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Research Article

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Studies on HPLC fingerprint of total glycosides of Bazhen decoction and quantitative analysis of three components

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ABSTRACT

To develop chromatographic fingerprint of total glycosides of Bazhen decoction and simultaneous determination of three components，including paeoniflorin , ginsenoside Rg_1 and ginsenoside Rb_1. Total glycosides of Bazhen decoction were separated and purified using a macroporous adsorption resins method, HPLC-DAD method was applied for fingerprint of total glycosides and simultaneous determination. The mobile phase, consisting of acetonitrile- phosphoric acid solution(0.05%),was programmed for a gradient elution. The flow rate was 1.0 mL/min, the detection wavelength was monitored at 203 nm, and the column temperature was maintained at 40 ℃. There were 15 common peaks in the chromatographic fingerprint，among these peaks, peak 1,9,13 were identified as paeoniflorin, ginsenoside Rg_1, ginsenoside Rb_1, respectively. The contents of 3 components were detected in the same chromatographic conditions. This study indicated that the combination of chromatographic fingerprint and quantitative analysis can be readily utilized as comprehensive evaluation of total glycosides of Bazhen decoction.

Keywords: total glycosides of Bazhen decoction, fingerprinting, determination

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INTRODUCTION

Ba zhen Tang is basic decoction from the Song Dynasty Prescription of peaceful benevolent dispensary, which is composed of sijunzitang and siwutang[1]. Radix ginseng and prepared rhizome of rehmannia were the monarch drug of Bazhentang, Angelicae Sinensis Radix, White Peony Root, atractylodes rhizome and Poria were the minister drug , Szechwan Lovage Rhizomeand Glycyrrhizae Radix were the assistant drug. Ba zhen Tang were widely used in medical department, surgical department, pediatrics department, gynecology department and blood department[2]. The active compounds reported in Bazhentang are glycosides, polysaccharides,flavones, alkaloids and steroids etc. The primary bioactive components of Radix ginseng are ginsenosides[3-5]. Paeoniflorin is the main active constituent of White Peony roots[6-7]. Herein, we developed methods for a systematic identification of total glycosides of Bazhen decoction. Total glycosides of Bazhen decoction have been shown to exhibit a wide range of biological activities like anti-ischemic , anti-tumor, anti-aging , enhance hematopoietic function and improve immune function. The popular methods used in identifying and analyzing herbal medicines is chromatographic technique[8]. In this study, a simple, reliable and sensitive analysis method by high-performance liquid chromatography coupled with diode array detection was developed for both fingerprint analysis of total glycosides of Bazhen decoction and quantitative determination of the three active compounds in Bazhen decoction.

EXPERIMENTAL SECTION

Instrumentation

Analytical HPLC was carried out using a Waters Alliance 2695 separations module-Waters 2996 photodiode array detector(Waters Technologies, USA), including a quaternary pump, an online vacuum degasser, an autosampler and

Empower software. KQ-250E ultrasonic cleaner was from Kunshan Ultrasonic Instrument Co., company; Germany Sartorius ACCULAB ALC-110.4 electronic balance.

Reagents and materials

Paeoniflorin reference (batch number: 110736-200320), Ginsenoside Rg_1 reference (batch number: 11610-200402), Ginsenoside Rb_1 reference (batch number: 110704-200420). All standard substance were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Acetonitrile were of HPLC grade and phosphoric acid were of analytical reagent grade. Double distilled water was used for all experiments.

Standard solution preparation

A mixed stock solution of standards, containing paeoniflorin (0.0515mg/mL), ginsenoside Rg_1 (0.0525 mg/mL) and ginsenoside Rb_1 (0.0520 mg/mL) were prepared in methanol. Working solutions of the low concentration were prepared by diluting the stock solutions with methanol in appropriate quantities.

Preparation of sample solutions

Concentrated extracts of Bazhentang were dispensed into suspension containing 0.25 g/mL crude drug, and adjusting pH value with hydrochloric acid to 6.0. 10 mL of the extractive of Bazhentang was added on a column of macroporous resin (D101, dry weight 2.0g). Total glycosides was enriched in the 40BV of 80% ethanol elution(in the speed of 3BV/min). The eluate was concentrated under reduced pressure, drying, and the content of total glycosides was 0.2253g. The residue was resolved with methanol to make exactly 10 mL, then were filtered through a 0.22μm filter before HPLC analysis.

Chromatographic Conditions

Separation was performed on a Waters Symmetry C_{18} Column (5 μm, 4.6 mm × 250 mm); The gradient HPLC elution was made using the mobile phase composed of 0.05% aqueous phosphoric acid (A) and acetonitrile (B); Detector wave length was set at 203 nm and the flow rate was kept constant at 1.0 mL/min; Column oven temperature was maintained at 40ºC. The gradient elution program were given in Table-1.

Table-1: Elution profile of gradient steps

T/min	Flow mL/min	A/%	B/%
0	1	85	15
25	1	65	35
40	1	60	40
50	1	50	50

HPLC Fingerprint of total glycosides of Bazhen decoction

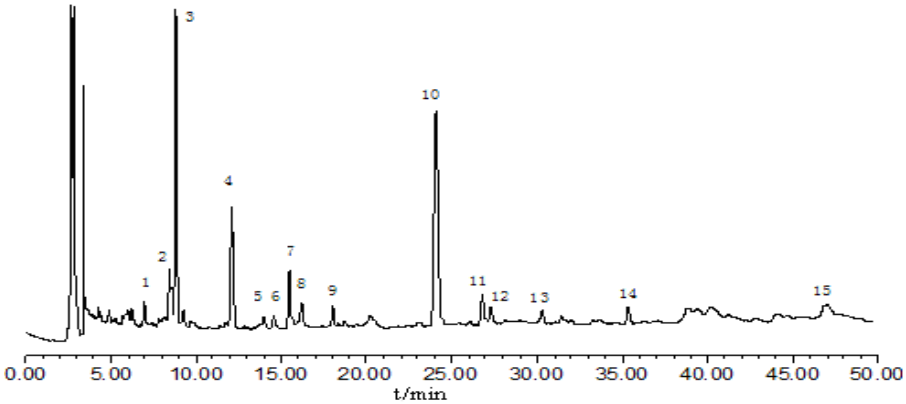
Method validation

Precision, repeatability and stability

For method validation study, the precision, stability and repeatability experiments were performed on total glycosides sample. The precision test was determined by replicating measurements of the same sample solution for five times. The RSD of the relative retention time of each common peak was found to range between 0.11% and 0.44%, and the RSD of the relative peak area of each common peak were between 0.15% and 1.92%. The stability test was determined by replicate analysis of the same sample solution during 48 hours(0、6、12、24、48h). The RSD of relative retention time and relative peak area were no more than 0.43 and 1.87%, respectively. The repeatability test was assessed by injecting five independently prepared samples,and the RSD of relative retention time and relative peak area were no more than 0.39 and 1.81%, respectively.All the RSD of precision, stability and repeatability of the sample is less than 3%, according with the regulation.

HPLC fingerprints development of total glycosides and identification of common peaks

In this study, the chromatographic fingerprints of total glycosides were obtained from 10 batches of samples. There were 15 peaks were selected as the common peaks to evaluate the similarities among different batches of total glycosides samples. Peak 3, which with highest response and symmetrical， was chosen as the internal reference peak to calculate the relative retention time and relative peak area of all other peaks. By comparing the retention time, UV absorption spectra and maximum absorption wavelengths of peaks in sample chromatogram with those of reference substance, 3 peaks were unambiguously identified as paeoniflorin(peak 1), ginsenoside Rg_1(peak 9) and ginsenoside Rb_1(peak 13).The chromatographic fingerprint shown in Fig-1, Fig-2 and Table-2.



1-paeoniflorin 9-ginsenoside Rg_1 13-ginsenoside Rb_1

Fig-1 Typical HPLC fingerprint of total glycosides in BaZhen Decoction

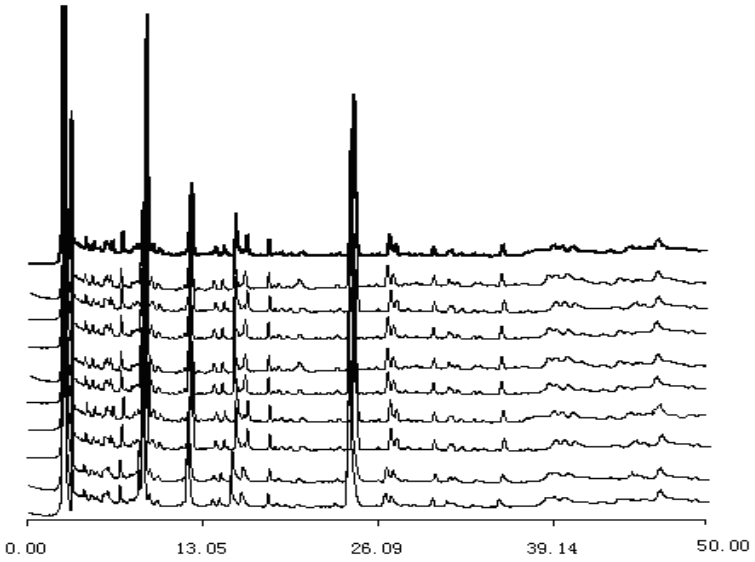


Fig-2 The common HPLC fingerprint of 10 batches of total glycosides in BaZhen Decoction

Table-2 Relative retention time and relative peak areas of common peaks（n=5）

Peak no	retention time	Peak area	relative retention time	relative peak area
1	7.011	504512	0.798	0.084
2	8.500	1157745	0.968	0.193
3	8.785	5994155	1.000	1.000
4	12.133	3374701	1.381	0.563
5	13.929	476625	1.586	0.080
6	14.576	374437	1.659	0.063
7	15.446	1399053	1.758	0.233
8	16.234	999521	1.848	0.167
9	18.001	534457	2.049	0.089
10	24.110	8526862	2.744	1.423
11	26.833	1021754	3.054	0.171
12	27.253	718861	3.102	0.120
13	30.311	673405	3.450	0.112
14	35.323	571890	4.021	0.095
15	46.967	17270881	5.346	2.881

Data analysis

The chromatograms of 10 batches of samples were compared by the software of Similarity Evaluation System for Chromatographic Fingerprint of TCM. The simulative mean chromatogram was generated as the representative standard fingerprint and the similarity of each chromatogram against the mean chromatogram was also calculated. All the similarity of the 10 samples are more than 90% and the results are shown in Table-3.

Table-3 The correlation coefficients of similarity in fingerprint （n=3）

No	1	2	3	4	5	6	7	8	9	10
similarity	0.914	0.944	0.956	0.923	0.971	0.901	0.963	0.937	0.985	0.939

Quantitative Analysis of paeoniflorin, ginsenoside Rg1, ginsenoside Rb1 in total glycosides

Linearity of quantification

The stock solutions containing three active constituents were diluted with methanol to yield a series of appropriate concentrations for the construction of calibration curves. The analyte solutions at five different concentrations were injected to the HPLC system in triplicate, and the regression curve were established by plotting the peak area (Y) against the concentration (x) of each constituent. The calculated results were listed in Table-4.

Table-4 Standard curve, correlation coefficient and linearity range of reference substances

Component	Standard curve	R	linearity range (mg/mL)
Paeoniflorin	Y=928433X+396	0.9999	0.0515-0.824
Ginsenoside Rg_1	Y =4104327X -1508	0.9997	0.0525-0.840
Ginsenoside Rb_1	Y =3755091X +19907	0.9998	0.0520-0.832

Precision, Stability, Reproducibility and Recovery

The intra-day precisions were assessed by analyzing known concentrations of three analytes in six replicates during one day. The RSD values of three active constituents were 0.05%, 0.60%, 0.46%, respectively. Precision results indicated satisfactory precision of the proposed methods. For the stability test, the same sample solution was re-analyzed at 0,3,6,12,24 hours. The stability (RSD, n = 3) of the measurements over 24h for the three compounds were 0.07%,0.71%,0.29%, respectively. Repeatability was confirmed by injecting five independently prepared analytical sample solutions in the proposed chromatographic conditions. the RSD under conditions of repeatability were less than 1%(0.04%,0.08%,0.24%, respectively). In the recovery test, three different quantities levels (low, medium and high) of the authentic standards were added to pre analyzed sample, then the mixtures were extracted as sample preparation described above. Contents for three compounds were subsequently calculated from its corresponding calibration curve. The mean recovery of paeoniflorin, ginsenoside Rg_1 and ginsenoside Rb_1 were 98.88%,97.43%,96.95%, and the RSD value were 0.18%,1.37%,1.21%, respectively.

Sample Analysis

The newly established HPLC analytical method was subsequently utilized for simultaneous determination of the bioactive components in 5 batches of total glycosides samples. Each sample was analyzed individually in triplicate to calculate the mean content (mg/g), and the results are summarized in Table-5.

Table-5 Determination result of 3 components in total glycosides of BaZhen Decoction (n=3)

constituent	1	2	3	4	5
paeoniflorin（mg/g）	24.2087	24.0996	24.1331	24.1719	24.0529
ginsenoside Rg_1（mg/g）	6.2063	6.1498	6.0972	6.1524	6.1581
ginsenoside Rb_1（mg/g ）	7.9097	7.7192	7.7815	7.8484	7.8564

CONCLUSION

The chromatogram of different wavelengths between 200 and 400 nm were compared，and a wavelength of 203 nm was selected as the detection wavelength for quantitative analysis at which all the index components showed absorption. Under above optimized chromatographic conditions, 15 chromatographic peak were well separated. The results indicated that the samples from different batches shared a satisfactory similarity, and the similarities of 10 batches samples were higher than 0.90. The present work provides a systematic quality control including chromatographic fingerprint analysis and simultaneous determination of multiple markers. The developed method lays a solid foundation for further comprehensive study on the pharmacodynamic-pharmacokinetic effects of Bazhen decoction.

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