Simultaneous Determination and Pharmacokinetic Studies on (3,4-Dihydroxyphenyl)-Lactic Acid and Protocatechuic Aldehyde in Rat Serum after Oral Administration of *Radix Salviae miltiorrhizae* Extract

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Abstract

A simple and sensitive high-performance liquid chromatography method is developed for the simultaneous determination of (3,4-dihydroxyphenyl)-lactic acid (Dhpl) and protocatechuic aldehyde (Pal) in rat serum after oral administration of *Radix Salviae miltiorrhizae* extract. Serum samples are acidified with hydrochloric acid and extracted with ethyl acetate. Analysis of the extract is performed on a reversed-phase column and a mobile phase of 0.02% phosphoric acid–acetonitrile (91:9, v/v) with UV detection at 280 nm. Standard curves are linear in the range of 1.47–456.96 µg/mL for Dhpl and 0.124–7.936 µg/mL for Pal. For both regression coefficients, r^2 is greater than 0.993. Mean recovery is determined to be 75.23% and 84.06%, respectively, by analyzing serum standard containing 7.14, 57.12, and 228.48 µg/mL of Dhpl and 0.124, 0.992, and 3.968 µg/mL of Pal. The intraday precision (relative standard deviation) ranges from 3.91% to 12.03% at concentrations of 1.43, 57.12, and 228.48 µg/mL for Dhpl and 3.79% to 8.12% at concentrations of 0.124, 0.992, and 3.968 µg/mL for Pal. The interday precision (relative standard deviation) ranges from 5.06% to 9.93% for Dhpl and 3.05% to 10.00% for Pal, respectively, at the same three concentrations. This validated assay is applied to the determination of Dhpl and Pal concentrations and used to take a limited view of the pharmacokinetic profile in rat serum after oral administration of *Radix Salviae miltiorrhizae* extract.

Introduction

Danshen, the roots of *Salvia miltiorrhiza* Bunge (Labiate), has been used in traditional Chinese medicine to treat coronary heart diseases, particularly angina pectoris and myocardial infarction (1). R-(+)-β-(3,4-dihydroxyphenyl)-lactic acid [named danshensu (Dhpl)] and protocatechuic aldehyde (Pal) have been considered the major active constituents of *Salvia miltiorrhiza*. They have been reported to exhibit anticogulant, antiarteriosclerotic, anti-inflammatory, and antihypoxic activities (2,3). There are some reported quantitative methods for the two compounds in biosamples (4,5). However, the samples were obtained only after administrating purified compounds or injections. Because most traditional Chinese medicines are administered orally as extracted powders or decoctions in clinics, the reported pharmacokinetcs could not be used as a suitable reference for clinical application. To obtain valuable pharmacokinetic data of the Dhpl and Pal, the current study describes the absorption and excretion of the two constituents, and their pharmacokinetic profiles were also evaluated and discussed.

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Experimental

Material and reagents

Danshen (the dried roots of *Salvia miltiorrhiza* Bunge) was purchased from the Zhongjiang Danshen Cultivation Base (Sichuan, 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.

The reference standard of Dhpl was purchased from Fudan University Medical School (Shanghai, China). Protocatechuic aldehyde and the internal standard (*p*-hydrobenzoic acid) were from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Hydrochloric acid and ethyl acetate of analytical grade and acetonitrile of high-performance liquid chromatography (HPLC) grade were obtained from Beijing Chemical Engineering Factory (Beijing, China).

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 an environmentally controlled breeding room for 3 days before starting the experi-

ments. They were fed with standard laboratory food and water (*ad libitum*) and fasted overnight before the test.

Chromatographic conditions

The HPLC system consisted of a Waters (Milford, MA) 600E pump, a Waters 2487 UV–vis detector set at 280 nm, a 100-µL injection loop, an 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 RP_{18} (5 µm) guard column (Dikma, Beijing, China), the mobile phase was 0.02% phosphoric acid–acetonitrile (91:9, v/v) filtered through a 0.45-µm milipore filter and degassed prior to use. The flow rate was 1 mL/min.

Content of Dhpl and Pal in the extract of *Radix Salviae miltiorrhizae*

To obtain the administered dose of Dhpl and Pal, their contents in the extract of *Radix Salviae miltiorrhizae* first had to be quantitated. The lyophilized extract of *Radix Salviae miltiorrhizae* was dissolved in distilled water and diluted to a concentration of 0.4 mg/mL, 2.0 mL of which was acidified with 1 mol/L HCl to pH 1–2. Then each portion was shaken with 4 mL of ethyl acetate for 5 min and centrifuged at 2500 rpm for 10 min, and then the organic layer was transferred into an empty tube. This procedure was repeated twice, and the collected organic layer was dried at 40°C under a nitrogen stream. The residue was dissolved in 1 mL of mobile phase, and 10 µL of this solution was injected into the HPLC column for analysis. The contents of Dhpl and Pal in the lyophilized extract of *Salvia miltiorrhiza* was determined to be 4.25% and 1.58%, respectively, from the peak area ratios using the equation for linear regression obtained from the calibration curve.

Standard solutions

Mixed stock solutions of Dhpl and Pal were prepared with 1% glacial acetic acid. These solutions were spiked into drug-free serum samples of rats to determine the recovery, precision, accuracy, and detection limit of the HPLC method. All standards were stored at 4°C before use.

Drug administration and blood sampling

Aqueous solution of *Radix Salviae miltiorrhizae* extracts were orally administrated to rats at a dose of 10 g/kg under anesthesia with sodium pentobarbital, and blood samples were collected from the abdominal aorta of each rat according to the specific schedule (0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, and 12 h after dosing). Thirty minutes after blood was withdrawn, 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.

Sample preparation

Aliquot portions (1.0 mL) of the serum sample were acidified with 1 mol/L HCl to pH 1–2. Then each portion was shaken with 4 mL of ethyl acetate (internal standard was dissolved in it at the concentration of 0.5 µg/mL) for 5 min and centrifuged at 2500 rpm for 10 min. Then the organic layer was transferred into an empty tube. This procedure was repeated twice and the organic layer collected was dried at 40°C under a nitrogen stream. The residue was dissolved in 1 mL mobile phase, and 10 µL of this solution was injected into the HPLC column for analysis.

Calibration procedure

The calibration curve was accomplished on the basis of the analysis of various concentrations of Dhpl (1.43, 7.14, 14.28, 28.56, 57.12, 114.24, 228.48, and 456.96 µg/mL) and Pal (0, 0.124, 0.248, 0.496, 0.992, 1.984, 3.968, and 7.936 µg/mL) spiked in rat serum by adding the same amount of internal standard to the serum by HPLC.

Recovery, precision, and accuracy

The recovery was determined by the standard addition method at concentrations of 1.43, 57.12, and 228.48 µg/mL for Dhpl and 0.12, 0.99, and 3.97 µg/mL for Pal. The precision (intraday and

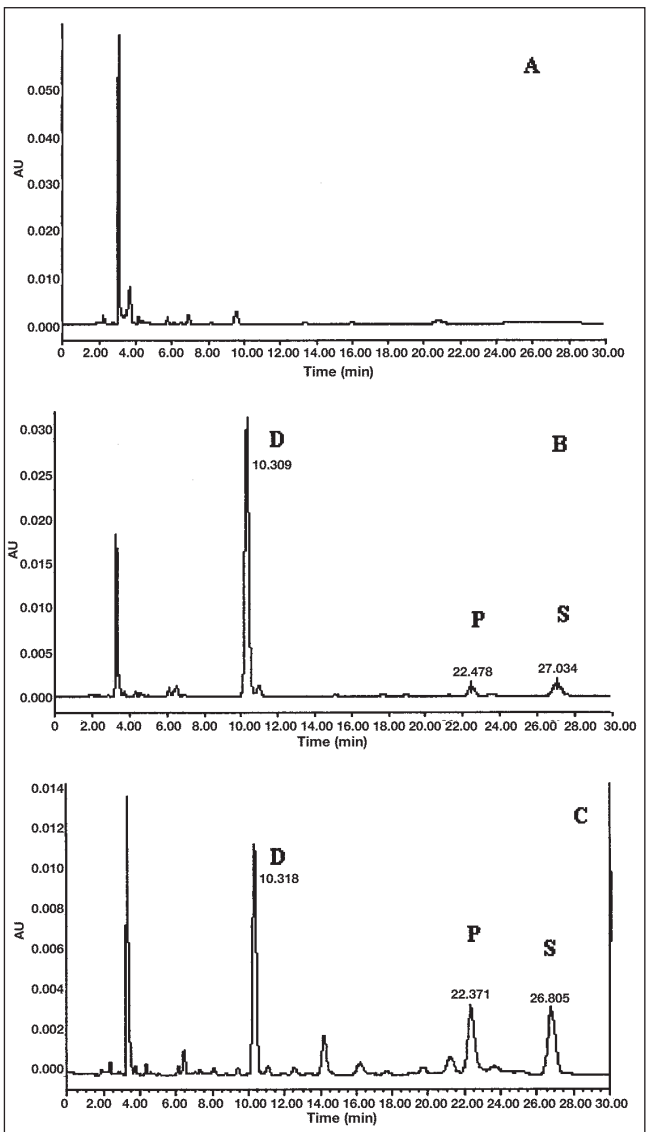

Figure 1. Typical chromatograms for the determination of Dhpl and Pal in serum samples: (A) chromatogram of a blank serum sample, (B) chromatogram of a serum sample spiked with Dhpl (D) and Pal (P) and internal standard (S), and (C) chromatogram of the serum sample of a rat taken 4 h after oral administration of the *Radix Salviae miltiorrhizae* extract.

interday) of the method was calculated at the same three concentrations. The variability of the peak-area ratio at each concentration was determined as a measure of the precision of the assay. The accuracy was determined by comparing the measured concentration with its true value.

Results

Selectivity

Under the described condition, the HPLC chromatograms of blank serum, serum spiked with Dhpl and Pal, and the serum obtained 4 h after oral administration of *Radix Salviae miltiorrhizae* extract are shown in Figure 1. The retention times of Dhpl, Pal, and *p*-hydrobenzoic acid (internal standard) were 10.3, 22.3, and 26.7 min, respectively. No interfering peaks were observed within the time frame in which Dhpl and Pal were detected.

Table I. Recovery of Dhpl and Pal Assay*

Compound	Conc. added (µg/mL)	%Recovery	%RSD	%Average
Dhpl	7.14	74.07 ± 4.90	6.62	
	57.12	74.97 ± 6.01	8.02	75.23
	228.48	76.87 ± 6.53	8.49	
Pal	0.124	84.73 ± 5.71	6.74	
	0.992	83.80 ± 4.65	5.55	84.06
	3.968	83.63 ± 4.72	5.64	

*Each value represents the mean ± SD (n = 3).

Table II. Validation of the Intraday Assay*

Compound	Conc. added (µg/mL)	Conc. measured (µg/mL)	%Accuracy	%RSD
Dhpl	1.43	1.33 ± 0.16	93.13	12.03
	57.12	54.97 ± 2.15	96.24	3.91
	228.48	214.82 ± 10.72	94.02	4.99
	0.124	0.124 ± 0.014	100.00	8.12
Pal	0.992	0.95 ± 0.05	96.4	5.4
	3.968	3.90 ± 0.15	98.3	3.79

* Each value represents the mean ± standard deviation (n = 5).

Table III. Validation of the Interday Assay*

Compound	Conc. added (µg/mL)	Conc. measured (µg/mL)	%Accuracy	%RSD
Dhpl	1.43	1.41 ± 0.14	98.60	9.93
	57.12	55.90 ± 3.00	97.86	5.37
	228.48	216.86 ± 10.97	94.91	5.06
	0.124	0.120 ± 0.012	96.77	10.00
Pal	0.992	0.95 ± 0.07	96.69	7.37
	3.968	3.93 ± 0.12	99.04	3.05

* Each value represents the mean ± standard deviation (n = 5).

Calibration curve

The calibration curve for the determination of Dhpl in rat serum was linear in the range of 1.43–228.48 µg/mL with a determination coefficient (r^2) of 0.996 (n = 8), and the limit of quantitation (LOQ) was 1.43 µg/mL. For Pal, the curve was linear in the range of 0.12–7.94 µg/mL with a determination coefficient (r^2) of 0.994 (n = 8) and its LOQ was 0.12 µg/mL. This linearity range would permit the use of this method for future pharmacokinetic studies.

Recovery test and reproducibility

The recoveries for Dhpl from rat serum were 74.07%, 74.97%, and 76.87% at the concentrations of 1.43, 57.12, and 228.48 µg/mL; and for Pal, the recoveries were 84.73%, 83.80%, and 83.63% at the concentrations of 0.12, 0.99, and 3.97 µg/mL, respectively (Table I). The intra- and interday precision assay also gave satisfactory results (Tables II and III).

Determinnation of Dhpl and Pal in serum

Serum samples from rats were taken at 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, and 12.0 h after the oral administration of the *Radix Salviae miltiorrhizae* extract. Figures 2 and 3 show the mean serum concentration versus time plot of Dhpl and Pal,

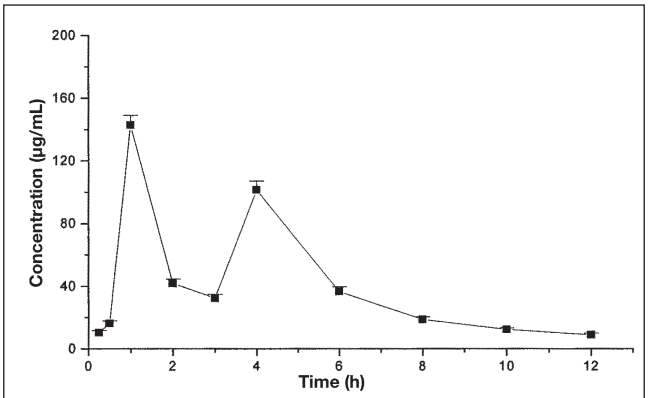

Figure 2. Plot of the concentration of Dphl in the serum of rats versus time after oral administration of *Radix Salviae miltiorrhizae* extract.

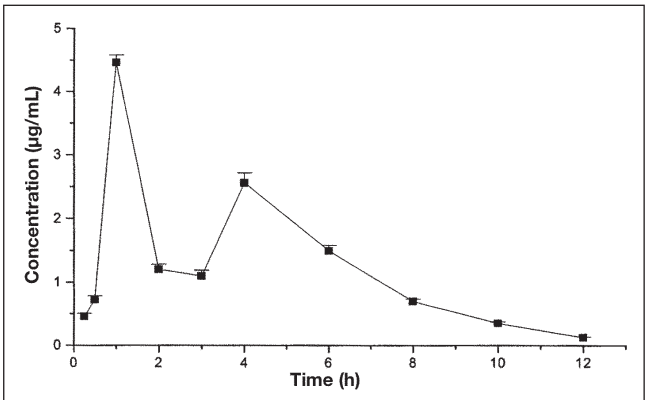

Figure 3. Plot of the concentration of Pal in the serum of rats versus time after oral administration of *Radix Salviae miltiorrhizae* extract.

respectively. The concentration was lower than the LOQ after 12 h. Based on the noncompartment analysis, the pharmacokinetic parameters were obtained [for Dhpl, mean residence time (MRT) = 3.43 ± 0.07 h, $AUC_{0-\infty}$ = 396.82 ± 17.27 µg • h/mL, for Pal, MRT = 4.31 ± 0.06 h, $AUC_{0-\infty}$ = 15.64 ± 0.66 µg • h/mL, respectively].

Discussion

In Figures 2 and 3, an obvious double-peak phenomenon could be observed at the times of 1 and 4 h. However, the MRT was enlarged greatly when compared with a previous investigation administered with the injections (4,5). The major factor that caused this difference was probably attributable to the different drugs and dosage forms administered. Because traditional Chinese medicines are usually administered orally in the form of crude extract in clinical practice, this study was designed to be consistent with this tradition. Therefore, the fate of the extract in the gastrointestinal tract should be considered. It is known that there are various polyphenolic acids in the aqueous extract of *Radix Salviae miltiorrhizae* (6). Among them, salvianolic acid A, B, C, D, E, H, I, and J; lithospermic acid B; isosalvianolic acid C; and rosemarinic acid all possess the Dhpl structural moiety (6–10). *Radix Salviae miltiorrhizae* was usually pretreated with either acid or alkali in previous studies (11), and the content of Dhpl in the extract would increase by a factor of two. In our procedure of preparing the extract, on the basis of direct HPLC chromatogram comparison, it was found that, as the refluxing time increases, the contents of Dhpl and Pal increase while the contents of polyphenolic acids decline. It was also proved that polyphenolic acids could be transformed to Dhpl and Pal under certain conditions. This mechanism might relate to the second peak that occurred in the concentration versus time plot. However, this was not the case in vivo, and there might have been some other mechanisms such as hepatoenteral circulation and redistribution in tissues.

Conclusion

This study describes a sensitive, specific, and rapid HPLC method with UV detection for the determination of Dhpl and Pal in rat serum. This method has been demonstrated to be suitable for use in pharmacokinetic studies of Dhpl and Pal in *Radix Salviae miltiorrhizae*.

Acknowledgments

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