Simultaneous Determination of the Contents of Three Stilbene Oligomers in *Caragana sinica* Collected in Different Seasons Using an Improved HPLC Method

Na SHU, Hong ZHOU, and Changqi HU*

Department of Chemistry of Natural Drugs, School of Pharmacy, Fudan University; Shanghai 200032, China.

Received August 18, 2005; accepted November 22, 2005

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 the roots, tubers, and leaves of *Caragana sinica* in various seasons. A HPLC method has been developed for efficiently quantifying the three analytes in the plant. Using this method, different samples of *Caragana sinica* were evaluated. The results showed that the contents of 1, 2, and 3 in the roots were much higher than those in the tubers, and the contents of stilbene tetramers were maximal in winter while the contents of the stilbene trimer were maximal in summer. Compounds 1, 2, and 3 could not be detected in the flowers of *Caragana sinica* in our detection ranges.

Key words *Caragana sinica*; carasinol B; kobophenol A; (+)- α -viniferin; HPLC; season

Caragana sinica (BUC'HOZ) REHD (Leguminosae) is widely distributed in China. Its dried roots (Chinese name: Jin Quegen) have been used in folk medicine for the treatment of asthenia syndrome, vascular hypertension, leukorrhagia, bruises, and contused wounds.¹⁾ In our previous study, we found that Jin Quegen 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 H₂ synthase.^{5,8)} In addition, the tubers of *Caragana sinica* were also abundant in biologically active stilbenoids and the flowers, which stimulates the appetite of children, can be used as a functional food according to the traditional folk usage.

Studies showed that stilbene oligomers promote the absorption of calcium,⁹⁾ and therefore *Caragana sinica* was developed and 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* which has 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 have drawbacks: capillary electrophoretic method has been described, but it has the marked drawbacks of interference and inaccurate results.¹²⁾

Moreover, the contents of compounds have shown considerable variations and appear to be dependent on many factors, such as cultivator, mineral ions, heavy metals, location, soil type and nutrition, pollution, herbicides, infection with fungi and bacteria, habitat and environmental conditions *in situ* as well as *in vitro*. Although they result from a large pool of very diverse environmental factors, the seasonal parameters of the environment stay within a relatively narrow range and are mostly repetitive from year to year. There has been no study so far on the corresponding relationships among the stilbene oligomers. A study of the aerial parts of *Caragana sinica* may be a logical method to extend the resource of the medicinal drug. In addition, the content of 1 was not determined in the previous study.¹³⁾

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 and then shows the changing trends in the contents of stilbene oligomers.

MATERIALS AND METHODS

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 Herbal samples were collected on the 25th of each month from March 2004 to February 2005 in

Fig. 1. Chemical Structures of Compounds 1–3

* To whom correspondence should be addressed. e-mail: 031103100@shmu.edu.cn

the same field in Huangmei, Hubei Province, P. R. China. The collection method was as follows: in the growing area, after the *Caragana sinica* with yellow flowers had been identified, the whole plants were collected and then separated into different parts. After collecting from the field, all samples were dried with a warm air (50 °C) stream until the water content reached $7.0 \pm 0.5\%$. All of the samples were authenticated as genuine *Caragana sinica* and genetically identified as being of the same age by experts before use. A specimen of the plant has been deposited at the College of Drugs, Fudan University, China. Dried samples were pulverized until all of resulting power passed through a No. 3 sieve (355 μm).

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- C_{18} column (150 $\times$ 4.6 mm, 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/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 .

Standard Solutions The three compounds isolated from *Caragana sinica* according to the reported methods were used as standards, and were confirmed by comparing their melting points, $[\alpha]_{\text{D}}$, $^1\text{H-NMR}$, IR, UV, and MS data with those in the literature.⁶⁾ Their purities were greater than 99.0%. Stock standard solutions were prepared at a concentration of 116.8 $\mu\text{g/ml}$, 398.7 $\mu\text{g/ml}$, and 71.3 $\mu\text{g/ml}$ in the mobile phase for **1**, **2**, and **3**, respectively. The stock solutions were diluted to yield a series of standard solutions for method validation of linearity.

Evaluation of Extraction Efficiency The extraction solvents and extraction methods were selected and determined according to the extraction efficiency of the three analytes in raw materials. Each of the samples was extracted with refluxing extraction, ultrasonic extraction, or direct extraction for 60 min with 100 ml of 50% ethanol or 95% ethanol.¹⁴⁾

Sample Pretreatment 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 90 °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 ethyl acetate three times. The extracting solutions were combined and distilled under vacuum to remove ethyl acetate. The residue was diluted to 50 ml in mobile phase.

Sample solutions and standard solutions were all filtered through a syringe cellulose acetate filter (0.22 μm) prior to further analysis. Each sample was repeated three times. The contents of **1**, **2**, and **3** were calculated from the integrated peak areas of the samples and the calibration curve of the standards.

RESULTS AND DISCUSSION

Evaluation of Extraction Method The results of preliminary extraction trials indicated that 60 min for extraction was the best, assuming that the extraction solvent was 50% ethanol as recommended by the *Chinese Pharmacopoeia* (2000 edition). Using 100 ml of 50% ethanol as the extraction solvent yielded the most analytes when samples were extracted for 60 min (Fig. 2). It was observed that the amounts of **1** and **3** extracted were not markedly affected by variations in the ethanol/water ratio, however, the greatest amount of **2** was extracted using ethanol/water (50 : 50) (v/v) and the amounts of **1** and **3** increased slightly under the same conditions. The efficiency of extracting analytes was investigated by repeated extraction. The samples were extracted three times with 100 ml of 50% ethanol using refluxing extraction for 60 min each time, and the cumulative extraction rate of **2** reached 97.15% after the first two extractions. Based on these results, the procedure of refluxing extraction for 60 min twice with 50% ethanol was deemed optimal for the efficient extraction of the three analytes from the raw materials.

Selection of Mobile Phase To determine the optimal elution conditions, a mobile phase system of methanol, acetonitrile, and sodium acetate-acetate buffer was investigated. When the proportion of acetonitrile/methanol was set at 13.0 : 16.0, the resolution of **1** was improved. Then, when the proportion of acetonitrile/methanol was changed to 12.8 : 16.2, a complete separation of **1** from other chemical compounds in the plants samples was successfully achieved (Fig. 3). This mobile phase achieved the complete elution of **1**, **2**, and **3** at 8.6 min, 16.2 min and 40.2 min, respectively. The entire chromatographic process required 45 min, which was much shorter than in the previous study.¹³⁾

The HPLC chromatogram of *Caragana sinica* extracts in the tubers was similar to that in the roots.

Method Validation The proposed method for quantitative analysis of **1**, **2**, and **3** was validated in terms of linearity, accuracy, repeatability, and stability when compared with those of standards.

Linearity was examined with a set of standard solutions in parallel with the samples. Injections in duplicate were made, and standard calibration curves of the three analytes were constructed by plotting analyte concentrations against peak

Fig. 2. Evaluation of Extraction Method

Fig. 3. HPLC Chromatogram of *Caragana sinica* in Roots

Table 1. Linearity and Linear Range

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

Table 2. Accuracy Studies

Compound	Roots recovery (%) ^{a)}	RSD (%)	Tubers recovery (%) ^{a)}	RSD (%)	Leaves recovery (%) ^{a)}	RSD (%)
1	98.8±1.34	1.36	100.5±0.80	0.80		
2	99.0±0.61	0.61	99.9±1.66	1.66	98.6±1.16	1.18
3	100.1±1.37	1.36	100.2±2.05	2.05		

^{a)} Mean value (±standard deviation), $n=3$.

Table 3. Repeatability, Stability and Limit Detection

Compound	Repeatability RSD (%)	Stability RSD (%)	Limit of detection (μg/ml)
1	4.33	4.27	0.04
2	2.78	3.56	0.01
3	1.23	2.96	0.07

areas. Linear relationships were obtained with the correlation coefficients greater than 0.999 for all three compounds (Table 1).

The accuracy was confirmed by performing a recovery experiment, where one sample was added with known amounts of the three analytes. The results are shown in Table 2. The recovery rates of added 1, 2, and 3 ranged from 97.4 to 101.7%, 98.4 to 102.4%, and 97.3 to 101.3%, respectively, and all the relative standard deviations (RSDs) were less than 4%.

Repeatability was investigated by analyzing five individual samples, and the samples were processed, as described above, in parallel (Table 3). Stability was determined with the same plant sample solutions at time intervals of 1, 2, 3, 7, and 30 d. No significance degradation of the sample solutions stored at 4 °C was observed. The limit of detection was defined as three times the level of noise. The precision was evaluated using a prepared sample, and the sample was subjected to HPLC analysis seven times on the same day to evaluate intra-day variation, and once each day for three consecutive days to assess inter-day variation (Table 4).

The results indicated that this protocol fulfilled the requirements for a validated HPLC method.

Table 4. Intra-day and Inter-day Precision

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

Comparison of the Contents of 1, 2, and 3 in Roots, Tubers, and Leaves of *Caragana sinica* in Different Seasons The contents of the three compounds were determined independently three times in duplicate. The results of the analysis are shown in Figs. 4, 5 and 6. It could be seen that there was a wide range of contents for 1, 2, and 3 in *Caragana sinica* in various seasons.

In contrast, the contents of 1, 2, and 3 in the roots were much higher than those in the tubers. In the roots, 2 was more abundant in November (9.28 mg/g) and lower in March (3.79 mg/g). The ratio between the maximum and minimum was 2.45, and the annual mean amount was 7.37. We found that 2 accounted for 0.38—0.92% of the dry weight. This conformed to the values recorded by Chen for the same species (0.7%).¹⁵⁾ For 1, the highest content was in November (1.43 mg/g) and the lowest in March (0.51 mg/g). The

Fig. 4. Contents of **1**, **2** and **3** in Roots of *Caragana sinica*

Fig. 5. Contents of **1**, **2** and **3** in Tubers of *Caragana sinica*

Fig. 6. Contents of **2** in Leaves of *Caragana sinica*

ratio between the maximum and minimum was 2.80, and the annual mean amount was 0.95. Interestingly, the changing trend of **1** was similar to that of **2**. However, the content of **3** varied little compared with that of **1** and **2**, and the changing trend of **3** was completely opposite to that of the other two. The content of **3** was maximal in August (0.84 mg/g) and minimal in December (0.54 mg/g). **3** accounted for 0.05—0.08% of the dry weight.

The contents of the three stilbene oligomers in the tubers of *Caragana sinica* also changed markedly with the season. The content of **2** in the tubers was dominant. The annual content of **2** was 0.96 mg/g, while that of **1** and **3** was 0.63 mg/g and 0.31 mg/g, respectively. The highest content of **2** was in December (1.48 mg/g). A significant decrease in the content of **2** occurred in August (0.67 mg/g). The content of **1** was the highest in December (0.43 mg/g) and the lowest in March (0.17 mg/g). In comparison with the roots, the content of **3** in the tubers varied widely. It was more abundant in July (0.76 mg/g) and lower in March (0.38 mg/g), and the maximal content was twice the minimal. Seasonal variations of **1**, **2**, and **3** in the tubers might be related to the growth stage, as

mentioned above. The contents of the three analytes increased within growth stages and decreased among growth stages. Since stilbene oligomers are considered to be associated with resveratrol, biosynthetic changes in resveratrol might affect the contents of the stilbene oligomers.

Results from assays carried out on the monthly collections of the leaves of *Caragana sinica* showed considerable fluctuations in the content of **2**, with the highest in August (0.84 mg/g) and the lowest in March (0.15 mg/g). Our data correlated well with the growth stages of leaves. The contents of **2** increased with the growth of the leaves from March to August, reaching a climax in August, and then declined with the defoliation from September to October, reaching a low level in October (0.23 mg/g), and successively increased with the appearance of new leaves from November (0.15 mg/g) to December (0.16 mg/g). The content of **2** in winter was at the same level as in spring. In addition, **1** and **3** could not be detected in the leaves of *Caragana sinica* in our detection ranges, in contrast with the chromatograms of the standards.

The elution time of the three compounds did not corre-

Fig. 7. HPLC Chromatogram of *Caragana sinica* in Flowers

sponded with that of the standards, and thus **1**—**3** could not be detected in the flowers of *Caragana sinica* in our detection ranges, as shown in Fig. 7.

In conclusion, a convenient and reliable HPLC method using a DAD detector has been developed in our laboratory for the quantitative analysis of **1**, **2**, and **3** contents in *Caragana sinica*. This analytical method was validated by its good linearity, accuracy, precision, and stability. Using this technology, we successfully determined the contents of the three analytes collected in different seasons. The results showed that the contents of **1**, **2**, and **3** in the roots were much higher than those in the tubers; however, the leaves did not contain **1** and **3** in our detection ranges and none of the three compounds described above could be detected in the flowers of *Caragana sinica* in our detection ranges. Both in the roots and in the tubers, the contents of the stilbene tetramers were the highest in winter, while the contents of the stilbene trimer were the highest in summer. The difference may be associated with temperature and sunlight hours. The contents of the stilbene oligomers may not change due to water stress, as indicated by the local weather reports. The reason is still unknown. It may be involved with many factors such as the activity of certain enzymes, microbes, *etc.* With regard to the industrial and chemical utilization of stilbene oligomers, it appears beneficial to collect the plants during winter.

Acknowledgments This project was sponsored by the National Natural Science Foundation of China (30270155). We are grateful to Professor Yunqiu Yu of Fudan University for her instruction in analysis experiments.

REFERENCES

- 1) Jiangsu New Medical College, "A Dictionary of Chinese Medicinal Material," Vol. 1, Shanghai People's Press, Shanghai, 1997, pp. 1402—1403.
- 2) Ma D.Y., Hu C. Q., *Chin. J. Chem.*, **22**, 207—211 (2004).
- 3) Kitanaka S., Takido M., Mizoue K., Kondo H., Nakaike S., *Chem. Pharm. Bull.*, **44**, 565—567 (1996).
- 4) Kitanaka S., Ikezawa T., Yasukawa K., Yamanouchi S., Takido M., Sung H. K., Kim I. H., *Chem. Pharm. Bull.*, **38**, 432—435 (1990).
- 5) Kulanthaivel P., Janzen W. P., Ballas L. M., Jiang J. B., Hu C. Q., Darges J. W., Seldin J. C., Cofield D. J., Adams L. M., *Planta Med.*, **61**, 41—44 (1995).
- 6) Luo H. F., Hu C. Q., *J. Chin. Pharm. Sci.*, **3**, 162—165 (2000).
- 7) Liang G. L., Hu C. Q., *Chem. Pharm. Bull.*, **52**, 1489—1491 (2004).
- 8) Xu G., Zhang L. P., Hu C. Q., *Acta Pharm. Sin.*, **29**, 818—822 (1994).
- 9) Lee S. H., Shin N. H., Kang S. H., Park J. S., *Planta Med.*, **64**, 204—207 (1998).
- 10) Cui Y. L., Hu C. Q., *Chin. Pharm. J.*, **39**, 173—175 (2004).
- 11) Wei W., Wang Y. M., *Acta Pharm. Sin.*, **32**, 476—480 (1997).
- 12) Chen G., Hu C. Q., *Talanta*, **54**, 1067—1075 (2001).
- 13) Wang S. G., Doctoral thesis, Fudan University, 2005.
- 14) Zhao Z. Z., Liu L., *Biol. Pharm. Bull.*, **28**, 105—109 (2005).
- 15) Chen G., Hu C. Q., *Planta Med.*, **67**, 665—668 (2001).