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Original article

Rapid Analysis of Polar Components in *Ophiocordyceps sinensis* by Conventional Liquid Chromatography System

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ABSTRACT

Objective To develop a rapid high-performance liquid chromatography coupled with diode array detection (HPLC-DAD) method for the simultaneous determination of six polar compounds in *Ophiocordyceps sinensis*. **Methods** A poroshell SB Aq column (50 mm × 4.6 mm, 2.7 μm) and gradient elution were used; The detection wavelength of compounds was set at 260 nm. The chromatographic peaks of the six investigated compounds in sample were identified by comparing their retention times with reference compounds. **Results** All calibration curves showed good linearity ($r > 0.999$) within the tested ranges. The intra- and inter-day precisions of the six analytes were less than 0.8% and 2.1%, respectively, and the recoveries of the six analytes were between 95% and 103%. The validated method was successfully applied to the determination of six polar compounds in *O. sinensis* samples. **Conclusion** The poroshell SB Aq column is suitable for the rapid analysis of polar components in Chinese materia medica on conventional HPLC system and the developed HPLC method is also helpful to the quality control of *O. sinensis*.

Key words

adenosine; cordycepin; guanosine; high-performance liquid chromatography; inosine; *Ophiocordyceps sinensis*; polar compounds; uracil; uridine

1. Introduction

The analysis on Chinese materia medica (CMM) is a challenge for modern liquid chromatography (LC) technology, since there are a lot of compounds in CMM. The rapid analysis and separation of polar compounds are hot research areas in analysis of CMM (Liang et al, 2009; Toyo'oka, 2008). Usually, analytical time for CMM is long (more than 30 min) due to the complex compounds in it. Therefore, several new LC column techniques such as sub 2 μm column had been applied in the analysis of CMM (Toyo'oka, 2008). However, high backpressures of sub 2 μm column restrict their applications on conventional LC system. Recently, Poroshell column was proved as a rapid LC separation technology with low backpressures, and was applied in the quality control of CMM (Du et al, 2011; Deng et al, 2011). However, the previous reports were mainly focused on the analysis of less polar compounds by conventional reverse phase material Poroshell column (Du et al, 2011; Deng et al, 2011; Song et al, 2013), which is difficult to separate the polar compounds. Fortunately, some special reverse phase columns such as SB Aq column are ideal for the separation of polar components in CMM with high ratio of aqueous mobile phases (Qian et al, 2008; Yang et al, 2010).

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Ophiocordyceps sinensis (Berk.) is a well-known CMM, which has been used as a tonic in China for hundreds of years. The nucleosides were believed as one kind of the major active components in *O. sinensis* (Li et al, 2006). In previous studies, many LC methods for analysis of nucleosides were reported, which included high performance liquid chromatography-diode array detection (HPLC-DAD) (Li et al, 2006), HPLC-mass spectrometry (HPLC-MS) (Yang et al, 2010; Li et al, 2006), and ultra-performance liquid chromatography (UPLC) (Yang et al, 2007). However, those methods mentioned above have disadvantages such as long analysis time or need of expensive LC system.

Therefore, a rapid HPLC analysis method for the determination of six nucleosides (uracil, uridine, inosine, guanosine, adenosine, and cordycepin) in *O. sinensis* on a Poroshell 120 SB Aq column (50 mm × 4.6 mm, 2.7 μm) by conventional HPLC system was developed in the present paper, and applied in *O. sinensis* samples.

2. Materials and methods

2.1 Materials

The samples of *Ophiocordyceps sinensis* were collected from Qinghai, Sichuan provinces, and Tibet Autonomous Region, and purchased from market of Guangdong province. The botanical origin of materials was identified by the correspondence author. The voucher specimens were deposited at HEC R&D Center, Dongguan, Guangdong, China.

Uridine, guanosine, and adenosine were obtained from Jinjinle Company (Shanghai, China). Uracil, inosine, and cordycepin were purchased from the National Institute for Food and Drug Control (Beijing, China). The purity of each compound was more than 98% and determined by HPLC. The chemical structures of these reference compounds are shown in Figure 1. Acetonitrile was of HPLC-grade from Merck (USA). Formic acid was purchased from Aladdin (Shanghai, China) and deionized water was purified by a Milli-Q Purification System (Millipore, USA).

2.2 Instruments

All separations were performed on an Agilent Series 1260 (Agilent Technologies, USA) liquid chromatograph, equipped with a vacuum degasser, a quaternary pump, an autosampler, and a diode array detection (DAD) system, connected to an Agilent OpenLab software.

2.3 HPLC analysis

2.3.1 Preparation of sample solution

The dried powder of samples (0.2 g) was suspended in 30 mL water, ultrasonically extracted for 30 min, and then cooled at room temperature; Water was added to compensate for the lost weight. The sample solution was filtered through a 0.45 μm membrane (Ameritech, Tianjin, China) prior to HPLC.

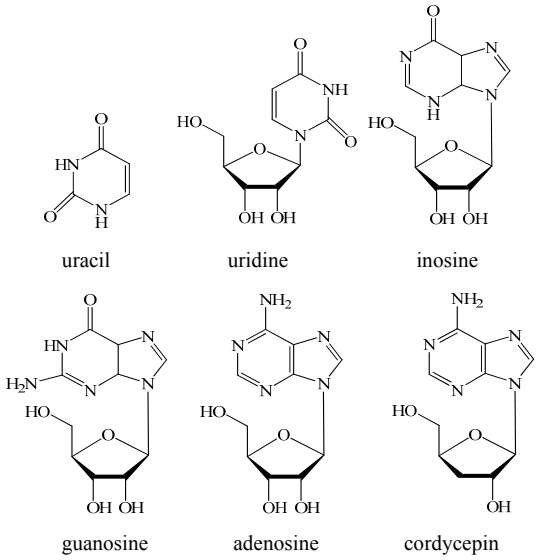

Figure 1 Chemical structures of investigated compounds

2.3.2 HPLC conditions

All separations were performed on an Agilent Poroshell 120 SB Aq Column (50 mm × 4.6 mm, 2.7 μm). The column temperature was maintained at 30 °C. The samples and reference compounds were separated using a gradient mobile phase consisting of 0.1% formic acid water solution (A) and acetonitrile (B). The gradient condition is: 0−3 min, 0% B; 3−8 min, 0−5% B; 8−10 min, 5%−7% B; 10−13 min, 7%−20% B; 13−15 min, 20% B. The flow rate was set at 0.4 mL/min. The UV detection wavelength was set at 260 nm and the injection volume was 2 μL.

2.3.3 Calibration curves

Uridine (33.44 mg), inosine (36.86 mg), guanosine (34.10 mg), and adenosine (33.34 mg) were accurately weighed, added to 100 mL volumetric flask, and diluted to the scale line with water to obtain stock solution 1. Uracil (20.04 mg) and cordycepin (20.06 mg) were accurately weighed, added to 100 mL volumetric flask, and diluted to the scale line with water to obtain stock solution 2. Stock solution 3 was obtained by accurately adding 10 mL stock solution 1 and 1 mL stock solution 2 to 20 mL volumetric flask and diluting to the scale line with water.

Stock solution 3 was diluted to appropriate concentration for construction of calibration curves. At least six kinds of concentration of the solution were analyzed, and then the calibration curves were constructed by plotting the peak area versus the concentration of each reference compound.

2.3.4 Limits of detection and quantification

The stock solution 3 containing six reference compounds was diluted to a series of appropriate concentration with water, and an aliquot of the diluted solutions was injected into HPLC for analysis. The limits of detection (LOD) and quantification (LOQ) under the present chromatographic conditions were determined at a signal-to-noise ratio (S/N) of about 3 and 10, respectively.

2.3.5 Precision

Intra- and inter-day variations were chosen to determine the precision of the developed HPLC method. For intra-day variability test, the mixed standard solution (uracil, uridine, inosine, guanosine, adenosine, and cordycepin as 1.002, 16.72, 18.43, 17.05, 16.67, and 1.003 μg/mL) was analyzed for six replicates within one day, while for inter-day variability test, the solution was examined in duplicates for consecutive 3 d. Variations were expressed by relative standard deviation (RSD).

2.3.6 Accuracy

The recovery was used to evaluate the accuracy of the method. The mixed standard solution (1 mL, including uracil, uridine, inosine, guanosine, adenosine, and cordycepin as 2.738, 100.32, 62.53, 68.87, 34.21, and 2.513 μg/mL) and 14 mL of water were accurately added into a certain amount (0.1 g) of *O. sinensis* sample. The mixture was extracted and analyzed using the method mentioned above. Six replicates were performed for the test.

2.3.7 Repeatability

To confirm the repeatability, six replicates of the same sample were extracted as "2.3.1" and analyzed as "2.3.2" above. The RSD value was calculated as a measurement of method repeatability.

3. Results and discussion

3.1 Optimization of extraction condition

As the analytes are polar compounds, the water was chosen as extract solvent. Then two common-used extraction methods (ultrasonic extraction and refluxing extraction) were compared, and the result showed that the ultrasonic extraction was better. In order to obtain the effective ultrasonic extraction conditions, the extraction time, and solvent volume were studied by univariate approach. The results were found that using 30 mL water and extracting for 30 min would give satisfactory extraction efficiency of sample.

3.2 Optimization of HPLC conditions

Different compositions of mobile phase were tried. As a result, 0.1% formic acid water solution and acetonitrile was chosen as the eluting solvent system to give the desired separation within the running time of 12 min. The different column temperatures (25, 30, and 35 ^{o}C) were also tested. The temperature has no significant effect on the separation of analytes, and 30 ^{o}C was used as column temperature. The detection wavelength was set at 260 nm based on the maximum UV absorption of analytes.

3.3 Validation of method

The linearity, regression, and linear ranges of six analytes were determined using the developed HPLC method. The correlation coefficient values (r > 0.999) indicated appropriate correlations between the investigated compound concentrations and their peak areas within the tested range (Table 1). The LOD and LOQ were less than 0.18 and 0.46 μg/mL, and the overall intra- and inter-day variations (RSD) of the six analytes were less than 0.8% and 2.1%, respectively. The developed method had good accuracy and repeatability. The recoveries were between 95% and 103% (RSD < 3.8%), and the overall repeatability (RSD) was less than 3.7% (Tables 2 and 3).

3.4 Sample analysis

This newly validated HPLC method was subsequently applied to simultaneous determination of the six polar components in *O. sinensis* samples. The analytical time of the new developed HPLC methods is less than 12 min, while more than 30 min was usually taken in the precious reports. The typical chromatograms of reference compounds and *O. sinensis* are shown in Figure 2, and their contents are listed in Table 4. The results showed that uridine, inosine, guanosine, and adenosine were the major nucleosides in *O. sinensis*, while the cordycepin was the component with lower content. These results were agreed with the previous reports, cordycepin was the major component in *Cordyceps militaris* and difficult to be found in *O. sinensis* by HPLC-UV methods. Furthermore, the contents of the investigated analytes in the different samples were variants. The content of adenosine in sample 3 (Qinghai) was 0.37 mg/g and that of sample 2 (Tibet) was 0.95 mg/g, which might be induced by the different growing conditions of *O. sinensis* in Qinghai Province and Tibet Autonomous Region. The contents of inosine, guanosine, and adenosine in sample 5 were the lowest, which might relate with poor processing or storage conditions.

Table 1 Calibration curves, LODs and LOQs of analytes

Analytes	Calibration curves[a]	r	Linear ranges / (μg·mL^{-1})	LOD / (μg·mL^{-1})	LOQ / (μg·mL^{-1})
uracil	Y = 42 888X + 41.279	1.0000	0.25－ 10.02	0.03	0.07
uridine	Y = 23 495X + 4516.4	1.0000	4.18－167.20	0.08	0.21
inosine	Y = 15 594X + 5873.6	1.0000	4.61－184.30	0.18	0.46
guanosine	Y = 21 834X + 11 051	0.9999	4.26－170.50	0.10	0.25
adenosine	Y = 31 030X + 3654.5	1.0000	4.17－166.70	0.11	0.28
cordycepin	Y = 33 269X − 1387.8	0.9999	0.25－ 10.03	0.05	0.13

[a] Y: peak area; X: concentration of analyte

Table 2 Precisions, recoveries, and repeatabilities of analytes (n = 6)

Analytes	Intra-day precision			Inter-day precision			Repeatability
	Actual concentration / ($\mu g \cdot mL^{-1}$)	Detected concentration / ($\mu g \cdot mL^{-1}$)	RSD / %	Actual concentration / ($\mu g \cdot mL^{-1}$)	Detected concentration / ($\mu g \cdot mL^{-1}$)	RSD / %	RSD / %
uracil	1.002	1.003	0.41	1.002	1.002	0.79	1.8
uridine	16.72	16.66	0.46	16.72	16.86	2.0	3.6
inosine	18.43	18.36	0.38	18.43	18.28	0.81	3.1
guanosine	17.05	16.98	0.40	17.05	16.98	0.72	2.6
adenosine	16.67	16.55	0.73	16.67	16.32	1.6	3.7
cordycepin	1.003	1.004	0.61	1.00 3	1.006	0.73	–

Table 3 Recoveries of analytes

Analytes	Contained / mg	Added / mg	Found / mg	Recovery / %	RSD / %
uracil	0.003 05	0.002 74	0.002 80	102.1	0.4
uridine	0.091 5	0.100 3	0.096 2	95.9	3.8
inosine	0.058 8	0.062 5	0.061 8	98.9	1.4
guanosine	0.071 4	0.068 9	0.069 0	100.1	4.9
adenosine	0.038 6	0.034 2	0.033 2	97.0	2.6
cordycepin	0.000 0	0.002 51	0.002 45	97.8	1.1

Table 4 Contents of analytes in *O. sinensis* samples

Sample No.	Collection places	Contents / ($mg \cdot g^{-1}$)					
		uracil	uridine	inosine	guanosine	adenosine	cordycepin
1	Sichuan	0.04	1.32	0.53	0.99	0.61	tr[a]
2	Tibet	0.03	1.21	0.43	1.05	0.95	tr
3	Qinghai	0.05	1.66	0.76	1.26	0.37	tr
4	commercial sample	0.04	0.99	0.62	0.89	0.53	tr
5	commercial sample	0.05	0.85	0.32	0.63	0.18	tr
6	commercial sample	0.03	0.74	0.53	0.64	0.37	tr

[a] tr: trace.

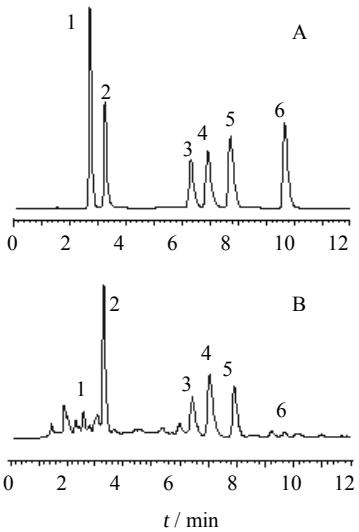

Figure 2 HPLC of reference compounds (A) and *O. sinensis* (B)
1: uracil; 2: uridine; 3: inosine; 4: guanosine; 5: adenosine; 6: cordycepin

4. Conclusion

In this study, a rapid method for the determination of six nucleosides in *O. sinensis* by conventional HPLC system is developed and applied to real samples. The results show that Poroshell SB Aq column can provide a rapid for the polar compounds in CMM by conventional HPLC system, and the developed method is helpful to the quality control of *O. sinensis*.

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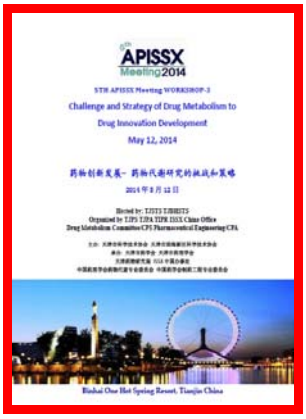

5th Asia Pacific ISSX Meeting Held on May 9-12, 2014, in Tianjin, China

The 5th Asia Pacific ISSX Meeting was organized by International Society for Study of Xenobiotics (ISSX) and Chinese Society for Study of Xenobiotics (CSSX) and co-organized by Tianjin Institute of Pharmaceutical Research and Committee of Pharmaceutical Engineering of Chinese Pharmaceutical Association (CPECPA). Professor Changxiao Liu was a co-Chair of meeting organizing committee and Dr Duanyun Si was a member of meeting organizing committee. In the opening ceremony, Professor Liu representatives the host to welcome experts and scholars from around the world came to Tianjin to attend the meeting. The meeting provided the academic exchanges and learning opportunities for new and old friends, and wish the meeting more successful than the APISSX meeting in Shanghai six years ago, and wish the guests happy and impressed in Tianjin.

During the meeting, nearly 30 researchers from Tianjin Institute of Pharmaceutical Research attended the meeting, and some experts were invited to report to the meeting and doing service. Professor Liu was Co-Chair of a Keynote Lecture and Symposium on Metabolism and safety of herbal medicines and natural products in preclinical and clinical toxicological significance. Professor Si was Co-Chair of Symposium on advances in Mass Spectrometry Application to DMPK Science. On workshop on the functional characterization of transporters, Dr Xiulin Yi take an oral presentation titled Drug transporter based pharmacodynamic screening of Chinese herbal medicines. Ten papers were exhibited with posters.

To demonstrate the needs of the biomedical industry in Tianjin development and innovation of new drugs, the APISSX meeting held a special conference: Workshop 3, co-sponsored by the Tianjin Science and Technology Association, and Tianjin Binhai Association of Science and Technology, and Tianjin Institute of Pharmaceutical Research. The meeting Invited Scientists from American Japan, China and Taiwan Region CDE to discuss issues of science and technology innovation in drug development and pharmaceutical industry representatives in Tianjin.