Quantitative Determination of Stilbene Oligomers in Jin Que-gen Collected from Different Regions by a HPLC Method

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The objectives of this research were to determine simultaneously the contents of two stilbene tetramers, carasinol B (1) and kobophenol A (2), and one stilbene trimer, (+)-α-viniferin (3), in Jin Que-gen in different regions. A HPLC method has been developed for efficiently quantifying the three analytes in the plant. Using this method, different samples of Jin Que-gen were evaluated. The results showed that contents of the three analytes varied significantly among different samples, and the contents of the three analytes in commercial Jin Que-gen were markedly lower than those in the plants collected directly from growing regions. And for the three analytes, the longer the cultivation time, the higher the contents.

Key words Jin Que-gen; carasinol B; kobophenol A; (+)-α-viniferin; HPLC; region

Caragana sinica (Buc’hoz) REHDI (Leguminosae) is widely distributed in China. Its dried roots (Chinese name: Jin Que-gen) have been used in folk medicine for the treatment of asthma syndrome, vascular hypertension, leukorrhagia, bruises, and contused wounds. In our previous study, we found that Jin Que-gen contains many stilbene oligomers such as carasinol B (1), kobophenol A (2), (+)-α-viniferin (3) (as shown in Fig. 1), pallidol, and miyabenol C, which had multifaceted bioactivities: 1 and 2 possessed estrogenic activity, and 3 inhibited prostaglandin H₂ synthase. Studies showed that stilbene oligomers promote the absorption of calcium, and therefore Jin Que-gen was developed as a active part and then marketed as a type of medicine for the treatment of osteoporosis for women after menopause. It has been reported, however, that other significantly different species of Caragana sinica are found in Chinese herbal markets and these species do not contain any of the active stilbene oligomers except for Caragana Rosea Tirez with red flowers; this seriously hinders proper application of the herbs. Therefore it is urgently needed to establish a quantitative and qualitative analytical method. Several technologies, including TLC and UV have been tried, but all methods thus far developed lead to inaccurate results. Capillary electrophoretic method has been described, but it has the marked drawbacks of interference and wrong results. In addition, Wang claimed that contents of 2 and 3 could be quality evaluation standard of Jin Que-gen. However, the activity of 1 was the best, so the content of 1 could be a factor of quality evaluation, and the relative research had not been made in the previous study.

In addition, the contents of compounds have shown considerable variations and appear to be dependent on many factors while the region parameters play an important role. There has been no study so far on the content variance of the stilbene oligomers in Jin Que-gen collected from different regions.

This work attempts to correct these deficiencies by establishing a modified HPLC system that yields a reliable, quantitative determination of three analytes in various plant samples.

Experimental

Chemicals Methanol, acetonitrile, and acetic acid were purchased from Dikma (Dima Technology Inc, U.S.A); all other chemicals were of analytical grade. Water was obtained with a Mili-Q (Millipore, Bedford, MA, U.S.A.) water purification system.

Plant Material Samples were collected at ten different locations in September 2005 in China. All of the samples were authenticated as genuine Jin Que-gen and identified as being of the same age by experts before being use. A specimen of the plant has been deposited at College of Drugs of Fudan University in China.

Standard Solutions The three compounds isolated from Jin Que-gen according to the methods we reported were used as standards, and had been confirmed by comparing their melting points, [α]D, 1H-NMR, IR, UV and MS data with those given in the literature. Their purities were above 99.0%. Stock standard solutions were prepared at a concentration of 116.8 µg/ml, 398.7 µg/ml and 71.3 µg/ml in the mobile phase for 1, 2 and 3 respectively. By varying injection volumes, different amounts of samples were chromatographed and analyzed. Graphs of peak area versus injected amount (seven points) were plotted and calibration curves were obtained by fitting the data linear regression analysis.

HPLC Analysis Quantitative analyses were performed on an Agilent 1100 series chromatography system (Agilent Tech., Germany) with a photodiode array detector (DAD). An ODS-2 RP-C18 column (150×4.6 mm,
July was extracted and analyzed in duplicate. The procedure was repeated
further analysis. Each sample was repeated three times.

Determination of Extraction Reproducibility Sample collected in
July was extracted and analyzed in duplicate. The procedure was repeated
time to evaluate the reproducibility of extraction protocol.15

Results and Discussion

Selection of Mobile Phase The HPLC conditions developed in this study produced full peak-to-baseline resolution of
the three analytes as shown in Fig. 2. The major advantage of the elution system is to fully resolve 1 from the intense
peaks at retention time below 9 min, as compared to the reported elution conditions.13 The mobile phase achieved the
complete elution of the three analytes as shown in Fig. 2. The major advantage
of the elution system is to fully resolve

Method Validation All three HPLC calibration curves of three analytes exhibited good linearity with excellent correlation
coefficient, and each compound gives a wide calibration range for routine analysis (Table 1). The precision of the results using the HPLC system was tested by five repeated injections of each standard and RSDs are less than 5% (Table 2). In order to test the reproducibility of results of extraction,
sample collected in July was extracted independently five times. The range of RSD of the three analytes (Table 3) indicates that the consistency of stilbene oligomer level extracted from an individual source is within 5%. Stability was determined with the same plant sample solutions at time intervals of 1, 2, 3, 7, and 30 d. The results (Table 3) show that there are no significance changes of the three compounds during the storage period.

The extraction recovery of each compound was determined as shown in Table 4. The recovery rates of added 1, 2, and
3 ranged from 92.5 to 101.7%, 98.4 to 102.4%, and 94.3 to 101.3%, respectively, and all the relative standard deviations
(RSD) were less than 4%. The results clearly indicated that the level of the three compounds did not affect the recovery provided the level was within the linear ranges.

Comparison the Contents of 1, 2, and 3 in Jin Que-gen in Different Regions The contents of the three analytes in Jin Que-gen collected in different regions were measured with the method developed above. The results were shown in Table 5. By contrast, we could definitely draw the conclusion that the contents of 2 were the most abundant stilbene oligomer in the three analytes, ranging from 0.31 to 0.93%. 1 was the next most abundant stilbene oligomer on average,
ranging from 0.07 to 0.14%, so 1 could be a factor of quality evaluation for Jin Que-gen. As 1 was an isomer of 2, the content variances of 1 and 2 would reflect the metabolism condition of the plant. The contents of 3 had a relatively lower variation from 0.04 to 0.12% than those of 1 and 2.

The assay results indicated that there was a big variation in the contents of stilbene oligomers among the plants from different regions. It could be seen that the contents of the three analytes collected from Hubei Province were much higher than those of the other ones. As all the samples were of the same species and age, and were collected in the same season, the difference might be related with the following factors: the type of the soil, the amount of irradiance, temperature, etc. The pH value of the soil in collecting place of Hubei Province ranges from 5.0 to 5.7, which may supply an appropriate medium for the biosynthesis of resveratrol. Therefore, plants from Hubei Province should be recommended for use as medicine.

**Comparison the Contents of 1, 2, and 3 in Jin Que-gen Collected from Wholesale Herbal Markets**

Samples of Jin Que-gen collected from wholesale herbal market were analyzed for the contents of the three stilbene oligomers. The results, as shown in Table 6, indicated that the average contents of 1, 2 and 3 were 0.56, 5.09 and 0.46 mg/g, respectively. Three samples were stored in different conditions: R-3 was stored in shade, ventilated place and R-2 was in shade but unventilated places while R-1 was stored in common places. The results indicated that plants stored in shade and ventilated place should be likely to have higher contents of stilbene oligomers. As other factors (such as origin, batch, species etc.) were the same, the difference should be associated with the process and storage conditions. So the storage way would affect the contents of the three analytes.

**Comparison the Contents of 1, 2, and 3 in Jin Que-gen of Different Cultivation Duration**

In order to compare the contents of 1, 2, and 3 in Jin Que-gen of different cultivation duration, samples of different cultivation periods were chosen at random from 500 individual plants. Figure 3 showed that the relative contents of the stilbene oligomers in three different samples (one from 2-year cultivation, one from 3-year cultivation and one from 5-year cultivation) normalized to the levels determined in the 2-year cultivation samples. Contents of 1 and 2 increased to 145 and 143%, respectively, in the 3-year cultivation sample, compared with the contents in the 2-year cultivation sample. With regard to the 5-year cultivation samples, the contents of the three analytes were all elevated. Although it was difficult to accurately compare the contribution of the cultivation time to contents of stilbene oligomers, contents were clearly affected by cultivation duration. For the three analytes, the longer the cultivation time the higher the contents.

In conclusion, a convenient and reliable HPLC method using a DAD detector has been developed in our laboratory for quantitative analysis of 1, 2 and 3 contents in Jin Que-gen. This analytical method was validated by its good linearity, precision, stability and accuracy. Using this technology, we have successfully determined the contents of the three analytes collected in different regions and from wholesale herbal markets. The results showed that contents of 2 in roots were much higher than those of 1 and 3, and 1 was the next most abundant stilbene oligomer on average. The contents of the samples collected from Hubei Province were much higher than those of the other ones. The concrete reason has been still unknown. As 1, 2 and 3 were of the same type of compounds and their extraction ways were of the same, the sum content of 1, 2 and 3 would be more comprehensive to evaluate the quality of Jin Que-gen than that of 2 and 3, and the variance of 1 and 2 would reflect the metabolism condition of plant. In addition, plants carefully selected and processed were likely to have higher contents of stilbene oligomers. Since the contents of the three stilbene oligomers are not necessarily directly associated, it seems appropriate to stipulate maximum levels for each of the three constituents separately. Such guidelines would help to guarantee the efficacy of stilbene oligomer preparations both in clinical applications and in pharmacological investigations. And for the three analytes, the longer the cultivation time, the higher the contents.

### Table 4. Extraction Recovery of Compounds 1—3

<table>
<thead>
<tr>
<th>Compound</th>
<th>Low level, mean±S.D.</th>
<th>Mid level, mean±S.D.</th>
<th>High level, mean±S.D.</th>
<th>Recovery, mean±S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg/g)</td>
<td>(mg/g)</td>
<td>(mg/g)</td>
<td>(mg/g)</td>
</tr>
<tr>
<td>1</td>
<td>94.5±1.09</td>
<td>100.5±0.80</td>
<td>98.8±1.34</td>
<td>97.9±3.09</td>
</tr>
<tr>
<td>2</td>
<td>98.6±1.16</td>
<td>99.9±1.66</td>
<td>99.0±0.61</td>
<td>99.2±0.54</td>
</tr>
<tr>
<td>3</td>
<td>96.7±2.01</td>
<td>100.2±2.05</td>
<td>100.1±1.37</td>
<td>99.0±1.99</td>
</tr>
</tbody>
</table>

a) $n=3$. b) $n=9$.

### Table 5. Contents of 1, 2 and 3 in Jin Que-gen Growing in Different Regions

<table>
<thead>
<tr>
<th>Number</th>
<th>Regions</th>
<th>1 (mg/g)</th>
<th>2 (mg/g)</th>
<th>3 (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ZJ, Hubei</td>
<td>0.96±0.01</td>
<td>9.24±0.03</td>
<td>0.71±0.01</td>
</tr>
<tr>
<td>2</td>
<td>XJ, Zhejiang</td>
<td>0.71±0.04</td>
<td>4.92±0.04</td>
<td>0.65±0.01</td>
</tr>
<tr>
<td>3</td>
<td>TMS, Zhejiang</td>
<td>0.90±0.01</td>
<td>5.87±0.01</td>
<td>0.64±0.02</td>
</tr>
<tr>
<td>4</td>
<td>HH, Hunan</td>
<td>0.05±0.00</td>
<td>6.03±0.00</td>
<td>0.50±0.01</td>
</tr>
<tr>
<td>5</td>
<td>HH, Hubei</td>
<td>0.43±0.01</td>
<td>9.28±0.01</td>
<td>0.78±0.03</td>
</tr>
<tr>
<td>6</td>
<td>ZJH, Hunan</td>
<td>0.89±0.02</td>
<td>3.05±0.02</td>
<td>1.21±0.08</td>
</tr>
<tr>
<td>7</td>
<td>TL, Anhui</td>
<td>0.05±0.00</td>
<td>4.64±0.03</td>
<td>0.54±0.04</td>
</tr>
<tr>
<td>8</td>
<td>NJ, Jiangsu</td>
<td>0.94±0.07</td>
<td>4.42±0.01</td>
<td>0.45±0.02</td>
</tr>
<tr>
<td>9</td>
<td>KM, Yunnan</td>
<td>0.87±0.00</td>
<td>7.73±0.01</td>
<td>0.55±0.01</td>
</tr>
<tr>
<td>10</td>
<td>NY, Henan</td>
<td>1.19±0.01</td>
<td>6.59±0.02</td>
<td>0.59±0.01</td>
</tr>
</tbody>
</table>

a) $n=3$.

### Table 6. Contents of 1, 2 and 3 in Jin Que-gen from Wholesale Herbal Markets

<table>
<thead>
<tr>
<th>Sample</th>
<th>1 (mg/g)</th>
<th>2 (mg/g)</th>
<th>3 (mg/g)</th>
<th>Sum (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-1</td>
<td>0.55±0.02</td>
<td>3.79±0.03</td>
<td>0.44±0.01</td>
<td>4.78</td>
</tr>
<tr>
<td>R-2</td>
<td>0.71±0.03</td>
<td>4.67±0.03</td>
<td>0.59±0.00</td>
<td>5.97</td>
</tr>
<tr>
<td>R-3</td>
<td>0.42±0.02</td>
<td>6.81±0.04</td>
<td>0.36±0.01</td>
<td>7.59</td>
</tr>
</tbody>
</table>

a) $n=3$. 

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References