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Purity assessment of ginsenoside Rg1 using quantitative ^{1}H nuclear magnetic resonance

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ABSTRACT

Ginseng herbs comprise a group of the most popular herbs, including *Panax ginseng*, *P. notoginseng* and *P. quinquefolius* (Family *Araliaceae*), which are used as traditional Chinese medicine (TCM) and are some of the best-selling natural products in the world. The accurate quantification of ginsenoside Rg1 is one of the major aspects of its quality control. However, the purity of the commercial Rg1 chemical reference substance (CRS) is often measured with high-performance chromatography coupled with an ultraviolet detector (HPLC–UV), which is a selective detector with unequal responses to different compounds; thus, this detector introduces probable error to purity assessments. In the present study, quantitative nuclear magnetic resonance (qNMR), due to its absolute quantification ability, was applied to accurately assess the purity of Rg1 CRS. Phenylmethyl phthalate was used as the internal standard (IS) to calibrate the purity of Rg1 CRS. The proton signal of Rg1 CRS in methanol-*d4* at 4.37 ppm was selected to avoid interfering signals, enabling accurate quantitative analysis. The relaxation delay, number of scans, and NMR windowing were optimized for data acquisition. For post-processing, the Lorentz/Gauss deconvolution method was employed to increase the signal accuracy by separating the impurities and noise in the integrated region of the quantitative proton. The method validation showed that the developed method has acceptable sensitivity, linearity, precision, and accuracy. The purity of the commercial Rg1 CRS examined with the method developed in this research was 90.34 ± 0.21%, which was obviously lower than that reported by the manufacturer (>98.0%, HPLC–UV). The cross-method validation shows that the commonly used HPLC–UV, HPLC–ELSD (evaporative light scattering detector) and even LC–MS (mass spectrometry) methods provide significantly higher purity values of Rg1 CRS compared with the qNMR method, and the accuracy of these LC-based methods largely depend on the amount of the sample that was loaded and the properties of the impurities.

1. Introduction

The term “ginseng herbs” commonly refers to three species of the genus *Panax* (Family Araliaceae), specifically *Panax ginseng*, *P. notoginseng* and *P. quinquefolius*. Over 80,000 tons per year of the three species is produced, which is worth more than 2 billion USD [1]. The growing popularity has resulted in a need for precise and accurate analytical methods for the quality control (QC) of ginseng herbs and related products. As one major active compound in ginseng herbs, ginsenoside Rg1 (Rg1), is reported to be effective in cancer prevention [2], neurodegenerative disorders [3], life-quality improvement [4], and cardiovascular disease [5]. Therefore, several countries and international organizations around the world have officially adopted Rg1 as the major quantitative marker compound for the QC of the ginseng herbs, including Chinese Pharmacopoeia (Edition 2015) [6], US Pharmacopoeia (USP 35) [7], Japanese Pharmacopoeia (JP XVI) [8], and CODEX STAN 295R-2009 Regional Standard for Ginseng Products [9].

The purity of a chemical reference substance (CRS) is crucial for the accurate quantification of Rg1 in ginseng herbs. Unfortunately, the purity of Rg1 CRSs purchased from commercial sources

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was found to be below the labeled value (98.0%). This situation is not rare for the CRS isolated from the herbs due to the technical difficulty of purifying the target compound from other homologous components with highly similar physicochemical properties. The low purity of several commercial CRSs has been reported in the recent literature, using quantitative nuclear magnetic resonance (qNMR) for calibration. The purity of daidzein [10], berberine hydrochloride [11], palmatine hydrochloride [11], tetrahydropalmatine [11], phosphoglycolate [12], and ginsenoside Rb1 CRS [13] was reported to range from 77.9% to 85.7%, significant lower than that reported by the manufacturers (>96.0%). The inaccurate purity value of the CRSs could cause error in the quantification results, and therefore the purity of these CRSs must be recalibrated with reliable method. In practice, the purity of CRS is commonly measured with high-performance liquid chromatography coupled with an ultraviolet detector (HPLC-UV). However, the UV detector is a selective detector and may not respond to some impurities that have no absorbance at the detected wavelengths and therefore would not give the correct purity of the CRS by using the area normalization method [14]. Other techniques, such as HPLC-ELSD (evaporative light scattering detector) and liquid chromatography coupled with mass spectrometry (LC–MS), have been reported for purity determinations of CRS, but their drawbacks, such as the nonlinear response in ELSD [15] and ionization efficiency in LC–MS [16], have greatly reduced the accuracy of these methods for purity determination. Differential scanning calorimetry (DSC) is an accurate method for purity determination; however, it is recommended to be applied only in the case of the CRS with high purity (>98%) [17] and is thus difficult apply to herbal medicine. Therefore, it is necessary to find a universal method that is suitable to accurately determine the purity of CRS that is isolated from plants.

^{1}H NMR is a universal technique to detect the protons in a compound. The fundamental principal of qNMR is that the intensity of the proton signal is directly proportional to the number of resonant nuclei (spins) [18]. By using qNMR, the accurate amount of an organic compound can be measured against the known amount of an internal standard (IS), which is usually a certified reference material (CRM) or substance corrected by the CRM. The purity of the target compound can then be accurately calculated by dividing the measured amount by its weight. Due to its high accuracy, qNMR is increasingly being applied in absolute quantification and was recently employed in the purity assessment of CRS isolated from plants [19,20]. Nevertheless, there are still some issues that may influence the accuracy of qNMR and should be overcome, such as selecting appropriate proton signals for quantification, eliminating interfering proton signals, and setting proper data-acquisition and post-processing parameters. In the current study, we aim to optimize the method of qNMR for quantifying the purity of Rg1 CRS and make use of phenylmethyl phthalate as the IS in the optimized method to measure the purity of Rg1 CRS.

2. Experimental

2.1. Chemicals and materials

Pyridine-d_5 (99.5%), methanol-d_4 (99.5%) and deuterium oxide (D_2O, 99.8%) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). HPLC-grade acetonitrile and formic acid were purchased from Sigma-Aldrich (Steinheim, Germany). The ultrapure water was prepared using a Millipore Milli-Q water purification system (Bedford, MA, USA). The potassium phthalate (purity: 100.00 ± 0.05%) was purchased from the Tianjin Chemical Agent Institute (Tianjin, China) and used as the CRM. Phenylmethyl phthalate was synthesized by Professor Shengyuan Xiao's group at the Beijing Institute of Technology (Beijing, P.R. China) using the CRM as precursor and then served as the IS. The commercial chemical reference substances of ginsenoside Rg1 (labeled HPLC purity >98%) and ginsenoside 20(S)-Rh2 (labeled HPLC purity >98%) were purchased from Shanghai Winherb Medical Technology Co, Ltd. (Shanghai, China). The chemical structures of the CRM, IS, Rg1, and 20(S)-Rh2 CRS are shown in Fig. 1. These chemicals were dried at 45 °C at 0.1 Mpa for 24 h before use.

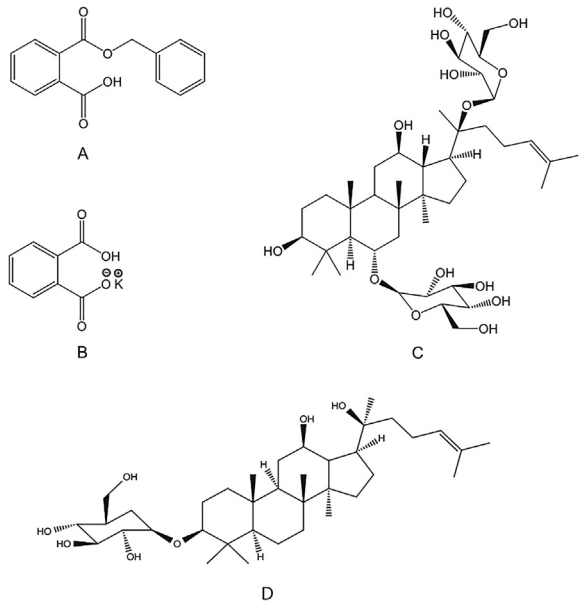

Fig. 1. Chemical structures of phenylmethyl phthalate (A), potassium phthalate (B), ginsenoside Rg1 (C), and ginsenoside 20(S)-Rh2 (D). Phenylmethyl phthalate was used as the internal standard (IS) to calibrate the purity of the commercial chemical reference substance (CRS) of ginsenoside Rg1. Phenylmethyl phthalate was synthesized using potassium phthalate, a certified reference material (CRM), and calibrated against potassium phthalate for purity. The phenylmethyl phthalate was designed to have a better solubility than the potassium phthalate in methonal-*d4* and then served as the internal standard for the purity assessment of Rg1 CRS. The 20(S)-Rh2 was used as a high purity analyte to cross-validate the purity assessment method established for ginsenoside Rg1.

2.2. ^{1}H NMR analysis

NMR data was obtained using a 5 mm CPTCI CryoProbe and recorded at 298 K (25 °C) on a Bruker Ascend 600 Spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) operating at a frequency of 600.13 MHz. All samples were analyzed by using a zg30 (flip angle 30°) pulse sequence (pulse length = 9.60 μsec), and 64 K data points were collected (corresponding to an acquisition time of 3.89 s at a spectral width of 8417 Hz) with a transmitter frequency (O1) = 6.17 ppm, receiver gain (RG) = 32, number of scans (NS) = 16, and relaxation delay (D1) = 3 s. Each sample was placed in the magnetic field at the same depth. The probe was tuned and matched to the specific frequency using the automatic tuning method (ATMA) from the Bruker BioSpin Corporation. The samples were field-frequency locked using the ^{2}H signal in the solvent. The automatic shimming method TopShim from the Bruker BioSpin Corporation was used. All the chemical shifts of proton signals in sample spectra were referenced to the solvent signal.

The spectra were processed using the Bruker TopSpin 3.1 software with standard parameter sets. For the quantification analysis, methanol-*d4* served as the field-frequency lock, and all the spectra were referenced to the signal from methanol-*d4* at 3.33 ppm. An exponential window function (line broadening) of 0.3 Hz and zero filling of 64 K data points were applied prior to Fourier Transformation of the free-induction delay (FID) data. Phase adjustment (APK)