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Association mechanism of four acetylcholinesterase inhibitors (AChEIs) with human serum albumin: A biochromatographic approach

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ABSTRACT

In this work, the interaction of a series of acetylcholinesterase inhibitors (AChEIs; donepezil, galanthamine, huperzine and neostigmine) with human serum albumin (HSA) immobilized on porous silica particles was studied using a biochromatographic approach. For all the tested AChEI molecules, linear retention plots were observed at all temperatures. An analysis of the thermodynamics (i.e. enthalpy (ΔH°), entropy ($(S^\circ*)$) of the interaction of the AChEI molecules with the immobilized human serum albumin was also carried out. The (H° and ($S^\circ*$ values for donepezil, galanthamine and neostigmine, were negative due to van der Waals interactions and hydrogen bonding which govern this association with albumin. Whereas the positive values of (H° and ($S^\circ*$ of huperzine binding on HSA indicated a predominance of hydrophobic interactions. The association of AChEIs with HSA was increased linearly with pH. A comparative thermodynamic study with benzodiazepine molecules was also done to determine the potential binding site of these drugs on HSA.

1. Introduction

Alzheimer's disease (AD) is a progressive brain disorder that gradually destroys a person's memory and ability to learn, reason, make judgments, communicate and carry out daily activities. The greatly reduced presence of acetylcholine in the cerebral cortex is a significant factor in AD [1,2]. The inhibition of acetylcholinesterase (AChE) activity may be one of the most realistic approaches to the symptomatic treatment of AD. AChE is responsible for degradation of the neurotransmitter acetylcholine (ACh) in the synaptic cleft of neuromuscular junctions and of neuronal contacts in the central nervous system [3,4]. Many medicinal agents, as donepezil, huperzine or rivastigmine, used for treatment of Alzheimer's disease, belong to the important class of acetylcholinesterase inhibitors (AChEIs) [5].

Age-related changes in physiology and organ function alter drug pharmacokinetics and pharmacodynamics. In addition, older persons take more medications in treating multiple disorders, increasing the risk of drug–drug and drug–disease interactions [6]. Thus, the expanded pharmacokinetics studies are important for drugs which are taken by aging patients as the drugs of Alzheimer's disease.

HSA is the most abundant protein in blood and can reversibly bind a large number of pharmacological substances, such as AChEI molecules. Few specific binding sites are present on HSA [7,8]. The most important sites are benzodiazepine and warfarin binding sites. He and Carter [8] have determined the three dimensional structure of HSA and have shown that these two binding sites are located in hydrophobic cavities in subdomains IIA and IIIA. Site I is formed as a pocket in subdomain IIA and involves the lone tryptophan of the protein (Trp214). The inside wall of the pocket is formed by hydrophobic side chains, whereas the entrance to the pocket is surrounded by positively charged residues. Site II correspond to the pocket of subdomain IIIA, which is almost the same size as site I, the interior of cavity is constituted of hydrophobic amino-acids residues and the cavity exterior presented two important amino-acids residues (Arg410 and Tyr411) [9,10]. HSA was the model protein used in a great number of studies [11]. The main advantage of using HSA is the data available for its interaction with a wide range of organic and inorganic compounds [12]. Affinity chromatography with HSA immobilized on the support is specially suited to the study of drug–protein interactions. The association constants of many ligands have been determined by zonal elution [13] or frontal analysis [14]. The thermodynamic process involved in the binding has already been studied [15–19]. The aim of this work was to study the association mechanism of four AChEIs (donepezil, galanthamine, huperzine, neostigmine) with the HSA using a biochromatographic approach, and to determine their potential binding site by comparative thermodynamic

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approach between these drugs and a group of five benzodiazepines. Moreover, the pH effect of the bulk solvent on the AChEI–HSA association was determined.

2. Theory

Single and multi-component isotherms are now measured by dynamic methods. The most widespread of this is frontal analysis, but this technique is time consuming and requires large amounts of pure compounds [20]. Another popular method, elution by characteristic point (ECP), derives the isotherm from the profile of the diffuse front of the band obtained in response to a single injection of a highly concentrated sample [21]. This method is fast and needs only small amounts of sample, but it requires accurate calibration of the detector and an efficient column. Distribution isotherms can also be apprehended using the perturbation technique originally developed for measuring gas-adsorbent equilibria. The perturbation technique makes possible the determination of adsorption isotherms by measuring the retention times of small sample sizes injected onto a column equilibrated with sample solutions at different concentration levels. The column used for the determination of the isotherm is first equilibrated with a solution containing the compound dissolved in a non-adsorbable solvent. Then a small sample volume containing higher concentration of the compound is injected onto the column. After the injection, the equilibrium condition is disturbed and the perturbation waves reache the column outlet, a peak is registered by the detector. In the case of single component equilibrium of a compound dissolved in a non-adsorbable solvent, one peak is observed and the distribution isotherm depends only on the concentration of a single solute [22,23]. The well-known Langmuir theoretical approach relates the total concentration of the sample in the stationary phase (C_s) and that in the mobile phase (C_m) [22–24]:

$$C_s = \frac{\alpha K C_m}{1 + K C_m} \tag{1}$$

where α is the column saturation capacity and K is the equilibrium constant for the distribution of ACEI between the mobile phase and the HSA stationary phase. The sample AChEI retention factor k' was directly proportional to the slope of its adsorption isotherm and can be thus given by the following equation [22–24]:

$$k' = \frac{t - t_{\grave{a}}}{t_{\grave{a}}} = \phi \frac{dC_s}{dC_m} = \frac{\phi \alpha K}{(1 + K C_m)^2} \tag{2}$$

where t is the retention time of the solute determined from the peak maximum, t_0 is the column hold-up time, i.e. the elution time of a non-retained compound, and ϕ is the column phase ratio (V_S/V_M) (V_S is the volume of the stationary phase in the column and V_M the void volume). By plotting the k' value versus the sample concentration in the bulk solvent C_m, the constant K can be determined using Eq. (2) [22–24] and a non-linear-regression. The main advantage of the perturbation technique consists in using a simpler instrumentation for the acquisition of the experimental data than frontal analysis method: the determination of the concentration of the individual compounds at the intermediate plateaus of the frontal analysis curves is no longer needed [22,23]. As well, using the HSA stationary phase, AChEI could tightly bind to residual silanol groups. Then if AChEI bound on two sites on the stationary phase, i.e. a specific site (site A with an adsorption constant K_A and a column saturation capacity α_A) and a second site which is non-specific (sites B corresponding to the residual silanol groups on the particles of the stationary phase with an adsorption constant K_B and a column saturation capacity α_B), then the AChEI retention factor (k') directly proportional to the slope of its adsorption isotherm is given by the following equation [22–24]:

$$k' = \frac{t - t_0}{t_0} = \phi \frac{dC_s}{dC_m} = \phi \left(\frac{\alpha_A K_A}{(1 + K_A C_m)^2} + \frac{\alpha_B K_B}{(1 + K_B C_m)^2} \right) \tag{3}$$

Eq. (3) was fitted to the solute retention factor k' by a non-linear regression and the adsorption constants K_A and K_B and the parameters $k'_A = \phi \alpha_A K_A$ and $k'_B = \phi \alpha_B K_B$ corresponding to the retention contributions of the two kinds of sites under linear conditions were calculated. Valuable informations about the processes driving the AChEI–HSA association mechanism can be further gained by examining the temperature dependence of AChEI retention [25,26]. Under linear conditions, the temperature dependence of the retention factor is given by the following relationship:

$$\text{Ln } k' = \left(-\frac{\Delta H^\circ}{RT} \right) + \Delta S^\circ * \tag{4}$$

with

$$\Delta S^\circ * = \left(\frac{\Delta S^\circ}{R} \right) + \ln \phi \tag{5}$$

where ΔH° and ΔS° are respectively the enthalpy and entropy of transfer of AChEI from the bulk solvent to the HSA stationary phase. T is the absolute temperature. If the HSA stationary phase, AChEI and solvent properties are independent of temperature, and ΔH° and ΔS° are temperature invariant, a linear van't hoff plot is obtained. From the slope and the intercept ΔH° and $\Delta S^{\circ *}$ can be calculated.

3. Experimental

3.1. Apparatus

The HPLC system consisted of a Shimadzu LC-10ATvp pump (Champs sur Marne-France), a Rheodyne 7125 injection valve (Cotati, CA, USA) fitted with a 20 μl sample loop, and a Shimadzu UV–visible detector. A chromtech HSA column (Interchim, Montluçon, France) (150 mm × 4 mm) was used in a controlled temperature oven TM701 (Interchim, Montluçon, France). The support was HSA immobilized onto spherical silica particles (diameter 5 μm; pore size 6 nm).

3.2. Solvents and samples

The four drugs AChEIs were depicted in (Fig. 1). Galanthamine, huperzine were purchased from Sigma (Paris, France), whereas neostigmine and donepezil were obtained from Interchim (Montluçon, France). Water was obtained from an Elgastat option water purification system (Odil Talant, France) fitted with a reverse osmosis cartridge.

Sodium dihydrogenophosphate and di-natriumhydrogenophosphate were obtained from Prolabo and Merck (Paris, France), respectively.

3.3. Operating conditions

The mobile phase consisted of 0.1 M sodium phosphate buffer. The phosphate buffer was prepared by mixing equimolar solutions of mono-and dibasic sodium phosphate to produce the desired pH value (between 5.0 and 7.0, i.e. 5.0, 5.5, 6.0, 6.5, and 7.0). Experiments were carried out over the temperature range 278–308 K (278, 283, 293, 298, 303, and 308 K). The detection wavelength was 254 nm, and the mobile phase flow-rate was 0.3 ml/min. AChEIs solutions were prepared in the mobile phase with a concentration of 7 μM and 20 μl was injected at least three times. For the

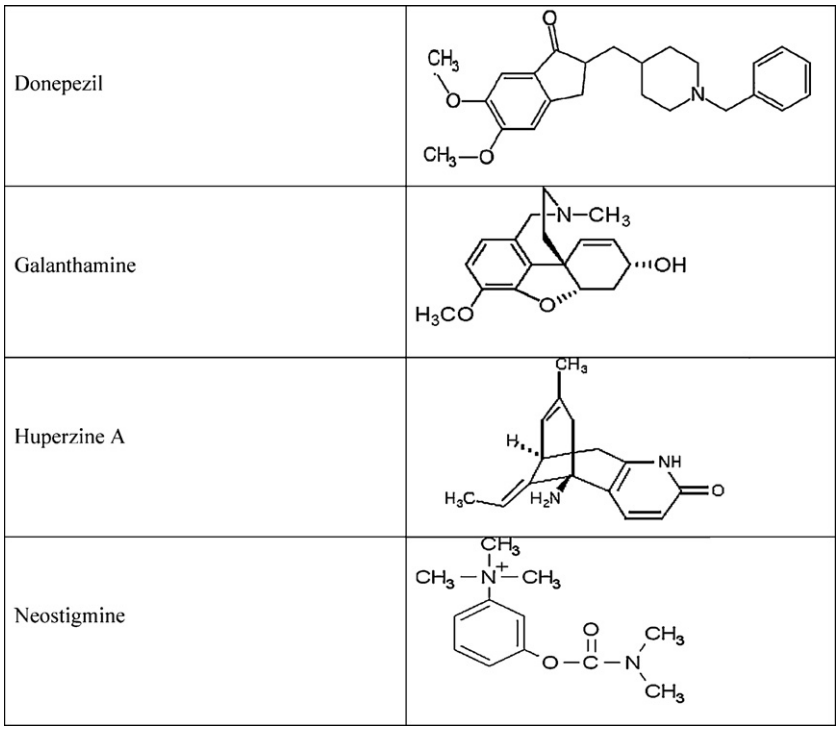

Fig. 1. Chemical structures of the studied drugs.

determination of the adsorption isotherms at pH 7 for each studied AChEI, the equilibration of the column was carried out with 15 concentrations of AChEI (0–7 μM) in the mobile phase to obtain a stable detection. 20 μl of the most concentrated AChEI sample was injected at least three times and the retention time was measured.

4. Results and discussion

4.1. Langmuir distribution isotherms

So as to calculate the adsorption constants of the AChEI with HSA, the Langmuir distribution isotherms were calculated at pH 7.0 and 298 K. For each AChEI and for each AChEI concentration in the bulk solvent, the most concentrated AChEI sample was injected into the chromatographic system and its retention factor was determined (see Section 3.3). The variation coefficients of the k' values were <0.4%, indicating a high reproducibility and a good stability for the chromatographic system. Using a weighted non-linear regression (WNLIN) procedure, the constants of Eq. (2) were used to estimate the retention factors. The slope of the curve representing the variation of the estimated retention factors (k') (Eq. (2)) versus the experimental values (0.999; ideal is 1.000) and r^2 (0.997) indicate that there is an excellent correlation between the predicted and experimental retention factors. The non-linear regression coefficient r^2 and the F value (from the Fisher test with the confidence level at 95%) were determined. These are shown in Table 1. The F value constitutes a more discriminating parameter than the r^2 value when assessing the significance of the model equation. From the full regression model, a student t-test was used to provide the basis for the decision as to whether or not the model coefficients were significant. Results of the student's t-test show that no variable can be excluded from the model. These results showed that the Langmuir model describes accurately the association behaviour of AChEI with HSA and the corresponding K values were given in Table 1. However, the immobilization of HSA on silica support could lead to non-specific interactions, i.e. association with the residual silanol groups. Using the non-linear regression, the retention contributions of the two kinds of sites k'_A and k'_B were determined from Eq. (3). The corresponding non-linear regression coefficient r^2 and F values of this bi-Langmuir model were determined and given in Table 1. The non-linear coefficient results ($r^2 > 0.99$) and the F values proved that the two-order Langmuir model described accurately the binding mechanism of AChEI with the HSA stationary phase. As well, the results showed that the interactions between AChEI and the residual silanol groups of the stationary phase were neglected (the k'_A and k'_B values were given in Table 1 and $k'_B \ll k'_A$).

4.2. Bulk solvent pH effect and possible thermodynamic origins of the AChEI binding to HSA

In this part, in all the experiments, the k' values were determined for a sample concentration in the mobile phase equal to zero; i.e.

Table 1
Values of the adsorption constant K, the retention contribution of the two kinds of binding sites k'_A and k'_B, the retention factor k' ($k' = k'_A + k'_B$) (extrapolated at $C_m = 0$), the relative bound percentage b%, the log P and the non-linear regression coefficients r^2 and F (Langmuir model: Lang and bi-langmuir model: bi-Lang), for the four AChEIs (at pH 7.0, $T = 298$ K). Standard deviations are in parentheses.

AchEI	K ($\times 10^4$ M^{-1})	k'_A	k'_B	k'	b (%)	Log P	r^2; F Lang bi-Lang
Donepezil	38.04 (0.12)	11.16 (0.03)	0.08 (0.01)	11.24 (0.03)	91.86 (0.12)	4.71	0.9994; 2880 0.9995; 5248
Galanthamine	3.20 (0.02)	0.89 (0.02)	0.02 (0.01)	0.91 (0.01)	47.64 (0.11)	1.75	0.9998; 8400 0.9997; 6220
Huperzine	2.82 (0.01)	0.73 (0.01)	0.01 (0.01)	0.74 (0.04)	42.53 (0.10)	0.71	0.9998; 7908 0.9999; 405120
Neostigmine	1.35 (0.03)	0.39 (0.01)	0.01 (0.01)	0.40 (0.01)	28.57 (0.09)	−3.03	0.9998; 9044 0.9999; 519876

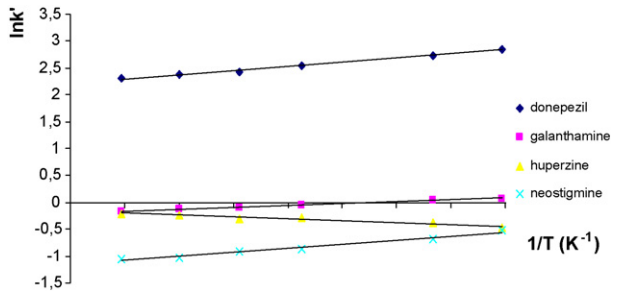

Fig. 2. Plot of ln k' vs. 1/T (Van't Hoff plot) for the four AChEIs at pH 7.

$C_m = 0$. The retention factors (k') for the four AChEIs were determined at various column temperatures (278–308 K) with various pH ($5 \leq pH \leq 7$) of the phosphate buffer (see Section 3.3). For example the k' values were given in Table 1 at $T = 298$ K and pH 7.0. From these k' values and the partition equilibrium constant K calculated above (Table 1) it was clearly shown that both partition equilibrium constant and AChEI elution order varied as follows:

neostigmine $<$ huperzine $\approx$ galanthamine $\ll$ donepezil.

Eq. (4) showed that with an invariant drug–albumin association mechanism over the temperature range being studied, the association enthalpy (H° remained constant and a plot of ln k' against $1/T$ leads to a straight line with an enthalpic slope and entropic origin. Linear van't Hoff plots were obtained (Fig. 2) with correlation coefficients r higher than 0.91 for all fits. In order to evaluate a possible change in the AChEI binding capacity with increasing temperature, the concentration dependencies of the solute retention factor (k') were measured for all the column temperatures. In order to compare the retention data, the normalized parameter $100 \times (k'/k'_{low})$ was used where k'_{low} represents the retention factor at the lowest solute concentration injected in the chromatographic system. For the column temperature range 278–308 K, the normalized parameter value was constant for each AChEI and ≈99.7. This behaviour is in accordance with no change in the number of binding sites when the temperature varied [27]. As well, the linear van't Hoff plot behaviour is thermodynamically expected when the albumin–AChEI association mechanism is independent of temperature. According to Eq. (4) these linear van't Hoff plots provided a conventional way of calculating the thermodynamic parameters. Both (H°, ($S^{\circ *}$ were negatives for donepezil, galanthamine and neostigmine (Table 2). Negative (H° indicates that it was energetically more favourable for these drugs to be linked to HSA rather to be in the bulk solvent. Negative entropies showed an increase in the order of the chromatographic system when these drugs are included in the HSA binding cavities. The negative values of the (H° and ($S^{\circ *}$ demonstrated that the binding was controlled enthalpically, and indicated that hydrogen bonding and van der Waals forces are the major interactions stabilizing the albumin–drug (i.e. donepezil, galanthamine, neostigmine) association [28–30].

In addition, many studies demonstrated that the hydrophobic effects play an important role in the solute molecule–albumin association [31]. The relative bound percentage (b) has been calculated at 298 K using the retention factor (k') on HSA for each compound according to the following equation:

$$b = \frac{k'}{1 + k'} \quad (6)$$

This equation has been shown to give a good correlation versus reference methods for compounds with medium-to-strong binding to HSA [32,33]. The b values were given in Table 1. The corresponding log P (drug partition coefficient octanol/water) values were exposed by many scientific sites (Pubchem, Drugbank, Chemspider, etc.). Table 1 presents the log P values which have been derived from an atomic fragment database using (ACD/Log P) software (http://www.chemspider.com/). Comparing the k' values (or the K values) and the b values of these AChEIs with log P (Table 1), it was shown that affinity enhanced with the increase in the molecule hydrophobicity and confirmed that the hydrophobic forces play as well a great role in the AChEI–albumin binding process.

Among the four AChEIs, donepezil was the most retained molecule on HSA, exhibiting negative entropy and the largest negative change in enthalpy. For example, at pH 7.0 ($H^\circ = -13.2$ kJ/mol and ($S^{\circ *} = -2.9$ (no units). This can be explained by the high hydrophobicity of this molecule due to the presence of hydrophobic aromatic groups (log $P = 4.71$ for donepezil). These aromatic groups of donepezil can be involved in strong π–π interactions with the aromatic amino acids of albumin molecule [34,35]. As well, all oxygen and nitrogen atoms of donepezil take part in the formation of hydrogen bonds, but mostly, donepezil forms hydrogen bonds with residues of albumin through its carbonyl oxygen atom of the dimethoxyindanone group [34]. For galanthamine, two principal hydrogen bonds can be formed with albumin, the hydroxyl group and the O-methyl group of galanthamine. The values of (H° and ($S^{\circ *}$ for galanthamine were less negative than for donepezil (Table 2), showing that HSA was less energetically stabilized with galanthamine than for the donepezil. Neostigmine exhibited the lowest association with the HSA, this can be explained by the lowest hydrophobicity of this drug molecule (log $P = -3.03$) due to its polar residues as the quaternary ammonium group which is highly charged at pH 5–7 (Fig. 1) [5,36]. The positive values of (H° and ($S^{\circ *}$ of huperzine binding to HSA (for example, at pH 7.0 ($H^\circ = +5.9$ kJ/mol and ($S^{\circ *} = +2.1$) indicated predominant hydrophobic forces between HSA and the huperzine, and draw attention to the role that solvent reorganization must be playing in determining the strength of the huperzine–HSA complex [37,31]. In addition to the hydrophobic interactions which govern the huperzine–albumin association, other interactions as the hydrogen bonds due to the electronegative atoms (O, N) can get involved in this association [38,39].

The logarithm of the retention factor k' was also plotted against the pH for each AChEI molecule and for a wide variation range of pH ($5.0 \leq pH \leq 7.0$). The plots log k' versus pH were linear for all studied AChEIs with correlation coefficients r higher than 0.95 for all fits (Fig. 3), and showed that the binding affinity was increased linearly with pH.

The concentration dependencies of the solute retention factors were also measured at different pH values. As reported above for the temperature experiments, the normalized parameter value was constant for each AchEI (around 99.8). Then, the binding capacity of the column was invariant when the pH changed and thus, the pH did not alter the number of binding sites of the immobilized HSA [27]. This increase of the binding affinity with pH probably came from two aspects of effects, one from the albumin and another from the drug. Many studies have demonstrated that pH-induced alterations in the binding sites of protein molecule play an important role in the changes of ligands binding to protein [40,41]. Although the influence of the buffer pH on the secondary structure of albu-

Table 2
Thermodynamic parameters (H° (kJ/mol) and ($S^{\circ *}$ for the four AChEIs (at pH 7.0). Standard deviations are in parentheses.

AchEI	(H° (kJ/mol)	($S^{\circ *}$
Donepezil	−13.21 (1.01)	−2.95 (0.05)
Galanthamine	−5.90 (0.08)	−2.51 (0.05)
Huperzine	+5.91 (0.05)	+2.11 (0.04)
Neostigmine	−12.42 (0.09)	−5.90 (0.03)

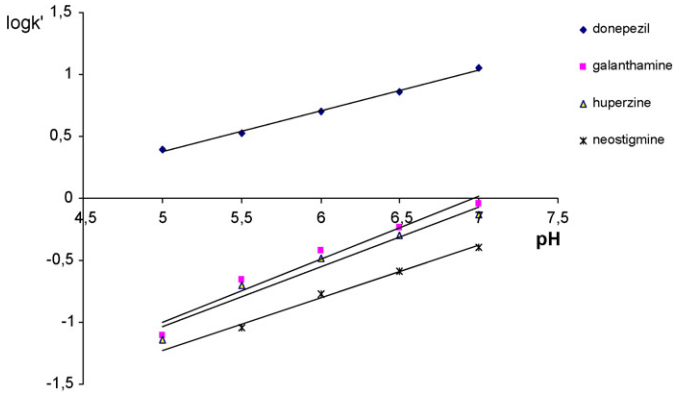

Fig. 3. Log k' vs. pH for the four AChEIs at $T = 298$ K.

min is small, the rigidity of the albumin molecule will be somewhat affected, and the changes of charge on the entrance of the binding pocket would influence in some extent the access of the drug to the binding site [42,43]. On the other hand the ionization state of the drug would be different with the variation of the bulk solvent pH, and thus, affected the binding affinity of the drug.

Enthalpy–entropy compensation (EEC) temperature is a useful thermodynamic approach to the analysis of physico-chemical data [44]. Mathematically the entropy–enthalpy compensation can be expressed by the following equation:

$$\Delta H^\circ = \beta \Delta S^\circ + \Delta G^\circ_\beta \quad (7)$$

ΔG°_β is the corresponding Gibbs free energy variation at the compensation temperature β. According to this last equation, when enthalpy–entropy compensation is observed with a group of compounds in a particular chemical interaction, all the compounds have the same free energy ΔG°_β at the temperature β [45,46] suggesting that all the solutes show an identical retention mechanism. The plot of (H° versus ($S^{\circ *}$ obtained for galanthamine, huperzine, neostigmine, and donepezil was linear at all pH values of the bulk solvent. The correlation coefficient of this plot was higher than 0.94, and this value can be considered adequate to verify enthalpy–entropy compensation [47]. Since different mechanisms could result in the same proportion of enthalpy and entropy relative to the overall free energy, it cannot be deduced rigorously that the association mechanism of huperzine, neostigmine galanthamine, and donepezil on the HSA was independent of the molecule structure and the pH bulk solvent. However, these molecules have similar biological activity. These two conditions (EEC and similar biological effects) seem to imply a similarity of properties of galanthamine, huperzine neostigmine, and donepezil. In order to gain further insight into the interaction process of these drugs with the albumin, a comparison with benzodiazepine molecules was carried out using the same chromatographic and thermodynamic approach. The benzodiazepine binding on HSA is well known since many years [48,49], and it is generally thought that benzodiazepines bind to site II (indole-benzodiazepine site) [50]. In a previous paper [39], the interaction of five benzodiazepines (nitrazepam, oxazepam, bromazepam, lorazepam, diazepam) with HSA was studied using the same experimental and operating conditions as those used for our present drugs. The plot of (H° versus ($S^{\circ *}$ was analyzed for this benzodiazepine group, and an enthalpy–entropy compensation was verified with a correlation coefficient higher than 0.99. This enthalpy–entropy compensation confirmed the fact that benzodiazepines bind on the same site on HSA, i.e. site II.

Moreover, the two (H° versus ($S^{\circ *}$ straight lines, for AChEIs and benzodiazepine group (for BZDs data were obtained from [39]), presented the same slope, and exhibited similar compensation temperature:

$$\text{AChEIs} : \Delta S^{\circ} * = 0.43\ \Delta H^\circ - 0.31 \qquad r^2 = 0.992 \quad (8)$$

$$\text{BDZs} : \Delta S^{\circ} * = 0.43\ \Delta H^\circ + 2.50 \qquad r^2 = 0.993 \quad (9)$$

According to this similarity of both, the AChEI retention dependence with relative bound percentage and EEC, huperzine, galanthamine, neostigmine and donepezil molecules seemed to be good candidates as ligands for the HSA site II (Indole-benzodiazepine site) [51].

5. Conclusion

The mechanism of donepezil, galanthamine, huperzine, and neostigmine binding to human serum albumin (HSA) was analyzed. The results demonstrated that binding of donepezil, galanthamine and neostigmine with albumin was temperature-independent, and governed principally by hydrogen bonding and van der Waals forces. The binding of huperzine with albumin was also temperature-independent and characterized by predominance of hydrophobic interactions. The albumin affinity of the four AChEIs enhanced slightly with the increase pH of the medium due to the ionization degree of both drug and albumin binding site. A comparative thermodynamic study with benzodiazepine molecules showed that huperzine, galanthamine, neostigmine and donepezil molecules seemed to be good candidates as ligands for the HSA site II.

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