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엉겅퀴에 함유된 Cirsimaritin 분석을 위한 HPLC 분석법 밸리데이션

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Validation of an HPLC/UV Analysis Method for Cirsimaritin in *Cirsium japonicum* var. *maackii*

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Abstract – *Cirsium japonicum* var. *maackii* (CM) has been used to treat certain medical conditions such as hemorrhage, hepatitis, and hypertension. Cirsimaritin was previously found as the major flavonoid in CM and is said to contribute to its pharmacological effects. There are currently no reports detailing the qualitative and quantitative detection of phytochemical indicators in the aerial parts of CM. Therefore, we developed a method to rapidly identify and quantify cirsimaritin in CM using HPLC/UV, and we optimized and validated this analytical method. The results showed good linearity in the concentration range tested (0.25-0.015 mg/mL, $r^2 \geq 0.9999$), accuracy (93.9-111.3%), and precision (RSD ≤ 0.59%). The developed method can therefore be used for the rapid evaluation of cirsimaritin in CM.

Keywords – *Cirsium japonicum* var. *maackii*, Cirsimaritin, HPLC/UV, Method validation

Plants contain numerous secondary metabolites that perform many functions in the plant system. They participate in plant defense and stress responses or act as pigments to provide coloration.[1,2)] Secondary metabolites have also attracted attention in natural products research because of their pharmacological activities. Among these secondary metabolites are flavonoids, a class of secondary metabolites with several different structures.[3)] They are widespread among plant species and have various metabolic functions, and they are important for the human diet.[4)] Reports have shown that flavonoids exhibit broad biological activities including anti-oxidant, anti-inflammatory, anti-bacterial, tumor suppressive, and cardioprotective effects.[5)]

Cirsium japonicum is a perennial herb in the Asteraceae family, and they are found across East Asia. The plant has traditionally been used as a folk medicine to prevent hemorrhage and high blood pressure. In China, it is used as a uretic agent.[6)] Recently, it has been included as an important ingredient in functional foods because of its extensive health benefits. Pharmacological reports have shown that it has anti-tumor, hepatoprotective, anti-inflammatory, and diuretic effects.[7,8)] Different phytochemicals have been isolated from *C. japonicum*, and among these are various flavonoids including luteolin, apigenin, hispidulin, cirsimarin, cirsimaritin, pectolinarin, and acacetin.[9-12)]

Among the flavonoids found in *C. japonicum*, there has been interest in isolating previously identified flavonoids

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from its leaves. The isolation of the flavone cirsimaritin is of particular interest because it is the major flavonoid constituent in *C. japonicum* var. *maackii* (CM) leaves.[12,13] Cirsimaritin possesses important biological activities including anti-oxidant, anti-cancer, hepatoprotective, anti-lipogenic, and anti-inflammatory activities[9,10,14,15] and the ability to alleviate the symptoms of menopause.[16,17] Cirsimaritin showed anti-cancer activity by inhibiting the proliferation of lung squamous cell lines by inducing apoptosis.[18]

Due to its flexibility and robustness, the use of HPLC technique is gaining popularity among various analytical techniques as the main choice for fingerprinting and assessing the quality control of herbal plants. To promote the pharmaceutical industrialization of CM extracts, it is very important to establish a standardized process to detect and quantify essential compounds in raw plant materials using validated analytical methods. Developing validated parameters for quality assessment of natural products is necessary to justify their acceptability and activity.[19] However, there are no reports available on validated analytical approaches for evaluating the phytochemical indicators in CM. The aim of this study was to develop and establish an optimized technique using HPLC/UV to detect and quantify the flavones in CM for use in the future development of its raw materials.

Materials and Methods

Plant materials – CM was collected in spring (May 2015) from Imsil Herbal Medicine Association and identified by Korea National Arboretum (KNA-1305), Korea. A sample voucher was deposited at the Herbarium of Department of Plant Science and Technology, Chung-Ang University, Korea.

Instrumentation and Chemicals – The Waters 1525 system equipped with a binary HPLC pump (Miami, FL, USA), an auto-sampler, and an INNO C18 (4.6 × 250 mm, 5 μm) column was used for HPLC analysis. A Waters 2489 UV/VIS detector (Miami, FL, USA) was used to perform chromatographic detection. The cirsimaritin standard (Fig. 1) was isolated from CM.[12] The HPLC-grade solvents acetonitrile and MeOH were purchased from J.T. Baker Chemicals (Radnor, PA, USA).

Preparation of Sample and Standard Solutions – The extraction of CM was performed under reflux using 30% EtOH for 3 h. The extract was then concentrated and lyophilized. A standard solution of cirsimaritin was prepared. To prepare the calibration curve, 1 mg of cirsimaritin was dissolved in 1 mL MeOH and then serially diluted. The peak area (Y), concentration (X, mg/mL) and mean values (n = 3) of the compound was also calculated for use in calibration.

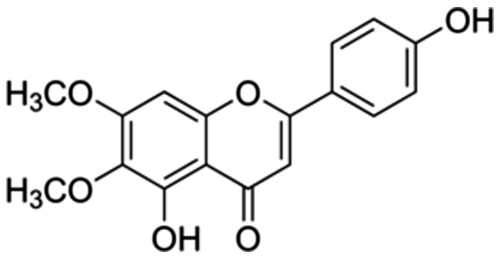

Fig. 1. Chemical structure of cirsimaritin.

HPLC/UV Analysis – Separation was performed under a gradient elution composed of 0.5% acetic acid in water (A) and acetonitrile (B) with a flow rate of 1 mL/min. The gradient elution was started with 83% (A) and was decreased to 70% (A) after 10 min and maintained until 25 min. Solvent A was further decreased to 20% at 30 min and to 0% at 35 min and maintained until 40 min. It was increased from 0% to 83% at 50 min and maintained for another 5 min. The total analysis time was 55 min. The flow rate of the mobile phase was 1 mL/min. The injection volume was 10 μL, and the detector was set at a UV absorbance value of 270 nm. The column was maintained at 30°C.

Validation of the HPLC Method – The HPLC method used to analyze cirsimaritin was validated in terms of its specificity, linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ). Specificity was assessed to detect any possible interference with the signals of the analytes. Linearity was confirmed using five concentration levels of standard mixtures (0.25-0.015 mg/mL) with three injections. A calibration curve was plotted using the peak areas measured at 270 nm on the chromatogram against the known concentrations of the standard solutions. The standard curve for cirsimaritin was analyzed using linear least-squares regression. LOD and LOQ were determined as the lowest concentration producing an appropriate peak shape and experimentally calculated by injecting a series of diluted solutions with known concentrations until the signal-to-noise ratio was 3:1 for LOD and 10:1 for LOQ. To assess inter-day precision, the % relative standard deviation (% RSD) of the standards was determined on three separate days. The accuracy of the

HPLC method was determined in recovery tests in which sample extracts spiked with three different standard mixture concentrations (mg/g) were analyzed and the % recovery was calculated. To validate accuracy and precision, standard mixtures with three concentrations of cirsimaritin were used for intra- and inter-day precision analyses. The peak areas and retention times of the three different concentrations of the standard solutions were calculated (expressed as % RSD) on the same day to determine intra-day precision.

Results and Discussion

An HPLC analytical method for assaying cirsimaritin was developed and validated. Cirsimaritin was analyzed using HPLC/UV, and the results are shown in Fig. 2. As depicted in Fig. 2, good separation was achieved with the HPLC method, and the retention time was estimated to be 31.9 min. A wavelength of 270 nm was determined to be the most effective wavelength for quantifying all impurities as well as the main compound cirsimaritin in a single run. No peak was detected close to the retention time of cirsimaritin, suggesting that the validated process has a high level of accuracy. The chromatogram in Fig. 2 shows the complete separation of cirsimaritin. These results suggest the successful validation of the HPLC method, as it achieved specificity for the analysis of cirsimaritin contained in CM.

The quantification parameters for cirsimaritin were examined using the aforementioned HPLC conditions. A calibration curve for the isolated compound was constructed by mapping the peak areas of the prepared concentrations, and linear regression was used to assess the linearity. For the evaluation of linearity, six solutions were used with a concentration range of 0.25-0.015 mg/mL (n = 3). The regression equation was calculated by plotting the peak area

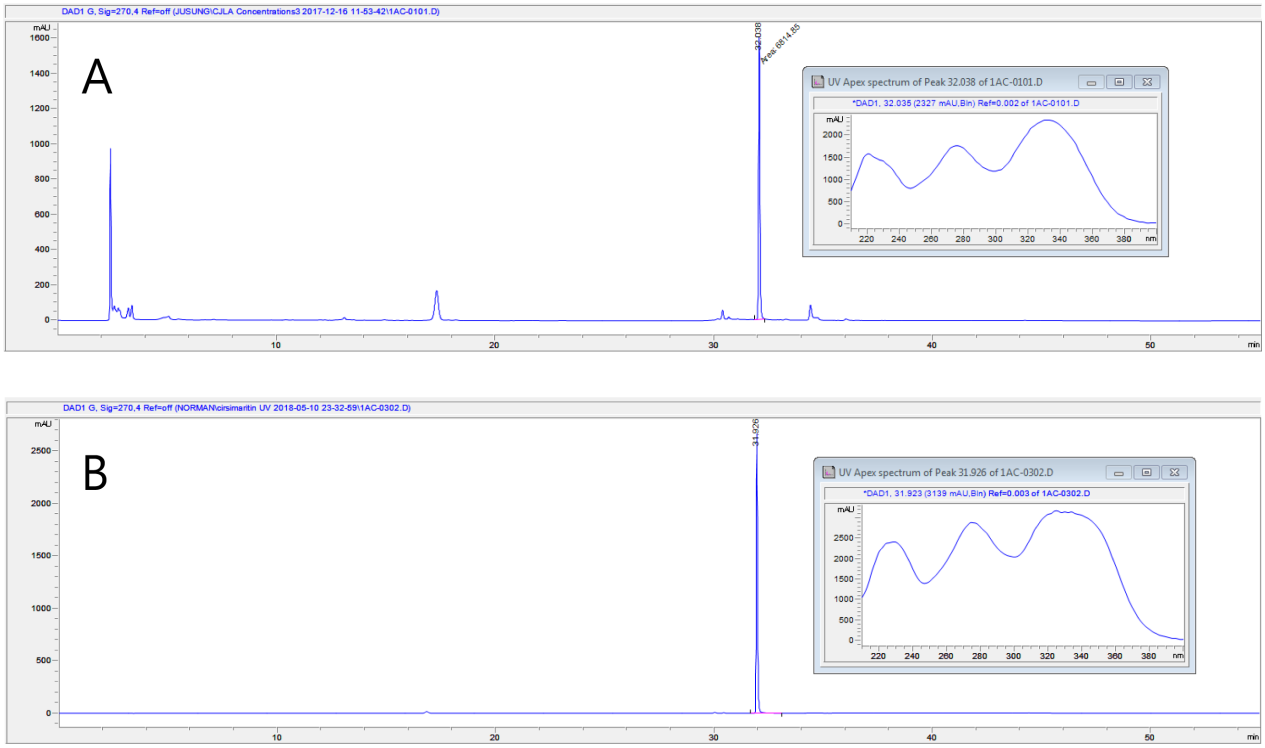

Fig. 2. HPLC chromatograms and specificity of CM (A) and cirsimaritin (B).

Table I. Linearity, LOD, and LOQ for cirsimaritin

Compound	Range (mg/mL)	Calibration equation[a]	r^{2b}	LOD (mg/mL)	LOQ (mg/mL)
Cirsimaritin	0.25-0.015	Y = 35942X + 49.6932	0.9999	0.006	0.018

[a]Y = peak area, X = concentration of standards (mg/mL).
[b]r^2 = correlation coefficient for five data points in the calibration (n = 3)

Table II. Accuracy for the determination of cirsimaritin

Compound	Concentration (mg)	Found content (mg)			Recovery (%)	RSD (%)
		1st	2nd	3rd		
Cirsimaritin	0.125	0.118	0.117	0.117	93.9	0.49
	0.25	0.260	0.259	0.259	103.7	0.22
	0.5	0.557	0.558	0.554	111.3	0.37
	1.0	1.070	1.079	1.048	106.6	1.50

Table III. Intra- and inter-day precision for the determination of cirsimaritin

Compound	Spiked concentration (mg/mL)	Intra-day (n = 5)		Inter-day (n = 5)	
		Found concentration (mg/g)	RSD (%)	Found concentration (mg/g)	RSD (%)
Cirsimaritin	5	37.32	0.51	37.47	0.59
	2.5	37.17	0.41	37.40	0.40
	1.25	38.50	0.39	37.26	0.39

(y) versus the cirsimaritin concentration (x) expressed in mg/mL. The correlation coefficient ($r^2 = 0.9999$) obtained for the regression line indicates a strong linear relationship between the peak area (Table I) and cirsimaritin concentration (37.13 mg/g from HPLC analysis). The LOD of cirsimaritin is the minimum concentration of the compound that can be analyzed, and it can be determined using an analytical method and the HPLC instrument. Conversely, the LOQ is the minimum concentration of the analyzed compound that can be quantified with great precision and accuracy using the instrument and the analytical method. The LOD value obtained in this analysis was 0.006 mg/mL, while the LOQ value was 0.018 mg/mL, suggesting that the analytical approach used to measure the three compounds extracted from CM achieved high sensitivity (Table I).

CM was spiked with cirsimaritin, and the cirsimaritin recoveries were assessed to verify the precision of the developed analytical method. Triplicate analyses were carried out using three injections. As shown in Table II, the recovery rates for cirsimaritin ranged over 93.9-111.3%. Thus, good recovery values were obtained, suggesting that the analytical method we used for quantifying cirsimaritin achieved good accuracy. The precision was validated by evaluating both the intra- and inter-day precision levels of the analytical method used for cirsimaritin. Table III summarizes the results of the analysis. The coefficient of variance for the intra-day and inter-day precision values for cirsimaritin ranges from 0.39 to 0.51% and 0.39 to 0.59%, respectively. The values obtained were lower than 2%, indicating that the corresponding results of the analytical method to quantify cirsimaritin derived from CM showed great reliability.

Several studies have demonstrated that flavonoids such as apigenin, kaempferol, rutin, and quercetin have anti-allergic, anti-inflammatory, hepatoprotective, anti-spasmodic, anti-thrombotic, and anti-cancer properties.[20] Cirsimaritin, the main flavonoid in CM,[13] is a marker compound and has various biological activities.[16,17,21-23]

Cirsimaritin was successfully quantified from CM using HPLC/UV. The validation techniques employed with the analytical methodology indicated that excellent results were obtained in terms of the accuracy, precision, specificity, and quantifying parameters. The findings of our analysis support the use of our method for the identification and accurate quantification of cirsimaritin from CM in routine analyses and large-scale extraction processes and for content determination. Moreover, our study provided an optimized technique that could be applied to the pharmaceutical industrialization of CM leaf extract.

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