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Original article

Synthesis and pharmacokinetic evaluation of novel *N*-acyl-cordycepin derivatives with a normal alkyl chain

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Abstract

For slowing down the too fast metabolic velocity and increasing the bioavailability of cordycepin, four *N*-acyl-(propionyl-, octanoyl-, lauroyl- and stearoyl-) cordycepin derivatives were synthesized chemically and their pharmacokinetic profiles were investigated in this study. The results show that time of maximum concentration (T_{max}) and half-life ($t_{1/2}$) would be elongated with the increase of the alkyl chain length, but maximum concentration (C_{max}) and area under concentration–time curve (AUC) increased initially, then decreased when the number of alkyl carbon exceeded eight. The T_{max}, C_{max} and AUC of *N*-octanoyl-cordycepin were nearly 4, 30 and 68 times, respectively, higher than that of cordycepin. All derivatives could be transformed into cordycepin *in vivo* and the concentration of transformed cordycepin was proportional to that of derivatives. It indicated that *N*-octanoyl modification could decrease the metabolic velocity and increase the bioavailability of cordycepin to the maximum, thus it might be a promising prodrug of cordycepin.

Keywords: Cordycepin; *N*-acyl modification; Pharmacokinetics

1. Introduction

Cordycepin is an important bioactive component of *Cordyceps militaris*, a traditional Chinese medicinal mushroom [1,2]. Reportedly, cordycepin has many important bioactivities, such as anti-bacterial [3], anti-metastatic action on some cell lines [4], stimulating effect on interleukin-10 production as an immune modulator [5], anti-leukemic activity [6], inhibition to the growth of various tumor cells [7,8], etc. But experiments *in vivo* indicated that it was hard for cordycepin to exert its pharmacological activities effectively, due to the rapid oxidation of its primary amine by adenosine deaminase (ADA) [9]. Although combination of cordycepin with ADA inhibitor could improve the bioactivity of cordycepin, the toxicity of ADA inhibitor cumbered its application in clinic [10].

Some previous works indicated that aminoacyl derives could be good prodrugs of many drugs [11], and increased lipophilicity of alkylated derivatives could improve bioavailablity of some drugs due to improved diffusion across biomembranes [12]. So, if cordycepin was changed chemically to its normal alkyl aminoacyl derivatives, not only might its primary amine be protected from fast oxidation but also its bioavailability changed with the length of its alkyl chain, thus the pharmacological activity of cordycepin might be improved greatly. So, four *N*-acyl-cordycepin derivatives with a normal alkyl chain were synthesized chemically and their pharmacological activities were investigated as compared with cordycepin in this study.

2. Chemistry

Aminoacyl derives of nucleosides have good stability in neutral or alkaline condition and can be synthesized by acylation

Abbreviations: ADA, Adenosine deaminase; C_{max}, Maximum concentration; AUC, Area under concentration–time curve; T_{max}, Time of maximum concentration; $t_{1/2}$, Half-life; C_n, Carbon number of alkyl chain in derivative.

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reaction. For example, as an aminoacyl prodrug of cytosine arabinoside (Ara-C), enotitabine can be synthesized with Ara-C and docosanoic chloride [11]. But the synthesis of cordycepins aminoacyl derives was not reported before. With cordycepin (**1**) as lead compound and pyridine as solvent, propionyl-, octanoyl-, lauroyl- or stearoyl-chloride as acylating agent, under strict control of reaction conditions, four novel aminoacyl derives of cordycepin, i.e. *N*-propionyl-cordycepin (**2**), *N*-octanoyl-cordycepin (**3**), *N*-lauroyl-cordycepin (**4**) and *N*-stearoyl-cordycepin (**5**) were synthesized according to Scheme 1.

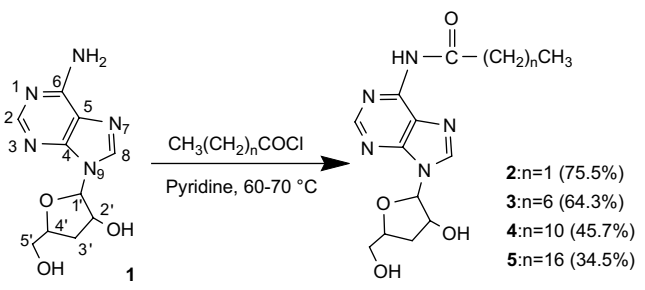
3. Pharmacology

Healthy C57BL/6 male mice (20 ± 2 g, 7 weeks old) were purchased from Laboratory Animal Centre of Chongqing Medical University. They were housed in stainless cages in a room with controlled temperature (23 ± 2 °C) and humidity (40–60%) and a 12 h light/dark cycle. The protocol complied with the guidelines of Chongqing City Laboratory Animal Administration Committee of China for the care and use of laboratory animals. They were randomly divided into five groups with 95 mice each. All compounds **1**–**5** were dissolved in 2% Tween-80 aqueous solution and given to mice by oral administration (500 μmol/kg body wt), respectively. After 0.5, 1.0, 1.5, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 16, 18 and 24 h, respectively, 0.4 mL blood samples were taken from the orbital veins of mice (each time five mice) for HPLC analysis to investigate the pharmacological activities of compounds **1**–**5**.

4. Results and discussion

4.1. Synthesis of new compounds

The spectra data and other characteristic parameters of compounds **1**–**5** are shown in Section 6.2. From the IR data, we could see the diagnostic IR absorbance of aminoacyl bond, aminoacyl I–III bands, emerged in the IR spectra of compounds **2**–**5**. Compared with cordycepin's ^{1}H NMR data, the peak data of $-NH_2$ disappeared and the peak data of 6-NH–CO– emerged in the ^{1}H NMR data of compounds **2**–**5**. All spectral data can be analyzed reasonably by their molecular structures. So, we concluded that compounds **2**–**5** were synthesized successfully.



Scheme 1. Synthesis of *N*-acyl-cordycepin derivatives (**2**–**5**).

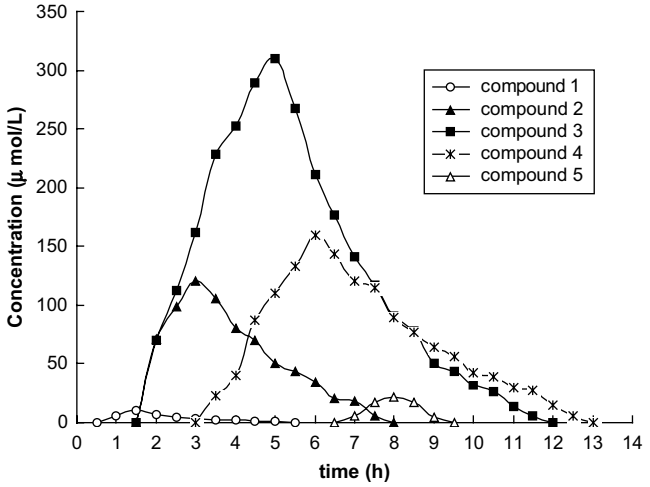


Fig. 1. Mean blood concentration–time profiles of compounds **1**–**5** after oral administrations (500 μmol/kg), each point represents the arithmetic mean ($n = 5$).

4.2. Pharmacokinetic profiles of new compounds

The mean blood concentration–time profiles of compounds **1**–**5** after oral administrations are shown in Fig. 1, and the pharmacokinetic parameters are listed in Table 1. The blood concentration of compounds **1**–**5** all increased initially with time, then decreased gradually after C_{max} (maximum concentration). The C_{max} and AUC (area under concentration–time curve) increased with the elongation of the alkyl chain (compounds **1**–**3**), and then declined when the number of alkyl carbon atoms was more than eight (compounds **4** and **5**). Compound **3** showed the highest C_{max} and AUC, which were, respectively, 30 and 68 times higher than that of compound **1**. In addition, T_{max} (time of maximum concentration) and $t_{1/2}$ (half-life) of compounds **1**–**5** were delayed significantly with the increase of alkyl chain length.

The tested results of pharmacokinetic experiments demonstrated that all derivatives **2**–**5** could be transformed to cordycepin *in vivo* after oral administration. The blood concentration–time profiles of transformed cordycepin are shown in Fig. 2, and the pharmacokinetic parameters are listed in Table 1. It was indicated that the concentration of transformed cordycepin was proportional to its derivative and had similar change tendency, C_{max} and AUC of transformed cordycepin increased as the elongation of alkyl chain, and then decreased when the number of alkyl carbon atoms exceeded eight. But T_{max} and $t_{1/2}$ of transformed cordycepin were postponed monotonically with the increase of alkyl chain length, and T_{max} of transformed cordycepin was delayed for about 2 h as compared with its derivative.

4.3. Correlation of molecular structure and bioavailability

From above, it was indicated that carbon number of alkyl chain in derivatives (C_n) would have important effect on the AUC of transformed cordycepin. Since AUC is the most

important index of bioavailability, the correlation between C_n and AUC of transformed cordycepin was analyzed to study the structure–activity relation of derivatives. The scatter diagram on the change trend of C_n and AUC of transformed cordycepin was shown in Fig. 3, from which it was estimated that the regression equation on AUC and C_n might fit a quadratic objective function model. So, selected C_n as independent variable, AUC as dependent variable, quadratic as objective function model, the curve fit was conducted with SPSS10.0 and the results are shown as follows: $R^2 = .99595$; adjusted $R^2 = .99190$; standard error = 3.06911; F = 245.79731, significant F = .0041.

Analysis of variance			
	DF	Sum of squares	Mean square
Regression	2	4630.5292	2315.2646
Residuals	2	18.8388	9.4194

Variables in the equation					
Variable	B	SE (B)	β (T)	T	σ (T)
C_n	14.711465	.749341	3.087621	19.633	.0026
C_n**2	−.873574	.040146	−3.422224	−21.760	.0021
(Constant)	21.006729	2.712895		7.743	.0163

From above, the regression equation on AUC and C_n can be expressed as Eq. (1), and the equation is reliable and can be applied to predict AUC under different C_n.

$$\mathrm{AUC} = -0.8736C_n^2 + 14.7115C_n + 21.0067 \quad (C_n = 0, 1, 2, \ldots, 18) \tag{1}$$

The differential equation of Eq. (1) was calculated as below.

$$d(\mathrm{AUC})/d(C_n) = -2 \times 0.8736C_n + 14.7115 \tag{2}$$

For Eq. (2), when $d(\mathrm{AUC})/d(C_n) = 0$, $C_n = 8.4$. So, when C_n was 8, AUC would be up to the maximal value 82.8 μmol h/L, which was very close to the experimental value 83.7 μmol h/L. Thus the optimal derivative of *N*-alkyl modification of cordycepin for high bioavailatility must be *N*-octanoyl-cordycepin.

5. Conclusion

Above results show that a small amount of cordycepin could be absorbed systemically being totally oxidized by ADA within 5 h after oral administration of cordycepin. But after *N*-alkyl modification with an normal alkyl chain, the T_{max} and $t_{1/2}$ of derivatives could be increased monotonically as the increase of its alkyl chain length, and the C_{max} and AUC increased initially and then decreased when the number of alkyl carbon atoms exceeded eight. It was suggested that alkyl modification would increase the hydrophobic activity of compound, thus increase the interaction between cell membrane and certain protein or lipid [12]. Therefore, the C_{max} and AUC of compounds **2** and **3** increased. However, too long alkyl could make the interaction among them stronger, causing the decrease of compound entering into blood. Consequently, the C_{max} and AUC of compound **5** were far lower than that of compound **1**. All derivatives could be transformed into cordycepin. The concentration of transformed cordycepin was proportional to that of its derivative. Present result shows that *N*-alkyl modification with a normal alkyl chain would protect NH_2 of cordycepin from fast oxidization *in vivo* and change the bioavailability of cordycepin greatly. *N*-octanoyl-cordycepin could decrease the metabolic velocity and increase the bioavailability of cordycepin to the maximum, thus it might be a promising prodrug of cordycepin.

6. Materials and methods

6.1. Chemicals and apparatus

Cordycepin standard was purchased from Sigma Chemical Co. (USA). Cordycepin used for synthesis was extracted from *C. militaris*, its purity was more than 98.5% analyzed by HPLC. Propionyl chloride, *n*-octanoyl chloride, lauroyl chloride and stearoyl-chloride were purchased from Generay Biotech (Shanghai) Co. Ltd. (China). All reagents were of analytical grade.

Melting points, UV, IR, ^{1}H NMR and TLC were used to identify structures of new compounds. Melting points were tested on a Buchi B-540 melting point apparatus. UV spectra were recorded on a Hitachi U-1800 UV–vis apparatus, IR spectra on a Perkin Elmer IR apparatus and ^{1}H NMR spectra on a Bruker Model Avance DMX300 Spectrometer (300M) using TMS as an internal standard and DMSO-d_6 or $CDCl_3$ as solvent. Element analysis was conducted on a Perkin Elmer 2400 element analyzer.

6.2. Synthesis of new compounds

Propionyl-, octanoyl-, lauroyl- or stearoyl-chloride (0.01 mol) was slowly dropped into compound **1** (0.009 mol) in 200 mL

Table 1
Pharmacokinetic parameters after oral administrations of 0.5 μmol/kg compounds **1**–**5**

Compound	Derivatives				Transformed cordycepin			
	T_{max} (h)	C_{max} (μmol/L)	AUC (μmol h/L)	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (μmol/L)	AUC (μmol h/L)	$t_{1/2}$ (h)
1	1.5^e	10.1^d	19.2^e	2.1^d	1.5^e	10.1^c	19.2^d	2.1^d
2	3.0^d	120.5^c	356.3^c	4.4^c	5.0^d	14.1^b	59.9^c	7.5^c
3	5.0^c	310.2^a	1307.5^a	6.8^b	7.0^c	20.6^a	83.7^a	9.4^b
4	6.0^b	160.1^b	671.5^b	8.3^a	8.0^b	15^b	69.1^b	10.5^a
5	8.0^a	22.1^d	32.5^d	8.6^a	10.0^a	2.2^d	3.7^e	10.8^a

Note: data in the table followed with different letters are significantly different at $P = 0.05$, F-test, $n = 5$.

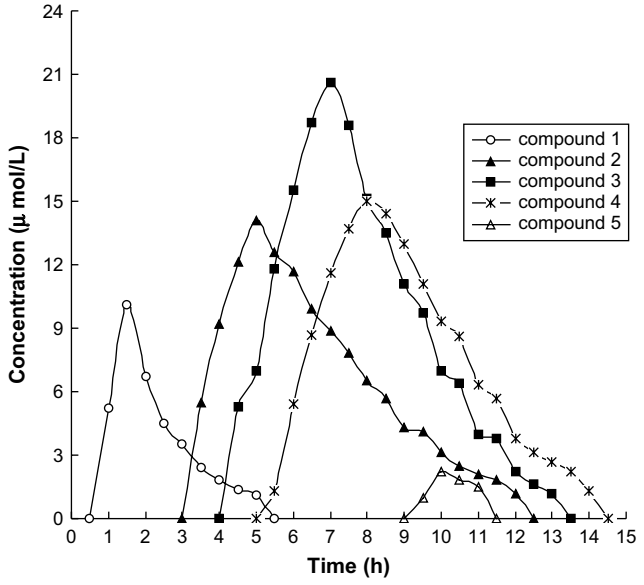


Fig. 2. Mean blood concentration–time profiles of transformed cordycepin from compounds **2**–**5** as compared with compound **1** after oral administrations (500 μmol/kg), each point represents the arithmetic mean ($n = 5$).

dehydrated pyridine at 60–70 °C under magnetic stirring and nitrogen protection. After 10 h of reflux, the reaction solution was concentrated and dissolved in 50 mL EtOAc with 5% HAc, then purified by a silica gel column (3 × 75 cm, 100 mL of 200–300 mesh silica gel, the eluent was 400 mL EtOAc with 5% HAc, followed by 200 mL MeOH). The resulting eluent was concentrated and crystallized at 0 °C to get the corresponding products.

Compound **1** was obtained as white needles or flakes from H_2O; mp 229–230 °C; R_f 0.25, silica gel-GF_{254}, EtOAc/MeOH/NH_4OH (99:10:1); UV (MeOH) λ_{max} (log ϵ) 213 (3.87), 260 (4.09) nm; IR (KBr)ν_{max} 3332, 3142, 1672, 1608, 1479, 1420, 1385, 1340, 1307, 1209, 1110, 1059, 1081, 1038, 1008, 727 cm^{-1}; ^1H NMR (DMSO-d_6, 300 MHz) δ 8.37(1H, s, 2-H), 8.15(1H, s, 8-H), 5.96–5.86 (3H, m, 1′-H, 2′-H, 4′-H), 5.17–5.13 (2H, d, 5′-OH, 2′-OH), 3.71–3.67 (2H, d, 6-NH_2), 2.29–2.20 (2H, m, 5′-CH_2), 1.92 (2H, m, 3′-CH_2); Anal. calcd for $C_{10}H_{13}N_5O_3$: C 47.81, H 5.22, N 27.88, O 19.10; found: C 47.75, H 5.16, N27.901, O19.16.

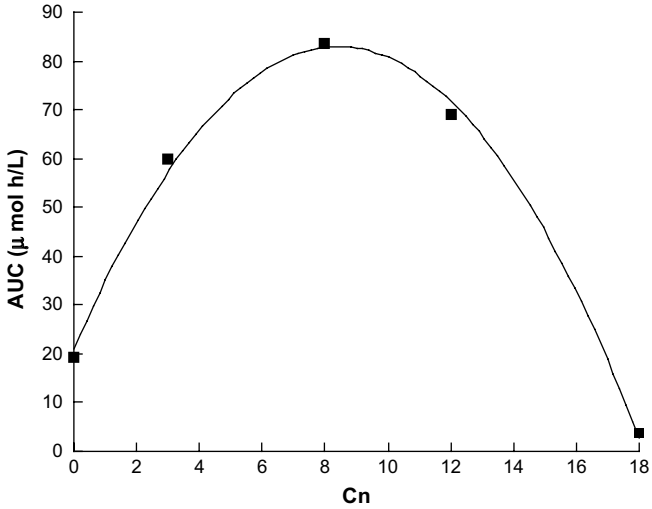


Fig. 3. Scatter diagram of AUC and C_n.

Compound **2** was obtained as white flakes from H_2O; mp 177–179 °C; R_f 0.37, silica gel-GF_{254}, EtOAc/MeOH/NH_4OH (99:10:1); Yield 75.5%; UV (MeOH) λ_{max} (log ϵ) 213 (4.05), 260 (3.87) nm; IR (KBr)ν_{max} 3378, 2933, 1741, 1632, 1544, 1478, 1375, 1287, 1099, 839, 722 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.62 (1H, m, 6-NH–CO–), 8.44 (1H, s, 2-H), 8.23 (1H, s, 8-H), 5.83–5.65 (3H, m, 1′-H, 2′-H, 4′-H), 4.36–4.23 (2H, m, 5′-OH, 2′-OH), 2.39–2.29 (4H, m, 3′-CH_2, 5′-CH_2), 1.59 (2H, m, –CH_2), 1.17–1.12 (3H, t, –CH3, $J = 7$ Hz); Anal. calcd for $C_{13}H_{17}N_5O_4$: C 50.81, H 5.58, N 22.79, O 20.82; found: C 51.05, H 5.51, N 22.81, O 19.95.

Compound **3** was obtained as white flakes from EtOAc; mp 139–141 °C; R_f 0.45, silica gel-GF_{254}, EtOAc/MeOH/NH_4OH (99:10:1); Yield 64.3%; UV (MeOH)λ_{max} (log ϵ) 213 (4.12), 260 (3.68) nm; IR (KBr)ν_{max} 3323, 2928, 1742, 1632, 1546, 1456, 1380, 1287, 1096, 842, 722 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.60 (1H, m, 6-NH–CO–), 8.22 (1H, s, 2-H), 8.01 (1H, s, 8-H), 5.92–5.86 (3H, m, 1′-H, 2′-H, 4′-H), 4.39–4.26 (2H, m, 5′-OH, 2′-OH), 2.32–1.23 (16H, m, –CH_2–), 0.87–0.85 (3H, t, –CH_3, $J = 8$ Hz); Anal. calcd for $C_{18}H_{27}N_5O_4$: C 57.28, H 7.21, N 16.96, O 18.56; found: C 57.37, H 7.17, N 17.03, O 18.48.

Compound **4** was obtained as ivory white needles or flakes from EtOAc; mp 105–106 °C; R_f 0.67, silica gel-GF_{254}, EtOAc/MeOH/NH_4OH (99:10:1); Yield 45.7%; UV (MeOH)λ_{max} (log ϵ) 213 (4.16), 261 (3.87) nm; IR (KBr)ν_{max} 3396, 2935, 1737, 1632, 1544, 1459, 1378, 1288, 1098, 899, 722 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.73 (1H, m, 6-NH–CO–), 8.34 (1H, s, 2-H), 8.19 (1H, s, 8-H), 5.87–5.66 (3H, m, 1′-H, 2′-H, 4′-H), 4.39–4.33 (2H, m, 5′-OH, 2′-OH), 2.36–1.25 (24H, m, –CH_2–), 0.87–0.85 (3H, t, –CH_3, $J = 6$ Hz); Anal. calcd for $C_{22}H_{35}N_5O_4$: C 60.95, H 8.14, N 16.15, O 14.76; found: C 60.88, H 8.11, N 16.05, O 14.55.

Compound **5** was obtained as ivory white flakes from EtOAc; mp 101–103 °C; R_f 0.68, silica gel-GF_{254}, EtOAc/MeOH/NH_4OH (99:10:1); Yield 34.5%; UV (MeOH)λ_{max} (log ϵ) 212 (4.07), 261 (3.98) nm; IR (KBr)ν_{max} 3424, 2924, 2854, 1745, 1632, 1545, 1454, 1374, 1284, 1096, 795, 721 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 8.66–8.64 (1H, m, 6-NH–CO–), 8.31 (1H, s, 2-H), 8.06 (1H, s, 8-H), 5.91–5.85 (3H, m, 1′-H, 2′-H, 4′-H), 4.39–4.22 (2H, m, 5′-OH, 2′-OH), 2.36–1.23 (36H, m, –CH_2–), 0.90–0.85 (3H, t, –CH_3, $J = 7.5$ Hz); Anal. calcd for $C_{28}H_{47}N_5O_4$: C 64.96, H 9.15, N 13.53, O 12.36; found: C 64.55, H 9.11, N 13.50, O 12.29.

6.3. HPLC analysis

To determine the recovery of compounds **1**–**5** from mice blood, 0.5, 1.0 or 1.5 μmol/L of standard compounds **1**–**5**

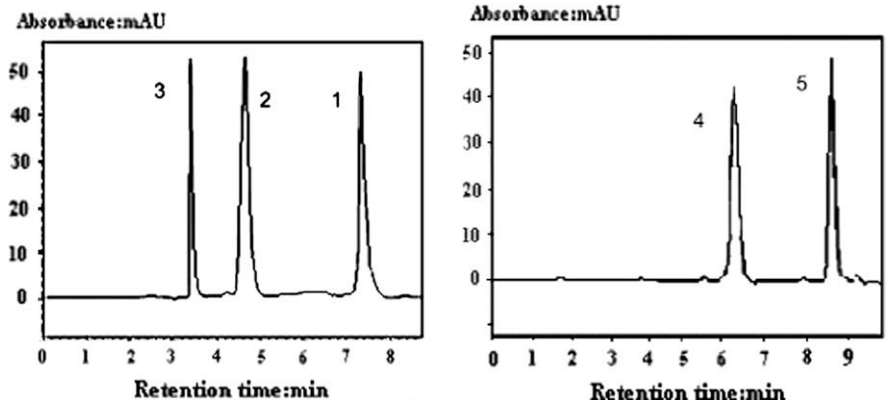


Fig. 4. HPLC spectra of cordycepin standard (**1**) and synthesized *N*-propionyl- (**2**), *N*-octanoyl- (**3**), *N*-lauroyl- (**4**) and *N*-stearoyl- (**5**) cordycepin.

were added to mice blood, respectively, and extracted with MeOH (for compounds **1** and **2**) or EtOAc (for compounds **3**–**5**). After centrifuged, the supernatant was used to measure their concentration by HPLC.

HPLC was carried out using an LC-6AD pump and a PREP-ODS C18 column (Shimadzu, Japan). Mobile phases for compounds **1**–**3** were the mixture of methanol/water (25:75, v/v), and those for compounds **4** and **5** were MeOH, flow rate 1 mL/min, column temperature at 30 °C. Absorbance was detected at 260 nm using a variable wavelength detector (SPD-M20A, Japan).

The HPLC spectra of cordycepin standard and synthesized compounds **2**–**5** are shown in Fig. 4. The extract recovery of compounds **1**–**5** was higher than 85%. So, the concentration of all blood samples could be measured by the above method.

6.4. Data analysis

The pharmacokinetic parameters were estimated using the Bioavailability Program Package (BAPP, Version 2.0, China Pharmaceutical University). C_{max} and T_{max} were obtained directly from the observed concentration–time data. The relationship between AUC and C_n was analyzed with SPSS10.0 statistical software.

Acknowledgements

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