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Sensitive and selective liquid chromatography–tandem mass spectrometry method for the determination of five ganoderic acids in *Ganoderma lucidum* and its related species

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ABSTRACT

The present paper describes a novel, sensitive and selective liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the simultaneous analysis of ganoderic acids C_2, B, A, H, D in *Ganoderma lucidum* and its related species. *Ganoderma* samples were prepared using simple ultrasonic extraction. Chromatographic separation was carried out on an Agilent Zorbax XDB C_{18} column (250 mm × 4.6 mm i.d., 5 μm) with an isocratic mobile phase consisting of acetonitrile, water and formic acid (42:58:0.5, v/v/v). Mass spectrometric detection was achieved by a triple-quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) interface operating in negative and positive ionization mode via a single within-run polarity switching. Quantitation of five ganoderic acids was performed using selective reaction monitoring (SRM) mode. The intra- and inter-day precision was less than 6.2% and the accuracy ranged from 90.0% to 105.7%. The limit of quantification (LOQ) was 20.0–40.0 ng/mL and the limit of detection (LOD) was 3.0–25.0 ng/mL. With this method, low levels of ganoderic acids in the fruiting bodies of *Ganoderma sinense* and *Ganoderma applanatum* were accurately quantified for the first time. Importantly, the method allows unequivocal quantification of the five ganoderic acids in the spores and fruiting bodies of *Ganoderma lucidum*, whereas the previously published methods have lacked the capability. The method presented will be a powerful tool for quality control of *Ganoderma lucidum* and its related species.

1. Introduction

Ganoderma lucidum, also known by the common name "Lingzhi" in Chinese, is a basidiomycete fungus which has been used as a folk remedy for the promotion of health in the orient. It was deemed as an elixir of life for thousands of years and now is widely revered as a valuable health supplement and "herbal" medicine worldwide. *G. lucidum* has been used for the prevention and treatment of a wide range of diseases, such as bronchitis, asthma, hypercholesterolaemia, hypertension, neurasthenia, hepatitis, leucopenia and cancer [1,2]. Modern research has revealed that triterpenes (mostly lanostane-type triterpenes) are one of the most important bioactive constituents of *G. lucidum* [3,4]. Triterpenes, such as ganoderic acids and alcohols, have been reported to possess diverse and potentially significant pharmacological activities, such as anti-tumor [5,6], anti-HIV-1 [7,8], hepatoprotection [9], anti-oxidation [10], anti-hypertension [11], cholesterol reduction [12], as well as inhibiting platelet aggregation [13]. These biologically active compounds have been considered as indices for estimation of quality of *G. lucidum* and its related species.

At present, high-performance liquid chromatography with UV detection (HPLC-UV) is the most widely used method for the simultaneous analysis of multiple triterpene constituents in *G. lucidum* and its related species [8,14–20]. Due to the lack of specificity of UV detection, accurate quantification of triterpenes often depends on definitive resolution of the analytes of interest from other sample components. However, the presence of a larger number of triterpenes with very similar chemical structure in *G. lucidum* usually resulted in severe peak overlap in chromatography, even if complex gradient systems with long run times were employed [16–23]. Therefore, HPLC-UV methods for triterpene analysis are prone to give erroneous results caused by concomitant determination of co-eluting compounds. In addition, the limits of quantification in previous methods were insufficient for the accurate determination of low levels of triterpenes present in some *Ganoderma* species that are commonly known to possess medicinal/nutritional values such as *G. applanatum* and *G. sinense* [17,18]. Therefore, a need existed

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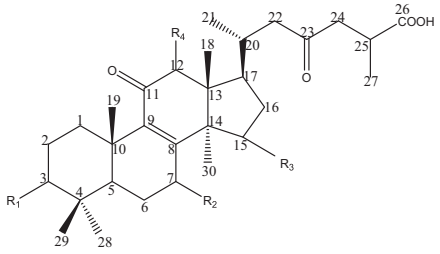
for a more reliable, sensitive and selective method for quantitative analysis of tritepenes in *G. lucidum* and its related species.

Liquid chromatography coupled with mass spectrometry has become the method of choice for both identification and quantification of analytes in complex samples owing to its high sensitivity and selectivity. Several qualitative methods have been published for the identification of triterpenes in crude extracts of *G. lucidum* by liquid chromatography–electrospray ionization–ion trap mass spectrometry [20,23–26]. To the best of our knowledge, quantitative analysis of tritepenes in *G. lucidum* using liquid chromatography coupled with mass spectrometry has not been reported. The present study describes a sensitive and selective method based on liquid chromatography–atmospheric pressure chemical ionization–triple-quadrupole mass spectrometry for simultaneous analysis of five ganoderic acids in *G. lucidum* and its related species. The superiority of the developed LC–MS/MS method over the previous HPLC-UV methods is demonstrated and emphasized for the first time.

2. Experimental

2.1. Chemicals and materials

Reference standards including ganoderic acid C_2, ganoderic acid B, ganoderic acid A, ganoderic acid H and ganoderic acid D were isolated from the fruiting bodies of *G. lucidium* in the Laboratory of Natural Products Chemistry, Shenyang Pharmaceutical University, Shenyang, China. The chemical structures of these ganoderic acids (shown in Fig. 1) were confirmed based on their UV, MS, ^{1}H NMR and ^{13}C NMR data [27] and by comparison of their spectral data with those reported previously in the literature. On the basis of UV, MS, NMR and HPLC, all reference compounds are considered to have a purity of 98% or more. Hydrocortisone was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and was used as an internal standard (I.S.). HPLC-grade acetonitrile and formic acid were purchased from Concord Corporation (Tianjin, China). All other reagents were of analytical grade. Doubly distilled water was used throughout the study.



Compound	R_1	R_2	R_3	R_4
Ganoderic acid C_2	β-OH	β-OH	α-OH	H
Ganoderic acid B	β-OH	β-OH	=O	H
Ganoderic acid A	=O	β-OH	α-OH	H
Ganoderic acid H	β-OH	=O	=O	β-OAc
Ganoderic acid D	=O	β-OH	=O	H

Hydrocortisone

Fig. 1. Chemical structures of five ganoderic acids and hydrocortisone (I.S.).

The fruiting bodies of *G. lucidum*, *G. sinense*, *G. applanatum* and whole spores of *G. lucidum* were collected from Beijing, Hebei, Jilin and Anhui provinces of China. The voucher specimens of these samples were identified by Professor Qishi Sun, School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, Shenyang, China.

2.2. Standard solutions

A mixed stock solution containing five reference standards was prepared in methanol. A series of working standard solutions were prepared by successive dilution of the mixed stock solution with methanol. Calibration standards were prepared by mixing each working standard solution with a 300 ng/mL I.S. working solution in the ratio of 1:1 to give final concentrations ranging from 20.0 to 2000 ng/mL for ganoderic acids C_2, B and A, from 25.0 to 2500 ng/mL for ganoderic acid D, and from 40.0 to 4000 ng/mL for ganoderic acid H. All the stock and working solutions were stored at 4 °C until use.

2.3. Sample preparation

Previous method reported by Wang et al. [17] was modified and used for extraction of ganoderic acids from different *Ganoderma* samples. A 1 g of sample powder of *G. sinense* or *G. applanatum* was extracted with 20 mL of chloroform in an ultrasonic water bath for 30 min. The extraction process was repeated twice. The extracts were filtered, combined and evaporated to dryness under reduced pressure at 40 °C. The resulting residue was dissolved and made up to 25 mL with methanol. A 2 mL aliquot of the extract was filtered through a 0.2 μm syringe filter and then mixed with the I.S. working solution in the ratio of 1:1 prior to analysis.

A 1 g of sample powder of *G. lucidum* was extracted as described above. A 2 mL aliquot of the extract was filtered through a 0.2 μm syringe filter and then diluted 10-fold with methanol before being mixed with the I.S. working solution in the ratio of 1:1 in order to achieve responses within the linear range of the calibration curve.

2.4. LC–MS/MS analysis

Chromatographic separation was carried out using a Shimadzu liquid chromatography system (Kyoto, Japan), consisting of a LC-10ADvp pump and a Shimadzu SIL-HT_A autosampler. An Agilent Zorbax XDB C_{18} column (250 mm × 4.6 mm i.d., 5 μm) and a C_{18} guard column (8 mm × 4 mm i.d., 5 μm) were used. The column temperature was held at 20 °C. The mobile phase consisted of acetonitrile, water and formic acid (42:58:0.5, v/v/v) and pumped isocratically at a flow rate of 0.5 mL/min. The injection volume was 20 μL.

Mass spectrometric analyses were performed on a Thermo Finnigan TSQ Quantum Ultra triple-quadrupole mass spectrometer (San Jose, CA, USA) equipped with an atmospheric pressure chemical ionization (APCI) interface in negative ionization mode for the first 20 min and then positive ionization mode for the rest of the total run. The corona discharge current was set at ±4 μA. The capillary temperature was maintained at 270 °C and the vaporizer temperature at 450 °C. Nitrogen was used as the sheath gas (35 Arb) and auxiliary gas (5 Arb) for nebulization and desolvation. Argon was used as the collision gas (1.0 mTorr) for collision-induced dissociation (CID). Quantitation was performed using selected reaction monitoring (SRM) mode. Both Q1 and Q3 were operated at unit res-

olution. Three time segments were set with MS/MS transitions of m/z 517 → 287 (ganoderic acid C_2), m/z 361 → 331 (I.S.) in segment 1 (0–11 min), m/z 515 → 249 (ganoderic acid B), m/z 515 → 300 (ganoderic acid A), m/z 571 → 467 (ganoderic acid H) in segment 2 (11–20 min) and m/z 497 → 237 (ganoderic acid D) in segment 3 (20–25 min). The collision energies for ganoderic acid C_2, B, A, H, D and I.S. were 35, 35, 35, 25, 35 and 15 eV, respectively. Data acquisition and processing was performed using Xcalibur 1.4 data system and LCquan 2.0 quantitation software.

3. Results and discussion

3.1. Method development

3.1.1. Optimization of HPLC conditions

The optimization of chromatographic conditions was mainly guided by the requirement for assuring assay specificity and reducing the analytical run time. The mobile phase components used in the previous HPLC methods were modified to be compatible with the LC–MS/MS system. Phosphoric acid (or acetic acid) was replaced by the more volatile formic acid. Isocratic elution was used in the present study because more stable mass spectral signals and reproducible results were obtained by isocratic elution than by gradient elution. Special emphasis was focused on the optimization of baseline separation of ganoderic acids A, B and C_2 in order to avoid cross-talk effects. The optimal result was achieved by using acetonitrile, water and formic acid (42:58:0.5, v/v/v) as a mobile phase. No detection interferences between co-eluting ganoderic acids A and H and between ganoderic acid C_2 and the I.S. were found.

3.1.2. Optimization of MS parameters

Ionization of the five ganoderic acids was attempted with electrospray ionization (ESI) as well as atmospheric pressure chemical ionization (APCI) sources. The results revealed that APCI provided more stable mass spectral signal in combination with considerably lower baseline noise compared with ESI. Therefore, APCI was used for the study. It was found that ganoderic acids C_2, B and A gave strong responses in negative APCI mode, whereas ganoderic acid D showed high sensitivity in positive APCI mode. Ganoderic acid H and the I.S. responded in both negative and positive mode, but they gave the better results in positive mode. In order to increase the sensitivity of MS/MS detection, both negative and positive ionization mode were used in one analytical run. Since the switching delay (0.2–0.3 s) between positive and negative APCI mode would decrease the stability of simultaneous detection of co-eluting compounds (i.e. ganoderic acid A and H, ganoderic acid C_2 and the I.S.) via polarity switching, negative ionization of ganoderic acid H and the I.S. was finally adopted to be compatible with the negative ionization of ganoderic acid A and C_2. For ganoderic acid D, the sensitivity obtained in negative mode was not sufficient to track signals with low levels of ganoderic acid D in some samples. Therefore, a single within-run polarity switching was applied to detect ganoderic acid D in positive mode.

In negative APCI mode, ganoderic acids C_2, B, A, H and the I.S. produced abundant deprotonated molecule ions $[M-H]^-$ at m/z 517, m/z 515, m/z 515, m/z 571 and m/z 361, respectively. In the case of positive APCI mode, ganoderic acid D formed predominantly the ion at m/z 497, which could be ascribed to the loss of one molecule of water from the protonated molecule ion. The $[M+H]^+$ ion at m/z 515 was also observed with weak intensity. The most abundant ions ($[M-H]^-$ in negative mode and $[M+H-H_2O]^+$ in positive mode) were selected as the precursor ions and subsequently fragmented in MS/MS mode. In order to identify fragment ions suitable for performing SRM quantitative analysis, collision-induced dissociation (CID) MS/MS spectra were recorded at various collision energies. It was found that when the collision energy was relatively low, only the $[M-H-H_2O]^-$ and $[M-H-2H_2O]^-$ of ganoderic acids C_2, B, A and H can be seen. When the collision energy was above 20 eV, some product ion fragments resulted from the rearrangement and cleavage of the rings appeared. Considering the stability and specificity of the product ions, the ion transitions chosen for quantitative analysis were m/z 517 → 287 for ganoderic acid C_2, m/z 515 → 249 for ganoderic acid B, m/z 515 → 300 for ganoderic acid A, m/z 571 → 467 for ganoderic acid H, m/z 497 → 237 for ganoderic acid D and m/z 361 → 331 for the I.S. Final optimization of collision energies was performed using the automatic tuning tool in order to obtain maximum sensitivity of SRM quantitation. Fig. 2 displays the product ion mass spectra of five ganoderic acids and the I.S. at the optimum collision energies.

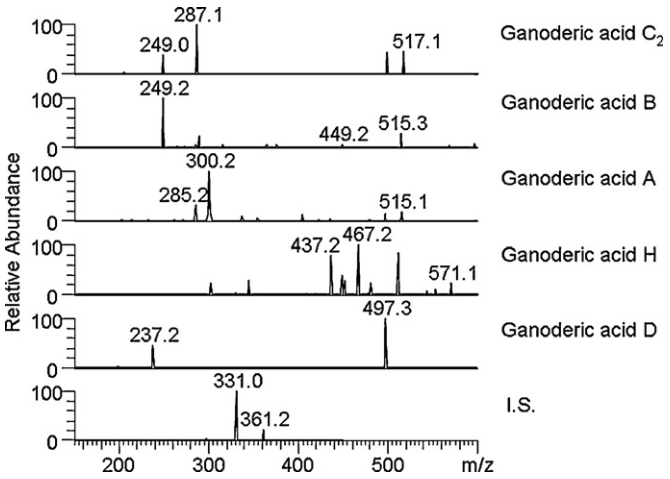


Fig. 2. Product ion mass spectra of five ganoderic acids and hydrocortisone (I.S.).

3.2. Method validation

3.2.1. Matrix effect

Matrix effect was determined using the method reported by Li et al. [28]. The matrix effect data ranged from 90.2 to 97.8% with RSDs of less than 4.3%. The results indicated that the co-eluting matrix components had little or no effect on the ionization of the analytes. In addition, no ionization suppression on the I.S. was observed.

3.2.2. Linearity, limits of detection and limits of quantification

The linearity of the method was studied by analyzing calibration standards in triplicate at six concentration levels. Calibration curves were conducted by weighted ($1/x^2$) least squares linear regression analysis of peak-area ratio (Y) of the analyte to I.S. versus the nominal concentration (X). The deviations of back-calculated concentrations from the nominal concentrations, expressed as percentage of the nominal concentration, were −2.3 to 5.5%. The correlation coefficient (r) was ≥0.999 for each calibration curve. The limit of quantification (LOQ) was defined as the lowest concentration point of calibration curve and was at least 10 times the noise level. The limit of detection (LOD) was defined as the lowest concentration of each analyte that could be recognized by the detector with a signal-to-noise ratio (S/N) of 3. Table 1 summarizes the linear regression data, LODs and LOQs of the five ganoderic acids.

3.2.3. Precision, accuracy and stability

The precision of the method was evaluated by six replicate analyses of the same *Ganoderma* sample over consecutive three days. The relative standard deviations (RSDs) of intra- and inter-day assay were 2.6–6.2% and 0.7–4.4%, respectively. The accuracy of

Table 1
Linear regression data, LODs and LOQs of five ganoderic acids.

Compound	Linear regression data			LOD (ng/mL)	LOQ (ng/mL)
	Linear range (ng/mL)	Regression equation	r^2		
Ganoderic acid C_2	20.0–2000	$Y = 4.834 \times 10^{-3}X + 1.366 \times 10^{-2}$	0.9990	5.0	20.0
Ganoderic acid B	20.0–2000	$Y = 2.657 \times 10^{-3}X + 2.779 \times 10^{-2}$	0.9993	5.0	20.0
Ganoderic acid A	20.0–2000	$Y = 2.232 \times 10^{-3}X + 1.092 \times 10^{-2}$	0.9998	3.0	20.0
Ganoderic acid H	40.0–4000	$Y = 5.032 \times 10^{-3}X + 7.342 \times 10^{-2}$	0.9992	25.0	40.0
Ganoderic acid D	25.0–2500	$Y = 6.836 \times 10^{-3}X + 3.729 \times 10^{-2}$	0.9996	10.0	25.0

the method was determined by analyzing the test samples spiked with three different levels of standards and calculating the percent recoveries of the analytes. Three replicate analyses were carried out at each spiked level. The original amounts of the analytes in the test samples were subtracted from the measured amounts of each spiked sample before calculating recoveries. The recoveries of the five ganoderic acids ranged from 90.0% to 105.7%. The stability of the analytes in the final extracts stored at room temperature was investigated by replicate injection of the sample solution at 0, 2, 4, 8, 12 and 24 h. The RSDs of the assay results at different time intervals were 1.6–3.5%, which indicated that the sample solution was stable at room temperature for at least 24 h.

3.3. Sample analysis

The validated analytical method was applied to 10 *Ganoderma* samples, including the fruiting bodies of *G. lucidum*, *G. sinense*, *G. applanatum* and the spores of *G. lucidum*. A representative SRM chromatogram of the extract of *G. Lucidum* is shown in Fig. 3. The quantitative results were summarized in Table 2. It can be seen that both the spores and fruiting bodies of *G. lucidum* were the best sources of the five ganoderic acids. For *G. sinense* and *G. applanatum*, the amount of each ganoderic acid ranged from 2.7 to 26.0 μg/g. It should be noted that previous HPLC methods failed to determine ganoderic acids at these levels due to the low sensitivity of UV detection. For example, Wang et al. [17] reported that *G. sinense* contained low levels of ganoderic acids, which were estimated mostly by extrapolation of standard curves below LOQ. No data about the amounts of ganoderic acids in *G. applanatum* were presented in the literature. In the present study, the five ganoderic acids in *G. sinense* and *G. applanatum* were not only detectable, but also measurable quantitatively by our LC–MS/MS method. The total contents of five ganoderic acids in these two species, as shown in Table 2, were about 10–40 times lower than those in *G. lucidum*. Since great variations in quantities of the major active ganoderic acids may lead to differences in usefulness and therapeutic efficacy of different *Ganoderma* species, further investigation is needed to determine whether *G. lucidum* and *G. sinense*, i.e. the only two official sources of Lingzhi recorded in Chinese Pharmacopoeia, are interchangeable with confidence in clinical practice needs.

As shown in Table 2, the contents of five ganoderic acids in both the spores and fruiting bodies of *G. lucidum* varied greatly from sample to sample, despite the same species of *Ganoderma*. Previously it was reported that the content of ganoderic acid H was significantly higher than those of the other ganoderic acids in *G. lucidum* [17]. In contrast, another study showed that ganoderic acid A was the most abundant ganoderic acid in *G. lucidum* [19]. Interestingly, the HPLC methods used in these two studies were similar and neither method involved the simultaneous analysis of ganoderic acids A and H. These findings indicate that the differing results in the two studies are probably due to the concomitant determination of co-eluting ganoderic acids A and H. To prove this speculation, we replicated two experiments under the same chromatographic conditions as described in the above two studies. It was found that ganoderic acids A and H appeared in an overlapping peak in both experiments (chromatograms not shown). It is clear that the co-elution resulted in significant overestimation of either ganoderic acid A or ganoderic acid H. In the present study, the high specificity offered by MS/MS running in SRM mode allows unequivocal quantification of co-eluting ganoderic acids A and H. The analytical results showed that neither ganoderic acid A nor ganoderic acid H was the dominant constituent in *G. lucidum*.

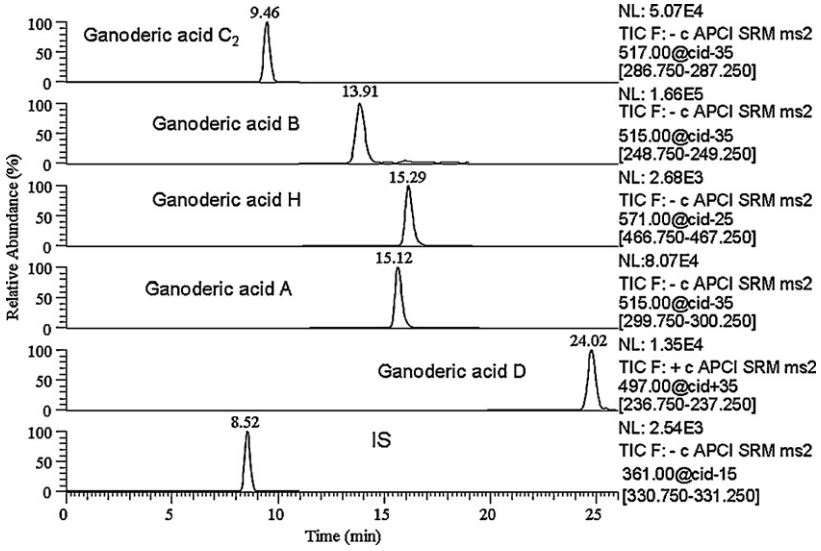


Fig. 3. SRM chromatogram of five ganoderic acids and the I.S. in an extract of *G. lucidum*.

Table 2
Contents of five ganoderic acids in different *Ganoderma* samples.

Sample no.[a]	Species	Origin	Content (μg/g)					
			Ganoderic acid C_2	Ganoderic acid B	Ganoderic acid A	Ganoderic acid H	Ganoderic acid D	Total
F1	*G. lucidum*	Jilin	72.2	576.0	987.2	569.6	729.0	2934.0
F2	*G. lucidum*	Jilin	36.1	130.7	57.7	327.3	639.0	1190.8
F3	*G. lucidum*	Hebei	77.2	628.6	948.7	204.1	1146.1	3004.7
F4	*G. lucidum*	Anhui	349.9	247.3	241.4	580.8	312.0	1731.4
F5	*G. sinense*	Hebei	2.7	18.6	20.0	10.9	26.0	78.2
F6	*G. applanatum*	Hebei	17.1	24.3	25.9	26.0	20.5	113.8
S1	*G. lucidum*	Beijing	38.1	278.6	346.8	215.1	257.4	1136.1
S2	*G. lucidum*	Hebei	34.5	33.4	53.7	320.2	70.3	512.1
S3	*G. lucidum*	Hebei	19.4	15.1	11.7	436.5	14.4	497.1
S4	*G. lucidum*	Beijing	11.3	95.9	173.4	489.2	587.1	1356.9

[a] F, fruiting body; S, spore.

4. Conclusions

A sensitive and selective LC–MS/MS method was developed and validated for simultaneous analysis of ganoderic acids C_2, B, A, H, D in *G. lucidum* and its related species for the first time. The method has the LOQs of 20.0–40.0 ng/mL and allows accurate quantification of low levels of ganoderic acids in *G. sinense* and *G. applanatum*. Moreover, unequivocal quantification of co-eluting ganoderic acids A and H in *Ganoderma* samples was achieved with the proposed method. An important finding was that previous HPLC-UV methods may suffer from concomitant determination of co-eluting ganoderic acids due to the lack of assay selectivity. The present work demonstrates the clear need for a sensitive and selective LC–MS/MS method for reliable determination of the analytes in highly complex samples.

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