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

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Received February 23, 2006; accepted March 25, 2006

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) REHD (Leguminosae) is widely distributed in China. Its dried roots (Chinese name: Jin Que-gen) have been used in folk medicine for the treatment of asthenia syndrome, vascular hypertension, leukorrhagia, brusises, and contused wounds.[1] 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,[2—6] which had multifaceted bioactivities: **1** and **2** possessed estrogenic activity,[7] and **3** inhibited prostaglandin H_2 synthase.[5,8] Studies showed that stilbene oligomers promote the absorption of calcium,[9] 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[10]; 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,[11] but all methods thus far developed lead to inaccurate results. Capillary electrophoretic method has been described,[12] but it has the marked drawbacks of interference and wrong results.[10] In addition, Wang claimed that contents of **2** and **3** could be quality evaluation standard of Jin Que-gen.[13] However, the activity of **1** was the best,[2] 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, $[\alpha]_D$, ^{1}H-NMR, IR, UV and MS data with those given in the literature.[6] 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 photo-diode array detector (DAD). An ODS-2 RP-C$_{18}$ column (150×4.6 mm,
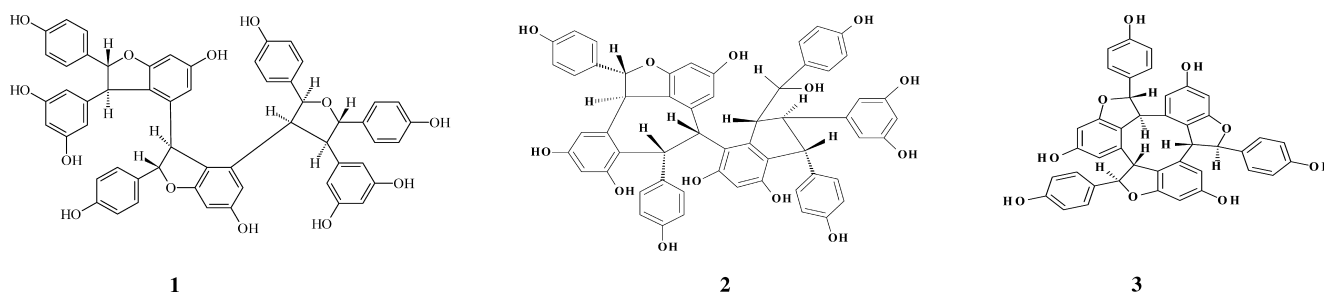

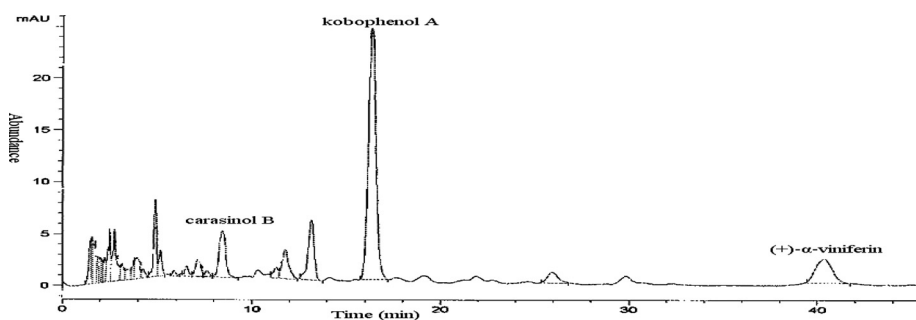Fig. 1.   Chemical Structures of Compounds **1**—**3**

Fig. 2. HPLC Chromatogram of Jin Que-gen

5 μm) (Thermo Electron Corp., U.K.) was used with column temperature set at 30 °C. The mobile phase was a mixture of methanol/acetonitrile/acetate buffer (16.2/12.8/71.0) (v/v/v) at a flow rate of 1.0 ml/min. The pH value of the buffer was 4.50. The detection wavelength was set at 284 nm. The injection volume was 20 μl.

Sample Pretreatment Samples (500 g) were collected and then got rid of stems. The roots were pulverized until all of powers passed through a No. 3 sieve (355 μm), and then all samples were dried with a warm air (50 °C) stream until the water content reached 7.0±0.5%. Each of the fine powdered samples was accurately weighed and extracted twice with 100 ml of 50% (v/v) ethanol for 60 min in a 75 °C water bath. After cooling, the mixture was filtered through a paper filter and the residue was washed twice with 50 ml of 50% (v/v) ethanol. The extract solutions and the wash were combined and evaporated to dryness under vacuum. The residue was dissolved in water, and then submitted to liquid–liquid partition with ethylacetate for three times. The extracting solutions were combined and distilled under vacuum to remove the ethylacetate. The residue was diluted to 50 ml with mobile phase. Sample solutions and standard solutions were all filtered through a syringe cellulose acetate filter (0.22 μm) prior to their further analysis. Each sample was repeated three times.

Determination of Extraction Recovery Extraction recovery was carried out by mixing a powered root sample (0.5 g) and thee control levels (high, mid and low levels of the standard solution) of the three analytes. 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 three analytes were calculated. The data were compared with those from the standard solutions (assuming 100% recovery) and those from the extracted samples (0.5 g powered root samples without addition of three compounds).

Determination of Extraction Reproducibility Sample collected in July was extracted and analyzed in duplicate. The procedure was repeated five times to evaluate the reproducibility of extraction protocol.[14]

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 **1**, **2**, and **3** at 8.6 min, 16.2 min and 40.2 min, respectively. In addition, the eluted time was much shorter than the previous study.[12,13] Apart from the elution conditions, the choice of detection at 284 nm provides an optimum signal to noise for quantitative analysis compared to that of 254 or 280 nm previously reported.[15]

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,

Table 1. Linearity and Linear Range

Compound	Linearity equation	Correlation coefficient	Linear range (μg/ml)	Limit of detection (μg/ml)
1	$Y=8.8x-50.343$	0.9996	6.6—116.8	0.04
2	$Y=8.2x-28.526$	0.9998	22.7—398.7	0.01
3	$Y=5.7x+229.910$	0.9994	15.1—71.3	0.07

Table 2. Intra-day and Inter-day Precision

Compound	1 RSD (%)[a]	2 RSD (%)[a]	3 RSD (%)[a]
Intra-day	1.89	0.45	0.33
Inter-day	3.79	4.48	3.27

a) $n=5$.

Table 3. Reproducibility and Stability

Compound	Reproducibility RSD (%)[a]	Stability RSD (%)[a]
1	4.33	4.27
2	2.78	3.56
3	1.23	2.96

a) $n=5$.

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,

Table 4. Extraction Recovery of Compounds 1—3

Compound	Low level, mean±S.D.[a] (RSD), %	Mid level, mean±S.D.[a] (RSD), %	High level, mean±S.D.[a] (RSD), %	Recovery (%), mean±S.D.[b] (RSD), %
1	94.5±1.09 (1.33)	100.5±0.80 (0.80)	98.8±1.34 (1.36)	97.9±3.09 (3.16)
2	98.6±1.16 (1.18)	99.9±1.66 (1.66)	99.0±0.61 (0.61)	99.2±0.54 (0.54)
3	96.7±2.01 (1.07)	100.2±2.05 (2.05)	100.1±1.37 (1.36)	99.0±1.99 (2.01)

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

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

Number	Regions	1 (mg/g)[a]	2 (mg/g)[a]	3 (mg/g)[a]
1	ZX, Hubei	0.96±0.01	9.24±0.03	0.71±0.01
2	XJ, Zhejiang	0.71±0.03	4.92±0.04	0.65±0.01
3	TMS, Zhejiang	0.90±0.00	5.87±0.01	0.64±0.02
4	HH, Hunan	0.05±0.00	6.03±0.00	0.50±0.01
5	HH, Hubei	0.43±0.01	9.28±0.01	0.78±0.03
6	ZJJ, Hunan	0.89±0.02	3.05±0.02	1.21±0.08
7	TL, Anhui	0.05±0.00	4.64±0.03	0.54±0.04
8	NJ, Jiangsu	0.94±0.07	4.42±0.01	0.45±0.02
9	KM, Yunnan	0.37±0.00	7.73±0.01	0.55±0.01
10	NY, Henan	1.19±0.01	6.59±0.02	0.59±0.01

a) $n=3$.

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

Sample	1 (mg/g)[a]	2 (mg/g)[a]	3 (mg/g)[a]	Sum (mg/g)
R-1	0.55±0.02	3.79±0.03	0.44±0.01	4.78
R-2	0.71±0.03	4.67±0.03	0.59±0.00	5.97
R-3	0.42±0.02	6.81±0.04	0.36±0.01	7.59

a) $n=3$.

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 comparison the contents of 1, 2, and 3 in Jin Que-gen of different cultiva-

tion duration, samples of different cultivation periods were choosed 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.
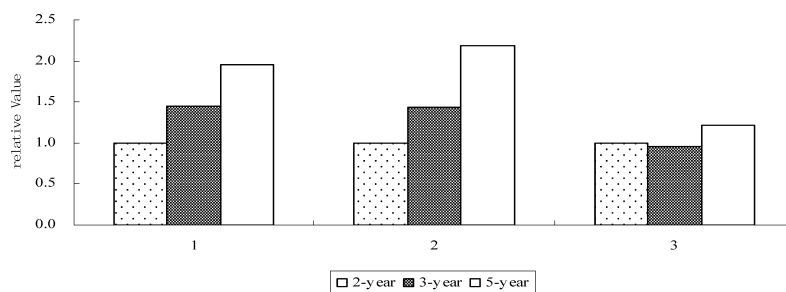
Fig. 3. Comparison the Contents of Stilbene Oligomers in Jin Que-gen of Different Cultivation Duration

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