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Analysis of the progesterone displacement of its human serum albumin binding site by β-estradiol using biochromatographic approaches: effect of two salt modifiers

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Abstract

The mechanisms of (i) the binding of two sex-hormones (i.e. progesterone and β-estradiol) to human serum albumin (HSA) and (ii) the progesterone displacement of its HSA binding cavity by β-estradiol were studied by biochromatography using three different methods. In the first time, zonal elution method was used to prove the direct competition effect between the two sex-hormone. In the second time, the competition effect between β-estradiol and progesterone to bound on the same HSA site was analysed by the competitive bi-Langmuir approach. Finally, the thermodynamic data of these two binding processes were studied. The Gibbs free energy value ($\Delta\tilde{G}^{\circ}$) of the displacement equilibrium was negative demonstrating that β-estradiol displaced progesterone of its HSA binding cavity. Moreover, the effect of two chloride modifiers (i.e. Na^{+}, Mg^{2+}) on these two binding processes were analysed. Results showed that in the salt biological concentration ranges, the Mg^{2+} cation enhanced strongly the bioavailable progesterone, whereas the Na^{+} cation interacted slowly on the progesterone displacement of its HSA binding site by β-estradiol. This study showed that it must be useful to carry out more in vivo test on the magnesium supplementation effect for women who suffer from estrogen dominance syndrome.

Keywords: Displacement; Salt modifiers; Progesterone; β-Estradiol

1. Introduction

Pre-menstrual syndrome (PMS) affects over 25 million women every month. This broad range of recurring symptoms usually appears between ovulation and the onset of menstruation when progesterone levels are low and estrogen (the most abundant and potent was β-estradiol) is dominant [1]. This hor-

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monal imbalance which produces a variety of symptoms such as bloating, anxiety, irritability, moodiness, food cravings, crying, breast tenderness, fatigue, depression and anxiety could be considered as a progesterone deficiency [2]. Although progesterone supplementation for women who suffer from PMS may resolve many of these symptoms, conventional pharmaceutical drugs are not necessarily the best choice of treatment for young women. Several studies have shown the interest to adapt the nutrition during the second stage of cycle (days 14–28) to reduce the PMS [3,4]. For example, Gleason's has previously reported that a Mg^{2+} supplementation can reduce many of estrogen dominance side effect (i.e. PMS) [4]. It is known that progesterone and estrogen were bound to the same site on human serum albumin (HSA) [5] and consequently a competition effect exist probably between these two steroid sex-hormones. Then, even if in vivo, the balance between the progesterone and the β-estradiol rate was controlled by multiple mechanisms, Gleason's observation could be explained by an enhancement of the progesterone displacement to its HSA binding site by β-estradiol when Mg^{2+} cation concentration increased [4]. Human serum albumin is the major plasma protein responsible for the reversible binding of a wide range of drugs [6–8]. Extensive studies on different aspects of drug–HSA interactions are still in progress because of the clinical significance of the process, especially in the case of tightly bound drugs ($K > 10^5\,M^{-1}$) [8,9]. Numerous analytical techniques are used for protein binding studies and they are continuously being added to, along with extending knowledge about the complex mechanisms involved in the drug–HSA binding process. The advantages and limitations of the various methods are discussed by Sebille et al. [10]. In recent years, high-performance liquid chromatography (HPLC) appears to be the optimal experimental strategy used in vitro binding studies. Several chromatographic methods were developed for the quantitative estimation of drug–HSA interactions, including varieties of high-performance size-exclusion techniques [11–15] and high-performance liquid affinity chromatography (HPLAC) [16–23]. In the last few years, HPLC was outlined as a powerful tool for the study of bimolecular interaction [22,23]. High-performance liquid affinity chromatography has already been used to study a variety of serum proteins. However, most of this work is focused on human serum albumin, bovine serum albumin (BSA) and α_1-acid glycoprotein (AGP). HSA or BSA support for HPAC can be prepared by covalently attaching albumin to diol-bonded silica activated with 1,1′-carbonyldiimidazole [24,25], by attaching albumin to silica through a two-step [26] or three-step Schiff base method (i.e. reductive amination) [25,27,28] or by using modified silica that has been activated with *N*-hydroxysuccinimide ester [29]. In addition, some studies have used albumin which is non-covalently adsorbed to ion-exchange columns [30] and HPLC-grade silica [28], or which is immobilized to HPLC supports based on agarose [31] or hydroxyethylmethacrylate (HEMA) [32,33]. Commercial column are prepared by crosslinking albumin in the presence of silica with such agent as glutardialdehyde or *N*,*N*′-disuccinimidyl carbonate [34]. For the immobilized albumin, several study showed that immobilized HSA or BSA can indeed provide good quantitative and qualitative agreement with the behavior seen for these proteins when they are in solution. For instance, it has been shown in numerous studies that displacement phenomena and allosteric interactions seen on HSA columns are similar to those observed for soluble HSA [35]. Also, the equilibrium constants measured by HPAC for immobilized albumins have close agreement with those reported in solution using methods like equilibrium dialysis or ultrafiltration under comparable temperature and buffer condition [35]. Several models have been used to describe the distribution of one sample component between the stationary phase and the mobile phase. For example, the association constant of many ligands can been determined by the thermodynamic process [36–38]. The solute association constant can be also measured by dynamic method [39,40]. The most widespread of these is frontal analysis, but this technique is time-consuming and requires large amounts of pure compounds, which are often very expensive or difficult to obtain, for example, in the case of pure enantiomers or proteins [41]. Another popular method, elution by characteristic points (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 [42]. This method is fast and needs only small amounts of sample, but requires accurate calibration of the detector and an efficient column. The binding

of solute to HSA can be also examined by using the technique of zonal elution [35]. The first use of zonal elution for the study of solute–ligand binding was in 1979 by Dunn and Chaiken [43], who examined the retention of staphylococcal nuclease on a low-performance affinity column containing immobilized thymidine-5′-phosphate-3′-aminophenylphosphate. By the late-1980s and early-1990s reports began to appear in which HPAC and zonal elution were used in quantitative studies of drug–protein interactions [44,45]. An important advantage of zonal elution is that requires only a small amount of solute per injection [35]. Another advantage of zonal elution is that it can easily be performed with standard HPLC equipment [35]. Another approach to study the distribution mechanism of a compound between the stationary phase and the bulk solvent is the perturbation technique, originally developed for measuring gas-adsorbent equilibria. The main advantage of the perturbation technique consist in using a simpler instrumentation for the acquisition of the experimental data than in frontal analysis method: the determination of the frontal analysis curves is no longer needed [46,47]. Furthermore, the determination of multi-component (i.e. competitive) isotherms was possible if the studied solute have the same column saturation and bound on the same HSA site. Then, this approach allowed to determine the association constant of two compounds under competitive binding condition [46,47].

In a first time, the zonal elution method was used to prove the direct competition between the two sex-hormones. In the second time, the study of the progesterone displacement of its HSA binding site by β-estradiol was investigated using the competitive bi-Langmuir equation in order to verify our hypothesis (i.e. the competition effect between progesterone and β-estradiol to bind on the HSA). Finally, the thermodynamic data on the mechanism of both (i) the HSA–sex-hormone (progesterone and β-estradiol) association and (ii) the progesterone displacement of its HSA binding site by β-estradiol were determined. Moreover, the effect of two salts (i.e. Mg^{2+}, Na^{+}) on these two thermodynamic processes was carried out. As well, enthalpy–entropy compensation was carried out for each salt to evaluate the main parameter controlling the binding mechanism.

2. Theory

2.1. Zonal elution approach [35]

The binding of solute to HSA can be also examined by using the technique of zonal elution. In this method, a known concentration of competing agent [A] is continuously applied to a column that contains an immobilized HSA while injections of small amount of analyte [B] are made. According to the simplest model for drug–HSA binding, the drug (analyte) interacts reversibly with a single type of equivalent binding site (i.e. direct competition). If a competing agent is added to the mobile phase, it influenced the concentration of free sites and, thus the chromatographic retention of the analyte. According to Zhivkova et al. the capacity factor of the compound B, k_B, declined with increasing the concentration of the competitive agent [A] as follows [35,44,45]:

$$\frac{1}{k_B} = \frac{K_A}{K_B[S_{tot}]}[A] + \frac{1}{K_B[S_{tot}]} \quad (1)$$

where K_B and K_A are the equilibrium affinity constants for the analyte and the competitive agent, respectively, $[S_{tot}]$ the effective concentration of common binding sites and [A] the compound A concentration.

2.2. Competitive bi-Langmuir approach

Single- and multi-component isotherms can be determined using the perturbation technique [46,47]. The column used for the determination of the isotherms is first equilibrated with a solution containing either a single hormone A (for the determination of a single-component isotherms) or a mixture of the hormones A and B (for the determination of a two-component isotherms) dissolved in a non-adsorbable solvent. Then, a sample volume containing different concentrations of the hormone A (or of the compounds A and B) is injected onto the column. After the injection, the equilibrium condition is disturbed and perturbation waves arise which migrate along the column [46,47]. When such a wave reaches the column oulet, a peak is registered by the detector. The case of a single-component Langmuir isotherm, the well-known Langmuir theoretical approach relates the total concentration of the sample in the HSA stationary phase (C_s) and that in the mobile phase (C_m)

[46,47]

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

where α is the column saturation capacity; K the adsorption constant between the studied hormone and the HSA stationary phase and ϕ the column phase ratio (volume of the stationary phase divided by the volume of the mobile phase). By plotting the k value versus the sample concentration in the bulk solvent, the constant K can be determined using Eq. (2) [46,47]. In contrast, the application of the perturbation technique to a two-component isotherms is less straightforward because although two peaks (peaks 1 and 2) were observed, the peaks cannot be attributed to the compound A or B as each peak corresponds to the perturbation of the concentrations of the two sample compounds [47]. The expected elution times of the two disturbances, t_{R1calc} and t_{R2calc}, can be obtained by the following relation [47]:

$$t_{R1calc} = t_o \left[1 + \phi \left(\frac{\partial C_{sA}}{\partial C_{mA}} + \frac{\partial C_{sA}}{\partial C_{mB}} \left(\frac{dC_{mB}}{dC_{mA}} \right)_1 \right) \right] \quad (3)$$

$$t_{R2calc} = t_o \left[1 + \phi \left(\frac{\partial C_{sA}}{\partial C_{mA}} + \frac{\partial C_{sA}}{\partial C_{mB}} \left(\frac{dC_{mB}}{dC_{mA}} \right)_2 \right) \right] \quad (4)$$

where t_o is the column hold-up time. Analogous equations are obtained with respect to the concentration of the hormone. For the two-component competitive Leangmuir isotherms, the following equations are obtained [46,47]:

$$\frac{\partial C_{sA}}{\partial C_{mA}} = \frac{\alpha_A K_A (1 + K_B C_{mB})}{(1 + K_A C_{mA} + K_B C_{mB})^2} \quad (5)$$

$$\frac{\partial C_{sA}}{\partial C_{mB}} = \frac{-\alpha_A K_A (K_B C_{mA})}{(1 + K_A C_{mA} + K_B C_{mB})^2} \quad (6)$$

Analogous equations was obtained for $\partial C_{sB}/\partial C_{mB}$ and $\partial C_{sB}/\partial C_{mA}$. After this substitution, Eqs. (3) and (4) enable the calculation of the expected retention times of perturbations using the estimated coefficients of the two-component Langmuir isotherm α, K_A and K_B. The retention times calculated in this way, t_{R1calc} and t_{R2calc} are compared with the experimental values of the retention times of the retention times for all perturbation experiments at different combinations of concentrations C_{mA} and C_{mB}. As there are more experimental retention times measured by perturbation injections of a mixed sample than unknown variables, numerical solution is employed to determine the isotherm coefficients yielding minimum squares of the differences between calculated and the experimental retention times, $t_{R1calc} - t_{R1exp}$ and $t_{R2calc} - t_{R2exp}$, at various combinations of C_{mA} and C_{mB}. To this aim, the initial estimates of the isotherm coefficients are subsequently corrected in repeated iterative calculation steps using the Marquardt method of minimization of the objective function OF

$$OF = \sum_{p=1}^{p} (t_{R1calc} - t_{R1exp})^2 + \sum_{p=1}^{p} (t_{R2calc} - t_{R2exp})^2 \quad (7)$$

where p is the number of all perturbation experiments. The values of the best fit isotherm coefficients corresponding to the minimum OF represent the desired solution for the competitive Langmuir isotherm. The initial estimated values of the isotherm coefficients for the calculation of t_{R1calc} and t_{R2calc} can be set equal to the single-component Langmuir or linear isotherm coefficient [47].

2.3. Thermodynamic approach

The retention factor k can be also expressed by the well-known equation [48]

$$\ln k = \frac{-\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} - \ln \phi \quad (8)$$

where R is the gas constant and T the absolute temperature, ϕ the phase ratio of the HSA column (volume of the mobile phase divided by the volume of the stationary phase), ΔH° and ΔS° are, respectively, the enthalpy and entropy changes accompanying sex-hormone binding to HSA. A plot of $\ln k$ against $1/T$ is called a van't Hoff plot [48]. For a linear plot, the slope and intercept are, respectively, $-\Delta H^\circ/R$ and $\Delta S^\circ/R - \ln \phi$. This provides a convenient way of calculating the thermodynamic constants ΔH° and ΔS°.

2.4. Wyman's parameters

It has been known for several years that increasing the ionic strength of a bulk solvent increases its sur-

face tension [49]. Considering Δn as the difference in the number of salt cation at the HSA/sex-hormone interface implied in the binding process between the two states of the binding equilibrium, k can be linked to the change in salt concentration, x, using the following equation [50,51]:

$$\ln k = \gamma - \frac{\Delta n}{\ln x} \quad (9)$$

where γ is a constant.

2.5. Diplacement equilibrium

The equilibrium constant of hormone A displacement of its HSA binding site by the hormone B can be given by the following equation:

$$\tilde{K} = \frac{K_B}{K_A} \quad (10)$$

$\tilde{K}$ can be also expressed as:

$$\ln \tilde{K} = \frac{-\Delta \tilde{H}^\circ}{RT} + \frac{\Delta \tilde{S}^\circ}{R} \quad (11)$$

where $\Delta \tilde{H}^\circ$ and $\Delta \tilde{S}^\circ$ are, respectively, the enthalpy and entropy of the displacement equilibrium.

3. Experimental

3.1. Chromatography

The chromatographic system consisted of a high-performance liquid chromatography 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 (Montluçon, France) (150 mm × 4.6 mm) was used with controlled temperature in a Interchim Crocodil oven TMN° 701 (Montluçon, France). The mobile phase flow-rate was fixed at 1 ml min^{-1} and the wavelength at 254 nm.

3.2. Reagents

The two steroid sex-hormones (i.e. progesterone and β-estradiol) were obtained from Sigma–Aldrich (Saint-Quentin, France). The chemical structure of these compounds are given by in Fig. 1. Sodium hydrogenphosphate and sodium dihydrogenphosphate were supplied by Prolabo (Paris, France). NaCl and $MgCl_2$ were obtained from Sigma–Aldrich (Saint-Quentin, France). It has been known that monovalent ions such as sodium are not able to bind to HSA [52]. Sodium nitrate (Merck) was used as a dead time marker (t_o = 0.89 min) [27]. This value was not modified by the eluent salt concentration. Water was obtained from an Elgastat option water purification system (Odil, Talant, France) fitted with a reverse osmotic cartridge.

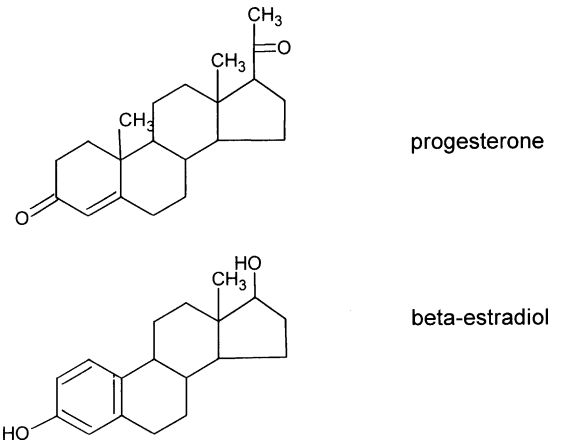

Fig. 1. Sex-hormone structure.

3.3. Operating condition for the zonal approach

All mobile phases (30/70 (v/v) methanol–sodium phosphate buffer (7×10^{-4} M) at pH = 7.3) [53], commercial model of mobile phase) were prepared by adding 0–10 μM of the desired competing agent (progesterone). Eleven progesterone concentration values were included in this concentration range (i.e. 0–10 μM).

All the experiments were carried out at 293 K.

3.4. Operating conditions for the Langmuir approach [46,47]

The mobile phases were prepared by mixing the components in the required ratios and degassed by ultrasonification before use. Single-component isotherms of progesterone and β-estradiol (each in the concentration range 0.01–0.1 mol l^{-1}) and

a two-component isotherms of a mixture of progesterone and β-estradiol (at a constant concentration ratio 0.01:0.01 to 0.1:0.1 mol l^{-1}) were measured in 30/70 (v/v) methanol–sodium phosphate buffer (7×10^{-4} M) at pH = 7.3, at 20 °C. Each isotherm data point was measured in 11 subsequent steps after equilibration of the column with a solution containing a sample compound (progesterone or β-estradiol (0, 0.0025, 0.005, 0.0075, 0.01, 0.0125, 0.015, 0.0175, 0.02, 0.0225, 0.025 mol l^{-1}) of or a mixture of progesterone and β-estradiol ($C_{\text{progesterone}} + C_{\beta\text{-estradiol}} = 0$, 0.0025, 0.005, 0.0075, 0.01, 0.0125, 0.015, 0.0175, 0.02, 0.0225, 0.025 mol l^{-1}) until a stable detector response was obtained. Small volume (5 μl) of the most concentrated sample (single or the mixture) was injected onto the column and the apparent retention time *k* were measured.

3.5. Operating condition for the thermodynamic approach

The bulk solvent consisted of a 30/70 (v/v) methanol– sodium phosphate buffer (7×10^{-4} M) at pH = 7.3 [53]. Hormone retention factors *k* were determined over the temperature range 293–318 K. The chromatographic system was allowed to equilibrate at each temperature for at least 1 h prior to each experiment. To study this equilibration, the retention time of progesterone was measured every hour for 5 h and again after 23 and 24 h. The maximum relative difference of the retention time of this compound was always 0.6%, making the chromatographic system sufficiently equilibrated for use after 1 h. The sex-hormones were injected three times at each temperature and salt concentration. Once the measurements were completed at the maximum temperature, the column was immediately cooled to ambient conditions to minimise the possibility of any unfolding of the immobilised HSA.

4. Results and discussion

4.1. Zonal elution approach

The binding of β-estradiol and progesterone to HSA will be examined in this work by injecting small amounts of β-estradiol into an HSA column while a known concentration of progesterone is applied to the column in the mobile phase (i.e. zonal elution method). By examining how the mobile phase concentration of the progesterone additive affects the retention of the β-estradiol injected solute, information will be gained on the type of competition (i.e. allosteric interaction or direct competition) which is occuring between the two sex-hormones. Fig. 2 presents the plot $1/k_{\beta\text{-estradiol}}$ versus the progesterone concentration at 293 K. As can be seen in Fig. 2, a decrease in retention of β-estradiol was observed as increasing amounts of progesterone were added to the mobile

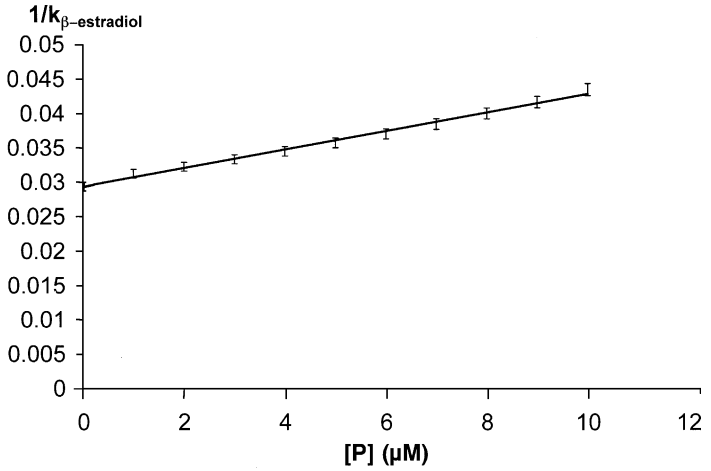

Fig. 2. van't Hoff plots ($\ln k'$ vs. $1/T$) when the Na^+ and the Mg^{2+} concentration was nil for the progesterone molecule.

phase. It appears that the plot $1/k_{\beta\text{-estradiol}}$ versus the progesterone concentration is linear ($r^2 > 0.989$). This plot gave a good agreement between the experimental intercept (i.e. $k_{\beta\text{-estradiol}}$ when no competing agent (progesterone) was present) and the intercepts which were determined by linear regression of the entire data set ($k_{\text{experimental}} = 33.76 \approx k_{\text{predicted}} = 33.80$). The agreement between these values is significant since it indicates that the displacement of progesterone by β-estradiol was though a direct, rather than an allosteric mechanism of competition (only one type of site) [35,44].

4.2. Competitive bi-Langmuir approach

The single-component distribution data of β-estradiol and progesterone were measured by the perturbation technique. The Langmuir model (Eq. (2)) was found to describe adequately the experimental data (non-linear coefficients were always higher than 0.987). The best values of the constants of Eq. (2) obtained by non-linear regression were calculated. Moreover, the difference of the column saturation factor for the two compounds was always lower than 0.04% justifying the use of the competitive Langmuir isotherm equation for this study [46]. The experiments were carried out with various progesterone and β-estradiol concentrations in the bulk solvent at $T = 293\,\text{K}$ (see Sections 2 and 3). The retention times of the two induced responses, t_{R1} and t_{R2} were obtained. For the evaluation of the coefficients of the two-component competitive Langmuir isotherms, the iterative Marquadt approach was used to fit the best isotherm coefficients values as shown in the Section 2. There is a good agreement between theoretical and experimental data also confirmed by the low standard deviation for all total isotherm derivative (ε, Table 1). These results indicated the importance of the competitive effect between progesterone and β-estradiol to bound on the same HSA site. $K_{\text{progesterone}}$ value (10.27×10^3) was lower than the one of $K_{\beta\text{-estradiol}}$ (31.02×10^3) confirming that DHEA can well displaced testosterone of its HSA binding site. The $\tilde{K}$ values were calculated from Eq. (10) (Table 1). At 25 °C, the $\tilde{K}$ value was equal to 3.02. The positive values confirmed the progesterone displacement of its HSA binding site by β-estradiol.

Table 1
$K_{\beta\text{-estradiol}}$, $K_{\text{progesterone}}$, α, ε

$K_{\beta\text{-estradiol}}$ ($\times 10^3$)	10.27
$K_{\text{progesterone}}$ ($\times 10^3$)	31.02
α	109
ε	1.35

4.3. Thermodynamic approach

The retention factors for progesterone and β-estradiol (k) were determined at various column temperature (293–318 K) with chlorure salts (sodium, magnesium) as mobile phase additive. Each experiments were repeated three times. The variation in the k values was less than 0.3% in most cases indicating high reproducibility and good stability for the chromatographic system.

4.3.1. Mechanism of (i) the HSA–sex-hormone binding and (ii) the progesterone displacement of its HSA binding cavity by β-estradiol when the salt concentrations were nil

van't Hoff plots ($\ln k$ versus $1/T$) were drawn for the two sex-hormones and for each concentration of the two salts. Linear plots were obtained with correlation coefficients r higher than 0.997 for all fits. For example, Fig. 2 represents the plot for progesterone when both the Na^+ and the Mg^{2+} concentration was nil in the mobile phase. These linear van't Hoff plots provided a conventional way of calculating the thermodynamic parameters. Both ΔH° and ΔS° values ($\Delta H^\circ_{\beta\text{-estradiol}} = -27.79\,\text{kJ mol}^{-1}$; $\Delta H^\circ_{\text{progesterone}} = -21.17\,\text{kJ mol}$; $\Delta S^\circ_{\beta\text{-estradiol}} = -69.72\,\text{J mol}^{-1}\,\text{K}^{-1}$; $\Delta S^\circ_{\text{progesterone}} = -52.41\,\text{J mol}^{-1}\,\text{K}^{-1}$) were negative, as was usually the case for several pharmacomolecule–HSA association [54,55]. Negative enthalpy indicated that it was energetically more favourable for the sex-hormone to be linked to HSA rather to be free. Negative entropy showed the loss of the degree of freedom of the sex-hormones when they are included in the HSA cavity. This association mechanism was enthalpically driven (magnitude of ΔH° was always greater than $T\Delta S^\circ$) and can be described by the replacement of weak sex-hormone–bulk solvent interactions by strong sex-hormone–HSA van der Walls inter-

actions. Moreover, the ΔH° and ΔS° values were smaller for β-estradiol than for progesterone. This result was corroborated by the fact that progesterone was eluted before β-estradiol and confirmed the importance of the hydrophobic effect on solute–HSA association [56]. Thus, the HSA–β-estradiol (E) binding (HSA–E) was more stabilised and more ordered than the HSA–progesterone (P) association (HSA–P). This result tends to confirm our hypothesis i.e. β-estradiol can displace progesterone of its HSA binding site. From Eq. (10), the $\tilde{K}$ value were calculated for six temperatures and when the Na^+ and the Mg^{2+} cation concentrations in the bulk solvent were nil (see Table 2). At 293 K, $\tilde{K}$ values was equal to 3.00. This value was similar to the one obtained with the competitive bi-Langmuir approach confirming well that the $\tilde{K}$ value can be considered as an index proportional to a competitive binding character. The plot $\ln \tilde{K}$ versus $1/T$ (van't Hoff plot) was reported in Fig. 3. The correlation coefficient for the linear fit was equal to 0.996. $\Delta \tilde{H}^\circ = -6.55\,\mathrm{kJ\,mol^{-1}}$ and $\Delta \tilde{S}^\circ = -17.22\,\mathrm{J\,mol^{-1}\,K^{-1}}$ were determined from the slope and intercept, respectively (Eq. (11)). The Gibbs free dissolution energy $\Delta \tilde{G}^\circ$ of this equilibrium were determined by the well-known formula:

$$\Delta \tilde{G}^\circ = \Delta \tilde{H}^\circ - T\,\Delta \tilde{S}^\circ \quad (12)$$

At the human temperature, $\Delta \tilde{G}^\circ$ value was equal to $-1.20\,\mathrm{kJ\,mol^{-1}}$. This negative value confirmed that β-estradiol well displaced progesterone of its HSA binding site. Moreover, $\Delta \tilde{H}^\circ$ was always higher than $T\,\Delta \tilde{S}^\circ$ value indicating that the mechanism of the progesterone displacement of its HSA binding cavity by β-estradiol was enthalpically controlled.

Table 2
$\tilde{K}$ values at six temperatures when the Na^+ and Mg^{2+} concentration was nil in the mobile phase

Temperature (K)	$\tilde{K}$
293	3.00
298	2.86
303	2.72
308	2.58
313	2.46
318	2.34

Standard deviation < 0.02.

4.4. Analysis of salt effects (i.e. Na^+ and Mg^{2+}) on the sex-hormone affinity to HSA

In order to study the salt concentration effect on the sex-hormone association to HSA, the plots $\ln k'$ versus $\ln x$ were drawn for each salt type (i.e. Na^+, Mg^{2+}). The Na^+ concentration varied from 5×10^{-4} to 0.15 M (biological Na^+ concentration range: 0.1–0.13 M). The Mg^{2+} concentration range varied from 5×10^{-4} to 4×10^{-3} M (biological concentration range 7.5×10^{-4} to 1×10^{-3} M) because above 4×10^{-3} M, the progesterone retention time can not be detected with sufficient accurate. Fig. 4 shows the

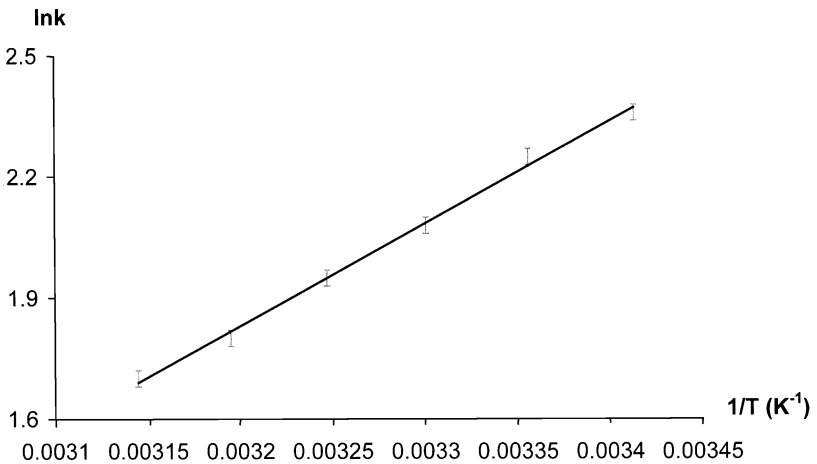

Fig. 3. Plot of $\ln \tilde{K}$ vs. $1/T$ (van't Hoff plot).

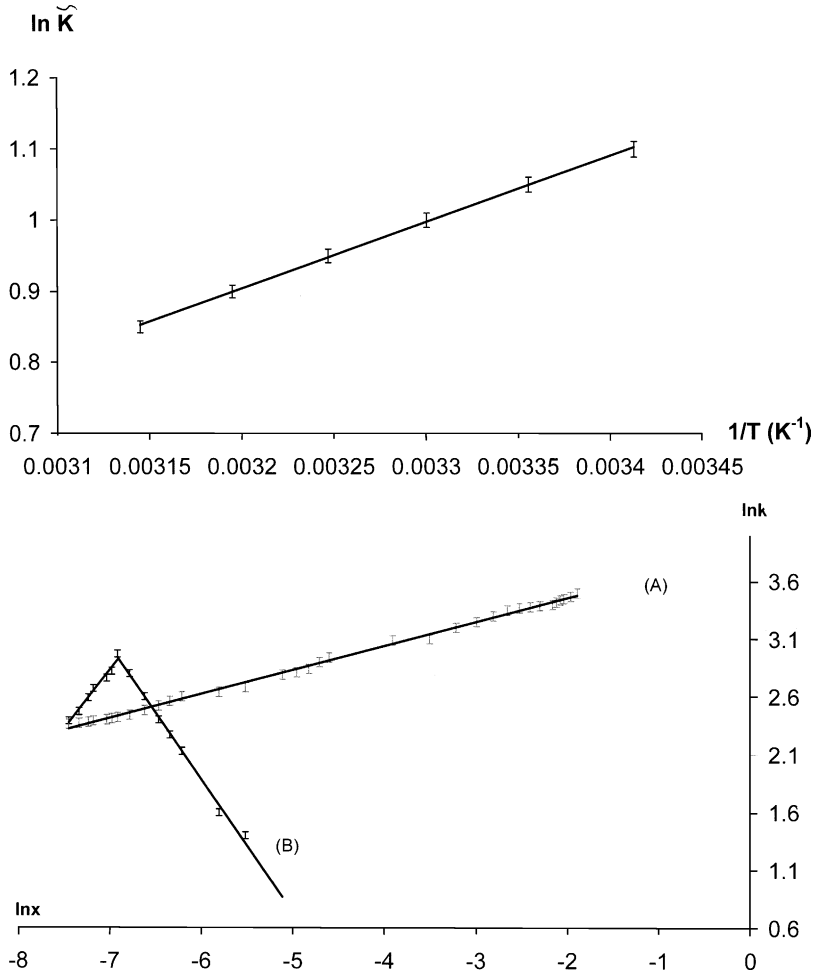

Fig. 4. Plots of $\ln k'$ vs. $\ln x$ for the progesterone and for the two salts: (A) sodium and (B) magnesium at 293 K.

plots $\ln k'$ versus $\ln x$ at 283 K for progesterone and for the two chlorure salts. Similar plots were observed for β-estradiol. As can be seen in Fig. 4, two retention behaviours were observed in relation to the salt type used. For the Na^+ cation, the HSA–sex-hormone affinity enhanced slowly over the total range of the Na^+ concentration. For the Mg^{2+}, the plot $\ln k$ versus $\ln x$ can be divided into two Mg^{2+} concentration domains demonstrating a change on the HSA–sex-hormone association. Such results have been previously observed by Gwillam's for other drug binding to HSA [57,58]. The decrease of sex-hormone retention factor when $x > x_c$ ($x_c = 1 \times 10^{-3}$ M) for the Mg^{2+} salt only can be explained by the fact that contrary to the Na^+ cation, the Mg^{2+} cation can interact with the HSA by means of electrostatic interaction between its charge and the negative charged surface of HSA (at pH = 7.3, the HSA was negatively charged) [56,57].

Then, for $x < 1 \times 10^{-3}$ M, whatever the salt used, the classical change in water activity with the salting-out agent (i.e. Na^+, Mg^{2+}) increased the sex-hormone affinity to HSA. This change of the surface tension (i.e. water activity) can be correlated with the salt concentration, x, by the use of Wyman's equation (Eq. (9)). Then, the Δn values, for both progesterone (P) and β-estradiol (E) were determined from the slope of the plot $\ln k$ versus $\ln x$ for each salt (Fig. 4) at 20 °C and are reported in Table 3.

Table 3
Δn_{Na^+} and $\Delta n_{Mg^{2+}}$ represented, respectively, the number of Na^+ and Mg^{2+} cation excluded during the β-estradiol (or progesterone) transfer from the bulk solvent to the HSA

Solute denomination	Δn_{Na^+}	$\Delta n_{Mg^{2+},x<x_c}$	$\Delta n_{Mg^{2+},x>x_c}$
β-Estradiol	−0.28	−1.89	1.15
Progesterone	−0.20	−1.09	1.15

Standard deviation < 0.02.

Whatever the salt used, Δn values varied similarly to the elution order. The magnitude of $\Delta n_{estradiol}$ was higher than the one of $\Delta n_{progesterone}$ confirming that Δn reflected the hydrophobic interactions. As well, for a given sex-hormone, it appeared that the number of magnesium cation excluded when the sex-hormone bound to HSA was higher than the one obtained with Na^+. This can be explained by the fact that Mg^{2+} cation is a more chaotropic agent than Na^+ cation (i.e. Hoffmeister series [59]). Moreover, these Δn values were in accordance with the one reported for the Na^+ cation number released when tryptophan was transferred from the bulk solvent to the HSA cavity [60].

In order to confirm this hypothesis (i.e. the change in water activity), the thermodynamic data of the HSA–sex-hormone binding process were determined at all the Na^+ and Mg^{2+} concentration studied. The plots ΔH° and ΔS° versus x were drawn for each salt and for the two sex-hormones. Two behaviours were observed in relation to the salt used. For example, Figs. 5 and 6 represent, respectively, the plot ΔH° versus the Na^+ concentration and the Mg^{2+} concentration for progesterone. Similar results were obtained for the entropy change versus x and for β-estradiol. Thus, for $x < x_c$ and for the two salts, the enhancement of the hydrophobic interactions between the HSA and the steroid sex-hormones led to an increase of the thermodynamic data (Figs. 5 and 6).

For $x > x_c$, with the Mg^{2+} salt only, the HSA–hormone binding decreased when the Mg^{2+} concentration increased. The non-specific binding mode of Mg^{2+} led to a competition effect between the sex-hormones and this divalent cation to bind to HSA and consequently a decrease of HSA–hormone affinity (Fig. 4). In this Mg^{2+} concentration domain, the decrease of the interactions between the hormones and HSA cavity due to the competition effect additive to the classical salt effect on the surface tension in the bulk solvent (i.e water activity) led to a strong increase of the thermodynamic data (Fig. 4). Moreover, the Δn_2, parameters related the decrease in the hormone–HSA affinity when the Mg^{2+} salt cation concentration increased were calculated. These values are shown in Table 3. These results were consistent with the one obtained when the salt interact with the stationary phase [61]. Thus, the positive values of Δn confirmed the competition effect between the magnesium cation and the sex-hormones to bind to HSA [62]. If the Na^+ cation was used instead of the Mg^{2+} cation, this competition effect would always be hidden.

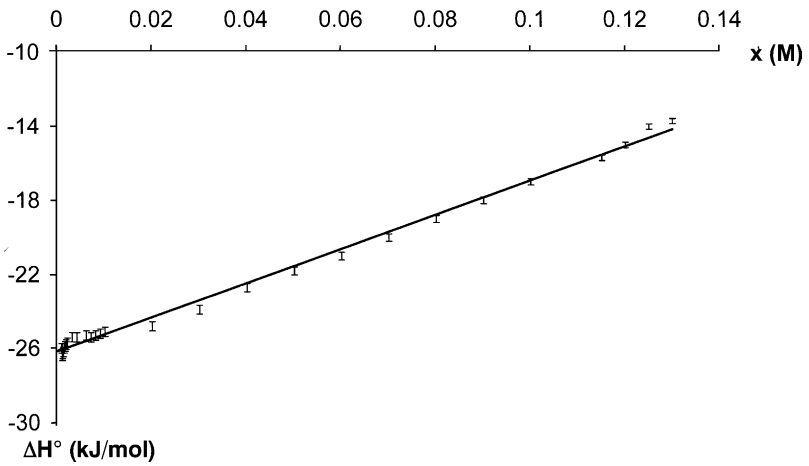

Fig. 5. Plot of ΔH° vs. sodium chlorure salt concentration (x) for progesterone molecule.

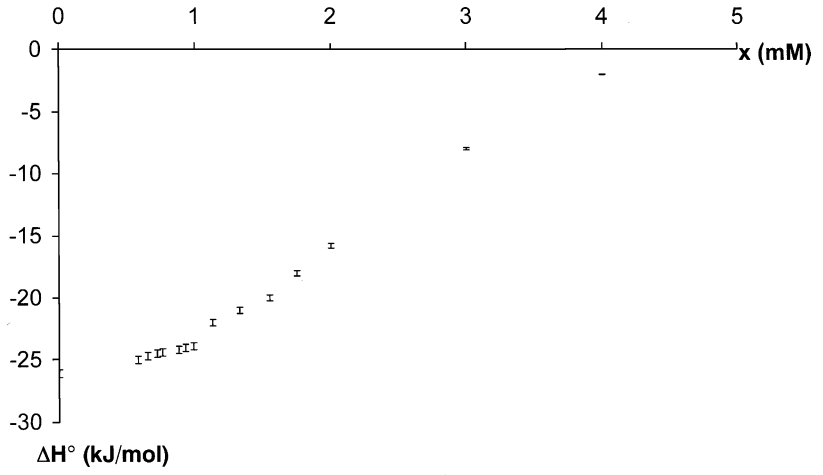

Fig. 6. Plot of ΔH° vs. magnesium chlorure salt concentration (x) for progesterone molecule.

4.4.1. Enthalpy–entropy compensation

In order to gain further insight into the HSA–sex-hormone binding mechanism with Na^{+} or Mg^{2+} in the bulk solvent, an enthalpy–entropy was investigated for the two salts. Enthalpy–entropy compensation is a term used to describe a compensation temperature which is a system independent for a class of similar experimental systems [48,55,61]. The enthalpy–entropy compensation can be expressed by the following equation [55,61]:

$$\ln k = \frac{-\Delta H^{\circ}}{R}\left(\frac{1}{T} - \frac{1}{\beta}\right) \frac{-\Delta G^{\circ}_{\beta}}{R\beta} - \ln \phi \qquad (13)$$

If the enthalpy–entropy compensation is observed (i.e. the plot $\ln k'$ against ΔH° is linear) then progesterone and β-estradiol are retained by an essentially identical interaction mechanism [55]. A first enthalpy–entropy compensation was carried out with the two sex-hormones and for the total range of the Na^{+} concentration (Fig. 7). A linear plot was observed with the following regression equation:

$$\ln k' = -0.1598\,\Delta H^{\circ} + 0.8705, \quad r = 0.996 \qquad (14)$$

This r value may be considered adequate to verify enthalpy–entropy compensation indicating that the

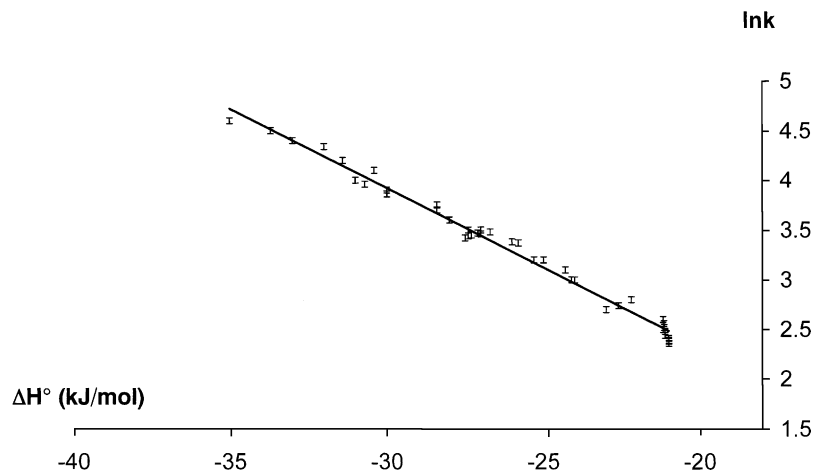

Fig. 7. Plot of $\ln k'$ vs. ΔH° for the two sex-hormones and for all the Na^{+} sodium concentrations at $T = 293\,K$.

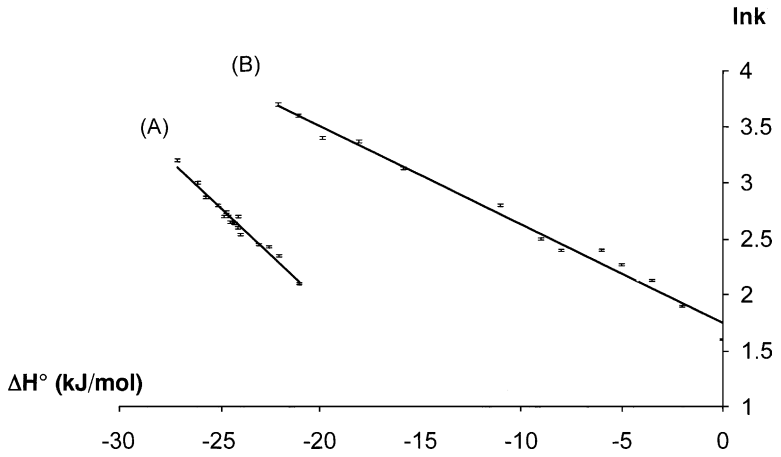

Fig. 8. Plot of $\ln k'$ vs. ΔH° for the two sex-hormones for a Mg^{2+} concentration $< x_c$ (A) and for a Mg^{2+} concentration $> x_c$ (B) at $T = 293\,K$.

sex-hormone retention on the HSA was independent to (i) the hormone structure and (ii) the Na^+ salt concentration. Moreover, this result confirmed that progesterone and β-estradiol bound to the same HSA site [5].

A second enthalpy–entropy compensation was carried out with the two sex-hormones and the Mg^{2+} salt (Fig. 8). Two distinct plots of $\ln k'$ versus x ($x = Mg^{2+}$ concentration) were observed (for $x < x_c$ and for $x < x_c$) confirmed well the change on the retention mechanism with the Mg^{2+} concentration. The regression equations for the two regions were:

$$\text{For } x < x_c: \quad \ln k' = -0.1601\,\Delta H^\circ + 1.4551, \quad r = 0.997 \quad (15)$$

$$\text{For } x > x_c: \quad \ln k' = -0.0881\,\Delta H^\circ + 1.7515, \quad r = 0.998 \quad (16)$$

These linear regressions (with $r^2 > 0.997$) proved that the HSA–sex-hormone association mechanism was in each Mg^{2+} concentration ($x < x_c$ and $x > x_c$) independent of the magnesium concentration. It was also interesting to note that the slope of the plot $\ln k'$ versus the Na^+ concentration (Eq. (11)) was similar to the one for the plot $\ln k'$ versus Mg^{2+} concentration when $x < x_c$ ($\beta_{Mg^{2+}, x<x_c} = \beta_{Na^+} \approx 294\,K$) confirming well that the HSA–sex-hormone association was governed by the hydrophobic interactions whatever the salt type used.

4.4.2. Salt influences on the progesterone displacement of its HSA binding site by β-estradiol

In order to determine the role of Mg^{2+} and the Na^+ cation on the displacement equilibrium, the plots $\ln \tilde{K}$ versus $\ln x$ were drawn at all the temperatures. Figs. 9 and 10 represent the curves for Na^+ and Mg^{2+} concentration in the bulk solvent at $T = 293\,K$. As previously observed for the sex-hormone retention mechanism, two different plots were observed. For the Na^+ cation, over the entire salt concentration range, the slow increase of surface tension in the bulk solvent (i.e. the water activity) cation, was weak favourable to the progesterone displacement of its binding cavity by β-estradiol (Fig. 9). The thermodynamic values of this displacement equilibrium were also plotted in relation to x. For example, Figs. 11 and 12 represent the curves of $\Delta \tilde{H}^\circ$ versus the Na^+ and Mg^{2+} concentration in the bulk solvent. Similar variation was obtained for the plot $\Delta \tilde{S}^\circ$ versus x. For the Na^+ cation, the weak enhancement of the hydrophobic interactions led to a weak increase of $\Delta \tilde{H}^\circ$ and $\Delta \tilde{S}^\circ$ values (Fig. 11). For the Mg^{2+} salt, under x_c ($x_c = 1 \times 10^{-3}\,M$), when the Mg^{2+} concentration increased, the free progesterone (not bound to HSA) concentration increased strongly due to the great enhancement of the hydrophobic interactions and then $\Delta \tilde{H}^\circ$ and $\Delta \tilde{S}^\circ$ values increased. Above x_c, for the Mg^{2+} salt only, β-estradiol less dis-

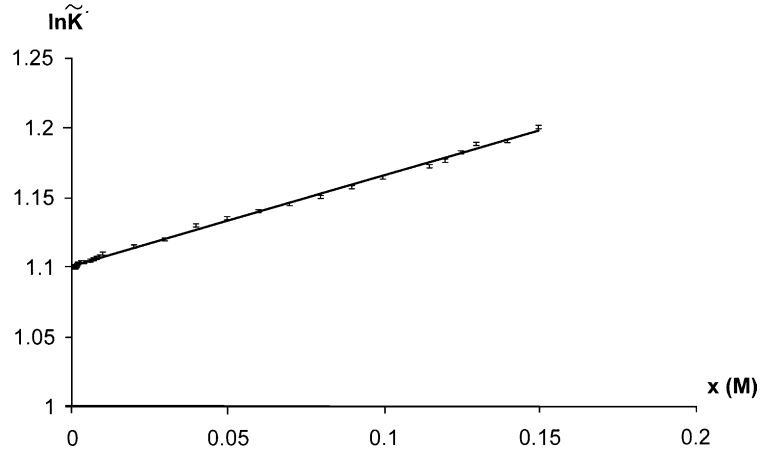

Fig. 9. Plot of $\ln \tilde{K}$ vs. sodium chlorure salt concentration (x) at $T = 293\,\mathrm{K}$.

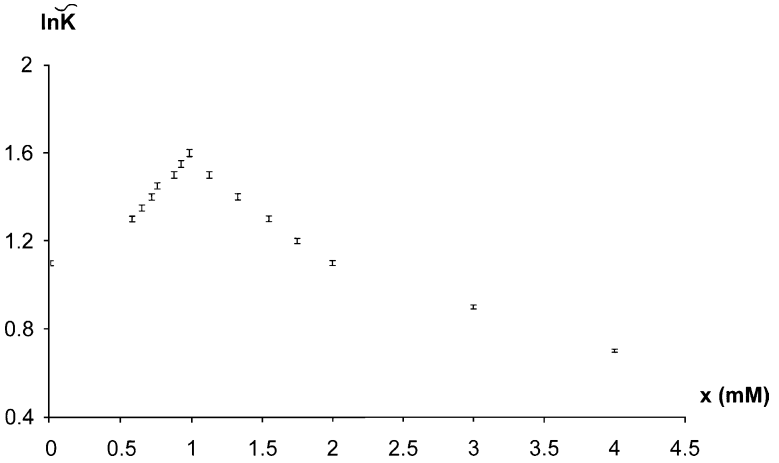

Fig. 10. Plot of $\ln \tilde{K}$ vs. magnesium chlorure salt concentration (x) at $T = 293\,\mathrm{K}$.

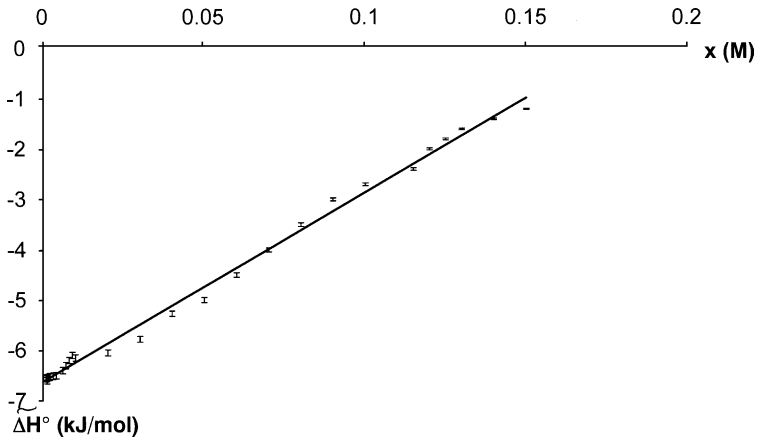

Fig. 11. Plot of $\Delta \tilde{H}^{\circ}$ vs. sodium chlorure salt concentration (x).

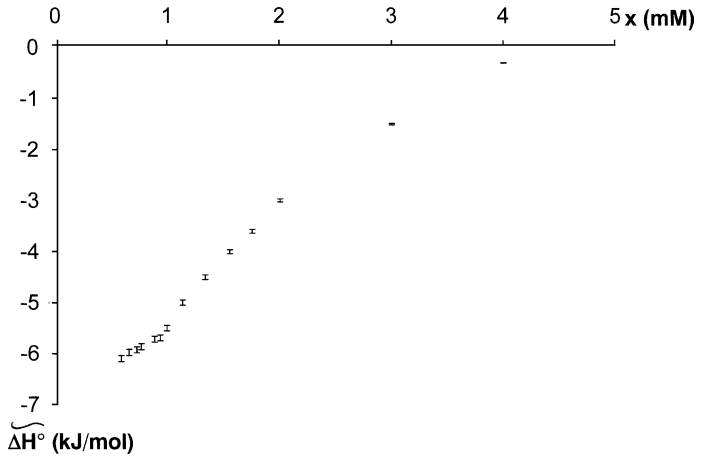

Fig. 12. Plot of $\Delta\tilde{H}^{\circ}$ vs. magnesium chlorure salt concentration (x).

placed the progesterone to its HSA binding cavity and then the progesterone rate decreased with the Mg^{2+} addition in the bulk solvent (Fig. 10). In this Mg^{2+} concentration domain, the thermodynamic data of this displacement equilibrium increased strongly (Fig. 12) due to the decrease of the interaction between the HSA and hormone (i.e. competition phenomena).

It was important to note that in the biological salt concentration (i.e. for the Na^{+}: 1.1–1.3 M; for the Mg^{2+}: 7.5×10^{-4} to 1×10^{-3} M), an increase of Mg^{2+} or Na^{+} concentration led an enhancement of the progesterone displacement of its HSA binding cavity by β-estradiol and consequently an increase of bioavailable testosterone. Then, more clinical study should be carry out to confirm our results and Gleason's observation [4].

5. Conclusion

In this paper, zonal elution, bi-Langmuir and thermodynamic approaches were used to analyse the progesterone displacement of its HSA binding site by β-estradiol. Moreover, the role of two cations i.e. Mg^{2+} and Na^{+} on the mechanism of the (i) HSA–progesterone or the HSA–β-estradiol binding and (ii) the progesterone displacement of its HSA binding site by β-estradiol was investigated. In the Mg^{2+} and Na^{+} biological concentration ranges, the data indicated that the increase of surface tension on the bulk solvent led an enhancement of both (i) the progesterone and β-estradiol affinity for HSA and (ii) the free progesterone rate.

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