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Quantitative determination of six major triterpenoids in *Ganoderma lucidum* and related species by high performance liquid chromatography

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Abstract

A reversed-phase liquid chromatographic method was developed for the quantitative determination of six triterpenoids, namely ganoderic acids C_2, B, AM_1, K, H and D in *Ganoderma lucidum* and its related species. Samples were extracted with chloroform in ultrasonic bath. The optimal conditions of separation and detection were achieved on an Agilent Zorbax SB-C_{18} column (250 mm × 4.6 mm, 5 μm), with a linear gradient of acetonitrile and 0.03% aqueous phosphoric acid (v/v), at a flow rate of 1.0 ml/min, detected at 252 nm. All calibration curves showed good linearity ($r^2 > 0.999$) within test ranges. The relative deviation of this method was less than 2% for intra- and inter-day assays, and the percentage recovery of the method was 93–103%, with relative standard deviation (R.S.D.) less than 5%. The current assay method was applied to quantitative determination of constituents of triterpenoids in 36 different samples of *G. lucidum* and its related species. The results indicated that the developed method could be readily utilized as a quality control method for *G. lucidum* and related species.

Keywords: *Ganoderma lucidum*; Triterpenoids; Reversed-phase high performance liquid chromatography

1. Introduction

Ganoderma lucidum (Leyss. ex Fr.) Karst (Polyporaceae), commonly called "Lingzhi" in China, is a well-known crude drug which has long been used in traditional Chinese medicine for the promotion of longevity and maintenance of vitality. In the 16th century, Lingzhi was cited in *Compendium of Materia Medica* (compiled by Li Shi-Zhen in Ming Dynasty) for enhancing "vital energy", increasing "intellectual capacity" and promoting "longevity", and this "mushroom of longevity" has been deemed as the sacred herb in China. Nowadays, it is still widely prescribed by traditional Chinese medical doctors for the treatment of debility and weakness, insomnia, hepatitis, cardiovascular diseases, cancer, etc. [1–4].

During the past two decades, more than 130 triterpenoids (including ganoderic acid derivatives) have been isolated from the fruiting bodies, cultured mycelia and spores of the *Ganoderma* [5,6]. Triterpenoids have received considerable attention owing to their conspicuous pharmacological activities. Some of these compounds showed anti-HIV-1 (ganoderic acids A, B, H, C_1) [7,8], antihistamine (ganoderic acids C_2, D) [9], antinociceptive (ganoderic acids A, B) [10], anticholesterol (ganoderic acids B, C_2) [11], and inhibitory activity of angiotensin converting enzyme (ganoderic acids K, F, S) [12]. Hence, the triterpenoids could be considered as the 'marker compounds' for the chemical evaluation or standardization of *G. lucidum*.

Owing to its satisfactory clinical effect, *Ganoderma* has been widely used as the major component of healthy foods and drugs for the time being in China. Therefore, development of quality control methods for *G. lucidum* and its related preparations is an essential issue for the effective clinical use of this medicinal herb. However, the previous studies on the quantitative analysis

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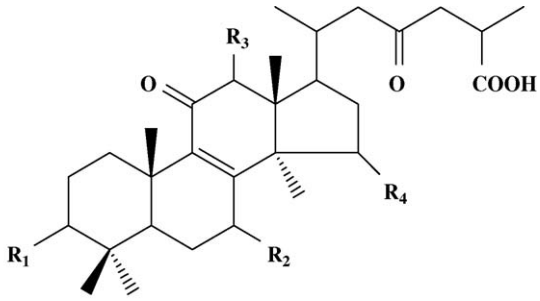

Ganoderic acid C_2 (1): R_1=R_2=β-OH, R_3=H, R_4=α-OH

Ganoderic acid B (2): R_1=R_2=β-OH, R_3=H, R_4=O

Ganoderic acid AM_1 (3): R_1=β-OH, R_2=R_4=O, R_3=H

Ganoderic acid K (4): R_1=R_2=β-OH, R_3= β-OAC, R_4=O

Ganoderic acid H (5): R_1=β-OH, R_2=R_4=O, R_3= β-OAC

Ganoderic acid D (6): R_1=R_4=O, R_2=β-OH, R_3=H

Fig. 1. Structures of six triterpenoids in *G. lucidum*.

of multiple triterpenoids in *G. lucidum* are insufficient [13–18] due to the difficulty to obtain the standard compounds. Furthermore, the authentication of commercial samples of *G. lucidum* was generally carried out by applying classical procedure performed by thin layer chromatography (TLC) [19]. In this study, a RP-HPLC method was developed for the simultaneous determination of six triterpenoids (shown in Fig. 1) with simple sample pretreatment methods. The developed method was successfully applied to the quantification of six major triterpenoids in 36 *Ganoderma* samples.

2. Experimental

2.1. Chemicals and materials

Acetonitrile and phosphoric acid were of HPLC grade (Burdick & Jackson, Honeywell International Inc., USA). HPLC grade water was prepared using a Milli-Q Water purification system (Millipore, MA, USA). The samples of *G. lucidum*, the major component of anticancer TCM formula SunRecome®, provided by Green Valley Pharmaceutical Co., China. And its related species were purchased from drug stores in different provinces of China.

The reference standards of triterpenoids **1**–**6** were isolated from the fruit bodies of *G. lucidum*. The dried fruit bodies (10 kg) were pulverized and extracted three times with 95% ethanol boiling in an immersion heater. The extract was concentrated under reduced pressure to yield residue (113 g), which was then suspended in hot H_2O and extracted with dichloromethane. The dichloromethane layer was concentrated to about 1/10 of its original volume and extracted with sat. aq. $NaHCO_3$ and the extract was acidified to pH 3–4 with 6 mol/l HCl at 0 °C. The resulting precipitate was dissolved in dichloromethane and then evaporated in vacuo to yield a residue (28 g), which was subjected to CC separation over silica gel (300 g) and eluted with a gradient of $CHCl_3$–MeOH (98:2–90:10, v/v) to afford 10 fractions (Fr. 1–10). Fr. 2 was further separated on silica gel column and eluted with $CHCl_3$–MeOH (95:5, v/v) to give three fractions (sFr. 1–3). Ganoderic acid D (17.3 mg) was deposited from sFr. 1 and recrystallized from acetone. sFr. 2 was further separated by preparative TLC (silica GF_{254}, 10–40 μm) with hexane–EtOAc–acetic acid (20:80:0.5) and two mixtures (M1 and M2) was obtained. M1 was separated by preparative HPLC and eluted with MeOH–0.5% HOAc (56:44, v/v) to give ganoderic H (38.9 mg) and ganoderic K (21.6 mg). M2 was subjected to preparative HPLC and eluted with MeOH–0.5% HOAc (53:47, v/v) to yielded ganoderic acid AM_1 (20.4 mg) and ganoderic acid B (24.1 mg). Ganoderic acid C_2 (41.8 mg) was deposited from sFr. 3 and recrystallized from MeOH. All these compounds were identified by direct comparison of their spectral data (UV, IR, NMR and MS) with those reported in the literature [12,20–24] and their purities were >98% determined by HPLC/UV analysis.

2.2. Apparatus and chromatographic conditions

An Agilent 1100 liquid chromatography system, equipped with a quaternary solvent deliver system, an autosampler and DAD detector, was used. A Zorbax SB-C_{18} column (250 mm × 4.6 mm, 5 μm) connected with a Zorbax SB-C_{18} guard column (20 mm × 4 mm, 5 μm) at temperature of 35 °C was applied for all analyses. Detection wavelength was set at 252 nm. The mobile phase consisted of (A) acetonitrile and (B) 0.03% apueous phosphoric acid (v/v) using a gradient elution of 30–32% A at 0–40 min, 32–40% A at 40–60 min. The flow rate was 1.0 ml/min and aliquots of 10 μl were injected.

2.3. Method validation

The method was validated for parameters such as linearity, precision, accuracy and stability following the International Conference on Harmonization (ICH) guideline [25].

2.3.1. Calibration curves

The mixture stock solution of ganoderic acids C_2 (**1**), B (**2**), AM_1 (**3**), K (**4**), H (**5**) and D (**6**) was prepared by dissolving the reference substances in methanol to final concentration of 510 μg/ml for ganoderic acid C_2, 555 μg/ml for ganoderic acid B, 540 μg/ml for ganoderic acid AM_1, 525 μg/ml for ganoderic acid K, 2010 μg/ml for ganoderic acid H and 675 μg/ml for ganoderic acid D, respectively, then diluted the mixture stock solution to appropriate concentration ranges for establishment of calibration curves. Linearity of each compound was determined with three injections for each concentration and plotted using linear regression of the mean peak area versus concentration.

2.3.2. Precision

The measurement of intra- and inter-day variability was utilized to determine the repeatability of the developed assay