

Original Article

Danshen-phospholipid complex for improved bioavailability: preparation, in vitro and in vivo evaluations

Yu Wang^{1*}, Yajun Shi^{1,2}, Suiling Zhang^{1*}, Junbo Zou^{1,2*}, Xiaofei Zhang^{1,2*}, Yulin Liang¹, Jia Tai¹, Dongyan Guo^{1,2}, Mei Wang^{1,2}

¹Department of Pharmaceutics, College of Pharmacy, Shaanxi University of Chinese Medicine, Xianyang 712000, Shaanxi, China; ²Key Laboratory of Basic and New Drug Research of Traditional Chinese Medicine, Shaanxi University of Chinese Medicine, Xianyang 712000, Shaanxi, China. *Equal contributors.

Received May 6, 2019; Accepted July 9, 2019; Epub August 15, 2019; Published August 30, 2019

Abstract: Object: In order to improve the oral bioavailability of *Salvia miltiorrhiza* extract (ESM), *S. miltiorrhiza* phospholipid complex (ESM-PC) was prepared by solvent evaporation. The relative proportions of Salvianolic acid B (Sal B) and Tanshinone IIA (Tan IIA) were investigated using the combination percentage as the standard. The physical and chemical properties of the phospholipid complex were studied by ultraviolet spectroscopy and infrared absorption. Solubility, octanol-water partition coefficient, dissolution rate and in vivo pharmacokinetics were also investigated. Results: ESM-PC with a drug to phospholipid ratio of 1:1 was successfully prepared in methanol at 50 °C for 1 h. The determined combination percentage was $55.25\% \pm 1.04\%$. Ultraviolet absorption and Fourier transform infrared spectroscopy confirmed the formation of ESM-PC. Relative to ESM, the solubility, octanol-water partition coefficient and in vitro dissolution rate of ESM-PC were improved to varying degrees in a pH-dependent manner. After intragastric administration in rats, the bioavailabilities of Sal B and Tan IIA in ESM-PC were 4.91- and 2.49-fold higher than those in ESM. Conclusion: The oral absorption of Sal B and Tan IIA were significantly improved using ESM-PC, mainly due to increased solubility and dissolution rate.

Keywords: *Salvia miltiorrhiza* extract, phospholipid complex, physicochemical properties, pharmacokinetics, microdialysis

Introduction

Salvia miltiorrhiza Bge. (Danshen) is a perennial herb that is used as a bulk commodity in Traditional Chinese Medicine [1]. The dried root and rhizome of Danshen are the most commonly used TCM in the clinic. The traditionally described effects of Danshen are removing stasis and relieving pain, cooling blood and invigorating circulation, nourishing blood and tranquilizing the mind [2]. Danshen has been used extensively in the clinic, primarily in the following areas [3-8]: (1) treatment of cardiovascular diseases. For example, Danshen injections dilate coronary arteries and increase coronary blood flow, which is used to prevent and treat cardiovascular diseases; (2) treatment of nervous system diseases. For example, compound Danshen injections have been shown to reduce

ischemia and hypoxia in brain cells and improve microcirculation; (3) treatment of digestive system diseases. Analysis has shown that Danshen improves microcirculation in the liver and promotes mucus secretion in the gastric mucosa. It is used to treat conditions including icteric hepatitis, cirrhosis, chronic gastritis and gastric ulcers [9]; (4) prevention of respiratory diseases. Compound Danshen tablets enhance resistance to upper respiratory tract infections and Danshen injections have been used to treat asthmatic bronchitis.

Danshen tablets are included in the 2015 edition of the Chinese Pharmacopoeia [10]. They have significant curative effects in the treatment of coronary heart disease, myocardial infarction and angina pectoris [11]. The active components of Danshen responsible for its

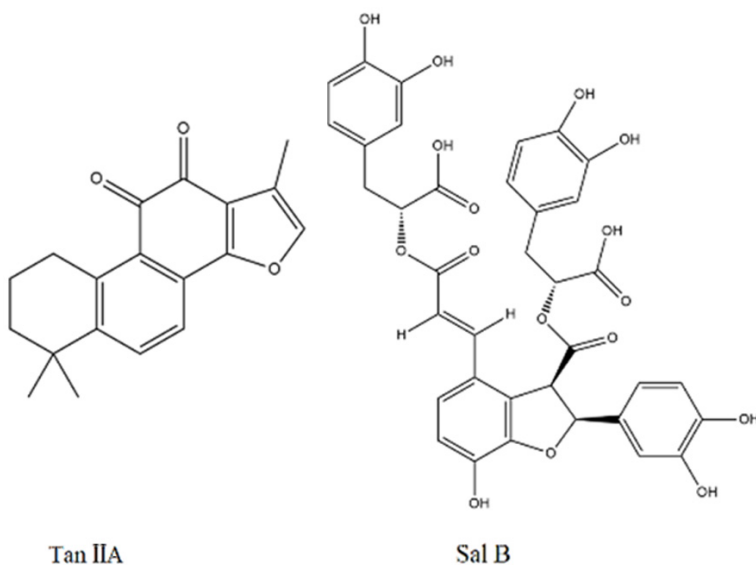


Figure 1. Structures of Tan IIA and Sal B.

therapeutic effects are divided into water-soluble (Sal B) and fat-soluble (Tan IIA) fractions [12, 13]. The poor water solubility of Tan IIA adversely affects oral absorption, resulting in a low bioavailability of 3.5% [14, 15]. The oral bioavailability of Sal B is only 2.3%, which seriously restricts its efficacy. Enhancing the oral bioavailability of active ingredients is one of the important aspects in modern research on Traditional Chinese Medicine [16]. To address the problems of low bioavailability and unreliable efficacies of TCM preparations, new dosage forms are needed to improve their clinical utilities. These problems have become a hot topic and challenging area of research for Chinese medicinal preparations [17].

Phospholipid complex (PC) is a drug-loading system first discovered by the Italian scholar, Bombardelli. It is formed by the interaction of drugs and phospholipids through intermolecular forces in a proton transfer solvent. The complex enhances drug solubility and facilitates absorption through the cell membrane [18]. To date, a variety of phospholipid complex products have been marketed globally [19, 20]. It has been reported that *Ginkgo biloba* extract phospholipid complex can be used for cardiovascular protection and as an anti-aging agent. Grape seed phospholipid complex can be used to prevent heart disease. Blueberry extract phospholipid complex is an antioxidant and improves vascular elasticity. Phospholipid complexes are also used in various formulations,

such as capsules, gels, granules, and emulsions. In addition, phospholipid complexes are widely used in cosmetics and health care products.

Significant achievements have been reported on improving the fat solubility of drugs and enhancing drug absorption [21]. Zhu studied the preparation, physicochemical properties and bioavailability of magnolol phosphatidylcholine complex [22]. Those results showed that the main pharmacokinetic parameters C_{max} , T_{max} , area under the curve (AUC) and mean residence time (MRT) were significantly changed in the complex. Fur-

thermore, oral bioavailability was increased by 97% and the complex exhibited sustained release characteristics. Ye studied the mechanism of gastrointestinal absorption and pharmacokinetics of baicalin-phospholipid complex in rats after oral administration [23]. The results showed that absorption of baicalin-phospholipid complex was about two-fold higher than that of baicalin. The plasma AUC value was nearly 3 times higher, the brain AUC value was nearly 1.5 times higher, and the oral bioavailability was nearly 4 times higher than that of baicalin.

In order to address the low oral bioavailability of the main medicinal components in Danshen tablets, this study applied phospholipid complex technology to prepare ESM-PC and assess its basic properties and pharmacokinetics. The mechanism by which the phospholipid complex enhanced fat solubility and bioavailability of ESM was also investigated.

Materials and methods

Materials

Sal B (batch number: 111562-201313) and Tan IIA (batch number: 110766-201520) were purchased from China Food and Drug Control Institute (Beijing, China). Danshen was purchased from Xi'an Shengxing Pieces Co. Ltd. (Xi'an, China). Soy phospholipids (95%) were purchased from Shanghai Taiwei Co. Ltd. (Shanghai, China). Purified water was purchased from Wahaha Group Co. Ltd. (Hangzhou,

Phospholipid complex for improved bioavailability

China). Methanol and acetonitrile were of HPLC grade, and all other chemical reagents employed in the experiments were of analytical grade.

Preparation of ESM-PC

Chromatographic separations were achieved by reverse phase high performance liquid chromatography (RP-HPLC, Waters 2695 separation module, Waters Co., MA, USA) using a C18 column (250 × 4.6 mm, 5 µm i.d.; Thermo Fisher Scientific Inc., USA). The mobile phase was formic acid solution (0.02 M): acetonitrile at a flow rate of 1 mL/min in isocratic mode. Sal B and Tan IIA were detected at 275 nm (λ_{\max}) using an ultraviolet (UV) detector.

Preparation of Danshen tablet extract

ESM was prepared according to the preparation method described for Danshen tablets in the 2015 Chinese pharmacopoeia [10]. A specific amount of *S. miltiorrhiza* Bge. was weighed and treated with 8 volumes of 90% ethanol solution. The mixture was heated at reflux for 1.5 h and then filtered. The retained solids were boiled in water for 1.5 h to obtain a decoction. The combined ethanol and aqueous extracts were then concentrated in a water bath (60°C). The concentrate was dried under reduced pressure to a dry powder and stored in a desiccator.

Preparation process of ESM-PC

Factors including reaction solvent, temperature, drug to phospholipid ratio, concentration and time were investigated.

ESM and phospholipids were combined by solvent evaporation at a mass ratio of 1:1 in methanol. The mixture was kept at 50°C for 1 h with magnetic stirring. The resultant solution was evaporated under reduced pressure. Petroleum ether was added to dissolve the phospholipids and ESM-PC, and the mixture was filtered. The filtrate was evaporated, and the residue was then dried and crushed in a mortar. The obtained ESM-PC was stored at room temperature.

ESM-PC combination percentage

The combination percentage of ESM-PC was determined as follows. An appropriate amount of the phospholipid complex was weighed and

dissolved in methanol. After filtration through a 0.22 µm microporous membrane, the content of Sal B in the ESM-PC was determined according to the content determination method. The combination percentage of ESM-PC was obtained using the following equation:

$$\text{combination percentage} = \frac{m_1}{m_2} \times 100\%$$

Where “ m_1 ” is the content of Sal B in the ESM-PC, and “ m_2 ” is the content of Sal B in the ESM.

Characterization of ESM-PC

Ultraviolet absorption spectroscopy

Samples of ESM, ESM-PC, phospholipid, physical mixture, Sal B, and Tan IIA were dissolved in methanol to prepare solutions of known concentration and then scanned over a wavelength range of 200-400 nm using a Shimadzu UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan).

Fourier transform infrared (FT-IR) spectroscopy

Potassium bromide (KBr) was ground through a sieve and dried at 120°C for more than 4 h. Infrared (IR) spectroscopic analyses of ESM, ESM-PC, phospholipid and physical mixture were performed using a Nicolet 640-IR spectrophotometer (Agilent, Waltham, MA, USA). Measurements were conducted in conventional transmission mode with KBr pellets, and the background spectrum was collected under identical conditions. Spectra were recorded in the range of 400-4000 cm^{-1} .

In vitro studies

Chromatographic separations were achieved by RP-HPLC (Waters 2695 separation module, Waters Co., MA, USA) using a C18 column (250 × 4.6 mm, 5 µm i.d.; Thermo Fisher Scientific Inc.). The mobile phase was formic acid solution (0.02 M):acetonitrile at a flow rate of 1 mL/min in isocratic mode. Sal B and Tan IIA were detected at 275 nm (λ_{\max}) using an ultraviolet detector.

Solubility

ESM (300 mg), ESM-PC (600 mg) and physical mixture (900 mg) were separately added to phosphate buffer solutions (1.5 mL) at different pH values (2.0, 5.0, 5.8, 6.8, 7.0, 7.6 and 8.0). The samples were vortexed, shaken for 24

Phospholipid complex for improved bioavailability

h at 37°C in a water bath oscillator and then centrifuged at 3000 rpm for 10 min. The concentrations of Sal B and Tan IIA in the supernatant were determined by HPLC.

Octanol-water partition coefficient

An appropriate amount of n-octanol was added to an equal volume of deionized water. The mixture was then separated completely, and the two phases were collected separately. Appropriate amounts of ESM and ESM-PC were added to the n-octanol solution. The concentration of the ESM in n-octanol solution was 1.0, 0.5, 1.0, 1.5 and 2.0 mg/mL. The n-octanol solution (2 mL) was mixed with 2 mL of the aqueous phase or phosphate buffer (pH = 2.0, 5.0, 5.8, 6.8, 7.0, 7.6 and 8.0), and ESM or ESM-PC was equilibrated between the two phases by shaking at 37°C for 12 h.

The prepared samples were centrifuged and then 500 µL water and octanol solution were diluted separately. The octanol-water partition coefficients of the two components in ESM or ESM-PC were calculated based on their concentrations in the two phases [24]. The concentrations of Sal B and Tan IIA in the supernatant were determined by HPLC.

Dissolution study

Appropriate amounts of ESM and ESM-PC were weighed and placed in a capsule for testing [25]. The dissolution media were HCl (0.1 mol/L) and pepsin (artificial intestinal juice), phosphate buffer (0.1 mol/L) and trypsin (artificial gastric juice) and water. The paddle was rotated at 100 rpm and the temperature of the dissolution medium was maintained at 37°C. Samples (2 mL) were taken at predetermined intervals (10, 20, 30, 40, 60, 90, 150 and 180 min) and replaced with the same volume of dielectric solution. Samples were analyzed by HPLC after filtration through a 0.22 µm microporous membrane. All assays were performed six times.

Pharmacokinetic study of ESM and ESM-PC in SD rats by the microdialysis technique

Instruments and UPLC-MS/MS conditions

The LC-MS system consisted of a 20A HPLC system and Q-trap 4500 mass spectrometer. The data were collected using Analysis 1.6.2 software. Tan IIA and its internal standard,

diphenhydramine, were scanned by electrospray ion source (ESI) in positive ion detection mode. Sal B and its internal standard, bergenin, were scanned in negative ion detection mode. The mass spectrometry conditions were optimized and included the following parameters: multi-reaction ion detection mode (MRM); spray voltage of 4.5 kV; capillary temperature of 550°C; auxiliary gas (nitrogen) pressure of 50 psi; and a collision gas (argon) pressure of 6 psi.

Chromatographic separation was achieved on a Hypersil GOLD column (100 × 2.1 mm, 1.9 µm) at 40°C. The mobile phase consisted of acetonitrile containing 0.2% formic acid with a gradient elution starting at 5% acetonitrile and progressing linearly to 30% acetonitrile over 3 min then to 70% acetonitrile from 3-6 min. The gradient was increased to 90% acetonitrile at 6.1 min, returning to 5% at 9.1 min. The flow rate was 0.3 mL/min and the total analysis time was 10 min. Sample solution (5 µL) was injected using a process to wash the outer wall of the sample needle after each injection.

Animals

Male Sprague-Dawley (SD) rats (220 ± 30 g) were obtained from the Chengdu Da Shuo Biological Co., Ltd. Rats were fasted for 24 h before the experiment and were given free access to water. Twelve animals were divided randomly into two groups (n = 6 each), including those dosed with ESM (53.9 mg/100 g) or ESM-PC (1:1, 107.8 mg/100 g). ESM-PC was dissolved in sodium carboxymethyl cellulose solution. The gastric irrigator was inserted along the angle of the rat mouth, entering the oesophagus from the tongue surface along the upper jaw. The drug solution was injected, followed by dialysate after 10 min. The jugular vein was isolated, the blood probe implanted, and the dialysate was collected after 0.5 h. Blood samples were collected at predetermined time points (0.08, 0.17, 0.25, 0.42, 0.58, 0.75, 0.92, 1.25, 1.58, 1.92, 2.42, 2.92, 3.42, 3.92, 4.92, 5.92, 7.92, 9.92 and 11.92 h). The research was approved by the Ethical Committee of Shaanxi University of Chinese Medicine.

UPLC-MS/MS method validation

Selectivity: Bergenin and diphenhydramine were precisely weighed and dissolved in Ringer's

Phospholipid complex for improved bioavailability

Table 1. Combination percentages at different reaction temperatures

Drug to phospholipid ratio	Combination percentage (%)		
	40°C	50°C	60°C
1:0.5	30.77 ± 1.02	31.66 ± 1.24	30.29 ± 1.95
1:1	51.57 ± 2.17	56.67 ± 2.31	54.72 ± 2.07
1:1.5	43.80 ± 1.68	59.12 ± 1.36	51.82 ± 1.54
1:2	52.93 ± 1.46	70.64 ± 1.81	49.70 ± 1.21

test solution to 100 mL as internal standard reserve solution. The internal standard stock solution was diluted to obtain mass concentrations of 12.5 µg/L and 0.000675 µg/L, respectively. The microdialysis probe was implanted into the jugular vein of the rat and perfused with 2 µL·min⁻¹ of blank Ringer's solution to obtain a rat blank dialysate. Sal B, Tan IIA, internal standard bergenin and diphenhydramine were added to the blank dialysate and blank dialysate, and the 90-minute sample was administered to the rats to examine the specificity. The sample was added with the internal standard solution, vortex-mixed and then analysed. The microdialysis probe was implanted into the jugular vein of the rat, and blank dialysate was obtained by perfusing with blank Ringer solution at 2.0 µL/min. The blank dialysate and blank dialysate containing Sal B, Tan IIA, and the internal standards bergenin and diphenhydramine were administered intragastrically to the rats. The specificity of the method was investigated.

Linearity, precision, stability, matrix effect: A series of Sal B reference solutions containing 39.06, 78.13, 156.25, 312.50, 625.00, 1250.00 and 2500.00 µg/L were prepared by dissolving Sal B standard in Ringer reagent. Similarly, solutions of Tan IIA containing 0.39, 0.78, 1.56, 6.25, 12.50, 25.00 and 50.00 µg/L were prepared. Calibration curves were constructed by analysing known concentrations in standard plasma.

Sal B (10 µL) and Tan IIA (10 µL) were accurately measured and then bergenin (10 µL) and diphenhydramine (10 µL) were added, followed by rat plasma. Quality control (QC) samples of Tan IIA and Sal B were prepared at concentrations of 78.13, 312.50, 1250.00 µg/L and 0.39, 1.56, 12.50 µg/L, respectively. Three batches of Sal B and Tan II A were analysed 6 times a day for 3 consecutive days and the RSD

values (%) in rat plasma were calculated.

The QC samples of Sal B and Tan IIA were analysed after storage at 4°C for 24 h, at room temperature for 24 h, at -20°C for 30 days and after three freeze-thaw cycles to determine stability and RSD.

The blank plasma of rats was taken to prepare three quality control samples of high, medium and low concentrations, each taking 30 µL, adding internal standard, and mixing the samples. Then 10% methanol solution was used to prepare the same concentration of reference solution, each taking 30 µL, adding internal standard, mixing sample analysis. Five samples were measured in parallel for each concentration.

In vivo recovery: After the microdialysis probes had been implanted into the jugular veins of normal rats, Ringer solutions containing Sal B and Tan IIA at low, middle and high concentrations (78.13, 312.50, 1250.00 µg/L and 0.75, 3.13, 6.25 µg/L) were perfused into the probes. The rats were perfused with 2 µL/min for 2 h. The dialysate was collected every other 10 min and the concentrations of Sal B and Tan IIA in the samples were determined. The *in vivo* recovery rate was calculated.

Pharmacokinetic analysis

The concentration of free drug should be corrected according to the *in vivo* recovery, so the concentration of free drug in the blood is the ratio of the sample concentration ($C_{\text{dialysate}}$) to the recovery rate (RL). Drug concentration-time curves for Sal B and Tan IIA were obtained by determining blood concentration and sampling the midpoint time of dialysate in each group. The pharmacokinetic parameters for Sal B and Tan IIA were estimated by non-compartmental modelling using DAS 3.2.8 software.

Results

Preparation of ESM-PC

In this study, solvent evaporation was used to prepare ESM-PC. The Sal B content in the ESM-PC was determined according to the content determination method. This method avoids

Phospholipid complex for improved bioavailability

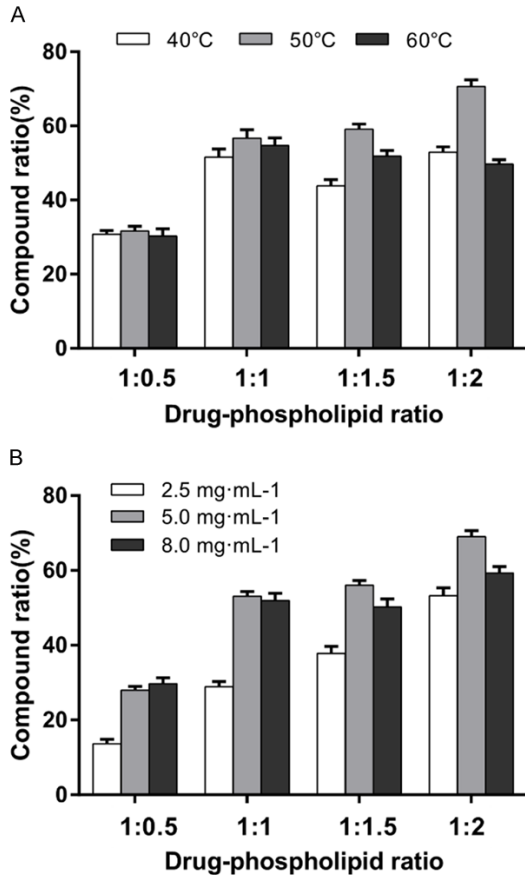


Figure 2. A. Combination percentages of Sal B from ESM-PC at different drug:phospholipid ratios and temperatures (n = 3). B. Combination percentages of Sal B from ESM-PC at different drug:phospholipid ratios and concentrations (n = 3).

the influence of adsorption by the filter paper on the measurement and enables calculation of the combination percentage of various components in the ESM-PC. Factors including reaction solvent, temperature, drug to phospholipid ratio, concentration and time were investigated.

The optimum temperature and concentration conditions were studied using drug to phospholipid ratios of 1:0.5, 1:1, 1:1.5 and 1:2. The results show that the combination percentage in all four complexes was higher when the reaction temperature was 50°C (Table 1, Figure 2A).

The combination percentage gradually increased and tended towards a plateau as the reaction concentration was increased to 5.0 mg/mL. Thus, the reaction concentration was set at 5.0 mg/mL (Table 2, Figure 2B).

The strong hygroscopicity of the phospholipids affected the stability of the complex as the proportion of phospholipids was increased. The stability of the drugs will also be affected, which is undesirable for production and storage. The results showed that at drug to phospholipid ratios of 1:0.5, 1:1, 1:1.5, 1:2, the combination percentages were 32.56% ± 0.67%, 55.25% ± 1.04%, 56.86% ± 1.38% and 69.09% ± 1.31%, respectively. The drug to phospholipid ratio was therefore set to 1:1. The optimum conditions for the reaction solvent and time were investigated when the drug to phospholipid ratio was 1:1. The combination percentage was used as a reference indicator. In the present study, four organic solvents with different dielectric constants were explored, including methanol, ethyl acetate, chloroform, and anhydrous ethanol. The combination percentages in these solvents were 52.40% ± 1.58%, 26.66% ± 2.04%, 47.32% ± 1.81% and 20.46% ± 2.17%, respectively. The combination percentage was highest in methanol, which was chosen as the reaction solvent. In addition, the reaction time was also studied. The combination percentages at 0.5, 1.0 and 1.5 h were 40.16% ± 1.62%, 53.72% ± 2.08% and 55.12% ± 2.23%, respectively. The combination percentage of Sal B in ESM-PC reached a comparatively high value when the reaction time was 1 h.

ESM and phospholipids were combined by solvent evaporation at a mass ratio of 1:1 in methanol. The mixture was kept at 50°C for 1 h with magnetic stirring. Three batches of phospholipid complexes were prepared according to the above preparation process for verification experiments. The resulting combination percentage was 55.25% ± 1.04%.

Characterization of ESM-PC

Ultraviolet absorption spectroscopy

The results from UV spectroscopy indicated that there were no absorption peaks for soybean lecithin at wavelengths of 200-400 nm. The absorption spectra of the physical mixture and ESM-PC were the same shape, and both had large absorptions at 206, 275, and 286 nm. The spectrum of Sal B standard had large absorptions at 206 and 286 nm, and that of Tan IIA had a large absorption at 275 nm. These observations suggested that there were no changes in the chromogenic structures of Sal B or Tan IIA in the ESM-PC.

Phospholipid complex for improved bioavailability

Table 2. Combination percentages at different concentrations

drug to phospholipid ratio	combination percentage (%)		
	2.5 mg·mL ⁻¹	5.0 mg·mL ⁻¹	8.0 mg·mL ⁻¹
1:0.5	13.64 ± 1.17	27.96 ± 1.03	29.67 ± 1.64
1:1	28.92 ± 1.35	53.09 ± 1.27	51.97 ± 1.96
1:1.5	37.82 ± 1.90	56.02 ± 1.31	50.21 ± 2.13
1:2	53.20 ± 2.17	69.00 ± 1.66	59.31 ± 1.75

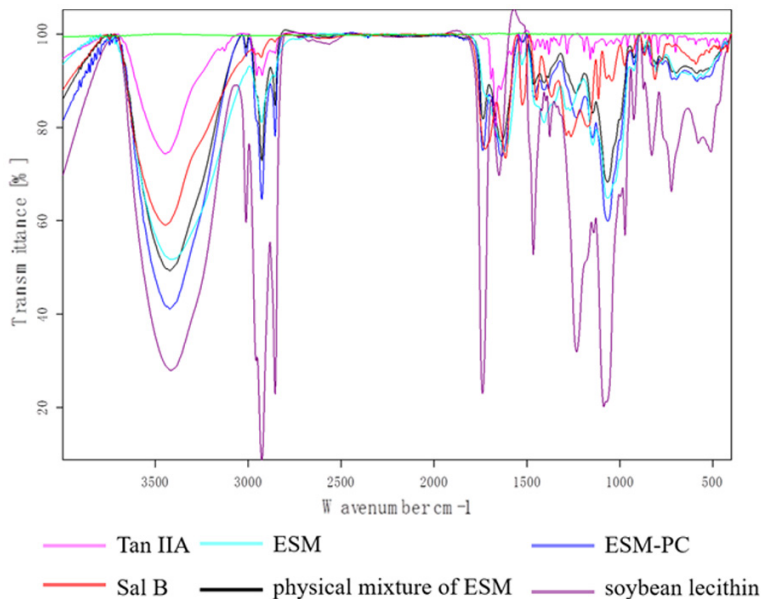


Figure 3. FT-IR spectra of Tan IIA, Sal B, ESM, physical mixture of ESM, ESM-PC and soybean lecithin.

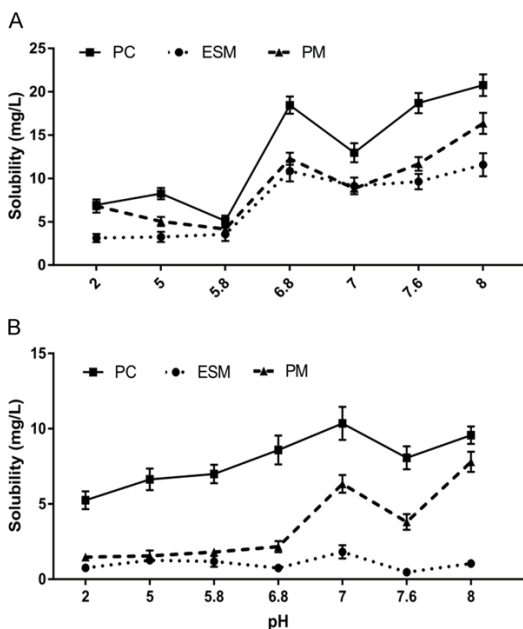


Figure 4. Equilibrium solubilities of Sal B (A) and Tan IIA (B) from ESM and ESM-PC. Data are expressed as mean ± SEM (n = 3).

FT-IR spectroscopy

The FT-IR spectra are shown in **Figure 3**. The characteristic absorption peaks for ESM were 3418 cm⁻¹ (-OH), 1659 cm⁻¹ (C = O), 1169 cm⁻¹ (C-O), and benzene ring vibrations near 1513 cm⁻¹. In the phospholipid spectrum a hydroxyl stretching band at 3380 cm⁻¹, C-H stretching of long fatty acid chains at 2924 and 2855 cm⁻¹, a carbonyl stretching band at 1738 cm⁻¹, a P=O stretching band at 1241 cm⁻¹, a P-O-C stretching band near 1124 cm⁻¹, and N⁺ (CH₃)₃ stretching at 969 cm⁻¹ were observed.

The infrared spectrum of the physical mixture had several characteristic absorption peaks for ESM and soybean lecithin, corresponding to the superposition of the main peaks from ESM and soybean lecithin spectra, indicating that there was no interaction between the mixture of ESM and phospholipid. Compared with the spectrum of ESM,

peak shapes and intensities were changed at 2800 and 1700 cm⁻¹ in the spectrum of ESM-PC. This suggested that some functional groups in the structure of ESM interacted with functional groups in the soybean lecithin molecule during phospholipid complexation. However, the basic chemical structures of Sal B, Tan IIA and soybean lecithin in ESM-PC were unchanged. The ESM-PC is not a new compound, nor a simple mixture, but a new state of bonding.

In vitro studies

Solubility

The solubilities of Sal B and Tan IIA in ESM, ESM-PC and physical mixture (PM) formulations are summarized in **Figure 4**. The solubilities of Sal B and Tan IIA varied slowly in the range of pH 2.0-5.8 and then increased from pH 5.8 to pH 6.8. The solubility of Sal B in ESM-PC was greatest (20.76 mg/L) at pH 8. Sal B is soluble in water, ethanol and methanol, and

Phospholipid complex for improved bioavailability

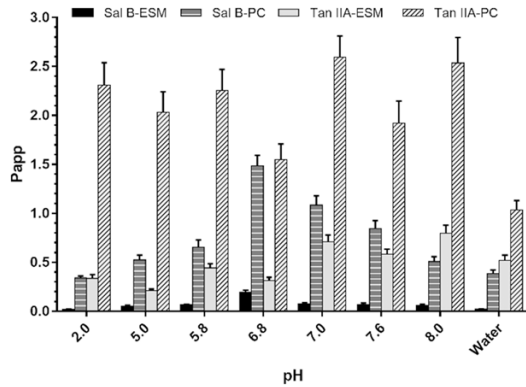


Figure 5. Octanol-water partition coefficients of Sal B and Tan IIA from ESM and ESM-PC. Data are expressed as mean \pm SD (n = 3).

has two carboxyl groups that form salts and are easily ionized under alkaline conditions. Tan IIA is soluble in ethanol, acetone, ether, benzene and other organic solvents, but only slightly soluble in water. The solubility of Tan IIA in ESM-PC was greatest (10.35 mg/L) at pH 7. The solubility of ESM was significantly improved in phosphate buffer after phospholipid modification, suggesting that *in vivo* drug absorption would be facilitated.

Octanol-water partition coefficient experiment

The octanol-water partition coefficients ($\log P$) for ESM and ESM-PC are shown in **Figure 5**. The octanol-water partition coefficient of Sal B has a normal distribution over a range of pH and reached a maximum at pH 6.8. The octanol-water partition coefficient of Tan IIA changes has the highest point in the range of pH. In water, the $\log P$ values of Sal B and Tan IIA in ESM were 0.018 and 0.522, respectively. Compared with the values in ESM, the octanol-water partition coefficients of Sal B and Tan IIA were higher in ESM-PC with values of 0.385 and 1.033, respectively. These results indicate that phospholipids enhance the fat-solubility of these components from ESM.

Dissolution study

The dissolution profiles of Sal B and Tan IIA from ESM and ESM-PC are shown in **Figure 6**. Sal B dissolved more rapidly from ESM than from ESM-PC when the pH was simulated gastric juice (pH 2) and intestinal juice (pH 6.8). After approximately 30 min, the dissolution from ESM-PC became more rapid and reached

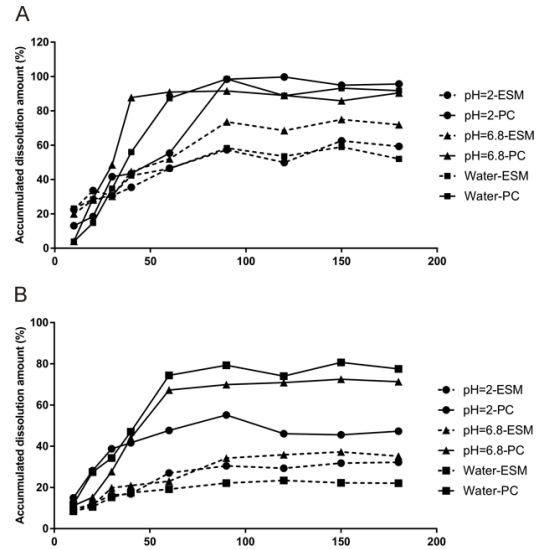


Figure 6. Dissolution profiles of Sal B (A) and Tan IIA (B) from ESM and ESM-PC. Data are expressed as mean \pm SD (n = 6).

80% at equilibrium after 90 min. This indicated that dissolution of Sal B from ESM-PC was slower than that from ESM, providing a sustained release and long-acting effect. In gastric juice (pH 2), the dissolution of Tan IIA from ESM-PC was slower than that from ESM. The dissolution rate from ESM-PC was significantly higher than that from ESM at pH 6.8 and aqueous intestinal liquid. The results showed that the phospholipid complex improved the dissolution of Tan IIA and Sal B from ESM.

Pharmacokinetics in rats

UPLC-MS/MS method validation [26]

Selectivity: Baseline noise in the blank dialysate was low. No endogenous interference was observed at the retention times of Sal B, Tan IIA or their internal standards.

Linearity: The Sal B and Tan IIA standard curves in plasma samples were linear over the concentration ranges of 39.06-2500.00 $\mu\text{g/L}$ and 0.39-50.00 $\mu\text{g/L}$, respectively. The regression equations for Sal B and Tan IIA were $Y = 0.8103X + 12.921$ ($R^2 = 0.9986$) and $Y = 159.36X + 172.42$ ($R^2 = 0.9991$).

Precision: The intra- and inter-day precision data for Sal B and Tan IIA are summarized in **Table 3**. The intra- and inter-day precisions for Sal B were < 3.25% and < 3.96%, respectively.

Phospholipid complex for improved bioavailability

Table 3. Precision and matrix effect for Sal B and Tan IIA in rat plasma (n = 5)

Compound	Concentration (μgL^{-1})	Intra-day RSD (%)	Intra-day RSD (%)	Matrix effect (% mean \pm SD)
Sal B	78.13	4.17	4.82	92.25 \pm 3.05
	312.50	3.25	4.71	87.19 \pm 2.19
	1250.00	3.51	3.96	96.57 \pm 4.71
Tan IIA	0.39	4.97	4.57	85.25 \pm 4.39
	1.56	3.12	4.25	93.96 \pm 5.28
	12.50	2.53	4.10	113.50 \pm 4.19

Table 4. Stability of Sal B and Tan IIA in rat plasma (n = 5)

Compound	Concentration (μgL^{-1})	4 h, room temperature	24 h, 4 °C	30 d, -20 °C	freeze-thaw cycles
		RSD (%)	RSD (%)	RSD (%)	RSD (%)
Sal B	78.13	4.27	2.14	1.27	5.61
	312.50	3.52	2.58	2.39	6.17
	1250.00	4.36	2.07	1.85	5.06
Tan IIA	0.39	3.19	1.96	1.07	7.06
	1.56	2.55	1.72	1.53	6.82
	12.50	3.24	1.03	1.29	6.17

The intra- and inter-day precisions for Tan IIA were < 2.53% and < 4.10%, respectively.

Stability, matrix effect: The results indicated that Sal B and Tan IIA were stable in plasma at -20 °C for 30 days, at 4 °C for 24 h and at room temperature for 24 h. The stability of the samples was decreased after three freeze-thaw cycles [27]. No significant matrix effect was observed for Sal B or Tan IIA (**Table 4**). Stability test data for Sal B and Tan IIA in rat plasma under different conditions are shown in **Table 5**.

In vivo recovery: During the sampling period, the recovery rate of the probe was stable, with an RSD of < 3%. The results showed that there was no significant difference in the recovery rate at the perfusion concentration, and that the method was suitable for pharmacokinetics study of extracts and their phospholipid complexes *in vivo*.

Application of the method in oral bioavailability experiments: The mean plasma concentration-time (c-t) curves for Sal B and Tan IIA are presented in **Figure 7**. Compared with ESM, the pharmacokinetic characteristics of Sal B and Tan IIA were enhanced in ESM-PC. As expected,

plasma concentrations of Sal B and Tan IIA from ESM-PC were much higher than those from ESM at 0.75 and 0.96 h. Interestingly, the four curves are characterized by a rapid increase and slow decrease with a single peak. The pharmacokinetic parameters are shown in **Table 6**. The T_{max} and MRT of ESM-PC were higher than those of ESM, indicating that the drug had a longer retention time *in vivo* and exhibited sustained release. The C_{max} of Sal B and Tan IIA increased from 1723.31 and 43.06 $\mu\text{g/L}$ after dosing with ESM to 4724.01 and 118.32 $\mu\text{g/L}$ after dosing with ESM-PC, respectively. The bioavailabilities of Sal B and Tan IIA from ESM-PC were 4.91 and 2.49 times higher, respectively, than those from ESM.

Discussion

Most Traditional Chinese medicines have been used in clinical practice for thousands of years. Modern research has found that many of these medicines have clear pharmacological activities and clinical efficacy. Some of the active ingredients have received significant scientific attention worldwide. Oral preparations of Traditional Chinese medicines are widely used because of their safety and convenience, especially in the treatment of chronic diseases that require long-term use of drugs. Flavonoids, triterpenoids, saponins, lactones and other active components are characterized by poor fat solubility, high polarity or high molecular weight. These properties lead to poor oral absorption and low bioavailability, thus, affecting their clinical application. Modification of these drugs is therefore necessary to improve the solubility of poorly soluble drugs and promote the permeability of drugs through biofilms. Ultimately, the goal is to improve the oral bioavailability of drugs [28, 29].

The fat-soluble component of ESM is the terpenoid Tan IIA, which has potential therapeutic effects in the prevention and treatment of cardiovascular and cerebrovascular diseases [30, 31]. The water-soluble component is the pheno-

Phospholipid complex for improved bioavailability

Table 5. In vivo recovery for analysis of Sal B and Tan IIA in rat plasma (n = 5)

Type of probe	Sal B				Tan IIA			
	C ($\mu\text{g}\cdot\text{L}^{-1}$)	RL (%)	Mean (%)	RSD (%)	C ($\mu\text{g}\cdot\text{L}^{-1}$)	RL (%)	Mean (%)	RSD (%)
Blood probe	78.13	29.37 ± 1.17	28.66 ± 1.34	1.20	0.75	19.80 ± 1.27	20.27 ± 1.29	2.61
	312.50	28.64 ± 0.94			3.13	20.16 ± 0.82		
	1250.00	27.97 ± 1.91			6.25	20.84 ± 1.78		

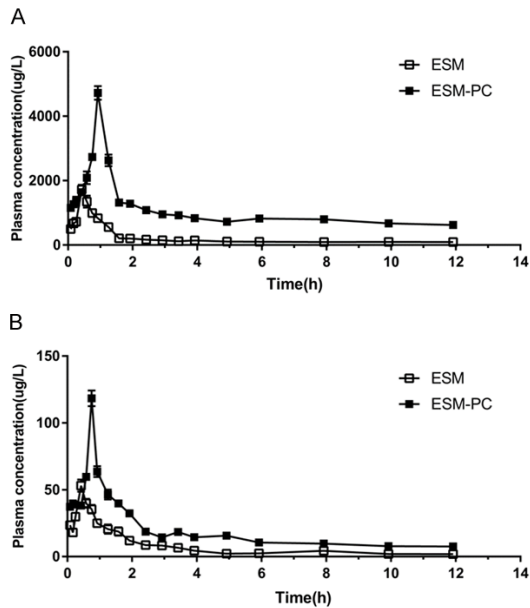


Figure 7. Plasma concentration-time profiles of Sal B (A) and Tan IIA (B) after oral administration of ESM and ESM-PC. Data are expressed as mean \pm SD (n = 5).

lic acid Sal B, which plays significant roles as an antithrombotic, anti-platelet aggregation and in cell protection [12, 32, 33]. According to the literature, the oral bioavailabilities of Sal B and Tan II A are relatively low [34, 35]. These drugs therefore have poor oral absorption, which affects their clinical efficacy. As a new drug delivery system, phospholipid complexation is increasingly used in Traditional Chinese medicine preparations and is regarded as a major breakthrough in traditional medicine research. Compared to ESM, ESM-PC has different physical and chemical properties, biological characteristics and pharmacological activities. The complex enhances the pharmacological action and improves the bioavailability of the component drugs. The complex can also delay the release of drug into the body and provide a sustained release effect. In addition, drug irritation and adverse reactions are effectively reduced

[36-38]. In this study, Sal B and Tan IIA from Danshen were used as research indicators to determine the basic properties and pharmacokinetic behaviours of ESM and ESM-PC.

The ultraviolet and infrared spectroscopic characteristics of ESM, ESM-PC, phospholipid and physical mixture were studied. The ultraviolet and infrared absorption spectra showed that the chromophore structures of Sal B and Tan IIA were unchanged after the formation of the complex. There were no new infrared absorption peaks, but a new binding state was formed. The equilibrium solubilities, octanol-water partition coefficients and *in vitro* dissolution rates from ESM and ESM-PC were assayed in phosphate buffer and n-octanol solution while varying the pH.

This study found that equilibrium solubility from ESM-PC was increased significantly compared to that from ESM, and it was pH-dependent in the physiological range. The octanol-water partition coefficients of Sal B and Tan IIA in ESM-PC were 21.39 times and 19.1 times of those in ESM, respectively. Different pH media affected the dissolution rate of ESM-PC and ESM, and the dissolution rate of ESM-PC was higher than that of ESM. An LC-MS/MS method was established and combined with a haemodialysis sampling technique for analysis of Sal B and Tan IIA *in vivo*. This method is effective for the determination of the *in vivo* profiles of Sal B and Tan IIA, greatly improving the analytical accuracy and reducing the damage to experimental animals. Suspensions of ESM and ESM-PC were administered intragastrically in rats. The main pharmacokinetic parameters of ESM-PC were significantly changed compared with ESM, exhibiting slow-release characteristics. The bioavailabilities of Sal B and Tan IIA from ESM-PC were 4.91 and 2.49 times of those from ESM, respectively. Thus, we can conclude that ESM-PC significantly improves the absorption of ESM, which provides a basis for the clinical

Phospholipid complex for improved bioavailability

Table 6. Pharmacokinetic parameters of Sal B and Tan IIA in ESM and ESM-PC ($x \pm s$, $n = 5$)

Parameters	Sal B		Tan IIA	
	ESM	ESM-PC	ESM	ESM-PC
C_{max} ($\mu\text{g}\cdot\text{L}^{-1}$)	1723.31 \pm 144.28	4724.01 \pm 185.94	43.06 \pm 4.59	118.32 \pm 5.89
T_{max} (h)	0.42 \pm 0.06	0.92 \pm 0	0.42 \pm 0.08	0.75 \pm 0
MRT_{0-t} (h)	3.26 \pm 2.15	4.59 \pm 0.05	2.96 \pm 0.91	3.67 \pm 1.29
AUC_{0-t} ($\mu\text{g}/\text{L}\cdot\text{h}$)	2463.09 \pm 56.88	12104.40 \pm 168.75	86.86 \pm 9.26	216.54 \pm 9.17
$T_{1/2}$ (h)	5.38 \pm 1.15	7.82 \pm 2.88	1.82 \pm 1.24	3.12 \pm 1.83

application of the new preparation. This may be useful for further development of oral preparations of ESM.

Conclusion

In this study, ESM-PC with a 1:1 mass ratio was prepared by solvent evaporation and characterized by UV and FT-IR spectroscopy. The solubilities and dissolution rates of Sal B and Tan II A were significantly increased from ESM-PC compared with ESM. Their oral bioavailabilities in rats were also improved. The drug phospholipid complexation technique has potential to improve the bioavailabilities of poorly soluble compounds.

Acknowledgements

This research was funded by the Key Research and Development Program of Shaanxi Province of China [grant number 2017ZDXM-SF-008]; the Chinese Medicine Pharmaceutical Key Discipline of Shaanxi Province [grant number 303061107]; and the Key Research and Development Plan of Shaanxi Province [grant number 2018SF-314].

Disclosure of conflict of interest

None.

Address correspondence to: Yajun Shi, Department of Pharmaceutics, College of Pharmacy, Shaanxi University of Chinese Medicine, Xianyang, Shaanxi, China. Tel: 029-38183689; E-mail: 20-51004@sntcm.edu.cn

References

- [1] Li ZM, Xu SW, Liu PQ. Salvia miltiorrhizaBurge (Danshen): a golden herbal medicine in cardiovascular therapeutics. *Acta Pharmacol Sin* 2018; 39: 802-824.
- [2] Committee of national pharmacopoeia. *Pharmacopoeia of P.R. China* 2015.

- [3] Cheng TO. Cardiovascular effects of Danshen. *Int J Cardiol* 2007; 121: 9-22.
- [4] Han J, Fan J, Horie Y, Miura S, Cui D, Ishii H, Hibi T, Tsuneki H and Kimura I. Ameliorating effects of compounds derived from Salvia miltiorrhiza root extract on microcirculatory disturbance and target organ injury by ischemia and reperfusion. *Pharmacol Ther* 2008; 117: 280-295.
- [5] Xiao ZH. Clinical application of Salvia miltiorrhiza. *Chinese And Foreign Medical Research* 2012; 10: 154-155.
- [6] Fang J, Little PJ and Xu S. Atheroprotective effects and molecular targets of tanshinones derived from herbal medicine danshen. *Med Res Rev* 2018; 38: 201-228.
- [7] Xu JW and FU CM. Progress of salvia miltiorrhiza in pharmaceutical action. *Medical Recapitulate* 2006; 1467-1469.
- [8] Wang L, Yu J, Fordjour P, Xing X, Gao H, Li Y, Li L, Zhu Y, Gao X and Fan G. Danshen injection prevents heart failure by attenuating post-infarct remodeling. *J Ethnopharmacol* 2017; 205: 22-32.
- [9] Peng Y, Yang T, Huang K, Shen L, Tao Y and Liu C. Salvia miltiorrhiza ameliorates liver fibrosis by activating hepatic natural killer cells in vivo and in vitro. *Front Pharmacol* 2018; 9: 762.
- [10] Committee of national pharmacopoeia. *Pharmacopoeia of P.R. China* 2015; 678.
- [11] Zhang C and Han XJ. Danshen medicinal materials-intermediates-HPLC fingerprints and correlation of danshen tablets. *Chinese Traditional Patent Medicine* 2010; 32: 1656-1662.
- [12] Wang X, Morris-Natschke S and Lee K. New developments in the chemistry and biology of the bioactive constituents of Tanshen. *Med Res Rev* 2007; 27: 133-148.
- [13] Zhou L, Zuo Z and Chow M. Danshen: an overview of its chemistry, pharmacology, pharmacokinetics, and clinical use. *J Clin Pharmacol* 2005; 45: 1345-1359.
- [14] Li SC, Liu JP, Zeng CZ and Zheng YX. Study on the solubility and permeability of tanshinone II a and on the excipients increasing the solubility and permeability. *Lishizhen Medicine and Materia Medica Research* 2008; 1724-1726.
- [15] Hao H, Wang G, Cui N, Li J, Xie L and Ding Z. Pharmacokinetics, absorption and tissue dis-

Phospholipid complex for improved bioavailability

- tribution of tanshinone IIA solid dispersion. *Planta Med* 2006; 72: 1311-1317.
- [16] Kim CK, Cho YJ and Gao ZG. Preparation and evaluation of salvianolic acid B microemulsions for oral delivery. *J Control Release* 2001; 149-155.
- [17] Sun J, Shi YJ, Zhang XF, Wang M and Cui CL. Preparations and dissolution study of solid dispersion of Danshen extract. *Journal of Chinese Medicinal Materials* 2017; 40: 164-166.
- [18] Gnananath K, Sri Nataraj K and Ganga Rao B. Phospholipid complex technique for superior bioavailability of phytoconstituents. *Adv Pharm Bull* 2017; 7: 35-42.
- [19] Gupta A, Saraf S and Saraf S. Phytosome: a novel approach for phytoconstituents. *Plant Archives* 2007; 7: 457-461.
- [20] Gupta NK, Dixit VK. Absorption enhancement of grape seed polyphenols by complexation with phosphatidyl choline. *Drug Deliv* 2011; 18: 312-319.
- [21] Kuche K, Bhargavi N, Dora CP, Jain S. Drug-phospholipid complex-a go through strategy for enhanced oral bioavailability. *AAPS PharmSci-Tech* 2019; 20: 43.
- [22] Zhu ZH, Li WH, Zhu YT, Xiao ZQ and Yang JG. Physicochemical characterization of magnolol-phospholipid complex and its oral bioavailability in rats. *Journal of Northwest University (Natural Science Edition)* 2012; 42: 415-421.
- [23] Wang C, Zhao X, Mao S, Wang Y, Cui X and Pu Y. Management of SAH with traditional Chinese medicine in China. *Neurol Res* 2006; 28: 436-444.
- [24] Zhao WN, Shang PP and Sun WJ. HPLC determination of apparent n-octanol/water partition coefficient of gentiopicoside. *Chinese Journal of Pharmaceutical Analysis* 2009; 29: 1093-1095.
- [25] Xue Q. Determination and evaluation of dissolution. *China Foreign Medical Treatment* 2008; 27: 73-74.
- [26] Rottbøll LA, Friis C. Microdialysis as a tool for drug quantification in the bronchioles of anaesthetized pigs. *Basic Clin Pharmacol Toxicol* 2014; 114: 226-232.
- [27] Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal Chem* 2003; 75: 3019-3030.
- [28] Zhang K, Zhang Y, Zhang M, Gu L, Liu Z, Jia J and Chen X. Effects of phospholipid complexes of total flavonoids from persimmon (*diospyros kaki L.*) leaves on experimental atherosclerosis rats. *J Ethnopharmacol* 2016; 191: 245-253.
- [29] Saoji SD, Raut NA, Dhore PW, Borkar CD, Popielarczyk M, Dave VS. Preparation and evaluation of phospholipid-based complex of standardized centella extract (SCE) for the enhanced delivery of phytoconstituents. *AAPS J* 2016; 18: 102-114.
- [30] Wu JF, Qui PS, Chen YW, Chen HN and Li HN. Preparation and dissolution study of solid dispersions of Danshen extract. *Chinese Journal of Hospital Pharmacy* 2010; 30: 900-905.
- [31] Lin R, Wang WR, Liu JT, Yang GD, Han CJ. Protective effect of tanshinone IIA on human umbilical vein endothelial cell injured by hydrogen peroxide and its mechanism. *J Ethnopharmacol* 2006; 108: 217-222.
- [32] Shi M, Huang F, Deng C, Wang Y and Kai G. Bioactivities, biosynthesis and biotechnological production of phenolic acids in *salvia miltiorrhiza*. *Crit Rev Food Sci Nutr* 2019; 59: 953-964.
- [33] Xu J, Wei K, Zhang G, Lei L, Yang D, Wang W, Han Q, Xia Y, Bi Y, Yang M and Li M. Ethnopharmacology, phytochemistry, and pharmacology of Chinese *Salvia* species: a review. *J Ethnopharmacol* 2018; 225: 18-30.
- [34] Wu Y, Chen Y, Hsieh Y, Jaw I, Shiao M and Tsai T. Bioavailability of salvianolic acid B in conscious and freely moving rats. *Int J Pharm* 2006; 326: 25-31.
- [35] Qi Q, Hao K, Li F, Cao L, Wang G and Hao H. The identification and pharmacokinetic studies of metabolites of salvianolic acid B after intravenous administration in rats. *Chin J Nat Med* 2013; 11: 560-565.
- [36] Shi YJ, Wang M, Wu X and Song XM. Optimization of the preparation technology of panax japonicus saponin-phospholipid complex using plackett-burman design and central composite design-response surface methodology. *China Pharmacy* 2015; 26: 518-521.
- [37] Zhou Y, Dong W, Ye J, Hao H, Zhou J, Wang R and Liu Y. A novel matrix dispersion based on phospholipid complex for improving oral bioavailability of baicalein: preparation, in vitro and in vivo evaluations. *Drug Deliv* 2017; 24: 720-728.
- [38] Zhang K, Gu L, Chen J, Zhang Y, Jiang Y, Zhao L, Bi K and Chen X. Preparation and evaluation of kaempferol-phospholipid complex for pharmacokinetics and bioavailability in SD rats. *J Pharm Biomed Anal* 2015; 114: 168-175.