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Short communication

Triazine–human serum albumin association: thermodynamic approach and sodium effect

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Abstract

Human serum albumin (HSA) serves as a carrier protein to transport triazine herbicides to molecular targets. In this paper, a theoretical treatment was developed to describe the HSA–triazine herbicides association. A determination of the association constant, K, as well as the degree of complexation n_c (the percent of complex guest) was carried out. Enthalpy–entropy compensation was also analyzed in relation to this mathematical model to confirm the herbicide complexation behavior with HSA. The role of the sodium cation (Na^+) on this association was investigated. It was expected that the sodium ion would act on the herbicide–HSA association process by modifying the surface tension of the bulk solvent and increase the K and n_c values. The results showed that for patients who suffer from Na^+ desequilibrium, the triazine–HSA binding would change and as well the toxicological effect of these herbicides.

Keywords: Thermodynamic; Sodium effect; Triazine; Human serum albumin

1. Introduction

Human serum albumin (HSA) is the most abundant protein in blood and can reversibly bind a large number of substances. Recently, the three dimensional structure of HSA was determined through X-ray crystallographic measurements [1]. The association

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constant of many ligands has been determined by zonal elution [2] or frontal analysis [3]. The thermodynamic process involved in the binding has already been studied [4–9]. The triazine herbicides are widely applied in agricultural practice. These herbicides are known to block electron transport in photosystem II [10]. The pernicious effects of these photosynthesis inhibitors apply to all species of the food chain. Thus, triazine herbicides are found not only in the plants, soil, and water but as well in the cultivated soil and in agricultural product such as fruit, milk, butter, sugar beet, etc. [11]. Triazine causes a significant inhibition of the specific binding of various proteins like estrogen and progesterone receptors [12,13] and also induces DNA damage [14,15]. It has been suggested that HSA serves as a carrier to transport some herbicides to molecular targets [16,17]. This paper describes the association of the atrazine herbicides with HSA and shows the role of sodium cation (Na^+) which had a great importance in the physiology of several pathogeneses such as diabetes or kidney insufficiency. Its effect on the triazine herbicides–HSA interaction was analyzed.

2. Theory

The reversible binding interaction between the human serum albumin (HSA) and the given herbicide, M, with an equilibrium constant K, is given by the following thermodynamic relationship:

$$M + HSA \leftrightarrow M \cdot HSA \quad (1)$$

2.1. When HSA was grafted on the stationary phase (HSA column)

In this case, the herbicide–HSA association is usually expressed in terms of the retention factor, k, using the well known equation:

$$k = \phi K \quad (2)$$

where ϕ is the column phase ratio (volume of the stationary phase divided by the volume of the mobile phase). k was linked with the thermodynamic constant of transfer of the herbicide from the bulk solvent to the HSA cavity following the equations:

$$\ln k = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln \phi \quad (3)$$

$$\Delta S^{\circ *} = \frac{\Delta S^\circ}{R} + \ln \phi \quad (4)$$

where ΔH° and ΔS° are, respectively, the enthalpy and entropy changes accompanying this transfer, T is the temperature and R the gas constant. If the herbicide binds to the HSA with a constant enthalpy of association, then the plot of $\ln k$ vs. $1/T$ should be linear with a slope of $-\Delta H^\circ/R$ and an intercept $\Delta S^{\circ *}$. This provide a convenient way of calculating the thermodynamic constants ΔH° and ΔS° if the phase ratio ϕ is known or can be calculated. Although ΔS° is not usually given, because of the ambiguity in the calculation of the phase ratio for commercial column, $\Delta S^{\circ *}$ varies identically with ΔS°.

2.2. When HSA was added in the mobile phase (C_{18} column)

In this case, reaction (1) took place in the bulk solvent. In a previous study [18], a mathematical model was presented to describe the variation of the retention factor, k, at a physiological pH, for a series of test solutes with an alkyl stationary phase (C_{18} column). By applying this model to the herbicide molecules, the following equation was obtained:

$$k = \frac{(k_M + k_{M\cdot HSA} K([HSA_t] - n_c[M_t]))}{1 + K([HSA_t] - n_c[M_t])} \quad (5)$$

where k_M and $k_{M\cdot HSA}$ are, respectively, the retention factor of the herbicide molecule in free solution (without HSA) and when it is completely complexed. $[HSA_t]$ and $[M_t]$ are the total concentrations of HSA and M in the mobile phase. n_c is the average number of HSA molecules bound per triazine molecule M (solute complexation degree). This non linear model involves no approximation of the concentration of the two species M and HSA and provides a convenient way of calculating the average number of HSA molecules bound per herbicide molecule.

2.3. Enthalpy–entropy compensation

A further thermodynamic approach to the analysis of physico-chemical data is enthalpy–entropy com-

pensation [19–23]. The enthalpy–entropy compensation can be described by the following equation:

$$\ln k_T = -\frac{\Delta H^\circ}{RT}\left(1-\frac{T}{\beta}\right)-\frac{\Delta G^\circ{}_\beta}{R\beta}+\ln\phi \qquad (6)$$

where $\Delta G^\circ{}_\beta$ is the Gibbs energy of a physicochemical interaction at a compensation temperature β. If enthalpy–entropy compensation is observed in liquid chromatography for a group of compounds, all the compounds will have the same net retention at the compensation temperature β, although their temperature dependences may differ. Eq. (6) shows that a plot $\ln k$ for different compounds at a constant temperature T, is a linear function of the corresponding ΔH°, and a compensation temperature β can be evaluated from the slope. Similarity of the values for the compensation temperature suggest that the solutes are retained by essentially identical interaction mechanisms. The results obtained with this method can be misleading, due to the cumulative errors associated with the determination of enthalpy [24,25]. According the analysis of Krug et al. [26], similar mechanisms could be mapped by thermodynamic studies if the correlation between $\ln k$ and ΔH° (Eq. (6)) was used at the harmonic temperature T_{hm} (the arithmethic mean of the independent variable $1/T$, the inverse experimental temperature, $\langle\frac{1}{T}\rangle$).

3. Experimental section

3.1. Apparatus

The chromatographic system consisted of a HPLC Waters pump 501 (Saint Quentin-Yvelines, France), an Interchim Rheodyne injection valve model 7125 (Montluçon, France) fitted with a 20-μl sample loop and a Merck 2500 diode array detector (Nogent-sur-Marne, France). An HSA protein chiral Shandon column 150×4.6 mm I.D. (Cergy-Pontoise, France) and an Interchim RP18 column 125×4 mm I.D. were used with a controlled temperature in an Interchim oven (TM No. 701). The mobile phase was fixed at 1 ml/min and the wavelength at 254 nm.

3.2. Reagents

All the triazine derivatives were obtained from Sigma–Aldrich (Saint-Quentin, France). The chemical structures of these compounds are given in Fig. 1. Fresh samples were prepared daily in water at a concentration varying from 0.10 to 1.00 m*M*. Deuterium oxide (Merck, Nogent-sur-Marne, France) was used as a dead time marker. Sodium chloride, sodium hydrogen phosphate and sodium dihydrogen phosphate were supplied by Prolabo (Paris, France). Water was obtained from an Elgastat option water purification system (Odil, Talant, France) fitted with a reverse osmosis cartridge. The mobile phase consisted of 2.5×10^{-3} *M* sodium phosphate buffer (pH 7.3). When the HSA (Aldrich, Paris, France) and sodium chloride were added to the buffer, their variation ranges were, respectively, 1.50–4.50 μ*M* and 80–160 m*M*, and no phosphate buffer pH difference was observed.

3.3. Temperature study

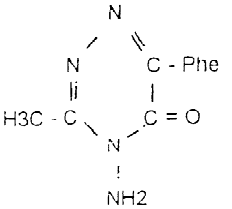
Compound retention factors were determined over the temperature range 25–45 °C (±0.1 °C). The chromatographic system was allowed to equilibrate at each temperature for at least 1 h prior to each experiment. To study this equilibration, the compound retention time of atrazine was measured every hour for 7 h and again after 22, 23 and 24 h. The maximum relative difference of the retention time of this compound was 0.6% making the chromatographic system sufficiently equilibrated for use after 1 h.

4. Results and discussion

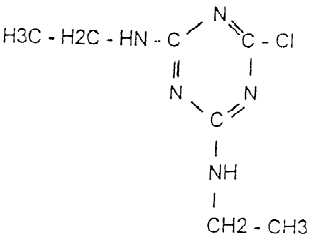
First, 20 μl of each solute solution were injected in triplicate. The variation in the coefficients of the k values was less than 1% in most cases indicating high reproducibility and good stability for the chromatographic system.

4.1. When HSA was grafted on the stationary phase (HSA Shandon column)

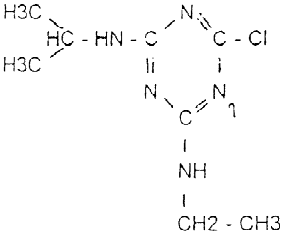
In this case, the concentration of triazine in each sample was equal to 1 m*M* and the concentration of



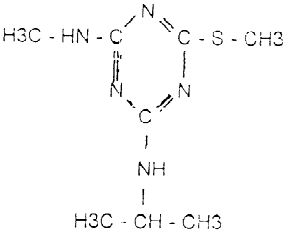
METAMITRON C10H10N4O



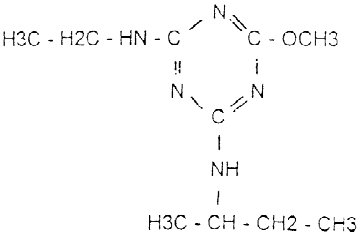
SIMAZINE C7H12ClN5



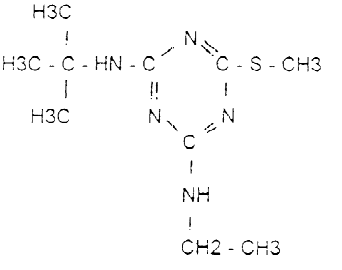
ATRAZINE C8H14ClN5



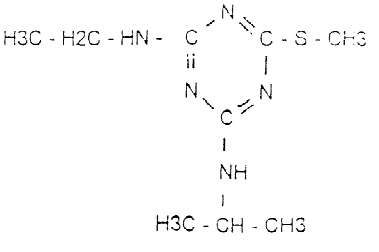
DESMETRYNE C8H15N5S



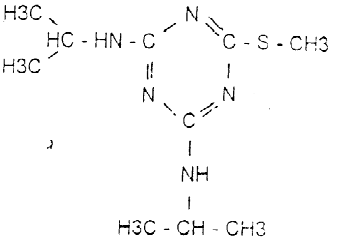
SECBUMETON C10H19N5O



TERBUTRYNE C10H19N5S



AMETRYNE C9H17N5S



PROMETRYNE C10H19N5S

Fig. 1. Triazine derivative structures.

Na^+ cation in the bulk solvent was equalled to zero. The *k* values were determined in the entire range of temperature, i.e. from 25 to 45 °C. Linear van't Hoff plots were obtained with correlation coefficients *r* higher than 0.997 for all fits. The thermodynamic data of the solute–HSA association process were determined according to Eq. (3) and were negative values (Table 1). In order to gain further insight into the herbicide–HSA association, the enthalpy–entropy compensation described in Eq. (6) was applied. An $\ln k_{T_{hm}} - \Delta H°$ plot determined at T_{hm} was made for the eight triazines derivatives. The determination coefficient r^2 for the linear fit was equal to 0.982 (Fig. 2). This degree of correlation can be considered adequate to verify enthalpy–entropy compensation.

4.2. When HSA was added to the mobile phase (RP 18 Interchim column)

In this case, the concentration range of each herbicide in the samples varied from 0.10 to 1.00 m*M*. To obtain the constant *K* and n_c at 37 °C (Eq. (5)) for each Na^+ cation concentration in the bulk solvent, the retention factors of all herbicides were determined for a wide range of total concentrations of HSA and herbicide M. With a weighted non-linear regression, used in early chromatographic studies [27,28], these data were fitted to Eq. (5). The WNLIN regression method was used to calculate the optimum parameter values by simultaneously minimizing the χ^2 function with respect to each of the parameters [28,29] (Eq. (5)). The correlation between the measured and predicted values from Eq. (5) exhibited a slope equal to 0.996 (ideal is 1.000) and an $r^2 = 0.995$ (Fig. 3). This correlation between the theoretical and the experimental values was considered to verify the model. The association mechanism of herbicides with the HSA cavity is controlled by classical primary and secondary interactions involved in the binding of the ligand to the receptor. The primary or non-specific interactions are dependent on long range forces which approach the solute near the HSA cavity. At the pH of the mobile phase used in this work (pH 7.3), the secondary or tertiary amine group ($pK_a > 10$) of the triazine derivatives were ionizied (cationic form) and the HSA was negatively charged [30]. Thus, these interactions consist principally of a combination of electrostatic attraction and hydrophobic effect. After this first contact step, the solute engages in strong secondary

Table 1
Values of the triazine–HSA association enthalpy $\Delta H°$ (kJ/mol) and entropy $\Delta S°^*$ (no unit as $\Delta S°^* = \frac{\Delta S°}{R} + \ln \phi$) (standard deviations <0.2)

Herbicides	$\Delta H°$ (kJ/mol)	$\Delta S°^*$ (no unit)
Metamitron	−9.59	−3.05
Simazine	−12.01	−3.77
Atrazine	−13.66	−3.99
Desmetryne	−19.01	−5.41
Secbumeton	−22.48	−6.48
Terbutryne	−24.01	−7.07
Ametryne	−27.44	−8.74
Prometryne	−36.98	−11.71

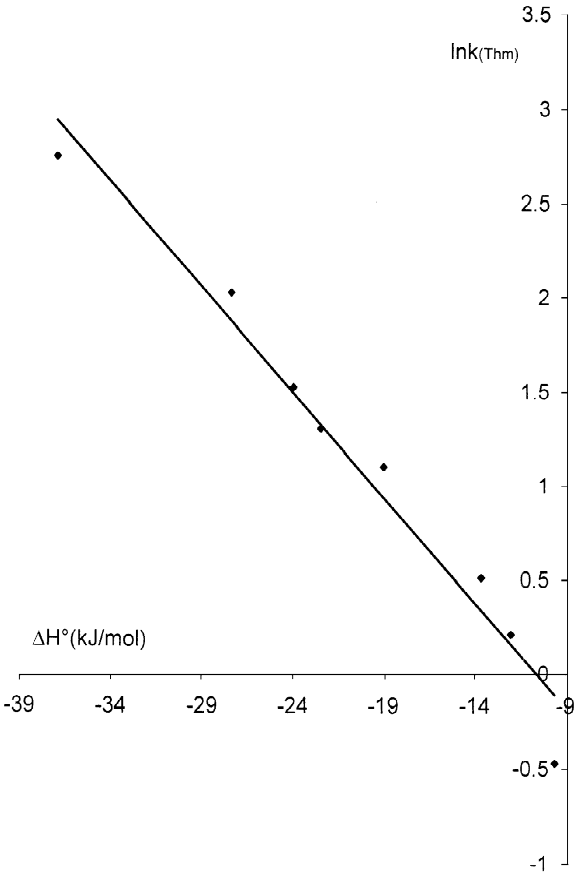


Fig. 2. Enthalpy–entropy compensation represented by the $\ln k_{T_{hm}} - \Delta H°$ plot for the eight triazine derivatives.

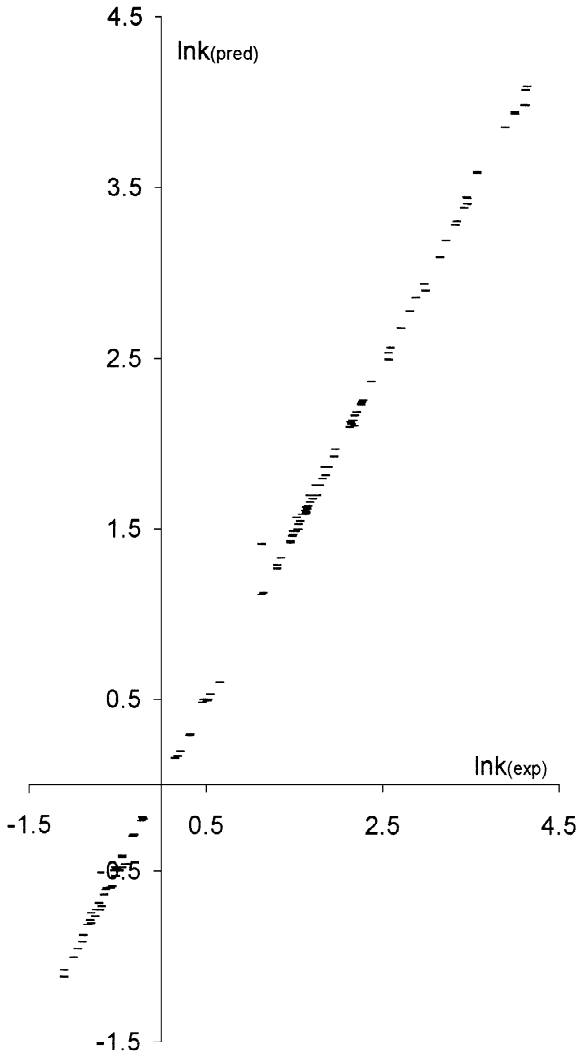


Fig. 3. Correlation between predicted (Eq. (5)) and experimental retention factors for the triazine herbicides at $T = 37\ ^{\circ}C$.

specific short-range interactions such as Van der Waal's interactions, steric repulsion or hydrogen bonding. The existence of the enthalpy–entropy compensation demonstrated that: (i) the herbicide–HSA association mechanism was independent of the herbicide molecular structure, (ii) the herbicide bound on the same site on HSA. The negative enthalpies (Table 1) indicated that it was energetically more favourable for the herbicide to be associated with the HSA cavity rather than to be free. This was classically accompanied by negative entropies (Table 1) due to the loss of the degree of freedom of the herbicide when it was included in the HSA cavity. This association mechanism was enthalpically driven (magnitude of ΔH° was always greater than that of $T\ \Delta S^{\circ}$) and can be described by the replacement of weak herbicide–bulk solvent interactions by strong herbicide–HSA Van der Waal's (London forces) and polar interactions. Prometryne, ametryne, terbutryne, secbumeton, and desmetryne had the highest formation constants and degree of complexation values and the lowest thermodynamic data in comparison with those of atrazine, simazine and metamitron (Table 2). Prometryne, ametryne, and desmetryne, differed only by the group R–NH– in position 2 in the 1,3,5 triazine ring (Fig. 1). The strength of the herbicide–HSA association, which varied identically as the K and n_c values (Table 2), was as follows: R = $-CH(CH_3)_2 > -CH_2-CH_3 > -CH_3$ showing the importance of the role of the hydrophobic effect on its association. These triazine derivatives exhibited the lowest enthalpy data because they had the strongest Van der Waal's and polar interactions with the HSA cavity. This was associated with the lowest entropy state classically attributed to the release of the water molecules surrounding the solute when it was transferred inside the HSA cavity [31–34]. Simazine and atrazine differed by the group R–NH– in position 2 in the 1,3,5-triazine ring (Fig. 1), and as well, R = $-CH(CH_3)_2 > -CH_2-CH_3$ confirming the importance of the hydrophobic effect on the herbicide–HSA association process. The substitution in position

Table 2
Values of the constants ln K and the degree of complexation n_c for the triazine–HSA association at 37 °C (standard deviations <0.1)

Herbicides	ln K	n_c
Metamitron	7.07	0.22
Simazine	7.53	0.27
Atrazine	8.54	0.33
Desmetryne	8.95	0.39
Secbumeton	9.62	0.49
Terbutryne	9.95	0.50
Ametryne	10.08	0.58
Prometryne	10.28	0.63

6 on the 1,3,5-triazine ring of a $-S-CH_3$ (or $-O-CH_3$) group (prometryne, ametryne, terbutryne, desmetryne and secbumeton) by a chloro group (atrazine, simazine) largely decreased the strength of the association. This variation can be explained by a large decrease in the hydrophobic effect. Metamitron (Fig. 1) which had a 1,2,4-triazine ring and in position 6, a phenyl group, had, among all the studied herbicides, the highest steric bulkiness. This decreased its association with the HSA cavity. Therefore, metamitron had the highest enthalpy value due to low Van der Waal's interactions with the HSA cavity. This was associated with the highest entropy state due to an increase of the degree of freedom of metamitron inside the HSA cavity.

4.3. Effect of the sodium cation (Na^+)

The constants K and n_c of the triazine–HSA association were determined for all the Na^+ concentrations in the bulk solvent at $T=37$ °C. All the triazine derivatives exhibited a similar variation for the K and n_c values with the NaCl concentration.

For example, Fig. 4A and B represent the increase in the K and n_c values with the NaCl concentration for atrazine. This variation was confirmed by the fact that the Na^+ cation increased the hydrophobic effect in the bulk solvent and thus the triazine–HSA association. These results showed the role of the salting out agent Na^+ as a co-enhancer of the association process. They corroborated the fact that,

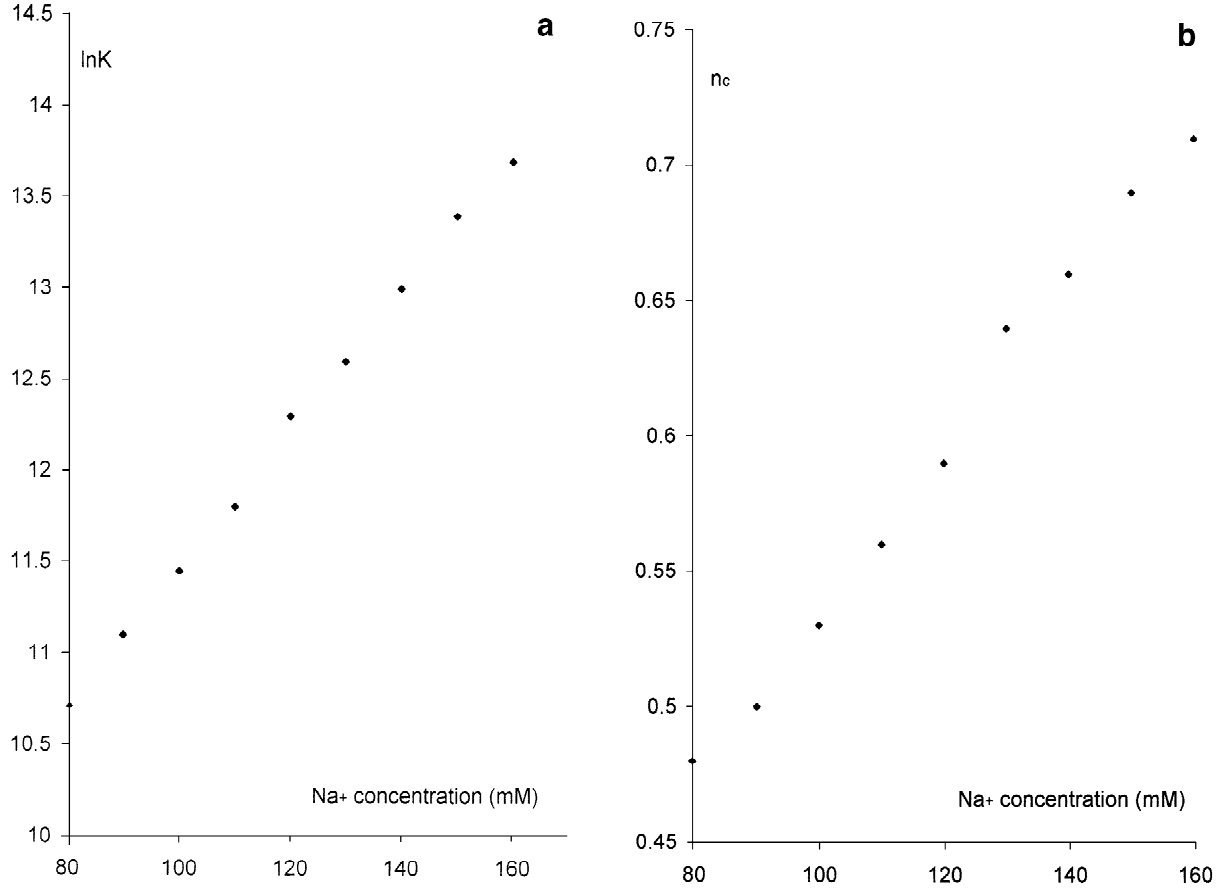


Fig. 4. Influence of the Na^+ concentration in the bulk solvent on the (A) K and (B) n_c values of atrazine at $T=37$ °C.

for patients who suffer from sodium desequilibrium, the binding of the herbicides with HSA changes, and, thus their toxicological effect.

5. Conclusion

Atrazine will be completely forbidden in France from 30 June 2002. On the basis of the results obtained, and by the fact that the toxicity effect of a substance is linked with its binding with HSA, the following remarks can be made:

(a) Prometryne, ametryne, terbutryne, secbumeton, and desmetryne had a higher affinity for HSA than atrazine. For simazine and metamitron the reverse was observed.

(b) Therefore, the delayed toxicity of prometryne, ametryne, terbutryne, secbumeton, and desmetryne was greater than that of atrazine. For simazine and metamitron, their immediate toxicity is greater than that of atrazine.

Also, the addition of an Na^+ cation to the bulk solvent increased the delayed toxicity for all triazines by increasing their binding constant with HSA.

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