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NOTE

Application of quantitative ^{1}H-NMR method to determination of gentiopicroside in Gentianae radix and Gentianae scabrae radix

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Abstract A quantitative ^{1}H-NMR method (qHNMR) was used to measure gentiopicroside content in Gentianae radix and Gentianae scabrae radix. Gentiopicroside is a major component of Gentianae radix and Gentianae scabrae radix. The purity of gentiopicroside was calculated from the ratio of the intensity of the H-3 signal at δ 7.44 ppm or the H-8 signal at δ 5.78 ppm in methanol-d_4 of gentiopicroside to that of a hexamethyldisilane (HMD) signal at 0 ppm. The concentration of HMD was corrected with SI traceability by using potassium hydrogen phthalate of certified reference material (CRM) grade. As a result, the gentiopicroside content in two lots of Gentianae radix as determined by qHNMR was found to be 1.76 and 2.17 %, respectively. The gentiopicroside content in two lots of Gentianae scabrae radix was 2.73 and 3.99 %, respectively. We demonstrated that this method is useful for the quantitative analysis of crude drugs.

Keywords Gentiopicroside · Quantitative ^{1}H-NMR · Gentianae radix · Gentianae scabrae radix · Reagent purity

Introduction

Gentianae radix (Gentiana in Japanese) and Gentianae scabrae radix (Ryutan in Japanese), the roots or rhizomes of *Gentiana lutea* Linne and *G. scabra* Bunge, as well as *G. manshurica* Kitagawa and *G. triflora* Pallas., are important sources of crude drugs [1, 2]. Gentianae radix is used throughout Europe for treating stomach ailments or stimulating the appetite. Gentianae scabrae radix is also used to treat stomach ailments or for appetite stimulation and is a part of many traditional "Kampo" formulas, such as Ryutan-shakan-to, Rikkou-san, and Sokei-kakketsu-to. The major physiologically active component of Gentianae radix and Gentianae scabrae radix is gentiopicroside, a secoiridoid glucoside (Fig. 1) [3–5] responsible for the pharmacological activity of the crude drug. Gentiopicroside exhibits antibacterial, antioxidant, smooth-muscle relaxing, and hepatoprotective activities [6–8]. Therefore, gentiopicroside content has been used as a chemical index for quality control of the crude drugs. However, the Japanese Pharmacopoeia has not adopted gentiopicroside determination as a measure of the quality of Gentianae radix and Gentianae scabrae radix preparations [1, 2]. High-performance liquid chromatography (HPLC) analysis is commonly used for the quantitative determination of active compounds in crude drugs. The use of this method depends upon the availability of standard compounds in order to identify the peaks in the chromatograms and to construct a calibration curve; however, a natural standard compound of appropriate purity is difficult to obtain.

In recent years, quantitative analysis using NMR spectrometry has been developed [9, 10]. In a previous paper, we reported the quantitative determination of components in crude drugs by proton-specific quantitative NMR (qHNMR) [11–14]. The qHNMR method has various advantages over other quantitative analysis methods, including HPLC. It requires neither reference compounds for establishing calibration curves nor sample pre-purification. This method also allows for the simultaneous determination of multiple constituents in a crude extract. Thus, qHNMR is a powerful alternative to other chromatographic methods for the chemical evaluation of crude drugs.

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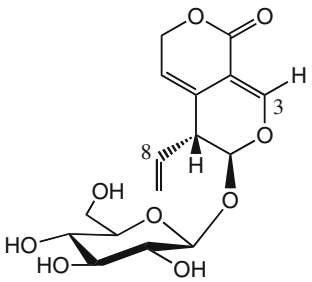

Fig. 1 The chemical structure of gentiopicroside

In this paper, we describe the application of qHNMR to quantitative determination of gentiopicroside content in Gentianae radix and Gentianae scabrae radix preparations (Fig. 1).

Materials and methods

Chemicals and crude drugs

HPLC-grade methanol was purchased from Wako Pure Chemicals (Osaka, Japan). HPLC-grade acetonitrile was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Methanol-d_4 (99.8 at. % D) and trifluoroacetic acid-*d* (TFA-*d*) (99.5 at. % D) were purchased from Isotec, Inc. (Miamisburg, OH, USA). Hexamethyldisilane (HMD, Sigma-Aldrich, Inc., St. Louis, MO, USA) was used as an internal standard for the NMR measurements. Potassium hydrogen phthalate (PHP; NMIJ CRM 3001-a) was purchased from Wako Pure Chemicals and used as a certified reference material (CRM). The purity of this compound is certified to be 100.00 ± 0.027 %. Gentianae radix and Gentianae scabrae radix samples were purchased from a market in Japan in 2012. The crude drugs were powdered using a Wonder Blender WB-1 (Osaka Chemical Co. Ltd.). The reagents were dried overnight in a desiccator under reduced pressure before they were weighed. Silica gel (BW-200, Fuji Silysia Chemical, Ltd., Kasugai, Japan) and ODS (Cosmosil 140C18-OPN, Nacalai Tesque, Inc.) were used for column chromatography. We used the AUW120D electric balance (Shimadzu) for weighing the reagent; capacity: max 120/42 g, readability: $d = 0.1$ mg/0.01 mg.

^{1}H-NMR spectrometry and parameters

The ^{1}H-NMR spectra were measured using a JEOL JMN-ECA500 (500 MHz) spectrometer. NMR acquisition and processing were performed essentially as described by Saito et al. [15]. For each sample, eight scans were recorded with a 90° pulse and a 60-s pulse delay, because high-precision qHNMR spectra can be obtained when the pulse angle is 90° and the pulse delay time is greater than five times the spin–lattice relaxation time (>5 × T_1). The beginning and end points of the peak areas that were integrated were manually selected. Chemical shifts are presented as values (ppm) relative to the HMD internal standard.

Isolation of gentiopicroside

Gentiopicroside was isolated from Gentianae scabra radix. Powdered Gentianae scabra radix (40.27 g) was sonicated with 100 ml methanol at room temperatures for 0.5 h. The extract was allowed to stand for 30 min before the supernatant was collected. The extraction procedure was repeated three times. The combined methanol extracts were concentrated by an evaporator under reduced pressure. The extract (13.43 g) was separated into six fractions by performing silica gel column chromatography and eluting with $CHCl_3$:MeOH:H_2O = 6:4:1. Fraction 3 (1.25 g) was purified by silica gel column chromatography ($CHCl_3$:MeOH = 20:1 → 6:1) and ODS column chromatography (H_2O:MeOH = 10:1 → 8:1) to yield gentiopicroside (698.8 mg).

Determination of HMD concentration in qHNMR reference stock solutions

HMD (20.0 or 5.0 mg) was dissolved in methanol-d_4 (10.0 ml) and used as a qHNMR-reference stock solution. An accurate measurement of the HMD concentration in the stock solution was determined using qHNMR, with PHP as an internal standard. PHP (10.0 mg) was weighed and dissolved in 2.0 ml of methanol-d_4. In the case of Gentianae radix, the PHP solution was added to 0.500 ml of the qHNMR reference stock solution, and 0.600 ml of the mixed solution was subjected to ^{1}H-NMR measurement. In the case of Gentianae scabra radix, the PHP solution was added to 0.500 ml of the qHNMR reference stock solution, and 0.600 ml of the mixed solution and TFA-*d* (20.0 μl) was subjected to ^{1}H-NMR measurement. The HMD concentration in the qHNMR reference stock solution was calculated by using the ratio of the signal integral at δ 0 ppm (HMD) to that at δ 7.50, 8.10 ppm (PHP in methanol-d_4) and δ 7.57, 7.72 ppm (PHP in methanol-d_4 + TFA-*d*). The concentration of HMD was calculated from Eq. (1):

$$C_{\mathrm{HMD}} = \frac{I_{\mathrm{HMD}}}{I_{\mathrm{PHP}}} \times C_{\mathrm{PHP}} \quad (1)$$

where C_{HMD} is the molar concentration of HMD in the qHNMR-reference stock solution, C_{PHP} is the molar concentration of PHP in the standard PHP solution, I_{HMD} is the signal intensity per proton at δ 0 ppm (HMD), and I_{PHP} is

the signal intensity per proton at δ 7.50, 8.10 ppm (PHP in methanol-d_4) or δ 7.57, 7.72 ppm (PHP in methanol-d_4 + TFA-*d*).

Quantitative determination of gentiopicroside in Gentianae radix by qHNMR

Powdered crude drug samples (150 mg) were sonicated in 1.00 ml methanol at room temperature for 30 min, followed by centrifugation for 5 min. The extraction procedure was repeated 3 times. The supernatants were combined and concentrated to dryness under vacuum. The residue was dissolved in 1.00 ml of the methanol-d_4 and was then added to 0.250 ml of the qHNMR-reference stock solution. 0.600 ml of the solution was then subjected to ^{1}H-NMR measurement. Gentiopicroside concentrations were calculated using Eq. (2):

$$C_{gp3} = \frac{I_{gp3}}{I_{HMD}} \times C_{HMD} \quad (2)$$

where C_{gp3} and C_{HMD} are the molar concentrations of the gentiopicroside and HMD, respectively, in the qHNMR-reference stock solution, I_{gp3} is the H-3 signal intensity per proton at about 7.44 ppm, and I_{HMD} is the signal intensity per proton at 0 ppm.

Quantitative determination of gentiopicroside in Gentianae scabrae radix by qHNMR

Powdered crude drug samples (150 mg) were sonicated in 1.00 ml methanol at room temperature for 30 min, followed by centrifugation for 5 min. The extraction procedure was repeated 3 times. The supernatants were combined and concentrated to dryness under vacuum. The residue was dissolved in 1.00 ml of the qHNMR-reference stock solution. The solution (0.600 ml) and TFA-*d* (20.0 μl) were then subjected to ^{1}H-NMR measurement of gentiopicroside. Gentiopicroside concentrations were calculated using Eq. (3):

$$C_{gp8} = \frac{I_{gp8}}{I_{HMD}} \times C_{HMD} \quad (3)$$

where C_{gp8} and C_{HMD} are the molar concentrations of the gentiopicroside and HMD, respectively, in the qHNMR-reference stock solution, I_{gp8} is the H-8 signal intensity per proton at about 5.78 ppm, and I_{HMD} is the signal intensity per proton at 0 ppm.

HPLC analysis of gentiopicroside in Gentianae radix and Gentianae scabrae radix

For HPLC determination of the gentiopicroside content of Gentianae radix and Gentianae scabrae radix, powdered crude drug samples (150 mg) were extracted in a similar fashion to the qHNMR samples. The methanol extracts were diluted with methanol to a final volume of 20.0 ml. Ten microliters of the resulting solution was subjected to HPLC analysis. The concentration of the standard solution was corrected on the basis of the purity of the reagent determined using qHNMR. Chromatographic separation was performed using a Capcell Pak C18 MG (4.6 × 150 mm; Shiseido Co., Ltd., Tokyo, Japan) HPLC cartridge. The mobile phase consisted of water–acetonitrile in a 9:1 mixture. The flow rate was 0.8 ml/min, and the elution was monitored at 274 nm [16].

Results and discussion

NMR analysis and the purity of gentiopicroside

Initially, we chose the H-3 signal as being suitable for qHNMR analysis, because this signal was fully separated from the other gentiopicroside peaks (Fig. 2). The H-3 signal of gentiopicroside in the methanol extract of Gentianae radix was detected independently. However, the H-3 signal of gentiopicroside overlapped with the signals from other Gentianae scabrae radix components. Therefore, we selected the H-8 gentiopicroside signal for qHNMR analysis of the methanol extract of Gentianae scabrae radix. This gentiopicroside signal was detected at 5.78 ppm and did not overlap with the other signals from other Gentianae scabrae radix components. Although the H-8 signal from Gentianae scabrae radix in methanol-d_4 overlapped with the foot of the H_2O signal, we found that the H_2O signal could be shifted downfield by the addition of TFA-*d* to the methanol-d_4 solution, to make the H-8 signal of gentiopicroside independent. From these results, the H-3 or H-8 signals were selected as target signals in the ^{1}H-NMR spectrum for the quantitative analysis of gentiopicroside.

In the present investigation, HMD was used instead of tetramethylsilane (TMS) as the internal standard in the ^{1}H-NMR measurement. We can accurately determine the HMD concentration in the reference stock solution by comparing the signal integration at δ 0 ppm with the methyl proton signals of PHP at δ 7.50, 8.10 ppm (in methanol-d_4) or δ 7.57, 7.72 ppm (in methanol-d_4 + TFA-*d*), because we have PHP standards, the purities of which are reliably confirmed. Thus, the qHNMR method afforded absolute quantification of HMD and gentiopicroside.

The ratio of the signal integration corresponding to the gentiopicroside concentration was proportional to that of HMD and in the range of 1.25–20.06 mg/ml (3-H in methanol-d_4) or 1.24–19.84 mg/ml (8-H in methanol-d_4 + TFA-*d*), as shown in Fig. 3. However, it should be noted that calibration curves are not necessary for the

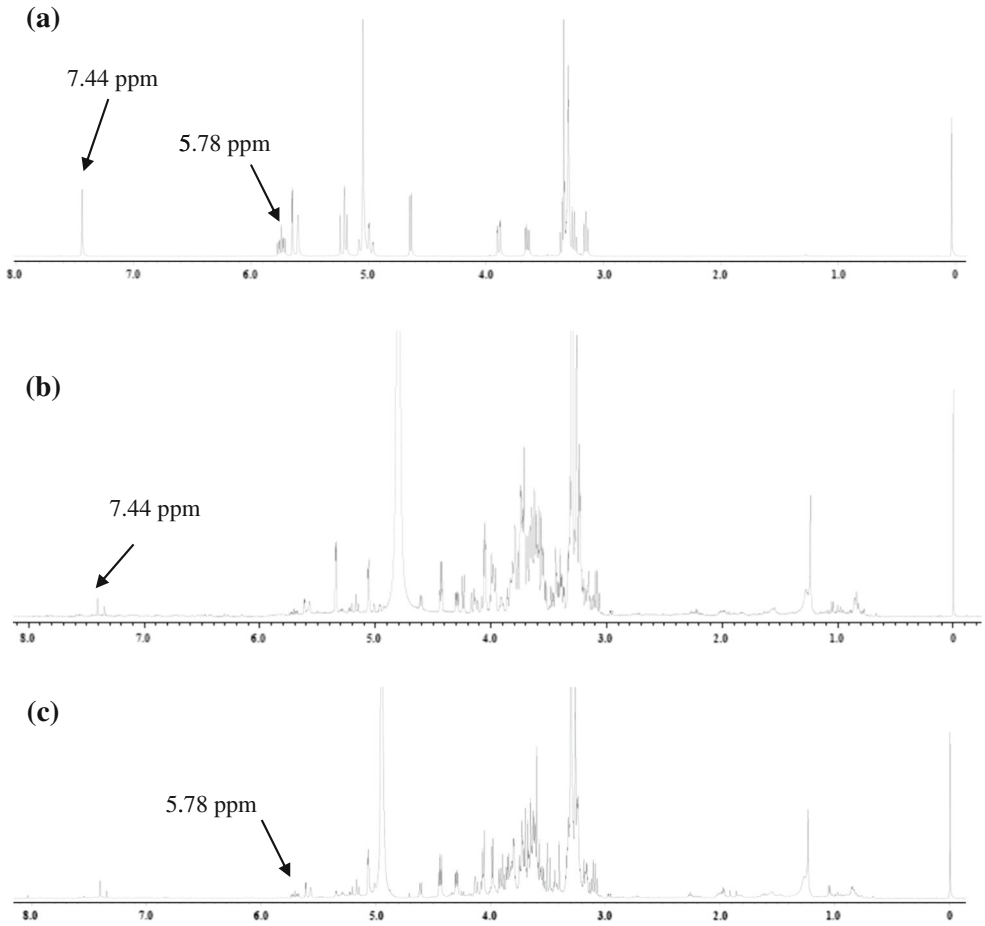

Fig. 2 The ^{1}H-NMR spectra of gentiopicroside and the crude drug extracts. **a** Gentiopicroside, **b** methanol extract of Gentianae radix, **c** methanol extract of Gentianae scabrae radix

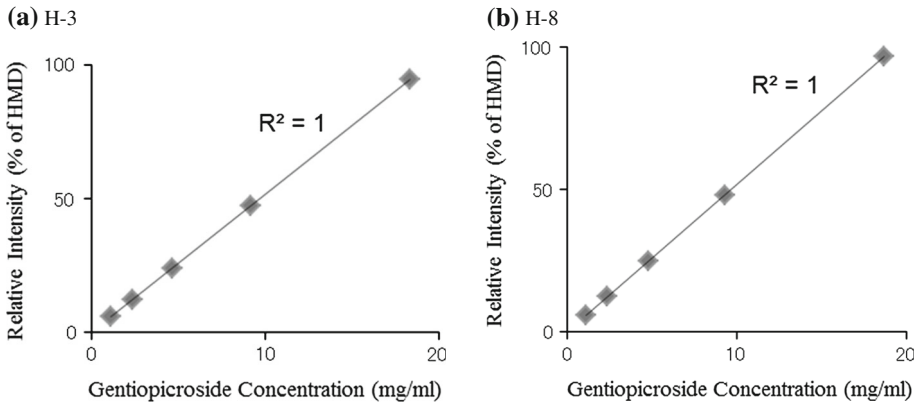

Fig. 3 The relationship between gentiopicroside concentration and the ratio of the integral of **a** the H-3 signal of gentiopicroside to that of the HMD signal in methanol-d_4, **b** the H-8 signal of gentiopicroside to that of the HMD signal in methanol-d_4 + TFA-*d*

quantitative determination of gentiopicroside, as the integration of a signal for one hydrogen atom is proportional to the amount of the test compound and is the same for all organic molecules. The quantification limit for gentiopicroside was 1.25 mg/ml (3-H in methanol-d_4) or 1.24 mg/ml (8-H in methanol-d_4 + TFA-*d*). To estimate the recovery rate of gentiopicroside by the qHNMR method, 5.0 mg gentiopicroside was spiked into 150 mg Gentianae radix and Gentianae scabrae radix, respectively. Three sets of the spiked samples and a blank extract (without additional gentiopicroside) were subjected to qHNMR analysis. The recovery rates in Gentianae radix or Gentianae scabrae radix were determined to be 92.7 and 99.1 %, respectively (Figs. 2 and 3).

Table 1 Gentiopicroside contents in Gentianae radix and Gentianae scabrae radix

Crude drug	Gentiopicroside content (%)	
	Determined by qHNMR	Determined by HPLC[a]
Gentianae radix		
A	1.76 ± 0.03	1.72 ± 0.03
B	2.17 ± 0.01	1.96 ± 0.04
Gentianae scabrae radix		
C	2.73 ± 0.18	2.59 ± 0.04
D	3.99 ± 0.01	3.95 ± 0.10

[a] Gentiopicroside isolated from Gentianae scabra radix was used as the standard material, the purity of which was determined by qHNMR

Determination of gentiopicroside contents in Gentianae radix and Gentianae scabrae radix by qHNMR

We used the qHNMR method to quantify gentiopicroside in the crude drugs. The gentiopicroside contents of Gentianae radix and Gentianae scabrae radix were estimated by comparing the intensities of the gentiopicroside H-3 or H-8 signal and the HMD signal (Fig. 2b). The gentiopicroside contents as determined by qHNMR were 1.76 and 2.17 % for 2 lots of Gentianae radix and 2.73 and 3.99 % for 2 lots of Gentianae scabrae radix (Table 1). We also determined the concentration of gentiopicroside in Gentianae radix and Gentianae scabrae radix by HPLC and corrected the results using the qHNMR-determined purity of the gentiopicroside reagents. As shown in Table 1, the gentiopicroside values determined using qHNMR were slightly higher than those determined using HPLC. This observation suggested that other peaks might overlap with the H-3 or H-8 gentiopicroside signals. Thus, ^{1}H-NMR spectra with 1000-times scans, and 2D COSY and HMQC spectra of methanol extracts of the crude drugs were measured in order to confirm the presence or absence of overlapping signals. Our results show that overlapping signals were not detected, and we confirmed the independence of the gentiopicroside H-3 or H-8 signals (Table 1).

In the future, the Japanese Pharmacopoeia may specify the required content of gentiopicroside in Gentianae radix and Gentianae scabrae radix. If so, the qHNMR method might be adopted for the determination of the purity of "standard" gentiopicroside reagents for HPLC analysis. This method may also be used to directly determine the gentiopicroside content in the crude drugs.

In conclusion, we determined the true purity of the gentiopicroside reagent and the gentiopicroside content in the crude drugs using a qHNMR method, and demonstrated that this method is sufficiently reliable for quality control of Gentianae radix and Gentianae scabrae radix. The further development of the qHNMR method for the quantitative analysis of crude drugs is now in progress.

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