Simultaneous determination of six isoflavonoids in commercial Radix Astragali by HPLC-UV

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Abstract

A HPLC-UV method for the quantification of six major isoflavonoids, calycosin 7-\textit{O-}\textbeta-D-glucoside (1), formononetin 7-\textit{O-}\textbeta-D-glucoside (2), (6\textalpha R, 11\textalpha R) 3-hydroxy-9,10-dimethoxypterocarpan-3-\textit{O-}\textbeta-D-glucoside (3), 7,2'\textit{V}-dihydroxy-3',4'-dimethoxyisoflavan-7-\textit{O-}\textbeta-D-glucoside (4), calycosin (5) and formononetin (6), in Radix Astragali (\textit{Huangqi}) was developed and validated. The method was proven to be sensitive, specific, accurate and precise, as well as effective and easy.

Keywords: Isoflavonoids; Radix Astragali; \textit{Astragalus membranaceus}; \textit{A. membranaceus} var. \textit{mongholicus}; HPLC-UV; Quantitative analysis

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1. Introduction

Radix Astragali, known as *Huangqi* in China, is one of the most popular herbal medicines known worldwide to reinforce "qi". It has been shown to have immunostimulant, tonic, antioxidant, hepatoprotective, diuretic, anti-diabetic, anti-cancer and expectorant properties [1–5]. Radix Astragali is traditionally prepared from the dried roots of *Astragalus membranaceus* and *A. membranaceus* var. *mongholicus*, which are grown mainly in north and northwest China. The roots have been shown to contain triterpene saponins, isoflavonoids, polysaccharides, and some trace elements [6–10]. Astragaloside IV, one of the triterpene saponins, has been reported to be an important component responsible for increasing T and B lymphocyte proliferation and antibody production in vivo and in vitro [11]. Isoflavonoids, in general, are known to be biologically active compounds and to possess antioxidant properties, able to reduce cardiovascular diseases [12–14].

The natural resources of *A. membranaceus* and *A. membranaceus* var. *mongholicus* are diminishing due to increasing demand for Radix Astragali and hence the bulk of the commercial supply is mainly taken from farming sources. The level of the active ingredients varies widely depending on the regions where the plants are grown and the period when they are harvested [15]. The isoflavonoids, considered “marker compounds” for the standardization of *Huangqi*, can be detected by HPLC analysis [10,16,17]. However the experimental conditions reported so far do not give well separated individual peaks.

In this study a HPLC-UV method is described which permits simultaneous quantitative analysis of six major isoflavonoids, calycosin 7-O-β-D-glucoside (1), formononetin 7-O-β-D-glucoside (2), (6αR, 11αR)-3-hydroxy-9,10-dimethoxypterocarpan 3-O-β-D-glucoside (3), formononetin 6,2′-dihydroxy-7-O-β-D-glucoside (4), daidzein 6,7-dihydroxy-3′-O-β-D-glucoside (5), and daidzein (6).
side (3), 7,2',6'-dihydroxy-3',4'-dimethoxyisoflavan-7-O-β-D-glucoside (4), calycosin (5) and formononetin (6) (Fig. 1), present in Radix Astragali. The variation of level of these isoflavonoids in commercial Radix Astragali obtained from traditional Chinese medicines wholesale centres and a variety of commercial outlets distributed throughout China is also presented.

2. Experimental

2.1. Materials

Reference compounds 1–6 were purified from *A. membranaceus* root extracts and characterized by ¹H-NMR and ¹³C-NMR spectroscopy. The purity of the six reference compounds was also evaluated by HPLC-UV. In all cases purity was found to be greater than 94% by peak area normalization method with detection at 230 nm. HPLC-grade MeCN was purchased from Sigma. Water was double distilled and subsequently filtered through a 0.45 µm membrane (Millipore, Bedford, MA, USA).

2.2. Sample preparation

Commercial samples were obtained from main wholesale traditional Chinese medicines markets and main city pharmacy in China. Plants were authenticated by Dr. Mian Zhang, Associate Professor in Pharmacognosy, China Pharmaceutical University, Nanjing, China.

The roots were powdered to a homogeneous size in a mill, sieved through a No. 40 mesh, and dried at 45 °C in the oven for 5 h. The powder sample (1.00 g) was extracted in a Büchi 811 extraction system with 60 ml of MeOH for 2 h. The resulting solution was evaporated to dryness in vacuo. Fresh MeOH (5 ml) was added, the solution was gently heated for 2 min, filtered, and the residue was washed with MeOH (5 ml). The combined filtrates were made up to exactly 10 ml with MeOH using a volumetric flask and filtered through a 0.45 µm membrane before injecting 15 µl samples.

2.3. Calibration

Stock solutions of reference standards 1 to 6 were prepared by dissolving accurately weighed samples of each compound in MeOH at a concentration of ca. 0.50 mg/ml. By varying injection sizes, different amounts of samples were chromatographed and analyzed. Graphs of peak area versus injected amount (six point) were plotted and calibration curves were obtained by fitting the data using linear regression analysis.

2.4. Chromatography conditions

HPLC was performed using an Agilent HPLC system model HP-1100 (Agilent, USA), consisting of a quaternary pump, a diode array spectrophotometric detector (DAD), a thermostatted column compartment and an auto-sampler, controlled by HP Chemstation software. The HPLC column was a Polaris (Metachem, Switzerland) C18 column (250×4.6
mm i.d.; 5 μm). A gradient elution regime was employed using water (eluent A) and MeCN (eluent B). The composition of the eluent was varied from 0% to 30% B in 15 min, while simultaneously the flow rate was changed from 1.2 ml/min to 1.0 ml/min; 30% to 40% B from 15 min to 30 min and 40% to 100% B from 30 min to 60 min, the flow rate was kept at 1.0 ml/min. Column temperature was kept constant at 40 °C. UV detection: 230 nm.

2.5. Determination of extraction recovery

Extraction recovery was carried out by mixing a powdered root sample (0.5 g) and three control levels (high, mid and low levels of the calibration range) of reference compounds 1 to 6. The experiments were repeated three times for each level. The extraction procedure was the same as above. The extracted samples were analyzed by HPLC and the amounts of the reference compounds (1 to 6) were calculated. The data were compared with those from the standard solutions (assuming 100% recovery) and those from the extracted sample (0.5 g powdered root samples without addition of reference compounds).

\[
\text{Recovery} = \left( \frac{\text{Amount from sample and added reference} - \text{Amount from sample}}{\text{Amount from added reference}} \right) \times 100
\]

2.6. Determination of stability over a three-day period

Stability of extracts at room temperature from a commercial sample obtained from Jiangxi Province was studied by HPLC. Injections were made at different time intervals, i.e. 0 h, 4 h, 8 h, 24 h, 2 days and 3 days.

2.7. Determination of extraction reproducibility

Commercial sample of *A. membranaceus* var. mongholicus from Jiangxi Province was extracted and analyzed in duplicate. The procedure was repeated five times to evaluate the reproducibility of extraction protocol.

3. Results and discussion

The HPLC conditions developed in this study produced full peak-to-baseline resolution of the six major isoflavonoids (1–6) present in Radix Astragali as shown in Fig. 2. The major advantage of the gradient elution system is to fully resolve isoflavonoid 1 from the intense peaks at retention time below 5 min, as compared to the reported isocratic elution conditions [15,17]. In addition, the separation can be achieved in one single run rather than two separate injections with different conditions [16]. Apart from the elution conditions, the choice of detection at 230 nm provides an optimum signal to noise for quantitative analysis compared to that of 254 or 280 nm previously reported. We have also noted that the use of 1% acetic acid in the mobile phase has drastically reduced the signal/noise ratio and the presence of
Acetic acid causes the baseline drift of the chromatogram. Chromatograms from extracts of six different commercial sources are presented in Fig. 2 showing the variations of samples’ qualities and individual isoflavonoid’s relative quantities.

All six HPLC calibration curves of isoflavonoids (1–6) exhibited good linearity with excellent correlation coefficient (Table 1). The common linear range for all six isoflavonoids is between 50.8 and 610 ng. In general each isoflavonoid gives a wide calibration range for routine analysis, from 0.144 μg to 1.479 μg (compound 1), from $4.95 \times 10^{-2}$ μg to 0.708 μg (compound 2), from $3.45 \times 10^{-2}$ μg to 0.366 μg (compound 3), from $5.40 \times 10^{-2}$ μg to 0.278 μg (compound 4), from $5.10 \times 10^{-2}$ g to 0.526 μg (compound 5), from $3.60 \times 10^{-2}$ μg to 1.023 μg (compound 6). The concentration of compound 5 in samples 5 and 10 are higher than the calibration range, so the injecting volume is reduced.

Table 1
Data from calibration curves of compounds 1–6, limits of detection and linear range

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Regression equation</th>
<th>Correlation coefficient</th>
<th>Linear range (µg)</th>
<th>Limits of detection (ng), (S/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$Y=2887.85502X-9.90785$</td>
<td>0.99999</td>
<td>$3.90 \times 10^{-3}$–$4.88$</td>
<td>9.76 (3.3)</td>
</tr>
<tr>
<td>2</td>
<td>$Y=2820.22967X+18.08548$</td>
<td>1.00000</td>
<td>$3.82 \times 10^{-2}$–$9.56$</td>
<td>7.65 (2.7)</td>
</tr>
<tr>
<td>3</td>
<td>$Y=2441.94342X-2.11299$</td>
<td>0.99989</td>
<td>$4.36 \times 10^{-3}$–$5.46$</td>
<td>10.89 (3.1)</td>
</tr>
<tr>
<td>4</td>
<td>$Y=1691.33816X+12.15156$</td>
<td>0.99998</td>
<td>$5.08 \times 10^{-2}$–$2.54$</td>
<td>11.42 (2.9)</td>
</tr>
<tr>
<td>5</td>
<td>$Y=3941.50652X-11.66629$</td>
<td>0.99997</td>
<td>$4.05 \times 10^{-2}$–$0.61$</td>
<td>10.14 (2.6)</td>
</tr>
<tr>
<td>6</td>
<td>$Y=3350.37732X+57.39224$</td>
<td>0.99996</td>
<td>$2.68 \times 10^{-2}$–$13.43$</td>
<td>13.43 (3.0)</td>
</tr>
</tbody>
</table>
to 8 μl to fit the range and injecting volume of 15 μl is used for determination of all the other compounds. The lowest LoD is 0.765 ng (2) and the highest is 1.343 ng (6). The precision of the results using the HPLC system was tested by six repeated injections of each standard (1–6) and the RSDs are less than 4% (RSD%: 1, 1.03; 2, 0.90; 3, 0.96; 4, 3.56; 5, 3.72; 6, 1.38).

Extraction recovery test was made by extracting a known amount of isoflavonoid from the Radix Astragali powder. A known amount at three different levels (low, medium and high from the linear range of the calibration curve) of each standard compound was mixed with the sample powder and extracted as described in the experimental section. The % recovery of each standard was determined and these range from 82% to 102% (Table 2). The consistency of the comparatively low recovery of 3 (82–87%) at all levels is noted for future reference in developing standardization assay. As a whole the results clearly indicate that the level of isoflavonoids present does not affect the % recovery provided the level is within the linear range.

In order to test the reproducibility of results of extraction, one commercial sample from Jiangxi Province was extracted independently six times. The results shown in Table 3 giving the range of RSD of the isoflavonoids (1–6) from 2.96% to 4.84% indicate that the consistency of the isoflavonoid level extracted from an individual source is within 5%. The stability profile of the extract from Jiangxi Province in 72 h was determined. The results (Fig. 3) show that there are no significance changes of these compounds during the 72 h storage period.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (mg/g)</th>
<th>RSD (%) a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.453</td>
<td>3.78</td>
</tr>
<tr>
<td>2</td>
<td>0.124</td>
<td>4.62</td>
</tr>
<tr>
<td>3</td>
<td>0.134</td>
<td>2.94</td>
</tr>
<tr>
<td>4</td>
<td>0.087</td>
<td>1.94</td>
</tr>
<tr>
<td>5</td>
<td>0.102</td>
<td>2.63</td>
</tr>
<tr>
<td>6</td>
<td>0.086</td>
<td>4.16</td>
</tr>
</tbody>
</table>

a N=5.
Twelve commercial samples were obtained from various provinces and cities in China and were analyzed for their isoflavonoids (1–6) content. The results are summarized in Table 4. The method can identify the species of *Astragalus* spp., which is a false or substitute and has not been medicinally used by China Pharmacopoeia. As shown in Fig. 2 sample from Sichuan Province, identified as *Astragalus tongonlensis*, is not *A. membranaceus* or *A. membranaceus* var. *mongholicus*. The data presented in Table 4 show that the total isoflavonoids content falls in the range 0.8 to 1.4 mg/g and the levels of

![Fig. 3. Stability profile of compounds 1–6 in commercial extracts.](image)

Table 4
Concentration of compounds 1–6 in commercial Radix Astragali (*Huangqi*)

<table>
<thead>
<tr>
<th>No.</th>
<th><em>Astragalus</em> spp.</th>
<th>Source</th>
<th>Concentration of compounds 1–6 (mg/g)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>A. membranaceus</em> var. <em>mongholicus</em></td>
<td>Jiangxi Province</td>
<td>0.453 0.124 0.134 0.087 0.102 0.086</td>
<td>0.986</td>
</tr>
<tr>
<td>2</td>
<td><em>A. membranaceus</em> var. <em>mongholicus</em></td>
<td>Guangxi Province</td>
<td>0.222 0.05 0.048 0.036 0.192 0.177</td>
<td>0.726</td>
</tr>
<tr>
<td>3</td>
<td><em>A. membranaceus</em> var. <em>mongholicus</em></td>
<td>HeBei Province</td>
<td>0.385 0.109 0.132 0.185 0.104 0.080</td>
<td>0.995</td>
</tr>
<tr>
<td>4</td>
<td><em>A. membranaceus</em> var. <em>mongholicus</em></td>
<td>Shanghai</td>
<td>0.210 0.045 0.086 0.079 0.261 0.224</td>
<td>0.905</td>
</tr>
<tr>
<td>5</td>
<td><em>A. membranaceus</em></td>
<td>Beijing</td>
<td>0.123 0.033 0.023 0.045 0.502 0.092</td>
<td>0.818</td>
</tr>
<tr>
<td>6</td>
<td><em>A. membranaceus</em></td>
<td>Anhui Province</td>
<td>0.096 + + + + 0.205 0.259</td>
<td>0.560</td>
</tr>
<tr>
<td>7</td>
<td><em>A. membranaceus</em></td>
<td>Henan Province</td>
<td>0.257 0.062 0.048 0.057 0.049 0.024</td>
<td>0.497</td>
</tr>
<tr>
<td>8</td>
<td><em>A. tongonlensis</em></td>
<td>Sichuan Province</td>
<td>+ + + 0.117 0.041 +</td>
<td>0.158</td>
</tr>
<tr>
<td>9</td>
<td><em>A. membranaceus</em> var. <em>mongholicus</em></td>
<td>Shanxi Province</td>
<td>0.389 0.082 0.092 0.047 0.150 0.099</td>
<td>0.859</td>
</tr>
<tr>
<td>10</td>
<td><em>A. membranaceus</em> var. <em>mongholicus</em></td>
<td>Guangdong Province</td>
<td>+ + + + 0.599 0.682</td>
<td>1.281</td>
</tr>
<tr>
<td>11</td>
<td><em>A. membranaceus</em> var. <em>mongholicus</em></td>
<td>HongKong</td>
<td>0.468 0.138 0.121 0.089 0.351 0.270</td>
<td>1.437</td>
</tr>
<tr>
<td>12</td>
<td><em>A. membranaceus</em></td>
<td>Shanxi Province</td>
<td>0.986 0.472 0.244 0.144 0.034 0.025</td>
<td>1.905</td>
</tr>
</tbody>
</table>

+: Can be detected, but cannot be quantified.
1–6 present in individual sample can vary considerably. For example Shanxi Province is known for the production of best Huangqi [15]. In this study the highest total concentration (1.905 mg/g) of isoflavonoids was found in the Shanxi Province commercial sample. Interestingly the concentration of 1 found in the sample obtained from this province is the highest (0.986 mg/g) and the concentration of 5 in the same sample is the lowest (0.034 mg/g) among all samples tested. The second highest total concentration of isoflavonoids was found in the sample obtained from Hong Kong. The level of each component is evenly distributed and also ranked second in the total content of 1 and 5. However sample from Guangdong Province has the third highest total concentration of isoflavonoids present, but the concentration of 1 to 4 was too low to be quantified and the content of the 5 and 6 was the highest among all the samples. The results strongly indicate the significance of isoflavonoid 1 or 5 in contributing to the biological activities of Huangqi.

4. Conclusion

The concentration of six major isoflavonoids in commercial Radix Astragali can be readily determined fairly accurately by the method described above. The use of a programmed gradient mobile phase in the absence of acetic acid instead of using isocratic elution has delayed the retention time of calycosin 7-O-β-D-glucoside (1) to 15 min which is well away from the intense peaks at around 1 to 4 min. The well-separated isoflavonoids peaks with a flat baseline allow good quantitation of commercial products and hence a means of quality control (for both qualitative and quantitative analysis) of the widely consumed Huangqi.

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References