Simultaneous Determination of 10 Active Components in Baizhu Shaoyao San and Its Single Herbs by High-Performance Liquid Chromatography Coupled with Diode Array Detection

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Baizhu Shaoyao San (BSS) is a famous traditional Chinese medicinal prescription, which is composed of Rhizome atractylodis macrocephalae, Radix Paeoniae Alba, Pericarpium Citri Reticulatae and Radix Saposhnikovia. In this study, a simple and sensitive high-performance liquid chromatography coupled with diode array detection method was established for the simultaneous determination of 10 marker compounds including atractylenolide I, gallic acid, albiflorin, paeoniflorin, narirutin, hesperidin, nobiletin, cimifugin, prim-O-glucosylcimifugin, 4'-O-β-D-glucosyl-5-O-methylvisamminol in BSS and its single herbs. The chromatographic separation was performed using a YMC C18 column with a gradient elution system of acetonitrile and 0.1% formic acid aqueous solution at a flow rate of 1 mL/min. The results demonstrated that the validated method was simple, reliable and successfully applied to evaluate the selected compounds in BSS and its single herbs for quality control. Moreover, the experimental data showed that the contents of two major active components detected in BSS decoction were significantly higher than those in the single herbs, which indicated that the combination of single herbs could result in the difference in the amount of active constituents.

Introduction

Traditional Chinese medicines (TCMs) and their preparations have been practised for thousands of years in China, and there is an increasing acceptance and usage of them owing to their undoubted protecting and therapeutic effects. TCMs are generally prescribed in combination in order to reach the effects of synergizing or to reduce potential adverse reactions (1). However, conventional research usually just use one or few chemical constituents for quality control of TCMs, which is unable to completely reveal the complex constituents and synergistic effects of both TCMs and their preparations (2). Therefore, establishment of a more comprehensive method, which could involve most of the active components, is crucial to control the quality of TCMs (3).

Baizhu Shaoyao San (BSS) is one of famous traditional Chinese preparations, comprising four herbs: Rhizome atractylodis macrocephalae, Radix Paeoniae Alba, Pericarpium Citri Reticulatae and Radix Saposhnikovia. The preparation is an effective prescription for the clinical treatment of diarrhea, especially for the irritable bowel syndrome in China (4). Modern pharmacological research has revealed some constituents such as atractylenolide I (AT-I) in Rhizome atractylodis macrocephalae gallic acid (GA), albiflorin (AF) and paeoniflorin (PF) in Radix Paeoniae Alba; narirutin (NR), hesperidin (HP) and nobiletin (NOB) in Pericarpium Citri Reticulatae; and cimifugin (CF), prim-O-glucoxylic acid (PG) and 4'-β-D-glucosyl-5-O-methylvisamminol (GM) in Radix Saposhnikovia, which were found to be responsible for the biological activities in the four single herbs and proved to be the active components (5–11). Their chemical structures are shown in Figure 1. However, the actual active constituents of BSS have still remained unclear.

Up to date, only a few analytical methods have been reported to determine the contents of active components in BSS, such as high-performance liquid chromatography/diode array detection/electrospray ionization tandem mass spectrometry (HPLC-DAD-ESI-MS/MS) (12) and reversed-phase high-performance liquid chromatography (RP-HPLC) (13). Besides, to the best of our knowledge, the methods used in previous reports only focused on one or several constituents of the whole preparation and lacked the quantitative determination of each single herb in BSS for quality control as well. Therefore, an accurate and reliable method is needed to quantify the chemical constituents in both BSS and its single herbs, which is helpful for controlling the quality, searching the multiple active components of this famous TCM recipe and revealing the meaning of combined use of single herbs.

In this study, a simple and sensitive analytical method for the simultaneous quantitative determination of 10 active components (including AT-I, GA, AF, PF, NR, HP, NOB, CF, PG and GM) present in BSS and its single herbs was developed by using HPLC-DAD. The developed method is quite simple and particularly suitable for the routine analysis of BSS and its quality control.

Experimental

Chemicals and reagents

Acetonitrile was of HPLC grade (Tedia Company, Inc., Fairfield, CT, USA). Ethanol, formic acid and other reagents were of analytical grade and purchased from Nanjing Chemical Reagent Co.,
Figure 1. Chemical structures of tested components in BSS.

Gallic acid (GA)  Albiflorin (AF)  Paeoniflorin (PF)

Prim-O-glucosylcimifugin (PG)  Cimifugin (CF)

Narirutin (NR)  4’-O-β-D-glucosyl-5-O-methylvisaminol (GM)

Hesperidin (HP)  Nobiletin (NOB)  Atractylenolide I (AT-I)
Reference compounds of GA and HP were purchased from the Control of Pharmaceutical and Biological Products (Beijing, China). AF, PF, PG, CF, NR, GM, NOB and AT-I were purchased from Nanjing Zelang Biological Technology Co., Ltd. (Nanjing, 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 normalization of the peak area detected by HPLC.

All the medicinal herbs were purchased from Nanjing Haiyuan Prepared Chinese Crude Drugs Co., Ltd. (Jiangsu, China) and authenticated by Prof. Jianwei Chen (Nanjing University of Chinese Medicine, Nanjing, China).

**Chromatographic conditions**

A Shimadzu LC-20A HPLC system (Shimadzu, Kyoto, Japan), equipped with LC-Solution software and comprised of a binary pump (LC-20AD), auto sampler (SIL-20A), column oven and diode array detector (SPD-M20A), was used for the HPLC analysis. Chromatographic separation was carried out at 35°C on a YMC-Pack ODS-A C18 column (250 × 4.6 mm, 5 μm). The mobile phase was composed of acetonitrile (A) and 0.1% formic acid aqueous solution (B) with gradient elution system (0–8 min, 5% A; 8–10 min, 5–15% A; 10–40 min, 15–25% A; 40–45 min, 25–50% A; 45–55 min, 50–70% A; 55–60 min, 70–85% A; 60–65 min, 85–5% A) at a flow rate of 1.0 mL/min. The injection volume was 10 μL. The detection UV wavelength of reference compounds was set at 280 nm.

**Preparation of standard and sample solutions**

Each standard stock solution of the 10 reference standards (GA, AF, PF, PG, CF, NR, GM, HP, NOB and AT-I) was prepared by dissolving them in methanol at a concentration of 0.8 mg/mL, respectively. Then each standard stock solution of the 10 reference standards was adequately taken, mixed and diluted to six concentrations for construction of calibration plots (GA, AF, PF, PG, CF, NR, GM, HP, NOB and AT-I) in the ranges of 4.59–45.95, 16.16–161.60, 8.66–86.60, 79.60–796.0, 4.10–41.0, 9.70–97.0, 8.23–82.30, 5.78–57.80, 1.21–12.11 and 1.22–12.21 μg/mL. Further dilution with the lowest concentrations in the calibration curves was carried out to afford a series of standard solutions for evaluating the limits of detection (LOD) and the limits of quantitation (LOQ) of the compounds. All the solutions were stored away from light at 4°C and brought to room temperature before use.

**Method validation**

Before performing any validation experiment, the system suitability test was performed by injecting a standard solution, in order to assure that the system and the procedure were capable of providing data of acceptable quality (14). Theoretical plates, retention factors, tailing and injection repeatability were determined. The developed chromatographic method was validated for specificity, linearity, precision and accuracy.

**Specificity**

The specificity was investigated by the determination of the peak purity with the aid of a DAD. The absorption spectrum of a single component remained little variable at each time point in one peak, which supported the specificity of each peak (15). The results clearly showed the specificity of each peak for 10 marker compounds by comparing the retention times with the noted standard references.

**Calibration curves, LOD and LOQ**

All calibration curves were plotted with seven different concentrations of standard solutions. The regression equations were calculated, and then the extract was transferred into a 10 mL volumetric flask with 75% methanol and filtered through a 0.22 μm nylon filter for HPLC analysis. All sample solutions were stored at 4°C and used at room temperature.

**Results**

**Optimization of HPLC conditions**

In general, a suitable chromatographic column, mobile phase, elution mode and detection wavelength are critically important for good separation. In this study, different columns packed with different materials, i.e., Kromasil C18 (250 × 4.6 mm, 5 μm), Lichrospher C18 (250 × 4.6 mm, 5 μm), Zorbax SB C18 (250 × 4.6 mm, 5 μm), Hypersil C18 (150 × 4.6 mm, 5 μm), Lichrosorb C18 (150 × 4.6 mm, 5 μm) and YMC-Pack ODS-A C18 (250 × 4.6 mm, 5 μm) were employed and compared. Various mobile phases consisting of acetonitrile–water and methanol–water with some modifiers including acetic acid, formic acid and phosphoric acid with different pH values were investigated under different gradient elution modes. The detection wavelength was selected according to the maximum absorption wavelengths of GA, AF, PG, CF, NR, GM, HP, NOB and AT-I at 273, 230, 280, 230, 297, 283, 292, 283, 328 and 220 nm, respectively, shown in UV spectra with three-dimensional chromatograms of DAD. The effect of the flow rate was concerned in the range of 0.7–1.5 mL/min and a flow rate of 1 mL/min was determined to be the optimum for good separation in a reasonable time. After the above tests, the YMC-Pack ODS-A C18 column with the acetonitrile–0.1% formic acid aqueous solution system using gradient elution was found suitable for the simultaneous separation and determination. Typical chromatograms of mixed standards and samples (including BSS and its single herbs) are shown in Figure 2. The peaks 1–10 represent GA, AF, PF, PG, CF, NR, GM, HP, NOB and AT-I, respectively.

Purified water was prepared using a Millipore water purification system (Milford, MA, USA) and filtered with a 0.22 μm membrane before use. Reference compounds of GA and HP were purchased from the Control of Pharmaceutical and Biological Products (Beijing, China). AF, PF, PG, CF, NR, GM, NOB and AT-I were purchased from Nanjing Zelang Biological Technology Co., Ltd. (Nanjing, 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 normalization of the peak area detected by HPLC.

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Figure 2. Typical chromatograms of mixed standards and samples. (A) mixed standards; (B) BSS solution; (C) Rhizome atractylodis macrocephalae solution; (D) Radix Paeoniae Alba solution; (E) Pericarpium Citri Reticulatae solution and (F) Radix Saposhnikovia solution. Peak 1: GA, 2: AF, 3: PG, 4: PF, 5: CF, 6: NR, 7: GM, 8: HP, 9: NOB, 10: AT-I.
Figure 2. Continued
calculated based on linear regression analysis of the integrated peak areas ($y$) versus concentrations ($x$, µg/mL) of the 10 marker constituents in the standard solution.

LOD and LOQ defined as the 3- and 10-fold of the ratio of the signal-to-noise ($S/N$) were also obtained. LOD and LOQ of 10 marker compounds were within a range of 0.01–14.65 and 0.03–44.40 µg/mL, respectively, which showed a high sensitivity under this chromatographic condition. Detailed information about calibration curves, linear ranges, LOD and LOQ are listed in Table I.

### Precision, repeatability and stability

The intra-day and inter-day precisions were validated by six replicate injections of three different concentrations of mixed standard solutions during a single day and on three consecutive days under the optimized conditions. Six independent sample solutions of BSS in parallel were prepared and analyzed for evaluating the repeatability. Stability was also tested by analyzing sample solutions at 0, 2, 4, 6, 8, 10, 12 and 24 h. As shown in Tables II and III, the intra-day, inter-day, repeatability and stability RSD values of the 10 marker compounds were all less than 3.74%.

### Recovery

Recovery was conducted by adding three accurately known quantities of the corresponding marker compounds to a sample of BSS that had previously been analyzed. Average recoveries of investigated targets ranged from 95.10 and 104.96%, and RSD values were all less than 3.45% for all the 10 compounds, indicating that the developed method was reliable and accurate enough for the measurement (Table IV).
BSS and its single herbs can be sufficiently resolved and separated. The results showed that three components (GA, AF and PF) in the formula were from the single herb *Radix Paeoniae Alba*, three components (NR, HP and NOB) from *Pericarpium Citri Reticulatae*, three components (GF, CF and GM) from *Radix Saposhnikovia* and one component (AT-I) from *Rhizome Atractylodis macrocephalae*. Furthermore, the concentrations of NR and HP in BSS were about 2 and 30 times higher than those in *Pericarpium Citri Reticulatae*, respectively, while other eight constituents in the prescription varied slightly in comparison with those in single herbs according to the quantitative analysis.

**Discussion**

The differences demonstrated above indicated that the application of prescription may change the concentrations of constituents in single herbs and then contribute to the different clinical effects. This may be based on some chemical reactions, such as hydrolysis and dehydration condensation, taking place during the process of decocting four single herbs together. Furthermore, much attention should be paid to physical effects such as solubilization effect and co-dissolving effect in decoction of single herbs, which are very important to TCM prescriptions (16).

**Conclusion**

Nowadays, more and more TCMs are extensively used worldwide for their obvious curative effects. Therefore, it is urgent to evaluate and control the quality of herbal products and their preparations by modern technology. This is the first report for simultaneous determination of the 10 marker compounds in BSS as well as in its single herbs. The established HPLC method has the advantages of simplicity, precision, accuracy and sensitivity, and it is proved to be suitable for controlling the quality of BSS.

Meanwhile, this is the first time to compare the separated chemical components of BSS with those of its single herbs quantitatively. The above results of chemical analysis via HPLC-DAD demonstrate that the contents of both HP and NR in single herbs are significantly increased after combing the single herbs together in the formula under the same experimental condition. Such information illustrates the significance of formula as well as the combination of single herbs and indicates that HP and NR may be part of the material basis of the therapeutic effects of BSS.

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