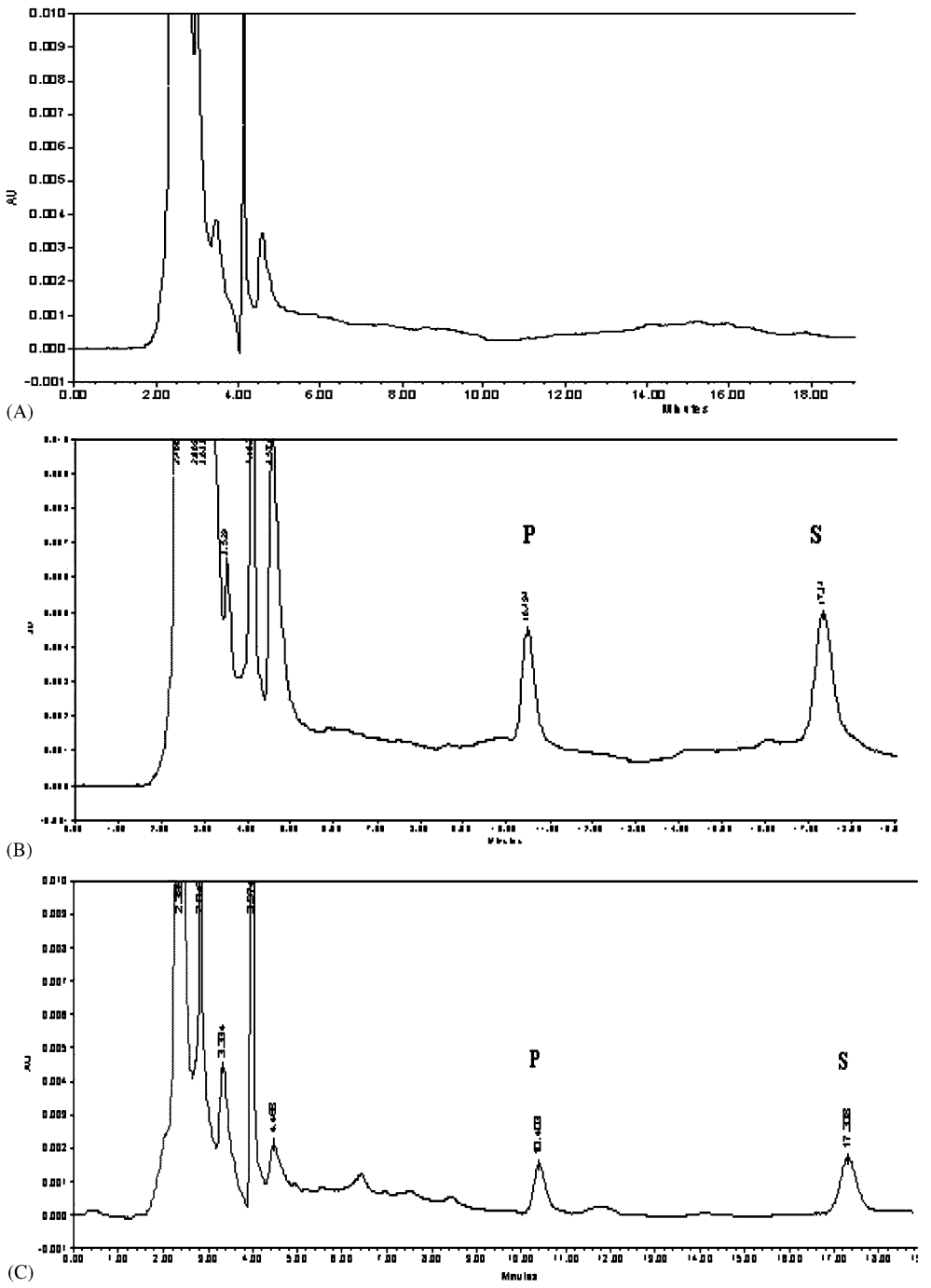

Fig. 3. Typical chromatograms for the determination of paeoniflorin in serum samples: (A) chromatogram of a blank serum sample; (B) chromatogram of a serum sample spiked with paeoniflorin (P) and internal standard (S); (C) chromatogram of the serum sample from a rat after 1 h of oral administration of the GXEH.

Table 2
Intra- and inter-day precision and accuracy of paeoniflorin in rat serum

Concentration added (ng ml^{-1})	Concentration measured (ng ml^{-1}) ± S.E.M.	Accuracy (%)	CV (%)
Intra-day reproducibility (n = 5)			
10	9.83 ± 0.91	98.312	9.3
80	80.24 ± 6.90	100.31	8.6
200	191.94 ± 12.28	95.97	6.4
800	791.24 ± 41.94	98.90	5.3
1200	1189.56 ± 49.96	99.13	4.2
Inter-day reproducibility (n = 5)			
10	10.02 ± 0.98	100.21	9.8
80	79.82 ± 7.26	99.78	9.1
200	194.76 ± 14.02	97.38	7.2
800	783.49 ± 30.56	97.94	3.9
1200	1178.94 ± 56.59	98.25	4.8

contained 125 mg kg^{-1} paeoniflorin, its concentrations in rat serum were extremely low. This implied that paeoniflorin has a high binding activity to organs and a low blood distribution, and another possible reason was the low bioavailability of paeoniflorin which is due to the fact that paeoniflorin is metabolized by gut wall, liver and lung on one hand, and on the other hand, it is poorly absorbed by the intestine and the unabsorbed paeoniflorin was degraded by the intestinal flora [27]. If an ideal vehicle could be found to increase absorption of paeoniflorin, great benefits for improving clinical efficacy of paeoniflorin will be obtained (Fig. 4).

4. Conclusion

Application of SPE method has permitted the determination of paeoniflorin with relatively high

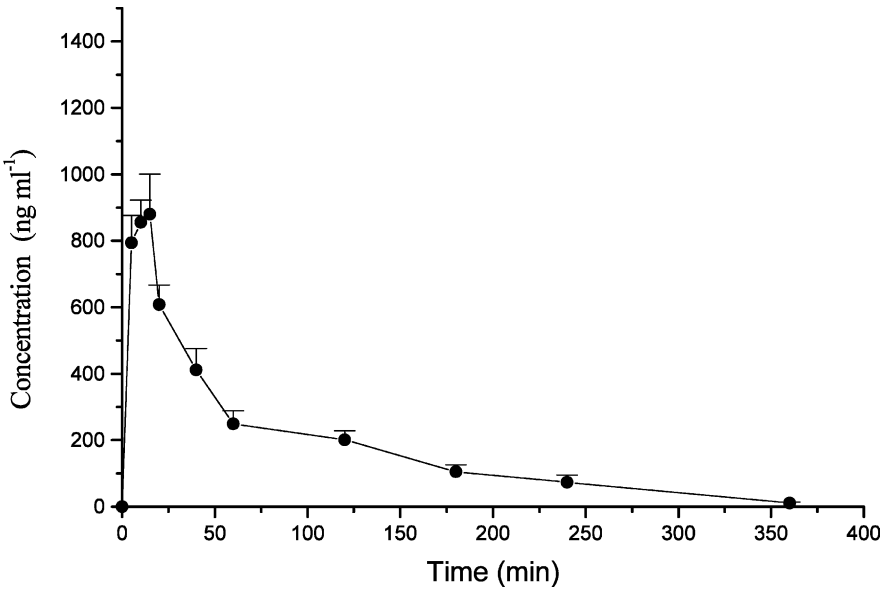

Fig. 4. Serum concentration–time curve of paeoniflorin in rats after oral administration of GXEH (at a dose containing 125 mg kg^{-1} paeoniflorin). Each point and bar represent the mean ± S.E.M. ($n = 5$).

Table 3
Pharmacokinetic parameters of paeoniflorin in rat serum ($n = 5$) after oral administration of extract of GXEH (at a dose containing 125 mg kg^{-1} paeoniflorin)

Parameter	Estimate (mean ± S.E.M.)
t_{max} (min)	14 ± 2.24
C_{max} (ng ml^{-1})	895.33 ± 88.35
$AUC_{0\rightarrow\infty}$ (ng min ml^{-1})	66496.03 ± 7079.72
$t_{0.5}$ (min)	66.26 ± 0.78
MRT (min)	92.91 ± 3.72

$AUC_{0\rightarrow\infty}$, the area under curve concentration–time; C_{max}, maximum concentration at t_{max}; $t_{0.5}$, elimination half life time; MRT, mean residence time.

recovery of extraction (over 90%) and elimination of the non-polar interfering impurities. The designed procedure fulfils the validation requirements and could be applied for in vivo studies.

Acknowledgements

We thank The Ministry of Science and Technology of China and the National Natural Science Foundation of China (39925040) for financial support.

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