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Quantitative Analysis of Coumarins from *Angelica gigas* Using ^{1}H-NMR

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Abstract ^{1}H-Nuclear magnetic resonance (NMR) spectrometry was applied to the quantitative analysis of coumarins in the roots of *Angelica gigas* without any chromatographic purification. The experiment was performed by the analysis of each singlet germinal methyl, which was well separated in the range of 1.0-2.0 ppm in the ^{1}H-NMR spectrum. The quantity of the compounds was calculated by the ratio of the intensity of each compound to the known amount of internal standard (dimethyl terephthalate). These results were compared with the conventional gas chromatography (GC) method. The contents of decursin and decursinol angelate in *A. gigas* were determined 1.98±0.07, 1.13±0.08% in quantitative ^{1}H-NMR method and 2.06±0.24, 1.17±0.24% in GC method, respectively. The advantages of quantitative ^{1}H-NMR analysis are that can be analyzed to identify and quantify, and no reference compounds required for calibration curves. Besides, it allows rapid and simple quantification for coumarins with an analysis time for only 10 min without any preprocessing.

Keywords: *Angelica gigas*, decursin, decursinol angelate, quantitative nuclear magnetic resonance (qNMR)

Introduction

There have been increasing interests in the natural products as sources of new drugs and functional foods in the past several years (1-4). For the quality control (QC) of these products, it needs to quantify the effective compounds in natural products and its products (5). Especially, when it uses natural products in functional foods, the quantity of reference compounds is necessary for safety, nutrition, and quality (6). Therefore, we must achieve the purpose using the quantitative analysis of reference compounds in the sources with the analytical instruments.

For the determination of the quantity of standard compounds in natural products, we have used high performance liquid chromatography (HPLC) and gas chromatography (GC) up to the present, generally (7,8). But, it needs to prepare organic solvents with degassing and filtration, preprocessing of samples and standard compounds using HPLC analysis (9). Moreover, GC analysis required elaborated clean up processes and derivatization procedures in order to enhance sensitivity and to remove compounds that can be interfere with the detection of the target compounds (10). For that reasons, an alternative method for the analysis of reference compounds from natural products would be highly desirable.

Nuclear magnetic resonance (NMR) spectrometry is one of the most important and widespread analytical method for elucidation of the molecular structure of purified compounds. Recently, NMR is applied as quantitative spectroscopic tool on the basis of theoretical backgrounds that the intensity of a resonance line is directly proportional to the number of resonant nuclei (spins) (1,11). The advantages of quantitative NMR (qNMR) are the ease of sample preparation, relatively short measuring time, and its non-destructive characters. Also, it is no need for intensity calibrations of reference compounds in case of determination of ratios (11). For example, some researchers have developed into quantitative analysis using ^{1}H-NMR spectrometry in recent years. Choi *et al.* (12) have analyzed the amount of ginkgolic acids from ginko leaves and products, and retinol and retinol palmitate in vitamin tablets (13). Song *et al.* (9) have quantified *t*-cinnamaldehyde in *Cinnamomum cassia* using ^{1}H-NMR spectrometry. In cider apple juices, Berregi *et al.* (14) have determined contents of (-)-epicatechin without any preparations. In the previous paper, we quantitatively analyzed paeoniflorin, the major component of the roots of *Paeonia lactiflora*, with direct extraction as deuterated solvent (15).

Angelica gigas (*danggwi*), which belongs to the family of Umbelliferae, has long been used as a traditional medicine not only for treatment of anemia but also as a sedative, and anodyne or a tonic agent (16,17). *A. gigas* has been studied extensively and are shown to contain a variety of substances including coumarins (18). Decursin and decursinol angelate, the structure isomer (pyranocoumarin) each other, are the main coumarin constituents in the roots of *A. gigas* (19). Decursin and decursinol angelate displayed toxic activity against various human cancer cell lines (20,21) and antagonized against the voluntary activity in mice (22). Decursin exhibited significant prolongation of hexobarbital-induced hypnosis as well as significant inhibition

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of hepatic microsomal drug metabolizing enzyme activities (23). The contents of decursin in domestic *A. gigas* is more than *A. acutiloba* which cultivated in Japan (24).

The methods for analysis of decursin and decursinol angelate from *A. gigas* include HPLC (19,25,26) and GC (27). However, HPLC and GC analysis wasted to equilibration time and organic solvents and more preprocessing. Therefore, an alternative method for the analysis of decursin and decursinol angelate from *A. gigas* would be highly desirable.

In this paper, ^{1}H-NMR spectrometry was applied to the quantitative analysis of coumarins from *A. gigas*. In addition, the results obtained from quantitative ^{1}H-NMR method were compared with those from conventional GC method.

Materials and Methods

Plant material The roots of *A. gigas* were purchased from a dispensary of herbal medicine, Suwon, Korea, in June 2005 and verified Prof. Dae-Keun Kim, Woosuk University, Jeonju, Korea. A voucher specimen (KN-0508) was reserved at the Laboratory of Natural Products Chemistry, Plant Metabolism Research Center, Kyung Hee University, Yongin, Korea.

Chemicals First grade ethyl acetate (EtOAc) was purchased from Daejung Chemicals (Siheung, Korea). $CDCl_3$ (99.9%) was obtained from Merck (Darmstadt, Germany). Internal standard, dimethyl terephthalate, was obtained from Sigma-Aldrich (St. Louis, MO, USA) and the reference compounds were isolated from the roots of *A. gigas*, previously. These reference compounds finally identified as decursin and decursinol angelate by comparison of several physical and spectroscopic data with those in the literatures (27-29).

Instruments ^{1}H-NMR spectra (400 MHz) were taken on a Varian Unity Inova AS 400 FT-NMR spectrometer (Palo Alto, CA, USA) in 0.5 mL of $CDCl_3$. For each sample, 128 scans were recorded with the following parameters: 0.45 Hz/point, spectral width 4,600.0 Hz (center of spectral width 5.02 ppm), acquisition time 2.5 sec, recycle delay 2.0 sec, flip angle 90° at 25°C. For quantitative analysis, peak area was used and the start and end point of the integration of each peak were selected manually. The area of dimethoxy signal of dimethyl terephthalate at 3.95 ppm was referred integral as 1,000 in ^{1}H-NMR spectrum. The amount of coumarins was calculated using the following equation (13).

$$\text{Quantity (mg)} = \frac{\text{integral (CO)}}{\text{integral (IS)}} \times \frac{\text{Mw (CO)}}{\text{Mw (IS)}} \times \text{weight (IS)}$$

where, integral (CO) = the peak area of the germinal methyls of coumarins; integral (IS) = the peak area of the dimethoxy of dimethyl terephthalate; Mw (CO) = the molecular weight of coumarins divided by 3 because there are 3 protons in case of germinal methyl signal (328.36 for coumarins); Mw (IS) the molecular weight of dimethyl terephthalate divided by 6 because there are 6 protons in case of dimethoxy signal (194.18 for dimethyl terephthalate); weight (IS) = the amount of dimethyl terephthalate added (1 mg in this paper).

Gas chromatography was performed using a GC (GC-14B; Shimadzu, Kyoto, Japan) as follows: Column-DB-5 capillary (length 30 meters, i.d. 0.25 mm, film thickness 0.25 mm, J&W Scientific, Folcom, CA, USA), retain temp. 250°C for 20 min; injection size 2 μL, temperature-injector 300°C, carrier gas (N_2, flow rate 1.0 mL/min); flame ionization detector (FID).

Extraction One-hundred mg of powdered plant material was weighed out and mixed with 3 mL EtOAc respectively and refluxed for 30 min (3 times). The extraction solvent, EtOAc, was selected by the group-contribution technique for the calculation of solubility parameters for the coumarin compounds (30). Actually, EtOAc was more suitable solvent than the other organic solvents (*n*-hexane, $CHCl_3$) on the respects of extraction mass and the coumarin compounds contents (data not shown). The combined extracts were made 10 mL volume and separated to equal volume fractions. One fraction was evaporated to dryness after addition of internal standard (dimethyl terephthalate). The dried sample was dissolved in 0.5 mL $CDCl_3$ and used for ^{1}H-NMR measurement. The other fraction was evaporated to dryness under reduced pressure and used for GC measurement (added also internal standard).

Results and Discussion

For the simple and rapid quantitative analysis of coumarins (Fig. 1) from the roots of *A. gigas*, quantitative ^{1}H-NMR was developed and confirmed by conventional GC analysis.

In our previous research (9), the temperature variations (19, 25, 30, 40, and 50°C) gave no effects on the chemical shift and the integration values of the signals on the ^{1}H-NMR spectra. So, all the sample solutions with various concentrations and extracts were measured for the ^{1}H-NMR at 25°C in this paper.

The quantitative ^{1}H-NMR method of coumarins as decursin and decursinol angelate in the roots of *A. gigas* requires that a target peak is chosen for the analysis. The chemical shift of the coumarins shows Table 1 comparing

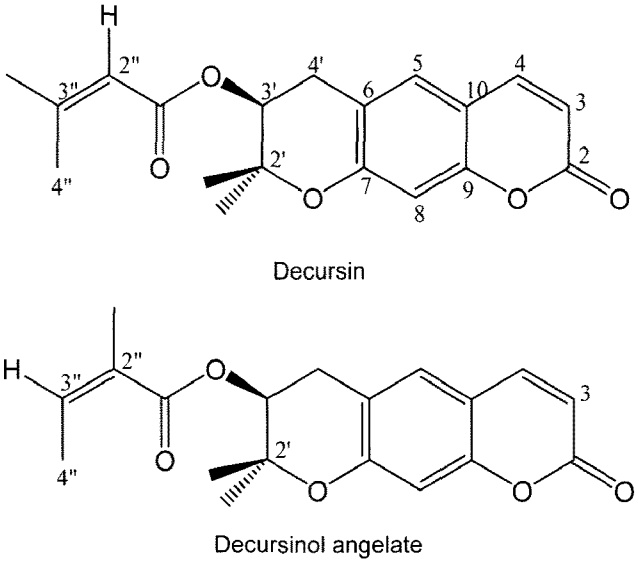

Fig. 1. Structures of decursin and decursinol angelate.

Table 1. Chemical shift of coumarins from *Angelica gigas* (400 MHz, $CDCl_3$)

Proton	Decursin	Decursinol angelate
3	6.20, 1H, *d* (9.5)	6.20, 1H, *d* (9.5)
4	7.58, 1H, *d* (9.5)	7.59, 1H, *d* (9.5)
5	6.77, 1H, *s*	6.78, 1H, *s*
8	7.15, 1H, *s*	7.15, 1H, *s*
3'	5.07, 1H, *t* (4.7)	5.14, 1H, *t* (4.9)
4'	2.90, 1H, *dd* (17.1, 4.7)	2.90, 1H, *dd* (17.0, 4.9)
	3.17, 1H, *dd* (17.1, 4.7)	3.24, 1H, *dd* (17.0, 4.9)
gem$(CH_3)_2$	1.35, 3H, *s*	1.38, 3H, *s*
	1.37, 3H, *s*	1.40, 3H, *s*
2"	5.65, 1H, *m*	-
3"	-	6.11, 1H, *qq* (7.2, 1.2)
2"-CH_3	-	1.85, 3H, *d* (1.2)
3"-CH_3	2.13, 3H, *d* (1.2)	-
4"	1.86, 3H, *d* (1.2)	1.89, 3H, *d* (7.2)

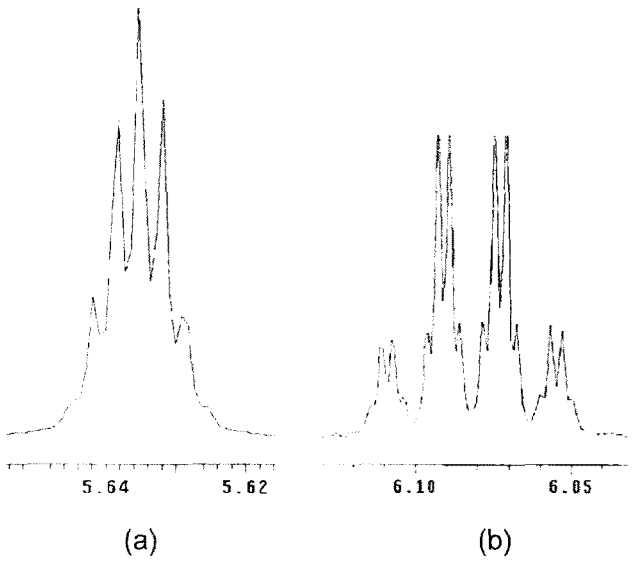

Fig. 3. ^{1}H-NMR spectra of H-2 of decursin (a) and H-3 of decursinol angelate (b).

the spectrum of decursin with decursinol angelate. At first, for the quantification of the coumarins, the spectral lines of H-2" of decursin and H-3" of decursinol angelate were selected as target signals in the ^{1}H-NMR spectra because these signals of the coumarins were separated from the EtOAc extracts. Figure 2 shows that peaks of H-2 (decursin) and H-3 (decursinol angelate) are separated from each other in non-crowded region. The chemical shifts of H-2" and H-3" are δ 5.65 (1H, *m*) and δ 6.11 (1H, *qq*, $J = 7.2, 1.2$), respectively, and the dimethoxy signal of internal standard, dimethyl terephthalate, indicates δ 3.95 (6H, *s*). But the signal of H-2" and H-3" were complicated to integrate (Fig. 3). Therefore, another peaks selected for the accuracy of quantification. Figure 4 shows that peaks of germinal methyls of decursin and decursinol angelate are separated from each other in high magnetic field region. The chemical shifts of germinal methyl signals of decursin are δ 1.35 (3H, *s*) and δ 1.37 (3H, *s*), respectively. However, the chemical shifts of germinal methyl signals of decursinol angelate are δ 1.38 (3H, *s*) and δ 1.40 (3H, *s*), respectively. In the ^{1}H-NMR spectrum of EtOAc extracts, these spectral lines separated from each other. So, the germinal methyls of coumarins with resonating in non-interfered region of the spectra were selected as target signals for quantification.

In the quantitative ^{1}H-NMR analysis, calibration curves are not needed for quantification of the compounds because integration of the peak is always proportional to the amount of the compound and the same for all compounds in ^{1}H-NMR spectra. However, calibration curves for coumarins using the ratio of the peak area of the compounds and the internal standard were determined in the range of 0.3125-5.0000 mg/mL in order to evaluate the accuracy of this method depending on the different concentrations. Each calibration curve is shown in Fig. 5. The linearity of the calibration curve of decursin and decursinol angelate by ^{1}H-NMR method was 0.9998 and 0.9999, respectively, and it was suitable to quantify.

A GC method was used to confirm the analysis results obtained by ^{1}H-NMR method. Decursin and decursinol angelate were separated at 15.99 and 15.38 min, respectively, and dimethyl terephthalate was detected at 3.36 min. The calibration curves for each compound using the ratio of peak areas of the reference compounds and the internal standard were prepared in the range of 0.25-2.5 mg/mL. The linearity of the calibration curves of decursin and decursinol angelate by GC were 0.9993 and 0.9996, respectively, and it was also suitable to quantify.

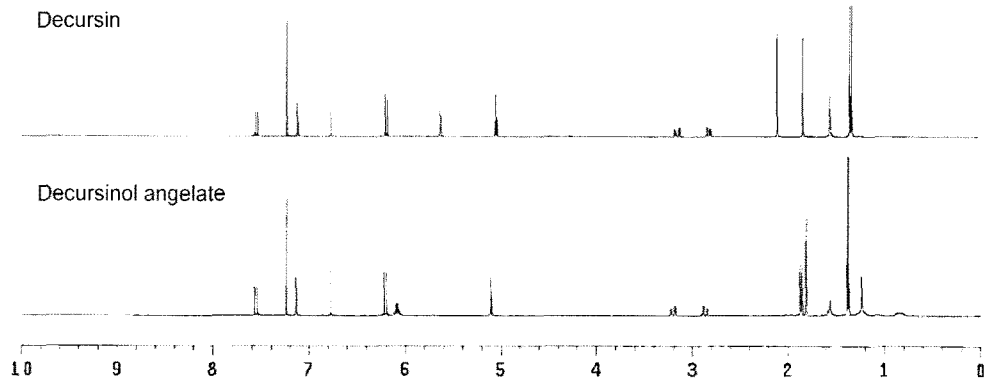

Fig. 2. ^{1}H-NMR spectra of decursin and decursinol angelate in $CDCl_3$.

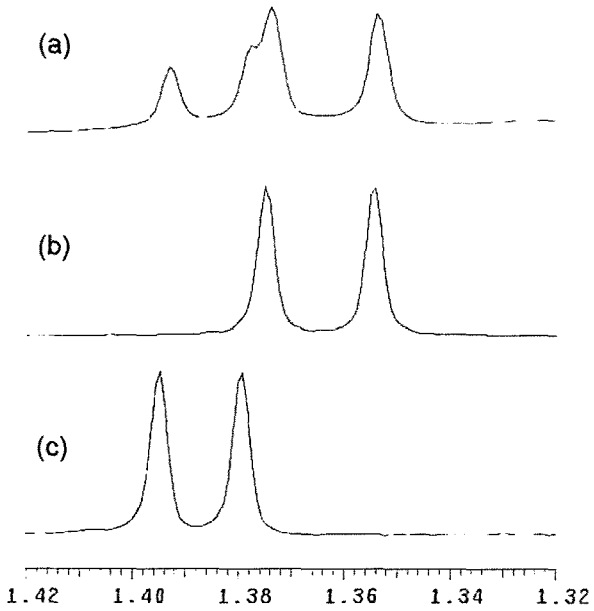

Fig. 4. δ 1.32-1.42 region of ^{1}H-NMR spectra. (a) EtOAc extracts; (b) decursin; (c) decursinol angelate.

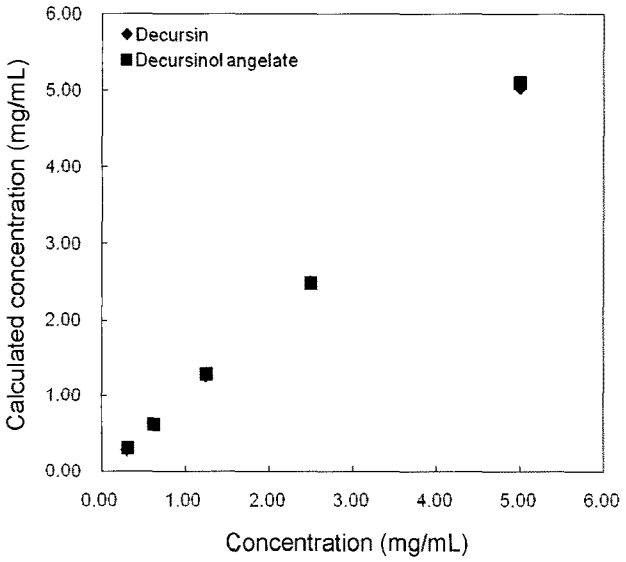

Fig. 5. Calibration curve-calibrations for decursin and decursinol angelate from the area of peak compared to internal standard (dimethyl terephthalate).

Table 2 showed the contents of decursin and decursinol angelate calculated from the peak integral in ^{1}H-NMR spectra as 1.98±0.07 and 1.13±0.08%, respectively, and the peak area in the GC chromatograms as 2.06±0.24 and 1.17±0.24%, respectively. These quantitative results showed a slight difference compared to the past research (27), which is thought to vary by the place that the roots of *A. gigas* were produced or purchased. The concentrations of coumarins in *A. gigas* showed similar results (relative error values <3%) as between the ^{1}H-NMR method and the GC method. Besides, ^{1}H-NMR method was more accurate because the standard deviation of ^{1}H-NMR method was smaller than GC method. Therefore, ^{1}H-NMR method was suitable with the quantification of coumarins from the roots of *A. gigas*.

Table 2. Comparison of the concentration (%, w/w) of decursin and decursinol angelate in the EtOAc extract as determined by integration of germinal methyls the ^{1}H-NMR spectrum and by peak area in the GC[1)]

Analysis method	^{1}H-NMR	GC
Decursin	1.98±0.07	2.06±0.24
Decursinol angelate	1.13±0.08	1.17±0.24

[1)]Mean±SD; all experiments were based on triplicate measurements.

In conclusion, the described quantitative ^{1}H-NMR analysis is a rapid and simple method for the identification and quantification of coumarins in the roots of *A. gigas*. Using the quantitative ^{1}H-NMR method of the contents of coumarins can be determined in much shorter time than the conventional GC measurements. Also, it didn't need to prepare any preprocessing of samples and reference compounds. As the previous method (15), natural products samples were extracted with deuterated solvents (e.g., $CDCl_3$) directly that is more fast and handy method. It is possible to apply the analysis of coumarins in liquid functional foods contained *A. gigas* by solvent fractionation without any pre-processing. In addition, the qNMR in combination with multivariate or pattern recognition techniques such as principal components analysis (PCA) and hierarchical cluster analysis (HCA) can be applied to metabolites profiling in foods for quality control.

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