Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 45 (2007) 141–144
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Determination of purine and pyrimidine bases in natural and cultured *Cordyceps* using optimum acid hydrolysis followed by high performance liquid chromatography

H. Fan [a,b], F.Q. Yang [a], S.P. Li [a,*]

[a] *Institute of Chinese Medical Sciences, University of Macau, Taipa, Macau SAR, China*
[b] *College of Pharmacy, Guangdong Pharmaceutical University, Guangzhou, China*

Received 6 December 2006; received in revised form 17 February 2007; accepted 19 February 2007
Available online 1 March 2007

Abstract

A method based on optimum acid hydrolysis followed by high-performance liquid chromatography (HPLC) with diode array detection was developed for quantitative determination of bio-available nucleosides, present as purine and pyrimidine bases including adenine, cytosine, guanine, hypoxanthine, thymine and uracil, in natural and cultured *Cordyceps*. It was found that the optimum conditions was hydrolyzing *Cordyceps* sample in eight folds of pure commercial perchloric acid for 1 h at 95–100 °C. The determination was achieved by using a Zorbax SB-AQ analytical column (250 mm × 4.6 mm i.d., 5 μm) at gradient elution with diode-array detection. All calibration curves showed good linearity ($r^2 > 0.999$) within test ranges. The developed method showed good repeatability for the quantification of six investigated nucleobases in *Cordyceps* with intra- and inter-day variations of less than 9.0 and 9.1%, respectively. The validated method was successfully applied to quantify bio-availbale nucleosides in natural and cultured *Cordyceps*, which is helpful to control their quality.

Keywords: Acid hydrolysis; HPLC; Purine; Pyrimidine; Bio-availbale nucleosides; *Cordyceps*

1. Introduction

Cordyceps sinensis, one of the well-known traditional Chinese medicines, is commonly used in China to replenish the kidney and soothe the lung for the treatment of multiple diseases [1,2]. To date, nucleosides are believed to be the active components in *Cordyceps*, and more than 10 nucleosides and its related compounds have been isolated from *Cordyceps* [3–6]. Actually, adenosine has been used as a marker for quality control of *Cordyceps sinensis* [7]. Indeed, nucleosides are involved in the regulation and modulation of various physiological processes in the central nervous system (CNS). However, fresh natural *C. sinensis* contains very little amount of nucleosides, as compared to dry and processed one [8], and more interesting is that cultured *Cordyceps* mycelium contains high level of nucleosides [9–13]. Furthermore, humidity and heat significantly increased the amount of nucleosides in natural *Cordyceps*. Storage of *Cordyceps* at 75% relative humidity and 40 °C for 10 days, the contents of nucleosides in natural *Cordyceps* are markedly increased about four folds. However, the effects of humidity and heat altering the contents of nucleotides could not be revealed in cultured *Cordyceps* mycelia [14]. Therefore, it is believed that the sources of nucleosides in natural *Cordyceps* may be different from that of cultured one. Thus, having nucleosides such as adenosine as a marker for good quality of *Cordyceps* may not be indicative.

The principal sources of dietary nucleosides are nucleic acids (DNA and RNA), which are digested by ribonucleases and deoxyribonucleases to nucleotides and then digested further by phosphatases to nucleosides for absorption from the small intestine [15]. Therefore, bio-available nucleosides should include nucleic acids as well as their metabolites such as nucleotides and nucleosides. For determination of bio-available nucleosides, a few methods were developed for quantification of their common units, such as Fiske–Subbarow method for phosphorus and colorimetric method for ribose and/or deoxyribose sugars of the backbone. But these methods have low selectivity and sensitivity. Several methods including HPLC [16,17], capillary

* Corresponding author. Tel.: +86 853 397 4692; fax: +86 853 2884 1358.
E-mail address: shaopingli@hotmail.com (S.P. Li).

0731-7085/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.jpba.2007.02.032

electrophoresis (CE) [18] and micellar electrokinetic chromatography (MEKC) [19] were developed for determination of purine and pyrimidine bases from nucleic acid hydrolysates. Nucleoside compounds in milk were also determined using enzymatic hydrolysis [20], but it was complicated because the multiple enzymes were need for hydrolyzing nucleic acid into nucleobases.

In present study, an optimum acid hydrolysis followed with HPLC method was developed for quantitative determination of bio-available nucleosides, represent as nucleobases, from natural and cultured *Cordyceps*, which is helpful to control the quality of *Cordyceps*.

2. Experimental

2.1. Chemicals, reagents and materials

Fish sperm DNA was purchased from Sigma (St. Louis, MO, USA). Triethylamine (TEA) and methanol for liquid chromatography were purchased from Merck (Damstadt, Germany). Commercial perchloric acid (70%), sulfuric acid (97%), formic acid (32%), phosphoric acid (85%) and hydrochloric acid (37%) were from Riedel-de Haën (AR, Seelze, Germany). Water was prepared using a Millipore Milli-Q Plus system (Millipore, Bedford, MA, USA).

Cytosine, uracil, hypoxanthine, guanine, thymine and adenine were purchased from Sigma (St. Louis, MO, USA). A stock solution of each standard (~0.5 mg/ml) except guanine was prepared in initial mobile phase (5 mmol/l aqueous TEA) and stored in a refrigerator. Guanine (0.1 mg/ml) was prepared in 0.1 mol/l HCl and stored in a refrigerator. A certain volume of the stock solution was transferred to a 2 ml volumetric flask which was made up to its volume with the same solvent so as to obtain the desired concentrations.

Natural *C. sinensis* was obtained from different provinces of China: Sichuan, Qinghai and two from Tibet. The identities of these natural *Cordyceps* were confirmed by the corresponding author. Cultured *C. sinensis* mycelium was obtained from Huadong, Jiangxi, Wanfeng, Hebei and Sizhuang. Cultured *C. militaris,* one of the substitutes for *C. sinensis*, was obtained from Aoli, Guobao, Xiankan and Quanxin. The species identities of these cultured *Cordyceps* were guaranteed by the corresponding author and/or State Food and Drug Administration of China. The voucher specimens of *Cordyceps* were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macau, China.

2.2. Acid hydrolysis and sample preparation

A certain amount of sample (0.5 g) was mixed with 4 ml specific acid in a glass tube, then sealed and hydrolyzed for 1 h at 95–100 °C water bath. Then the hydrolysate was adjusted to pH 7 with 2 mol/l NaOH. Finally, the solution was filtered into a 25 ml volumetric flask, which was made up to its volume with water and filtered through a 0.45 μm Econofilter (Agilent Technologies) prior to injection into the HPLC system.

2.3. HPLC analysis

All separations were performed on an Agilent Series 1100 (Agilent Technologies, USA) liquid chromatograph, equipped with a vacuum degasser, a quaternary pump, an autosampler, and a diode array detector (DAD) system, connected to a Agilent ChemStation software. A Zorbax SB-AQ column (250 mm × 4.6 mm i.d., 5 μm), which is an alkyl reversed-phase bonded phase designed to retain hydrophilic and other compounds when using highly aqueous mobile phases, including 100% water, with a Zobax SB-C18 guard column (20 mm × 3.9 mm i.d., 5 μm) were operated at 25 °C. Solvents that constituted the mobile phase were A (5 mmol/l aqueous TEA) and B (methanol). The elution conditions applied were: 0–8 min, linear gradient 0–20% B; 8–12 min, linear gradient 20–60% B; 12–20 min, linear gradient 60–100% B. Washing the column with isocratic 100% B for 5 min, and finally, reconditioning step of the column was 0% B isocratic for 15 min. The flow rate was 1 ml/min, and the injection volume was 10 μl. The analytes were monitored at 254 nm.

3. Results and discussion

3.1. Optimization of acid hydrolysis

An ideal acid for hydrolysis should completely hydrolyze nucleic acids into nucleobases without degradation of the products. Based on the method mentioned above, fish sperm DNA (10 mg) was used for investigation of hydrolysis of different acids, including perchloric acid, sulfuric acid, formic acid, phosphoric acid and hydrochloric acid. The result showed that perchloric acid had a good ability for hydrolysis of nucleic acids. However, high concentration of perchloric acid could induce the degradation of nucleobases. Therefore, the concentration of acid for hydrolysis of sample should be optimized.

Cultured *C. sinensis* (Hebei) was used to optimize acid concentration for hydrolysis. Fig. 1A shows the content of nucleobases in the cultured *C. sinensis* hydrolyzed with different concentration of perchloric acid for 1 h. It is noteworthy that the contents of uracil and cytosine significantly enhanced with increased concentration of perchloric acid though degradation of nucleobases could be induced at high concentration of acid, which suggested that high concentration of acid is necessary for complete hydrolysis of *Cordcyeps* sample. However, the degradation of nucleobases was dominant when the hydrolysis was finished (Fig. 1B).

Considering the mentioned above, the optimum acid hydrolysis conditions for *Cordyceps* sample is as follows: *Cordyceps* sample was hydrolyzed in eight folds of 100% commercial perchloric acid for 1 h at 95–100 °C.

3.2. Validation of the method

The linearity, regression and linear ranges of six nucleobases were performed using the developed HPLC method. The correlation coefficient ($r^2 > 0.999$) values indicated good correlations between investigated compounds concentrations and their peak

Table 1
Linear regression data, LOD and LOQ of six investigated nucleobases

Analytes	Linear regression data			LOD (μg/ml)	LOQ (μg/ml)
	Linear range (μg/ml)	Regression equation	r^2		
Cytosine	0.7–44.5	$y = 19.38x - 0.03$	1.000	0.05	0.10
Uracil	3.8–245.1	$y = 31.34x + 4.66$	1.000	0.07	0.14
Thymine	1.1–17.1	$y = 6.85x + 0.34$	0.999	0.06	0.11
Hypoxanthine	0.4–12.6	$y = 11.28x + 0.65$	0.999	0.02	0.04
Guanine	1.3–81.0	$y = 9.37x - 0.32$	1.000	0.02	0.04
Adenine	3.1–201.0	$y = 13.51x - 8.99$	0.999	0.06	0.13

r^2, squares of correlation coefficients for the standard curves; LOD, limit of detection; LOQ, limit of quantification.

areas within the test ranges. The limits of detection and quantification were lower than 0.02 and 0.14 μg/ml, respectively (Table 1). Intra- and inter-day variations were chosen to determine the precision of the method. For intra-day variability test, the standard solutions were analyzed in triplicates for three times within 1 day, while for inter-day variability test, the samples were examined in triplicate for consecutive 3 days. The repeatability present as the relative standard deviation for intra- and inter-day variations of less than 9.0 and 9.1%, respectively. Recovery was performed by adding known amounts of six investigated components into a certain amount (0.25 g) of cultured *C. sinensis* (Hebei) material. The mixture was hydrolyzed and analyzed using the method mentioned above. The recoveries of cytosine, uracil, thymine, hypoxanthine, guanine and adenine are 105.5, 106.1, 95.2, 96.2, 89.7 and 89.6%, respectively.

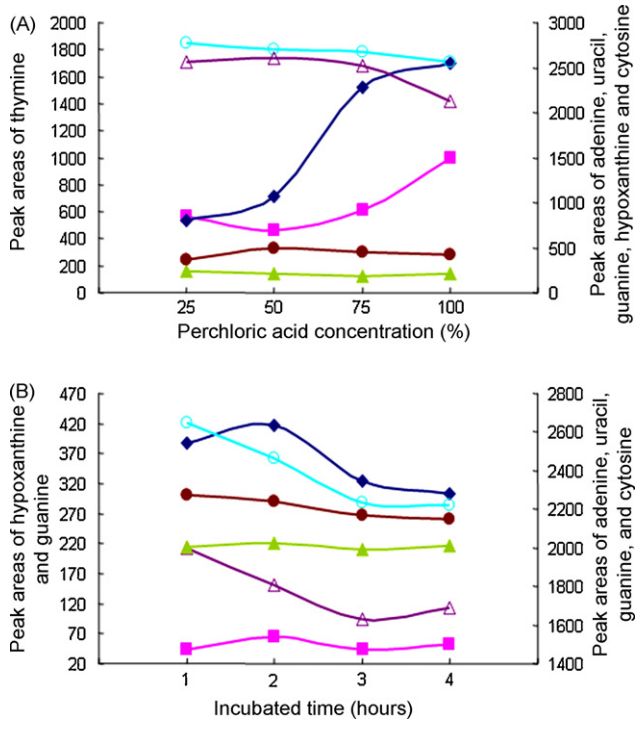

Fig. 1. Effects of (A) perchloric acid concentration and (B) incubated time on hydrolysis of Hebei cultured *Cordyceps* material. (A) The sample was hydrolysed for 1 h at different concentration of perchloric acid; (B) the sample was hydrolysed using pure commercial perchloric acid (70%) for different time. Cytosine (■), uracil (♦), thymine (●), hypoxanthine (▲), guanine (◯) and adenine (△).

3.3. Quantitative determination of purine and pyrimidine bases in Cordyceps

The typical HPLC chromatograms of purine and pyrimidine bases in natural and cultured *Cordyceps* were shown in Fig. 2. Using the calibration curve of each investigated compound, 13 samples of natural and cultured *Cordyceps* were analyzed. Table 2 shows the summary results. The total nucleobases

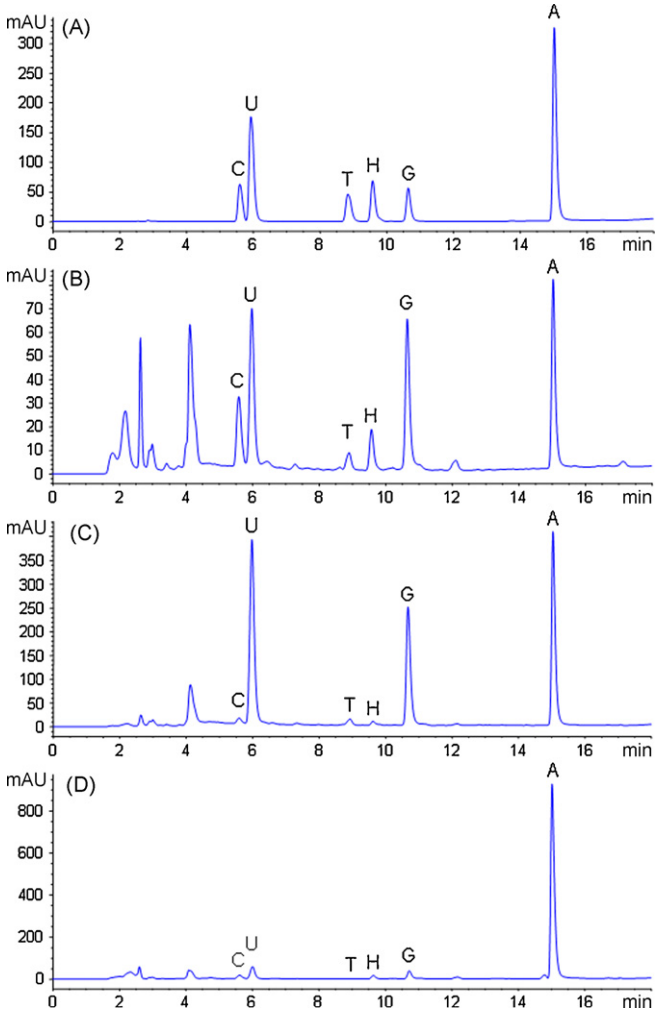

Fig. 2. HPLC profiles of (A) mixed nucleosbases standards, hydrolysates of (B) natural and (C) cultured *Cordyceps sinensis*, and (D) cultured *Cordyceps militaris*. C, cytosine; U, uracil; H, hypoxanthine; G, guanine; T, thymine; A, adenine.

Table 2
The contents (μg/g) of six investigated components in *Cordyceps*

Samples	Cytosine	Uracil	Thymine	Hypoxanthine	Guanine	Adenine	Total amounts
Natural *C. sinensis*							
Sichuang	493.2	629.6	122.5	91.5	560.3	478.5	2375.6
Tibet 1	345.1	567.7	102.7	147.7	428.5	334.3	1925.8
Tibet 2	449.7	736.7	119.0	190.3	693.9	547.3	2736.8
Qinghai	539.6	709.7	117.0	151.0	766.0	601.3	2884.6
Cultured *C. sinensis*							
Hebei	1265.1	2212.8	187.3	196.2	2457.6	2181.3	8500.3
Wanfeng	204.2	4132.2	223.7	69.0	2959.3	2969.4	10557.9
Jiangxi	621.2	1567.8	281.6	86.7	1322.1	1198.5	5077.9
Huadong	1625.3	1841.0	504.7	50.7	2389.8	2431.1	8842.7
Sizhuang	761.2	2158.3	203.4	110.7	1904.6	1625.0	6763.2
Cultured *C. militaris*							
Aoli	242.1	285.5	90.9	38.0	455.3	2358.2	3470.0
Guobao	339.6	641.6	234.4	338.2	151.5	7000.8	8706.0
Xiankang	276.6	753.6	19.0	41.2	362.3	1097.3	2549.9
Quanxin	490.3	997.9	34.7	53.7	569.5	1400.4	3546.5

The data were presented as average of three replicates (RSDs < 6%).

amounts in natural and cultured *Cordyceps* are in accordance with their contents of nucleosides [13]. It means that the content of total nucleobases in natural *Cordyceps* is much lower than that in cultured *Cordyceps*. It is noteworthy that acid hydrolysis also significantly increase total nucleobases amount in cultured *Cordyceps*, though heat and humidity has no the effects. The results seem contrary to the hypothesis of the source of nucleosides is different between natural and cultured *Cordyceps*. But the results suggest that there may be difference between acid hydrolysis and heat and humidity effects on *Cordyceps*. It is suspected that the hydrolysis under heat and humidity may derive from enzyme effect which has high specificity, while acid hydrolysis is universal. The details need further investigation.

4. Conclusion

The principal sources of dietary nucleosides are nucleic acids (DNA and RNA). Therefore, total purine and purimidine bases may be the reasonable marker for evaluation of the nutrition of the materials contain nucleosides. The developed method based on optimum acid hydrolysis and HPLC analysis is simple and feasible for quantitative determination of purine and pyrimidine bases in natural and cultured *Cordyceps*, which is helpful to control their quality correctly.

Acknowledgements

We are grateful to Leon Lai and Sandy Lao from our institute for their expert technical assistance. The research was supported by grant from Macao Science and Technology Development Fund (028/2006/A2 and 077/2005/A) and University of Macau (RG086/04-05S) to S.P. Li.

References

[1] J.S. Zhu, G.M. Halpern, K. Jones, J. Altern. Complement Med. 4 (1998) 289–303.
[2] J.S. Zhu, G.M. Halpern, K. Jones, J. Altern. Complement Med. 4 (1998) 429–457.
[3] W.H. Xu, Z. Xue, J.M. Ma, Bull. Chin. Mater. Med. 13 (1988) 226–228.
[4] S. Frederiksen, H. Malling, H. Klenow, Biochim. Biophys. Acta 95 (1965) 189–193.
[5] A.J. Guarino, N.M. Kredich, Biochim. Biophys. Acta 68 (1963) 317–319.
[6] T. Furuya, M. Hirotani, M. Matsuzawa, Phytochemistry 11 (1983) 2509–2510.
[7] Pharmacopoeia Commission of PRC (Ed.), Pharmacopoeia of the People's Republic of China, vol. 1, Chemical Industry Press, Beijing, P.R. China, 2005, p. 75–76.
[8] S.P. Li, P. Li, T.T.X. Dong, K.W.K. Tsim, Electrophoresis 22 (2001) 144–150.
[9] L. Yu, J. Zhao, S.P. Li, H. Fan, M. Hong, Y.T. Wang, Q. Zhu, J. Sep. Sci. 29 (2006) 953–958.
[10] Y.X. Gong, S.P. Li, P. Li, J.J. Liu, Y.T. Wang, J. Chromatogr. A 1055 (2004) 215–221.
[11] F.Q. Guo, A. Li, L.F. Huang, Y.Z. Liang, B.M. Chen, J. Pharm. Biomed. Anal. 40 (2006) 623–630.
[12] S.P. Li, P. Li, C.M. Lai, Y.X. Gong, K.K.W. Kan, T.T.X. Dong, K.W.K. Tsim, Y.T. Wang, J. Chromatogr. A 1036 (2004) 239–243.
[13] H. Fan, S.P. Li, J.J. Xiang, C.M. Lai, F.Q. Yang, J.L. Gao, Y.T. Wang, Anal. Chim. Acta 567 (2006) 218–228.
[14] S.P. Li, P. Li, H. Ji, P. Zhang, T.T.X. Dong, K.W.K. Tsim, Acta Pharm. Sin. 36 (2001) 436–439.
[15] J.D. Carver, W.A. Walker, J. Nutr. Biochem. 6 (1995) 58–71.
[16] X.L. Huang, L.Q. Li, Y.H. Chen, H.Y. Zhang, Se Pu. 18 (2000) 500–502.
[17] C.Y. Wang, F.Z. Hu, Z.X. Shi, Se Pu. 20 (2002) 348–349.
[18] W.R. Jin, H.Y. Wei, X. Zhou, Electroanalysis 9 (1997) 770–774.
[19] G. Chen, X. Han, L. Zhang, J. Ye, J. Chromatogr. A 954 (2002) 267–276.
[20] R.L. Tressler, M.B. Ramstack, N.R. White, B.E. Molitor, N.R. Chen, P. Alarcon, M.L. Masor, Nutrition 19 (2003) 16–20.