Simultaneous quantification of six main active constituents in Chinese Angelica by high-performance liquid chromatography with photodiode array detector

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ABSTRACT

Background: Angelica sinensis is a famous traditional Chinese medicinal herb, which is predominantly used in the treatment of gynecological conditions. It is the first report for the simultaneous determination of six major active components in Chinese Angelica, which is important for quality control. Objective: A validated HPLC-PAD method was first developed to evaluate the quality of crude and processed Radix Angelica through simultaneous determination of six bioactive compounds, namely ferulic acid, senkyunolide I, senkyunolide H, coniferyl ferulate, Z/E-ligustilide and Z/E-butylidenephthalide. Materials and Methods: Samples were separated on a Ximate™ C₁₈ column (250 x 4.6 mm, 5 μm) and detected by PAD. Mobile phase was composed of (A) aqueous phosphoric acid (0.02%, v/v) and (B) acetonitrile (MeCN) (including 10% tetrahydrofuran, v/v) using a gradient elution. Analytes were performed at 30°C with a flow rate of 1.0 mL/min. Results: All calibration curves showed good linear regression (r² ≥ 0.9963) within the tested ranges, and the recovery of the method was in the range of 91.927–105.859%. Conclusion: The results demonstrate that the developed method is accurate and reproducible and could be readily utilized as a suitable quality control method for the quantification of Radix Angelica. Key words: Chinese Angelica, high performance liquid chromatography, photodiode-array detector, quality control, simultaneous determination

INTRODUCTION

Angelica sinensis (Apiaceae, Angelica, A. sinensis), called “Danggui” in Chinese, has been used in the treatment of gynecological conditions, namely dysmenorrhea, amenorrhea, menopausal syndromes[¹] for thousands of years in traditional Chinese, Korean and Japanese medicines, which was first cited in Shenlong Bencao Jing (200–300 A.D, Han Dynasty).[²][³] Besides that, it has been widely applied to treat anemia, abdominal pain, migraine headaches, cardiovascular disease and hepatic fibrosis.[⁴]

Many kinds of compounds have been isolated and identified from Angelica sinensis, including essential oils (mainly including monomeric phthalides as well as phthalide dimmers), coumarins, organic acids and their esters, polysaccharides, amino acids, and others.[⁵][⁶] But it is quite distinct in different crude resources. Avula et al have quantitative determined eight coumarin constituents from Angelica sinensis.[⁷] Instead of coumarins in Korean Angelica, phthalides are the principal components in Chinese Angelica, and even coumarins have not been found in the latter.[⁸] Based on a large amount of pharmacological research, the constituents such as ferulic acid, senkyunolide I, senkyunolide H, coniferyl ferulate, Z/E-ligustilide, Z/E-butylidenephthalide were found to be responsible for the biological activities in Chinese Angelica. Their chemical structures are shown in Figure 1.

Since curative effect of TCMs is an integrative result of a number of bioactive compounds, quantitative determination not only for the quality control of crude drugs but also for elucidating the therapeutic principle, which lead quality control of herbal medicines is necessary and important. Currently, only a few analytical studies have been reported to determine the active components in Chinese Angelica. Guang-Hua Lu et al developed a high-performance liquid chromatographic fingerprints which could analyse Chinese...
Angelica a squalitative. But they only focused on the qualitative analysis of chemical constituents in Chinese Angelica and lacked the information of quantitative determination for quality control. To date, the methods for the simultaneous separation and quantitative determination of multiple active components in a single running for Chinese Angelica are still not available. Therefore, an accurate and reliable method is needed for the quality control of this famous traditional Chinese medicinal.

The HPLC-PDA method applied for the simultaneous quantitative determination of 6 active components (including ferulic acid, senkyunolide I, senkyunolide H, coniferyl ferulate, Z/E-ligustilide and Z/E-butylidenephthalide) contained in Chinese Angelica is the first time reported in this work. The developed HPLC–PDA coupled method is very simple, accurate and reliable for the routine analysis and quality control of Chinese Angelica.

**MATERIALS AND METHODS**

**Plant materials**

Eleven commercial products were purchased from different provinces’ herbal market on July, 2011 in China, which were crude plant material. The identity, sampling part and sample source of 11 tested samples are summarized in Table 1, and those samples have been authenticated by Dr. H. Zhao, from the Institute of Materia Medica, The Fourth Military Medical University. The 11 samples were cut into smaller pieces, further ground into powder, and stored at desiccator before use.

**Chemicals and Reagents**

All the solvents used in this experiment were HPLC-grade. Methanol (MeOH) was purchased from Burdick and Jackson (SK Chemical, Ulsan, Korea), acetonitrile (MeCN) from Honeywell (Muskegon, MI, USA). The deionized water was prepared from Millipore water purification system (Milford, MA, USA) and filtered with a 0.22-µm membrane. Other reagents were all of analytical grade. A membrane filter (diameter-13 mm, pore size-0.22 µm, Advantec, CA, USA) was used to filter each sample.

The standards of ferulic acid, senkyunolide I, senkyunolide H, coniferyl ferulate, Z/E-ligustilide and Z/E-butylidenephthalide were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China), and the purity was shown to be greater than 98%.

**Instrumentation and HPLC conditions**

An Waters 2695 Alliance HPLC system (Waters Corp, Milford, MA, USA), equipped with Empower™ Software and comprised of a quaternary solvent delivery system, an on-line degasser, an autosampler, a thermostated compartment and a 2996 photodiode array detection, was used for the chromatographic analysis. All separations were performed on a Xttimate™ C18 column (250×4.6 mm, 5 µm), and the solvent gradient conditions are shown in Table 2. The temperature was maintained at 30°C, and the flow rate was 1.0 ml/min. The injection volume was 20 µL, and re-equilibration duration was 10 min between individual runs. Monitoring of the analytes and quantitation was performed at the wavelength of maximum absorbance for each analyte. Ferulic acid and Z/E-ligustilide were monitored at 322 nm, senkyunolide I and senkyunolide H at 277 nm, coniferyl ferulate at 216 nm, and Z/E-butylidenephthalide at 237 nm. Peak identification was performed both by retention times and by spectral information provided by the PAD. The components were quantified based on peak areas at the maximum wavelength in their UV spectrum.

**Preparation of standard solutions and sample solutions**

Stock solutions for standard compounds were prepared with HPLC-grade methanol as solvent and stored away.
from light at 4°C. Working calibration solutions containing the six compounds were prepared by appropriate serial dilution of the stock solution with methanol and the final concentrations were 6.4, 60, 200, 20, 1200 and 300 µg/ml.

Accurately weighed about 1.0 g dried and powdered samples of Chinese *Angelica*, added 20 mL of methanol, weighed the mixture and sonicate it for 30 min, made up the weight loss with methanol after cooling down to ambient temperature. The extract was then filtered with a 0.22-µm microporous membrane into an amber glass HPLC vial prior to analysis.

### RESULTS AND DISCUSSION

#### Optimization of chromatographic conditions

Regarding the choice of solvent for optimal extraction, methanol was the preferred choice of extraction solvent in the present study as a variety of compounds with different polarity can be coextracted effectively. Extraction efficiency of methanol–water at ratios of 100:0, 90:10, 80:20, 70:30, 60:40 and 50:50 were examined, by ultrasonic extraction 0.5 h and 1 h, respectively. It was observed that compounds included coniferyl ferulate, Z/E-ligustilide, and Z/E-butylideneephthalide were decreasing with ratios of methanol went down and those compounds of adenosine and ferulic acid were opposite, and the influence of time is ignorable. Considering the content and pesticide effect of water-solubility compounds is tiny, purely methanol was chosen in this study. Besides, the interference from sugars in the raw herbs could also be minimized by extraction using methanol.

In general, a suitable chromatographic column, mobile phase, elution mode and detection wavelength are critically important for good separation. In the present study, different columns, such as *Xtimate*™ C₁₈ column, *Yilite Hypersil BDS* C₁₈ column, *Yilite SinoChrom ODS-BP C₁₈* column and Phenomenex Luna 5µ C₁₈ column were employed. Various mobile phases consisting of MeCN–water, methanol–water, methanol–MeCN–water, methanol–THF–water and MeCN–THF–water with different gradient elution modes were tested. In addition, the water modified by phosphoric acid, acetic acid and formic acid with different pH values were tested. The flow rate of 0.8 ml/min and 1.0 ml/min were also optimized. Since the structures of Z/E-ligustilide and Z/E-butylideneephthalide are extremely similar, the separating degree of them in chromatograms presented were not so good. After add THF into the mobile phases, the separating degree had improved obviously.

The detection wavelength was selected according to the maximum absorption wavelengths of 216, 237, 277, 322 nm, respectively, shown in UV spectra with three-dimension chromatograms of photodiode array detection. The desired components from Chinese *Angelica* were identified by comparing both the retention times and UV spectra with those of the authentic standard.

After many tests, *Xtimate™ C₁₈* column with the MeCN–THF–phosphoric acid solution system using gradient elution was found suitable for the simultaneous separation and determination. Excellent agreement between standard and sample spectra was found in all analyzed samples, indicating that under the proposed analytical conditions, the six marker constituents were sufficiently resolved and successfully separated. Typical chromatograms of the authentic standards and Chinese *Angelica* are shown in Figure 2.

### METHOD VALIDATION

The HPLC method was validated by defining the linearity, limits of detection, identification and quantification of the precision, stability and recovery.

Calibrations working standard solutions were freshly prepared in methanol by appropriate dilution of the stock solutions. All calibration curves were constructed by analysis of a mixture containing six standard substances at various concentration levels and plotting peak area against the concentration of each reference standard. A good correlation was found between the peak area (y) and the concentrations (x) (r² ≥ 0.9963) for all the compounds in the range of concentration tested at their detected wavelengths.

The limits of detection (LOD) were determined according to International Conference on Harmonization (ICH) recommendations. The LOD value was calculated by means of serial dilution based on a signal-to-noise (S/N) ratio of 3:1, which confirmed the applicability of the proposed method. The regression equations, correlation coefficients, and linear ranges and LODs for the analysis of the six marker constituents are shown in Table 3.

The intraday and interday precisions were determined by assaying standard solutions at three concentrations during a single day and on three consecutive days, respectively. The results are shown in Table 4. From Table 4, it appears that the RSDs of intra day were not exceeding 2.429%, while the

### Table 2: Solvent gradient conditions for HPLC–PAD

| Final time (min) | Flow rate (ml/min) | Aqueous phosphoric acid (0.02%, v/v) | MeCN (including 10% THF, v/v) |
|-----------------|--------------------|-------------------------------------|------------------------------|
| 0               | 1                  | 85                                  | 15                           |
| 15              | 1                  | 70                                  | 30                           |
| 32              | 1                  | 38                                  | 62                           |
| 40              | 1                  | 35                                  | 65                           |

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Table 3: Regression equation, linear range and LODs of calibration curves

| Constituent           | Regression equation | Regression (r²) | Linearity range (µg/mL) | LOD (µg/mL) |
|-----------------------|---------------------|-----------------|-------------------------|-------------|
| Ferulic acid          | y = 2158.2x+92.049  | 0.9979          | 0.4–6.4                 | 0.02        |
| Senkyunolide I        | y = 152.20x—307.68  | 0.9963          | 3.75–60                 | 0.75        |
| Senkyunolide H        | y = 7.4797x+1.640   | 0.9974          | 12.5–200                | 2.45        |
| Coniferyl ferulate    | y = 688.63x+156.73  | 0.9972          | 1.25–20                 | 0.06        |
| Z/E-ligustilide       | y = 18.213x+77.513  | 0.9988          | 75–1200                 | 3.72        |
| Z/E-butylidenephthalide | y = 42.323x+128.64 | 0.9976          | 18.75–300               | 0.96        |

Figure 2: Chromatograms of the authentic standards and Chinese Angelica Typical chromatograms of the standard mixture (a) and Chinese Angelica (b) at 270 nm (1) ferulic acid, (2) senkyunolide I, (3) senkyunolide H, (4) coniferyl ferulate, (5) Z/E-ligustilide, (6) Z/E-Butylidenephthalide

interday precisions were not all less than 5.0%. Since some constituents in Chinese Angelica were chemical instability mostly, which could decompose at high temperature and direct sunlight. It was proposed that the concentration of compounds converted during the storage period of sample solution. Some studies have authenticated Z/E-ligustilide and Z/E-butylidenephthalide were volatile and unstable compounds, which can be changed to other phthalides through oxidation, isomerization, dimerization, etc. [11]

Recovery test was used to evaluate the accuracy of this method, showed in Table 5. An appropriate amount of Chinese Angelica powder was weighed and spiked with a known amount of each standard compound. They were then extracted and analyzed as described above. The recovery percentage was calculated by using the formula: Recovery(%) = (amount found — original amount)/amount spiked × 100. Therecoveries were found in the range of 91.927–105.859%.

Stability was tested with Chinese Angelica at room temperature and analyzed at 0, 2, 4, 8, 12, and 24 h, respectively. The results are shown in Table 6. The RSDs were less than 2.798% for all analytes. The similarity of these results indicated that the sample remained stable within 24 h.

Analysis of commercial products by HPLC–PAD

The newly established method has been applied to the determination of six marker constituents in 11 batches of Chinese Angelica samples, and the results are shown in Table 7. Altogether 11 Chinese Angelica samples including 6 whole roots, 2 root head, 1 rootlet and 2 prepared slices were analyzed which were collected from a variety of sources and conditions. These included different cultivation areas, various cultivating environments, different processing methods and different parts of roots, etc.

The results indicated that their chromatographic patterns were generally consistent although the absorption
intensity of some peaks was different. As shown in Table 7, the contents of compounds in whole roots and root head were higher than rootlet and prepared slices, and some kind of compounds with low abundance cannot be found in prepared slices, which suggested to maintain the active components, the whole roots of Chinese *Angelica* should

### Table 4: Analytical results of intra and inter-day test

| Components | Concentration (µg/mL) | Intra-day (n= 3) | Inter-day (n= 3) |
|------------|----------------------|----------------|----------------|
|            | Mean ± SD (µg/mL)    | RSD (%)        | Mean ± SD (µg/mL) | RSD (%) |
| Ferulic acid | 0.53                | 0.490 ± 0.0004 | 0.120          | 0.489 ± 0.002 | 0.422 |
|            | 1.60                | 1.382 ± 0.008  | 0.583          | 1.376 ± 0.020 | 1.477 |
|            | 4.00                | 3.878 ± 0.039  | 1.190          | 3.817 ± 0.086 | 2.665 |
| Senkyunolide I | 5.00                | 4.695 ± 0.006  | 0.152          | 4.669 ± 0.044 | 1.095 |
|            | 15.00               | 13.966 ± 0.005 | 0.032          | 14.027 ± 0.280 | 1.994 |
|            | 37.50               | 35.815 ± 0.374 | 1.045          | 35.520 ± 0.657 | 1.851 |
| Senkyunolide H | 16.67               | 14.144 ± 0.043 | 0.303          | 14.276 ± 0.285 | 1.998 |
|            | 50.00               | 48.270 ± 1.172 | 2.429          | 47.685 ± 1.256 | 2.635 |
|            | 125.00              | 122.536 ± 1.189| 0.971          | 120.988 ± 2.899 | 2.396 |
| Coniferyl ferulate | 1.67                | 1.256 ± 0.009  | 0.787          | 1.253 ± 0.006 | 0.565 |
|            | 5.00                | 4.251 ± 0.051  | 1.195          | 4.102 ± 0.200 | 4.888 |
|            | 12.50               | 9.396 ± 0.102  | 1.085          | 9.069 ± 0.383 | 4.220 |
| Z/E-ligustilide | 100.00              | 89.854 ± 0.045 | 0.065          | 89.490 ± 0.334 | 0.480 |
|            | 300.00              | 279.958 ± 1.917| 0.767          | 274.162 ± 8.708 | 3.566 |
|            | 750.00              | 730.933 ± 7.031| 1.232          | 714.341 ± 22.257 | 4.015 |
| Z/E-butylidenephthalide | 25.00       | 22.503 ± 0.022 | 0.119          | 22.370 ± 0.141 | 0.770 |
|            | 75.00               | 65.591 ± 0.664 | 1.012          | 63.385 ± 2.900 | 4.554 |
|            | 187.50              | 156.055 ± 1.953| 1.251          | 147.680 ± 9.537 | 6.458 |

### Table 5: Recoveries of the analyte

| Components | Spiked amount (µg/mL) | Measured amount (µg/mL) | Recovery (%) | RSD (%) |
|------------|----------------------|-------------------------|--------------|---------|
| Ferulic acid | 0.80                | 0.767 ± 0.019          | 96.333       | 2.199 |
|            | 1.00                | 0.939 ± 0.021          | 93.888       | 2.273 |
|            | 1.20                | 1.168 ± 0.005          | 97.004       | 0.484 |
| Senkyunolide I | 10.00               | 10.152 ± 0.201         | 101.522      | 1.978 |
|            | 12.00               | 11.161 ± 0.313         | 93.011       | 2.801 |
|            | 15.00               | 14.146 ± 0.083         | 94.304       | 0.590 |
| Senkyunolide H | 40.00               | 37.847 ± 1.509         | 94.617       | 3.986 |
|            | 45.00               | 42.337 ± 1.068         | 95.521       | 2.523 |
|            | 50.00               | 52.934 ± 0.498         | 105.859      | 0.940 |
| Coniferyl ferulate | 5.00               | 5.007 ± 0.102          | 100.140      | 2.030 |
|            | 5.50                | 5.461 ± 0.155          | 99.293       | 2.835 |
|            | 6.00                | 5.907 ± 0.012          | 98.455       | 0.198 |
| Z/E-ligustilide | 350.00              | 338.636 ± 7.693        | 96.753       | 2.271 |
|            | 400.00              | 370.299 ± 7.577        | 92.575       | 2.046 |
|            | 500.00              | 459.635 ± 12.875       | 91.927       | 2.801 |
| Z/E-butylidenephthalide | 40.00          | 38.736 ± 0.904         | 96.839       | 2.333 |
|            | 45.00               | 41.872 ± 0.716         | 93.049       | 1.711 |
|            | 50.00               | 52.131 ± 0.337         | 104.262      | 0.646 |

### Table 6: Stability of the analyte

| Time (h) | Ferulic acid | Senkyunolide I | Senkyunolide H | Coniferyl ferulate | Z/E-ligustilide | Z/E-butylidenephthalide |
|----------|--------------|----------------|----------------|-------------------|----------------|-------------------------|
| 0        | 1.150        | 14.000         | 55.000         | 8.500             | 600.00         | 48.000                  |
| 2        | 1.066        | 13.611         | 53.073         | 8.429             | 580.684        | 45.979                  |
| 4        | 1.092        | 13.7488        | 53.826         | 8.301             | 592.045        | 46.484                  |
| 8        | 1.137        | 13.892         | 54.632         | 8.235             | 570.875        | 46.527                  |
| 12       | 1.102        | 13.537         | 53.947         | 8.208             | 576.076        | 47.724                  |
| 24       | 1.123        | 13.688         | 56.095         | 8.258             | 587.474        | 46.850                  |
| RSD      | 2.798%       | 1.265%         | 1.941%         | 1.404%            | 1.838%         | 1.663%                  |
Table 7: Content of the 6 active components in 11 batches of Chinese Angelica

| No. | Ferulic acid | Senkyunolide I | Senkyunolide H | Coniferyl ferulate | Z/E-ligustilide | Z/E-butylidenephthalide |
|-----|--------------|----------------|----------------|-------------------|----------------|-------------------------|
| 1   | 0.808        | 6.767          | 17.844         | 6.078             | 329.408        | 39.837                  |
| 2   | 0.809        | 11.123         | 31.490         | 12.307            | 644.024        | 49.211                  |
| 3   | 0.598        | 10.521         | 23.324         | 10.033            | 649.043        | 43.672                  |
| 4   | 0.878        | 5.706          | 26.142         | 6.265             | 428.417        | 37.355                  |
| 5   | 0.971        | 5.347          | 19.710         | 6.006             | 330.288        | 39.860                  |
| 6   | 1.037        | 15.994         | 56.125         | 8.179             | 598.340        | 43.901                  |
| 7   | 1.768        | 16.313         | 61.220         | 9.586             | 789.617        | 40.868                  |
| 8   | 1.110        | 8.408          | 29.542         | 4.456             | 526.424        | 35.684                  |
| 9   | 1.009        | 7.597          | 23.488         | 3.396             | 455.446        | 26.062                  |
| 10  | 1.176        | 7.827          | 33.574         | 3.492             | 469.987        | 41.198                  |
| 11  | 1.196        | 7.906          | 33.328         | 3.547             | 478.665        | 47.124                  |

be a better choice than prepared slices for medicine trade, which was demonstrate by previous study. In addition, Chinese Angelica which is cultivated in Minxian County, Gansu Province, China, is regarded as the authentic herb according to traditional experience; however, the contents of active components in substitute herbs cultivated in Yunan Province proved to be similar with the authentic herb in Gansu Province.

CONCLUSIONS

An accurate and reliable HPLC method to simultaneously determine multiple active components in Chinese Angelica was developed. This is the first report for the simultaneous determination of six major active components in Chinese Angelica by using reverse phase high performance liquid chromatography coupled with photodiode array detection. The results demonstrate that the developed method is accurate and reproducible and could be readily utilized as a suitable quality control method for the quantification of Chinese Angelica. It also suggest that the analytes should be test as soon as possible since some components can be changed to other phthalides, and the content of major active components in whole roots of Chinese Angelica would be higher than prepared slices, which demonstrates the whole roots of Chinese Angelica should be a better choice for medicine trade.

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