Simultaneous Determinations of Eleven Bioactive Components in Suanzaoren Decoction Granules by High-Performance Liquid Chromatography and Its Application to the Quality Control in Productive Processes

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A simple and reliable method using high-performance liquid chromatography coupled with a photodiode array detector (HPLC-PDA) was firstly established for the determinations of eleven bioactive compounds (neomangiferin, mangiferin, spinosin, liquiritin apioside, liquiritin, fumalic acid, 6′′′-feruloylspinosin, senkyunolide I, isoliquiritin, glycyrrhizic acid and senkyunolide A) in Suanzaoren decoction (SZRD) extract and its granules. The chromatographic analysis was performed on a C18 column at 30°C. Excellent linear behaviors over the investigated concentration ranges were observed with the values of R² being higher than 0.9990 for all analytes. The developed method showed good precision and accuracy with overall intra- and inter-day variations of less than 2.0%, and overall recoveries in the range of 97.2 – 102.1%. The validated method was successfully applied to the determination of eleven components in SZRD samples from different production batches, including SZRD extract, lab-made SZRD granules and clinical medicine. This accurate and reliable HPLC-PDA method will be helpful for improving the quality evaluation of SZRD granules and its quality control in productive processes.

Keywords HPLC-PDA, Suanzaoren decoction granules, quality control, productive process

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Introduction

Insomnia, defined as persistent difficulty to fall or stay asleep, is a highly prevalent sleep disorder, which has a negative impact on the quality of life.1–3 The damages of insomnia not only involve vitality and energy, but also extend to other aspects of mental, social, and physical functioning.4 Current treatments for insomnia cover sleep hygiene measures, behavioral therapies and pharmacological treatments.5 A quite large proportion of insomnia patients would rather resort to the last therapeutic method.6 To some degree, many chemical medicines are limited by concerns regarding various adverse effects, such as tolerance, dependence and impaired cognitive function.5 Traditional Chinese medicines (TCM) have the characteristics of multiple pharmacological effects and relatively low potential adverse reactions in disease treatment.7

As one of most famous traditional Chinese herbal prescriptions with the effects of hypnosis, sedation and anti-convulsion,8,9 Suanzaoren decoction (SZRD), has been efficiently and widely used as a tranquilizing agent to treat insomnia for thousands of years in Asia.10 SZRD, a compound prescription composed of Ziziphi Spinosae Semen [dried and ripe semen of Ziziphus jujuba Mill. var. spinosa (Bunge) Hu ex H. F. Chou.], Poria (dried sclerotium of Poria cocos (Schw.) wolf.), Anemarrhenae Rhizoma (dried rhizome of Anemarrhena asphodeloides Bge.), Chuanxiong Rhizoma (dried rhizome of Ligusticum chuanxiong Hort.) and Glycyrrhizae Radix Et Rhizoma (dried root and rhizome of Glycyrrhiza uralensis Fisch., Glycyrrhiza inflata Bat. and Glycyrrhiza glabra L.), was firstly documented in the classical Chinese medical book JinGuiYaoLue (Synopsis of Prescriptions of the Golden Chamber) by Zhang Zhongjing (Han Dynasty).11 Compared with the traditional decoction form or raw materials, granules are easier to store and transport.12 In addition, granules are convenient for oral administration, and conducive to improving patient compliance. As a result, SZRD granules have enormous market potential. Such granules were manufactured by extracting five medicinal herbs with proper solvent and temperature, and then refining and granulation with a fluid-bed granulation technique. Thus, the quality of the granules is being challenged by many factors, such as the quality of raw herbal materials, different manufacturing processes, etc. The most critical problem is that no reliable quality-control method for granules and herbal extract is currently available.

It is widely accepted that the therapeutic effects of TCM prescription are integrative results of multiple bioactive components.13 Pharmacological studies on SZRD have further linked saponins, flavonoids, organic acids, phthalide and polysaccharides to their pharmacological activities and therapeutic efficacy.14–21 Many analytical methods have been established for the quantitative analysis of SZRD and SZRD granules, but most of them have relied on a few constituents as chemical markers.22–26 An analytical method for SZRD granules, capable of the detection of multi-components for quality control, is therefore needed.
In this paper, a selective, sensitive and reliable HPLC-PDA method for simultaneous determinations of eleven components, including neomangiferin (NM), mangiferin (M), spinosin (S), liquiritin apioside (LA), liquiritin (L), fumalic acid (FA), 6′′-teruloylspinosin (FS), senkyunolide I (SI), isoliquiritin (IL), glycyrrhizic acid (GA) and senkyunolide A (SA) in SZRD, was firstly established, and then successfully applied to SZRD extract, lab-made SZRD granules, and clinical medicine compound SZR granules (clinical granules) from different production batches. Moreover, the method was also used in analyzing the intermediate products of SZRD granules so as to control the quality in the productive process. The qualification of multiple ingredients offers a more reliable method for the routine analysis of SZRD granules and quality control in the productive process.

**Experimental**

**Reagents and chemicals**

The standards of FA, L and S were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), NM, M, LA, SI, IL and GA were prepared from Jiangsu Yongjian Pharmaceutical Technology Co., Ltd. (Jiangsu, China). FS, SA were bought from Tianjin Shilan Technology Co., Ltd. (Tianjin, China). The purities of the standards were all above 98.0%; the structures of the eleven compounds are shown in Fig. 1.

HPLC-grade acetonitrile and methanol were purchased from J&K Scientific Co., Ltd. (Beijing, China). Glacial acetic acid for HPLC was obtained from Shandong Yu Wang Industrial Co., Ltd. (Shandong, China). High-purity water (for HPLC) was obtained from Shandong Yu Wang Industrial Co., Ltd. (Shandong, China). Glacial acetic acid for HPLC was obtained from Shandong Yu Wang Industrial Co., Ltd. (Shandong, China). Glacial acetic acid for HPLC was obtained from Shandong Yu Wang Industrial Co., Ltd. (Shandong, China). Glacial acetic acid for HPLC was obtained from Shandong Yu Wang Industrial Co., Ltd. (Shandong, China). Glacial acetic acid for HPLC was obtained from Shandong Yu Wang Industrial Co., Ltd. (Shandong, China). Glacial acetic acid for HPLC was obtained from Shandong Yu Wang Industrial Co., Ltd. (Shandong, China).

**Ziziphi Spinosae Semen, Poria, Chuanxiong Rhizoma, Anemarrhenaes Rhizoma and Glycyrrhizae Radix Et Rhizoma were purchased from GuoDa Pharmacy (Shenyang, China), and identified by Prof. Ying Jia (Shenyang Research Center for Modernization of Traditional Chinese Medicine, Shenyang Pharmaceutical University). Voucher specimens were deposited at Shenyang Pharmaceutical University. The SZRD extract, intermediate products and finished Suanzaoen decoction granules were prepared by our laboratory in a previous study. Clinical granules were provided by General Hospital of Shenyang Military Region (Shenyang, China).

**Apparatus**

Analysis was performed by a Shimadzu 20A HPLC System (Shimadzu Corporation, Japan), consisting of a binary solvent delivery system, an on-line degasser, an auto-sampler, a column temperature controller and a photodiode array detector coupled with Lab solution software. An AB 135-S electronic balance (Mettler Toledo Co., Ltd., Switzerland) was prepared for sample weighing.

**HPLC analytical conditions**

Chromatographic separation was achieved with a Kromasil C18 column (250 × 4.6 mm, 5 μm) protected by a C18 guard column with a sample injection volume of 10 μL. The detection wavelength was set at 275 nm, and the flow rate was 1.0 mL min⁻¹. The column temperature was maintained at 30°C. The mobile phase consisted of 1% glacial acetic acid in water (A) and acetonitrile (B). The gradient program was as follows: 10 - 17% (B) in 0 - 6 min, 17 - 19% (B) in 6 - 11 min, 19 - 30% (B) in 11 - 30 min, 30 - 70% (B) in 30 - 40 min, 70 - 75% (B) in 40 - 50 min, 75 - 10% (B) in 51 - 55 min.

**Standard solution of SZRD extract**

Mixed stock solutions of eleven standard substances containing NM (296.6 μg mL⁻¹), M (271.5 μg mL⁻¹), S (83.2 μg mL⁻¹), LA (235.6 μg mL⁻¹), L (207.6 μg mL⁻¹), FA (32.60 μg mL⁻¹), FS (46.50 μg mL⁻¹), SI (95.5 μg mL⁻¹), IL (50.90 μg mL⁻¹), GA (217.9 μg mL⁻¹) and SA (77.60 μg mL⁻¹) for the determination were prepared in methanol.

**Standard solution of SZRD granules**

Mixed stock solutions of eleven standard substances containing NM, M, S, LA, L, FA, FS, SI, IL, GA and SA were prepared by accurately dissolving weighed standards with methanol to yield concentrations of 64.90, 58.20, 93.6, 178.8, 190.1, 16.30, 46.50, 119.4, 101.9, 305.1, 38.80 μg mL⁻¹, respectively.

Working standard solutions for establishing calibration curves were prepared by dilutions of the mixed standard solutions to appropriate concentrations with methanol, and stored at 4°C before use.

**Sample solutions**

Sample solutions of SZRD extract and negative control of SZRD extract were prepared by diluting 1.0 mL of SZRD fluid extract and SZRD negative control fluid extract (without the target herb) to 10 mL in 30% aqueous ethanol, respectively.

After 3.0 g of clinical granules and 1.5 g of lab-made SZRD granules were crushed into powder and accurately weighed respectively, they were transferred into an Erlenmeyer flask and extracted with 100 mL of methanol in an ultrasonic bath at room temperature for 30 min. After cooling, methanol was added into the solutions for the loss, and then the solutions were...
shaken and filtered. Finally, every solution was filtered through a syringe filter (0.45 μm) before being injected into the HPLC system for analysis.

Results and Discussion

Optimization of sample preparation

Considering the productive process of the SZRD extract, various ratios of ethanol (30, 50, 70%) and water were screened so as to optimize the dilution solvent. The total extract contents of the eleven bioactive compounds were 9.02, 8.49, 6.520 and 3.410 mg g⁻¹, respectively, and 30% aqueous ethanol was regarded as being the preferred choice, which provided the highest responses of the 11 markers with a broad range of polarity.

In terms of SZRD granules, a high proportion of organic solvents (75% ethanol, ethanol, 75% methanol and methanol) were tried so as to reduce the columns pollution from water-soluble excipients. The results revealed that ethanol did not benefit efficient extraction due to the low solubility in ethanol. However, methanol exhibited less interfering peaks, and allowed for the most efficient extraction with the total yield contents being nearly triple as much as ethanol did. Then, the sample to solvent ratios (1:10, 3:100, 1:50, 3:200, and 1:100, w/v), time (15, 30, 45 min) of sonication were also investigated to optimize the extraction procedure. The results demonstrated that the total extraction values of lab-made granules increased to over 10% when the extraction time varied from 15 to 30 min, and showed no obvious change when the extraction time was extended to 45 min, and the similar situation appeared in the extraction of clinical granules. There is 100 mL of methanol for the extraction of 1.5 g of SZRD lab-made granules and 3.0 g clinical granules in 30 min was the appropriate condition owing to operational convenience, and a relatively higher value achieved.

Optimization of chromatographic conditions

Optimization of the separation conditions for HPLC analysis was performed, including the chromatographic column, mobile phase composition, and gradient elution program. The resolutions of these compounds were tested and compared with different reversed-phase conditions using a variety of analytical columns, such as a Merck Purospher STAR RP-18 (250 × 4.6 mm, 5 μm) and Kromasil C₁₈ (250 × 4.6 mm, 5 μm). Then Kromasil C₁₈ was selected for the following experiments for appropriate resolution and response.

Different compositions of the mobile phase were tried to obtain good resolution and sharp peaks. Various mixtures of water and methanol were used as the mobile phase, but the separation was not satisfactory. However, when methanol was replaced by acetonitrile, the situation was greatly improved, and satisfactory resolution was obtained. Considering most of the compounds are slightly acidic, the addition of acid in the mobile phase was found to enhance the resolution and to eliminate any peak tailing of the target compounds. Thus, the type and concentration of acids (phosphoric acid, formic acid and glacial acetic acid) were examined. As a result, acetonitrile and water containing 1% glacial acetic acid gave the best resolution and eliminated the tailing of all investigated compounds.

Different maximum adsorption wavelengths of analytes were shown in UV spectra with three dimension chromatograms of PDA. Specifically, NM, M, S, LA, L, FA, FS, SI, IL, GA and SA were at 256, 258, 336, 275, 274, 323, 330, 360, 276, 249 and 275 nm, respectively. However, all components showed an appropriate absorption at 275 nm simultaneously with a smooth baseline. Concerning the simplicity and versatility of the method, a wavelength of 275 nm was selected as the detection wavelength for multi-ingredient determination, which is more suitable for routine work.

Furthermore, other chromatographic variables were also optimized, including analytical column temperatures (25, 30, 35°C), and flow rates (0.8, 1.0, 1.2 mL min⁻¹). It was suggested that a column temperature of 30°C and a flow rate of 1.0 mL min⁻¹ could give the desired separation within a shorter time.

Validation of the HPLC method

The specificity was assessed by comparing the chromatograms obtained from the analysis of standard solution, SZRD sample solutions and negative control samples. The integration peak in the chromatogram of the sample solution corresponded in time to the peak in the chromatogram of standard solution. As shown in Fig. 2, a good separation was obtained under the determination condition, and no interfering peaks appeared in negative control samples. The purities of the investigated peaks were confirmed to be pure through PDA purity studies.

Comparative studies were performed by using the reference compounds, SZRD samples of full herbs and negative control solutions to identify the origin of these target compounds from each herb. Chromatograms indicated that NM and M were from Anemarrhenae Rhizoma; S and FS were from Ziziphi Spinosae Semen; FA, SI and SA were from Chuanxiong Rhizoma; (H) negative control sample of SZRD extract without Glycyrrhize Radix Et Rhizoma; (I) negative control sample of SZRD extract without any herb. The peaks marked with 1 – 11 are: 1, neomangiferin; 2, mangiferin; 3, spinosin; 4, liquiritin apioside; 5, liquiritin; 6, fumaric acid; 7, 6′′-feruloylspinosin; 8, senkyunolide I; 9, isoliquiritin; 10, glycyrrhizic acid; 11, senkyunolide A.
appropriate concentrations for plotting the calibration curves. Good relationships between the concentrations and the peak areas of the analytes within the test ranges were achieved ($R^2 \geq 0.9990$). The limit of detection (LOD) and limit of quantification (LOQ) values of individual compounds were determined at a signal-to-noise ratio of about 3 and 10, respectively. Detailed information regarding the calibration curves, linear ranges, LOD and LOQ are listed in Table 1.

For intra-day variability tests, the SZRD samples were analyzed by three replicates at three different concentrations (low, medium, high). The inter-day variability of the precision was analyzed by three replicates each day on three consecutive days, respectively. The accuracy was further investigated with a recovery test performed by adding three concentration levels (low, medium and high) of the mixed standard solutions to known the amounts of the SZRD samples. The results of the precision and recovery tests are recorded in Table 2.

To further evaluate the stability of the developed assay, samples were analyzed in triplicate at different times (0, 2, 4, 8, 12, 24, 48 h). The relative standard deviation (RSD) value of the peak areas of each compound was calculated. The RSD values were not more than 2.0% for all components, indicating that the analytes were relatively stable in sample solutions at room temperature for 48 h.

**Sample analysis**

The validated analytical method was successfully applied to the simultaneous determinations of the 11 compounds in different SZRD samples, i.e. SZRD extract, lab-made SZRD granules and clinical granules. The results are given in Table 3, which indicate that the contents of the analyzed compounds from different production batches in all SZRD samples varied a lot. In addition, the contents of analytes in lab-made SZRD granules were much higher than the clinical granules. This variation might have resulted for several reasons. First, different originals and harvest times of the compositional herbal materials may cause their quality discrepancy. Second, different manufacturing processes, e.g. heating temperature and heating time, could affect the contents of some unstable compounds.

The method was also used for the quality control of SZRD granules in the productive process from the extract and intermediates to the finished granules (the granules were manufactured by extracting the five medicinal herbs to extract, refining to intermediates and granulation to finished granules).
The contents and the transport rates of each target compound were calculated; the results were summarized in Table 4. It can be seen that the transfer rate of fumalic acid from SZRD extract to the intermediate product was the lowest, which may arise from the instability of FA in high-temperature environments.27 Conversely, the transfer rate of IL was the highest, and even surpassing 100%. It might be due to the conversion of some other ingredients to IL. Since certain structurally/uni00A0similar compounds from liquorice could convert into each other.28

Conclusions

Increasing numbers of TCM are being used worldwide. Efficient methods to evaluate and control the quality of TCM products are urgently needed. In this study, an accuracy and sensitive HPLC-PDA analytical method was firstly established and validated for the determination of 11 major components in SZRD, and then successfully applied to quality evaluations of clinical granules and different SZRD samples collected from different steps in the productive progress of SZRD granules, including SZRD extract, intermediate product and finished SZRD granules. The developed method has the advantages of simplicity, precision, accuracy and sensitivity, and therefore could make a contribution to the quality control of SZRD granules in the productive process.

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