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

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Submitted: 19-03-2012 Revised: 26-05-2012 Published: 30-04-2013

ABSTRACT

Background: *Angelica sinensis* is a famous traditional Chinese medicinalherb, 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 Xtimate™C_{18} column (250 × 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 ($r^2 \geq 0.9963$) 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

Access this article online

Website: www.phcog.com

DOI: 10.4103/0973-1296.111255

Quick Response Code:

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 isquite 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*, andeven 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 resultof a number of bioactive compounds, quantitative determination not only for the qualitycontrol of crude drugs but also for elucidating the therapeuticprinciple, 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 analyseChinese

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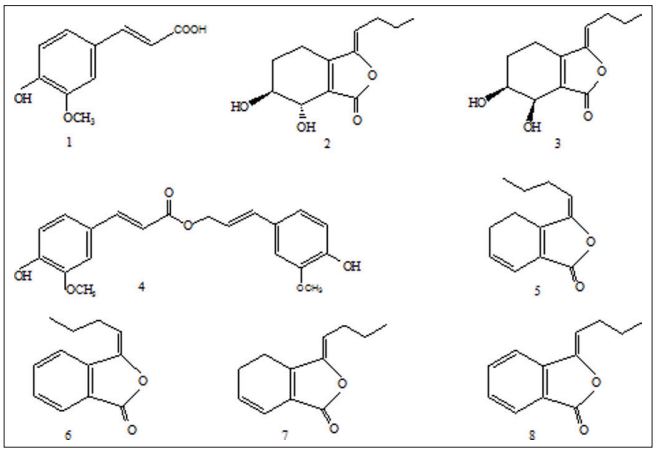

Figure 1: Chemical structures of the 6 active components contained in Chinese Angelica (1) ferulic acid, (2) Senkyunolide I, (3) Senkyunolide H, (4) Coniferyl ferulate, (5) E-Ligustilide, (6) E-Butylidenephthalide, (7) Z-Ligustilide, (8) Z-Butylidenephthalide

Angelica a squalitative.[9] 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

Elevencommercial products were purchased from different provinces' herbal marketon July, 2011 in China, which werecrude 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 theInstitute of Materia Medica, The Fourth Military Medical University. The 11 samples were cut into smaller pieces, further ground into powder, and stored at desiccatorbefore use.

Chemicals and Reagents

All the solvents used in this experiment were HPLC-grade. Methanol (MeOH) was purchased from Burdick and Jackson (SK Chemical, UIsan, 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, poresize-0.22 μm, Advantec, CA, USA) was used to filter eachsample.

Table 1: A summary of the tested samples

Sampleno.	Source	Sampling part
1	Minxian, Gansu, China	prepared slices
2	Minxian, Gansu, China	whole roots
3	Minxian, Gansu, Chin	whole roots
4	Minxian, Gansu, China	prepared slices
5	Minxian, Gansu, China	rootlet
6	Lijiang, Yunan, China	whole roots
7	Lijiang, Yunan, China	Root head
8	Dali, Yunan, China	Root head
9	Dali, Yunan, China	whole roots
10	Shennongjia, Hubei, China	whole roots
11	Shennongjia, Hubei, Chinan	whole roots

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 Xtimate™ C_{18} column (250 × 4.6 mm, 5 μm), and the solvent gradient conditions areshown 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 237nm. Peak identification was performed both by retention timesand 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 withHPLC-grade methanol as solvent and stored away

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

from light at 4°C. Working calibration solutionscontaining the six compounds were prepared byappropriate serial dilution of the stocksolution with methanol and the final concentrations were 6.4, 60, 200, 20, 1200 and 300 μg/ml.

Accurately weighed about 1.0g dried and powdered samples of Chinese *Angelica*, added 20 mL ofmethanol, weighed the mixture andsonicate 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 membraneinto 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 thepreferred choice of extraction solvent in the present studyas a variety of compounds with different polarity can be coextracted effectively. Extraction efficiencyof methanol–water at ratios of 100:0, 90:10, 80:20, 70:30,60:40 and 50:50 were examined, by ultrasonic extraction 0.5h and 1h, respectively. It was observed that compounds included coniferyl ferulate, Z/E-ligustilide, and Z/E-butylidenephthalide 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 inthis 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™ C_{18} column, Yilite Hypersil BDS C_{18} column, Yilite SinoChrom ODS-BP C_{18} column and Phenomenex Luna 5u C_{18} 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 modifiedby phosphoric acid, aceticacid and formic acid with different pH values were tested. The flow rate of 0.8 ml/minand 1.0 ml/min were also optimized. Since the structures of Z/E-ligustilide,and Z/E-butylidenephthalide 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 adsorption 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™ C_{18} 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 andquantification of the precision, stability and recovery.

Calibrations working standard solutions were freshlyprepared in methanol by appropriate dilution of the stocksolutions. All calibration curves were constructedby analysis of a mixture containing sixstandard substances atvarious concentration levels and plotting peak area againstthe concentration of each reference standarda good correlation was found betweenthe peak area (y) and the concentrations (x) ($r^2 \leq 0.9963$) forall the compounds in the range of concentration tested attheir detected wavelengths.

The limits of detection (LOD) were determined according to International Conference onHarmonization (ICH) recommendations.[10] LODvalue was calculated by means of serial dilution based on a signal-to-noise (S/N)ratio of 3:1, which confirmed the applicabilityof 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 intradayand interday precisions were determinedby assaying standard solutions at three concentrations during a single day and on three consecutive days, respectively. The resultsare shown in Table 4. From Table 4, it appears that the RSDs of intra day were not exceeding 2.429%, while the

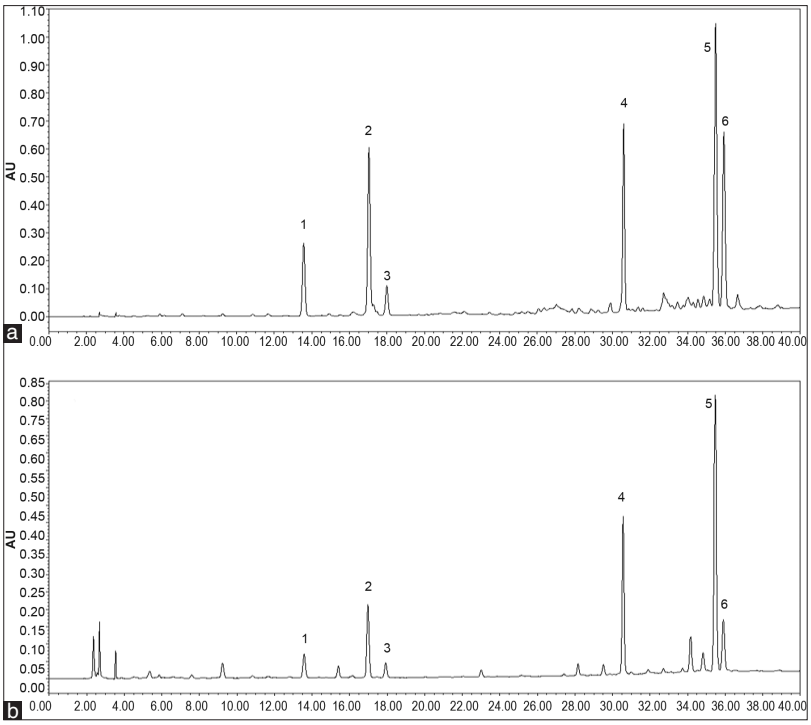

Figure 2: Chromatograms of the authentic standards and Chinese Angelica Typical chromatograms of the standard mixture (a) and Chinese Angelica (b) at 270 nm(1) ferulic acid, (2) senkyunolide I, (3) senkyunolide H, (4) coniferyl ferulate, (5) Z/E-ligustilide, (6) Z/E-Butylidenephthalide

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

Constituent	Regression equation	Regression (r^2)	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

interdayprecisions were not allless than 5.0%. Since some constituents in Chinese *Angelica* were chemical instability mostly, which could decomposeat 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 bechanged to other phthalides through oxidation, isomerization, dimerization, etc.[11]

Recovery test was used toevaluate the accuracy of this method, showed in Table 5. Anappropriate amount of Chinese *Angelica* powder wasweighed and spiked with a known amount of each standardcompound. They were then extracted and analyzed as describedabove. The recovery percentage wascalculated by using the formula: Recovery(%) = (amountfound—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, 12and24 h, respectively. The results are shown in Table 6. The RSDs were less than 2.798% for all analytes. The similarityof 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 varietyof sources and conditions. These included different cultivationareas, 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

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%

intensityof 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 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	599.340	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.[12] 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 bereadily 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 componentscan bechanged 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.

ACKNOWLEDGEMENTS

This work was supported by National Natural Science Foundation of China (30701081 and 81173513).

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Cite this article as: Ying L, Si-Wang W, Hong-Hai T, Wei C. Simultaneous quantification of six main active constituents in Chinese Angelica by high-performance liquid chromatography with photodiode array detector. Phcog Mag 2013;9:114-9.

Source of Support: National Natural Science Foundation of China (30701081 and 81173513). **Conflict of Interest:** None declared.