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Determination of Six Major Compounds in Shaoyao-Gancao-Tang and its Single Herb Decoctions

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Shaoyao-Gancao-Tang is a traditional Chinese formula containing *Radix paeoniae alba* and *Radix glycyrrhizae.* An ultra performance liquid chromatography-photo diode array detection method was employed for simultaneous quantitative determination of major constituents *i.e.*, gallic acid, albiflorin, paeoniflorin, benzoic acid, glycyrrhizin and liquiritigenin in Shaoyao-Gancao-Tang and its single herb decoctions. Chromatographic separation and determination were performed on an Acquity UPLC BEH C_{18} (100 mm × 2.1 mm, i.d., 1.7 μM) column by gradient elution using a mobile phase of acetonitrile-0.5 % aqueous acetic acid at a flow rate of 0.3 mL/min. The method was validated for selectivity, linearity, sensitivity, precision, accuracy and stability. It was successfully applied to the comparison of quantification of major active components between Shaoyao-Gancao-Tang and its single herb decoctions. The concentrations of gallic acid, glycyrrhizin and liquiritigenin increased and that of albiflorin, paeoniflorin and benzoic acid decreased significantly ($p < 0.01$). The experimental data indicated that drug interactions during decocting process could result in the changes of the amount of active components.

Key Words: Shaoyao-Gancao-Tang, *Paeoniae radix*, *Glycyrrhizae radix*, Ultra performance liquid chromatography, Drug interactions.

INTRODUCTION

Traditional Chinese medicines (TCMs) has been playing an important role on the prevention and treatment of diseases for a long history. Traditional Chinese medicines mostly are prescribed by a unique methodology with a specific combination of different herbs as formulae and prepared by boiling the mixed crude drugs in water. In the decocting process, crude drugs may influence each other so that the amount of constituents in the formula could change[1,2]. Consequently, the process may enhance solubility, facilitate absorption, increase pharmacological action, reduce toxic side-effects and remove odours, *etc*.[3]. Increasing attention is currently being paid to scientific evaluation of herb-herb interaction for better understanding of composition mechanism of traditional Chinese mediciness.

Shaoyao-Gancao-Tang (SGT, Shakuyaku-Kanzo-to in Japanese), a representative Chinese medicinal preparation, is traditionally used for leg cramps, stomach ache and menstrual colic pain[4,5]. The formula consists of two medicinal herbs including *Radix paeoniae alba* and *Radix glycyrrhizae* with the ratio of 1:1. *Radix paeoniae alba* have long been used in Chinese medicine for treating hepatic diseases and women's diseases[6]. The main constituents of *Radix paeoniae alba* are gallic acid, albiflorin, paeoniflorin and benzoic acid[3]. *Radix glycyrrhizae* are widely used to treat diseases of the respiratory tract, gastrointestinal and cardiovascular system, *etc*.[7]. Glycyrrhizin and liquiritigenin are two most important bioactive constituents in *Glycyrrhizae radix*[8]. Therefore, the six compounds (Fig. 1) should be considered as markers for quantitative analysis of Shaoyao-Gancao-Tang, single herb *Paeony* decoction and single herb *Glycyrrhiza* decoction.

Recently, ultra-performance liquid chromatography (UPLC) has attracted considerable attention for pharmaceutical, toxicological and biochemical analysis because of its speed and sensitivity[9]. Ultra-performance liquid chromatography combined with photodiode array detection (PDA) technique has also been proven to be a powerful tool for the simultaneous determination of the constituents in botanic extracts and traditional Chinese mediciness[10,11]. In this paper, we first developed a reliable and convenient UPLC-PDA method to simultaneously quantify the six constituents including gallic acid, albiforin, paeoniflorin, glycyrrhizin, benzoic acid and liquiritigenin in Shaoyao-Gancao-Tang and compare the differences in concentration of the chemical components between Shaoyao-Gancao-Tang and its single herb decoctions during the decocting process. The study focuses on the change of major components during

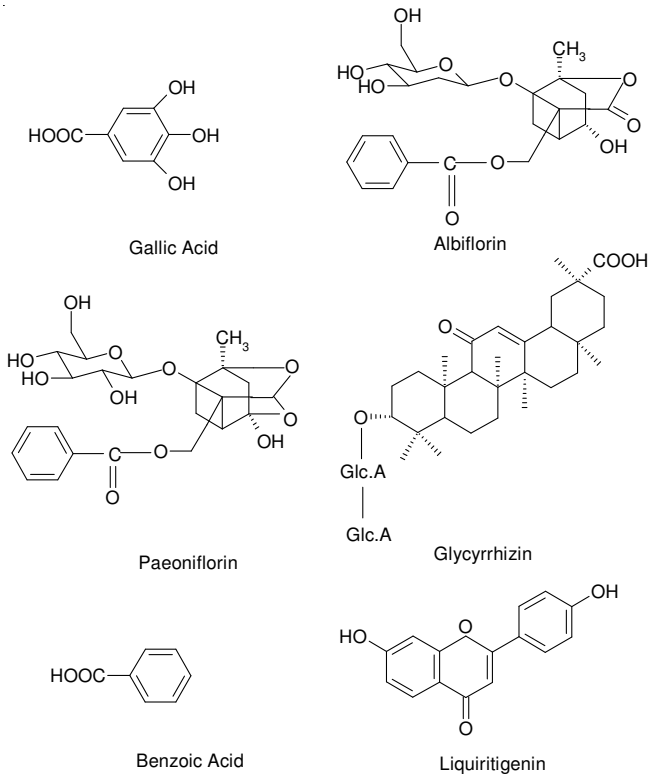

Fig. 1. Structures of six constituents present in Shaoyao-Gancao-Tang and its single herb decoctions

the decocting process in Shaoyao-Gancao-Tang and its single herb decoctions by using UPLC-PDA. It is highly possible that the quantity changes of these markers originated from the drug interactions during decocting process.

EXPERIMENTAL

Radix paeoniae alba and *Radix glycyrrhizae* were obtained from Laobaixing Pharmacy (changsha, China). Standards of gallic acid, albiflorin, peoniflorin, glycyrrhizin, benzoic acid and liquiritigenin (purity ≥ 98 %) were obtained from National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, China). Methanol and acetonitrile were LC-grade (Tedia, USA). Acetic acid was obtained from Sinopharm Chemical Reagent Company (Shanghai, China) and high purity water was obtained from Wahaha Co., Ltd. (Hangzhou, China).

Samples preparation: The formula (Shaoyao-Gancao-Tang, 80 g), *Radix paeoniae alba* (40 g) and *Radix glycyrrhizae* (40 g) were weighed and boiled two times with a ten-fold mass of distilled water for 1 h, respectively. Each hot water soluble mixture obtained was filtered and freeze dried to obtain the powder form of samples, which were then stored at 4 ºC until use. For UPLC analysis, the dried powder was dissolved in distilled water with a final concentration of 20 mg/mL for *Radix paeoniae alba* and *Radix glycyrrhizae* in Shaoyao-Gancao-Tang and its single herb decoctions. The solutions were extracted with an equal volume of methonal, respectively. After centrifuged at 12000 × g for 10 min, the supernatants diluted with an equal volume of water and subsequently centrifuged as above. The supernatant solutions were filtered through a 0.22 μM filter before injection.

Preparation of standard solutions: The six reference compounds were accurately weighed and dissolved together in methanol to prepare stock solution. The stock solution diluted with high purity water to obtain a four-fold dilute mixed solution. Five different concentrations of working standard solutions were prepared every day by diluting the mixed solution with 25 % aqueous methanol. The calibration curves were prepared with the mixed solution and the series of working solutions. All solutions were stored at 4 ºC in dark brown calibrated flasks, respectively.

Instrumentation and separation conditions: Chromatographic separation was performed on a waters acquity ultra peance liquid chromatography system, consisting of binary solvent manager, sample manager, photodiode array detector and Empower™ Software. The chromatographic column was an Acquity UPLC BEH 2.1 × 100 mm, 1.7 μM C_{18} column (Waters, Milford, MA, USA). Mobile phase contained a mixture of acetonitrile and 0.5 % aqueous acetic acid at a flow-rate of 0.3 mL/min. Optimum separation was carried out by gradient program as follows: 0-2 min, 2 % A, 2-3 min, 2-13 % A, 3-10 min, 13-18 % A, 10-14 min, 18-23 % A. The column temperature was held at 40 ºC and the autosampler was maintained at 25 ºC. The sample volume injection was 3 μL. The peaks were detected at the maximum absorbance wavelength of each analyte.

Method validation: The UPLC method was validated for selectivity, linearity, sensitivity, precision, accuracy and stability according to FDA guideline. Selectivity was investigated by comparison two blank samples. Linearity was determined by preparing six different concentrations of standard solutions. The calibration curves were obtained by linear regression analysis of the peak area (y) *versus* concentration (x, μg/mL). The limit of detection (LOD) and limit of quantification (LOQ) were estimated as signal-to-noise ratio of 3 and 10, respectively. Intra-day and inter-day precision were assayed by analyzing standard solutions at three different concentrations (five replicates) during a single day and on five consecutive days, respectively. Accuracy was expressed as recovery test by the addition of known amount of reference compounds to the known Shaoyao-Gancao-Tang samples of five replicates, followed by extraction and analysis as described above. Stability was carried out by determining the analytes in sample and reference standards solutions stored at 4 ºC and room temperature (*ca.* 25 ºC) at 0, 24 and 48 h, respectively.

Statistical analysis: Data was presented as mean ± standard deviation. Differences between two groups were assessed by one-way ANOVA using SPSS Statistical Software (Chicago, USA). A probability value less than 0.05 was considered to indicate statistical significance.

RESULTS AND DISCUSSION

Chromatography: Gradient elution was used to successfully separate the markers with a mobile phase of acetonitrile and 0.5 % acetic acid water in less than 14.0 min. The compounds were identified by comparison both the retention time and UV spectra with those of authentic standards. The peaks of markers were separated significantly from biological noise and the shape was symmetrical (Fig. 2a-b).

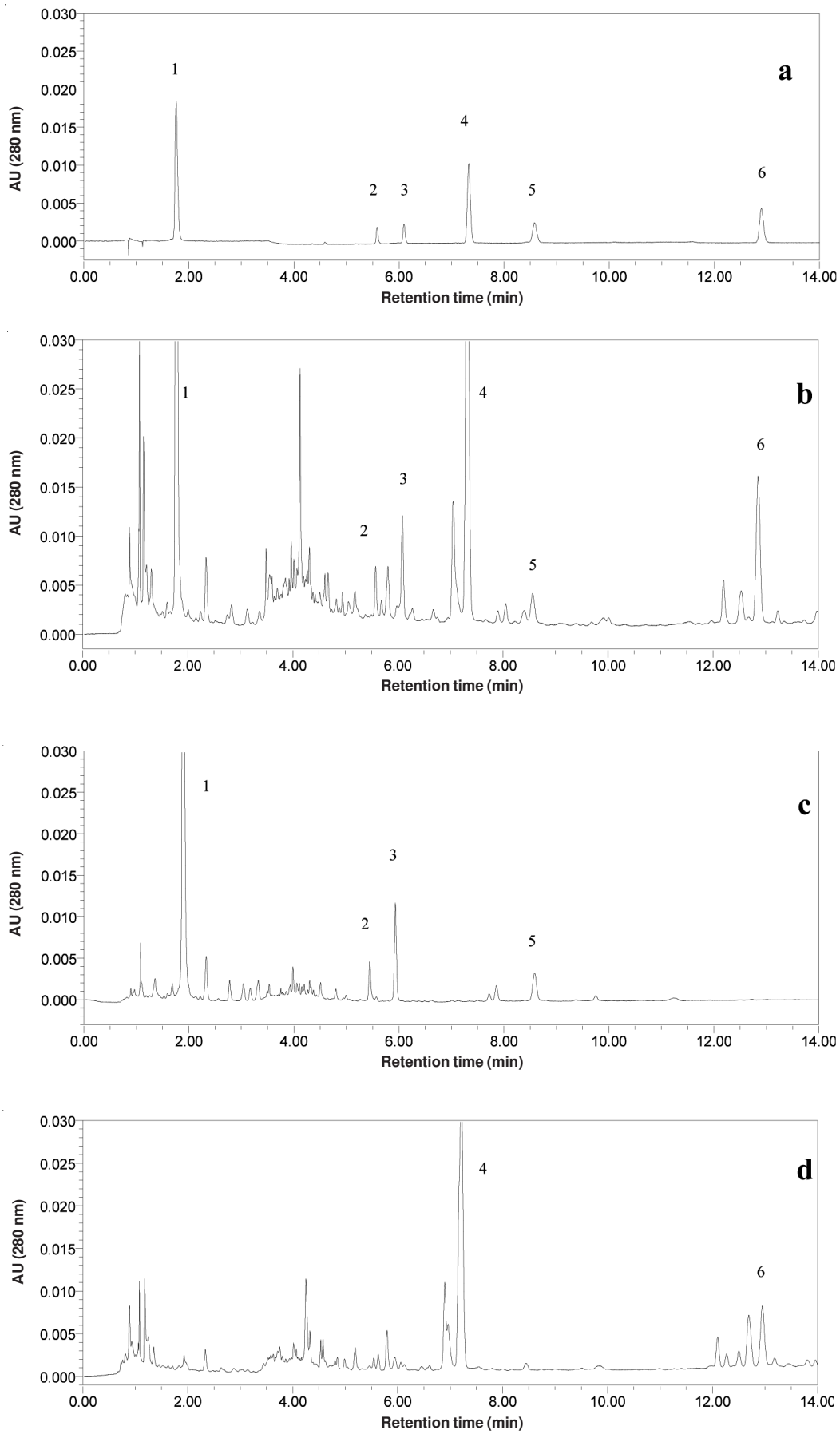

Fig. 2. Representative UPLC-PDA chromatograms obtained after analysis of Shaoyao-Gancao-Tang. (a) standard mixture; (b) Shaoyao-Gancao-Tang test sample; (c) single herb *Paeony* decoction test sample; (d) single herb *Glycyrrhiza* decoction; 1 = gallic acid, 2 = albiflorin, 3 = peoniflorin, 4 = glycyrrhizin, 5 = benzoic acid, 6 = liquiritigenin

TABLE-1
CALIBRATION CURVES, LODS AND LOQS FOR UPLC ANALYSIS

Analytes	Regression equation	r^2	Test Range (μg mL^{-1})	LOD (ng)	LOQ (ng)
Gallic acid	y = 22136x - 11158	0.9995	0.38259-27.546	0.0120	0.0399
Albiflorin	y = 804.9x - 78.757	0.9999	0.52941-38.117	0.0403	0.1343
Peoniflorin	y = 955.75x -356.49	0.9997	0.57870-41.667	0.0434	0.1446
Glycyrrhizin	y = 15873x - 1567.9	0.9999	0.34705-24.988	0.0277	0.0923
Benzoic acid	y = 3702x - 240.33	0.9999	0.27186-19.574	0.0278	0.0927
Liquiritigenin	y = 29796x - 988.41	0.9999	0.066730-4.8045	0.0232	0.0774

TABLE-2
PRECISION OF THE SIX ANALYTES FOR UPLC METHOD

Analytes	Nominal concentration (μg mL-1)	Intra-day (n = 5)		Inter-day (n = 5)	
		Observed concentration (μg mL^{-1})	Precision (RSD %)	Observed concentration (μg mL^{-1})	Precision (RSD %)
Gallic acid	1.1478	1.1995	3.2	1.1988	4.5
	6.8866	6.5803	3.4	6.7042	1.1
	27.546	26.610	1.4	27.713	1.0
Albiflorin	1.5882	1.5758	3.4	1.5348	4.7
	9.5293	9.2913	1.4	9.5114	4.7
	38.117	37.559	2.2	38.028	3.1
Peoniflorin	1.7361	1.7949	4.3	1.7955	4.2
	10.417	10.030	1.5	10.013	2.9
	41.667	41.522	1.2	41.648	3.2
Glycyrrhizin	1.0412	1.0888	1.1	1.0766	4.8
	6.2469	6.2153	2.6	6.2584	3.5
	24.988	24.888	2.8	24.984	1.1
Benzoic acid	0.81559	0.82921	4.5	0.82505	3.9
	4.8935	4.9601	2.4	4.9495	4.3
	19.574	19.702	2.3	19.606	1.7
Liquiritigenin	0.20019	0.210997	2.9	0.20569	4.6
	1.2011	1.2052	1.6	1.1819	3.5
	4.8045	4.8999	2.4	4.8087	1.3

Validation of the method: Fig. 2c-d showed there was no interference at the retention time of the six markers in blank samples. The regression equation was expressed as Y = aX + b, where Y and X were the peak area and concentration (μg/mL), with excellent correlation as assessed by the R^2 values (Table-1). The LOD and LOQ ranges were 0.0120-0.0434 and 0.0399-0.1446 ng (Table-1), respectively. The intra-day and inter-day precision (RSD) were in the range of 1.1-4.8 % indicating good precision (Table-2). Recoveries ranged from 96.20-101.1 % with RSD less than 4.7 % (Table-3), characterizing satisfactory reliability and accuracy of the method. The RSD values of peak areas obtained from stability test were lower than 4.9 %. Solutions were therefore regarded as stable for at least 48 h.

TABLE-3
RECOVERY OF THE SIX ANALYTES IN SGT (n = 5)

Analytes	Original (ng)	Added (ng)	Determined (ng)	Recovery (%)	RSD (%)
Gallic acid	50.574	20.660	70.689	97.36	1.1
Albiflorin	43.842	28.588	72.376	99.81	4.7
Peoniflorin	91.968	31.250	122.03	96.20	2.9
Glycyrrhizin	38.250	18.741	57.026	100.2	3.5
Benzoic acid	11.605	14.681	26.453	101.1	4.3
Liquiritigenin	7.6076	3.6034	11.159	98.56	3.5

Samples analysis: The developed UPLC-PDA method was subsequently applied to determine gallic acid, albiflorin, peoniflorin, glycyrrhizin, benzoic acid and liquiritigenin in samples. The comparison of concentrations of main active components between Shaoyao-Gancao-Tang and the single herb decoctions were shown in Fig. 3. The results showed that the concentration of six markers detected in the individual herbs were different from those in Shaoyao-Gancao-Tang ($p < 0.01$). The amount of gallic acid increased while albiflorin, paeoniflorin and benzoic acid decreased after *Radix paeoniae alba* combining with *Radix glycyrrhizae* in Shaoyao-Gancao-Tang. Glycyrrhizin and liquiritigenin had a higher quantity in Shaoyao-Gancao-Tang than in single herb *Glycyrrhiza* decoction. The differences in concentrations of major components between Shaoyao-Gancao-Tang and its single herb decoctions might be induced by the interaction of crude drugs to change the solubility of the active components during decocting process[12-14]. When decocting together, a series of chemical reactions might happen such as solubilization, complexation, salification, oxidation, hydrolytic decomposition and reduction[2]. This could account for the differences in concentration between the single herb decoctions and Shaoyao-Gancao-Tang.

Traditional Chinese medicines, mostly prescribed in combination and prepared by boiling the mixed crude drugs in water, are aimed to obtain the synergistic effects or to diminish the possible adverse reactions[15,16]. Therapeutic and pharmacological effects of traditional Chinese mediciness are usually attributed to synergism among multiple herbs and constituents[17]. Several studies indicated that there have been optimal combinations of crude drugs to achieve the best chemical composition and biological responses[18-20]. As for Shaoyao-Gancao-Tang, the

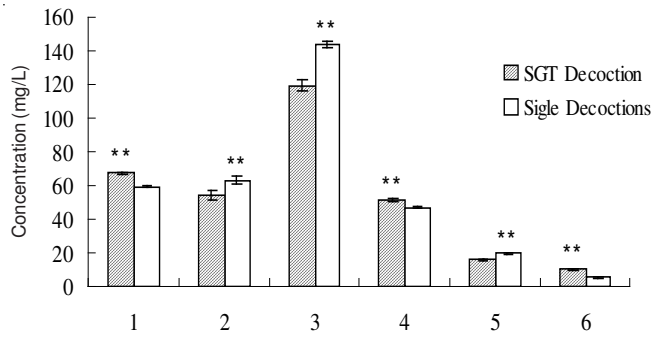

Fig. 3. Comparison study of the concentrations of six bioactive compounds in Shaoyao-Gancao-Tang decoction and its single herb decoctions. Data were presented as means ± standard deviations (n = 5). 1 = gallic acid, 2 = albiflorin, 3 = peoniflorin, 4 = glycyrrhizin, 5 = benzoic acid, 6 = liquiritigenin. (**$p < 0.01$)

most effective combination ratios of *Radix paeoniae alba* and *Radix glycyrrhizae* is 1:1[5]. Gallic acid, glycyrrhizin and liquiritigenin were three effective constituents whose some properties resemble Shaoyao-Gancao-Tang such as analgesic, antiinflammatory, antioxidant, relaxation and neuroprotection activities[8,21-26]. Benzoic acid was harmful if it was at higher than permitted safety level[27,28]. Albiflorin and paeoniflorin decreased so that a stronger efficacy of *Radix glycyrrhizae* is obtained[29]. According to the results, it indicated that the curative effects might be enhanced and the toxic side-effects be reduced by compatibility.

Conclusion

In this paper, the validated UPLC-PDA method was firstly applied for the comparison of concentrations of the major active components in Shaoyao-Gancao-Tang and its single herb decoctions. The concentrations of the six markers changed after decocting process. The contents of gallic acid, glycyrrhizin and liquiritigenin increased and that of albiflorin, paeoniflorin and benzoic acid decreased significantly ($p < 0.01$). The results indicated that drug interactions during decocting process could change the solubility of the active constituents.

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