Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis
33 (2003) 1155–1162
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Simultaneous separation and determination of active components in Cordyceps sinensis and Cordyceps militarris by LC/ESI-MS

Lan-Fang Huang [a], Yi-Zeng Liang [a,*], Fang-Qiu Guo [a], Zhi-Feng Zhou [a], Ben-Mei Cheng [b]

[a] *Research Center of Modernization of Chinese Herbal Medicines, College of Chemistry and Chemical Engineering, Central South University, Changsha 410083, PR China*
[b] *Analytical Testing Center of Xiangya, Medical College of Central South University, Changsha 410078, PR China*

Received 3 June 2003; received in revised form 5 July 2003; accepted 9 July 2003

Abstract

A simple and rapid isocratic LC/MS coupled with electrospray ionization (ESI) method for simultaneous separation and determination of adenine, hypoxanthine, adenosine and cordycepin in Cordyceps sinensis (Cs) and its substitutes was developed. 2-Chloroadenosine was used as internal standard for this assay. The optimum separation for these analytes was achieved using the mixture of water, methanol and formic acid (85:14:1, v/v/v) as a mobile phase and a 2.0 × 150 mm Shimadzu VP-ODS column. Selective ion monitoring (SIM) mode ($[M+H]^+$ at *m/z* 136, 137, 268, 252 and 302) was used for quantitative analysis of above four active components. The regression equations were liner in the range of 1.4–140.0 μg ml^{-1} for adenine, 0.6–117.5 μg ml^{-1} for hypoxanthine, 0.5–128.5 μg ml^{-1} for adenosine and 0.5–131.5 μg ml^{-1} for cordycepin. The limits of quantitation (LOQ) and detection (LOD) were, respectively 1.4 and 0.5 μg ml^{-1} for adenine, 0.6 and 0.2 μg ml^{-1} for hypoxanthine, 0.5 and 0.1 μg ml^{-1} for adenosine and cordycepin. The recoveries of four constituents were from 93.5 to 107.0%. The nucleoside contents of various types of natural Cs and its substitutes were determined and compared with this developed method.

Keywords: High performance liquid chromatography; Electrospray ionization (ESI) interface; Mass spectrometry; Nucleoside; Cordyceps sinensis

1. Introduction

Due to the merits of low toxicity and rare complications, traditional Chinese medicines (TCMs) has been extensively used to prevent and cure many diseases that have inflicted humans for over a millennium. The pertinent investigations

* Corresponding author. Tel.: +86-731-882-2841; fax: +86-731-882-5637.
E-mail address: yzliang@public.cs.hn.cn (Y.-Z. Liang).

0731-7085/03/$ - see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0731-7085(03)00415-1

include the analysis of active ingredients and major components of the medicine, treatment of diseases and the search for alternative drugs. Cordyceps sinensis, a black blade fungus highly valued in China that grows at high altitude, has been used in China as food and herbal medicine for a variety of diseases. Cordyceps sinensis extract has been used for treatment of hyperglycemia, respiratory and liver diseases, renal dysfunction, renal failure [1,2] and for its antioxidant [3]. Cordyceps sinensis is also an anti-cancer agent [4]. In addition to its usage as a crude drug, it is used extensively as a folk tonic food or invigorant by the Chinese. Because of its scarcity in nature and high price, some studies have been carried out in order to find substitutes for Cordyceps sinensis. Fortunately, some fruitful work and progress have been made and gone steady forward [5–8]. It has been demonstrated that adenine, hypoxanthine, adenosine and cordycepin are the major bioactive components in Cordyceps sinensis. Their molecular structures are shown in Fig. 1. In particular, the two most important active components, adenosine and cordycepin, may be considered as indices for estimation of quality of Cordyceps sinensis and its substitutes [9]. So far, several methods are available for the determination of nucleosides in Cordyceps sinensis and cultured Cordyceps, including thin layer chromatography (TLC) [8,10], high performance liquid chromatography (HPLC) [11–14] and capillary electrophoresis [5,6]. Among these methods, HPLC might be the most useful one. However, HPLC methods mentioned above suffer from limitation such as low sensitivity, poor selectivity and time-consuming. For example, for a good separation of each component of interest from the sample matrix, complex gradient HPLC systems are usually required [11]. Liquid chromatography–mass spectrometric detection (LC/MS) has been used for identification and determination of active components in TCMs. Because it often provides low limit of detection, high selectivity and short analysis time, LC/MS has been accepted by more and more people as a useful method for identification and determination of active components in TCMs [15–18]. To the best of our knowledge, there are no reports on separation and determination of the four active components in Cordyceps sinensis and its substitutes by LC/MS. The purpose of the present study is to develop a simple, fast and sensitive LC/MS method for simultaneous separation and determi-

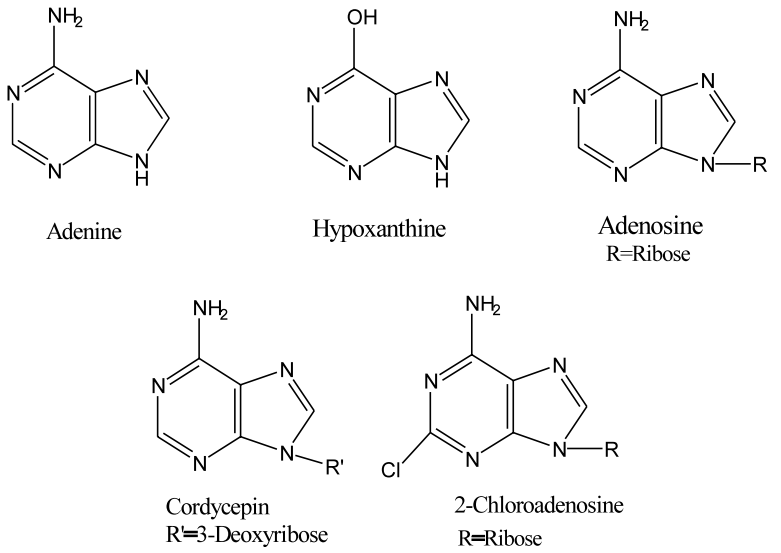

Fig. 1. Molecular structures of adenine, hypoxanthine, adenosine, cordycepin and 2-chloroadenosine.

nation of active components in these Chinese traditional medicines mentioned above. Based on this work, the contents of bioactive nucleosides in various types of natural Cordyceps and cultured Cordyceps were determined and compared.

2. Experimental

2.1. Apparatus

All experiments were carried out on a Shimadzu LCMS-2010 equipment (Kyoto, Japan), which consists of an LC-10ADvp solvent delivery pump, an FCV-10ALvp low pressure gradient unit, a DGU-14A degasser, a CTO-10Avp column oven, a SPD-M10Avp photodiode array detector, a quadrupole mass spectrometer. The column utilized for separation was a 2.0×150 mm Shimadzu VP-ODS column with a particle size of 5 μm. The analytical column was protected by a C_{18} guard-pak cartridge (Waters, Milford, MA, USA).

2.2. Materials and standards

Adenine, hypoxanthine, adenosine, cordycepin and internal standard 2-chloroadenosine (IS) were purchased from Sigma (St. louis, MO, USA). A stock solution of each standard (0.2 mg ml^{-1}) was prepared in methanol and stored in a refrigerator. All solutions were filtered through a 0.45 μm membrane and degassed. Working standard solution (20 μg ml^{-1}) was prepared by diluting the stock solution with methanol. Formic acid used was analytical grade. HPLC-grade methanol was obtained from Hanbang Science and Technology CO. (Jiangsu, PR China) and Milli-Q quality water was used in the preparation of the mobile phase. Samples of Cordyceps sinensis and Cordyceps militarris from different sources were obtained from local drug stores.

2.3. Sample preparation

Nucleosides, the water soluble components, were extracted with water [19]. The dried Cordyceps sinensis was grinded into powder (approximately 20 mesh) at first. Then, about 0.5 g of Cordyceps sinensis was exactly weighed and added to 20 ml of distilled water for extraction of nucleosides. The sample-water mixture was placed into an ultrasonic bath for 2 h. Then the sample mixture was filtered and the filtrate was vacuum-dried. The residue was dissolved in 10 ml of methanol. Similar procedure of extraction was also performed on about 0.5 g of Cordyceps militarris (Cm).

2.4. Chromatographic conditions

The mixture of water, methanol and formic acid (85:14:1, v/v/v) as a mobile phase was degassed ultrasonically before use. Each component of the mobile phase was filtered through a 0.22 μm membrane. All separations were at room temperature and a flow-rate of 0.2 ml min^{-1}. The wavelength of photo-diode array detector was 200–300 nm. The amount of injecting was 5 μl.

2.5. Mass spectrometric detection conditions

MS coupling with ESI interface was used in positive ion mode and SIM at *m/z* 136, 137, 268, 252 and 302. $[M+H]^+$ was selected as the SIM ion in quantification. ESI temperature was 380 °C. Curved desolvation line (CDL) and block temperature were 250 and 200 °C, respectively. Probe voltage was +4.0 kV. Detector voltage was 1.5 kV. CDL voltage was −18 V. Q-array Bios was 45 V. Nebulizing gas flow was 4.5 l min^{-1}.

2.6. Validation

A set of seven non-zero calibration standards of each component, ranging from 1.4 to 140.0 μg ml^{-1} for adenine, 0.6–117.5 μg ml^{-1} for hypoxanthine, 0.5–128.5 μg ml^{-1} for adenosine and 0.5–131.5 μg ml^{-1} for cordycepin was prepared in order to calculate the standard curves, which were calculated by plotting peak area ratio (Y) of each analyte and IS in total ion chromatogram (TIC) of LC/ESI-MS versus concentration (X, μg ml^{-1}) with least-squares linear regression. The retention times of adenine, hypoxanthine, adenosine, cordycepin and IS in LC/ESI-MS TIC were 2.57, 3.78, 4.86, 6.12 and 6.84 min, respectively. The method

has been validated for selectivity, linearity, precision, accuracy and recovery. Quality control samples of each analyte at concentrations of 2.0, 20.0 and 100 μg ml^{-1} were prepared by diluting the stock solution with the mobile phase. The precision and accuracy were determined by six replicate analyses of quality control samples. 75.0 μg adenine, 30.0 μg hypoxanthine, 75.0 μg adenosine and 10.0 μg cordycepin at sample level were added to Cordyceps sinensis and performed as section 2.3. This spiked sample was also used to evaluate precision and accuracy. The selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Due to using SIM mode in this method, no interference at the retention times of adenine, hypoxanthine, adenosine and cordycepin was observed. Various standard concentrations at sample level of adenine, hypoxanthine, adenosine and cordycepin (see Table 3) were added to about 0.5 g actual samples, after extracting as section 2.3, the concentration of these components was determined and recoveries were calculated. The recovery was evaluated by comparing the peak area response of analytes in extracted samples and standard added samples.

3. Results and discussion

3.1. Optimization of separation

The choice of the chromatographic conditions was guided by the need to obtain chromatograms and TIC with better resolution of adjacent peaks within a short analysis time, especially when numerous samples were to be analyzed. The separation of nucleosides is very difficult due to their similar structure (see Fig. 1). For the determination of nucleosides in Cordyceps sinensis and its substitutes, some reports [11,14] used the mobile phase including (A) KH_2PO_4 buffer and (B) methanol with complex gradient HPLC systems. However, the separation is poor and the elution time is as long as 30 min not including the equilibration time between runs. In a preliminary study, it was found that separation was achieved on a 2.0 × 150 mm Shimadzu VP-ODS column by using the mobile phase consisting of water, methanol and formic acid. Separation conditions such as percentage of water, methanol and formic acid were optimized. Of the optimizable parameters, the pH value of mobile phase and organic modifier percentage had the greatest effect on the separation, peak shape and detection sensitivity of these four compounds. The percentage of formic acid was the most important factor. With the concentration of water and methanol being optimized, different percentage of formic acid ranging from 0.1 to 2% were used in order to study the effect of formic acid on the separation and ionization. HPLC-UV chromatogram with a DAD detector set at 260 nm was shown in Fig. 2(A). The four analytes and IS were completely separated when the concentration of formic acid was in the range of 0.2–1.2%. However, when the concentration of formic acid was below 0.9%, the ionization was poor. Considering both resolution and ionization,

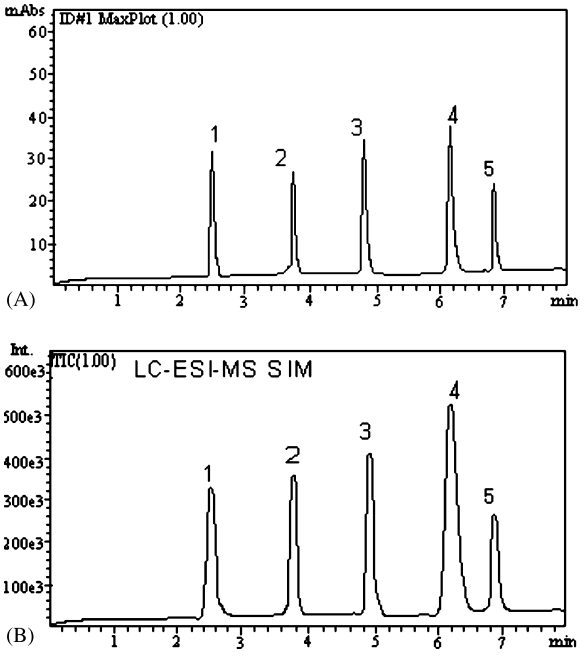

Fig. 2. Simultaneous HPLC-UV chromatogram detected with a photo-diode array detector set at 260 nm (A) and LC/ESI-MS TIC of mixture of nucleoside standards (B). Chromatographic conditions are described in Section 2.4 and SIM mode parameters are listed in Section 2.5. The concentration of each standard is 20 μg ml^{-1}. (1) adenine; (2) hypoxanthine; (3) adenosine; (4) cordycepin; (5) 2-chloroadenosine(10 μg ml^{-1}).

the optimized formic acid concentration of 1% was chosen for all subsequent experiments.

3.2. Optimization of the ESI-MS conditions

The aim of the present work is to develop a method for simultaneous separation and determination of nucleosides in Cordyceps sinensis and its substitutes by LC/MS. Although the mixture of adenine, hypoxanthine, adenosine and cordycepin standards can be separated at the optimal chromatographic conditions and monitored at 260 nm with a photo-diode array detector (see Fig. 2A), it is quite difficult to determine adenosine, cordycepin, adenine and hypoxanthine simultaneously with HPLC-DAD because of the presence of other components in Cordyceps sinensis and its substitutes with different polarities and consequently, different retention times. Furthermore, some components with very low contents in real samples (such as cordycepin in Cordyceps sinensis) cannot be detected with HPLC-DAD due to the low inherent UV absorbance of these components. Here a first combined isocratic LC/MS method based on coupling with the ESI interface was developed. In positive ion mode, LC/ESI-MS mass spectra of adenine, hypoxanthine, adenosine and cordycepin standards were obtained by scanning between m/z 50 and 350 per second (see Fig. 3). The mass spectra of adenine, adenosine and cordycepin obtained from scan mode were characterized by a protonated molecular ion $[M+H]^+$ as base peak. However, the mass spectrum of hypoxanthine was characterized by a Na adduct two molecular ion $[2M+Na]^+$ as base peak and the protonated molecular ion $[M+H]^+$ was the second peak. In additional to $[M+H]^+$ or $[2M+Na]^+$ as a base peak, the MS spectra of adenosine, hypoxanthine and cordycepin obtained from scan mode had an $[M+Na]^+$ or $[2M+H]^+/[2M+Na]^+$. The results were summarized in Table 1. SIM mode involved the use of the $[M+H]^+$ ions at m/z 136, 137, 268, 252 and 302 was chosen for simultaneous determination of the four active components. A mixture containing four standard compounds and IS was injected into the LC/MS system. They were monitored using SIM mode. TIC of adenosine, adenine, hypoxanthine, cordycepin standard mixture and IS in SIM mode was shown in Fig. 2B. It was observed that adenine, hypoxanthine, adenosine, cordycepin and IS were

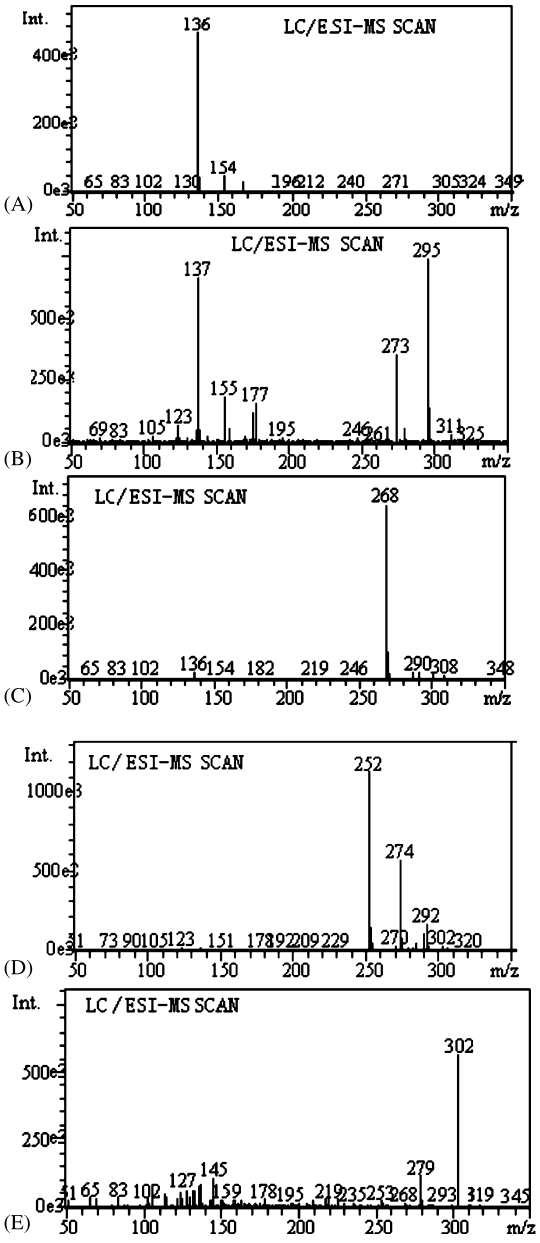

Fig. 3. LC/ESI-MS mass spectra of mixture of nucleoside standards in positive ion and scan mode between m/z 50 and 350 per second. Chromatographic conditions are described in Section 2.4. (A) Adenine; (B) hypoxanthine; (C) adenosine; (D) cordycepin; (E) 2-chloroadenosine.

Table 1
The values of t_R in TIC $[M+H]^+$, $[2M+H]^+$, $[M+Na]^+$, $[2M+Na]^+$ in MS spectra of nucleoside standard compounds

Peak number	Compound	t_R (min)	UVλ_{max} (nm)	$[M+H]^+$ (m/z)	$[M+Na]^+$ (m/z)	$[2M+H]^+$ (m/z)	$[2M+Na]^+$ (m/z)
1	Adenine	2.57	207,261	136			
2	Hypoxanthine	3.78	198,252	137		273	295
3	Adenosine	4.86	206,260	268	290		
4	Cordycepin	6.12	207,260	252	274		

baseline resolved. With an isocratic HPLC, the compounds were separated in less than 7.5 min. Resolution was superior to those determination methods of nucleosides, including HPLC [11–14] and capillary electrophoresis [5,6]. Sensitivity optimization was performed by injection of adenosine standard (20 μg ml^{-1}). The best ESI-MS conditions were listed in Section 2.5. UV spectrum and mass spectrum of standard of each component were used for comparison with the chromatograms of extracts of Cordyceps sinensis and its substitutes for identification of components. Their retention time(t_R), $[M+H]^+$, $[2M+H]^+$, $[M+Na]^+$, $[2M+Na]^+$ and UVλ_{max} values were shown in Table 1.

3.3. Analytical performance

SIM mode was used to exam linearity using a set of seven non-zero calibration standards of each components. The linear relationships between the concentrations of the four active components and the corresponding peak area ratio of each analyte and IS were found in the range of 1.4–140.0 μg ml^{-1} for adenine, 0.6–117.5 μg ml^{-1} for hypoxanthine, 0.5–128.5 μg ml^{-1} for adenosine and 0.5–131.5 μg ml^{-1} for cordycepin. The regression equations of these curves and their coefficients were calculated as follows: adenine, $Y=0.05968X+0.00487$ (0.9967); hypoxanthine, $Y=0.1245X+0.0135$ (0.9981); adenosine, $Y=0.1689X+0.0157$ (0.9990) and cordycepin, $Y=0.2247X+0.0208$(0.9986). Because extracts of Cs and Cm contain analytes, no real blank was available for preparation of standards or controls. A solvent blank was analyzed for determining limit of detection and no peaks at m/z 136, 137, 268, 252 and 302 were observed in the blank. Limit of detection, which determined from signal-to-noise ratio of 3:1, was 0.5 μg ml^{-1} for adenine, 0.2 μg ml^{-1} for hypoxanthine, 0.1 μg ml^{-1} for adenosine and cordycepin, respectively. Nevertheless, after applying the method to actual samples, the limits of quantitation (LOQ) for these components were considered more important. The lower limits of the range of the calibration curves can be considered the LOQ of the method. Six replicate analyses of quality control samples and a spiked sample were used to calculate the precision and accuracy. The results were listed in Table 2. The accuracy was in the order of −2.6–3.5% for adenine, −4.0–3.3% for hypoxanthine, −2.5–3.3% for adenosine and −0.6–4.0% for cordycepin. The precision was in the order of 1.6–6.2% for adenine, 1.8–5.7% for hypoxanthine, 1.5–5.3% for adenosine and 1.8–6.3% for cordycepin. No major differences were observed when using standard solutions or a spiked sample for the calculations of accuracy and precision. The recoveries of four constituents were from 93.5 to 107.0% (see Table 3).

3.4. Application

The method has been applied to simultaneous separation and determination of active components in Cordyceps sinensis and Cordyceps militarris. The typical TICs of real samples were shown in Fig. 4. Peaks were identified by comparison UV and mass data. The four components were baseline resolved and hypoxanthine in Cordyceps militarris was not detected, indicating that the content of the hypoxanthine is very low. The analytical results of two typical samples were summarized in Table 3. The nucleoside contents of many other Cordyceps sinensis and Cordyceps

Table 2
Summary of precision and accuracy of adenine, hypoxanthine, adenosine and cordycepin

Analyte	Concentration added (μg ml^{-1})	Concentration found (μg ml^{-1})	RE (%)	RSD (%, $n=6$)
Adenine	2.0	2.07	3.5	6.2
	20.0	19.8	−1.0	3.4
	15.0[a]	14.6	−2.6	1.8
	100.0	101.4	1.4	1.6
Hypoxanthine	2.0	1.92	−4.0	5.7
	6.0[a]	6.2	3.3	4.2
	20.0	20.3	1.5	3.2
	100.0	99.2	−0.8	1.8
Adenosine	2.0	1.95	−2.5	5.3
	20.0	20.4	2.0	3.6
	15.0[a]	15.5	3.3	1.9
	100.0	99.0	−1.0	1.5
Cordycepin	2.0	2.06	3.0	6.3
	2.0[a]	2.08	4.0	4.3
	20.0	20.5	2.5	2.9
	100.0	99.4	−0.6	1.8

[a] Water extract of a Cordyceps sinensis sample spiked with adenine, hypoxanthine, adenosine and cordycepin standards.

militarris were also determined and compared (the results were not listed in this paper). There were differences between the nucleoside contents of Cordyceps sinensis and its substitutes. The content of adenine and hypoxanthine in Cordyceps sinensis is higher than in its substitutes, Cordyceps militarris. However, the content of adenosine and cordycepin in Cordyceps militarris is much higher than in Cordyceps sinensis. The contents of the four determined nucleosides obtained from different sources, both in Cordyceps sinensis and its substitutes, also varied considerably from locality to locality. Since nucleosides are important biologically active components, therefore, quality control of this commonly used drug is in great demand. Determination of the four principal

Table 3
Results for the determination of the four active components in Cordyceps sinensis and Cordyceps militarris

Samples[a]	Components	Found[b] (μg g^{-1}, $n=5$)	Added (μg g^{-1})		Increased (μg g^{-1})		Recovery (%)	
Cs	Adenine	328	100	200	94.6	187.0	94.6	93.5
	Hypoxanthine	12.6	10	20	10.3	21.2	103.0	106.0
	Adenosine	246	100	200	101.3	206.5	101.3	103.3
	Cordycepin	4.8	5	10	5.2	10.7	104.0	107.0
Cm	Adenine	29	10	20	9.4	19.2	94.0	96.0
	Hypoxanthine	nd[c]						
	Adenosine	1240	200	500	187.5	508.4	93.8	101.7
	Cordycepin	750	200	500	206.8	487.5	103.4	97.5

[a] Cs is Cordyceps sinensis from the Tibet Autonomous Region of the PR China and Cm is Cordyceps militarris, which is a newly cultured substitute of Cordyceps sinensis and was provided by Yunnan Silkworm and Bee Institute (Kunming, China).
[b] Mean value.
[c] Not detected.

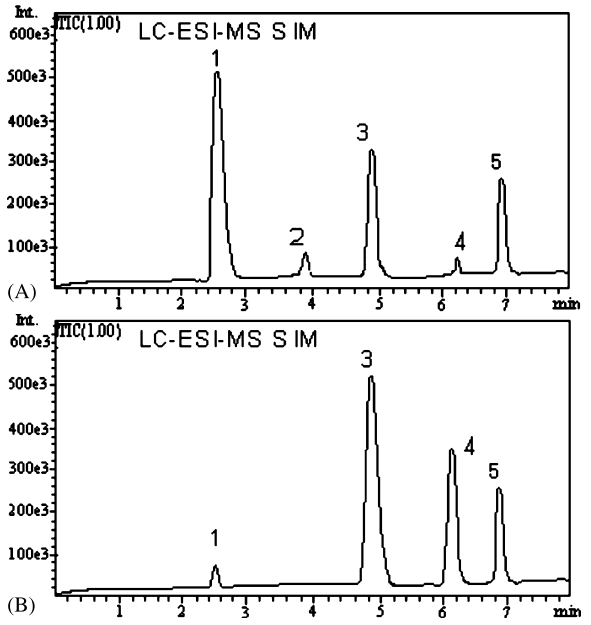

Fig. 4. Typical LC/ESI-MS TICs of water extracts of Cordyceps Sinensis (A) and Cordyceps Militarris (B). Chromatographic conditions and SIM mode parameters are described in Fig. 2. Peaks are identified as in Fig. 2.

nucleosides can be of great importance for the identification, differentiation and quality evaluation of Cordyceps sinensis and its substitutes.

4. Conclusions

In this paper, a simple, rapid and specific LC/MS coupled with ESI method to separate and determine simultaneously four active components in Cordyceps sinensis and its substitutes was developed. The mass spectra of nucleosides obtained from the extracts of actual samples were characterized by $[M+H]^+$ or $[2M+Na]^+$ as base peak. SIM mode was used for quantitative analysis of four active components. The successfully simultaneous separation and determination of the four components in Cordyceps sinensis and its substitutes shows that LC/MS method is a powerful technique for the analysis of bioactive components in TCMs. The developed method also proves to be applicable to the quality control of TCMs.

Acknowledgements

This research work is financially supported by National Natural Science Foundation of China (Grant No. 20175036 and 20235020).

References

[1] J.S. Zhu, G.M. Halpern, K. Jones, J. Alternative Complementary Med. 4 (1998) 289–303.
[2] J.S. Zhu, G.M. Halpern, K. Jones, J. Alternative Complementary Med. 4 (1998) 429–457.
[3] S.P. Li, P. Li, T.T. Dong, K.W. Tsim, Phytomedicine 8 (2001) 207–212.
[4] K.C. Huang, The Pharmacology of Chinese Herbs, CRC Press, Boca Raton, 1993, pp. 207–208.
[5] S.P. Li, P. Li, H. Ji, P. Zhang, Tina T.X. Dong, Karl W.K. Tsim, Acta Pharmaceutica Sinica 36 (2001) 436–439.
[6] S.P. Li, P. Li, H. Ji, P. Zhang, Tina T.X. Dong, Karl W.K. Tsim, Electrophoresis 22 (2001) 144–150.
[7] H. Jiang, K. Liu, S. Meng, Z.Y. Chu, Acta Pharmaceutica Sinica 35 (2000) 663–668.
[8] H.Z. Wu, X.H. Dong, D.E. Ma, Chinese Traditional and Herbal Drugs 31 (2000) 576–578.
[9] H.Z. Zheng, Z.H. Dong, J. She, Modern Study of Traditional Chinese Medicine, vol. 6, Xue Yuan Press, Beijing, 1999, p. 99.
[10] M. Cheng, Y. Zhang, Chin. Traditional Patent Med. 17 (1995) 40–41.
[11] C. Guo, J. Zhu, L.J. Zhang, China J. Chinese Materia Media 23 (1998) 236–237.
[12] X.Q. Li, Y. Wang, T.T. Bao, China J. Chinese Materia Media 24 (1999) 12–14.
[13] C.M. Wu, R.P. Ye, Chinese Traditional and Herbal Drugs 30 (1999) 658–660.
[14] X.N. Chu, Q. Zhang, Q.M. Chen, J.Y. Luo, J.M. Yuan, J. Pharmaceutical Analysis 16 (1996) 249–251.
[15] Z. Cai, F.S. Lee, X.R. Wang, W.J. Yu, J. Mass Spectrom. 37 (2002) 1013–1024.
[16] M. Brolis, B. Gabetta, N. Fuzzati, R. Pace, F. Panzeri, F. Peterlongo, J. Chromatogr. A 825 (1998) 9–16.
[17] D. Ryan, K. Robards, S. Lavee, J. Chromatogr. A 832 (1999) 87–96.
[18] L.Z. Lin, X.G. He, M. Lindenmaier, G. Nolan, J. Yang, M. Cleary, S.X. Qiu, G.A. Cordell, J. Chromatogr. A 876 (2000) 87–95.
[19] A.K. Leung, F. Gong, Y.Z. Liang, F.T. Chau, Anal. Lett. 33 (2000) 3195–3211.