Simultaneous quantitation of eight active components in crude and processed Radix Scrophulariae extracts by high performance liquid chromatography with diode array detector

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[ABSTRACT] AIM: To determine eight major components in crude and processed Radix Scrophulariae, several samples from different arrears were examined. METHODS: The eight components were separated on an Agilent Zorbax Extend C18 column (250 mm × 4.6 mm, 5 μm) and detected by diode array detector (DAD). Mobile phase was composed of (A) aqueous phosphoric acid (0.03%, V/V) and (B) acetonitrile using a gradient elution. Analytes were performed at 30 °C with a flow rate of 1.0 mL·min⁻¹ and UV detection at 280 nm and 210 nm. RESULTS: All calibration curves showed good linear regression (r² ≥ 0.999 2) within tested ranges. The limits of detection and limits of quantification were 0.07–0.32 μg·mL⁻¹ and 0.16–0.93 μg·mL⁻¹, respectively. Overall intra-day and inter-day variations were less than 0.45%, and the average recoveries were 99.31%–100.19%, with RSD ranging from 0.29% to 1.28% for the analytes. CONCLUSION: The developed method can be applied to the intrinsic quality control of crude and processed Radix Scrophulariae. [KEY WORDS] HPLC-DAD; Radix Scrophulariae; Processed; Quality control

1 Introduction

Radix Scrophulariae (Xuanshen in Chinese), derived from the dried root of Scrophularia ningpoensis Hemsl., is one of the oldest and most frequently used Chinese herbs for oriental medicine in China [1]. Pharmacological studies and clinical practice have demonstrated that Radix Scrophulariae possesses various bioactivities, including anti-chronic inflammatory, antihypertensive, abirritative, antispasmodic, anti-HBV and immunological enhancement [2-4]. The crude drug and its processed products of Zheng Zhi Pin (ZZP) are used clinically [5].

In the last decades, Radix Scrophulariae has been extensively investigated in phytochemistry, and the results indicated that 5-HMF, acteoside, angoroside C, harpagoside, cinnamic acid, catalpol, harpagide and geniposide were the main components in Radix Scrophulariae [6-8]. Besides, pharmacological studies on the components show that they have various biological activities, including inhibiting macrophage functions involved in the inflammatory process and antioxidative activity for reducing the oxidized OH...
adducts of dAMP and dGMP [9-12]. These reports indicate that these potentially bioactive compounds may be responsible for the various biological activity of Radix Scrophularia.

To our knowledge, previously reported analytical methods were developed to quantify only several components in crude Radix Scrophulariae [13-16]. In our current study, an HPLC-DAD method was developed to quantify eight potentially bioactive components simultaneously in both crude Radix Scrophulariae and its processed products, which was extracted by methanol. The method is simple, quick, and cheap with good reproducibility. It offers a new method that can be employed for the quality control of not only crude Radix Scrophulariae, but also its processed product.

2 Experimental

2.1 Materials and reagents

Radix Scrophulariae was collected from eight suppliers in Henan, Hunan, Anhui, Zhejiang, Sichuan, Shandong, Jiangxi and Hubei provinces of China. Reference standards of 5-HMF (1), acteoside (2), angoroside C (3), harpagoside (4), cinnamic acid (5), catalpol (6), harpagide (7) and geniposide (8) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The structures of these eight compounds are shown in Fig. 1. The purity for each standard compound was greater than 98% by HPLC analysis. All reagents with high grade were obtained from others. Milli-Q water (Millipore, Bedford, MA, USA) was used throughout the study.

2.2 Apparatus and chromatographic conditions

Analyses were performed using HPLC system Agilent 1200 (Agilent Technologies, Palo Alto, CA, USA) with diode array detector. Detection wavelengths were set at 280 nm for 5-HMF (1), acteoside (2), angoroside C (3), harpagoside (4) and cinnamic acid (5), 210 nm for catalpol (6), harpagide (7) and geniposide (8). An Agilent Zorbax Extend C18 (250 mm × 4.6 mm, 5 μm) was used with a flow rate of 1.0 mL·min−1. The injection volume was 10 μL and the column temperature was maintained at 30 °C. The mobile phase was composed of (A) aqueous phosphoric acid (0.03%, V/V) and (B) acetonitrile using a gradient elution of 3%-6% B at 0-8 min, 6%-15% B at 8-18 min, 15%-20% B at 18-25 min, 20%-35% B at 25-35 min, 35%-47% B at 35-38 min, 47% B at 38-40 min, 47%-75% B at 40-5 min, 75%-80% B at 45-50 min.

![Fig. 1 Structures of eight active components in Radix Scrophulariae](image)

2.3 Preparation of standard solutions and calibration curve

Primary stock standard solutions of the eight compounds were prepared by dissolving them with methanol, respectively, to get a concentration of 0.034 mg·mL−1 (5-HMF), 0.054 3 mg·mL−1 (acteoside), 0.028 4 mg·mL−1 (angoroside C), 0.020 4 mg·mL−1 (harpagoside), 0.027 5 mg·mL−1 (cinnamic acid), 0.049 2 mg·mL−1 (catalpol), 0.023 2 mg·mL−1 (harpagide) and 0.005 24 mg·mL−1 (geniposide). Mixed standard working solutions were prepared daily by mixing and diluting the stock solutions with methanol. The standard stock and working solutions were all prepared in calibrated flasks and stored at 4 °C. All calibration curves were constructed from peak areas of the reference standards versus their concentrations. The solutions were filtered.
through a 0.45 μm membrane (Automatic science, Tianjin, China) prior to injection.

2.4 Preparation of sample solutions

The powder of crude and processed Radix Scrophulariae samples, was precisely weighed (0.500 g), and transferred into dark brown calibrated flasks. They were extracted with 50 mL of 50% methanol in an ultrasonic bath for 45 min at room temperature. Additional 50% methanol was added to make up the loss. The supernatants were filtered through a 0.45 μm membrane prior to injection.

2.5 Method validation

The method was validated for linearity, precision (inter- and intra-day precision and intermediate precision), accuracy, stability, specificity and selectivity following the International Conference on Harmonization (ICH) guideline and some reports on determination analysis.

3 Results and Discussion

3.1 Optimization of HPLC chromatography conditions

The aim of this study was to develop an HPLC method using DAD detection for the simultaneous determination of 5-HMF, acteoside, angoroside C, harpagoside, cinnamic acid, catalpol, harpagide and geniposide in Radix Scrophulariae. In order to obtain better chromatograms, different mobile phase compositions, including water-acetonitrile; water-methanol; aqueous phosphoric acid (0.01%, V/V)-acetonitrile; aqueous phosphoric acid (0.03%, V/V)-acetonitrile were tested. As a result, the combination of aqueous phosphoric acid (0.03%, V/V)-acetonitrile for mobile phase was the best for separation. Furthermore, other chromatographic variables were optimized, including analytical columns (Hanbon Lichrospher C18 and Agilent Zorbax Extend C18), the column temperatures (20, 25 and 30 °C) and the flow rates (0.5, 0.8 and 1.0 mL·min⁻¹). Eventually, the optimal separation was achieved on an Agilent Zorbax Extend C18 column (250 mm × 4.6 mm, 5 μm) at a column temperature of 30 °C with a flow rate of 1.0 mL·min⁻¹.

3.2 Calibration curves, limits of detection and quantification

Linear regression analysis for each of the 8 compounds was performed by external standard method. All the calibration curves showed good linearity (r² ≥ 0.999 2). The limit of detection and quantification under the chromatographic conditions were determined by injecting a series of standard solutions until the signal-to-noise (S/N) ratio for each compound was 3 for LOD (limit of detection) and 10 for LOQ (limit of quantification). The results are given in Table 1.

Table 1  Calibration curves of 8 components

<table>
<thead>
<tr>
<th>Marker compound</th>
<th>Regression equation</th>
<th>r²</th>
<th>Linear range (μg·mL⁻¹)</th>
<th>LOQ (μg·mL⁻¹)</th>
<th>LOD (μg·mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>y = 1.3 058 x + 1.513 2</td>
<td>0.999 5</td>
<td>4.25–51.00</td>
<td>0.36</td>
<td>0.10</td>
</tr>
<tr>
<td>2</td>
<td>y = 1.718 x + 0.932 3</td>
<td>0.999 4</td>
<td>6.78–81.45</td>
<td>0.62</td>
<td>0.15</td>
</tr>
<tr>
<td>3</td>
<td>y = 3.140.8 x + 1.244 1</td>
<td>0.999 9</td>
<td>3.55–42.60</td>
<td>0.24</td>
<td>0.09</td>
</tr>
<tr>
<td>4</td>
<td>y = 19.413 x + 3.059 1</td>
<td>0.999 9</td>
<td>2.55–30.60</td>
<td>0.16</td>
<td>0.05</td>
</tr>
<tr>
<td>5</td>
<td>y = 14.301 x + 1.784 3</td>
<td>0.999 2</td>
<td>3.44–41.25</td>
<td>0.30</td>
<td>0.08</td>
</tr>
<tr>
<td>6</td>
<td>y = 1.252.1 x + 14.328</td>
<td>0.999 6</td>
<td>6.15–98.40</td>
<td>0.60</td>
<td>0.21</td>
</tr>
<tr>
<td>7</td>
<td>y = 1.372 x + 2.705 9</td>
<td>0.999 8</td>
<td>11.60–46.40</td>
<td>0.93</td>
<td>0.32</td>
</tr>
<tr>
<td>8</td>
<td>y = 3.348.7 x – 0.183 3</td>
<td>0.999 7</td>
<td>2.62–10.48</td>
<td>0.18</td>
<td>0.07</td>
</tr>
</tbody>
</table>

3.3 Precision, repeatability and stability

The intra- and inter-day precision was determined by analyzing calibration samples during a single day and on three different days, respectively. The intra-day variation was determined by analyzing the six replicates on the same day and inter-day variation was determined on three consecutive days. Overall intra- and inter-day variations were less than 0.45%.

To further evaluate the repeatability of the developed assay, Radix Scrophulariae was analyzed in six replicates as described above. The contents of eight compounds in Radix Scrophulariae were calculated from the corresponding calibration curves. The relative standard deviations (RSDs) were taken as measures of repeatability. Stability was tested with Radix Scrophulariae at room temperature and analyzed at 0, 2, 4, 8, 12, 24 and 48 h within 2 days, respectively. RSDs of repeatability test and stability were not more than 3.93% for all analytes.

3.4 Accuracy

Accuracy was determined by the recovery test. An appropriate amount of Radix Scrophulariae powder was weighed and spiked with known amount of each standard compound. They were then treated and analyzed as described above. Each sample was analyzed in six replicates. The total amount of each analyte was calculated from the corresponding calibration curve. Mean recoveries of eight compounds were 99.31%–100.19%, with RSD ranging from 0.29% to 1.28% (n = 6).

3.5 Sample analysis

The established HPLC-DAD method was successfully
applied to the simultaneous determination of eight compounds in crude and processed Radix Scrophulariae commercial samples from different places. Representative chromatograms of the extracts of crude and processed Radix Scrophulariae samples are shown in Fig. 2. All the contents are summarized in Table 2. The results showed that the total amounts of eight components determined in crude drug and ZZP varied significantly. For instance, for Radix Scrophulariae collected from Zhejiang, 5-HMF was hardly detected in crude drug, but higher in ZZP (2.14 mg·g⁻¹); the contents of catalpol, harpagide and geniposide (6.27, 6.43 and 1.25 mg·g⁻¹) were higher than those in ZZP (1.15, 1.39 and 0.98 mg·g⁻¹); the contents of acteoside, angoroside C, harpagoside and cinnamic acid were lower in crude drug (1.50, 2.20, 1.36 and 1.35) mg·g⁻¹, but higher in ZZP (2.70, 4.11, 1.49 and 2.97) mg·g⁻¹. A number of factors may contribute to the variation of contents, such as plant origin, genetic variation, growth circumstance, processing, storage conditions and so on. The variation in contents of the “marker compounds” could certainly lead to the variation of therapeutic effects. That is why each aspect involving procedure needed to be standardized. Compared with the reported analytical methods of Radix Scrophulariae, this newly established method provided much higher specificity, precision and accuracy. By

Table 2 Contents of the eight components in crude and processed Radix Scrophulariae (x ± s, n = 5)

<table>
<thead>
<tr>
<th>Samples</th>
<th>Suppliers</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude drug</td>
<td>Henan</td>
<td>a</td>
<td>3.33 ± 0.08</td>
<td>4.10 ± 0.05</td>
<td>1.55 ± 0.05</td>
<td>1.77 ± 0.01</td>
<td>0.65 ± 0.00</td>
<td>4.61 ± 0.01</td>
<td>0.87 ± 0.01</td>
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<tr>
<td></td>
<td>Hunan</td>
<td>a</td>
<td>2.11 ± 0.01</td>
<td>3.35 ± 0.03</td>
<td>1.85 ± 0.00</td>
<td>1.58 ± 0.01</td>
<td>2.32 ± 0.00</td>
<td>6.13 ± 0.01</td>
<td>0.80 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Anhui</td>
<td>a</td>
<td>2.13 ± 0.03</td>
<td>2.90 ± 0.01</td>
<td>2.18 ± 0.00</td>
<td>2.19 ± 0.05</td>
<td>0.35 ± 0.00</td>
<td>5.66 ± 0.01</td>
<td>0.57 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Zhejiang</td>
<td>a</td>
<td>1.50 ± 0.01</td>
<td>2.20 ± 0.03</td>
<td>1.36 ± 0.01</td>
<td>1.35 ± 0.03</td>
<td>6.27 ± 0.02</td>
<td>6.43 ± 0.01</td>
<td>1.25 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Sichuan</td>
<td>a</td>
<td>4.20 ± 0.00</td>
<td>3.13 ± 0.04</td>
<td>1.42 ± 0.01</td>
<td>2.02 ± 0.01</td>
<td>1.33 ± 0.01</td>
<td>4.44 ± 0.06</td>
<td>1.09 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Shandong</td>
<td>a</td>
<td>2.78 ± 0.00</td>
<td>2.93 ± 0.00</td>
<td>1.41 ± 0.07</td>
<td>2.23 ± 0.08</td>
<td>0.46 ± 0.04</td>
<td>3.85 ± 0.02</td>
<td>0.98 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>Jiangxi</td>
<td>a</td>
<td>4.53 ± 0.00</td>
<td>3.33 ± 0.02</td>
<td>2.45 ± 0.02</td>
<td>2.77 ± 0.04</td>
<td>0.80 ± 0.08</td>
<td>4.43 ± 0.03</td>
<td>1.19 ± 0.01</td>
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<td></td>
<td>Hubei</td>
<td>a</td>
<td>4.60 ± 0.01</td>
<td>2.81 ± 0.07</td>
<td>2.33 ± 0.02</td>
<td>2.48 ± 0.02</td>
<td>_ a</td>
<td>4.58 ± 0.01</td>
<td>0.79 ± 0.02</td>
</tr>
<tr>
<td>ZZP</td>
<td>Henan</td>
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<td>2.26 ± 0.01</td>
<td>1.35 ± 0.02</td>
<td>2.73 ± 0.02</td>
<td>0.73 ± 0.06</td>
<td>2.25 ± 0.03</td>
<td>0.84 ± 0.08</td>
<td>1.21 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>Hunan</td>
<td>0.79 ± 0.00</td>
<td>1.54 ± 0.06</td>
<td>3.15 ± 0.01</td>
<td>0.99 ± 0.00</td>
<td>1.54 ± 0.00</td>
<td>0.68 ± 0.05</td>
<td>0.97 ± 0.00</td>
<td>0.39 ± 0.08</td>
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<tr>
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<td>3.35 ± 0.03</td>
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<td>3.03 ± 0.00</td>
<td>0.27 ± 0.03</td>
<td>2.17 ± 0.00</td>
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<td>1.27 ± 0.01</td>
<td>2.69 ± 0.08</td>
<td>0.73 ± 0.03</td>
<td>2.21 ± 0.01</td>
<td>1.51 ± 0.01</td>
<td>1.05 ± 0.01</td>
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<tr>
<td></td>
<td>Shandong</td>
<td>1.59 ± 0.01</td>
<td>3.79 ± 0.02</td>
<td>4.12 ± 0.01</td>
<td>1.49 ± 0.01</td>
<td>2.64 ± 0.01</td>
<td>0.37 ± 0.01</td>
<td>0.83 ± 0.03</td>
<td>0.76 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>Jiangxi</td>
<td>1.36 ± 0.01</td>
<td>3.37 ± 0.00</td>
<td>3.97 ± 0.01</td>
<td>1.50 ± 0.02</td>
<td>2.94 ± 0.01</td>
<td>5.87 ± 0.02</td>
<td>_ a</td>
<td>1.44 ± 0.02</td>
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<tr>
<td></td>
<td>Hubei</td>
<td>1.16 ± 0.01</td>
<td>2.95 ± 0.00</td>
<td>3.55 ± 0.01</td>
<td>1.60 ± 0.05</td>
<td>2.96 ± 0.01</td>
<td>0.92 ± 0.03</td>
<td>_ a</td>
<td>0.79 ± 0.01</td>
</tr>
</tbody>
</table>

*Not detected

4 Conclusion

The method described in this paper is a specific, accurate, and sensitive HPLC-DAD method for simultaneous quantification of eight compounds in crude and processed Radix Scrophulariae samples. It can be used as a valid analytical method for intrinsic quality control of Radix Scrophulariae. The characteristic compounds such as compounds 1, 4 and 7 are suggested as the reasonable markers for the quality control due to their strong bioactivities. The present study laid a solid foundation for the establishment of a comprehensive quality control method of Radix Scrophulariae and its related herbal products in Chinese Pharmacopoeia.
HPLC-DAD 法同时测定玄参炮制前后 8 个有效成分的含量

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【摘 要】目的: 同时测定不同来源玄参生品与炮制品中 8 个主要成分的含量; 方法: 采用 Agilent Zorbax Extend C18 色谱柱 (250 mm × 4.6 mm, 5 μm), 二极管阵列检测器; 以 0.3%磷酸水溶液-乙腈为流动相, 梯度洗脱, 流速为 1.0 mL·min⁻¹, 检测波长为 280 nm 和 210 nm, 柱温为 30 ℃。结果: 8 个主要成分均呈现良好的线性关系(R²≥0.999 2); 检测限与定量限分别为 0.07–0.32 和 0.16–0.93 μg·mL⁻¹; 日间与日内偏差小于 0.45%; 平均回收率在 99.31%–100.19%, RSD 范围为 0.29%–1.28%。结论: 本方法可用于玄参生品与炮制品内在质量控制。

【关键词】高效液相色谱-二极管阵列检测器; 玄参; 炮制; 质量控制

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