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Discussion

Huperzine A–human serum albumin association: Chromatographic and thermodynamic approach

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Abstract

The synthesis of six new huperzine analogues was reported. Each product presents an amidification of the free amine on huperzine A. The synthesis strategy of these new huperzine A derivatives is based on a condensation with an acyl anhydride. The binding on HSA of two molecule series (huperzine and benzodiazepine, respectively) was investigated with high performance liquid affinity chromatography (HPLAC) using an HSA column. A thermodynamic approach showed that binding huperzine A on HSA involved hydrophobic and Van der Waals interactions. A comparative thermodynamic study with benzodiazepine molecules was carried out to determine the potential binding site of huperzine derivatives on HSA.

Keywords: Thermodynamic; Huperzine; HPLC; Human serum albumin

1. Introduction

Alzheimer's disease is a common age-related neurodegenerative pathology with neurological and psychiatric manifestations. There is an acetylcholine decrease in the brain of sick person. One treatment approach was to inactivate acetylcholinesterase (AchE). This enzyme degrades synaptic acetylcholine. Many medicinal agents, as donepezil or rivastigmine, used for treatment of Alzheimer's disease, belong to the important class of acetylcholinesterase inhibitors [1].

Huperzine A, an alkaloid extracted from Huperzia serrata, is a novel generation of powerful and selective AchE inhibitor. This well tolerated Lycopodium was discovered in Chinese folk medicine and its properties may be especially appropriate for Alzheimer's disease healing [2–5].

The drug–protein binding in blood plays an important role in pharmacokinetic and pharmacodynamic profiles of drugs, since only the unbound form (particulary for drugs as huperzine A crossing the blood brain barrier [6,7]) is expected to reach the target tissues and exhibit a therapeutic effect.

The human serum albumin (HSA) is particulary involved in such binding processes. Indeed HSA is the major soluble constituent of the blood system implicated in the blood pressure, the transport of drugs, ions and other small biological molecules. Many sites on HSA are involved in drug–HSA bindings, but the most important interactions mainly occur inside of the two cavities, also called, respectively, warfarin-binding site (site I) and benzodiazepine-binding site (site II).

The aim of this work was: (i) to test seven cholinesterase inhibitors, huperzine A and its analogues with regard to their binding on HSA using the affinity chromatography method and (ii) to determine their potential binding site by comparative thermodynamic approach between this huperzine series and a group of five benzodiazepines.

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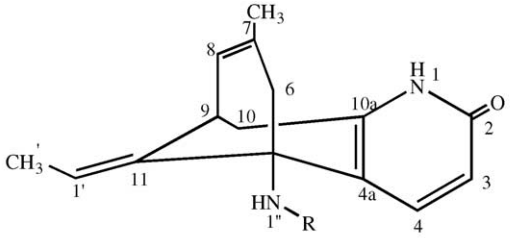
2. Material and methods

2.1. Chemicals

Water was obtained from an Elgastat water purification system (Odil, Talant, France) fitted with a reverse-osmosis cartridge. Disodium hydrogenphosphate and sodium dihydrogenophosphate were obtained from Prolabo (Paris, France). Huperzine A (M1) was obtained from Aldrich (Courtaboeuf, France), and other derivatives were synthesised in the laboratory: *N*-acetyl-huperzine A (M2), *N*-isobutyryl-huperzine A (M3), *N*-benzoyl-huperzine A (M4), *N*-propionyl-huperzine A (M5), *N*-valeryl-huperzine A (M6), *N*-butyryl-huperzine A (M7). Their chemical structures were given in Fig. 1. The preparation of these compounds was made by condensation of huperzine A with the corresponding acyl anhydride in the dichloromethane (Fig. 2). Their structures were established by ^{1}H NMR. Benzodiazepines (bromazepam, oxazepam, nitrazepam, diazepam, lorazepam) (Fig. 3) were provided by Aldrich (Courtaboeuf, France).

2.2. Apparatus

The chromatographic apparatus was equipped with a constant flow pump model LC10-AT and a C-R6A Chromatopac integrator (Shimadzu, Croissy Beaubourg, France), an Interchim Rheodyne injection valve model 7125 (Montluçon, France) fitted with a 20 μL sample loop and a SPD-10 A vp UV–vis detector (Shimadzu, Croissy Beaubourg, France).



5-N-*R*-amino-11-ethylidène-5,6,9,10-tetrahydro-7-methyl-5,9-methanocycloocta[b]pyridine-2(1H)-one

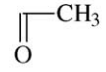
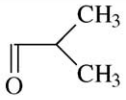
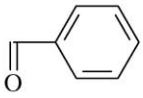
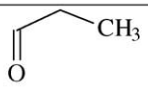
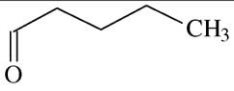
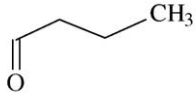
R	Name
H	huperzine A = M1
	N acetyl-huperzine A = M2
	N isobutyryl-huperzine A = M3
	N Benzoyl-huperzine A = M4
	N propionyl-huperzine A = M5
	N valeryl-huperzine A = M6
	N butyryl-huperzine A = M7

Fig. 1. Structure of huperzine series.

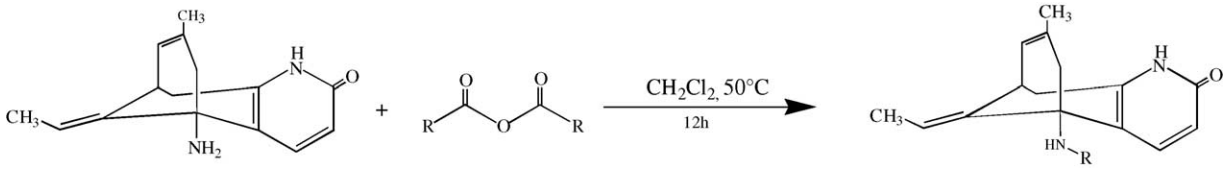


Fig. 2. General synthesis scheme of huperzine A derivatives.

Melting points were determined on a Kofler bench and were not corrected.

^{1}H NMR spectra were recorded on a Bruker AC 300 spectrometer at 300 MHz using tetramethylsilane as the internal reference. Chemical shifts are reported in parts per million.

2.3. Chromatographic conditions.

The mobile phase flow rate was maintained at 0.9 mL/min. The mobile phase was a phosphate buffer (10 mM) at pH 7. A ChromTech HSA column (Interchim, Montluçon, France; 150 mm × 4 mm I.D., 5 μm particle size) was used with controlled temperature in an Interchim Crococil oven TM701 (Monluçon, France). Experiments were repeated three times and carried out at a 230 nm detection wavelength and with the temperature ranging from 20 to 35 °C (20, 25, 30, 35 °C).

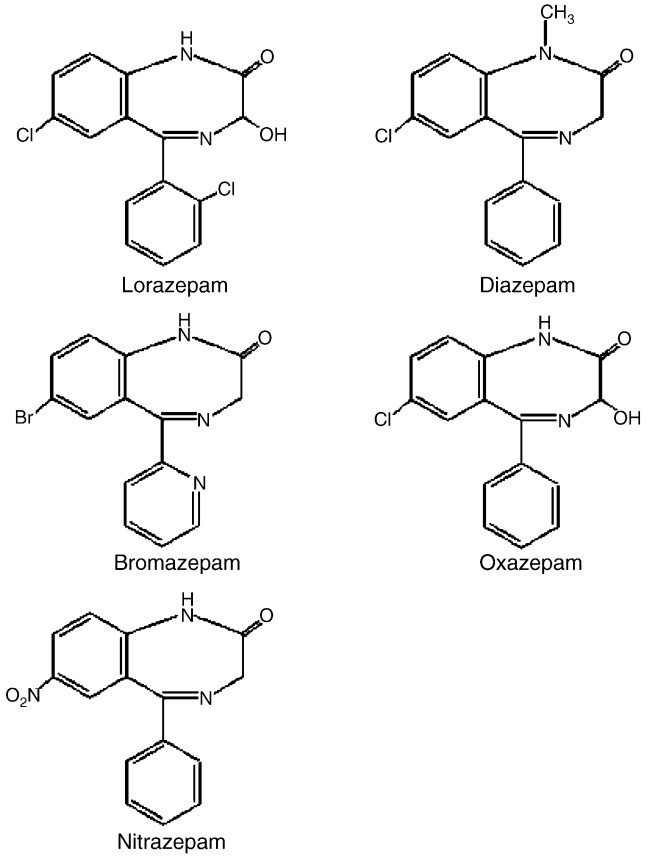


Fig. 3. Structure of benzodiazepine series.

2.4. General procedure of preparation of huperzine A derivatives M2–M7

Huperzine A (100 mg; 0.41 mmol) was dissolved in 5 mL of dichloromethane and 0.41 mmol of corresponding acyl anhydride was added at 45 °C with stirring for 12 h. After cooling, the solvent was evaporated under reduced pressure. The residue was dissolved in 15 mL of ethylacetate and washed with 3 × 10 mL of water. The organic layer was dried over sodium sulphate and evaporated. The crude product was purified with silica gel column. The ^{1}H NMR was given in Table 1.[1] Fusing points and reaction yields were, respectively, equal to 139 °C, 80% for M2, 143 °C, 59% for M3, 132 °C, 62% for M4, 135 °C, 65% for M5 145 °C, 70% for M6 and 148 °C, 30% for M7.

2.5. Thermodynamic relationships

The affinity chromatography with immobilized HSA on the stationary phase allows to study the solute HSA interactions on the HSA stationary phase. Indeed the retention factor (k') is proportional to the association constant K of the solute on HSA and can be described by the following equation [8]:

$$K = \frac{k'}{\Phi} \quad (1)$$

where Φ is the HSA column phase ratio (volume of the stationary phase divided by the volume of the mobile phase).

The binding of solute to HSA can be expressed in terms of retention factor k' using the well know Van't Hoff equation:

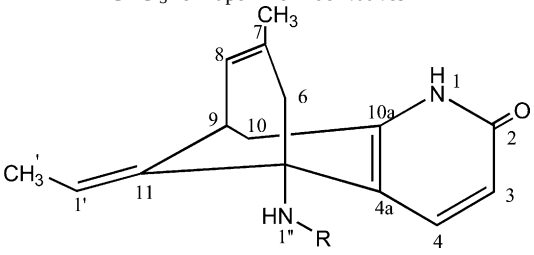
$$\ln k' = \frac{-\Delta H^\circ}{RT} + \Delta S^{\circ *} \quad (2)$$

$$\Delta S^{\circ *} = \frac{\Delta S^\circ}{R} + \ln \Phi$$

where R is the gas constant, T is the column temperature, ΔH° and $\Delta S^{\circ *}$ are, respectively, the solute enthalpy and entropy changes accompanying the transfer of the solute molecules from the bulk solvent to the HSA stationary phase. ΔH° and $\Delta S^{\circ *}$ can be calculated from the slope and intercept of linear Van't Hoff plot. This provides a suitable way to estimate the thermodynamic constants ΔH° and ΔS° if the phase ratio is known or can be calculated. Although ΔS° is not usually provided because of the ambiguity in the phase ratio for commercial use, $\Delta S^{\circ *}$ varies identically with ΔS°.

[1] The analytical data for M2–M7 was given in Table 2.

Table 1
H^1 NMR in $CDCl_3$ for huperzine A derivatives



Molecules	M2[a]	M3	M4	M5	M6	M7
Heterocycle						
H1	4.85[b]	4.60	4.90	4.84	4.92	4.91
H3	6.85	6.82	6.82	6.89	6.88	6.88
H4	8.1	8.14	8.17	8.2	8.11	8.2
H6 and H′6	2.12; 2.15	2.10; 2.13	2.09; 2.12	2.09; 2.11	2.11; 2.13	2.12; 2.13
H8	5.42	5.42	5.40	5.46	5.44	5.43
H9	3.45	3.46	3.48	3.41	3.40	3.48
H10 and H′10	2.7; 2.75	2.71; 2.77	2.69; 2.73	2.71; 2.79	2.72; 2.79	2.76; 2.8
H′1	4.78	4.79	4.77	4.8	4.79	4.8
CH_3	1.55	1.48	1.45	1.47	1.51	1.52
CH_3'	1.71	1.71	1.73	1.72	1.72	1.70
H″1	5.17	5.19	5.23	5.21	5.21	5.24
Substitution R						
CO–CH_3	2					
CO–$CH(CH_3)_2$		1.2; 2.4				
CO–C_6H_5			7.5; 8.2			
CO–C_2H_5				1; 1.8		
CO–C_4H_9					0.9; 1.4; 1.7; 2.3	
CO–C_3H_7						1; 1.5; 2.3

[a] See also, Section 2.1 and Fig. 1.
[b] δ in ppm.

3. Results and discussion

The binding of huperzine A and its analogues to HSA has been examined in this work by injecting huperzines into a HSA column. All the experiments were repeated three times and the variation coefficients of the k' values were all less than 3%, indicating high reproducibility and good stability for chromatographic systems.

In order to understand the retention mechanism of the huperzine series, the retention factor k' representing binding intensity was measured for both compound groups (huperzine and benzodiazepine series, respectively) at several column temperatures. A comparative thermodynamic study with benzodiazepine molecules was thus carried out to visualize more clearly the association mechanism of huperzine A and its derivatives with HSA.

The Van't Hoff plots ($\ln k'$ versus $1/T$) of Eq. (2) were drawn for all the solute molecules. Linear plots were obtained with correlation coefficients (r) higher than 0.97 for all the fits. Fig. 4 shows the Van't Hoff plot for huperzine-CO–$CH(CH_3)_2$. These linear behaviours agreed with that expected thermodynamically if there was no change in the interactions over the temperature range. The linear Van't Hoff plots allowed to calculate values of the thermodynamic parameters (ΔH° and $\Delta S^{\circ *}$) (Eq. (3)). Table 3 shows ΔH° and $\Delta S^{\circ *}$ values for the seven huperzines and the five benzodiazepines.

On the HSA protein, the retention of compound family was related to size, polarity and deformability of the molecules [9]. The site II cavity seems to be a good choice for huperzine binding, since the interior of cavity is constituted of hydrophobic amino-acids residues (Pro384, Leu387, Ile388, Phe395, Leu407, Leu423, Leu430, Val433, Ala449, Leu453, Phe488, Val473 and Leu460) and the cavity exterior presented two basic amino-acids residues (Arg410 and Tyr411). It appears that at pH 7.4, Tyr411 was unionized and Arg410 was totally ionized (positive charge) and can participate to

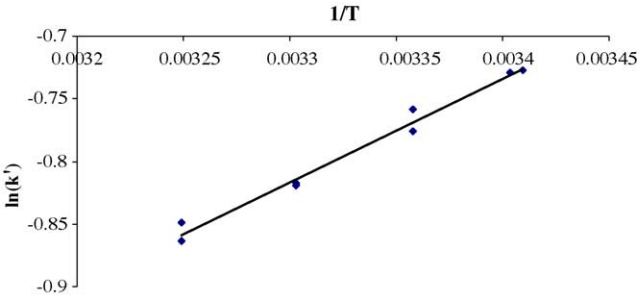


Fig. 4. Van't Hoff plot ($\ln(k')$ vs. $1/T$) for isobutyryl-huperzine A (M3).

Table 2
Analytical data for M2 to M7

	Analytical data calculated			Analytical data found		
	%C	%H	%N	%C	%H	%N
M2	71.80	7.09	9.85	71.59	7.13	9.97
M3	73.04	7.74	8.97	72.77	7.85	9.05
M4	76.27	6.40	8.09	76.34	6.22	8.17
M5	72.45	7.43	9.39	72.21	7.55	9.58
M6	73.58	8.03	8.58	73.78	7.92	8.64
M7	73.04	7.74	8.97	73.15	7.68	8.78

the bond [9]. Indeed the huperzine derivatives show a potentially negative charge, which can interact with Arg410, on the oxygen in position 2 formed by tautomery. Moreover, electronegative atoms (O, N) are able to form hydrogen bonds with HSA and the apolar group fixed on nitrogen 1 of huperzine A can interact with hydrophobic cavity of site II. According to these characteristics, huperzine molecules seem to be good candidates as ligands for the HSA site II.

The elution order of the seven huperzine derivatives at all the temperatures was invariable for the studied R groups (Fig. 1): huperzine-A–CO–CH_3 < huperzine-A–CO–CH_2–CH_2–CH_2–CH_3 ≈ huperzine-A–CO–CH_2–CH_2–CH_2–CH_3 ≈ huperzine-A–CO–CH_2–CH_3 ≈ huperzine-A-H ≈ huperzine-A–CO–$CH(CH_3)_2$ < huperzine-A–CO–C_6H_5. All the derivative molecules presented negative ΔH° values (Table 3) indicating that it was energetically more favourable for the huperzine A derivatives to be in the HSA stationary phase than in the bulk solvent. The corresponding negative entropy values showed an increase in the order of the chromatographic system when the huperzine molecules were transferred from the bulk solvent to the HSA stationary phase. These negative thermodynamic data were consistent with results reported in the literature for various chromatographic systems [10,11]. The negative values of ΔH° and $\Delta S^{\circ *}$ indicate predominant hydrophobic forces between HSA and the Huperzine series. That is illustrated in particular for the Huperzine-A–CO–C_6H_5 which exhibited the lowest thermodynamic data showing that this huperzine A derivative had the strongest hydrophobic and London interactions with the HSA. This was associated with the decrease in the entropic term classically attributed to a large immobilization of the solute molecule when solute is transferred into the HSA cavity. Except for huperzine-A–CO–C_6H_5 and huperzine-A–CO–CH_3, the studied huperzine presented both similar ΔH° values and retention factors. These results indicated that the amino-substituted groups (R groups) of the huperzine-A, huperzine-A–CO–$CH(CH_3)_2$, huperzine-A–CO–C_2H_5, huperzine-A–CO–C_3H_7 and huperzine-A–CO–C_4H_9 had a similar retention on HSA protein. In this case, the retention was not governed by the R group substituted on the heterocycle (Fig. 1). Only the substitutions of huperzine-A–CO–C_6H_5 and huperzine-A–CO–CH_3 on the amine function involved a significant difference for the retention on HSA.

Table 3
Values of the transfer enthalpy (ΔH°) and entropy ($\Delta S^{\circ *}$) for the seven huperzines (M1–M7) and the five benzodiazepines studied

Molecules	ΔH° (kJ/mol)	ΔS°[a]
M1[a]	−11.4	−4.7
M2	−5.5	−3.5
M3	−12.6	−5.2
M4	−16.7	−5.5
M5	−10.8	−4.5
M6	−11.9	−5.0
M7	−9.6	−4.1
Bromazepam	−10.4	−1.8
Oxazepam	−16.5	−4.8
Diazepam	−3.5	1.0
Lorazepam	−2.4	1.4
Nitrazepam	−25.5	−8.5

[a] See also, Section 2.1 and Fig. 1.

The $\Delta S^{\circ *}$ values were approximately identical for all huperzines. The huperzine-A–CO–C_6H_5 and huperzine-A–CO–$CH(CH_3)_2$ presented the highest values. Therefore, the binding of these two molecules with HSA was more ordered than the other huperzine molecules. That is due to the highest steric hindrance of isobutyl and benzoyl groups which induce a lower freedom degree.

In order to gain further insight into the interaction process, the enthalpy–entropy compensation was examined. This approach has been previously used in chromatographic procedures to analyse and compare the retention mechanism for a group of compounds [12,13]. The enthalpy–entropy compensation can be described by the following relation:

$$\Delta H^\circ = \beta \Delta S^\circ + \Delta G^\circ_\beta \quad (3)$$

ΔG°_β is the Gibbs free energy variation at the compensation temperature β.

If a plot of ΔH° against $\Delta S^{\circ *}$ is linear, the compensation temperature β can be determined. According to Eq. (3), enthalpy–entropy compensation is observed for a group of compounds, all the compounds have the same ΔG°_β at the compensation temperature β, suggesting that all the solutes show an identical retention mechanism.

The plot of ΔH° versus $\Delta S^{\circ *}$ obtained for the huperzine A, huperzine A–CO–$CH(CH_3)_2$, huperzine A–CO–C_2H_5, huperzine A–CO–C_4H_9 and huperzine A–CO–C_3H_7 was linear. The correlation coefficient of this plot was higher than 0.989, and this value can be considered adequate to verify an enthalpy–entropy compensation [14,15]. On the other hand, if the huperzine-A–CO–C_6H_5 and huperzine-A–CO–CH_3 were integrated into this compensation, the correlation coefficient was lower and both plots exhibited a different slope (higher than 10%). Consequently the binding mechanism with HSA appeared to be identical for huperzine A, huperzine-A–CO–$CH(CH_3)_2$, huperzine-A–CO–C_2H_5, huperzine-A–CO–C_3H_7 and huperzine-A–CO–C_4H_9, but huperzine-A–CO–C_6H_5 and huperzine-A–CO–$CH(CH_3)_2$ seemed to bind on HSA with a different mechanism from the other huperzines.

The benzodiazepine binding on HSA is well known since many years [16,17]. To understand more clearly the binding mechanism of huperzine A, a comparison with five benzodiazepine molecules was carried out, using the same chromatographic and thermodynamic approach. The elution order of the five benzodiazepines at all the temperatures was invariable: nitrazepam < oxazepam < bromazepam < lorazepam < diazepam. Structures are given in Fig. 3. The negative enthalpies (Table 3) indicated that it was energetically more favourable for the benzodiazepines to be in HSA stationary phase than in the bulk solvent. These negative values of enthalpy changes were due to Van der Waals and hydrogen bonding interactions. Two molecule groups were correlated with the entropy values (corresponding to the $\Delta S^{\circ *}$, Table 2): (i) for the nitrazepam, oxazepam and bromazepam ($\Delta S^{\circ *} < 0$) an increase was observed in the order of the chromatographic system when the benzodiazepine molecules were transferred from the bulk to the HSA stationary phase; (ii) for bromazepam and lorazepam ($\Delta S^{\circ *} > 0$), a strong contribution of hydrophobic interactions was associated with an increased of entropy.

Benzodiazepines were generally thought to bind to site II (indole-benzodiazepine site) [18], which is located at subdomain III A of the protein. However, differences in the binding characteristics of benzodiazepine drugs with HSA have been reported for flunitrazepam [19]. In order to compare the binding mechanism between the five benzodiazepine molecules studied, an enthalpy–entropy compensation was examined. The plot of ΔH° versus $\Delta S^{\circ *}$ obtained for benzodiazepine group was linear and verified the enthalpy–entropy compensation with a correlation coefficient higher than 0.999. In opposite to flunitrazepam [19], our enthalpy–entropy compensation confirmed that the binding mechanism was identical for the five benzodiazepines studied.

4. Conclusion

The similarity of binding mechanism for the two molecule groups was examined by comparing the both previous enthalpy–entropy compensations. The two ΔH° versus $\Delta S^{\circ *}$ straights obtained, respectively, for huperzine and benzodiazepine group, presented the same slope. According to the literature [20], identical compensation temperatures (identical slopes) did not allow to conclude that benzodiazepines and huperzines bound on HSA with an identical association mechanism. Consequently, in order to verify if benzodiazepine and huperzine binds on the same HSA binding site, an elution zonal approach must be used [21] in our future work.

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