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Anti-coagulant rodenticide binding properties of human serum albumin: a biochromatographic approach

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Abstract

In this paper, the anti-coagulant rodenticide-human serum albumin (HSA) binding was investigated using a perturbation method to calculate the solute distribution isotherms. It was shown that rodenticide can bound either on the benzodiazepine HSA site with low affinity (site I) or on the warfarin HSA site with high affinity (site II). The thermodynamic parameters of this association were calculated for the two HSA binding sites. For the site II, the rodenticide-HSA association was governed enthalpically whereas for the site I, this one was driven entropically. Moreover, the role of the magnesium (Mg^{2+}) and calcium (Ca^{2+}) on this association was carried out. It was clearly demonstrated that the rodenticide affinity for the site I was not affected by modifying the bulk solvent surface tension whereas for the site II the association constant increased strongly with the Mg^{2+} or the Ca^{2+} concentration in the bulk solvent. These results showed that the rodenticide-HSA affinity and thus the rodenticide toxicological effect depends on the Mg^{2+} or Ca^{2+} concentration.

Keywords: Human serum albumin; Rodenticide

1. Introduction

Rodenticides specially designed to kill rodents, pose particular risks for accidental poisoning for several reasons. Since they have been designed to kill mammals, they are also toxic to humans. Because rodents usually share human environments, use of rodenticides poses an inherent risk of exposure to people, particularly children and their pets, as well as other non-target species [1]. In addition, as rodents have developed resistance to these chemicals, there continue to be a need to develop new and potentially more toxic rodenticides [2,3]. Multiple feed baits are the most commonly used type of rodent poisons. Typically these poisons act as anti-coagulants, literally causing the victim to bleed to death internally [4,5]. The fact that these poisons must be made available to the pest animal over time makes them very hazardous as children, pets and other non-target animals have an extended opportunity to get into them. The Environmental Protection Agency (EPA) recognizes that anti-coagulant rodenticides, are responsible for a high number of human incidents and accidental exposures each year [2]. There are two classes of anti-coagulant type rodent poisons, the coumarins and the indandiones [2]. Coumarins include some very common rodent poisons such as warfarin, bromadiolone, and coumafuryl. Indandiones include the rodent poison chlorophacinone. Both of these classes of toxic materials work by blocking vitamin K-dependent synthesis of the blood clotting substance prothrombin, which predisposes the animal to widespread internal bleeding [4,5]. Animals suffering from exposure to anti-coagulant rodenticides suffer from the following list of immediate toxic effects: nosebleeds, bleeding gums, blood in urine and feces; bruises due to ruptured blood vessels; and skin damage. Exposure to these poisons also has long-term health effects. The coumarin, warfarin rodenticides, for example, caused paralysis due to cerebral hemorrhage and is teratogenic (causes birth defects). Long-term exposure to the indandione, diphacinone causes nerve, heart, liver, and kidney damage as well as damage to skeletal muscles [2]. Human serum albumin (HSA) is a single non glycosylated polypeptide that organizes to form a heart-shaped protein with approximatively 67% α-helix but no β-sheet [6]. The protein is composed of three homologous

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domain (I–III); each domain has two subdomains (A and B) possessing common structural elements [7–9]. HSA is able to bind reversibly a large number of endogenous and exogenous compounds. The capacity of binding aromatic and heterocyclic compounds depends largely on the existence of two major binding regions namely Sudlow's site I (or benzodiazepine site) and site II (or warfarin site), which are located within specialized cavities in subdomains IIA and IIIA, respectively [9]. It is known that site II is more versatile, because it can bind ligands that are chemically very different with a high affinity. Several examples of independent, anti-cooperative and competitive binding to this region have been published [10–12]. It has been suggested that HSA serves as a carrier to transport rodenticides to molecular targets and their toxicity effect is directively linked with their HSA binding. Then, this paper describes the association of six rodenticides (i.e. bromadiolone, warfarin, difenacoum, chlorophacinone, diphacinone brodifacoum) with HSA and shows the role of magnesium cation (Mg^{2+}) and calcium cation (Ca^{2+}) which their plasma concentrations are linked to different factors such as stress, pathogenese, nutrition [13,14]. Their effects on the rodenticide-HSA interaction was analysed using the perturbation technique, originally developed for measuring gas-adsorbent equilibria [15–17].

2. Theory

The non-linear chromatography determinates the sample adsorption isotherms using the perturbation technique [18–21] which consists in the determination of the retention times of small sample amounts injected onto the column equilibrated with sample solutions at different concentration levels. 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 isotherms is first equilibrated with a solution containing the sample dissolved in a non-adsorbable solvent. Then a small sample volume containing different (lower or higher) concentration of the sample is injected onto the column. After the injection, the equilibrium condition is disturbed and a perturbation wave arise which migrate along the column [18–21]. When such a wave reaches the column outlet, a negative or a positive peak is registered by the detector, depending on whether the concentrations of the sample compounds injected are higher or lower than their equilibrium concentrations at the start of the experiment. The well-known Langmuir theoretical approach relates the total concentration of the sample in the HSA (C_s) and in the mobile phase (C_m) [18–21]:

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

where α is the HSA column saturation capacity and K is the association constant between the studied sample and the HSA. The sample retention factor k was directly proportional to the slope of its adsorption isotherm and can be thus given by the following equation [18,20]:

$$k = \frac{\phi \alpha K}{(1 + K C_m)^2} \tag{2}$$

where ϕ is the column phase ratio (volume of the stationary phase divided by the volume of the mobile phase; equal to 0.22 for the HSA stationary phase, commercial data). By plotting the k value versus the sample concentration in the bulk solvent, the constant K can be determined using Eq. (2). If the sample bound on two sites on the HSA, i.e. a site with a lower affinity (site I; with an adsorption constant K_I and a column saturation capacity α_I) and a high affinity site (site II; with an adsorption constant K_{II}, and a column saturation capacity α_{II}), then the rodenticide retention factor (k) directly proportional to the slope of its adsorption isotherm is given by the following equation:

$$k = \phi \left(\frac{\alpha_I K_I}{(1 + K_I C_m)^2} + \frac{\alpha_{II} K_{II}}{(1 + K_{II} C_m)^2} \right) = \frac{\bar{k}_I}{(1 + K_I C_m)^2} + \frac{\bar{k}_{II}}{(1 + K_{II} C_m)^2} \tag{3}$$

where $\bar{k}_I$ (equal to $\phi \alpha_I K_I$) and $\bar{k}_{II}$ (equal to $\phi \alpha_{II} K_{II}$) are the apparent retention factors (retention factor when the rodenticide concentration in the mobile phase was nil) of respectively the rodenticide association on the low affinity HSA site (site I) and high affinity site (site II). Then, using a non-linear regression analysis, by studying the variation of the k values versus the sample concentration in the mobile phase, the apparent retention factors $\bar{k}_I$, $\bar{k}_{II}$ and the association constant K_I, K_{II} can be calculated using Eq. (3).

The Gibbs free energy (ΔG_i° of the solute molecule adsorption either on the low affinity site (site I; i = I) or with the high affinity site (site II; i = II) can be linked to the adsorption constant K_i according to the well-known equation [22]:

$$\ln(K_i) = \frac{-\Delta G_i^\circ}{RT} \tag{4}$$

where R is the gas constant and T is the column temperature.

As well ΔG_i° can be linked to the enthalpic (ΔH_i°) and entropic (ΔS_i°) terms for the two adsorption processes (i.e. rodenticide adsorption on the HSA site of low affinity (site I; i = I) and high affinity (site II; i = II) by the following equation:

$$\Delta G_i^\circ = \Delta H_i^\circ - T \Delta S_i^\circ \tag{5}$$

The relationship between the apparent retention factor of the HSA site i (i = I or II) $\bar{k}_i$, and the thermodynamic parameters (ΔH_i° and ΔS_i°) is given by the following equations [22]:

$$\ln(\bar{k}_i) = \frac{-\Delta H_i^\circ}{RT} + \frac{\Delta S_i^\circ}{R} + \ln(\alpha_i \phi) \tag{6}$$

where ΔH_i° and ΔH_i° are, respectively, the enthalpy and entropy changes accompagning the rodenticide adsorption on the HSA site i.

The plot of $\ln(\bar{k}_i)$ against $1/T$ is called a van't Hoff plot. For linear plot, the slope and intercept are, respectively, $-\Delta H_i^\circ/R$ and $\Delta S_i^\circ/R + \ln(\alpha_i\phi)$. Knowing the values of $\bar{k}_i$ and K_i (thank to Eq. (3) (non-linear regression analysis) and ϕ (equal to 0.22; commercial data)) the values of α_i can be calculated ($\alpha_i = \bar{k}_i/\phi K_i$).

This provides a convenient way of calculating the thermodynamic constants ΔH_i° and ΔS_i°. Investigation of the enthalpy–entropy compensation temperature is a useful thermodynamic approach to the analysis of physicochemical data [23–27]. Mathematically, the enthalpy–entropy compensation can be expressed by the following equation [23–27]:

$$\Delta H_i^\circ = \Delta G_{i\beta}^\circ + T\,\Delta S_i^\circ \tag{7}$$

In this equation ΔH_i° and ΔS_i° are respectively the enthalpic and entropic changes during the rodenticide adsorption on the site i. $\Delta G_{i\beta}^\circ$ 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 $\Delta G_{i\beta}^\circ$ at the temperature β [23–27].

Combining Eqs. (6) and (7) the following equation is obtained:

$$\ln(\bar{k}_i)_T = \frac{-\Delta H_i^\circ}{R}\left(\frac{1}{T} - \frac{1}{\beta}\right) - \frac{\Delta G_{i\beta}^\circ}{R\beta} + \ln(\alpha_i\phi) \tag{8}$$

Eq. (8) shows that if a plot $\ln(\bar{k}_i)_T$ versus ΔH_i° is linear, all the rodenticide will have the same apparent retention factor $\bar{k}_i$ at the temperature β and are retained by essentially identical adsorption mechanism.

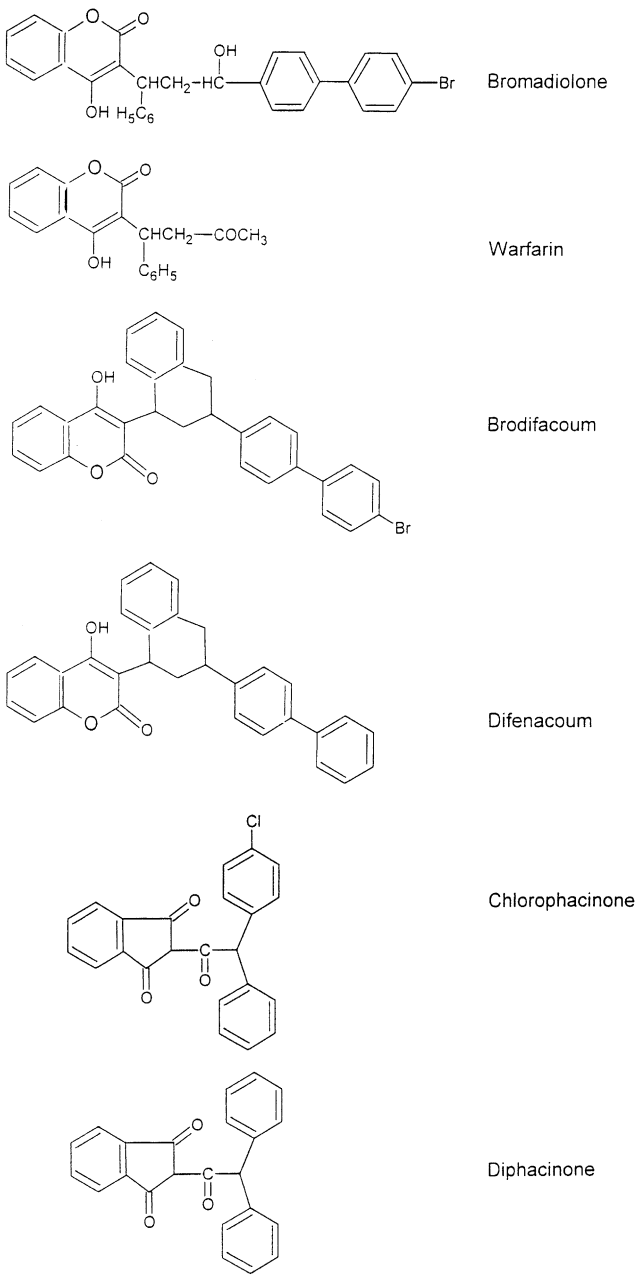

Fig. 1. Anticoagulant rodenticide structures.

3. Experimental section

3.1. Apparatus

The chromatographic system consisted of an HPLC Water pump 501 (Saint-Quentin, France), an Interchim Rheodyne injection valve model 7125 (Montlucon, 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 mm × 4.6 mm (Cergy-Pontoise, France) was used with a controlled temperature in an Interchim oven (TM No. 701). After each utilisation, the column was stored at 277 K until further use. The mobile phase was fixed at 1 ml/min and the wavelength at 254 nm.

3.2. Reagents

All the rodenticides were obtained from Sigma–Aldrich (Saint-Quentin, France). The chemical structure of these compounds are given in Fig. 1. Although some of rodenticides used in this study have one or two asymetric center (s), no enantiomer rodenticide can be separated. Natrium sodium was used as a dead time marker. 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; human plasma pH). The concentration range of $MgCl_2$ and $CaCl_2$ varied, respectively, from 0 to 2 mM (including its biological concentration range (0.70–0.90 mM)) and from 80 to 160 mM (including its biological concentration range (110–130 mM)). 12 $MgCl_2$ values (i.e. 0, 0.2, 0.3, 0.5, 0.7, 0.9, 1.1, 1.2, 1.4, 1.6, 1.8 and 2.0 mM) and 16 $CaCl_2$ values (i.e. 80.0, 85.0, 90.0, 100.0, 105.0, 110.0, 115.0, 120.0, 125.0, 130.0 135.0, 140.0, 145.0, 150.0, 155.0, 160.0 mM) were included in these ranges. Experiments were run over the temperature range 278 to 313 K. For all the experiments, no phosphate buffer pH difference was observed. The equilibration of the column was carried out with 15 different concentrations of rodenticide studied (0–7 mM) in each mobile phase used to obtain a stable detection. 20 μl of the most concentrated rodenticide sample was injected three times and the retention time was measured.

3.3. Temperature study

Compound retention factors were determined over the temperature range 278–313 K. The chromatographic system was allowed to equilibrate at each temperature for at least 1 h prior to each experiment. To study this equilibrium process the retention time of bromadiolone was measured after 20, 21 and 23 h. The maximum relative difference between retention times of this compound was 0.7% meaning that after 1h the chromatographic system was sufficiently equilibrated for use.

4. Results and discussion

4.1. Rodenticide-HSA binding process when the Mg^{2+} and Ca^{2+} concentration in the mobile phase was nil

For each temperature and for each rodenticide concentration in the bulk solvent, the most concentrated sample was injected and its retention factor were determined at five temperatures, i.e. 278, 288, 298, 310 and 313 K. The variation coefficients of the k values were <0.3%, indicating a high reproducibility and a good stability for the chromatographic system. The plot of the k values versus the rodenticide concentration in the mobile phase was similar for all the rodenticide studied. An example of plot was given in Fig. 2. Using a weighted non linear regression (WNLIN) procedure, the constants $\bar{k}_I$, $\bar{k}_{II}$ and K_I, K_{II} values of Eq. (3) were estimated. The slope (0.999; ideal is 1.000) and r^2 (0.997) indicated that there is an excellent correlation between the predicted and experimental retention factors. 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 variables can be excluded from the model. These results showed that the Langmuir model (Eq. (3)) describes accurately the association behavior of rodenticide with HSA. For each temperature, the corresponding K_I and

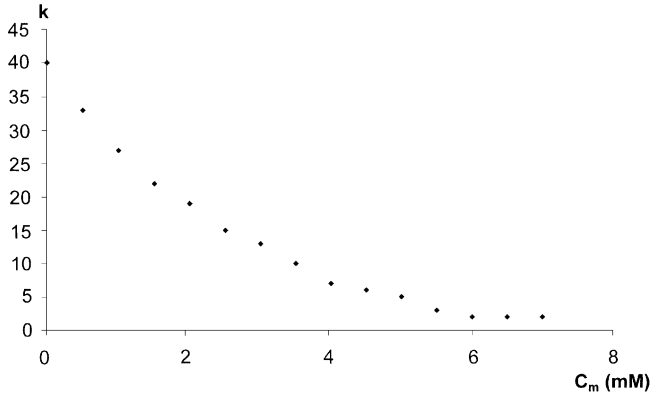

Fig. 2. k values vs. the bromadiolone concentration in the bulk solvent at 298 K.

K_{II} values were calculated. For example, the K_I and K_{II} values were given in Table 1 for all rodenticide molecules at 310 K. The K_I values were always lower than the K_{II} values (Table 1; for example, for warfarin, $K_I = 1.9 \times 10^3 \ll K_{II} = 4.8 \times 10^4$). Similar warfarin HSA association constant was previously observed by Loun and Hage [28], but here an additional and important conclusion can be drawn: anti-coagulant rodenticides can bound either on the low affinity site (site I, benzodiazepine site) or the high affinity site (site II also named warfarin site). The thermodynamic parameters for the two HSA sites (I and II) were calculated from van't Hoff plots. $\ln \bar{k}_i$ versus $1/T$ were drawn for the six rodenticides. Linear van't Hoff plots were obtained for all the compounds with r higher than 0.998. For example, Fig. 3 shows the plots for bromadiolone and for both the site I and II. The ΔH° and ΔS° values for the site I and II are given in Table 2. For the site I, the ΔH° and ΔS° values were positive whereas for the site II, these values were negative. Then, the transfer of the rodenticides from the bulk solvent to the HSA site I was entropically driven (i.e. governed by steric hindrance) whereas the one to the HSA site II was enthalpically controlled (i.e. governed by hydrophobic effect). Rodenticide coumarin classe (i.e. bromadiolone, brodifacoum, difenacoum, warfarin) presents HSA association constant higher than indandione classe (i.e. chlorophacinone, diphacinone) (Table 1) due to its high hydrophobic character [29,30]. Among the coumarin family, bromadiolone exibited the lowest thermodynamic data for the site II.

Table 1
K_I and K_{II} (M^{-1}) at T = 310 K when the magnesium and calcium concentration in the mobile phase was nil

Rodenticides	K_I (10^3)	K_{II} (10^4)
Diphacinone	1.51	2.22
Chlorophacinone	1.57	2.27
Warfarin	1.90	4.81
Difenacoum	2.54	6.21
Brodifacoum	2.78	6.98
Bromadiolone	2.81	7.01

Standard deviation < 0.07.

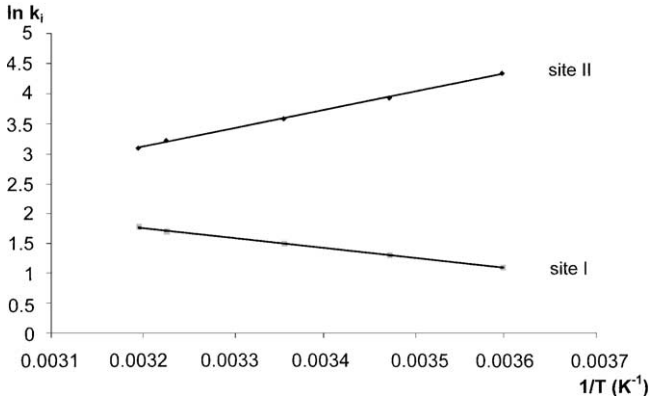

Fig. 3. Van't Hoff plots for the sites I and II for the bromadiolone compound.

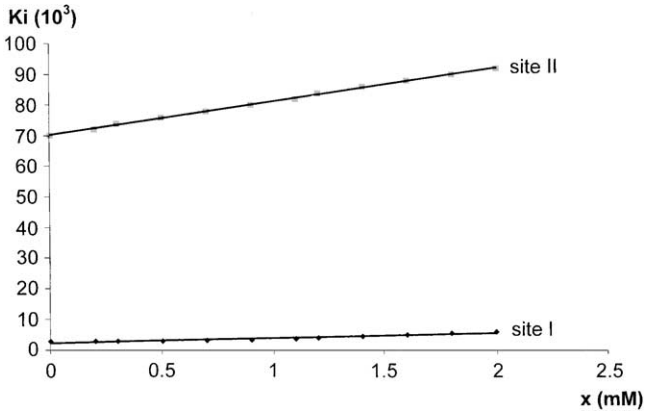

Fig. 4. K vs. the magnesium concentration in the mobile phase (x) for the sites I and II for the bromadiolone compound at 310 K.

This can be explained by (i) the high hydrophobicity of this rodenticide and (ii) the –Br substituent. Indeed, as previously observed by Guillaume's group, the polar substituent on the compound enhanced the HSA affinity by increasing van der Walls interactions between the solute and the rim of the HSA sites [12]. This was associated with the lowest entropy state classically attributed to the release of the water molecules surrounding the solute when the rodenticide was transferred inside the HSA site [12,26]. 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, it can be deduced that the immediate toxicity of rodenticide indandione classe was greater than rodenticide coumarin family. Among this last classe, bromadiolone presents the greatest delayed toxicity. This was consistant with litterature [1–5]. The first anticoagulants (warfarin, diphacinone and chlorophacinone) action required multiple feedings over several days to a week or more to produce death [1]. In order to achieve this multiple feeding, the bait must be made available on a continuous basis until the desired control is reached. Where these anticoagulants have been used over long periods of time at a particular location, there is a potential increase for a population to become somewhat resistant to the lethal effects (i.e. warfarin resistance) [3,4]. This led to the development of the second generation (brodifacoum, bromadiolone, difenacoum). These compounds are much more potent than the first generation anticoagulant rodenticides (i.e. they are effective against warfarin resistant rats and mice) [4,5].

Table 2
ΔH_i° (kJ mol^{-1}) and ΔS_i° (J mol^{-1} K^{-1}) values for the sites I and II for the six rodenticides when the magnesium and calcium concentrations in the mobile phase was nil

Rodenticides	ΔH_I°	ΔS_I°	ΔH_{II}°	ΔS_{II}°
Diphacinone	11.09	50.81	−19.77	−49.91
Chlorophacinone	11.12	50.96	−19.79	−49.96
Warfarin	11.21	50.98	−19.81	−49.99
Difenacoum	13.80	57.10	−24.91	−55.02
Brodifacoum	13.85	58.21	−24.98	−55.45
Bromadiolone	13.86	58.94	−25.43	−55.47

Standard deviation < 0.5.

4.2. Mg^{2+} and Ca^{2+} effect on the rodenticide-HSA association at 310 K

To determine the influence of magnesium or calcium concentration on the rodenticide-HSA binding mechanism, the previous experiments were carried out at 310 K with a various magnesium concentration (0–2.0 mM) or calcium concentration (80–160 mM) including their biological concentration ranges. For each herbicide rodenticide and magnesium concentration (or calcium concentration) the non-linear regression coefficient of the bi-Langmuir equation (Eq. (3)) were determined ($r^2 > 0.995$) and the corresponding K_I and K_{II} values were calculated. The curves K_i versus the magnesium concentration (or calcium concentration) in the mobile phase were similar for each herbicide molecule. For example, Figs. 4 and 5 give the plots obtained for bromadiolone for the site I (low affinity) and II (high affinity) with respectively different magnesium and calcium concentrations. A general phenomenon in early studies on HSA is its ability to bind divalent inorganic cations which led to a competition with the solute to bind on the same HSA site and then a decrease of the solute-HSA association

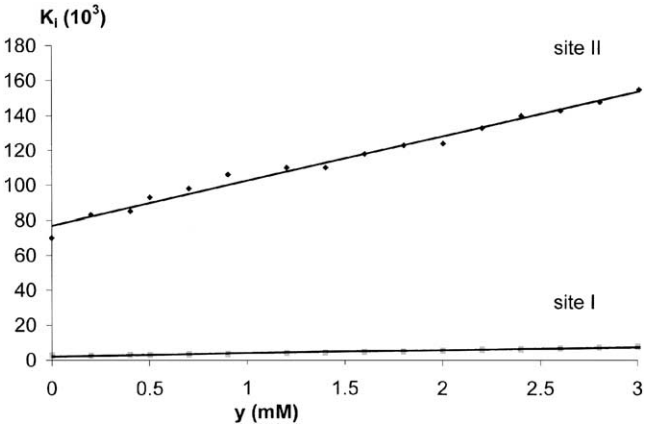

Fig. 5. K vs. the calcium concentration in the mobile phase (y) for the sites I and II for the bromadiolone compound at 310 K.

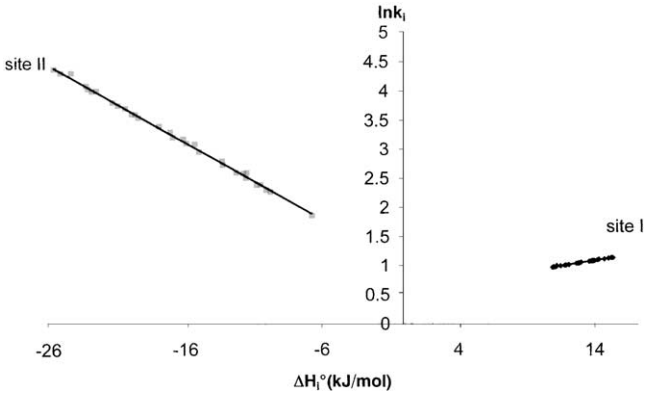

Fig. 6. $\ln \bar{k}_i$ vs. DHi plot determined for the sites I and II, for all the rodenticides and (i) the magnesium and (ii) the calcium concentration in the bulk solvent at $T = 310$ K.

[31–34]. In contrast, with the great majority of cationic ligands such as Ni^{2+}, Cd^{2+}, which are bound specifically to albumin with the binding mode involving the formation of multiple chelate ring [35], Ca^{2+} and Mg^{2+} interact preferentially with several sites, which differ only slightly in their affinity towards the ligand [36]. For example, Pederson et al. demonstrated that magnesium constant association of magnesium to HSA was equal to 10^2 and the number of binding sites was 12 [37]. The other process observed with magnesium and calcium cations was an increase of the hydrophobic effect and thus the solute-HSA association [31,38]. Figs. 4 and 5 showed that for the two HSA sites, increasing the magnesium or the calcium concentration in the bulk solvent led an increase of the rodenticide-HSA association. Then, for this calcium and magnesium concentration, it was the increase of the hydrophobic effect which was predominant and governed the rodenticide-HSA association. However, the K_{II} values increased strongly with increasing Mg^{2+} or Ca^{2+} concentration in the bulk solvent whereas K_I values increased very slowly. This observation showed that the rodenticide association mechanism governed by the hydrophobic effect principally took place in the HSA site II.

In order to gain further insight into this binding mechanism, an enthalpy–entropy compensation was also investigated. The plot $\ln \bar{k}_i$ versus ΔH_i° determined for the site I and II were drawn for all the rodenticides and for all the (i) magnesium and (ii) calcium concentrations in the bulk solvent at $T = 310$ K (Fig. 6). The correlation coefficient for the four linear fits were higher than 0.997.

The regression lines for the magnesium concentration were

- for the site I: $\ln \bar{k}_I = -0.1327\,\Delta H_I^\circ + 1.0123$;
- for the site II: $\ln \bar{k}_{II} = -0.0378\,\Delta H_{II}^\circ + 0.5612$.

The regression lines for the calcium concentration were

- for the site I: $\ln \bar{k}_I = -0.1329\,\Delta H_I^\circ + 3.5896$;
- for the site II: $\ln \bar{k}_{II} = -0.0380\,\Delta H_{II}^\circ + 0.4578$.

According to these regression analyses and the correlation coefficients, the following conclusions can be drawn [23–27,29,30]:

- The binding mechanism for the two HSA sites was independent of (i) the anti-coagulant rodenticide structure and (ii) the Mg^{2+} or the Ca^{2+} concentration in the mobile phase.
- Whatever the cation used, the slopes of these linear plots were different for the two HSA sites (I and II) confirming a change in the rodenticide-HSA association mechanism in these two binding sites (for example, $\beta_{I,Mg^{2+}} \approx 470\,K \neq \beta_{II,Mg^{2+}} \approx 345\,K$).

5. Conclusion

The Environmental Protection Agency (EPA) recognizes that anti-coagulant rodenticides are responsible for a high number of human incidents and accidental exposures. Although the toxicity effect of a substance is linked with its binding with HSA, no publication reported the rodenticide-HSA association process. In this manuscript, using a non linear chromatography method, it was demonstrated that two sites were implied in the rodenticide association with HSA. The magnesium and calcium cation effect on this association was also investigated. As well, it was clearly demonstrated that, in the biological concentration ranges, both magnesium and calcium effect increased the rodenticide-HSA association.

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