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Validations of Analysis Methods for Decursin and Decursinol Angelate of Angelicae gigantis Radix by Reversed-Phase Liquid Chromatography.

Jong-Pill Lee, Seung-Yeup Chang, and Sang-Yong Park[1]*

Department of Natural Medicines Evaluation, KFDA, Seoul 122-704, Korea
[1]College of Pharmacy, Kyung Hee University, Seoul 130-701, Korea

Abstract – A reversed-phase liquid chromatographic method for decursin and decursinol angelate of Angelicae gigantis Radix, an important crude drug in Korean traditional medicine, was developed and validated. Decursin and decursinol angelate, the structure isomer (pyranocoumarin) each other, are the main organic constituents in Angelicae gigantis Radix. This method was developed using a RP-18 column, UV detector at 280 nm and 50% acetonitrile solution containing 0.01 M sodium dodecyl sulfate and 25 mM sodium dihydrogen phosphate (pH 5.0) as the mobile phase. Various validation parameters were included and evaluated satisfactorily. Linearity was established in range 2-75 mg/ml of decursin and decursinol angelate (correlation coefficient = 0.9997 and 0.9995, respectively). This analytical method showed good accuracy (98.1% and 99.5%, respectively). Precision (repeatability) revealed a relative standard deviation value of 1.71% (decursin) and 3.19% (decursinol angelate). For intermediate precision measure the considered variables were equipment and days. A robustness test showing the influence of deferent counter-ion concentration in mobile phase was also performed.
Keywords – Angelica gigantis Radix, decursin, decursinol angelate, HPLC, validation

Introduction

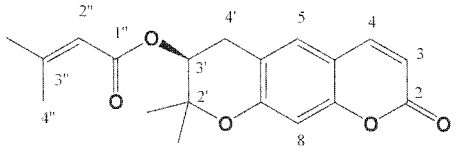
Angelicae gigantis Radix, an important Korean herbal drug, is the dried root of *Angelica gigas* Nakai (Um belliferae) and the substitute for Angelicae sinensis Radix on Chinese traditional medicine used for blood-activator having the effects of invigorating the blood, promoting blood circulation, and regulating menstruation (Yook *et al.*, 2003). This herbal drug, called "Danggui", is described as different original plants in Korea Pharmacopoeia (KP), Japan Pharmacopoeia (JP), and China Pharmacopoeia (CP) each other; *Angelica gigas* Nakai, *A. acutiloba* Kitagawa, A. *chinensis* Diels, respectively. The analytical method of reference constituents for quality evaluation about this drug is not established in any Pharmacopoeias. Angelicae gigantis Radix contains pyranocoumarin, decursin and decursinol angelate as main constituents, and these compounds are the structure isomer having a same formula (Fig. 1, Ryu and Yook, 1967; Chi., 1967; Chi., 1969).

The establishment of analytical method for reference constituents is an essential procedure for the quality evaluation for medicinal herbal drug. There are many reports about analysis method of Angelicae gigantis Radix, but the validation of analytical method for Angelicae gigantis Radix has not been established yet (Ryu *et al.*, 1990; Ko and Ko., 1979; Yook and Kim., 1990; Kang *et al.*, 2003). Actually, it is unusual to find the study about the validation of analytical method for crude drugs (Gambaro *et al.*, 2002; Toker *et al.*, 2001). A reversed-phase HPLC method was thus developed and then validated for the quantitative assay of decursin and decursinol angelate in the crude drug as a new, simple and rapid method.

Experimental

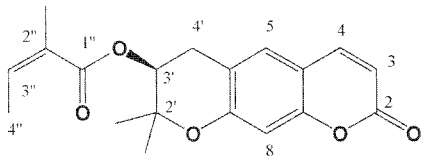
Instrumental and operating condition – HPLC ana lysis was carried out on Shiseido liquid chromatographic system (Nanospace SI-2 chromatograph, Shiseido, Japan) equipped with autosampler and double pumping system connected to a photodiode-array detector. Peak area integration was performed by using a chromatographic data system (ChromoQuest ™ chromatography workstation). A applied column was a reversed-phase C_{18}, Capcell Pak C_{18}

*Author for correspondence
Fax: +82-966-3885; E-mail: dragonpsy@khu.ac.kr



decursin

$C_{19}H_{20}O_5$
Exact Mass: 328.13
Mol. Wt.: 328.36
C, 69.50; H, 6.14; O, 24.36



decursinol angelate

$C_{19}H_{20}O_5$
Exact Mass: 328.13
Mol. Wt.: 328.36
C, 69.50; H, 6.14; O, 24.36

Fig. 1. Chemical structurs of decursin and decursinol angelate.

(type UG120, 5 μm, 250 × 4.6 mm I.D.), thermostated at 35°C A mobile phase composition was 50% acetonitrile solution with 0.01 M sodium dodecyl sulfate and 25 mM sodium dihydrogen phosphate, at pH 5.0. Flow rate was 1.2 ml/min, detection wavelength 280 nm, and injection volume 10 μl. Instruments and chromatographic data were controlled by S-MicroChrom-21 (Shiseido).

Another HPLC instrument equipped with an auto-sampler (Waters 717 plus), a Series 600 LC pump and Waters 996 photodiode array detector operating at 280 nm was used for the study of the intermediate precision. A applied column was μ-bondapack C18 (10 μm, 250 × 4.6 mm I.D.). The instruments and chromatographic data were fully managed by Millenium[32] Chromatographic manager (Waters). The other chromatographic conditions, such as mobile-phase, flow-rate, and injection volume, were the same conditions used in the Shiseido HPLC system.

Reagents – The standards of decursin and decursinol angelate were provided by Professor Young-Choong Kim in Seoul National University (Seoul, Korea). Sodium dihydrogen phospate dihydrate and phosphoric acid were purchased from Wako Pure Chemical (Osaka, Japan) and sodium dodecyl sulfate was from Sigma (St. Louis, MO, USA). Lichrosolc® acetonitrile was purchased from Merk (Darmstadt, Germany). Water used for the mobile phase was deionized, distilled and filtered through a 0.22 μm Millipore (Bedford, USA) before use. All samples were filtered through a Nylon Membrane of 0.20 μm pore size (13 mm Millex®-GN, Millipore, Bedford, USA) before injection. Angelicae gigantis Radix was purchased from circulating Korean market at random.

Preparation of standard solution – The 101.9 mg and 108 mg quantity of decursin and decursinol angelate were accurately weighed and dissolved in 100 ml of methanol. Standard solutions were prepared by the dilution of the above stock solution with mobile phase to five different concentrations over the range from 2~76 μg/ml (decursin: 76.425, 50.95, 20.38, 10.19 and 2.038 μg/ml, decursinol angelate: 76.35, 50.9, 20.36, 10.18 and 2.036 μg/ml) which were used to make calibration curves.

Preparation of sample solution – Ten samples of crude drug, Angelicae gigantis Radix, were grounded to fine powders. The powder 1.0 g was dissolved in 80 ml methanol, refluxed on water bath for 30 minutes, and massed up to 200 ml by methanol, and diluted to 10 times by methanol. After the filtration through 0.20 μm Nylon membrane (Millex® -GN), the solution was used as working solution in method development.

Chromatographic conditions – A phosphate buffer of pH 5.0 was prepared by adding 250 mM sodium dihydrogen phosphate to 250 mM phosphoric acid. The solution after diluting 10 times was mixed with acetonitrile (1:1, v/v). Sodium dodecyl sulfate 2.88 g was dissolved in 1000 ml of the above mixture and used as the mobile phase of HPLC. The other conditions of HPLC were flow rate of 1.2 ml/min, detection wavelength at 280 nm, and column temperature at 35°C in the method.

Results and Discussion

The procedure and parameters used for the chroma tographic method are according to ICH-Guidelines. The applied chromatographic conditions permitted to obtain a good separation of decursin and decursinol angelate (Fig. 2). The LC method was validated for its linearity, accuracy, robustness and precision as the reported below

Specificity – The chromatogram obtained from the sample solution is shown in Fig. 2. The last-elution compound was decursinol angelate (t_R about 26min), which was separated from its neighboring peak of decursin. Resolution between decursin and decursinol angelate attained 1.66, which was beyond the requirements of complete separation (up to 1.5). UV spectra of peak 8 and 9 were well overlaid with each other and with those of the standards, decursin and decursinol angelate. (Fig. 3 a)

Fig. 3 shows the contour map by 3-dimensional map for the chromatogram of Fig. 2. On the basis of interpretation for chromatogram and 3-dimensional map, we supposed that the main compounds were decursin and

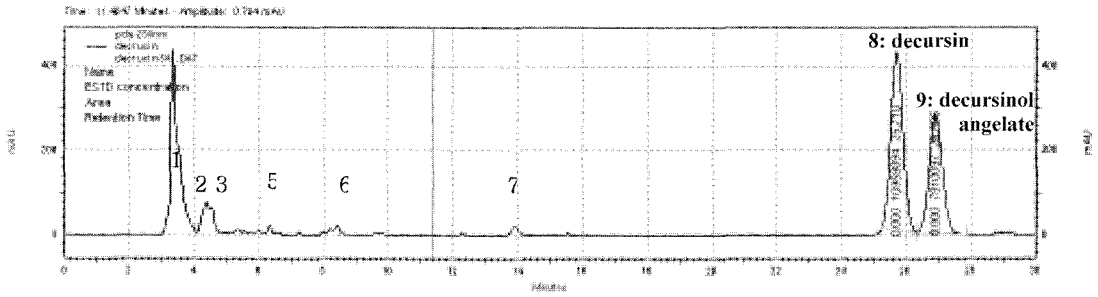


Fig. 2. Chromatogram of a solution containing Angelicae gigantis Radix at a concentration of 1.00 mg/ml (peak No.8: decursin t_R 25.71 and peak No. 9: decursinol angelate t_R 26.91).

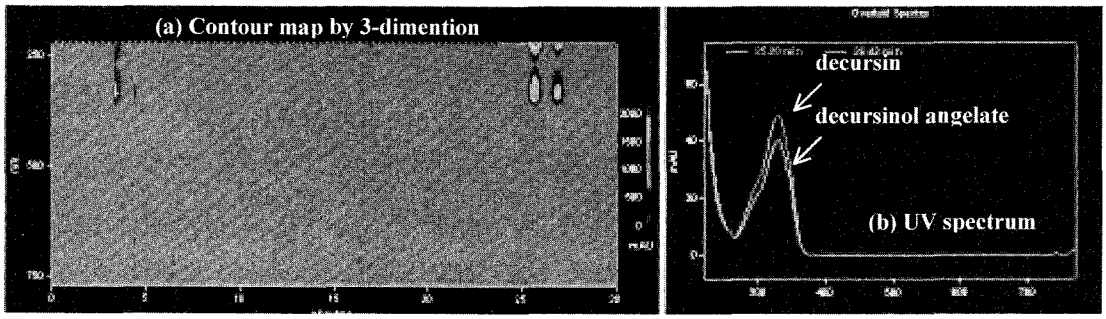


Fig. 3. The UV contour map by 3-dimentional for the chromatogram (a) and the UV spectrum of decursin and decursinol angelate (b).

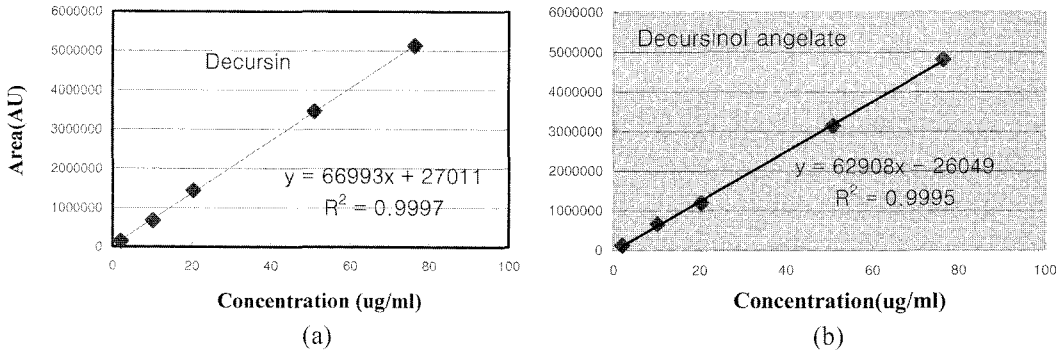


Fig. 4. Calibration curves for decursin (a) and decursinol angelate (b).

decursinol angelate, and other compounds were contained as low concentration in Angelicae gigantis Radix.

Linearity – Each five standard solution of decursin and decursinol angelate were run for the calibration curve using three replicate injections. Their coefficients of correlation (r) were 0.9997 and 0.9995, respectively. The equations were $y = 66993x + 27011$ and $y = 62908x - 26049$ for decursin and decursinol angelate, respectively (y for peak area and x for concentration μg/ml, Fig. 4).

Accuracy – The sample solution was prepared to contain *Angelicae gigantis* Radix 1.0009 mg/ml methanol solution, and certificated the contents of decursin and decursinol angelate to 39.3214 μg/mg and 29.1548 μg/mg by the same HPLC condition. The samples for accuracy test were prepared by mixing the standard solutions of decursin and decursinol angelate into the sample of crude drug. The accuracy results, expressed as recovery and relative standard deviation (RSD), were summarized in Tables 1 and 2. The recoveries of decursin were between 96.08 and 100.29% (1.4% RSD), and decursinol angelate showed the recoveries range form 96.28 to 102.44% (2.09 % RSD)

Repeatability – Three sample solutions of 0.5, 0.75 and 1.0 mg/ml were prepared by extraction of about 1.0 g, 1.5 g, and 2.0 g of crude drug with methanol reflux on the water bath and dilution, respectively, and assayed for

Table 1. Results of accuracy determination for decursin of Angelicae gigantis Radix

Sample	Theoretical concentration of decursin (μg/ml)	No.	Experimental concentration of decursin (μg/ml)	Recovery (%)
Crude drug soln. 5ml		1	57.4678	99.23
(decursin 39.3214 μg/ml)	57.8732	2	55.6067	96.08
+decursin standard soln. 5 ml (76.425 μg/ml)		3	55.6298	96.12
Crude drug soln. 5ml		1	43.9160	97.30
(decursin 39.3214 μg/ml)	45.1357	2	43.9384	97.35
+decursin standard soln. 5 ml (50.95 μg/ml)		3	44.3852	98.34
Crude drug soln. 5 ml		1	29.5598	99.60
(decursin 39.3214 μg/ml)	29.6797	2	29.3134	98.77
+decursin standard soln. 5 ml (20.38 μg/ml)		3	29.7668	100.29
average				98.12
%RSD				1.4425

Table 2. Results of accuracy determination for decursinol angelate of Angelicae gigantis Radix

Sample	Theoretical concentration of decursinol angelate (μg/ml)	No.	Experimental concentration of decursinol angelate (μg/ml)	Recovery (%)
Crude drug soln. 5 ml (decursinol angelate		1	51.5394	97.69
29.1548 μg/ml) + decursinol angelate	52.7524	2	50.7958	96.28
standard soln. 5 ml (50.9μg/ml)		3	52.4386	99.41
Crude drug soln. 5 ml (decursinol angelate		1	40.2944	100.67
29.1548 μg/ml) + decursinol angelate	40.0274	2	41.0006	102.43
standard soln. 5 ml (50.9μg/ml)		3	40.1432	100.23
Crude drug soln. 5 ml(decursinol angelate		1	25.3606	102.44
29.1548 μg/ml) + decursinol angelate	24.7574	2	24.6158	99.43
standard soln. 5 ml (20.36μg/ml)		3	24.0454	97.01
average				88.51
% RSD				2.0916

Table 3. Results of repeatability determinations

Contents of Crude drug	Experimental concentration of decursin (mg/ml)	Calculated % contents of decursin in crude drug	Experimental concentration of decursinol angelate (mg/ml)	Calculated % contents of decursinol angelate in crude drug
0.5206 mg/ml	21.4110	4.1128	16.6485	3.1403
	20.9929	4.0324	16.1981	3.1114
	20.8805	4.0109	16.2016	3.1121
0.7524 mg/ml	30.8158	4.0957	22.8759	3.0404
	30.6049	4.0676	22.8579	3.0380
	30.4440	4.0463	22.8824	3.0412
1.0009 mg/ml	39.4609	3.9425	29.1323	2.8872
	39.2361	3.9201	29.1319	2.8872
	39.2673	3.9232	29.2003	2.8940
average	-	4.0168	-	3.0169
% RSD	-	1.7168	-	3.1939

contents of decursin and decursinol angelate (three replicates each). The % RSD value (1.7 and 3.2) indicated that this HPLC method shows acceptable repeatability in crude drug (Table 3).

Intermediate precision – The same samples injected into second HPLC system were also the same as the ones used for the repeatability study. The results from both HPLC equipments were similar. Run-to-run repeatability and day-to-day reproducibility were used to assess the intermediated precision of repeated injection. Nine injections were made each day and this was repeated for 3 consecutive days. The % RSD values of each concentration

(0.5, 0.7 and 1.0 mg/ml of crude drug) were 3.24, 4.35 and 2.39 about decursin, respectively, and, 3.46, 3.84, and 2.94 about decursinol angelate, respectively. Table 4 indicated the individual values obtained from this study, indicating the proposed HPLC method shows acceptable intermediate precision.

Range – The method developed on our laboratory showed its suitability for in the concentration range from 2 to 75 μg/ml

Stability of solution – The stability of solution was tested with standard solutions and sample solutions that were stored at room temperature (25°C) and 4°C for 24 h. No significant changes in concentrations of decursin and decursinol angelate were observed.

Robustness – The counter-ion concentration (0.005 M, 0.01 M, 0.02 M sodium dodecyl sulfate) in mobile phase did not show any significant changes in the system's suitability parameters (retention time, capacity factor k', asymmetry and number of theoretical plates). The obtained results were detailed in Table 5. However, change of percent of acetonitrile contents in mobile phase and column brand have influence over their resolution and retention time.

Consequently, the analytical method developed in this study is specific for decursin and decursinol angelate, which are the structure isomers and the major constituents of Angelicae gigantis radix, an important herbal drug in Korean.

Results of robustness test showed that the method was susceptible mainly to change of column brand (manufa cturer) and acetonitrile percentage in mobile phase. Whenever this analytical method is applied, these factors should be selected carefully. Study of validation parameters according to ICH recommendation was demonstrated that the method is specific linear, accurate and precision within the established range. When linearity of peak area was tested in the range 2-75 mg/ml of decursin and decursinol angelate, their correlation coefficient were

Table 4. Results of run-to-run repeatability and day-to-day reproducibility determinations

	Contents of crude drug	Experimental concentration of decursin (μg/ml)[a]	% RDS (n=3)	Experimental concentration of decursinol angelate (μg/ml)[b]	% RDS (n=3)
1 day	0.5 mg/ml	21.1282	1.16	16.2494	0.53
	0.7 mg/ml	30.6217	0.61	22.8721	0.06
	1.0 mg/ml	39.2673	0.31	29.1548	0.14
2 day	0.5 mg/ml	19.7535	0.75	15.1074	0.11
	0.7 mg/ml	28.1928	0.48	21.2087	0.56
	1.0 mg/ml	38.686	1.25	28.9105	0.46
3 day	0.5 mg/ml	19.7269	0.35	15.1152	0.24
	0.7 mg/ml	27.7696	0.53	21.0152	0.18
	1.0 mg/ml	37.1019	0.66	27.2706	0.40
	average		0.68		0.30

[a] %RSD of each concentration on multiple day: 3.24%(0.5mg/ml), 4.35%(0.7 mg/ml), 2.39%(1.0 mg/ml)
[b] %RSD of each concentration on multiple day: 3.46%(0.5mg/ml), 3.84%(0.7 mg/ml), 2.94%(1.0 mg/ml)

Table 5. Results of Robustness (effect of counter-ion concentration in the mobile phase) study

	Sample	Counter-ion concentration	Retention time (min)	k' (capacity factor)	Asymmetry	N (theoretical plate)	Area
crude drug	decursin ST	0.012M	22.31	5.60	1.03	85.17	3340089.76
	decursinol angelate ST		23.40	5.91	1.02	86.12	3264106.98
	decursin		22.30	5.46	1.02	84.04	2817969.40
	decursinol angelate		23.38	5.78	1.02	86.45	1898028.22
crude drug	decursin ST	0.010M	23.57	6.01	1.03	85.60	3378033.90
	decursinol angelate ST		24.66	6.41	1.30	87.87	3315439.46
	decursin		23.58	5.96	1.02	85.51	2850925.55
	decursinol angelate		24.72	6.29	1.02	86.53	1931790.59
crude drug	decursin ST	0.008M	23.42	6.08	1.16	86.54	3426047.75
	decursinol angelate ST		24.37	6.34	1.02	88.71	3311877.33
	decursin		23.16	5.85	1.03	87.92	2881814.76
	decursinol angelate		24.23	6.17	1.02	88.03	1959376.56

were between 96.08 and 102.44%, showing 1.44% (decursin) and 2.09%(decursinol angelate) of RSD value. Precision (repeatability) revealed a relative standard deviation value of 1.71% (decursin) and 3.19% (decursinol angelate). The intermediate precision obtained by analysis of three deferent concentrations on multiple days showed acceptable RSD (2.4~4.4%)

Therefore the developed analysis method was proved to be suitable for the assays of decursin and decursinol angelate in crude drugs.

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