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RESEARCH ARTICLE

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^{1}H nuclear magnetic resonance relaxation investigation of huperzine E binding to acetylcholinesterase

Abstract In order to search for better acetylcholinesterase (AchE) inhibitors, the binding properties of AchE with huperizine E, which is a derivative of huperzine A, were investigated with ^{1}H nuclear magnetic resonance (^{1}H NMR) method. The nonselective, selective and double-selective spin-lattice relaxation rates of some protons in huperzine E were acquired in the absence and presence of AchE at a concentration ratio of [ligand]/[protein] $= 1:0.005$. The enhancements of selective relaxation rates of these protons were obvious after adding AchE. The molecular motional correlation times of two pairs of protons, H-1a/H-1b and H-2/H-3, in the bound state at $T = 298$ K were 11.7 and 9.46 ns respectively, while they were 27.7 and 35.2 ps in the free state. All of these show that huperzine E has high binding affinity with AchE.

Keywords huperzine E, acetylcholinesterase, proton spin-lattice relaxation rate, molecular motional correlation time, nuclear magnetic resonance (NMR)

1 Introduction

Alzheimer' disease (AD) is a kind of disease which causes the loss of capabilities of memory, cognition and behavior by its harmful effect on the brain [1]. At present, the inhibitors of acetylcholinesterase (AchE) have been one of the most significant drugs in clinic treatment of AD (at the extent of light to middle) [2]. Tacrine (Fig. 1(a)), the first drug as AchE inhibitor to treat AD, offered in the American market in 1993, was found to have a toxic side effect severely on the liver of human body during its following application. In 1997, donepefil (E2020) (Fig. 1(b)), as a similar drug of Tacrine, had been launched in the market of UK and America under the cooperation of American and Japanese scientists. However, it still showed the toxic effects to some extent. Therefore, developing high effective but low poisonous inhibitor for AD has been the global focus of the scientific research.

Huperzine A (Fig. 1(c)), a natural product separated first from the Chinese traditional medicine moss *Huperzia serrata* of *Lycopodium* species (*Huperziaceae*) by Chinese Scientists, has been identified as an effective, low poisonous, reversible and high selective inhibitor against AchE, with its inhibition constant $Ki = 0.024\ \mu\text{mol}\cdot\text{L}^{-1}$ [3]. Due to its better performance on therapy and toxicity than two former counterparts, Tacrine and E2020, huperzine A could be ranked as one ideal candidate on AD motif. The binding mechanism between huperzine A and AchE had been reported in previous literature, and the key binding sites in active pocket of AchE had also been described in the well-resolved crystal structure in their bound state [4] (Fig. 2).

Nevertheless, the fact of side effect at certain degree on the human body involving huperzine A accelerates many nations over the world, by taking it as the leading compound, to investigate its total synthesis technique, and develop its derivates and analogues for the sake of obtaining potential drug molecules of much better attributes. Under this motivation, it is necessary to employ a ready approach for screening valuable inhibitors of AchE from a vast number of huperzine A analogues. Ellman's colorimetry is one method widely adopted in present research. Yet, it defeats in the higher cost of enzyme and the lack of stability during the procedure of colorimetry [5]. Besides the traditional approach, some newer ones have been developed already by many research groups. For instance, Han et al. [6] studied the interaction between complex of huperzine A and AchE by calculating their binding free energy.

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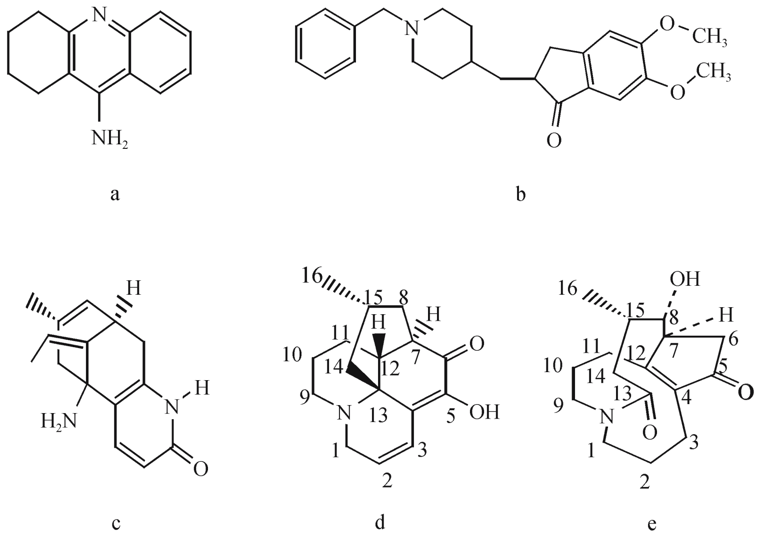

Fig. 1 Structures of compounds

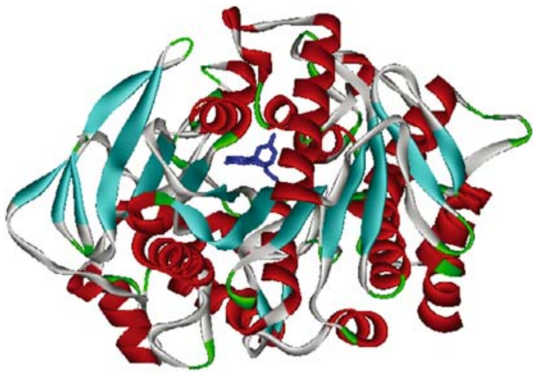

Fig. 2 Crystal structure of AchE with Huperzine A (PDB code 1GPK)

In this paper, the ^1H nuclear magnetic resonance (^1H NMR) approach was used to screen the potential AchE inhibitors by investigating the binding state of sample compound with AchE. The advantages of this approach could be as follows. (1) It is easy and prompt to be manipulated. It needs only to mix the compound molecule with enzyme in solution for direct NMR analysis, which avoids rigid experimental conditions and other reagents being used. And the result could be obtained within short time period. (2) The screening of several components against enzyme could be realized via various NMR signals of sample in the absence and presence of enzyme, to figure out the compound bound to enzyme. (3) It needs less consumption of enzyme, which has been proved that, even if the concentration of macromolecule is 0.5% relative to small molecule ligand, the ^1H spin-lattice relaxation rate of ligand is still a very suitable parameter to evaluate the interaction between the ligand and macromolecule [7]. Recently, this parameter has been widely applied to investigate the interactions of Tacrine derivatives and huperzine A with AchE [8,9]. This work applied NMR method to study huperzine E (Fig. 1(d)), a new analogue of huperzine A, binding to AchE, so as to verify the method to be a screening way of *Lycopodium* alkaloids as AchE inhibitors.

2 Experimental

2.1 Reagents and apparatus

The ^1H NMR spectrum was acquired on a Bruker Avance 500 MHz NMR spectrometer, Switzerland. Samples were weighed in a DT-100 optical balance, Beijing Optical Instruments Factory. Huperzine E was provided by Shanghai Institute of Materia Medica, Chinese Academy of Science. AchE was purchased from Sigma and used without further purification. D_2O (99.8%) was from Beijing Chemical Factory. K_2HPO_4, in AR grade, was from YiLi Refined Chemical Limited Corporation of Beijing. KH_2PO_4, whose purity is larger than 99.5%, was from Shanghai Sangon Biological Engineering Technology & Service Co. Ltd.

2.2 Sample preparation

Huperzine E was dissolved in 50 mM phosphate D_2O buffer at pH = 7 with its concentration being 1.0 mM in solution. Two huperzine E samples were prepared: one without AchE was set as reference, another with AchE was set to keep the concentration of AchE at 5.0 μM. Then the samples were moved to 5 mm NMR tube with a label each.

2.3 NMR analysis

All measurements were performed on a Bruker Avance 500 MHz NMR spectrometer operating at 500 MHz. The acquisition temperature was set at 298 K. The ^1H 90° pulse width was 9.0 μs with pulse repeat interval time 8.3 s. The ^1H NMR spectrum width was 5,000 Hz, and 16 scans were collected into 32 K data points. To process the data and calculate relaxation times, the XWinnmr program package was used on a Microsoft Windows PC. Chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). After ^{1}H NMR spectrum of pure huperzine E sample was acquired, the resonances signals were assigned. Their chemical shifts are consistent with the reported values [10]. In Fig. 3(a), the peaks ranging from δ 1.6 to δ 1.7 are overlap signals of protons H-10, H-12 and H-15. For another sample with concentration ratio of [protein]/[ligand] = 0.005 : 1, the NMR spectrum has also been acquired (Fig. 3(b)). The peak $\delta = 3.65$ in Fig. 3(b) is assigned as hydroxymethyl protons of Tris component from AchE sample.

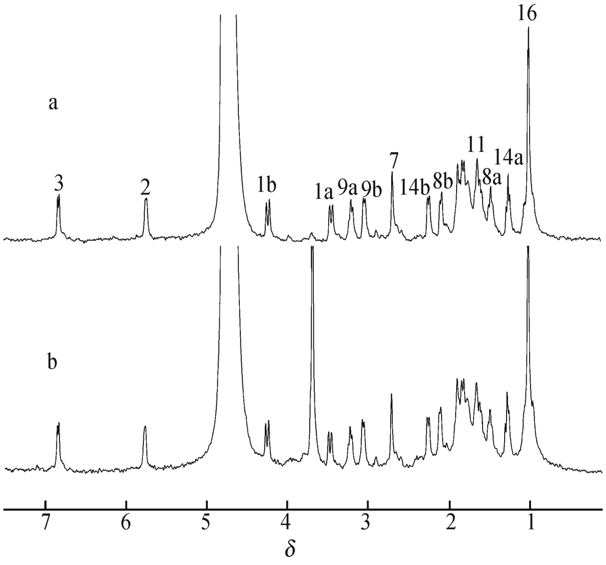

Fig. 3 ^{1}H NMR spectra of huperzine E and peaks assignments
a) Free huperzine E; b) huperzine E with AchE

2.4 Measurements of spin–lattice relaxation rates

The inversion recovery NMR experiment was utilized to observe the nonselective spin-lattice relaxation rates (R^{ns}) of huperzine E for both huperzine E samples (with or without AchE). And the 180° selective pulse was used to monitor the selective spin–lattice relaxation rates (R^S) of huperzine E at both states for protons H-1a, H-1b H-2, H-3 and M-16. In consideration of peaks splitting and the existence of dipole-dipole interaction, the two proton pairs, H-1a/H-1b and H-2/H-3, were observed. Besides, only the protons of M-16 is selected to observe from the rest of proton signals.

2.5 Molecular motional correlation time (τ_C)

The nonselective spin–lattice relaxation rates were measured using the 180°-τ-90° inversion recovery pulse sequence. The τ values used for the nonselective and selective experiments were: 0.01, 0.1, 0.2, 0.3, 0.5, 0.8, 1, 2, 3, 6 and 10.0 s sequentially, and the delay time t was 5 s. The exponential equation, $I = I_o[1 - \exp(-R^{ns} \cdot t)]$, was adopted to perform the single exponent regression and obtain the proper R^{ns}. The item I in equation is the peak integral area of inversion recovery regarding the definite value of τ.

The selective spin-lattice relaxation rates were measured using the 180°-τ-90° inversion recovery pulse sequence. The width of 90° pulse for ^{1}H is 9.0 μs. The 180° selective inversion of the proton spin population was obtained by a selective Gauss1.100 shape pulse with a length of 47 ms corresponding to an excitation width of about 45 Hz. The other parameters used were the same as in R^{ns} experiments.

τ_C, relating to the disordered molecular motion, could be considered as the average time of interval during collisions when the molecules are in translation, or as the average time of rotating a radian when molecules are in tropism. In solution, the molecular motional correlation time τ_C relies on a series of factors, such as the bulk and the symmetry of molecule, also the viscosity of solution, and so on. τ_{ij} is the motional correlation time of vector, according to Eq. (1), it could be obtained by determining crossing relaxation rate of Hi/Hj proton pair at an invariable distance [10].

$$\sigma_{ij} = R_i^{ij} - R_i^{s} = \frac{1}{10}\frac{\gamma^4\hbar^2}{r_{ij}^6}\left\{\frac{6\tau_{ij}}{1+4\omega^2\tau_{ij}^2} - \tau_{ij}\right\} \tag{1}$$

where γ is the proton magnetogyric ratio, $\hbar$ is the reduced Plank's constant ($= h/2\pi$), r_{ij} is the Hi-Hj internuclear distance, ω is the proton Larmor frequency ($\omega = 3.141\,6 \times 10^9$ rad·s^{-1},when magnetic field intensity is 500 MHz).

To further study the behavior of huperzine E interacting with AchE, the double irradiation method was applied to determine the molecular crossing relaxation rate, then to obtain the motional correlation time by Eq. (1).

2.6 Negative control experiment

In the case of small molecule ligand adhering to the surface of protein other than binding into its inner active site, the relaxation property could be influenced through the change of molecular motility due to the existence of protein. To clarify whether the ligand has truly bound to the active site of protein, and consequently exclude false relaxation phenomenon arising from the adhesion of ligand to protein, it is necessary for us to measure the proton relaxation change in the presence and absence of AchE for another molecule, 8-exo-hydroxyphlegmariurine (Fig. 1(e)), similarly separated from the *huperzia serrata* as huperzine E and sharing the approximate bulk but distinct structure between the two.

3 Results and discussion

3.1 Optimization of huperzine E concentration for NMR measurements

The various states of huperzine E self-aggregation in solution will lead to the drift of chemical shift of compound, and then influence the relaxation time of protons in huperzine E, which interfere with the determination of relaxation rate in the presence of AchE.

Therefore, it needs to determine the chemical shift at various concentration of huperzine E in the absence of AchE to identify the aggregation state of small molecule. Focused on the change of chemical shift of H-3 proton of huperzine E at a range of compound concentration from 0.2 to 30.0 mM, it showed that the chemical shift of H-3 moves to upfield gradually with the increase of the concentration, which indicates that there is an aggregation at high concentration of huperzine E. However, below the concentration of 1 mmol · L^{-1}, there is no obvious change of chemical shift. Hence, the compound concentration of 1 mM was selected for NMR measurements so that the relaxation rate will not be perturbed by self-aggregation of the compound in solution. For the 8-exo-hydroxyphlegmariurine, the same concentration was employed as for huperzine E.

3.2 Determination of molecular motional rate

The nonselective relaxation rate of proton (R^{ns}) is the relaxation rate of each proton in one molecule under simultaneous irradiation. The selective relaxation rate (R^{s}) is the one when a specific proton is only irradiated. The relationship between R^{ns} and R^{s} could be described as follows

$$R_i^{\mathrm{ns}} = \sum_{j\neq i} \rho_{ij} + \sum_{j\neq i} \sigma_{ij} + \rho_i^* \tag{2}$$

$$R_i^{\mathrm{s}} = \sum_{j\neq i} \rho_{ij} + \rho_i^* \tag{3}$$

In Eqs. (2) and (3), ρ_{ij} is the auto-relaxation rate contributed from H_{i} energy transfer after irradiation to other protons which have dipole-dipole interaction with H_{i}. σ_{ij} is the cross relaxation rate contributed from two dipole-dipole interactive protons after both of them are irradiated. ρ_i^* is the H_{i} auto-relaxation rate contributed from other kinds of relaxation mechanism. By comparing Eqs. (2) with (3), the H_{i} is only irradiated for R^{s} in Eq. (3), there is not the item σ_{ij} which reflects the influence of other irradiated protons on H_{i}.

In this work, the relaxation rates of H-1a, H-1b, H-2, H-3 and M-16 of huperzine E are observed when the compound is free or bound to AchE. For other protons, due to overlap of peaks and multiple split, they could not be detected well by suitable 180° soft pulse. The chemical shifts and the relaxation rates of observed protons are listed in Table 1. The experimental results show that, after adding enzyme to huperzine E solution, the chemical shifts of protons keep constant, which indicate that chemical shifts are not influenced in the presence of AchE. However, the relaxation rates of all the protons experience a significant change after mixing with AchE. However, all the protons relaxation rates have changed at different extent. Moreover, the increase in R^{s} is obviously stronger than R^{ns}. This is because the binding of small molecule with macromolecule leads the σ_{ij} to be minus, which could cancel the enhancement of relaxation rate arising from the ρ_{ij}. The clear increase in R^{s} manifests that huperzine E has bound to the enzyme. The influence order of R^{s} is: H-1b > H-1a > H-2 > H3 > M-16 (Fig. 4), which illustrates that the unsaturated conjugated ring portion containing acylamide group of huperzine E might interact more strongly with AchE than other parts.

Table 1 500 MHz ^{1}H NMR parameters of huperzine E (1 mmol · L^{-1}) in D$_2$O buffered at pH 7.0, $T = 298$ K in the absence and presence of AchE (5 μmol · L^{-1})

Proton	Huperzine E				Huperzine E + AchE			
	δ	R^{ns}/s^{-1}	R^{s}/s^{-1}	R^{ij}/s^{-1}	δ	R^{ns}/s^{-1}	R^{s}/s^{-1}	R^{i}/s^{-1}
H-1a	3.37	2.331	1.639	2.042	3.37	2.140	2.051	2.203
H-1b	4.17	2.284	1.546	1.958	4.17	2.031	2.283	2.518
H-2	5.71	0.710	0.583	0.631	5.71	0.707	0.773	0.808
M-3	6.82	0.491	0.405	0.476	6.82	0.526	0.565	0.625
M-16	0.92	1.934	1.894	–	0.92	1.758	1.962	–

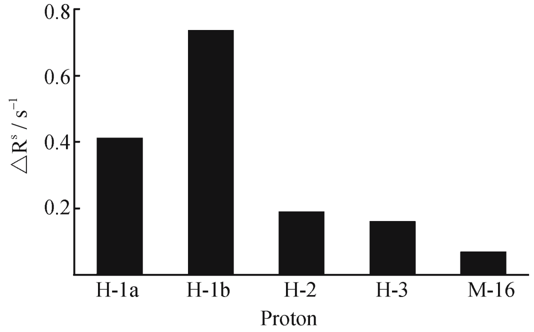

Fig. 4 Increments of selective relaxation rate of huperzine E (1 mmol · L^{-1}) in the presence (5 μmol · L^{-1}) and absence of AchE (500 MHz, solvent: D$_2$O, pH 7.0, $T = 298$ K)

3.3 Molecular motional correlation time (τ_{ij})

As shown in Eqs. (2) and (3), the cross relaxation rate of protons $\sigma_{ij} = R_i^{ij} - R_i^{\mathrm{s}}$. R_i^{ij} is the relaxation rate measured when both H_{i} and its coupled proton H_{j} are irradiated. R_i^{s} is the relaxation rate that only proton H_i is irradiated. Hence, according to Eq. (1), the motional correlation time $\boldsymbol{\tau_{ij}}$ for proton pair $H_{\mathrm{i}}/H_{\mathrm{j}}$ could be obtained.

It has been found that when enzyme interacts with small molecule, a rapid dynamic equilibration is set up, the system is indicative of quick binding and slow dissociation. Then Eq. (4) can be established

$$\sigma_{\mathrm{obs}}^{ij} = p_{\mathrm{free}} \sigma_{\mathrm{free}}^{ij} + p_{\mathrm{bound}} \sigma_{\mathrm{bound}}^{ij} \tag{4}$$

where p_{free} is the fraction of unbound ligand in solution; p_{bound} is the bound fraction; $\sigma_{\mathrm{obs}}^{ij}$ is the cross relaxation rate in the presence of enzyme; and $\sigma_{\mathrm{free}}^{ij}$ is the cross relaxation rate which is observed without enzyme. That means, $\sigma^{ij}_{\text{obs}} = R_i^{ij}(\text{hupE}+\text{AchE}) - R_i^{s}(\text{hupE}+\text{AchE})$, $\sigma^{ij}_{\text{free}} = R_i^{ij}(\text{hupE}) - R_i^{s}(\text{hupE})$, $\sigma^{ij}_{\text{bound}}$ is the calculated cross relaxation rate in the case of the complete binding state.

If $\sigma^{ij}_{\text{bound}}$ and $\sigma^{ij}_{\text{free}}$ are considered by the most extreme situation, which means small molecule binds to enzyme completely, $p_{\text{bound}} = (\text{protein})/[\text{ligand}] = 0.5\%$, $p_{\text{free}} = 1 - p_{\text{bound}} \approx 1$, and the $\sigma^{ij}_{\text{bound}}$ is figured out by Eq. (4) (see Table 2). Then, simultaneously combining with Eq. (5), the motional correlation time ($\boldsymbol{\tau_{ij}}$) for a small molecule after its bound to enzyme could be obtained.

$$\sigma^{ij}_{\text{bound}} = \left(\sigma^{ij}_{\text{obs}} - \sigma^{ij}_{\text{free}}\right)/p_{\text{bound}} = \frac{1}{10}\frac{\gamma^4\hbar^2}{r_{ij}^6}\left\{\frac{6\tau_{ij}}{1+4\omega^2\tau_{ij}^2} - \tau_{ij}\right\} \quad (5)$$

The τ_{ij} is solved out, when setting the corresponding value of σ_{ij} and the other constants based on Eq. (1).

Table 2 500 MHz ^{1}H NMR molecular motion rates of huperzine E (1 mmol·L^{-1}) in D_2O buffered at pH 7.0, $T = 298$ K in the free and in the presence of AchE (5 μmol·L^{-1})

Proton/Proton	r_{ij} /nm	$\sigma^{ij}_{\text{free}}$ /s^{-1}	τ^{ij}_{free} /ps	σ^{ij}_{obs} /s^{-1}	$\Delta\sigma^{ij}$ /s^{-1}	$\sigma^{ij}_{\text{bound}}$ /s^{-1}	τ^{ij}_{bound} /ns
H-1a/H-1b	0.163	0.412	27.7	0.235	−0.177	−35.4	11.7
H-2/H-3	0.243	0.048	35.2	0.035	−0.013	−2.6	9.46

It can be seen from the data that the correlation relaxation rate σ_{ij} has been varied from a smaller positive value when it is free of binding to enzyme, to a larger negative value when it binds to the enzyme. It reveals that, after huperzine E binds to AchE, its motional correlation rate has slowed down, and resulted in the change of $\omega\tau$ from the value less than 1 to the value larger than 1. Herein, the proton pairs H-1a/H-1b and H-2/H-3 are selected as representations to calculate the motional correlation time. The results show that their τ_{ij} have changed from 27.7 ps for H-1a/H-1b and 35.2 ps for H-2/H-3 in the absence of enzyme to 11.7 ns for H-1a/H-1b and 9.46 ns for H-2/H-3 in the presence of enzyme at $T = 298$ K, which demonstrates that huperzine E has bound to AchE.

In huperzine E, H-2 and H-3, a coupling proton pair, link to adjacent carbons located at the conjugated ring. After binding to AchE, the τ_{ij} of this proton pair is 9.46 ns at $T = 298$ K. By comparison, the τ_{ij} of same proton pair in huperzine A is 40.5 ns [9] after binding to the enzyme. It illustrates that the binding affinity of huperzine E to enzyme is weaker than huperzine A in *vitro*. The question, whether it is feasible to compare the $\tau_{2,3}$ of counterpart proton pair between different molecules so as to elucidate the binding constant, still needs to be proved by kinetic study on inhibition mechanism of huperzine E to AchE.

In huperzine E, H-1a and H-1b are the homocarbon protons. For the well distinguished chemical shifts of them, it is available to employ the proper 180° selective pulse to irradiate the two resonances. The nonselective, selective and double selective spin lattice relaxation rates of H-1a and H-1b are observed. Furthermore, according to Eq. (5), the motional correlation time $\boldsymbol{\tau_{ij}}$ after binding to enzyme is solved out at $T = 298$ K. The $\tau_{1a,1b} = 11.7$ ns ($\sigma^{1a,1b} = -35.4$ s^{-1}, $r_{1a,1b} = 0.163$ nm) for this proton pair, and the value is reasonable in comparison with $\tau_{2,3}$. This is the first example to use the coupled proton pair locating at homocarbon to investigate the motional correlation time by NMR relaxation method.

3.4 Measurement of negative control experiment

To discern whether huperzine E has truly bound to the active site of protein, and exclude the probability that adhesion results in the change of relaxation rate, the 8-exo-hydroxyphlegmariurine, a compound similarly separated from the *huperzia serrata*, was used as negative control to observe the property of relaxation rate in the presence and absence of AchE (data shown in Table 3 and Fig. 5). The

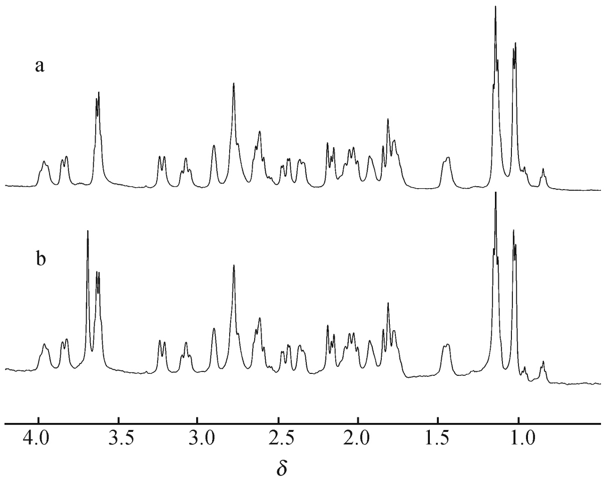

Fig. 5 ^{1}H NMR spectra of 8-exo-hydroxyphlegmariurine
a) Free 8-exo-hydroxyphlegmariurine; b) 8-Exo-hydroxyphlegmariurine with AchE

Table 3 500 MHz ^{1}H NMR parameters of 8-exo-hydroxyphlegmariurine (1 mmol·L^{-1}) in D_2O buffered at pH 7.0, $T = 298$ K in the free and in the presence of AchE (5 μmol·L^{-1})

Proton	8-exo-hydroxyphlegmariurine			8-exo-hydroxyphlegmariurine + AchE		
	δ	R^{ns}/s^{-1}	R^{s}/s^{-1}	δ	R^{ns}/s^{-1}	R^{s}/s^{-1}
H-7	3.08	1.542	1.211	3.08	1.556	1.119
M-16	1.05	1.721	1.584	1.05	1.707	1.546

experimental data disclose that the relaxation properties of H-7 and M-16 in this molecule do not change obviously in the presence or absence of AchE. The R^s of H-7 in 8-exo-hydroxyphlegmariurine is (1.211 ± 0.052) s^{-1} in the absence of AchE, and for M-16, $R^s = (1.584 \pm 0.064)$ s^{-1}; however, in the presence of AchE, R^s is $(1.119 \pm 0.034\,6)$ s^{-1} for H-7, and R^s is (1.546 ± 0.076) s^{-1} for M-16, which show that there is no significant change observed. Therefore, it is concluded that 8-exo-hydroxyphlegmariurine does not bind to AchE.

4 Conclusions

This work has reported that the presence of AchE (5 μmol · L^{-1}) leads to the significant increase in the selective spin-lattice relaxation rates (R^s) and its motional correlation time (τ_{ij}) of huperzine E (1 mmol · L^{-1}), which indicates that huperzine E is of stronger binding with AchE, and worthy of further pharmacological research. Meanwhile, it suggests that the application of NMR spin-lattice rate is a promising approach in the study on interactions between small molecule and protein, and the screening of potential AchE inhibitors. This method is simple and sensitive. It only needs to analyze mixture solution of compound and AchE by NMR, and the results of experiments come out promptly. Hence, it is advisable to apply this method to study the interactions of other enzymes and their inhibitors.

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