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Quality assessment of Zaoren-an-shen granule by High-performance liquid chromatography (HPLC) fingerprinting and quantitative analysis

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To control the quality of Zaoren-an-shen granule (ZRG), a simple, reliable and reproducible method of High-Performance Liquid Chromatography equipped with Photodiode Array Detector (HPLC-DAD) was developed for fingerprint analysis and quantitative determination of three major classes of constituent’s namely phenolic acids, flavonoids and lignanoids. In the fingerprint analysis, nineteen peaks were selected as characteristic peaks and the chemical characteristics of three herbs in ZRG were presented in the HPLC chromatographic file. In the quantitative analysis, one flavonoid spinosin, four phenolic acids including danshensu, protocatechuic acid, protocatechuic aldehyde and salvianolic B and four lignanoids including schisandrin, gomisin A, deoxyschizandrin and gomisin N were successfully separated on an Ultimate™ XB-C₁₈ column (250 × 4.6 mm I.D., 5.0 µm) at 35°C. The mobile phase was acetonitrile-water (containing 0.03% phosphoric acid) employing a gradient elution at a flow rate of 1.0 mL/min, and the detection wavelength was set at 280 nm. Regression equations revealed good linear relationship between the peak areas of the analytes and their concentrations (r > 0.9991) and the recoveries were in the range of 95.5~103.7%. The results indicated that the developed assay method could be considered as a suitable quality control method for ZRG.

Key words: Zaoren-an-shen granule, high-performance liquid chromatography, quality control, fingerprint.

INTRODUCTION

Based on its own advantages and characters, the modernization of Traditional Chinese Medicine (TCM) aims at researching and developing medicines which can be legally traded in the international pharmaceutical market. By making full use of modern scientific techniques and referring to international pharmaceutical standards and norms, the modernization will greatly increase the competitiveness of TCM in the international market. Therefore, the quality control is essential for the modernization of TCM. Great progress has been made in the quality control of TCM. A combination of fingerprint analysis and multi-components quantitative analysis is the most efficient method for controlling the efficacy and stability of TCM.

Zaoren-an-shen granule (ZRG) is a TCM formulation recorded in the ministerial standards of Ministry of Health (Chinese Pharmacopoeia Commission, 1998), which has been clinically used for the treatment of agrypnia, morbid forgetfulness and cardiopalmus caused by nervous exhaustion. The formula, on which the preparation is based, is composed of three Chinese medicinal herbs,
Suanzaoren (Semen Ziziphi Spinossae), Danshen (Radix et Rhizoma Salviae Miltiorrhizae), and Wuweizi (Fructus Schisandrae Chinensis) which are all listed in the Chinese Pharmacopoeia (edition, 2005, volume 1) and have been commonly used for treating insomnia diseases for a long time (Peng et al., 2000; Fang et al., 2010; Huang et al., 2007). Our recent studies showed that ZRG significantly and dose-dependently augmented pentobarbital-induced sleep in super-threshold (40 mg/kg, i.p.) and sub-threshold dosages (30 mg/kg, i.p.), reflected by increased sleeping duration and sleeping numbers of mice. By correlation analysis of chemical and pharmacological data, three major classes of constituents, that is, flavonoid (spinosin) from Suanzaoren, phenolic acids (danshensu, protocatechuic acid, protocatechuic aldehyde and salvianolic acid B) from Danshen and lignanoids (schisandrin, gomisin A, deoxyschizandrin and gomisin N) from Wuweizi were selected as therapeutic components. Such methodology for elucidating the therapeutic material basis and quality control indices of TCM was started since 1990s by Luo and Bi et al. (1993) in our laboratory and applied successfully for studying Suan-Zao-Ren decoction (Li and Bi, 2006) and Lin-Gui-Zhu-Gan decoction et al. Hence the nine compounds can be regarded as important indicators of quality variation and inconsistency in the pharmaceutical processes. Until now, quality control of ZRG using chromatographic fingerprint techniques has not been reported. Most of the previous reports focused on quantitative analysis of one or two constituents in ZRG using Thin Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC) (Ding et al., 2007; Li et al., 2008; Wang et al., 2009). The aim of this study was to develop a reliable HPLC method that could be used for both quantitative determinations of nine bioactive compounds and fingerprint analysis to control the quality of ZRG.

**MATERIALS AND METHODS**

**Chemicals procurement**

Acetonitrile and methanol were HPLC grade from Yu-wang Chemical Factory (Shandong, China). Phosphoric acid was HPLC grade from Beijing Reagent Company (Beijing, PR China). Double distilled water was used throughout. Standard substances of danshensu, protocatechuic acid, protocatechuic aldehyde and salvianolic acid B (Figure 1) were all purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Spinosin, schisandrin, gomisin A, deoxyschizandrin and gomisin N (Figure 1) were obtained from Shenyang Pharmaceutical University and their structures were fully characterized by chemical and spectroscopic methods Ultraviolet (UV), Infrared (IR), Nuclear magnetic resonance (NMR) and MS. It was proved that their purities were above 98%.

**Plant materials and manufacturing procedures of ZRG pilot products**

The intact herbal materials used for preparation of ZRG were obtained from Liaoning academy of Chinese medicine. The pilot products of ZRG (Batch No. Z-001, Z-002, Z-003, Z-004) were prepared as blow: Amounts of crude drugs (Suanzaoren 250 g, Danshen 50 g and Wuweizi 50 g) equivalent to the dose of Zaoren-an-shen granules were mixed. A 10-fold mass of water was added and the mixtures were boiled on an electric heater for two hours. The extraction was repeated twice; then, the extracted solutions were combined together, concentrated and dried under reduced pressure to produce dried extract. Finally the extract was mixed thoroughly with pharmaceutical adjuvant of dextrin to produce the ZRG.

**High-performance liquid chromatograms system**

Chromatographic analysis was performed on an Agilent series 1100 HPLC instrument equipped with a quaternary pump, a diode-array detector, a column compartment, an autosampler and an Agilent LC chemistation. An Ultimate™ XBD C18 column (250 × 4.6 mm I.D., 5.0 µm) at 35°C was applied for all analytes. The mobile phase was composed of acetonitrile (A) and water containing 0.03% phosphoric acid (B). The elution program was designed as follows: 0 -10 min, 6 -14%(A); 10 - 35 min, 14 - 25%(A); 35 - 40 min, 25 - 40%(A); 40 - 50 min, 40 - 70%(A); 50 - 70 min, 70 - 100%(A), the flow rate was 1.0 mL min⁻¹. The optimum wavelength was set at 280 nm.

**Preparation of standard solutions**

The reference standards of the target compounds, that is, danshensu (1), protocatechuic acid (2), protocatechuic aldehyde (3), salvianolic B (4), spinosin (5), schisandrin (6), gomisin A (7), deoxyschizandrin (8) and gomisin N (9) were accurately weighted and dissolved in methanol: water (70:30 v/v) to prepare working standard solutions for the establishment of calibration curves.

**Sample solutions and negative control samples preparation**

The sample solution for LC analysis was prepared by suspending exact ZRG (1.0 g) in 10 mL 70% methanol (v/v), ultrasonically extracted for 30 min, and then cooled at room temperature; 70% methanol were added to compensate for the lost weight. After filtered with a 0.45 µm Millipore membrane, the filtrate was ZRG sample. Suanzaoren, Danshen, Wuweizi and the combination of whichever two crude drugs, with the amounts equivalent to the dose in ZRG, were weighed and prepared according to the process of ‘ZRG’ to produce S (Suanzaoren extract), D (Danshen extract), W (Wuweizi extract), SD (ZRG without Wuweizi), SW (ZRG without Danshen) and DW (ZRG without Suanzaoren). The extracts mentioned above were used as negative control samples and for investigating the interaction of the crude drugs as well.

**RESULTS AND DISCUSSION**

**Optimization of the extraction method**

In order to achieve the satisfactory extraction efficiency, extraction method, extraction solvent and extraction time were investigated. Since danshensu and salvianolic B were thermally labile (Chen et al., 2007; Mao et al., 2003; Ni et al., 2006), it was found out that the ultrasonic extraction was better than refluxing. Various extraction
solvents including water, methanol-water (30: 70, v/v; 50: 50, v/v; 70: 30, v/v) and methanol were screened. By comparing the sum numbers and areas of characteristic peaks in each chromatogram, the most suitable condition
for extraction solvent was methanol-water (70:30, v/v). Then the optimal extraction time was studied: samples were extracted with 70% methanol in ultrasonic bath for 20, 30, 40, 50 min, respectively. At last, all analyzed compounds were almost completely extracted within 30 min. It turned out that second extraction step was unnecessary.

**Optimization of chromatographic conditions**

To achieve chromatograms with adequate chemical information and better resolution within a reasonable analysis time for both quantitative determination and qualitative fingerprint analysis, the column, mobile phase, detection wavelength, column temperature and gradient elution conditions were investigated in this study. Different types of chromatographic column were investigated, more chemical information and good baseline separation could not obtain on Hypersil C18 column. Analysis time of constituents was too long on Diamonsil C18 column. The Ultimate™ XB-C18 column was found to be more suitable for good separation and adequate chemical information. Since the phenolic acids, flavonoids and lignanoids have great disparity in physicochemical property, a gradient elution mode was needed for complete separation of the nine target compounds. A low concentration acid was used to restrain the ionization of phenolic acids and flavonoids. Because these wavelengths showed maximum absorption for lignanoids, flavonoids, and phenolic acids respectively. Only 280 nm was found to be more suitable for both adequate characteristic peaks in the fingerprint chromatogram and sensitive detection. Therefore, 280 nm was selected as detection wavelength. The effect of temperature on the separation was investigated in the range of 25 - 40°C, 35°C was better than the others.

**Method validation of quantitative analysis**

Method validation for the quantitation of nine compounds was performed in terms of specificity, linearity, limits of detection and quantification (LODs and LOQs), intra-day and inter-day precisions, repeatability, stability and recovery test. Nine target compounds from ZRG were identified by comparing the retention times and UV spectra with those of reference standards. The analytes were further identified by spiking the actual samples with the standards. Moreover, according to the HPLC chromatograms of different negative control samples, there were no interferences for determination of danshensu, protocatechuic aldehyde, salvianolic B, spinosin, schisandrin, gomisin A, deoxyxyschizandrin and gomisin N at 8.3, 12.9, 24.1, 39.1, 50.4, 51.8, 59.6 and 61.3 min, respectively.

Calibration curves were plotted based on the integrated peak areas versus six different concentrations of standard solutions. All 9-calibration curves showed good linearity ($r > 0.9991$) within test ranges. The LODs and LOQs were calculated at signal-to-noise (S/N) of 3 and 10, respectively. All the results were summarized in Table 1.

Intra- and inter-day variations were utilized to assess the precision. The intra-day variation was performed on six individual samples within 1 day and inter-day variation was determined on three consecutive days. To confirm the repeatability, six different working solutions prepared from the same sample (Batch No. Z-001) were analyzed. The Relative Standard Deviation (R.S.D.) was taken as a measure of precision and repeatability. Table 1 showed the results which indicated that the R.S.D. values of the overall intra- and inter-day variations were less than 3.8%.

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**Table 1.** Calibration curves, detection limits, quantification limits, precision and repeatability of the analytes (n = 6) by HPLC-DAD.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Regression equation</th>
<th>Linear range (µg/ml)</th>
<th>R (n = 6)</th>
<th>LOD (µg/ml)</th>
<th>LOQ (µg/ml)</th>
<th>Precision R.S.D. (%)</th>
<th>Repeatability R.S.D. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$y = 9.04x + 16.19$</td>
<td>13.24 ~ 132.4</td>
<td>0.9993</td>
<td>0.04</td>
<td>0.13</td>
<td>0.25 ~ 3.43</td>
<td>2.1</td>
</tr>
<tr>
<td>2</td>
<td>$y = 34.05x + 10.45$</td>
<td>2.38 ~ 23.8</td>
<td>0.9991</td>
<td>0.01</td>
<td>0.04</td>
<td>0.26 ~ 3.14</td>
<td>1.7</td>
</tr>
<tr>
<td>3</td>
<td>$y = 61.09x + 7.727$</td>
<td>1.24 ~ 12.4</td>
<td>0.9994</td>
<td>0.01</td>
<td>0.03</td>
<td>0.34 ~ 3.10</td>
<td>1.2</td>
</tr>
<tr>
<td>4</td>
<td>$y = 10.03x + 75.17$</td>
<td>72.19 ~ 721.9</td>
<td>0.9993</td>
<td>0.07</td>
<td>0.23</td>
<td>0.30 ~ 2.81</td>
<td>2.1</td>
</tr>
<tr>
<td>5</td>
<td>$y = 8.44x - 5.747$</td>
<td>60.24 ~ 361.5</td>
<td>0.9999</td>
<td>0.07</td>
<td>0.22</td>
<td>2.12 ~ 2.34</td>
<td>1.6</td>
</tr>
<tr>
<td>6</td>
<td>$y = 9.19x + 6.685$</td>
<td>23.57 ~ 141.4</td>
<td>0.9999</td>
<td>0.04</td>
<td>0.14</td>
<td>1.81 ~ 1.04</td>
<td>0.9</td>
</tr>
<tr>
<td>7</td>
<td>$y = 10.93x - 0.3254$</td>
<td>3.45 ~ 20.72</td>
<td>0.9999</td>
<td>0.03</td>
<td>0.11</td>
<td>1.86 ~ 1.97</td>
<td>1.3</td>
</tr>
<tr>
<td>8</td>
<td>$y = 9.14x - 0.5628$</td>
<td>3.47 ~ 20.81</td>
<td>0.9994</td>
<td>0.07</td>
<td>0.25</td>
<td>1.84 ~ 1.85</td>
<td>0.7</td>
</tr>
<tr>
<td>9</td>
<td>$y = 10.26x - 1.565$</td>
<td>5.35 ~ 30.89</td>
<td>0.9997</td>
<td>0.05</td>
<td>0.17</td>
<td>1.63 ~ 3.85</td>
<td>0.5</td>
</tr>
</tbody>
</table>

*The notation for analytes refer to Figure 1, y is the peak area, x refers to the concentration of compound (µg/ml), R is the correlation coefficient of the equation. LOD, limit of detection (S/N = 3), LOQ, limit of quantification (S/N = 10).*
Table 2. Recoveries of nine compounds in ZRG.

<table>
<thead>
<tr>
<th>Analyte&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>Added (µg)</th>
<th>Measured (µg)&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Recovery&lt;sup&gt;d&lt;/sup&gt;</th>
<th>R.S.D. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>137.7</td>
<td>141.3</td>
<td>102.7</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>169.5</td>
<td>163.2</td>
<td>96.4</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>211.9</td>
<td>209.2</td>
<td>98.7</td>
<td>1.6</td>
</tr>
<tr>
<td>2</td>
<td>24.75</td>
<td>25.47</td>
<td>102.9</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>45.21</td>
<td>44.60</td>
<td>98.7</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>59.96</td>
<td>61.03</td>
<td>101.8</td>
<td>2.3</td>
</tr>
<tr>
<td>3</td>
<td>10.17</td>
<td>10.16</td>
<td>99.9</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>17.11</td>
<td>16.89</td>
<td>98.7</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>26.04</td>
<td>26.18</td>
<td>100.5</td>
<td>1.9</td>
</tr>
<tr>
<td>4</td>
<td>1011</td>
<td>980</td>
<td>96.7</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>1516</td>
<td>1450</td>
<td>95.5</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>2022</td>
<td>2020</td>
<td>99.6</td>
<td>0.8</td>
</tr>
<tr>
<td>5</td>
<td>662.7</td>
<td>659.7</td>
<td>99.5</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>843</td>
<td>824</td>
<td>97.7</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>1024</td>
<td>1026</td>
<td>100.2</td>
<td>0.9</td>
</tr>
<tr>
<td>6</td>
<td>282.8</td>
<td>270.3</td>
<td>95.6</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>353.5</td>
<td>341.4</td>
<td>96.6</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>424.2</td>
<td>416.4</td>
<td>98.2</td>
<td>1.1</td>
</tr>
<tr>
<td>7</td>
<td>34.54</td>
<td>35.43</td>
<td>102.6</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>44.90</td>
<td>45.68</td>
<td>101.7</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>55.26</td>
<td>57.28</td>
<td>103.7</td>
<td>1.0</td>
</tr>
<tr>
<td>8</td>
<td>38.15</td>
<td>39.22</td>
<td>102.6</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>48.55</td>
<td>48.52</td>
<td>99.9</td>
<td>1.1</td>
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<td></td>
<td>58.96</td>
<td>57.91</td>
<td>98.7</td>
<td>2.6</td>
</tr>
<tr>
<td>9</td>
<td>58.82</td>
<td>57.39</td>
<td>97.6</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>74.86</td>
<td>74.88</td>
<td>100.0</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>90.9</td>
<td>90.5</td>
<td>99.6</td>
<td>2.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> The notation for analytes refer to Figure 1.<br><sup>b</sup> Triplicate assay at each concentration level.<br><sup>c</sup> Calculated by subtracting the total amount after spiking from the amount in the ZRG before spiking.<br><sup>d</sup> Calculated as detected amount/added amount × 100%.

and the repeatability was less than 2.1% for the analytes. The stability studies (RSDs < 3.9%) manifested that the sample solutions were stable during 2 days when stored at room temperature. To evaluate the accuracy of the method, recovery experiments were carried out by adding three concentration levels of the mixed standard solutions to the samples in which the contents of marker compounds were known. Then the spiked samples were treated according to the above preparation procedure. The ratios of measured and added amounts were calculated. As a result, the recoveries of this method were in the range of 95.5 - 103.7% as shown in Table 2. Therefore, the verified HPLC-DAD method was precise, accurate and sensitive enough for simultaneous evaluation of nine active compounds in ZRG.

Method validation of high-performance liquid chromatography fingerprint

The Relative Retention Times (RRT) and Relative Peak Areas (RPA) of “characteristic peaks” were used for HPLC fingerprint analysis as the parameters evaluated and validated. Chromatographic peaks with relatively larger areas and good separation were selected as characteristic peaks for identification of the ZRG. After carefully analyzing the fingerprint profiles of the samples, nineteen characteristic peaks (Figure 2) meet the requirements. Among these components, No. 12 peak (salvianolic B), one of the bioactive compounds in ZRG, was used as the reference peak and the RRT and RPA of all characteristic peaks were obtained with reference to this substance. The relative standard deviations (R.S.D.s) of RRT and RPA in the precisions test not exceeded 0.09 and 2.59% (n = 6), respectively. Repeatability of the RRT and RPA of all characteristic peaks was below 0.16 and 3.74% (n = 6), respectively. And for stability tests, the R.S.D.s of RRT and RPA were less than 0.31 and 3.41% (n = 6), respectively, which demonstrated that the conditions used in the HPLC fingerprint analysis were stability and reproducibility.
Table 3. Contents of nine bioactive compounds in four batches of the ZRG product.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Four phenolic acids</th>
<th>Flavonoid</th>
<th>Four lignanoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-001</td>
<td>0.505 0.101 0.048 2.710</td>
<td>1.160 0.457 0.076 0.066</td>
<td>0.108</td>
</tr>
<tr>
<td>Z-002</td>
<td>0.504 0.103 0.058 2.441</td>
<td>1.179 0.471 0.081 0.064</td>
<td>0.111</td>
</tr>
<tr>
<td>Z-003</td>
<td>0.488 0.099 0.056 2.364</td>
<td>1.142 0.457 0.079 0.062</td>
<td>0.107</td>
</tr>
<tr>
<td>Z-004</td>
<td>0.544 0.111 0.062 2.621</td>
<td>1.268 0.507 0.087 0.069</td>
<td>0.119</td>
</tr>
</tbody>
</table>

a The value = the content of bioactive compound / ZRG product amount, b The notation for analytes refer to Figure 1.

Quantitative analysis of the marker compounds in ZRG and its negative control samples

The verified analytical method was successfully applied for simultaneous determination of danshensu, protocatechuic acid, protocatechuic aldehyde, spinosin, salvianolic B, schisandrin, gomisin A, deoxyschizandrin and gomisin N in four batches of ZRG (Table 3). The content of target compounds in ZRG and its negative control samples for investigating the interaction of the crude drugs were showed in Table 4. In order to compare easily, the amount of negative control samples should be equally converted into those of corresponding crude drugs in ZRG. The results suggested that the content of phenolic acids in ZRG, including danshensu, protocatechuic acid, protocatechuic aldehyde and salvianolic B varied greatly compared with the other groups. The decrease of danshensu content in DW was more noticeable than those in D, SD and ZRG, while the salvianolic B content displayed a distinguished reversed tendency which was higher in D and DW. It suggested that danshensu was decomposed to rosmarinci acid under acidic condition which provided by Wuweizi; so the content of danshensu was the lowest in DW. Salvianolic B has two ester bonds which are not stable and easily influenced by temperature and pH. During the decoction and concentration process, a small number of salvianolic B was easily hydrolyzed to lithospermic acid and danshensu; but acid environment provided by Wuweizi could help to slow the decomposition rate (Guo et al., 2007 and Xu et al., 2005).

Another notable phenomenon was that the contents of
Table 4. Contents of the nine bioactive compounds in ZRG, combination of each two crude drug (SD, SW and DW) and corresponding single herb (S, D, W).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Four phenolic acids</th>
<th>Flavonoid</th>
<th>Four lignanoids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>ZRG</td>
<td>2.22</td>
<td>0.45</td>
<td>0.24</td>
</tr>
<tr>
<td>S</td>
<td></td>
<td>0.78</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>2.34</td>
<td>0.30</td>
<td>17.50</td>
</tr>
<tr>
<td>W</td>
<td>0.17</td>
<td></td>
<td>1.74</td>
</tr>
<tr>
<td>SD</td>
<td>2.15</td>
<td>0.05</td>
<td>0.54</td>
</tr>
<tr>
<td>SW</td>
<td>0.31</td>
<td>0.24</td>
<td>0.46</td>
</tr>
<tr>
<td>DW</td>
<td>1.29</td>
<td>0.16</td>
<td>0.12</td>
</tr>
</tbody>
</table>

- The value = the content of bioactive compound corresponding crude drug amount.
- The notation for analytes refer to Figure 1.
- S = Semen Ziziphi Spinosae; D = Radix Salviae Miltiorrhizae; W = Fructus Schisandrae; SD = ZRG without Fructus Schisandrae; SW = ZRG without Radix Salviae Miltiorrhizae, DW = ZRG without Semen Ziziphi Spinosae.

Lignanoids from Wuweizi changed significantly in different preparations. ZRG and SW had a higher content of schisandrin, lower content of deoxyschizandrin and gomisin N than those in W. Yue et al. (2006) reported that saponins might be helpful to increase the dissolution of schisandrin which was the active component for treating insomnia (Huang et al., 2007), and interestingly, Suanzaoren contains this kind of saponins. While the decrease of the content of deoxyschizandrin and gomisin N in ZRG and SW suggested that the variation of the two components was caused by interaction with the components in Suanzaoren rather than Danshen. The variation phenomenon was also observed in DW. We found that combinations of Wuweizi with Danshen could make all the contents of lignanoids increase. The results suggested that it was reasonable for many prescriptions combining with the two drugs to treat insomnia. In addition, it is widely reported that spinosin plays an important role in sedation and hypnosis (Shin et al., 1978; Wang et al., 2008; Kawashima et al., 1987); our research also confirmed that the content of spinosin was the highest in ZRG. The results mentioned above proved that TCM preparations had general therapeutic effect compared with single medicinal material. The effective matter exerting efficacy is the complicated constituents which may be compatible or may antagonize with each other, and illustrated the importance of evaluating the quality of ZRG by simultaneous determination of multi-components.

Correlation between ZRG preparations and their raw herbal medicines

Using HPLC method, four batches of ZRG were analyzed under the given chromatographic conditions. As shown in Figure 2, the chromatograms of ZRG from four samples consisted of 19 characteristic peaks within 65 min. Among these components, nine characteristic peaks including danshensu, protocatechuic acid, protocatechuic aldehyde, spinosin, salvianolic B, schisandrin, gomisin A, deoxyschizandrin and gomisin N (peak number 3, 4, 6, 8, 12, 15, 16, 17 and 19, respectively), were identified by comparing the relative retention time with those of the authentic standards.

To analyze the possible individual contributions from the corresponding raw herbs to the general chromatographic profile, a correlation study was carried out between ZRG and the negative control samples in the work. The chromatograms of S, D, W and ZRG preparation were merged in one chart, shown in Figure 3. Comparing the relative retention time and DAD spectra in ZRG fingerprint with those in S, D and W, it could be found that there were 6 peaks in ZRG fingerprint provided by S, 8 peaks provided by D, and 7 peaks provided by W. In addition, peak 10 was contributed by the common constituent of S and D, and peak 18 was attributed to D and W. The attribution of the characteristic peaks in the HPLC chromatographic fingerprints were shown in details in Table 5.

Conclusions

This is the first report on the fingerprint analysis and simultaneous determination of nine bioactive compounds in ZRG by HPLC. The simple, sensitive and reliable HPLC method is suitable for routine quantitative analysis and quality control of ZRG. Moreover, based on the method developed in this study, further studies of pharmacodynamics should be carried out.

REFERENCES

Table 5. The attribution of the characteristic peaks in the HPLC chromatographic fingerprints.

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Compound names</th>
<th>Corresponding herbs*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1, 5, 7, 9</td>
<td>-</td>
<td>S</td>
</tr>
<tr>
<td>8</td>
<td>Spinosin</td>
<td>S</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>S and D</td>
</tr>
<tr>
<td>3</td>
<td>Danshensu</td>
<td>D</td>
</tr>
<tr>
<td>6</td>
<td>Protocatechuic aldehyde</td>
<td>D</td>
</tr>
<tr>
<td>12</td>
<td>Salvianolic B</td>
<td>D</td>
</tr>
<tr>
<td>11, 13, 14</td>
<td>-</td>
<td>D</td>
</tr>
<tr>
<td>4</td>
<td>Protocatechuic acid</td>
<td>W</td>
</tr>
<tr>
<td>18</td>
<td>-</td>
<td>D and W</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>W</td>
</tr>
<tr>
<td>15</td>
<td>Schisandrin</td>
<td>W</td>
</tr>
<tr>
<td>16</td>
<td>Gomisin A</td>
<td>W</td>
</tr>
<tr>
<td>17</td>
<td>deoxyschizandrin</td>
<td>W</td>
</tr>
<tr>
<td>19</td>
<td>Gomisin N</td>
<td>W</td>
</tr>
</tbody>
</table>

*a S = Semen Ziziphi Spinoseae, D = Radix Salviae Miltiorrhizae, W = Fructus Schisandrae, (-) Unknown.


