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Quantitative Analysis of Paeoniflorin from *Paeonia lactiflora* Using ^{1}H-NMR

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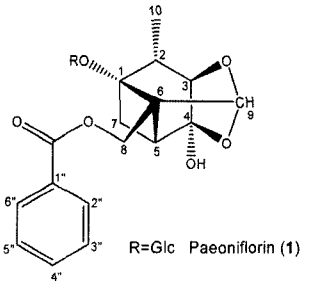
Abstract – Paeoniflorin, the major component of the root of *Paeonia lactiflora*, was quantitatively analyzed using ^{1}H-NMR spectrometry. The quantity of paeoniflorin was calculated by the ratio of the intensity of the signals (H-9, H-10, H-2", 6") to the aldehyde peak of the known amount of internal standard, 2,4,6-trihydroxybenzaldehyde. These results were compared with the conventional HPLC method. The contents of paeoniflorin in *P. lactiflora*, which were respectively calculated by H-9, H-10, H-2", 6" in the ^{1}H-NMR spectra and HPLC, were determined 2.60 ± 0.07, 2.44 ± 0.09, 2.77 ± 0.12 and 2.46 ± 0.16%. 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 paeoniflorin with an analysis time for only 20 min without any preprocessing.

Keywords – ^{1}H-NMR, quantitative analysis, *Paeonia lactiflora*, paeoniflorin

Introduction

The root of *Paeonia lactiflora* (Paeoniae Radix) is one of the most important crude drugs (Kang *et al.*, 1991) containing monoterpene glycosides such as paeoniflorin (Fig. 1), oxypaeoniflorin, benzoylpaeoniflorin, albiflorin and lactiflorin (Ikeda *et al.*, 1996; Aimi *et al.*, 1969; Kaneda *et al.*, 1972), and has been used as drug for gynecological problems and for cramp, pain and giddiness (Liu *et al.*, 2006). The main quality index of Paeoniae Radix as the major component is paeoniflorin (Kim & Kim, 2004; Kim *et al.*, 2006) which has pharmacological activities such as analgesia (Sugishita *et al.*, 1984), anti-inflammatory (Yamahara *et al.*, 1982), anti-hyperglycemic (Hsu *et al.*, 1997) and anti-thrombotic effect (Ye *et al.*, 2001). The concentration of paeoniflorin varies from 0.12 to 9.61% in the root of *P. lactiflora* in accordance with cultivated locations (Ikeda *et al.*, 1996), harvesting seasons (Kim *et al.*, 2006) and cultivated years (Kim *et al.*, 1996), and it must be 2% or over for the quality control. Therefore, it is necessary to quantify the content of paeoniflorin. The methods for analysis of paeoniflorin from *P. lactiflora* include thin layer chromatography (TLC) (Okamoto & Noguchi, 1986), high performance liquid chromatography (HPLC) (Ikeda *et al.*, 1996; Kim *et al.*, 2006), capillary electrophoresis (CE) (Wu & Sheu, 1996), but up to now HPLC has been used more often for paeoniflorin quantification. However, HPLC analysis wasted to equilibration time and organic solvents and more preprocessing. Therefore, an alternative method for the analysis of paeoniflorin from *P. lactiflora* would be highly desirable.



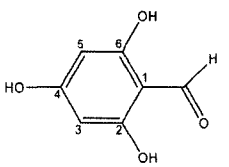


Fig. 1. The chemical structure of paeoniflorin (**1**).

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Conventionally, nuclear magnetic resonance spectrometry (NMR) has been used to elucidate the molecular structure of purified compounds as an analytical tool. But, NMR is possible to quantitative spectroscopic tool, because the intensity of a resonance line is directly proportional to the number of resonant nuclei (spins). Quantitative NMR (qNMR) has particularly due to specific advantages like, (i) the possibility to determine structures, (ii) no need for intensity calibrations in case of determination of ratios, (iii) relatively short measuring time, (iv) its non-destructive character, (v) no prior isolation of the analyte in a mixture, (vi) the possibility of a simultaneous determination of more than one analyte in a mixture (Malz & Jancke, 2005). So, some researchers have been developed into quantitative analysis using ^{1}H-NMR spectrometry in recent years, however, it is nothing but several reports. Choi has analyzed the amount of ginkgolic acids from *Ginkgo* leaves and products (Choi *et al.*, 2004a) and retinol and retinol palmitate in vitamin tablets (Choi *et al.*, 2004b), and Song has quantified *t*-cinnamaldehyde in *Cinnamomum cassia* using ^{1}H-NMR (Song *et al.*, 2005).

In this paper, we described the quantitative analysis of paeoniflorin from *Paeonia lactiflora* using ^{1}H-NMR spectrometry. Besides, the results obtained from ^{1}H-NMR method were compared with those from conventional HPLC method.

Experimental

Plant material – The root of *Paeonia lactiflora* was purchased from a dispensary of herbal medicine, Suwon, Korea in April 2006 and verified by Prof. Dae-Keun Kim, Woosuk University, Jeonju, Korea. A voucher specimen (No. KN-0611) has been deposited at the Natural Products Chemistry Laboratory, Plant Metabolism Research Center, Kyung Hee University, Suwon, Korea.

Chemicals – All organic solvents were HPLC grade and purchased from Fisher Scientific. Deuteriated water (D_2O, 99.9%) was obtained from Merck (Damstadt, Germany). Internal standard, 2,4,6-trihydroxybenzaldehyde, was obtained from Aldrich (St. Louis, U.S.A.) and the reference compound (paeoniflorin) was purchased from Waco Pure Chemical Industries (Osaka, Japan).

Instrumentation – ^{1}H-NMR spectra (400 MHz) were recorded in D_2O (99.9%) using a Varian UNITY Inova AS 400 FT NMR spectrometer (California, U.S.A.). For each sample, 128 scans were recorded with the following parameters: resolution, 0.45 Hz; spectra width, 4600.0 Hz (center of spectral width 5.02 ppm); pulse width, 4.0 μs; relaxation delay, 1 s; acquiring time, 2.5 s; temperature, 25 °C. For quantitative analysis, peak area was used and the start and end point of the integration of each peak were selected manually.

HPLC was performed on a Shimadzu LC-20 (Tokyo, Japan) system using an Atlantis dC18 column (3.0 × 250 mm, 5 μm, Waters). The mobile phase using isocratic elution was a solvent mixture of water (mobile phase A) and acetonitrile (mobile phase B) as 4 versus 1. The total flow rate was 0.4 ml/min with a sample volume of 20 μl and UV detection at 254 nm.

Extraction – One hundred milligram of powdered plant material were weighed out and mixed with 1 ml of D_2O and sonicated for 30 min (room temperature). The extracts were filtrated through a 0.45 μm PVDF filter (Pall, USA) and 0.5 ml of filtrated extract used for ^{1}H-NMR measurement (added 2 mg of 2,4,6-trihydroxybenzaldehyde as internal standard). The other remainder was used for HPLC measurement (added also internal standard).

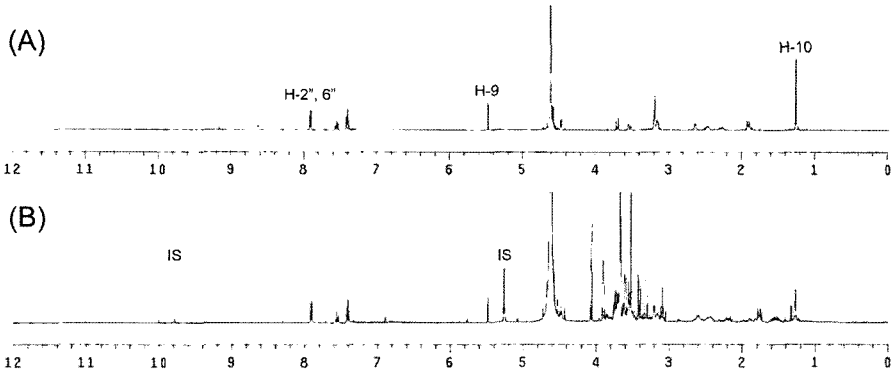
Results and Discussion

For the simple and rapid quantitative analysis of paeoniflorin from *Paeonia lactiflora*, quantitative ^{1}H-NMR was developed and confirmed by conventional HPLC analysis.

The ^{1}H-NMR quantification of the paeoniflorin in the root of *P. lactiflora* requires that a target peak is chosen for the analysis. The spectral lines of H-9, H-10 and H-2", 6" were selected as a target signal in the ^{1}H-NMR spectra because these signals of paeoniflorin were separated from the D_2O extracts. Fig. 2 shows that peaks of H-9, H-10 and H-2", 6" are detected in non-crowded region, well separated from each other. The chemical shift of H-9, H-10 and H-2", 6" are δ 5.48 (1H, s), δ 1.26 (3H, s) and δ 7.91 (2H, d, J = 8.0 Hz), respectively and the aldehyde of internal standard, 2,4,6-trihydroxybenzaldehyde, indicates δ 9.91 (1H, s). When we used CD_3OD as extraction solvent, the water peak from CD_3OD and the signals of other compounds interfered with H-9 and H-10 signals of more than D_2O extract. For these reasons, D_2O was used.

The amount of paeoniflorin was calculated using the following equation (Choi *et al.*, 2004b): Quantity (mg) =

$$\frac{\text{integral (PA)}}{\text{integral (IS)}} \times \frac{\text{MW (PA)}}{\text{MW (IS)}} \times \text{weigt (IS)}$$ where integral (PA) = the peak area of the H-9, H-10 and H-2", 6" of paeoniflorin; integral (IS) = the peak area of the aldehyde of 2,4,6-trihydroxybenzaldehyde; MW (PA) = the molecular weight of paeoniflorin divided by 3 because there



IS: internal standard (2,4,6-trihydroxybenzaldehyde)

Fig. 2. ^{1}H-NMR Spectra of (A) paeoniflorin, (B) the D_2O extract of the root of *Paeonia lactiflora* in D_2O in the range of δ 0.0-12.0 after 128 scans.

Table 1. Comparison of the concentration (%w/w) of paeoniflorin in the D_2O extract as determined by integration of H-9, H-10 and H-2", 6" in the ^{1}H-NMR spectrum and by peak area in the HPLC

analysis method	^{1}H-NMR			HPLC
peak	H-9	H-10	H-2", 6"	
paeoniflorin content	2.60 ± 0.07	2.44 ± 0.09	2.77 ± 0.12	2.46 ± 0.16

Mean (± standard deviation). All experiments were based on triplicate measurements.

are three protons in case of H-10 (480.46 for paeoniflorin); MW (IS) the molecular weight of 2,4,6-trihydroxybenzaldehyde (MW: 154.12); weight (IS) = the amount of 2,4,6-trihydroxybenzaldehyde added (2 mg in this report).

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. However, calibration curve for paeoniflorin using the ratio of the peak area of the compound and the internal standard were determined in the range of 0.25-4.0 mg/ml in order to evaluate the accuracy of this method depending on the different concentrations. Each calibration curve is shown in Fig. 3. The linearity of H-9, H-10 and H-2", 6" were found to be higher than 0.9990 (0.9999, 0.9999, 0.9995, respectively). All calibration curves were suitable to quantify.

An HPLC method was used to confirm the analysis results obtained by ^{1}H-NMR method. The paeoniflorin was eluted at retention time 8.9 min, and the internal standard, 2,4,6-trihydroxybenzaldehyde, was at 14.7 min. And the calibration curves for each compound using the ratio of peak areas of the reference compound and the internal standard were prepared in the range of 0.25-4.0 mg/ml. The linearity of the calibration curve by HPLC was 0.9994 and it was suitable to quantify, also. Table 1.

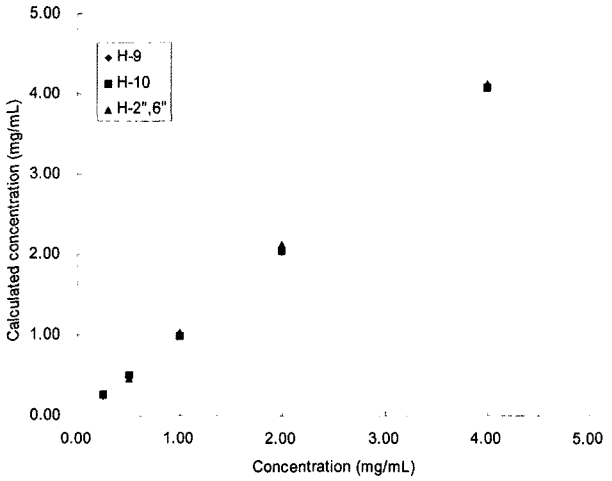


Fig. 3. Calibrations for H-9, H-10 and H-2", 6" of paeoniflorin from the integral of aldehyde compared to internal standard (2,4,6-trihydroxybenzaldehyde).

showed the content of paeoniflorin calculated from the integral of H-9, H-10 and H-2", 6" in the ^{1}H-NMR spectra, and peak area in the HPLC chromatograms (relative error values < 2%) as 2.60 ± 0.07, 2.44 ± 0.09, 2.77 ± 0.12 and 2.46 ± 0.16%, respectively. Using the quantitative ^{1}H-NMR analysis method, the content obtained by measuring H-9 and H-2", 6" were generally higher than the content calculated from H-10 and HPLC analysis method. This was probably due to the H-9 and H-2", 6" peaks being

interfered by the similar monoterpene glycosides in *P. lactiflora*, such as albiflorin, lactiflorin, benzoylpaeoniflorin, oxypaeoniflorin and benzoyloxypaeoniflorin. In the range of δ 9.0-5.0, the olefinic proton signal and acetal proton signal of these compounds were overlap with the H-9 and H-2", 6" of paeoniflorin. However, in case of H-10 signal, several references indicated that H-10 signal didn't overlap with other signals (Kaneda *et al.*, 1972; Yu *et al.*, 1990; Kang *et al.*, 1993; Son & Park, 2002; Lee *et al.*, 2005). So, H-10 signal is suitable for quantification of paeoniflorin in the extract by ^{1}H-NMR analysis method which compared with former literatures (Ikeda *et al.*, 1996; Kim *et al.*, 2006; Kim *et al.*, 1996).

Using the quantitative ^{1}H-NMR the contents of paeoniflorin can be determined in much shorter time than the conventional HPLC measurements. Besides, it didn't need to prepare organic solvents with degassing and filtration, preprocessing of samples and reference compounds. In conclusion, the described quantitative ^{1}H-NMR analysis is a rapid and simple method for the identification and quantification of paeoniflorin in the root of *P. lactiflora*.

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