Simultaneous Determination of Six Major Active Furocoumarins in *Radix Glehniae* by HPLC–DAD

Xuguang Zheng[1], Xiaowei Zhang[2], Xiaona Sheng[2], Hong Zhu[1], Zhifang Yuan[1], and **Lantong Zhang[1,*]**

[1]Department of Pharmaceutical Analysis, School of Pharmacy, Hebei Medical University, Shijiazhuang, 050017, P.R. China and [2]Department of Metabolic Regulation, Institute of Aging and Adaptation, Shinshu University Graduate School of Medicine, 3-1-1 Asahi, Matsumoto, Nagano, 390-8621 Japan

Abstract

A rapid, reliable, and sensitive method for the simultaneous determination of 6 furocoumarins (psoralen, xanthotoxin, bergapten, imperatorin, cnidilin, and isoimperatorin) in *Radix glehnia*e was developed using reversed-phase high-performance liquid chromatography (RP-HPLC) coupled with diode array detection. The HPLC assay was performed on an Ultimate C_{18} column (5 µm, 250 mm × 4 mm) with gradient elution of acetonitrile and water within 20 min. The detection wavelength was set at 310 nm. All compounds showed good linearity (r^2 > 0.999). The RSD of intra-day and inter-day variations ranged from 0.2% to 2.7% and 0.3% to 1.7%. The recovery of the assay was in the range of 91.7–107.6%. The method was successfully applied to the simultaneously determination of 6 furocoumarins in *Radix glehnia*e from different areas.

Introduction

Radix glehniae (Bei Sha Shen), the dried radix of *Glehnia littoralis Fr. Schmidt ex Miq*., is a well known traditional Chinese medicine (TCM) (1). It belongs to Umbelliferae family and was found in China, Japan, Canada, and the United States. As reported previously, the chemical composition of *Radix glehniae* mainly includes furocoumarins, volatile oil, and glycoside, among which furocoumarins are generally considered as the most abundant components, such as bergapten, imperatorin, cnidilin, isoimperatorin, and xanthotoxol (2–6). Until now, pharmacological studies and clinical practice demonstrated that the major active compounds of *Radix glehniae* were furocoumarins, which had exhibited several pharmacological activities including antihistamine (7), inhibition of insulin induced lipogenesis (8), anticancer (9), and antibacterial (10). So the constituents listed above could be considered as the marker compounds for the chemical evaluation or standardization of *Radix glehniae*. In the Chinese Pharmacopoeiaes, there have not been any compounds used as the chemical marker for quality evaluation of *Radix glehniae*. However the therapeutic effects of TCM are based on the complex interaction of numerous ingredients in combination, which are totally different from that of chemical drugs. Therefore, simultaneous determination of major furocoumarins in *Radix glehniae* could be a better strategy for the comprehensive quality evaluation of *Radix glehniae*.

To date, there has been some preliminary research about the quantitative analysis of the furocoumarins in the different plant material. For example, simultaneous determination monocoumarins by capillary electrophoresis (CE–UV) or CE with indirect laser-induced fluorescence detection (11–12), simultaneous determination some mono-coumarins and pyranocoumarins by high performance liquid chromatography diode array detector coupled with electrospray ionization–mass spectrometry (HPLC–DAD–ESI–MS) (13). But there has been no simultaneous quantitative analysis for several furocoumarins in *Radix glehniae* by HPLC–DAD. Reversed-phase HPLC methods are generally used to quantify furocoumarins with DAD detector because furocoumarins have strong UV chromophore. They usually possess same UV spectra and make the single-wavelength detection more selective and sensitive.

As a TCM, *Radix glehniae* has been recorded in all editions pharmacopoeia of China. The processing method recorded in Pharmacopoeiaes is to soak the plant in boiling water, peel the root bark, and dry in the sunshine. The purpose of this processing method is to improve the appearance, dry it easily, reduce the odour, and prevent pests. This is the process used for most of the *Radix glehniae* purchased at drug stores. Therefore, it is an important issue to comprehensively evaluate the difference of processing method of *Radix glehniae*, so as to ensure the clinical efficacy of this Chinese herbal drug.

Up to now, there has been no simultaneous quantitative analysis for furocoumarins in *Radix glehniae*. Therefore, it is very important to establish such a method for quality control of these furocoumarins, which could help evaluate the quality of *Radix glehniae*. In this study, a HPLC method was developed and validated for simultaneous determination of 6 major furocoumarins, namely psoralen (1), xanthotoxin (2), bergapten (3), imperatorin (4), cnidilin (5), and isoimperatorin (6).

*Author to whom correspondence should be addressed: email zhanglantong@263.net.

Experimental

Chemicals and reagents

Acetonitrile and methanol were of HPLC grade and obtained from Tedia (Tedia, Fairfield, OH). The distilled water was prepared from demineralized water and used throughout the study. Other reagents were all of analytical grade. For samples, 14 batches of samples were collected from different fields of Hebei, 6 batches of samples were purchased from local drug stores in different provinces. Psoralen, imperatorin, and isoimperatorin were provided from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Xanthotoxin, bergapten, and cnidilin were extracted and purified by our laboratory. All these compounds were identified by direct comparison of their ^{1}H NMR, ^{13}C NMR, and MS spectral data with those reported in the literature (14–15), and their purities were no less than 98% by HPLC analysis. Their chemical structures of the six furocoumarins were shown in Figure 1.

Instrumentation and analytical conditions.

Experiments were performed on the Agilent 1200 series HPLC system (Agilent, Santa Clara, CA) consisting of a quaternary pump, an autosampler, a degasser, an automatic thermostatic column compartment, a DAD and controlled by the chemstation software (Agilent). The analytical column used was an Ultimate C_{18} column (5 µm, 250 mm × 4 mm; Welch Materials, Ellicott City, MD) for ultimate performance. The mobile phase for HPLC analysis consisted of acetonitrile–water using gradient elution (0–5 min, 40–45% acetonitrile; 20 min, 80% acetonitrile). The flow rate was 1.0 mL/min and the column temperature was 30°C. The detection wavelength was set at 310 nm and the sample injection volume was 20 µL. The peak identification was based on the retention time and the DAD spectrum against the standards presented in the chromatogram.

Standard solution preparation

A stock solution containing the 6 standards (Psoralen 0.026 mg/mL, xanthotoxin 0.045 mg/mL, bergapten 0.016 mg/mL, imperatorin 0.013 mg/mL, cnidilin 0.0024 mg/mL, and isoimperatorin 0.0088 mg/mL) was prepared in 75% methanol. The standard stock solution was further diluted with methanol to make 6 different concentrations including 1/50, 4/50, 8/50, 12/50, 16/50, and 20/50 of the original concentration, All solutions were stored in a refrigerator at 4°C before analysis.

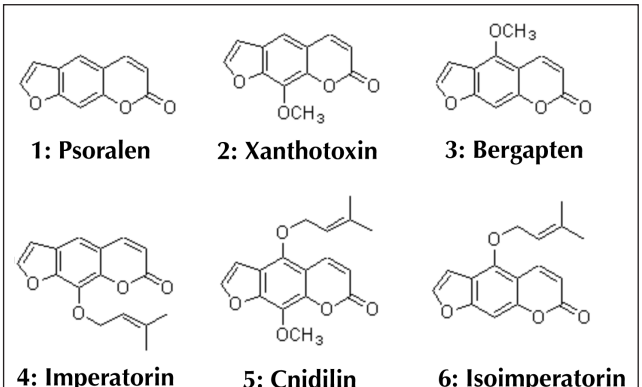

Figure 1. Chemical structures of six furocoumarins: psoralen, 1; xanthotoxin, 2; bergapten, 3; imperatorin, 4; cnidilin, 5; and isoimperatorin, 6.

Sample preparation

The dried powders of *Radix glehniae* samples (0.5 g, 75 mesh) were accurately weighed and extracted by ultrasonic with 20 mL 75% methanol solution for 30 min. Then the resultant mixture was adjusted to the original weight and aliquots of the supernatant were filtered through 0.45 µm membrane before HPLC injection.

Results and Discussion

Extraction method

In order to obtain satisfactory extraction efficiency, extraction method, extraction solvent, and extraction time were investigated. It suggested that ultrasonic extraction was better than refluxing, so ultrasonic extraction was used in further experiments. Water, 30% methanol, 50% methanol, 75% methanol, 90% methanol, and methanol were performed as extraction solvents to analyze the effect of the solvent on extraction efficiency. The results showed that 75% methanol was the most suitable extraction solvent, which allowed extraction of all the major constituents in high yields. To determine optimal extraction time, 0.5 g samples were extracted with 20 mL 75% methanol by ultrasonic extraction for 10, 20, 30, 40, and 60 min, respectively, the compounds were almost completely extracted within 30 min. Hence, 30 min was chosen as optimal extraction time.

Optimization of chromatographic conditions

In order to obtain good resolution, different mobile phases (CH_3OH–H_2O, CH_3OH–H_2O–Acid, CH_3CN–H_2O, CH_3CN–H_2O–Acid, CH_3CN–CH_3OH–H_2O) were attempted to elute the investigated 6 components. It was found that psoralen and xanthotoxin could hardly be resolved when methanol was used. However, when methanol was replaced by acetonitrile, the situation was greatly improved and satisfactory resolution was obtained. The type and concentration of acids (0.05% formic acid, 0.05% acetic acid and 0.05% phosphoric acid) were examined. Addition of acid in mobile phase seemed to have no serious effect on the separation. Considering the total resolution of the chromatographic separation and the running time, the mobile phase CH_3CN–H_2O was chosen for the separation. It was also suggested that separation was better when column temperature was kept at 30°C than 20, 25, 35, or 40°C. As the six compounds showed the same UV absorption properties, the monitoring wavelength was set at 310 nm, where all of the compounds have sufficient absorption. The typical chromatographic profiles of standard solution and the samples were shown in Figure 2.

Calibration curves, limits of detection, and quantitation

Standard stock solutions containing 6 analytes were prepared and diluted to appropriate concentrations for plotting the calibration curves. At least six concentrations of the 6 analyte solutions were analyzed in triplicate, and then the calibration curves

were constructed by plotting the peak areas versus the concentration of each analyte. Limits of detection (LOD) and quantification (LOQ) under the chromatographic conditions used were separately determined at signal-to-noise ratios (S/N) of 3 and 10, respectively. The results are given in Table I. All the analytes showed good linearity ($r^2 > 0.999$) in a relatively wide concentration range.

Precision, accuracy, repeatability, and stability

The precision of the method was validated by determination of intra- and inter-day variance. The intra-day precision was determined by replicate analysis ($n = 5$) of standard solutions of the 6 furocoumarins at low, medium, and high concentrations in a single day, and the inter-day values were carried out over three consecutive days. The concentration of each solution was determined using a calibration curve prepared on the same day. The results are presented in Table II. The intra- and inter-day precisions calculated as the relative standard deviation (RSD) were within the range of 0.2–2.7% and 0.3–1.7%.

Recovery was used to further evaluate the accuracy of the method. Known amounts of each standard solution (20 mL, 75% methanol) at three different concentrations levels were mixed with known amounts of *Radix glehniae* samples (0.25 g), the samples were extracted and analyzed with the previously-established method. The experiments were repeated three times for each level. For comparison, a blank sample (not spiked with standard compounds) was prepared and analyzed. The results are presented in Table III.

Stability of sample solution was tested at room temperature. The sample solution was analyzed within 24 h. The analytes were found to be very stable in 75% methanol solution (RSD < 0.8%) over the tested period.

Six samples of *Radix glehniae* from the same source were extracted and analyzed with the above-established method. The

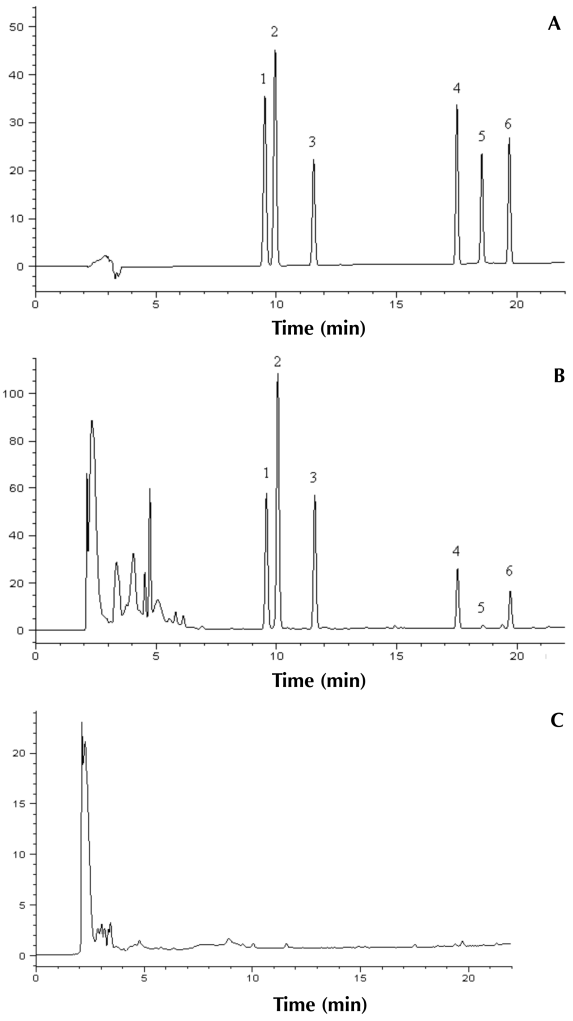

Figure 2. HPLC chromatograms of standard solution and the samples: (A) standard mixture; (B) Radix glehniae (Xiwangqi, Anguo, Hebei); (C) Radix glehniae (Neimeng, China).

Table I. Calibration Curves of the 6 Furocoumarins

Regression equation	r^2	Linear range (µg/mL)	LOD (ng/mL)	LOQ (ng/mL)
Psoralen				
y = 54.708x – 8.0662	0.9996	0.52–10.42	148.5	215.3
Xanthotoxin				
y = 59.404x – 4.0231	0.9998	0.89–17.84	76.1	125.3
Bergapten				
y = 81.006x – 7.2049	0.9997	0.32–6.40	95.1	167.2
Imperatorin				
y = 48.853x – 3.138	0.9994	0.26–5.27	74.4	102.3
Cnidilin				
y = 40.925x – 1.0962	0.9992	0.05–0.96	25.0	39.9
Isoimperatorin				
y = 68.376x – 2.736	0.9995	0.17–3.49	42.3	67.1

Table II. Intra- and Inter-day Precision of the 6 Furocoumarins

	Intra-day ($n = 6$)		Inter-day ($n = 3$)	
Spiked conc. (µg/mL)	Measured conc. (µg/mL, mean ± SD)	RSD (%)	Measured conc. (µg/mL, mean ± SD)	RSD (%)
Psoralen				
2.08	2.07 ± 0.02	1.0	2.08 ± 0.01	0.5
6.25	6.19 ± 0.03	0.5	6.16 ± 0.03	0.5
10.42	10.31 ± 0.02	0.2	10.32 ± 0.07	0.7
Xanthotoxin				
3.57	3.50 ± 0.02	0.6	3.53 ± 0.03	0.9
10.70	10.66 ± 0.06	0.6	10.64 ± 0.07	0.7
17.84	17.73 ± 0.03	0.2	17.66 ± 0.05	0.3
Bergapten				
1.28	1.27 ± 0.01	0.8	1.27 ± 0.01	0.8
3.84	3.82 ± 0.02	0.5	3.78 ± 0.02	0.5
6.40	6.34 ± 0.06	1.0	6.35 ± 0.05	0.8
Imperatorin				
1.05	1.04 ± 0.01	1.0	1.05 ± 0.01	1.0
3.16	3.12 ± 0.02	0.6	3.12 ± 0.02	0.6
5.27	5.21 ± 0.02	0.4	5.25 ± 0.03	0.6
Cnidilin				
0.19	0.19 ± 0.01	2.7	0.19 ± 0.00	1.7
0.58	0.56 ± 0.01	1.1	0.57 ± 0.01	1.3
0.96	0.93 ± 0.01	0.6	0.93 ± 0.01	1.0
Isoimperatorin				
0.70	0.69 ± 0.01	1.5	0.67 ± 0.01	1.5
2.09	2.06 ± 0.02	1.0	2.03 ± 0.02	1.0
3.49	3.45 ± 0.01	0.3	3.45 ± 0.03	0.9

RSD value was calculated as a measurement of method repeatability. The RSD values of 6 compounds were from 0.5% to 2.8%, which showed high repeatability of the method.

Table III. Recoveries of the Six Furocoumarins

Initial amount (µg)	Added amount (µg)	Detected amount (µg)	Recovery (%, mean ± SD)	RSD (%)
Psoralen				
81.3	62.5	141.8	96.8 ± 1.5	2.5
	83.3	169.0	105.3 ± 1.4	1.6
	104.2	183.6	98.2 ± 2.3	2.3
Xanthotoxin				
137.3	107.0	245.6	101.2 ± 2.2	2.0
	142.7	284.0	102.8 ± 2.5	1.7
	178.4	314.1	99.1 ± 2.7	1.5
Bergapten				
53.7	38.4	95.0	107.6 ± 0.7	1.7
	51.2	104.5	99.2 ± 1.4	2.8
	64.1	120.1	103.6 ± 1.3	2.0
Imperatorin				
36.2	29.9	64.7	95.3 ± 0.7	2.5
	39.9	76.7	101.5 ± 1.1	2.7
	49.9	85.3	98.4 ± 0.7	1.4
Cnidilin				
2.9	2.4	5.1	91.7 ± 0.1	4.6
	3.2	6.0	96.9 ± 0.1	3.2
	4.0	7.1	105.0 ± 0.1	2.4
Isoimperatorin				
18.1	13.5	31.9	102.2 ± 0.4	2.9
	17.9	35.2	95.5 ± 0.3	1.8
	22.4	40.8	101.3 ± 0.4	1.8

Sample analysis

The optimized HPLC–DAD method was used to evaluate the contents of 20 samples of *Radix glehniae* from different parts of China. The contents of coumarin for the samples from different origin were significantly different. The analytical results (Table IV) showed that the sample from Xiwangqi in Hebei Province, was of relatively good quality, with the highest contents of psoralen (332.5 µg/g), xanthotoxin (566.9 µg/g), bergapten (217.5 µg/g), imperatorin (154.5 µg/g), cnidilin (10.7 µg/g), and isoimperatorin (71.1 µg/g). Among the 20 samples, the content of the total furocoumarins fell in the range of 338.3–1353.2 µg/g. It also showed that xanthotoxin was the highest component, whose mean content was 173.0 µg/g, followed by psoralen, whose mean content was 113.6 µg/g. Cnidilin was the lowest ingredient with the concentration of about 11.6 µg/g. The data also presented that the contents of furocoumarins in plant (1–14 samples) differed from those in cut crude drug (15–20 samples) significantly, which indicated the processing method might affect the stability of these components.

The plants collected from different fields of Hebei were sliced and dried in the sunshine while the cut crude drug purchased from local drug stores in different provinces were processed by soaking in boiling water, peeling the root bark and drying in the sunshine. Several papers have reported that the contents of furocoumarins in *Radix glehniae* had serious loss after being processed (16). Comparing the two kinds of processing methods, the peeling, in the traditional manufacture process has no practical value. Therefore, to improve the processing of *Radix glehniae*, establish the quality standard of *Radix glehniae*, ensure the drug safety and efficacy, it should be modified.

Table IV. Amounts (µg/g) of 6 Furocoumarins in 20 Samples from Different Parts of China

No.	Source	Date	1†	2	3	4	5	6	Total
1*	Liushuang Hebei	Nov 2007	101.2	158.8	105.4	108.1	11.6	77.2	562.3
2	Chezhangzhuang Hebei	Oct 2007	55.2	133.4	55.5	51.0	11.9	44.9	351.9
3	Dongwangqi Hebei	Oct 2007	165.4	211.8	135.0	109.9	12.4	100.4	734.9
4	Magu Hebei	Nov 2007	54.5	113.1	67.0	36.7	10.7	56.3	338.3
5	Liuchang Hebei	Nov 2007	74.2	103.0	104.9	58.5	16.3	60.0	416.9
6	Xiwangqi Hebei	Nov 2007	332.5	566.9	217.5	154.5	10.7	71.1	1353.2
7	Xixu Hebei	Nov 2007	81.2	91.4	118.1	46.5	9.4	59.2	405.8
8	Changzhuang Hebei	Oct 2007	53.8	76.4	72.9	89.4	13.4	55.9	361.8
9	Liutou Hebei	Nov 2006	66.6	87.9	76.9	66.3	11.3	83.7	392.7
10	Guanyintang Hebei	Oct 2007	56.3	85.1	78.2	95.2	12.4	63.3	390.5
11	Fengbai Hebei	Nov 2007	154.9	224.1	169.8	85.0	10.7	65.2	709.7
12	Zhongsong Hebei	Nov 2007	131.0	144.4	91.2	43.3	11.6	45.1	466.6
13	Sixia Hebei	Oct 2007	170.3	286.0	202.7	146.4	11.1	77.6	894.1
14	Jiaozhuang Hebei	Nov 2006	93.8	140.2	83.0	56.7	9.1	59.5	442.3
15	Hebei-1	Oct 2007	–	–	–	–	–	–	
16	Hebei-2	Nov 2007	–	–	–	–	–	–	
17	Hebei-3	Nov 2007	–	–	–	–	–	–	
18	Shandong Prov	Nov 2006	–	–	–	–	–	–	
19	Neimong Prov	Oct 2007	–	–	–	–	–	–	
20	Liaoning Prov	Nov 2007	–	–	–	–	–	–	
	Average (1–14)		113.6	173.0	112.7	82.0	11.6	65.7	558.6

* Numbers 1–14 were collected from different fields of Hebei, and dried in the sunshine. Numbers 15–20 were purchased from local drug stores in different provinces. The processing method was to soak the plant in boiling water, peel the root bark, and allow to dry in the sunshine.
† The compounds numbers are the same as in Figure 1.

Conclusions

In this paper, a rapid, reliable and sensitive method was developed for the determination of bioactive constituents in by HPLC–DAD. It was the first time to report the simultaneous quantification of 6 major furocoumarins in *Radix glehniae*. The samples were divided into two clusters based on their source and processing methods, which were significantly different in the contents of furocoumarins. The cut crude drug could be easily differentiated from the plant by its low total furocoumarins. The newly established method could be applied as a reliable and sensitive quality control procedure for *Radix glehniae*.

Acknowledgement

The work received financial support from the scientific and technological project of Hebei Province, China (No.09276423D).

References

1. *The Pharmacopoeia of the People's Republic of China,* The Pharmacopoeia Commission of PRC, Part I, Beijing, 2005, pp. 66.
2. Z.Yuan, Y. Tezuka, W. Fan, S. Kadota, and X. Li. Constituents of the underground parts of *Glehnia littoralis*. *Chem Pharm Bull*. **50**: 73–77 (2002).
3. N. Hiraoka, J.I. Chang, L.R. Bohm, and B.A. Bohm. Furanocoumarin and polyacetylenic compound composition of wild Glehnia littoralis in North America. *Biochem. System. Ecol*. **30**: 321–325 (2002).
4. Z. Yuan, M.F. Zhao, and F.K. Chen. The chemical constituents of *Radix Glehniae*. *J. Tradit. Chin. Med*. **33**: 1063–1065 (2002).
5. H. Sasaki, H. Taguchi, T. Endo, and I. Yoshioka. The constituents of *Glehnia littoralis* Fr. Schmidt et Miq. Structure of a new coumarin Glycoside, Osthenol-7-O-β-gentiobioside. *Chem. Pharm. Bull*. **28**: 1847–1852 (1980).
6. J. Kitajma, C. Okamura, T. Ishikawa, and Y. Tanaka. Coumarin glycosides of Glehnia littoralis root and rhizome. *Chem. Pharm. Bull*. **46**: 1404–1407 (1998).
7. Y. Kimura, and H. Okuda. Histamine-release effectors from. Angelica dahurica vat. dahurica root. *J. Nat. Prod*. **60**: 249–251 (1997).
8. Y. Kimura, H. Ohminami, H. Arichi, H. Okuda, K. Baba, M. Kozawa, and S. Arichi. Effects of various coumarins from roots of Angelica dahurica on actions of adrenaline, ACTH and insulin in fat cells. *Planta Med*. **45**: 183–187 (1982).
9. T. Okuyama, M. Takata, H. Nishino, A. Nishino, J. Takayasu, and A. Iwashima. Inhibition of tumor-promoter-enhanced phospholipid metabolism by umbelliferous materials. *Chem. Pharm. Bull*. **38**: 1084–1086 (1990)
10. T. Smyth, V.N. Ramachandran, and W.F. Smyth. A study of the antimicrobial activity of selected naturally occurring and synthetic coumarins. *Int. J. Antimicrob. Agents* **33**: 421–426 (2009).
11. R.J. Ochocka, D. Rajzer, P. Kowalski, and H. Lamparczyk. Determination of coumarins from *Chrysanthemum segetum* L. by capillary electrophoresis. *J. Chromatogr., A*. **709**: 197–202 (1995).
12. W.P. Wang, J.H. Tang, S.M. Wang, L. Zhou, and Z.D. Hua. Method development for the determination of coumarin compounds by capillary electrophoresis with indirect laser-induced fluorescence detection. *J. Chromatogr. A*. **1148**: 108–114 (2007)
13. M.J. Ahn, M.K. Lee, Y.C. Kim, and S.H. Sung. The simultaneous determination of coumarins in Angelica gigas root by high performance liquid chromatography–diode array detector coupled with electrospray ionization/mass spectrometry. *J. Pharmaceut. Biomed. Anal*. **46**: 258–266 (2008).
14. T.K. Razdan, B. Qadri, S. Harkar, and E.S. Waicht. Chromones and coumarins from Skimmia laureola. *Phytochemistry* **26**:2063–2069 (1987).
15. S. Harkar, T.K. Razdan, E.S. Waight. Steroids, chromone, and coumarins from *Angelica officinalis*. *Phytochemistry* **23**: 419–426 (1984).
16. B.G. Li, and J.Y. Shi. Determination of coumarins in Radix Glehniae by high performance liquid chromatography. *J. Shandong Uni. TCM* **29**: 383–384 (2005).

Manuscript recieved July 9, 2009;
revision received November 5, 2009