Quality Evaluation of Yanghuo Sanqi Tablet through a Simultaneous Determination of Five Major Active Flavonoids and Three Main Saponins by HPLC–DAD–ELSD

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Summary. Yanghuo Sanqi tablet (YST), combined prescription mainly derived from the leaves of Herba epimedii and the roots of Panax notoginseng, is a traditional Chinese medicine (TCM). Flavonoids (icarrin, epimedin A, epimedin B, epimedin C, and baohuoside I) and saponins (notoginsenoside R1, ginsenoside Rgl, and ginsenoside Rbl) are considered as the main bioactive compounds of YST. However, there is no report on quality control of TCMs by simultaneous determination of above-mentioned flavonoids and saponins so far. In this work, for the first time, a high-performance liquid chromatography–diode array detector–evaporative light scattering detector (HPLC–DAD–ELSD) method was developed to evaluate the quality of YST through a simultaneous determination of five major active flavonoids and three main saponins. Optimum separations were obtained with a Zorbax SB-C18 column by gradient elution with acetonitrile–water as the mobile phase. The drift tube temperature of ELSD was set at 105 °C, and the nebulizing gas flow rate was 2.5 L min⁻¹. The fully validated method was successfully applied to quantify the eight bioactive components in three lot products. This simple, low-cost, and reliable HPLC–DAD–ELSD method provided a new basis for assessing the quality of traditional Chinese medicinal compound preparations (TCMCPs) consisting of many bioactive components.

Key Words: Yanghuo Sanqi, quality evaluation, flavonoid, saponin, icarrin, ginsenoside, HPLC–DAD–ELSD

Introduction

Traditional Chinese medicines (TCMs), especially in China, applied by TCMs practitioners for thousands of years, have played an important role in clinical therapy and have been attracting more and more attention for their complementary therapeutic effects to western medicines with low toxicity and few or even no complication [1, 2]. Yanghuo Sanqi tablet (YST), combined prescription mainly derived from the leaves of Herba epimedii and the roots of Panax notoginseng, is newly used Chinese herbs. Pharmacological studies and clinical practice have demonstrated that YST possesses many biological functions, including rising blood flow of arteria coronaria, ameliorating myocardial ischemia, distended cardiovascular, cutting down
myocardial consumption of oxygen, inhibiting thrombogenesis, improving microcirculation, and resisting cardiac arrhythmia. It is used for the treatment of stethalgia, chest distress, cardiopalms, and hypodynamia [3]. According to prescription of YST, two kinds of bioactive components, namely flavonoids (epimedii) and saponins (notoginseng), are related to effects on human health. Epimedii (or epimedium), Yinyanghuo in Chinese, is a well-known Chinese herbal medicine. It has been used to treat multiple diseases in China for over 2000 years [4]. Flavonoids, mainly icarrin (4), are thought to be the major active components of Yinyanghuo. Besides icarrin (4), epimedin A (1), epimedin B (2), epimedin C (3), and baohuoside I (5) are usually co-existing in Yinyanghuo [5]. The main bioactive compounds in P. notoginseng (Sanqi in Chinese) are saponins, including notoginsenoside R1 (6), ginsenoside Rgl (7), and ginsenoside Rbl (8), which can antagonize thrombosis, dilate blood vessel, and protect cardiac microvessels [6, 7]. Therefore, an accurate measurement of bioactive components, e.g., flavonoids and saponins in these products, becomes essential for quality control of their therapeutic efficacy.

Recently, the prevailing methods for determining flavonoids in Yinyanghuo and its herbal preparations are high-performance liquid chromatography–diode array detector (HPLC–DAD) or ultra-performance liquid chromatography (UPLC)–DAD [8, 9]. On the other hand, HPLC–evaporative light scattering detector (ELSD) [10–13] is usually used for quantification of saponins in Sanqi owing to the low UV absorptivities of saponins. However, the reported methods could only employ flavonoids (in Yinyanghuo) or saponins separately as chemical markers to evaluate the quality of TCMs. In addition, it is well known that interaction of multiple chemical compounds contributes to the therapeutic effects of TCM. As a result, simultaneous determination of above-mentioned flavonoids and saponins would be a better strategy for the comprehensive quality evaluation of YST. However, up to now, there is no literature concerning this issue. Although HPLC–mass spectrometry (MS) may qualify the simultaneous determination of flavonoids and saponins, it is not suitable and readily available for routine analysis with respect to cost, time, and complex sample preparation. Therefore, low-cost and reliable method needs to be established for the quality evaluation of YST through a simultaneous determination of flavonoids and saponins.

In recent years, an on-line coupled HPLC–DAD–ELSD method has attracted ever-increasing attention and has been successfully applied to simultaneous quantification of multi-components in traditional Chinese herbal medicines and their preparations [14–23]. This method is potentially ideal for routine analysis and quality evaluation of YST since UV and ELSD signals contain complementary information for each other. The sensitive UV
Quality Evaluation of Yanghuo Sanqi Tablet

Fig. 1. Chemical structures of the eight bioactive components determined.
detection reveals strongly UV absorbing compounds such as flavonoids (icarrin, epimedin A, etc.), while the versatile ELSD reveals none or poor UV absorbing compounds like saponins (notoginsenoside R1, ginsenoside Rgl, and ginsenoside Rbl) providing a stable and flat baseline even with gradient elution.

In this paper, for the first time, using on-line coupled HPLC–DAD–ELSD, an improved quality control method for YST was developed through simultaneous determination of eight bioactive components, including five major flavonoids (compounds 1–5) and three major saponins (compounds 6–8). The structures of these eight compounds were given in Fig. 1.

**Experimental**

**Reagents and Materials**

YST (batch nos. 20090201, 20090401, and 20090601) was purchased from Changyuan Pharmaceutical Corporation of Jilin Province (Changchun, China). Reference compounds 4 and 6–8 were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); reference compounds 1–3 were bought from Chengdu Pusi Bio. Sci. and Tec. Co. Ltd. (Chengdu, China); 5 was isolated previously from the dried epimedii, and its structure was elucidated by comparison of spectral data (UV, insulin receptor (IR), MS, hydrogen-1 nuclear magnetic resonance (1H-NMR), and carbon-13 nuclear magnetic resonance (13C-NMR)) with those published references [24]. The purity of each reference compound was determined to be above 99% by normalization of the peak area detected by HPLC–DAD–ELSD. HPLC grade acetonitrile was purchased from Merck (Darmstadt, Germany); deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA); analytical grade methanol was purchased from Hanbang Science and Technology (Nanjing, China).

**Preparation of Standard Solutions**

Stock solution of the mixture of the eight reference compounds was prepared by dissolving accurately weighted portions of the standards in methanol. The stock solution was further diluted to give serial concentrations in the range of 0.036–0.29 μg for compound 1, 0.068–0.55 μg for 2, 0.017–0.13 μg for 3, 0.24–1.88 μg for 4, 0.014–0.11 μg for 5, 0.15–1.22 μg for 6, 0.30–2.37 μg for 7, and 0.30–2.41 μg for 8, respectively.
Sample Preparation

Twenty YSTs were scraped off the coating and ground into powder. A 1000-mg portion of the powder was transferred into the calibrated flask and extracted with 30 mL of pure methanol, refluxing for 4 h, then the decoction was filtered through a colander while hot, and the volume was adjusted to 50 mL with methanol after cooling. The adjusted decoction was centrifuged at 14,000 rpm for 15 min and analyzed as the decoction. The sample solution was filtered through 0.45-μm filter before injecting into HPLC system.

Instrument and Chromatographic Conditions

Analyses were performed on an Agilent Series 1100 liquid chromatograph system (Agilent Technologies, Palo Alto, CA), consisting of a quaternary pump, an auto sampler, a DAD coupled with an ELSD (Alltech Associates, Deerfield, USA), an HP ChemStation software (Agilent Technologies, USA), and a Zorbax SB-C18 column (4.6 mm × 250 mm, 5 μm). The column temperature was kept constant at 30 °C, and the mobile phase flow rate was 1.0 mL min⁻¹. The drift tube temperature for ELSD was set at 105 °C, and the nebulizing gas flow rate was 2.5 L min⁻¹. The mobile phases consisted of water (A) and acetonitrile (B) using a gradient elution of 10–25% B at 0–15 min, 25% B at 15–38 min, 25–71% B at 38–61 min, and 71% B at 61–70 min, and the re-equilibration time of gradient elution was 15 min.

Results and Discussion

Optimization of Extraction Procure

In order to obtain quantitative extraction, parameters involved in the procedure such as solvent and extraction time were optimized. Pure and aqueous methanol or ethanol solutions were selected as the extraction solvent. The best results in terms of complete extraction of all isoflavonoids and saponins in high yield were obtained with 100% pure methanol. In addition, soxhlet extraction was compared with refluxing. It was found that refluxing extraction was simpler and more effective for extraction of isoflavonoids and saponins. The effect of the extraction time (2, 3, 4, and 5 h) on the efficiency of extraction was also investigated. The results suggested that the highest amount of isoflavonoids and saponins was obtained with the extraction time of 4 h. After extraction, the residue was further extracted with methanol for additional 60 min, and almost no isoflavonoids and saponins were detected by HPLC–DAD–ELSD.
Optimization of HPLC Conditions

The optimization of HPLC conditions was carried out using the mixed reference compound solutions. In trial, the isocratic elution with acetonitrile–water or methanol–water using different analytical columns (Zorbax SB-C18 and Zorbax ODS) was used, which failed to achieve the separation of mixed reference compounds. Then the resolutions of investigated compounds were tested with different gradient elution programs in consideration of the polarity differences of these eight compounds. The results showed that Zorbax SB-C18 column with gradient elution of acetonitrile–water could achieve better separation on the investigated compounds.

Under selected HPLC conditions, nebulizing gas flow rate and evaporating temperature are the two major instrumental adjustments available for maximizing the detector response efficiency. Varying gas flow rates of 1.0, 1.5, 2.0, 2.5, and 3.0 L min\(^{-1}\) were investigated. The results revealed that the

![Fig. 2. Typical HPLC–DAD–ELSD chromatograms of eight reference compounds](image)

(A and B) and YST sample (C and D)

(A) UV chromatograms of eight reference compounds monitored at 270 nm; (B) ELSD chromatograms of three reference compounds; (C) UV chromatograms of methanol extraction of sample; (D) ELSD chromatograms of methanol extraction of the sample.

6: notoginsenoside R1; 7: ginsenoside Rgl; 8: ginsenoside Rbl
noise was decreased with the flow rate increased, but the responses would be weakened if the flow rate increased too much. Thus, a moderate flow rate of 2.5 L min\(^{-1}\) was adopted to achieve the best results. With respect to the drift tube temperature, solvent evaporation is not completed at low temperature, and the detector response is decreased at high temperature. Therefore, the optimal drift tube temperature was determined to be 105 °C according to the data computed with the ELSD software. Typical HPLC–DAD–ELSD chromatograms for all of the reference compounds are shown in Fig. 2 (A, B).

**Method Validation**

**Calibration Curves, Limits of Detection, and Quantification**

The calibration curve was constructed with at least six appropriate concentrations in triplicate, and then constructed by plotting the peak areas versus the concentration of each analyte. For the five flavonoids by UV detection, their regression equations were calculated in the form of \( Y = AX + B \), where

\[
\begin{array}{cccccc}
\text{Compound} & \text{Regression}^a & R^2 & \text{Linear range (μg)} & \text{LOD}^b (\text{ng}) & \text{LOQ}^c (\text{ng}) \\
1 & Y = 24937X + 0.6898 & 0.9997 & 0.036–0.29 & 12.78 & 32.08 \\
2 & Y = 15707X + 0.1258 & 0.9998 & 0.068–0.55 & 22.48 & 56.02 \\
3 & Y = 21172X – 0.7205 & 0.9998 & 0.017–0.13 & 15.02 & 38.15 \\
4 & Y = 31285X + 18.812 & 0.9996 & 0.24–1.88 & 53.05 & 134 \\
5 & Y = 38333X – 0.2611 & 0.9997 & 0.014–0.11 & 10.05 & 25.13 \\
6 & y = 1.0026x + 6.1371 & 0.9997 & 0.15–1.22 & 109 & 162 \\
7 & y = 0.9963x + 5.9988 & 0.9998 & 0.30–2.37 & 247 & 489 \\
8 & y = 1.0151x + 6.3899 & 0.9993 & 0.30–2.41 & 123 & 307 \\
\end{array}
\]

\(^a\)Y is the peak area in UV chromatograms monitored at 270 nm, \( X \) is the compound concentration injected, and \( y \) and \( x \) are the logarithmic values of area and concentration injected in ELSD chromatograms.

\(^b\)LOD refers to the limits of detection.

\(^c\)LOQ refers to the limits of quantification.
Y and X were peak area and compound nominal concentration, while for the three saponins by ELSD detection, their regression equations could be described as \( Y = AX^b \), so the calibration curves should be obtained in double logarithmic coordinates. The dilute solution of the reference compounds was further diluted to a series of concentrations with methanol to obtain the limits of detection (LOD) and quantification (LOQ). The LOD and LOQ under the selected chromatographic conditions were determined at a signal-to-noise \((S/N)\) ratio of 3 and 10, respectively. All results were shown in Table I.

**Precision, Repeatability, and Stability**

Intra- and inter-day variations (presented as relative standard deviation (RSD)) were chosen to determine the precision of the developed method. For intra-day variability test, the calibration sample solutions were analyzed for five replicates within 1 day, while for inter-day variability tests, the solutions were examined in duplicates for three consecutive days. To evaluate the repeatability, five different working solutions prepared from sample were analyzed. The RSD was taken as a measure of precision and repeatability. The stability test was performed with one sample solution which was stored at room temperature and analyzed at 0, 2, 4, 8, 12, 24, and 48 h within 2 days. Variations were expressed by RSD (Table II).

**Table II.** Precision, repeatability and stability for the eight compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precision</th>
<th>Repeatability</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-day ((n = 5))</td>
<td>Inter-day ((n = 3))</td>
<td>((n = 5))</td>
</tr>
<tr>
<td></td>
<td>Mean (\text{mg g}^{-1})</td>
<td>RSD (%)</td>
<td>Mean (\text{mg g}^{-1})</td>
</tr>
<tr>
<td>1</td>
<td>5.13</td>
<td>0.98</td>
<td>5.27</td>
</tr>
<tr>
<td>2</td>
<td>12.75</td>
<td>1.24</td>
<td>13.29</td>
</tr>
<tr>
<td>3</td>
<td>2.73</td>
<td>1.94</td>
<td>3.04</td>
</tr>
<tr>
<td>4</td>
<td>28.70</td>
<td>1.50</td>
<td>29.05</td>
</tr>
<tr>
<td>5</td>
<td>0.68</td>
<td>0.64</td>
<td>0.69</td>
</tr>
<tr>
<td>6</td>
<td>5.85</td>
<td>0.44</td>
<td>5.83</td>
</tr>
<tr>
<td>7</td>
<td>34.68</td>
<td>1.51</td>
<td>34.67</td>
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<tr>
<td>8</td>
<td>35.11</td>
<td>2.70</td>
<td>35.11</td>
</tr>
</tbody>
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Accuracy

Recovery test was used to evaluate the accuracy of this method. Appropriate amounts of powder sample of YST (batch no. 20090201) were weighted and spiked with known amount of each reference compound by adding standard stock solutions of five flavonoids and three saponins, and then analyzed as described in section “Quantification of Eight Compounds in YST.” Each sample was analyzed in sextuple. The total amount of each analyte was calculated from the corresponding calibration curve, and the average recoveries were counted by the formula: recovery (%) = (amount found − original amount)/amount spiked × 100%, and RSD (%) = (SD/mean) × 100%. The results were given in Table III.

Table III. Recovery for the eight compounds from YST (n = 6)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Original mean (mg g⁻¹)</th>
<th>Spiked mean (mg g⁻¹)</th>
<th>Found mean (mg g⁻¹)</th>
<th>Recovery mean (%)</th>
<th>RSD (%)</th>
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<tr>
<td>1</td>
<td>2.49</td>
<td>2.50</td>
<td>5.00</td>
<td>100.3</td>
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<tr>
<td>2</td>
<td>5.65</td>
<td>5.67</td>
<td>11.32</td>
<td>100.3</td>
<td>1.07</td>
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<tr>
<td>3</td>
<td>1.37</td>
<td>1.38</td>
<td>2.75</td>
<td>100.4</td>
<td>0.57</td>
</tr>
<tr>
<td>4</td>
<td>15.00</td>
<td>15.05</td>
<td>30.06</td>
<td>100.3</td>
<td>0.94</td>
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<tr>
<td>5</td>
<td>0.33</td>
<td>0.33</td>
<td>0.65</td>
<td>98.2</td>
<td>1.68</td>
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<tr>
<td>6</td>
<td>2.94</td>
<td>2.95</td>
<td>5.89</td>
<td>100.2</td>
<td>0.60</td>
</tr>
<tr>
<td>7</td>
<td>17.27</td>
<td>17.30</td>
<td>34.57</td>
<td>100.2</td>
<td>0.42</td>
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<tr>
<td>8</td>
<td>17.50</td>
<td>17.52</td>
<td>35.03</td>
<td>100.1</td>
<td>0.36</td>
</tr>
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Quantification of Eight Compounds in YST

Samples of three lots were prepared as described in section “Sample Preparation.” An aliquot of 10-μL filtrate was injected into the HPLC system. Each sample was determined in triplicate. The content of each analyte was calculated from the corresponding calibration curve.

Sample Analysis

The eight components in three batches of YST from the same manufacturer were simultaneously determined by the proposed HPLC–DAD–ELSD method. The results are shown in Table IV. The representative chromatograms sample solutions were shown in Fig. 2 (C, D). Lot number shows the manufacture date by the form of year–month–day. As listed in Table IV, the content of eight bioactive components in the three batches was consistent.
Furthermore, the icarrin and epimedin B are the main flavonoids, and ginsenosides Rg1 and Rb1 are main saponins in YST, which could be considered as the marker components when it comes to quality control of YST.

Table IV. Contents of eight compounds in YST (batch nos. 20090201, 20090401, and 20090601) sample (n = 3)

<table>
<thead>
<tr>
<th>Compound</th>
<th>20090201</th>
<th>20090401</th>
<th>20090601</th>
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<tbody>
<tr>
<td>1</td>
<td>4.83</td>
<td>5.01</td>
<td>5.10</td>
</tr>
<tr>
<td>2</td>
<td>12.07</td>
<td>12.51</td>
<td>12.44</td>
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<tr>
<td>3</td>
<td>2.32</td>
<td>2.47</td>
<td>2.47</td>
</tr>
<tr>
<td>4</td>
<td>27.34</td>
<td>27.86</td>
<td>27.89</td>
</tr>
<tr>
<td>5</td>
<td>0.75</td>
<td>0.77</td>
<td>0.78</td>
</tr>
<tr>
<td>6</td>
<td>5.57</td>
<td>5.66</td>
<td>5.50</td>
</tr>
<tr>
<td>7</td>
<td>32.79</td>
<td>33.25</td>
<td>32.68</td>
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<tr>
<td>8</td>
<td>33.16</td>
<td>33.60</td>
<td>33.09</td>
</tr>
</tbody>
</table>

Conclusion

In present work, for the first time, an HPLC-DAD-ELSD has been established and successfully applied for simultaneous determination of the two types of bioactive compounds in YST samples. The results demonstrated that the proposed method could be readily utilized as a routine analysis and effective quality control method for YST and other TCM with similar preparations.

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References


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