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Identification and Determination of Absorbed Components of Danggui-Shaoyao-San in Rat Plasma

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[ABSTRACT] AIM: To investigate the absorbed components of Danggui-Shaoyao-San (DSS) in rat plasma and their pharmacokinetics. **METHODS:** Serum samples from rats with DSS extract administrated orally were analyzed by HPLC-DAD-MS^n and an HPLC-DAD method was established for the determination. **RESULTS:** Seven prototypes and one metabolite were identified in rat serum, and albiflorin, paeoniflorin, ferulic acid and ligustilide in rat plasma were determined for pharmacokinetic study. The proposed method was validated with a linear range of 0.025–5.60 μg·mL^{-1} and limit of quantitation (LOQ) at 0.022–0.180 μg·mL^{-1}, respectively. The RSD of intra- and inter-day precision variations were less than 10.1% and the accuracies ranged from 92.5%–111.5%. The overall recovery was over 80%. **CONCLUSION:** Paeoniflorin sulfonate, albiflorin, paeoniflorin, ferulic acid, senkyunolide I, ligustilide and butylidenephthalide were found to be the potentially effective constituents in DSS. The method for determination is simple and sensitive, which is proved to be suitable for pharmacokinetic study of albiflorin, paeoniflorin, ferulic acid and ligustilide in DSS.
[KEY WORDS] Danggui-Shaoyao-San; Serum pharmacochemistry; Pharmacokinetics

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1 Introduction

Danggui-Shaoyao-San (DSS) is a widely used traditional Chinese medicine (TCM) formula composed of Radix *Paeoniae* Alba, Radix *Angelica sinensis*, Rhizoma *Chuanxiong*, *Poria cocos*, Rhizoma *Atractylodis macrocephalae* and Rhizoma *Alismatis*. It has been used in China as a blood-activating and stasis-eliminating drug to treat gynecological disorders such as dysmenorrhea, amenorrhea and infertility for thousands of years. Current pharmacological studies focus on its neuroprotective effects and the formula is applied as a remedy for some neurodegenerative disorders in Japan [1].

Although this formula contains hundreds of constituents, it remains unclear which constituents are responsible for the actions of the drug. Reports showed that albiflorin, paeoniflorin, ferulic acid and ligustilide are representative bioactive components of DSS [2]. Moreover, their pharmacological activities indicated that they may play an important role in the treatment of dysmenorrhea [3-5]. Serum pharmacochemistry is an efficient tool for screening of the potentially active constituents in TCMs when combined with LC-MS/MS, while there are few reports regarding the physiological disposition of the components in DSS. Previous studies mainly focused on the constituents of R. *Paeoniae* [6-7]. In the present study the absorbed components were analyzed by HPLC-DAD-MS^n after oral administration of DSS extract in rats, and four major components were determined in rat plasma for the pharmacokinetic study.

2 Experimental

2.1 *Reagents, materials and animals*

HPLC grade acetonitrile was purchased from Tedia (Fairfield, OH, USA); water for HPLC analysis was purified by a Milli-Q academic water purification system (Milford, MA, USA). Methanol and formic acid were of analytical grade (Jiangsu Hanbon Sci. & Tech. Co., Ltd., Huaiyin, China).

Albiflorin, paeoniflorin, ferulic acid and kaempferol were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

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Ligustilide, senkyunolide I and butylidenephthalide were isolated from the essential oil of *Angelica sinensis* in the authors' laboratory; their structures were elucidated on the basis of their spectral data (MS, ^{1}H NMR and ^{13}C NMR). Each compound was determined to be greater than 98% in purity by HPLC.

Male Sprague–Dawley rats (180−220 g) were obtained from the Animal Center of China Pharmaceutical University (Nanjing, China) and the studies were approved by the Animal Ethics Committee of China Pharmaceutical University. The animals were taken care of according to the regulations of the Animal Committee under controlled conditions [(20 ± 2) °C, RH (50 ± 20)%, a natural light-dark cycle and 10-15 air changes per hour]. They were fasted with only access to water for 12 h prior to the experiment.

2.2 *Preparation of standard solutions and DSS extract*

The stock solution of albiflorin (1.1 mg·mL^{-1}), paeoniflorin (2.0 mg·mL^{-1}), ferulic acid (0.20 mg·mL^{-1}), ligustilide (0.38 mg·mL^{-1}) and kaempferol (IS, 0.256 mg·mL^{-1}) was prepared in methanol. All solutions were stored at 4 °C and brought to room temperature and further diluted to prepare working solutions. The powdered crude drugs compounded according to the formula in "Jin Gui Yao Lue" were refluxed twice with 10 folds (*W*/*V*) 70% ethanol for 1 h. After removing ethanol under reduced pressure, the residue was then diluted with distilled water to give the DSS extract of 5 g crude drug/mL and stored at 4 °C until use.

2.3 *Drug administration and sample collection*

Serum pharmacochemistry experiments were performed on 6 rats. A 10 mL·kg^{-1} dosage of DSS extract was administered orally to rats three times at a one-hour' interval. The blood was collected at 0.5 h after the last dosage, and then centrifuged at 3 000 r·min^{-1} for 10 min to separate serum. The serum was mixed and stored at −70 °C before analysis.

Pharmacokinetic experiments were performed on 12 rats, which were divided randomly into 2 batches. DSS extract was administered orally to rats by gavage at a dosage containing 250 mg·kg^{-1} paeoniflorin (1.7 mL per 100 g body weight). The contents of abiflorin, paeoniflorin, ferulic acid and ligustilide in DSS extract were determined as 9.96, 14.8, 1.26 and 13.17 mg·mL^{-1}, respectively. After administration, the blood samples were collected at 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, 360 and 480 min, respectively, while those at 5–60 min and 90–480 min were collected from the first and the second batches of rats, respectively. The blood samples were centrifuged at 3 000 r·min^{-1} for 10 min to obtain the plasma sample and stored at −70°C until analysis.

2.4 *Sample preparation*

After mixed with 10 μL of IS solution, a plasma sample of 200 μL was extracted for 1 min with 0.8 mL ethyl ester by vortex and then demixed by centrifugation at 3 000 r·min^{-1} for 5 min. This process was repeated three times. After the upper ethyl ester phase was collected into a glass centrifuge tube by aspiration, 0.8 mL acetonitrile containing 1% (*V*/*V*) acetic acid was added into the plasma and vortexed for 30 s. After centrifugated at 9 000 r·min^{-1} for 5 min, the supernatant was transferred into the above glass centrifuge tube and allowed to evaporate to dryness under a nitrogen gas stream in a 37 °C water bath. The residue was dissolved in 100 μL methanol and centrifuged at 12 000 r·min^{-1} for 5 min. An aliquot (20 μL) of the supernatant was injected to HPLC analysis.

2.5 *Assay condition*

An Agilent 1100 series HPLC with a diode array detector was used for analysis. The column was an Alltima C_{18} (250 mm × 4.6 mm, i.d., 5 μm, Alltech, USA). The above HPLC system was interfaced with an Agilent 1100 LC/MSD Trap XCT ESI (Agilent Technologies, MA, USA). The qualitative assay was performed using a previous established method [2].

The quantitative assay was performed on an HPLC-DAD system using a gradient program of A (CH_3CN) and B (HCOOH–H_2O 0.1 : 100, *V*/*V*): 10%–35% A at 0–20 min and 35%–87% A at 20–40 min. The flow rate was 1.0 mL·min^{-1}, and the column temperature was set at 30 °C. The monitor wavelengths were 231 nm for albiflorin and paeoniflorin, whilst 320 nm for ferulic acid and ligustilide.

3 Results and Discussion

3.1 *Sample collection and preparation*

The profiles of the physiological disposition of components were investigated. The results showed that the number of peaks and the corresponding peak areas in chromatograms were different when collected at different periods, and tha the most abundant components in serum were found within 1 h.

For the extraction methods, liquid-liquid extraction (LLE), deproteinization and solid phase extraction (SPE) were tried, and deproteinization followed by LLE was found to give good extraction efficiency for most analytes. In addition, acetic acid was also added in acetonitrile to improve the recovery of ferulic acid.

3.2 *Identification of absorbed components in rat serum by HPLC-DAD-MS^n*

Previous study on DSS showed that monoterpene glycosides, phenolic compounds and phthalides are representative components of DSS [2]. Therefore, the *in vivo* analysis focused on searching these components and their metabolites. After oral administration of DSS, rat serum samples were analyzed by HPLC-DAD-MS^n in both positive and negative modes and compared with the blank samples. The base peak and UV chromatograms of the sample are presented in Fig. 1. Seven prototype components, namely paeoniflorin sulfonate (**1**), albiflorin (**3**), paeoniflorin (**4**), ferulic acid (**5**), senkyunolide I (**6**), ligustilide (**7**) and butylidenephthalide (**8**) were detected by comparing their retention times, UV and MS spectra with those of reference compounds or literature data [2]. One metabolite was also observed in MS spectrum,