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Determination of components in *Radix paeoniae rubra* based on QAMS

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Abstract: The current study aimed to establish simple and quick quality evaluation method of Chishao based on QAMS. Oxypaeoniflorin is used as a marker in the Chishao root. Based on it, the content of other components could be obtained by establishing the mathematical relationship. UPLC method was used to collect data, and the detection wavelengths were 230nm (benzoic acid, paeoniflorin), 263nm (hydroxy paeoniflorin) 274nm (gallic acid, paeoniflorin, catechin), respectively. The stationary phase was an Agilent ZORBAX SB-C_{18} and the mobile phase was acetonitrile -0.1% formic acid-water. The gradient elution method was adopted at the certain flow rate (0.3 mL/min). The column temperature set 40ºC, and the injection volume was 1μL. Multiple reaction monitoring mode was selected for data collection. The linear ranges of benzoic acid, paeoniflorin,hydroxy-paeoniflorin, gallic acid, catechin, and paeoniflorinhad good linearity (r ≥0.9995). The UPLC method was established to determine the content of paeoniflorin, benzoic acid, catechin, gallic acid, paeoniflorin, andhydroxy-paeoniflorin in *Radix Paeoniae Rubra*. In the current study, the method for the chemical components in *Radix Paeoniae Rubra* to provide the evaluation basis of medicinal effects.

Keywords: QAMS, radix *paeoniae* rubra, quantitative analysis.

INTRODUCTION

Radix Paeoniae Rubra, namely, Chishao in Chinese, which is a common traditional Chinese medicine (TCM), which is the dried root of *Paeonia lactiflora* Pall and *Paeonia veitchii* Lynch. (National Pharmacopoeia Commission 2020; Zhang *et al*., 2020) In China, Chishao is widely distributed from north to south. Due to various types of chemical components, such as monoterpene glycosides, tannins, *etc*., Chishaohave many pharmacological activities, including anti-tumor, antioxidant, and anti-inflammatory activities, and so on (Ren *et al*., 2021; Dong 2017; Darío *et al*., 2019; Zhang *et al*., 2020). It's known that different environments have different growth conditions, such as temperature, climate, light and other factors, which would result in uneven quality (Sun *et al*., 2021). Therefore, the compositions have distinct in Chishao from different regions. It's essential to ensure the consistency of the quality of Chishao by certain methods and technologies.

At present, paeoniflorin is usually used to be an indicator as the evaluation basis for quality control in both the national drug standards and drug standards in several provinces. However, it is slightly insufficient for assessing the quality of multiple chemical components in Chishao. Chishao have been widely utilized in various prescriptions and decoction pieces, meanwhile, various quality evaluation methods have been emerged to guarantee uniformity and stability, which have been widely used in the quality evaluation of Chishao medicinal materials and Chishao decoction pieces. For example, atomic absorption spectrophotometry (AAS), thin layer chromatography (TLC) andhigh-performance liquid chromatography (HPLC) (Chen *et al.*, 2017; Lu *et al.*, 2019; Ma *et al.*, 2020).

In the study on quality control, reference substances are always needed to assist test. However, the existing control substances would be not sufficient for research. The problem could be solved by the quantitative analysis of multi-components by a single marker method (QAMS), while reference materials have as shortage and high detection costs in multi-component quantitative analysis. One reference substance can be used to identify the content of multiple components by QAMS, which reduces the cost of testing and experimentation, more comprehensively and accurately controls the quality of TCM and improves work efficiency. Moreover, the QAMS could save experimental consumables, simplify the operation steps, and save the determination time, which is appropriate for the efficacy of medicines. In conclusion, QAMS has characters of highly accurate, good reproducible, and low-cos

Based on the deficiencies of the current quality control standards for Chishao, the study used the one-test-multiple-evaluation method to determine the content of 6 components in Chishao and investigate the composition of different types of chemical components,

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with a view to analyzing the chemical components of Chishao and its efficacy. Research on the material basis can provide an effective basis.

MATERIALS AND METHODS

Instrument and material

Waters 4695 ultra-high performance liquid chromatograph (Waters company, USA), KQ-1500VDB ultrasonic cleaner (Kunshan Ultrasonic Instrument Co., Ltd.), AE-240 electronic balance (Mettler-Toledo International Trade (Shanghai) Co., Ltd. company), Sorvall LYNX 6000high-speed centrifuge (U.S. Thermo Fisher Scientific), KDM type thermostat electric heating mantle (Heze Mudan District Junteng Electronic Instrument Co., Ltd.), MDF-492 ultra-low temperature refrigerator (Japan Sanyo Company), SK-1 Quick mixer (Jiangsu Jintan Medical Equipment Factory).

Benzoic acid (purity ≥98%, batch number G139784, abbreviated as BJS, China Food and Drug Control Institute). Paeoniflorin (purity ≥98%, batch number YZ050421, abbreviated as SYG). hydroxypaeoniflorin (purity ≥98%, batch number 200820, referred to as QJSYG). Gallic acid (purity ≥98%, batch number 200810, referred to as MSZS). Catechin (purity ≥98%, batch number 200718, referred to as ECS). Paeoniflorin (purity ≥98%, batch number 200923, referred to as SYNZG). Methanol (chromatographically purity, thermo fisher, USA). Acetonitrile (chromatographically pure, thermo fisher, USA); water (Watsons drinking water, Guangzhou Watsons Food and Beverage Co., Ltd.). Other reagents were analytical grade.

Chishao is the dry root of *Paeonia ladiflora* Pall. It washarvested in Jubao village, Chahayang, Neimenggu, Jubao town, Gannan, Nianzishan, Gugao Town, Baiquan, Moqi and Gannan Changshan, which were recorded as No.1-No.8, which were all identified by associated professor Sun Jikai of Qiqihar medical university.

Preparation

Preparation of reference solution

Reference substances, namely, benzoic acid, paeoniflorin, hydroxy paeoniflorin, gallic acid, catechin, and paeoniflorin were weighed accurately. Then, place them in a 10mL volumetric flask, and added methanol to prepare stock solution within concentration of 1mg/mL. Before determination, measure accurately the reference substance storage solution and place it in a 10mL volumetric flask and add methanol to the mark to obtain the reference substance stock solution. The concentration of paeoniflorin was 1000μg/mL and the concentration ofhydroxy paeoniflorin was 800μg/mL. The concentration of benzoic acid was 1000μg/mL and the concentration of catechin was 160μg/mL. The concentration of gallic acid and paeoniflorin were 640μg/mL and 1000μg/mL. The reference substance solution was stored in a refrigerator at -20ºC.

Preparation of sample solution

Crude Chishao powder was taken and weighed accurately 0.5g. Then, 25mL methanol was added precisely to dissolve it. After sonication, the solution was cooled for 20 minutes and methanol was used to fill lost weight. After filtering, take the additional filtrate to get it.

Chromatography conditions

The chromatography column was ZPRBAX SB-C C_{18} (100mm × 2.1 mm, 1.8μm). Flow rate was 0.3mL/min; detection wavelength was 230nm and column temperature set at 40ºC. Injection volume was chosen 1μL. The mobile phase was acetonitrile (A) with 0.1% formic acid aqueous solution (B) in gradient elution. Program was as follows: 0~5 min, ~5% A; ~7min, ~9%; ~11 min, 20% A; ~11.5 min, ~90% A.

RESULTS

Methodological investigations

Linearity

In this part, we needed to take each reference solution and accurately measure it to be in the 10 mL volumetric flasks. Then, methanol was the solution. Finally, the mixture contains benzoic acid, paeoniflorin, hydroxy paeoniflorin, gallic acid, and catechin and concentration of 6 chemical compositions were 200.0μg/mL, 80.00μg/mL, 160.0μg/mL, 64.00μg/mL, 200.0μg/mL, respectively. After dilution, the contents of the six components were 3.125~200.0μg/mL, 3.125~80.00μg/mL, 2.000~128.0μg/mL, 2.500~160.0μg/mL, 1.000~64.00μg/mL, 3.125~200.0μg/mL. Precisely draw 1.0μL of a series of mixed reference substance solutions in sequence, and determine according to chromatographic conditions. Take the mass concentration of the reference substance as the abscissa and the peak area integral value as the ordinate, perform regression processing to obtain the regression equation. As shown in table 1, the linear relationship of the six reference substances was good under the analysis conditions.

Repeatability

Six samples were taken from the same Chishao sample powder to prepare the test solution according to "1.2.2", and were measured accurately. Then, 1μL sample solution was precisely drawn and injected to test, which aimed to determine the average content of the components in different patches. RSD value meets the requirements by calculation. The required RSD value was <3% and the RSD values of benzoic acid, paeoniflorin, hydroxy paeoniflorin, gallic acid, catechin and paeoniflorin were 1.6%, 0.55%, 0.84%, 2.5%, 1.8%, 0.81%, respectively.

Table 1: Linearity determination of six chemical components in Chishao by UPLC method

Compound name	Abbreviation	Standard curve	r^2
Benzoic acid	BJS	A=37729 × C+24322	0.9990
Paeoniflorin	SYG	A =868.74 × C +6173	0.9975
Hydroxypaeoniflorin	QJSYG	A =4883.1 × C +7801.9	0.9997
Paeoniflorin	SYNZG	A =261.37 × C +267.42	0.9990
Catechin	ECS	A =2446.1 × C +1926.1	0.9996
Gallic acid	MSZS	A =10498 × C +23039	0.9979

Table 2: Determination the recovery rate of six chemical components of Chishao by UPLC method

Compound name	1	2	3	4	5	6	Recovery rate (%)
Benzoic acid	2222935	2221267	2219901	2226583	2225453	2246962	110.0
Paeoniflorin	23722	19247	22025	22293	22354	22929	98.00
Hydroxypaeoniflorin	12701	10337	12509	12906	12232	12347	102.5
Paeoniflorin	52215	52018	51053	50615	50273	51487	117.9
Catechin	16679	14227	14883	14122	14900	15042	114.6

Table 3: Determination the stability of six chemical components of Chishao by UPLC method

No.	BJS	SYG	MSZS	ECS	SYNZG
1	7.6425	0.2423	2.4115	0.5106	0.0465
2	7.6482	0.2443	2.4152	0.5293	0.0508
3	7.5957	0.2399	2.3408	0.5170	0.0503
4	7.5547	0.2314	2.3423	0.4989	0.0499
5	7.6302	0.2282	2.351	0.5337	0.0508
6	7.6024	0.2202	2.4074	0.5206	0.0504
7	7.5285	0.2309	2.3804	0.5207	0.0504
Average	7.5213	0.2339	2.37830	0.5188	0.04994
RSD (%)	0.6	3.0	1.3	1.9	2.6

Limit of quantification

When the S/N was 10, the limit of quantification was measured for benzoic acid, paeoniflorin, hydroxy paeoniflorin, gallic acid, catechin, and paeoniflorin. Based on S/N=10, the limits of quantification of were determined to be 4μg/mL, 2.5μg/mL in sequence, 4μg/mL, 5μg/mL, 5μg/mL, 4μg/mL.

Recovery test

Take an appropriate amount of Chishao from the certain batch and divide them equally into 6 parts within equal reference solution. Then, methanol was put into solution to be tested by the method in the "1.2.2". After collection chromatogram, we could calculate recovery rate (%) after the determination. Component with the corresponding concentration. Under the analysis conditions, the contents of the four components were measured in the six test solutions that had been added to the reference substance. According to the calculation formula, the sample recovery rates were shown in table 2.

Stability test

A certain batch of Chishao was prepared to be sample solution and data were collected under the same measurement conditions at 0h, 2h, 4h, 6h, 10h, 12h and 24h, respectively. Under the condition of analysis condition, six chemical components were determined by UPLC-MS/MS and the peak area of benzoic acid, paeoniflorin, hydroxy paeoniflorin, gallic acid, catechin, and paeoniflorin were calculated to evaluate the stability value. The RSD was relatively larger at 24h. According to the analysis in table 3 and the sample solution was relatively stable 4h after the preparation. Therefore, the experiment should be completed within 4h after the solution preparation was accomplished. Standard RSD value was <3%.

Relative correction factors

1.0μL reference substance solution was precisely drawn and injected into the UPLC. The relative correction factors were calculated between peak area of hydroxy paeoniflorin and other compounds, including benzoic acid, paeoniflorin, gallic acid, catechin, and paeoniflorin, and the RSD <2.0% was qualified. Relative correction factors were 7.521, 0.2339, 2.378 0.5188, 0.04994, respectively. Injection volume had no significant effect on the relative correction factor.

Content determination

According to the standard curve, the contents of benzoic acid, paeoniflorin, hydroxy paeoniflorin, gallic acid, catechin, and paeoniflorin were calculated from No.1 to No. 8, which were listed in table 4. It's showed that the contents of benzoic acid and paeoniflorin were higher in No. 6 and the level of paeoniflorin was higher in No.8. In addition, the content of catechin was relatively higher in No. 5.

Comparison between QAMS and external standard method

8 batches of Chishao were prepared by the preparation method, and were determined under the chromatogram condition according to "1.3". Both the external standard method (ESM) and the one-test-multiple-assessment method (QAMS) were used to calculate the contents. Finally, the SPSS 20.0 was used to perform *t test* on two content analysis methods. It's shown that there was no significantly different between the two methods (p>0.05).

Table 4: Determination the six chemical components of Chishao by UPLC method

No.	Content Determination (%)					
	BJS	SYG	QJSYG	MSZS	ECS	SYNZG
1	0.4570	0.7747	1.609	0.1637	0.7890	0.6543
2	0.4833	0.2434	1.704	0.0488	0.0189	0.2090
3	0.3522	0.0681	0.7662	0.0284	0.0141	0.4485
4	0.6533	0.4773	1.384	0.0278	0.0311	0.3172
5	0.3593	0.0677	2.689	0.0087	0.0495	0.1940
6	0.1662	0.0079	0.4253	0.0091	0.0142	0.2071
7	0.2831	0.2671	0.4788	0.0136	0.0144	0.1603
8	0.2175	0.0332	0.3794	0.0094	0.0143	0.1702

DISCUSSION

The main active ingredients of Chishao are monoterpenoids and monoterpene glycosides, *etc*. Before determination, the CAD detector was chosen as the signal collector, which had the high sensitivity. The chromatogram conditions needed to screen, for example, the UV absorption wavelength of benzoic acid and paeoniflorin should be 230nm based on the content of the literature. However, hydroxy paeoniflorin's test wavelength was 263nm to keep hopeful intensity. Otherwise, ester glycosides and catechins had the high response intensity when the ultraviolet absorption wavelength was 274nm. Methodological verification tests had been shown that the UPLC method established could be used for six components' determination of Chishao in the current study, including benzoic acid and paeoniflorin, *etc*. The method had good repeatability and high sensitivity, which indicated that the method was reliable and accurate. In addition, this study also investigated the influence of different column temperatures, different instruments and columns on the relative correction factor and relative retention time (t_R). The results showed that both relative correction factor and relative retention time was repeated under different conditions.

The QAMS method was used to analyze multiple ingredients based on vital correction factors, which could complete the evaluation of the content of 6 components in Chishao from different origins at the same time. Compared with the external standard method, there were no significant differences. Therefore, the established QAMS method was quick, convenient and accurate, and provided the more comprehensive reference for the comprehensive evaluation and quality control of Chishao.

CONCLUSION

Hydroxy paeoniflorin was chosen as the internal reference material in the one-test-multiple-assessment method established in this study. By establishing a functional relationship with other components, the content of other components in Chishao could be determined more quickly and simply. It could be used to identify the content of various components in Chishao and provided reference for the quality control and effective substances study of Chishao.

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