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Short communication

Qualitative and quantitative analyses of nucleosides and nucleobases in *Ganoderma* spp. by HPLC–DAD-MS

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Abstract

A high-performance liquid chromatography–diode array detector-mass spectrometry (HPLC–DAD-MS) analytical method was developed for detection of the nucleosides and nucleobases in two species of Lingzhi, the dried sporophore of *Ganoderma lucidum* and *G. sinense*. The method, combining advantages of both DAD and MS, was successfully used to qualitatively identify for six nucleosides namely, adenosine, cytidine, guanosine, inosine, thymidine, uridine and five nucleobases namely, adenine, guanine, hypoxanthine, thymine and uracil in Lingzhi samples. Quantitative analyses showed that uridine was the most abundant nucleoside in these Lingzhi samples and the contents of nine target analytes were found to be different in pileus and stipes of the fruiting bodies and among the different species of *G.* spp. The established method might apply as an alternative approach for the quality assessment of Lingzhi.

Keywords: Nucleoside; Nucleobase; HPLC–DAD-MS; *Ganoderma lucidum*; *G. sinense*; Quantitative analysis

1. Introduction

Lingzhi, the dried sporophore of *Ganoderma lucidum* (Leyss.ex Fr.) Karst. or *G. sinense* Zhao, Xu et Zhang, is well-known as a health supplement in promoting longevity and have been widely used for a long time all over the world especially in the Orient. It is given the name of Lingzhi in China, Reishi in Japan, Yongzhi in Korea, Lucidium Lingzhi in America, etc. Lingzhi has traditionally been used to treat dizziness, insomnia, palpitation, shortness of breath, asthenic cough and asthma [1]. Pharmacological studies demonstrated that Lingzhi could lower blood pressure, blood sugar, inhibit platelet aggregation, enhance immune function, protect liver, and exhibit anticancer, antioxidant, anti-inflammatory and anti-HIV effects [2]. Chemical analyses revealed that Lingzhi contained a variety of pharmacologically active constituents including nucleosides [3,4]. It was reported that nucleosides and their bases exhibited various bioactivities. For instance, all purines can inhibit the activity of mononamine oxidase and show anti-oxidant effect [5]; nucleoside analogues have been used as anti-HIV drugs [6]. Furthermore, nucleosides and bases in Lingzhi were reported to be capable of inhibiting platelet aggregation and lowering the elevated serum aldolase level of experimental model mice [7]. Therefore, it is of interest to develop a method to determine the contents of these nucleosides and nucleobases in Lingzhi.

Several methods have been developed to analyze nucleosides and nucleobases in Chinese medicines including Lingzhi. For example, Li et al. determined nucleosides and their bases in *Cordyceps* by HPLC [8]; Jin et al. identified adenine, guanosine, uridine, cytidine and adenosine in Banlangen injection (a Chinese patent drug) by using HPLC–DAD-MS–MS [9]. Jiang detected the amounts of uracil, adenine and adenosine in Lingzhi product (Lingzhi capsule, a Chinese patent drug) by TLC [10]. However, there still exists a void for rapid and precise determination of nucleosides and bases in Lingzhi. In light of this, we employed HPLC–DAD-MS to analyze for most nucleosides and their bases in Lingzhi.

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In this study, LC–MS was used for the qualitative analysis of eleven nucleosides and nucleobases in the samples of *G. lucidum* and *G. sinense*. The analyzed compounds could be satisfactorily separated in their aqueous extracts. Furthermore, with the developed HPLC–DAD-MS method, nine nucleosides and nucleobases in Lingzhi samples could be simply and accurately quantified. This is the first report about the simultaneous determination of nucleosides and nucleobases in Lingzhi by HPLC–DAD-MS, which provide an alternative, feasible approach for the quality assessment of Lingzhi in addition to the methods using polysaccharides and terpenoids as the markers [1,11].

2. Experimental

2.1. Materials and reagents

HPLC grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany); ammonium acetate was obtained from Riedel-de Haën (AR, Seelze, Germany); uridine, uracil, thymidine, thymine, guanosine, hypoxanthine, cytidine, adenosine and inosine were obtained from Sigma (St. louis, MO, USA). Milli-Q water was prepared using a Grand III, Millipore, Milli-Q & Rios Systems (Millipore, Bedford, MA, USA).

Six cultivated Lingzhi samples, belong to *G. lucidum* and *G. sinense,* were collected from Guangxi, Zhejiang, Shandong, Sichuan and Xinjiang provinces of China. The samples were collected in the period of February 2004–March 2005.

2.2. Instrumentation

Quantitative analyses were performed on an Agilent 1100 series LC/MSD VL trap system (Agilent, USA) equipped with an autosampler, a high-voltage power supply, a diode array detector, an electrospray ionization (ESI) source and an ion trap analyzer. The signals from the mass detector were recorded and analyzed on LC/MSD trap software (version 4.2). An Agilent ZORBAX Eclipse XDB C18 column (3.5 μm, 4.6 mm × 150 mm) with a ZORBAX Eclipse XDB C8 guard column (5.0 μm, 3.9 mm × 20 mm) was used.

2.3. LC condition

The separation was achieved using gradient elution with 5 mM ammonium acetate solution and methanol: 0–5% methanol in 0–10 min; 5–20% methanol in 10–30 min. The flow rate was set at 0.5 ml min^{-1}. The column temperature was maintained at 25 °C. The DAD detection wavelength was set at 254 nm for acquiring chromatograms and quantitative analysis. An aliquot of 10 μl solution obtained from above was injected for HPLC analysis.

2.4. MS method

The MS spectra were acquired in positive ion mode. The capillary voltage was set at −4 kV. The full scan mass spectra were obtained from *m/z* 50 to 400. Nebulizer pressure was 30 psi. The flow rate of dry gas was maintained as 10 l min^{-1}. Dry gas temperature was 350 °C. Collision energy was set at 2 V.

2.5. Preparation of standard solutions for linearity studies

Calibration curves were established for quantitative analysis. Compound solutions were prepared in methanol–water (1:1): uracil, 199 μg ml^{-1}; cytidine, 199 μg ml^{-1}; hypoxanthine, 200 μg ml^{-1}; uridine, 400 μg ml^{-1}; thymine, 199 μg ml^{-1}; inosine, 204 μg ml^{-1}; guanosine, 195 μg ml^{-1}; thymidine, 204 μg ml^{-1}; adenosine, 201 μg ml^{-1}. All solutions were filtered through a 0.45 μm membrane filter and stored at 4 °C. After appropriate dilution, working solutions of various concentrations were freshly prepared. 200 μl of each standard solution were then transferred into a 2 ml volumetric flask and made up to the volume with 50% methanol.

2.6. Sample preparation

One gram of pulverized pileus or stipe of the fruiting body was accurately weighed. 15 ml Milli-Q water was added and then extracted for 45 min at room temperature using an ultrasonic processor (Transsonic 700/H). The supernatant was collected, concentrated to about 1 ml under a gentle stream of nitrogen and filtered through a 0.45 μm millipore filter. The solution was then ready for HPLC analysis.

3. Results and discussion

3.1. Qualitative analysis

Based on the retention time as well as the fragmentation patterns of molecular ions in positive mode, nine compounds were unequivocally identified as uracil (**1**), cytidine (**2**), hypoxanthine (**3**), uridine (**5**), thymine (**6**), inosine (**8**), guanosine (**9**), thymidine (**10**) and adenosine (**11**). Another two nucleobases were tentatively identified as guanine (**4**) and adenine (**7**) (see Fig. 1 for their structures) in the chromatograms of Lingzhi's fruiting body.

Nine compounds, their chemical standards being available, were unequivocally identified. In general, the molecular ions of these target compounds were exhibited as $[M+H]^+$, $[M+Na]^+$ and $[M+H\text{-ribose}]^+$. Fragments involving loss of $–CH_3$ and NH_3 were also observed in the MS–MS spectra. All the fragmentation patterns of these molecular ions in positive mode are summarized in Table 1. Moreover, these compounds were further identified based on the extracted ion chromatogram (EIC) of ions at *m/z* 112, 113, 127, 136 and 152. The EICs of the five selected ions and the corresponding HPLC chromatogram are shown in Fig. 2. The results indicated that EIC could be applied in the qualitative analysis of nucleosides and nucleobases in Lingzhi.

Owing to the unavailability of authentic standards, peaks 4 and 7 were tentatively assigned as guanine and adenine, respectively, by comparing with the reported HPLC and MS–MS data [9,12]. Their parent ions 152 and 136 *m/z* were observed in EIC, respectively (Fig. 2d). The product ions $[M+Na]^+$

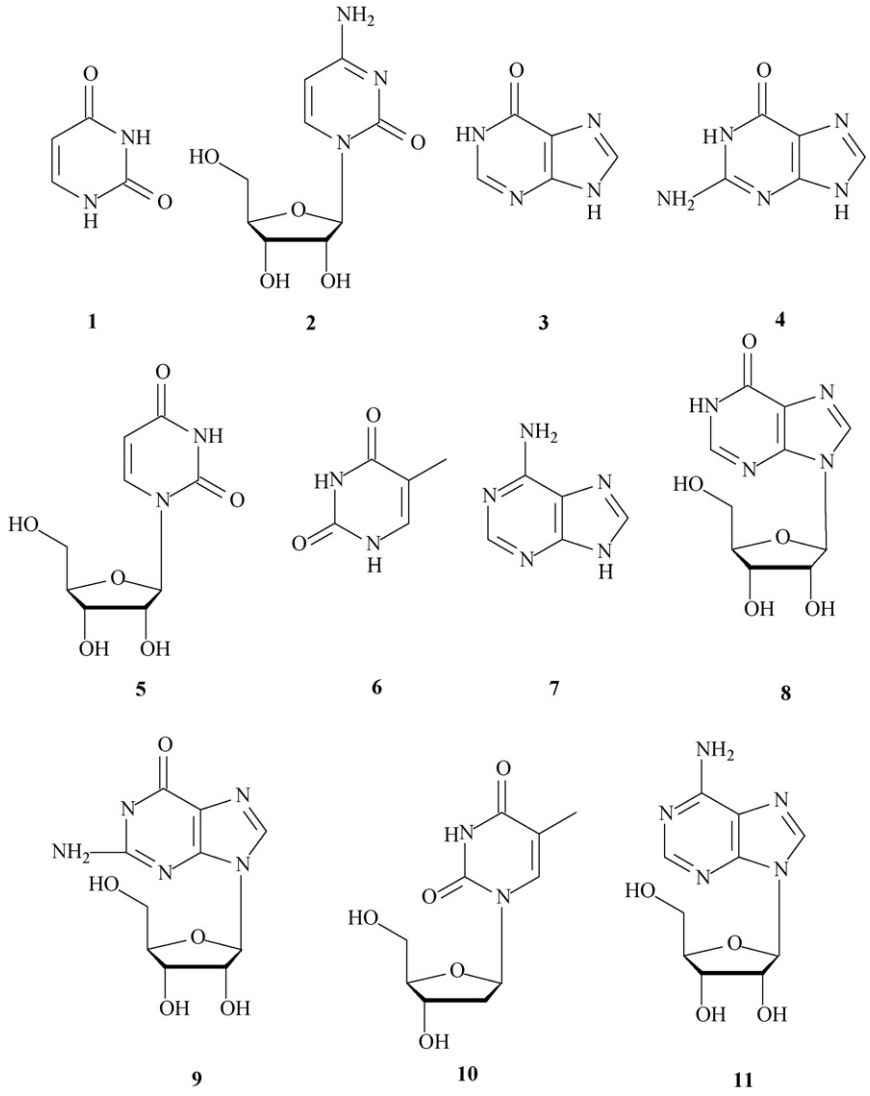

Fig. 1. Chemical structures of the nucleosides and nucleobases. **1**, Uracil; **2**, cytidine; **3**, hypoxanthine; **4**, guanine; **5** uridine; **6**, thymine; **7**, adenine; **8**, inosine; **9**, guanosine; **10**, thymidine and **11**, adenosine.

and $[M+H–NH_3]^+$ were also available in the MS–MS spectra (Table 1).

During the analysis, it was observed that the sensitivity of uracil and uridine in mass detection was fairly low. It was likely that the ESI ionization could not generate adequate ions in the interface for those compounds of interest [9,13,14]. In ESI-MS, analytes containing basic sites on the molecule, such as basic nitrogens, usually demonstrated higher sensitivity in slightly acidic conditions (pH <7). On the other hand, those without basic nitrogens generally produced a much lower response in positive

Table 1
Molecular mass, molecular ion and relative fragment ions of target compounds

Compound	t_R (min)	MW (*m/z*)	Molecular ion	Product ions
Uracil (**1**)	5.18	112.1	113 $[M+H]^+$	227 $[2M+H]^+$, 135 $[M+Na]^+$, 96 $[M+H\text{-}NH_3]^+$
Cytidine (**2**)	7.57	243.2	244 $[M+H]^+$	112 $[M+H\text{-ribose}]^+$, 266 $[M+Na]^+$, 282 $[M+K]^+$
Hypoxanthine (**3**)	8.06	136.1	137 $[M+H]^+$	266 $[2M+Na\text{-}NH_3]^+$, 109 $[M+H\text{-}28]^+$
Guanine (**4**)	8.62	151.1	152 $[M+H]^+$	174 $[M+Na]^+$
Uridine (**5**)	10.26	244.2	267 $[M+Na]^+$	113 $[M+H\text{-ribose}]^+$, 283 $[M+K]^+$, 135 $[M+Na\text{-ribose}]^+$
Thymine (**6**)	11.21	126.1	127 $[M+H]^+$	110 $[M+H\text{-}NH_3]^+$, 149 $[M+Na]^+$
Adenine (**7**)	14.86	135.1	136 $[M+H]^+$	119 $[M+H\text{-}NH_3]^+$
Inosine (**8**)	16.38	268.2	292 $[M+Na]^+$	139 $[M+H\text{-ribose}]^+$, 159 $[M+Na\text{-ribose}]^+$, 307 $[M+K]^+$
Guanosine (**9**)	17.28	283.3	306 $[M+Na]^+$	322 $[M+K]^+$, 174 $[M+Na\text{-ribose}]^+$, 152 $[M+H\text{-ribose}]^+$
Thymidine (**10**)	20.73	242.2	265 $[M+Na]^+$	127 $[M+H\text{-ribose}]^+$, 281 $[M+K]^+$, 149 $[M+Na\text{-ribose}]^+$
Adenosine (**11**)	25.14	267.2	268 $[M+H]^+$	136 $[M+H\text{-ribose}]^+$, 290 $[M+Na]^+$

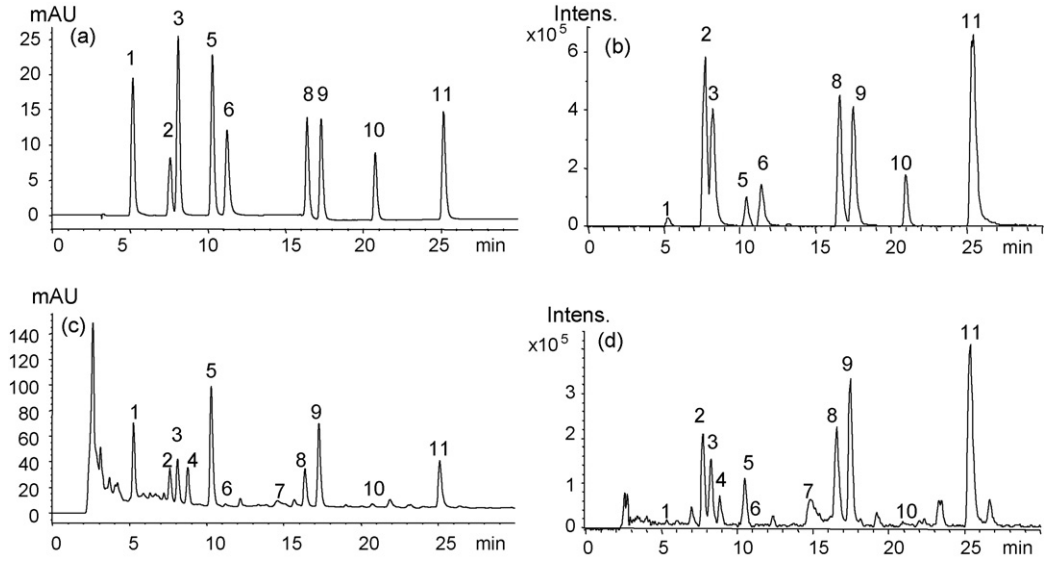

Fig. 2. DAD-UV chromatogram and extracted ion chromatogram (EIC) of standards and water extract of *G. lucidum* (sample L2). (a) DAD-UV chromatogram of standards. (b) EIC of standards. (c) DAD-UV chromatogram of *G. lucidum* water extract. (d) EIC of *G. lucidum* water extract. The ions at *m/z* 112, 113, 127, 136 and 152 were extracted in the EIC mode (width = 1 *m/z*). **1**, Uracil; **2**, cytidine; **3**, hypoxanthine; **4**, guanine; **5** uridine; **6**, thymine; **7**, adenine; **8**, inosine; **9**, guanosine; **10**, thymidine and **11**, adenosine.

ion mode. Among the list of compounds in the present study, the basicity of uracil was the lowest and therefore demonstrated the lowest response in ESI-MS detection.

3.2. Quantitative analysis

3.2.1. Linearity and calibration

Calibration curves of the target compounds were obtained as $Y = 4108.1X + 10.033$ for uracil, $Y = 1853.7X + 5.8387$ for cytidine, $Y = 4998.5X + 5.5428$ for hypoxanthine, $Y = 2495.3X - 20.910$ for uridine, $Y = 2931.7X - 3.1404$ for thymine, $Y = 2834.8X - 1.8817$ for inosine, $Y = 3106.1X - 12.067$ for guanosine, $Y = 1970.4X + 0.7181$ for thymidine and $Y = 3488.6X - 12.300$ for adenosine. Where Y and X were the value of peak area (mAU) and the concentration of standard solution ($\mu g\,ml^{-1}$), respectively. Linear regressions of these standard compounds showed good linearity in the range of 0.030–1.20 μg with correlation coefficient (R^2) ranged from 0.9994 to 0.9999. This allows the determination of these compounds over a wide range of concentrations. The contents of these compounds in the test samples were then calculated using the established regression equations.

3.2.2. Method validation

To test for the repeatability of above method, a sample powder was repeatedly analyzed. The relative standard deviations (R.S.D.s) of the contents of the nine compounds in repeatability tests were found within 0.97–4.26% ($n = 6$).

For stability investigation, the compound contents of a *G. lucidum* sample solution (sample L2) stored at room temperature were determined at 0, 2, 4, 8, 12 and 24 h after extraction. The results showed that the R.S.D.s of peak areas ranged from 0.38 to 3.85% ($n = 6$) and the R.S.D.s of retention time lied within 0.19–0.36% ($n = 6$). It indicated that the extract was stable within 24 h at room temperature.

To verify the method precision, standard mixtures of the nine compounds were analyzed with six replicates on the same day and then over three consecutive days. The R.S.D.s of the intraday assay were 0.10–0.58% ($n = 6$) while the R.S.D.s of the inter-day assay were 0.77–1.95% ($n = 3$).

The recoveries of the marker compounds were determined using spiked samples. Altogether seven trials were conducted. The recoveries were found within 93.83–102.04% ($n = 7$). All the above indicated that the performance of the established method was satisfactory.

The LOD and LOQ for each compound were determined at a signal-to-noise ratio (*S/N*) of about 3 and 10, respectively. LOD were found within the range of 0.1–0.3 $\mu g\,ml^{-1}$ while the LOQ were within 0.3–1.0 $\mu g\,ml^{-1}$.

3.2.3. Comparison of compound contents in the pileus and stipe of Lingzhi

Nowadays, both of the pileus and stipe of the fruiting body of Lingzhi are used for medicinal purposes. Previously it was reported that contents of triterpenes in the pileus and stipe were distinctive [15]. However, little is known about the contents of nucleosides and nucleobases in the two parts. Therefore, the contents of each target compound in both the pileus and stipe of Lingzhi were investigated (Table 2). The results revealed that the distribution contents between the two parts of Lingzhi were varied. In general, the pileus contains relatively higher content of target compounds than the stipe.

3.2.4. Comparison of target compounds in G. lucidum and G. sinense

G. lucidum and *G. sinense* are the two official sources of Lingzhi recorded in Chinese Pharmacopoeia and used in the

Table 2
Contents of nine nucleosides and nucleobases in different parts of six Lingzhi samples ($\mu g\ g^{-1}$)

Sample[a]	Part[b]	**1**	**2**	**3**	**5**	**6**	**8**	**9**	**10**	**11**	Total
L1	P	32.52	94.98	110.83	264.93	7.89	4.54	4.18	34.46	ND	554.31
	S	9.41	48.06	47.74	135.57	ND	13.47	18.63	19.26	4.11	326.23
L2	P	67.47	117.78	30.40	252.83	4.67	96.06	174.91	25.10	97.89	867.11
	S	22.69	43.11	13.64	117.14	ND	27.48	63.34	17.14	29.35	333.89
L3	P	7.38	39.19	52.77	185.22	5.81	ND	ND	9.60	3.85	303.81
	S	ND	ND	ND	ND	ND	ND	11.44	10.37	ND	21.81
L4	P	73.17	200.57	38.34	310.71	17.19	58.03	214.62	36.02	269.03	1217.68
	S	31.88	50.70	13.96	113.91	ND	16.66	39.51	27.57	17.34	311.52
S1	P	ND	3.04	1.33	67.06	ND	7.25	10.81	6.62	ND	96.11
	S	ND	8.67	2.54	61.49	ND	8.28	10.64	5.51	1.76	98.89
S2	P	62.96	15.04	25.37	118.43	11.88	17.04	33.62	ND	22.19	306.53
	S	37.99	6.22	12.80	102.65	3.62	1.83	17.21	ND	10.16	192.48

ND: not detected/below LOQ. **1**, Uracil; **2**, cytidine; **3**, hypoxanthine; **5** uridine; **6**, thymine; **8**, inosine; **9**, guanosine; **10**, thymidine; **11**, adenosine.
[a] L1, *G. lucidum* from Zhejiang; L2, *G. lucidum* from Shandong; L3, *G. lucidum* from Guangxi; L4, *G. lucidum* from Sichuan; S1, *G. sinense* from Xinjiang; S2, *G. sinense* from Sichuan.
[b] P: pileus; S: stipe.

treatment of similar diseases. Four samples of *G. lucidum* and two samples of *G. sinense* were qualitatively and/or quantitatively analyzed for the 11 compounds. The results showed that the amounts of the nucleosides and nucleobases in *G. lucidum* were always more abundant than those in *G. sinense*, in particular for cytidine (Table 2). This finding could be useful for tracing reliable sources of these compounds. Moreover, the observed trend could be elaborated as a means for differentiation among different Lingzhi species.

4. Conclusions

A HPLC–DAD-MS method for the qualitative and quantitative analyses of nucleosides and nucleobases in Lingzhi was established. The method is simple, sensitive and reliable. From the results, uridine was found to be the most abundant compound among the target analytes in the two species of Lingzhi. Although *G. lucidum* and *G. sinense* are now interchangeably used as Lingzhi for clinical purposes in China, the present results revealed that the former always contained more abundance of respective nucleosides and nucleobases than the latter. Despite the fact that little is known about the synergistic pharmacological activities of these compounds, the study provided valuable insight for the distribution and thus alternative supplying sources for these compounds. The distribution trend of the studied nucleosides and nucleobases could also be developed as a reliable means to differentiate *G. lucidum* and *G. sinense*, or even other members of ganoderma spp. in future studies. The developed HPLC–DAD-MS method in the present study could be employed as an alternative approach for quality assessment of Lingzhi using nucleosides and nucleobases as the markers.

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