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Rodenticide–humic acid adsorption mechanisms and role of humic acid on their toxicity on human keratinocytes: chromatographic approach to support the biological data

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Abstract

Humic substances are the most important soil components affecting the behaviour and performances of herbicides in the soil–water–organism system. In this paper, a chromatographic approach was used for analysis of anticoagulant rodenticide–humic acid adsorption mechanisms. Using an equilibrium perturbation method, it was clearly shown that: (i) humic acid can be adsorbed on the C18 stationary phase, and (ii) all the rodenticides can be adsorbed on the humic acid adsorbed on the C18 stationary phase. This approach allowed the determination of the adsorption constant values between the anticoagulant rodenticides and humic acid as well as the corresponding thermodynamic data of this adsorption mechanism. The role of humic acid on the toxicity of these rodenticides on human keratinocytes was also clearly described in relation to these physico-chemical data.

Keywords: Rodenticide; Humic acid; Keratinocytes

1. Introduction

The increase in the population of pest rodents population in urban and rural areas is tackled by dissemination of baits poisoned with anticoagulant compounds, such as warfarin. However, this strategy carries a non-negligible health hazard because anticoagulants are toxic also for humans [1–12]. The danger has grown bigger with the introduction of a second generation derivatives, also called superwarfarins, because of their very long biological half lives [3–10]. The discovery of the anticoagulant activity prompted a wide research effort since the 1940s. It quickly became clear that the most promising compounds were those chemically related to 4-hydroxycoumarin (i.e. bromadiolone, brodifacoum, difenacoum) and to 1,3-indandione (i.e. chlorophacinone, diphacinone) [13,14]. Anticoagulant compounds are known to break the Vitamin K cycle [15–21]. Animals suffering from exposure to anticoagulant 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. Moreover, particularly for bromadiolone, residues of anticoagulants which are present in the bodies of dead or dying rodents can cause toxic effects to scavengers, predator and indirectly to humans [2–4]. Massive and repetitive rodenticide use may possibly cause modifications of general status and functions of native soil organic matter, and particularly, of its most biologically and chemically reactive fractions, humic substances [22]. Humic substances are the most important soil components affecting the fate, behaviour and performances of herbicides in the soil–water–organism system [23,24]. Among the various ways humic acid (HA) may interact with herbicides in soil, adsorption is considered to be the most relevant. The

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prevalent type (s) of binding mechanisms occurring in the adsorption processes mainly depend on the chemical, structural and functional properties of the interacting species and ground waters [25]. In our previous manuscript the interaction of anticoagulant rodenticides with human serum albumin (HSA) was studied in order to determine the delay of the toxicity of these poisons [26]. The objective of this work was to evaluate the rodenticide–humic acid (HA) adsorption process and the role of HA on the rodenticide toxicity on human keratinocytes.

2. Materials and methods

2.1. Apparatus

HPLC was performed with an Hitachi L7100 pump (Merck, Nogent sur Marne, France), a Rheodyne (Interchim, Montluçon, France) 7125 injection valve fitted with a 20 μl sample loop, and a Hitachi L4500 diode-array detector. The RP18 column used was a Nucleosil NAUTILUS (150 mm × 4.6 mm; 5 μm particle size) model (Macherey Nayel Hoerdt, France) which can support a total aqueous mobile phase. The column temperature was controlled by mean of an Interchim TM 701 for high temperature and an Osi Julabo FT 200 cryoimmerser (Elancourt, France) for low temperature. The mobile phase flow-rate was 0.8 ml/min and the detection wavelength 254 nm.

2.2. Solvents and samples

Water was obtained from an Elgastat water purification system (Odil, Talant, France) fitted with a reverse-osmosis cartridge. All the anticoagulant rodenticides were obtained from Sigma–Aldrich (Saint-Quentin-Fallavier, France). Their chemical structures were given in Fig. 1. HA is a generic term to describe various materials, according to their aquatic or terrestrial origin. In this context, Lee et al. [27] highlighted that it was impossible to predict the toxicological effects of humic organic substances, because of their heterogeneity. Thus, for our study, a commercial and standardized humic material was used (Fluka (Saint-Quentin-Fallavier, France). This HA (molecular mass 40,500) could form very stables complexes with metals and solutes [27,28], which were easily ingested by biological systems [29–32]. HaCaT cells were kindly given by Dr N. Gault (CEA, Bruyère Le Chatel, France). Dubelcco's modified Eagle's minimum essential medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), dimethylsulfoxide (DMSO) were purchased from Sigma–Aldrich; Costar culture flasks, microtiter plates, fetal calf serum (FCS) and trypsin were from D. Dutscher (Brumath, France). Phosphate-buffered saline (PBS without calcium and magnesium), trypsine and HEPES were obtained by VWR International (Cergy-Pontoise, France).

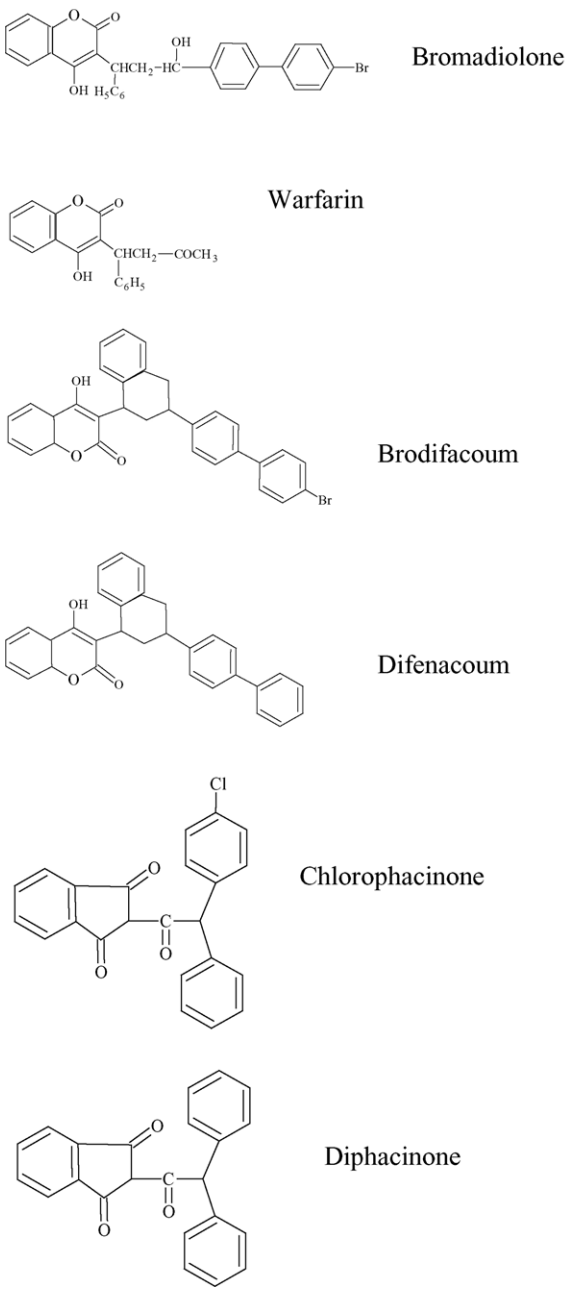

Fig. 1. Anticoagulant rodenticide structures.

2.3. Chromatographic operating conditions

The mobile phase consisted of an HA solution prepared by adding different weights of HA into deionized water and gently heating to 60 °C in order to accelerate the dissolution of HA. Then, the HA solutions were cooled down to room temperature gradually and was filtered through a 0.45 μm Millipore syringe filter. The HA residue on the filter was dried in an oven at 105 °C until its weight stabilized. The HA concentration in clear solution (filtrate) was calculated as ($[HA]_{initial} - [HA]_{residue}$) by the gravimetric method. The mobile phase pH was adjusted at pH 5 using a 0.05 M phos-

phate buffer solution [33]. The range of HA concentration was 0.01–0.1 M. For each rodenticide used, the equilibration of the column was carried out with 13 different concentrations of rodenticides (0–6 mM) in each mobile phase used to obtain a stable detection. Twenty μl of the most concentrated anticoagulant rodenticide samples were injected three times and the retention time was measured. The compound retention factors were determined over the temperature range −5 °C to 45 °C. The chromatographic system was left to equilibrate at each temperature for at least 1 h before each experiment. To study this equilibration, the retention time of bromadiolone was measured after 22, 23, 24 h. The maximum relative difference between retention times of this compound was never more than 0.7%, meaning that after 1 h the chromatographic system was sufficiently equilibrated for use.

2.4. Biological operating condition

The HaCaT cells were routinely grown in 75 cm^2 Costar plastic flasks in monolayer cultures in DMEM medium supplemented with 10% (v/v) FCS, and 5 M of HEPES [34,35]. They were grown in a humidified atmosphere of 5% CO_2 in air. The medium was renewed routinely 2, 4 and 6 days after passage and when confluence was attained, cells were trypsinized and split for subcultures (seeding density 3500 cells/cm^2 in a 75 cm^2 flask) or used for cytotoxicity assays. Cells were used for experiments within ten passages to ensure cell line stability.

3. Theory

3.1. Non-linear chromatography method

This process determines the sample adsorption isotherms using the perturbation technique. Indeed, the perturbation technique allowed 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 anticoagulant rodenticide dissolved in a non-adsorbable solvent. Then, a small sample volume containing different concentrations of the rodenticide is injected onto the column. After the injection, the equilibrium condition is disturbed and a perturbation wave arise which migrate along the column. When such a wave reaches the column outlet, 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 rodenticide in the stationary phase (C_s) and that in the mobile phase (C_m) by the following equation [36–38]:

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

where α is the column saturation capacity and K is the adsorption constant between the anticoagulant rodenticide and the C18 stationary phase. The rodenticide retention factor k was directly proportional to the slope of its adsorption isotherm and can be thus given by the following equation [36–38]:

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

where ϕ is the column phase ratio (volume of the stationary phase divided by the volume of the mobile phase) and $\bar{k}$ (equal to $\phi\alpha K$) is the rodenticide apparent retention factor (i.e. retention factor when the anticoagulant rodenticide concentration in the mobile phase was nil). Then, for each HA concentration in the bulk solvent, by the plot of the k value versus the rodenticide concentration in the bulk solvent, the constant $\bar{k}$ can be determined using Eq. (2). These initial relations of the Langmuir theory are limited by the fact that the experimental data are evaluated only through the assumption that the HA do not modify the solute binding site. Recently, Duan et al. [33] have demonstrated that HA can be adsorbed by powdered activated carbon in saline water conditions. Then, if the rodenticide bound either on the free C18 stationary phase (no HA adsorbed on the surface, adsorption constant K_1, column saturation capacity α_1) or either on HA adsorbed on the C18 stationary phase (adsorption constant K_2, column saturation capacity α_2), then the rodenticide concentration C_s in the C18 stationary phase was given by the equation [36–38]:

$$C_s = \frac{\alpha_1 K_1 C_m}{1 + K_1 C_m} + \frac{\alpha_2 K_2 C_m}{1 + K_2 C_m} \tag{3}$$

Then, in this case the anticoagulant rodenticide retention factor directly proportional to the slope of its adsorption isotherm is given by the following equation:

$$k = \phi \left(\frac{\alpha_1 K_1}{(1 + K_1 C_m)^2} + \frac{\alpha_2 K_2}{(1 + K_2 C_m)^2} \right) = \frac{\bar{k}_1}{(1 + K_1 C_m)^2} + \frac{\bar{k}_2}{(1 + K_2 C_m)^2} \tag{4}$$

where $\bar{k}_1$ (equal to $\phi\alpha_1 K_1$) and $\bar{k}_2$ (equal to $\phi\alpha_2 K_2$) are the apparent retention factor (retention factor when the rodenticide concentration in the mobile phase was nil) of respectively the rodenticide adsorbed on the free C18 stationary phase and on the HA adsorbed on the C18 stationary phase. Then, using a non-linear regression analysis, by studying the variation of the k values versus the rodenticide concentration in the mobile phase, the apparent retention factors $\bar{k}_1$, $\bar{k}_2$ and the corresponding adsorption constants K_1 and K_2 can be calculated.

3.2. Treatment of cultures and cell viability

HaCaT cells were seeded at a density of 6×10^4 cells per well in 100 μl culture medium containing 10% FCS in 96-well microtiter plates and incubated overnight for adherence. The following day, the medium was discarded and the cells were incubated in FCS-free medium containing increasing concentrations of anticoagulant rodenticide (with less than 0.5% ethanol vehicle) and/or HA (0–600 mg/l). The treated cells were then cultured for an additional 24-h period. Control cells were not exposed to drugs (anticoagulant rodenticide or HA). Cell survival was assayed by measuring mitochondrial activity with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [34]. This assay is based on the reduction of the yellow tetrazolium salt MTT by the mitochondrial succinate dehydrogenase to form an insoluble blue formazan product. Only viable cells with active mitochondria reduce significant amounts of MTT. After treatment, the reaction medium was removed and the adhering cells were washed with PBS. One hundred μl MTT solution (0.5 g/l in medium) was added to the culture wells and after a 4-h incubation, the medium/MTT mixture was replaced with 100 μl of DMSO. Blue formazan formation was quantified with a spectrophotometer at 570 nm. Values of absorbance were converted into percentage of residual viability (Y). Usually, inhibition concentration 50% (IC_{50}) was chosen as the best biological marker of cytotoxicity. For the determination of the 50% inhibitory concentration (IC_{50} values) of each substance tested, seven different concentrations were tested on cells and at least three experiments were carried out. The comparison of the cytotoxic effects of rodenticide present with different concentrations of HA and the cytotoxic effects of rodenticide administrated alone was performed by one-way analysis of variance (ANOVA) [39]. After the ANOVA yielded a significant result ($P < 0.05$), the groups differing from one another were identified by Scheffé test [39].

4. Result and discussion

For each anticoagulant rodenticide and HA concentration in the mobile phase, the most concentrated anticoagulant rodenticide solution was injected on the C18 stationary phase and its retention time was determined at all the temperatures studied (i.e. −5, 0, 10, 25, 37, 45 °C). For a diphacinone concentration in the mobile phase equal to 0 mM, Fig. 2 presented the diphacinone chromatogram for an humic acid concentration equal to: (A) 0, (B) 100, and (C) 600 mg/l. All the experiments were repeated three times. The variation coefficient of the k values were always <0.3% indicating a high reproducibility and a good stability for the chromatographic system. The variation of the k values versus the anticoagulant rodenticide concentration in the mobile phase was similar for all the anticoagulant rodenticides. For example, Fig. 3 presents the plot obtained for the brodifacoum compound when the HA concentration was nil in the mobile phase at 37 °C. Using a non-linear regression and for each HA concentration, the non-linear regression coefficients of Eq. (2)

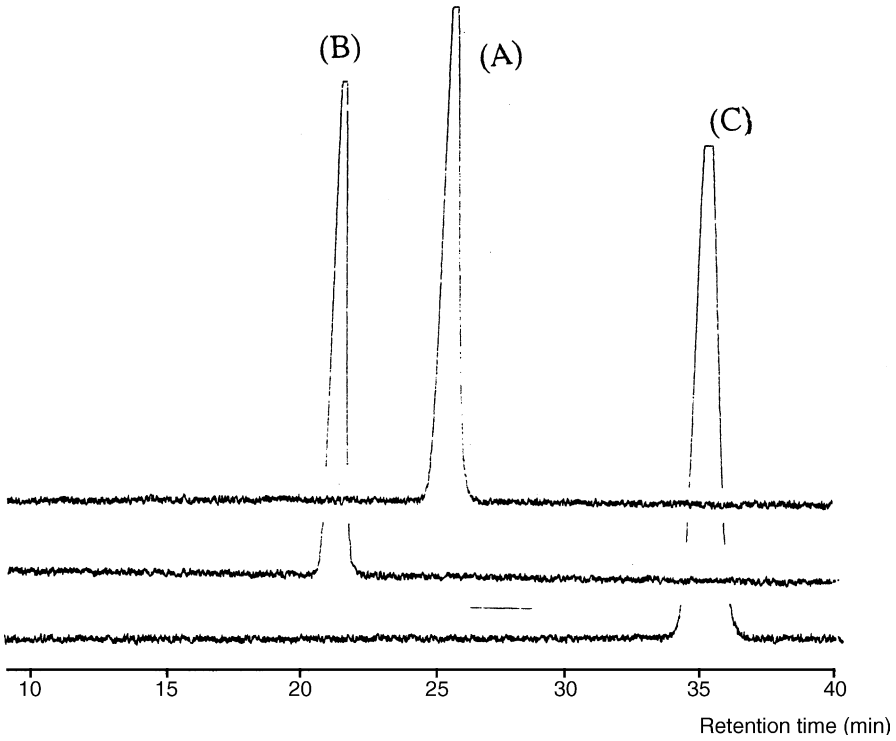

Fig. 2. Diphacinone chromatograms for rodenticide concentration in the mobile phase equal to 0 mM and an humic acid concentration in the mobile phase equal to: (A) 0, (B) 100, and (C) 600 mg/l.

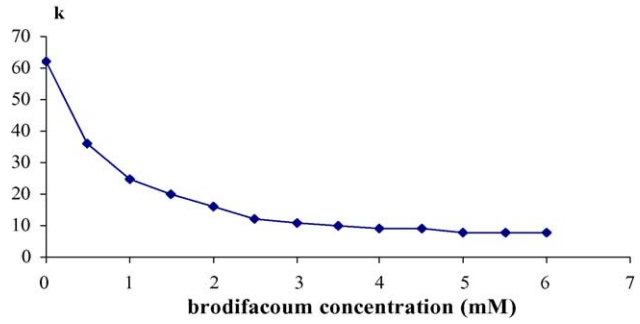

Fig. 3. Plot of the k values vs. the brodifacoum concentration at 37 °C.

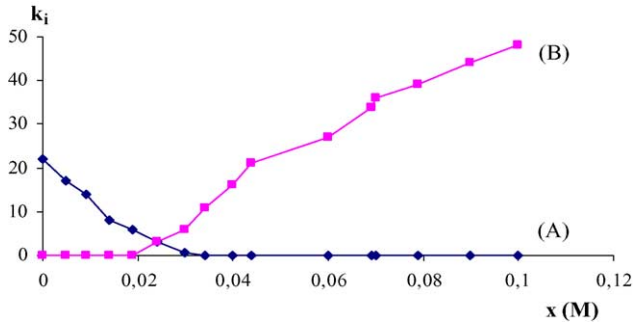

Fig. 4. Plot of: (A) $\bar{k}_1$, (B) $\bar{k}_2$, values vs. the HA concentration in the bulk solvent x for the bromadiolone compound at 25 °C.

were determined for all the rodenticides. Table 1 gives the values obtained for all the anticoagulant rodenticides at 37 °C. The values of the non-linear regression coefficients of Eq. (2) showed that the simplified Langmuir equation was not sufficient accurate to fit the experimental data (Table 1). Using a non-linear regression, the $\bar{k}_1$, and $\bar{k}_2$, values (i.e. the apparent retention factor respectively of the rodenticide adsorbed on the free C18 stationary phase and on the HA adsorbed on the C18 stationary phase) were determined from Eq. (4) at all the HA concentrations in the bulk solvent and for all the anticoagulant rodenticides. The non-linear regression coefficient results proved that the two-order Langmuir model described accurately the binding mechanism of rodenticide with the C18 stationary phase (Table 1). These data clearly shown that: (i) HA interacted on the C18 stationary phase and (ii) the anticoagulant rodenticide can be associated on HA adsorbed on the C18 stationary phase. The adsorption of HA on powdered activated carbon was previously observed by Duan et al. [33]. This C18-HA adsorption phenomena can be explained by the hydrophobic effect [42]. The $\bar{k}_1$ and $\bar{k}_2$, values were plotted against the HA concentration (x) in the bulk solvent. Fig. 4 presents the plots obtained for bromadiolone at 25 °C. Similar plots were obtained for the other anticoagulant rodenticides and for the other temperatures. When HA concentration, x, was lower than a critical values, x_c ($x_c = 0.03$M), the $\bar{k}_1$ values decreased whereas the $\bar{k}_2$ values increased with x. However, the apparent retention factor corresponding to the anticoagulant rodenticide adsorption on the free C18 stationary phase ($\bar{k}_1$) was always much higher than the one obtained for the rodenticide bound to the HA adsorbed on the C18 stationary phase ($\bar{k}_2$). These data demonstrated that below x_c, the rodenticide adsorption on the HA adsorbed on the C18 stationary phase was negligible in relation to the rodenticide bound to the free C18 stationary phase. At high HA concentration ($x > x_c$), the $\bar{k}_2$ values were always higher than the $\bar{k}_1$ values demonstrating that rodenticide principally bound on the HA adsorbed on the RPC18. In order to confirm these results the plot corresponding to the $\bar{k}_1 + \bar{k}_2$ values versus the HA concentration in the bulk solvent was drawn for all the rodenticides. Fig. 5 gives the curve obtained for the chlorophacinone compound at 25 °C. The curve of the $\bar{k}_1 + \bar{k}_2$ values versus the HA concentration (x) presents a break at a certain HA concentration in the bulk solvent, x_c. The decrease in the rodenticide-C18 stationary phase adsorption at low HA concentration ($x < x_c$) in the bulk solvent corresponds to a competition effect between the HA molecule and the rodenticide to bind on the C18 stationary phase (Figs. 2 and 5). Such a competition effect, between lithium perchlorate salt and phenol derivatives was previously by Guillaume's group observed on a PGC surface [40]. At high HA concentration in the bulk solvent ($x > x_c$), the rodenticide adsorption on the C18 stationary phase increased with x (Figs. 2 and 5). This result was attributed to the binding of the rodenticide molecules on the HA adsorbed on the C18 stationary phase. The adsorption constants of these rodenticides associated respectively to the free C18 stationary phase (K_1) and to the HA adsorbed on the C18 stationary phase (K_2) were calculated for the six anticoagulant rodenticides ($K_1 = \bar{k}_1/\phi\alpha_1$ and $K_2 = \bar{k}_2/\phi\alpha_2$). For example, Table 2 presents the K_1 and K_2 values obtained for all the anticoagulant rodenticides at 25 °C. For example, the adsorption constants of, respectively, bromadiolone-C18 stationary

Table 1
Non-linear regression coefficient (r^2) of Eqs. (2) and (4)

Rodenticides	r^2 (Eq. (2))	r^2 (Eq. (4))
Bromadiolone	0.612	0.985
Brodifacoum	0.675	0.978
Chlorophacinone	0.692	0.986
Difenacoum	0.642	0.983
Diphacinone	0.625	0.991
Warfarin	0.653	0.931

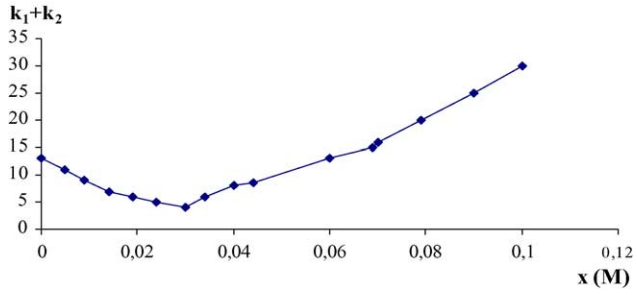

Fig. 5. Plot of $\bar{k}_1 + \bar{k}_2$ values vs. the HA concentration in the bulk solvent x for the chlorophacinone compound at 25 °C.

Table 2
K_1, K_2 values at 25 °C and ΔH_i° (kJ/mol) determined for all the anticoagulant rodenticides

Rodenticides	K_1 ($\times 10^3$)	K_2 ($\times 10^4$)	ΔH_1° (kJ/mol)	ΔH_2° (kJ/mol)
Bromadiolone	3.12	6.01	−56.25	−115.33
Brodifacoum	2.98	5.65	−52.34	−98.25
Chlorophacinone	1.86	4.78	−39.98	−68.98
Difenacoum	2.66	5.23	−49.56	−86.56
Diphacinone	1.65	4.26	−35.69	−65.32
Warfarin	2.23	4.95	−45.36	−79.26

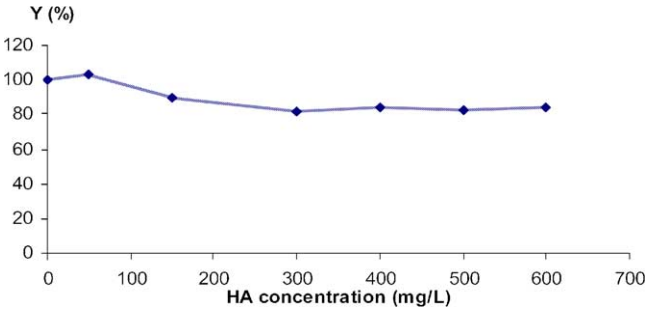

Fig. 6. HA cytotoxicity profile: percentage of residual viability (Y) vs. the HA concentration.

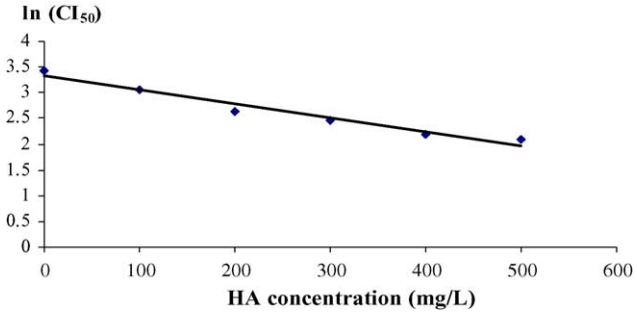

Fig. 7. Plot of the ln(IC_{50}) values vs. the HA concentration in the cellular culture medium for the bromadiolone compound.

phase and bromadiolone-HA adsorbed on the C18 stationary phase were equal to 3.12×10^3 and 6.01×10^4 at 25 °C. It has also been observed that the affinity of the coumarin rodenticide class (i.e. bromadiolone, warfarin, brodifacoum difenacoum) for both HA adsorbed on the C18 stationary phase and the free C18 stationary phase were higher than that determined for the indandione anticoagulant class (diphacinone, chlorophacinone). This result can be explained by the greatest hydrophobic character of the coumarin class. Similar results were previously observed by Guillaume's group for the adsorption of these rodenticides with HSA [26].

The role of humic acid on the toxicity of the anticoagulant rodenticides on a human keratinocyte cell line (HaCaT) was then studied. First, it was proved that HA did not alter the cell viability of HaCaT culture in the concentration range studied (i.e. 0–600 mg/l) (Fig. 6). For the first time, the IC_{50} values were determined for the six anticoagulant rodenticides and for different concentrations of HA in the cellular culture medium. Table 3 gives the IC_{50} values of all the anticoagulant rodenticides on HaCaT cells when the HA concentration was nil in the medium. Fig. 7 presents the ln(IC_{50}) for bromadiolone obtained for different concentrations of HA (the HA concentration varied from 0 to 600 mg/l). Similar variations were observed for the other anticoagulant rodenticides. The addition of HA in the medium led to an increase of the rodenticide toxicity on human keratinocytes. Our results were contradictory to most other studies which demonstrated the relative cytoprotection induced by humic substances [41,43]. In many biological models, the toxicity of the solute or the metal ions is related to the concentration of the free forms (i.e. uncomplexed) [42,43]. Addition of humic acid to the cellular culture medium led to an increase of stable rodenticide humate chelates which cannot enter into cells and consequently the solute toxicity decreased [42]. According to Sauvant et al. [44], a solute toxicity increase with humic acid can be explained because HaCaT may incorporate the rodenticide humate chelate by phagocytosis rather than by simple membrane diffusion. Thus, a rodenticide bioconcentration was induced which facilitated the enzymatic lysis of the rodenticide humate chelates in the food vacuoles and the liberation of rodenticide under a free and toxic form into the cells. In this case, HA may act only as a carrier [45]. In such a case, the more the rodenticide associates with HA, the more the rodenticide penetrates into the cells and consequently is toxic for the HaCaT.

Table 3
Values of IC_{50} (μM) when the HA concentration was nil in the cellular culture medium

Rodenticides	IC_{50} (μM)
Bromadiolone	31
Brodifacoum	36
Chlorophacinone	66
Difenacoum	41
Diphacinone	75
Warfarin	55

Moreover, plots of K_2 versus the IC_{50} values were drawn for all the rodenticides and at different AH concentrations. Fig. 8 presents the linear plot ($r^2 = 0.96$) obtained for the six rodenticides and an HA concentration equal to 200 mg/l. The rodenticide toxicity was directly proportional to its HA adsorption constant, i.e. its K_2 value. For example, bromadiolone which had the greatest adsorption constant with HA ($K_2 = 6.01 \times 10^{-4}$) presented the greatest toxicity on HaCaT.

In order to gain further insight into these adsorption mechanisms, a thermodynamic study was undertaken on the roden-

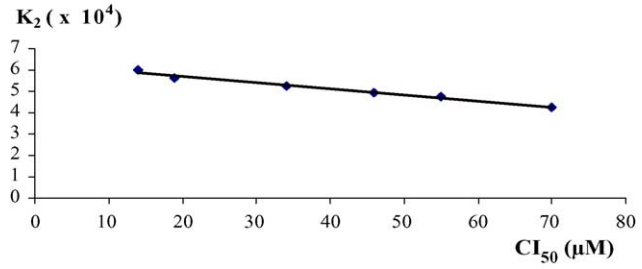

Fig. 8. Plot of the K_2 values vs. the IC_{50} values at 25 °C for a HA concentration equal to 200 mg/l.

ticide adsorption mechanism with: (i) the free C18 stationary phase and (ii) the HA adsorbed on the C18 stationary phase. The Gibbs free energy (ΔG_i°) of the rodenticide adsorption either with the free C18 stationary phase (site 1; $i = 1$) or with the HA adsorbed on the C18 stationary phase (site 2; $i = 2$) can be linked to the adsorption constant K_i according to the well-known equation [46]:

$$\ln(K_i) = \frac{-\Delta G_i^\circ}{RT} \tag{5}$$

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 by the following equation:

$$\Delta G_i^\circ = \Delta H_i^\circ - T\,\Delta S_i^\circ \tag{6}$$

Combining Eqs. (5) and (6), the relationship between the K_i ($i = 1$ or 2) and the thermodynamic parameters (ΔH_i° and ΔS_i°) is given by the following equation [46,47]:

$$\ln(K_i) = \frac{-\Delta H_i^o}{RT} + \frac{\Delta S_i^\circ}{R} \tag{7}$$

where ΔH_i° and ΔS_i° are, respectively, the enthalpy and entropy changes accompaning the anticoagulant rodenticide adsorption on the site i.

The plot of $\ln(K_i)$ against $1/T$ is called a van't Hoff plot. For linear plots, the slope and intercept are respectively $-\Delta H_i^\circ/R$ and $-\Delta S_i^\circ/R$. The van't Hoff plots were all linear for the six rodenticides and for the two sites. The correlation coefficients for the fits were over 0.991. The ΔH° values corresponding to the rodenticide adsorption with the free C18 stationary phase (ΔH_1°) or with the HA absorbed on the RPC18 (ΔH_2°) was given in Table 2 for six rodenticides. Negative enthalpies indicated that it was energetically more favourable for the rodenticide to be in the free C18 stationary phase or in the HA adsorbed on the C18 stationary phase. Negative entropies showed a freedom loss for the rodenticide when it was transferred from the mobile to the C18 stationary phase or to the HA adsorbed on the C18 stationary phase. For all the rodenticides, these transfers were enthalpically driven [40]. Bromadiolone which had the greatest affinity for both the free C18 stationary phase and the HA adsorbed on the C18 stationary phase had the lowest enthalpy and entropy values. This confirmed the importance of the hydrophobic interaction between the rodenticide and: (i) the free C18 stationary phase and (ii) the HA adsorbed on the C18 stationary phase.

A further thermodynamic approach to the analysis of physicochemical data is enthalpy–entropy compensation. This approach has been previously used in chromatographic procedures to analyze and compare the retention mechanism for groups of compounds. Enthalpy–entropy compensation can be described by the following equation [46–48]:

$$\Delta H_i^\circ = \beta\,\Delta S_i^\circ + \Delta G_{i\beta}^\circ \tag{8}$$

where $\Delta G_{i\beta}^\circ$ is the free Gibbs energy of a physicochemical interaction at a compensation temperature β for the site i ($i = 1$ or 2), ΔH_i° and ΔS_i° are the corresponding standard enthalpy and entropy respectively. According to Eq. (8), when enthalpy–entropy compensation is observed for a group of compounds in a particular chemical interaction, all of the compounds have the same free energy change ΔG° at the temperature β. For example, 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. However, the results obtained with this method can be misleading [48]. A better method can be obtained by the use of the following equation [37,38]:

$$\ln(K_i)_T = \frac{-\Delta H_i^\circ}{R}\left(\frac{1}{T} - \frac{1}{\beta}\right) - \frac{\Delta G_{i\beta}^\circ}{R\beta} \tag{9}$$

Eq. (9) shows that if a plot $\ln(K_i)_T$ versus ΔH_i° is linear, all the anticoagulant rodenticides will have the same adsorption constant K_i at the temperature β. Fig. 9 presents the two linear plots (K_i ($i = 1$ or 2) versus ΔH_i) obtained at 25 °C for all the studied rodenticides. The linear regression coefficients were higher than 0.978 corroborating the existence of an enthalpy–entropy compensation. According to Carr and co-workers [49,50], the only conclusion that can be drawn is that the fraction of the total free energy that arises from the enthalpy contributions is the same for all the rodenticide adsorption: (i) on the free C18 and (ii) on the humic acid. Similarly, the fraction of the total free energy arising from the entropy contributions is the same. Since different mech-

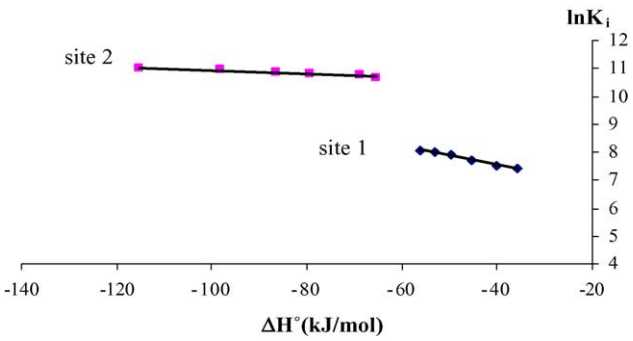

Fig. 9. $\ln K_i$ vs. ΔH_i° plot determined for the sites 1 and 2, for all the rodenticides at $T = 25$ °C.

anisms could result in the same proportion of enthalpy and entropy relative to the overall free energy, it cannot be deduced rigorously that the rodenticide adsorption mechanisms on the free C18 or on the humic acid was independent of the rodenticide structure [49,50]. However, application of Occam's Razor suggests that it is reasonable to conclude that the adsorption mechanisms on the free C18 or on the humic acid were identical for all the rodenticides. Moreover, all the studied molecule have similar biological activity. This seems to imply a similarity of properties of all the studied rodenticides.

5. Summary

In this paper, it was demonstrated that above an HA critical concentration value x_c ($x_c = 0.03$ M), HA was adsorbed on the C18 stationary phase, which the anticoagulant rodenticide molecules can be adsorbed. Moreover, an unusual increase of rodenticide toxicity for human keratinocytes when HA was added in the medium was described.

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