·化学与分析·

气血双补酊剂 HPLC 指纹图谱及含量测定

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［摘要］ **目的**：采用 HPLC-DAD 方法，对气血双补酊剂进行指纹图谱分析和 10 种活性成分同时测定来进行质量控制。**方法**：通过优化提取、分离和分析条件，采用 Merck C_{18} 色谱柱，流动相乙腈-0.1% 磷酸水溶液，波长 203 nm。**结果**：通过分析 10 批样品指纹图谱，确立了 32 个共有峰作为特征峰来进行评价，10 批样品相似度高，10 种活性成分含量同时测定的结果稳定可靠。**结论**：该多成分含量测定的方法，结合色谱指纹图谱分析，具有实际应用，可以全面对气血双补酊剂进行质量进行控制。

［关键词］ 指纹图谱分析；高效液相-二极管阵列检测；多组分测定；质量控制；气血双补酊剂；相似度评价
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Determination and Fingerprint Analysis in Quality Control of Qixue Shuangbu Tincture

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［**Abstract**］ **Objective**: Using a simple and reliable HPLC-DAD method, for Qixue Shuangbu tincture (QST) of fingerprint analysis and simultaneous determination of 10 bioactive constituents to carry out quality control. **Method**: Through optimal extraction, separation and selection of analytical conditions, Merck C_{18} chromatographic column was adopted at wavelength of 203 nm, with acetonitrile and 0.1% phosphoric acid as mobile phase. **Result**: According to fingerprint analysis on 10 batches of samples, 32 peaks were adopted as the characteristic peaks and evaluation indexes. The similarity of the 10 batches of samples was high, and the determination of content of ten active components was stable and reliable. **Conclusion**: The multicomponent determination method combined with the chromatographic fingerprint analysis is a practical and meaningful method, and can be used for overall quality control of QST.

［**Key words**］ fingerprint analysis; HPLC-DAD; multicomponent determination; quality control; Qixue Shuangbu tincture; similarity evaluation

Nowadays, more and more traditional Chinese medicines (TCM) have been widely used worldwide due to their obvious curative effects[1-2]. Therefore, it is urgent to evaluate the quality control of herbal products and their preparations by modern technology. However, in conventional studies, only one or few chemical constituents are adopted for the quality control of TCM, which fail to completely reveal

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complex constituents and synergistic effects of both TCM and their preparations[3]. Therefore, a more comprehensive method shall be established to include most of active components, which is crucial for the quality control of TCM.

Qixue Shuangbu tincture (QST), a TCM prescription developed by professor Meng Jing-chun of Jiangsu Provincial Hospital of TCM to replenish blood and raise "Qi" (vital energy), is composed of nine common Chinese herbs, namely Ginseng Radix et Rhizoma, Astragali Radix, Angelicae Sinensis Radix, Paeoniae Radix Alba, Citri Reticulatae Pericarpium, Polygoni Multiflori Radix, Polygonati Odorati Rhizoma, Polygonati Rhizoma and Lycii Fructus[4]. Nowadays, QST is used not only as an efficacious medicinal prescription, but also as healthcare nourishment for relieving men's ailments in Asia. Indeed, recent publications have demonstrated that QST had many attractive pharmacological activities, including immune-regulatory effect, estrogenic effect, anti-inflammatory effect, cardio-protective effect and anti-diabetic nephropathy[5]. However, the actual active constituents of QST still remain unclear. Therefore, an accurate and reliable method shall be established to quantify the chemical constituents in QST, which is helpful for controlling the quality, studying the multiple active components of this famous TCM recipe and defining the combined administration of the single herbs.

In this study, a simple and sensitive analytical method for the simultaneous quantitative determination of 10 active components [namely paeoniflorin (PA), ferulic acid (FA), calycosin 7-*O*-*β*-*D*-glucoty pyranosid (CA), stilbene glucoside (SG), hesperidin (HE), ginsenoside Rb_1 (RB), astragaloside IV (AS), rhein (RH), emodin (EM) and physcion (PH)] in QST was established by HPLC-DAD. The established method is quite simple and particularly suitable for routine analysis on QST and its quality control.

1 Materials

Acetonitrile was adopted as the HPLC-grade reagent (manufactured by Tedia Company, Inc., Fairfield, CT, USA). Methanol, phosphoric acid and other reagents were adopted as analytical-grade reagents and purchased from Nanjing Chemical Reagent Co. Ltd. (Jiangsu, China). Purified water was prepared by using a Millipore water purification system (Milford, MA, USA) and filtered with a 0.22 μm membrane before use.

Reference substance of PA (batch number 110736-201337), HE (batch number 110722-201111), FA (batch number 110773-200611), CA (batch number 111920-201001), SG (batch number 110844-200505), RB (batch number 110704-200420), AS (batch number 110781-201314), RH (batch number 110757-200206), EM (batch number 110756-200110) and PH (batch number 110758-200611) were purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). Their structures were completely elucidated by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). The purity of each compound was determined to be more than 98% by HPLC based on the peak area normalization detected method. The 10 batches of QST samples (batch number 20150102, 20150202, 20150302, 20150402, 20150503, 20150602, 20150702, 20150801, 20150902, 20151003) were purchased from Nanjing Haichang Co. Ltd. (Jiangsu, China).

2 Methods and Results

2.1 Chromatographic Conditions HPLC-DAD system (Shimadzu, Kyoto, Japan), which is equipped with LC-solution software and comprised of a binary pump (LC-DAD), auto sampler (SIL-DAD), column oven and diode array detector (SPD-M20A), was used for the HPLC analysis. Chromatographic separation was carried out at 35 ℃ on a Merck C_{18} column (4.6 mm × 250 mm, 5 μm). The mobile phase was composed of acetonitrile (A) and 0.1% phosphoric acid aqueous solution (B) in a gradient elution mode (0-12 min, 2%-15% A; 12-20 min, 15%-42% A; 20-45 min, 42%-55% A; 45-63 min, 55%-70% A; 63-82 min, 70-86% A) at a flow rate of 1.0 mL·min^{-1}. The injection volume was 10 μL. The detection wavelength of the reference compounds was set at 203 nm.

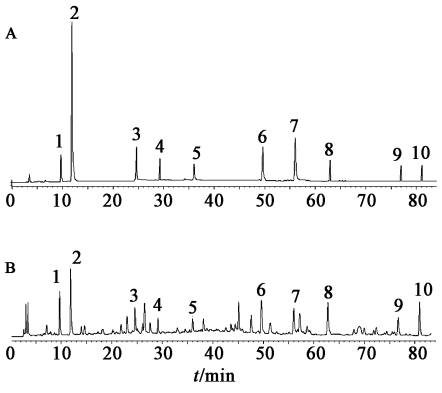
2.2 Preparation of Standard and Sample Solutions Each of the 10 reference standard stock solutions was prepared by being dissolved in methanol at a

concentration of 0.6 g·L^{-1}, respectively. Then each standard stock solution was adequately mixed and diluted to develop calibration plots at six concentrations (specifically, PA: 51.20, 114.6, 178.0, 241.4, 304.8, 368.2 mg·L^{-1}; HE: 3.590, 18.05, 32.51, 46.97, 61.43, 75.90 mg·L^{-1}; FA: 5.360, 17.74, 30.13, 42.52, 54.91, 67.30 mg·L^{-1}; CA: 1.401, 7.601, 13.80, 20.00, 26.20, 32.40 mg·L^{-1}; SG: 1.100, 5.080, 9.060, 13.04, 17.02, 21.01 mg·L^{-1}; RB: 1.210, 3.390, 5.570, 7.750, 9.930, 12.11 mg·L^{-1}; AS: 10.02, 23.67, 37.32, 50.98, 64.63, 78.32 mg·L^{-1}; RH: 6.230, 19.24, 32.25, 45.27, 58.28, 71.30 mg·L^{-1}; EM: 1.221, 3.418, 5.615, 7.812, 10.01, 12.21 mg·L^{-1}; PH: 4.281, 22.96, 41.65, 60.33, 79.01, 97.71 mg·L^{-1}). The lowest concentrations in the calibration curves was further diluted to get a series of new standard solutions for evaluating the limits of detection (LOD) and the limits of quantity (LOQ) of the compounds.

The QST samples were transferred into a 10 mL volumetric flask, mixed with 80% methanol, and filtered through a 0.22 μm filter membrane before being injected into the HPLC system for analysis.

2.3 Data Analysis To evaluate the changes in the 10 batches of QST, the similarity analysis was performed by the professional software *Similarity Evaluation System for Chromatographic Fingerprint of TCM* (Version 2004 A), which was recommended by the SFDA of China for evaluating similarities of chromatographic profiles of TCM.

2.4 Optimization of Detection Wavelength Conditions[6-7] The detection wavelength was defined by scanning the spectrum within the range of 200-800 nm. The 203 nm wavelength was superior to other wavelengths based on the maximum absorption and the minimum baseline noise, and therefore selected as the optimum detection wavelength for determination. Using the optimized chromatographic analysis on the conditions mentioned above, the determination of the selected compounds combined with fingerprint analysis was carried out in less than 82 min as shown in Fig 1. The 10 chromatographic peaks were identified by comparing their retention time with that of each reference compound, which was eluted in parallel with a series of mobile phases.



1. AS; 2. PA; 3. HE; 4. CA; 5. RB; 6. FA; 7. SG; 8. RH; 9. EM; 10. PH

Fig. 1 Typical chromatograms of mixed standards (A) and QST solution (B)

2.5 Method Validation[8-10] The HPLC method was validated with a high linearity, precision (both inter-day and intraday precision), accuracy, stability, specificity and selectivity, but a lower LOQ. The calibration curves were constructed by comparing the peak areas with the concentrations of the series of standard solutions. The correlation coefficient was determined by a linear regression model. The LOD was defined with a signal-to-noise (S/N) of 3, and the LOQ was defined with an S/N of 10. As shown in Table 1, all of the calibration curves showed a good linear regression ($r \geqslant 0.999\ 5$), and the LOD was less than 0.11 mg·L^{-1}, indicating that this HPLC-DAD method is precise and sensitive for the quantitative evaluation of major active components in QST.

By adding standards to real samples at three different concentrations and performing the extraction and analysis as above described, the recoveries were monitored to examine the accuracy of the method. According to the test results, the recoveries were calculated based on the differences between spiked and unspiked samples under the same conditions, and ranged between 96.3%-102.6%, with RSDs of less than 2.8%. It was clear that the established method was reliable and accurate for the determination of the 10 bioactive compounds. All of the above results indicated that the HPLC-DAD method was accurate, sensitive and precise for the quantification of the 10 compounds in QST.

The HPLC fingerprint method was verified under

Table 1 Linear regression data, LOD and LOQ of 10 constituents using HPLC-DAD

Component	Regression equation	Correlation factor	Linear range /mg	LOD /mg	LOQ /mg
AS	$Y = 6\ 137X - 992.2$	0.999 5	10.02 – 78.32	1.05	5.27
PA	$Y = 12\ 549X - 101.89$	0.999 6	51.20 – 368.2	8.85	23.5
FA	$Y = 9\ 629X + 223.58$	0.999 5	5.360 – 67.30	1.06	3.12
RH	$Y = 9\ 574X - 426.12$	0.999 8	6.230 – 71.30	0.47	2.41
PH	$Y = 11\ 744X + 941$	0.999 7	4.281 – 97.71	0.74	2.24
HE	$Y = 18\ 974X - 5\ 938$	0.999 9	3.590 – 75.90	0.32	0.96
SG	$Y = 12\ 479X + 572$	0.999 8	1.100 – 21.01	0.73	2.20
CA	$Y = 9\ 083X - 2\ 893$	0.999 9	1.401 – 32.40	0.69	3.60
RB	$Y = 12\ 074X + 891$	0.999 8	1.210 – 12.11	0.28	0.94
EM	$Y = 31\ 028X + 826$	0.999 8	1.221 – 12.21	0.11	0.42

the guidance of the Chinese Pharmacopoeia Commission. Its precision and reproducibility were evaluated by the analysis of six batches of the same sample solutions and six replicates of the same sample, respectively. The RSDs of retention time (t_R) and peak area of characteristic peaks in the precision test ranged between 1.2%-2.9%, with RSDs of t_R and peak area below 1.5% and 3.9%, respectively, in the reproducibility test. Meanwhile, the analysis of the same sample solutions at intraday different time points (0, 2, 6, 12, 18, 24 h) were evaluated as in a stability test, and with RSDs of t_R and peak area below 0.8% and 2.9%, respectively. These results indicated that the HPLC fingerprint analysis method was consistent with the requirements.

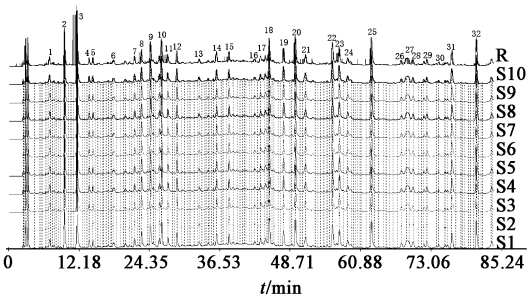
2.6 Quantitative Determination of QST The proposed method was applied in the simultaneous determination of the 10 active components in 10 batches of QST samples. According to the chromatograms of standards and samples shown in Fig 2, it was evident that the investigated components and other compounds in QST samples were separated appropriately by the established HPLC-DAD method. The quantitative analytical results are shown in Table 2.

Table 2 Contents of 10 components in QST $mg \cdot L^{-1}$

No.	AS	PA	FA	RH	PH	HE	SG	CA	RB	EM
S1	71.23	176.49	78.62	77.92	73.52	56.19	46.21	27.02	22.51	37.11
S2	72.27	183.73	77.28	75.03	72.35	55.37	45.41	25.94	22.72	32.63
S3	70.97	171.53	75.29	78.77	71.37	57.84	42.55	27.53	21.62	35.55
S4	69.52	169.63	75.03	78.02	72.83	58.62	46.26	28.52	24.72	37.73
S5	71.95	175.67	70.26	76.47	70.25	57.83	45.52	24.52	20.93	38.33
S6	76.29	159.82	72.77	76.63	73.83	54.02	49.12	27.93	22.36	34.73
S7	61.37	192.13	73.95	74.73	71.72	56.83	44.51	26.83	24.65	34.66
S8	71.27	185.25	75.72	77.72	74.82	53.51	44.28	28.26	23.63	36.45
S9	70.15	197.28	76.83	75.94	73.32	55.93	43.82	27.74	22.79	35.73
S10	68.48	167.82	75.62	78.13	74.61	57.63	45.92	27.24	23.72	34.62

2.7 Fingerprint Analysis of QST[11-15] A total of 10 batches of QST were investigated. The standardized HPLC fingerprint was generated with the average method based on the general comparison of these different batches by using the professional software of *Similarity Evaluation System for Chromatographic Fingerprint of TCM* (Version 2004 A). The similarity index of each sample was calculated by comparing the correlation coefficient value with simulated standard fingerprint. The similarity of the fingerprints was calculated based on the relative ratio of peak area and retention time. The selected peaks were automatically compared with chromatographic peaks on a matching template, and then showed differences in peak performance evaluation and overall evaluation on a standard template. Thirty-two peaks were chosen as 'characteristic peaks' in fingerprints of QST samples, which presented a good resolution most of the time.

The similarities of the 10 batches of QST samples exceeded 0.93, indicating that the samples were consistent to some extent. Among them, based on comparison for retention time with reference standards, 10 peaks, namely peak 2, 3, 9, 12, 14, 20, 22, 25, 31, and 32, were assigned to compound astragaloside IV, paeoniflorin, hesperidin, calycosin 7-*O*-*β*-*D*-glucoty pyranosid, ginsenoside Rb_1, ferulic acid, stilbene glucoside, rhein, emodin and physcion, respectively. Peak 20 (ferulic acid) was assigned as the reference peak, because it was one of the most intense peaks in the chromatograms. According to the HPLC fingerprints, the similarities of the QST samples were further quantified. The similarities of characteristic peaks from the 10 samples are shown in Table 3. The SA results indicated that the samples at different time points had similar correlation coefficients, suggesting that these fingerprint chromatograms could be used for the quality control of QST.



2. AS; 3. PA; 10. HE; 12. CA; 14. RB; 20. FA; 21. SG; 25. RH; 28. EM; 31. PH

Fig. 2 Chromatographic fingerprints of 10 batches of QST through similarity evaluation system for chromatographic fingerprint of TCM software

Table 3 Similarities among different batches of QST and the common mode

No.	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	R
S1	1	0.938	0.941	0.933	0.937	0.939	0.933	0.932	0.938	0.941	0.950
S2	0.938	1	0.999	0.983	0.999	0.999	0.983	0.982	0.999	0.998	0.996
S3	0.941	0.999	1	0.989	0.998	1	0.988	0.988	0.999	0.999	0.998
S4	0.933	0.983	0.989	1	0.982	0.989	0.997	0.998	0.985	0.987	0.994
S5	0.937	0.999	0.998	0.982	1	0.999	0.981	0.981	0.999	0.998	0.996
S6	0.939	0.999	1	0.989	0.999	1	0.988	0.988	0.999	0.999	0.998
S7	0.933	0.983	0.988	0.997	0.981	0.988	1	0.999	0.984	0.986	0.994
S8	0.932	0.982	0.988	0.998	0.981	0.988	0.999	1	0.984	0.986	0.994
S9	0.938	0.999	0.999	0.985	0.999	0.999	0.984	0.984	1	0.998	0.997
S10	0.941	0.998	0.999	0.987	0.998	0.999	0.986	0.986	0.998	1	0.997
R	0.950	0.996	0.998	0.994	0.996	0.998	0.994	0.994	0.997	0.997	1

3 Discussion

In this study, a new HPLC-DAD quantitative method and fingerprint analysis combined with chemometric methods were established and applied in the quality control of QST. This is the first report for the simultaneous determination of the 10 marker compounds in QST. To define optimal separation, optimal retention time and maximal chemical information in the chromatograms, such chromatographic conditions as mobile phase, flow rate, column temperature, chromatographic column, detection wavelength, and gradient elution profiles were investigated in this study. Various mobile phases consisting of acetonitrile-water and methanol-water with some modifiers including acetic acid, formic acid and phosphoric acid of different pH values were investigated in different gradient elution modes. Column temperatures at 25, 30, 35 ℃ and flow rates at 0.8, 1.0, 1.2 $mL \cdot min^{-1}$ were also investigated. Different columns packed with different materials, namely Kromasil C_{18} (4.6 mm × 250 mm, 5 μm), Lichrospher C_{18} (4.6 mm × 250 mm, 5 μm), Merck C_{18} (4.6 mm × 250 mm, 5 μm) and YMC-Pack ODS-A C_{18} (4.6 mm × 250 mm, 5 μm) were employed and compared. After the above tests, the Merck C_{18} column with the acetonitrile-0.1% phosphoric acid aqueous solution in a gradient elution mode was found suitable for the simultaneous separation and determination.

After column lifetime and analytical time were taken into account, a constant flow rate at 1.0 mL·min^{-1} and column temperatures at 35 ℃ were chosen for HPLC analysis. The results clearly demonstrated that the described HPLC-DAD analytical method could qualitatively and quantitatively determine the content of the 10 major components in QST samples. The established HPLC method has the advantages of high simplicity, precision, accuracy and sensitivity, and is proved to be suitable for controlling the quality of QST.

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