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Testimony of the correlation between DHEA and bioavailable testosterone using a biochromatographic concept: effect of two salts

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Abstract

In a previous paper (C. André et al., submitted to J. Chromatogr. B) a mathematical model based on the Langmuir theory was developed to visualize the competition effect between testosterone and deshydroepiandrosterone (DHEA) for their identical human serum albumin (HSA) binding cavity. In this work, the thermodynamic mechanisms of (i) the binding of two hormones, DHEA and testosterone to HSA and (ii) the testosterone displacement of its HSA binding cavity by DHEA was studied by biochromatography. The Na^+ cation effect used as physico-chemical marker of these binding processes was clearly described. The Gibbs free energy value ($\Delta\tilde{G}^\circ$) of the displacement equilibrium was always negative demonstrating that DHEA well displaced testosterone of its HSA binding cavity. The thermodynamic data also showed that this displacement equilibrium was enthalpically controlled. Moreover, the effect of Mg^{2+} concentration (x') on the two binding mechanisms was analyzed. It appeared that for old men with a deficit of testosterone, Mg^{2+} supplementation during treatment with DHEA can increased the free testosterone concentration and its biological effect. All these results must be confirmed by in vivo test.

Keywords: DHEA; Testosterone displacement; Human serum albumin

1. Introduction

Much of the popular and scientific interest in dehydroepiandrosterone (DHEA) stems from our culture's emphasis on youth. If levels of this hormone decline with age, the thinking goes, we

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could avoid the health problems that accompany aging by keeping DHEA levels high [1–5]. One of the most exciting studies on the importance of DHEA for older men was published by Morley [6]. He found that DHEA, in turn, correlated well with the levels of bioavailable testosterone (also known as "free testosterone", not bound to albumin). Although it was known that DHEA can be easily converted into testosterone, and in that sense DHEA and its sulfated form serve as precursors for testosterone, the values obtained in the Morley's study indicated a larger involvement of DHEA in the regulation of the levels of free testosterone. It has been previously observed by fluorescence that all the steroid hormones bound on the warfarin HSA site (also named site II) [7]. Then, Morley suggested that DHEA decreased the binding of testosterone to albumin and thus increased the delivery of free testosterone to tissue receptors [6]. If this hypothesis is correct, then DHEA would be a very welcome addition to testosterone replacement for old men who suffered of andropause (partial testosterone deficiency in the aging male). More recently, Guillaume's group demonstrated the competition effect between testosterone and DHEA to bound on the same HSA cavity using the perturbation equilibrium concept [8]. The association constant of HSA and testosterone were determined at 37 °C (respectively, equal to 28.10^3 and 19.10^3) [8].

Human serum albumin (HSA) is the most abundant protein in blood plasma and possesses a capacity of reversible binding of a great number of substances including bilirubin, hormones, drugs and ions [8,9]. HSA is a globular protein (molecular mass = 66 000) consisting of a single chain of 585 amino acid residues, which is formed into subdomains by paired 17 disulfides bonds. Equilibrium dialysis is specially suited to the study of drug–protein interactions [10]. Several high performance liquid chromatographic (HPLC) separation methods have been also introduced. Hummel and Dryer [11] used a single component added to the mobile phase for the HPLC determination of the equilibrium constant of the drug–protein association. Affinity chromatography with protein immobilized on the chromatographic support is equally used to study the mechanism of this

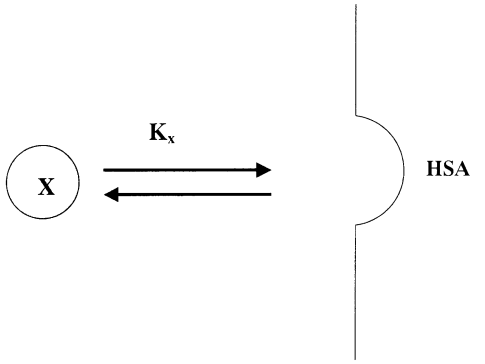

Fig. 1. Hormone (X = DHEA or testosterone) binding to HSA cavity.

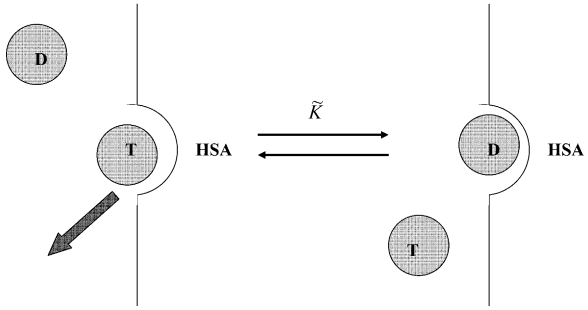

Fig. 2. Testosterone (T) displacement of its HSA binding cavity by DHEA (D).

association [12–16]. Then, in order to confirm Morley's hypothesis, the mechanism of both (i) the DHEA and testosterone binding to HSA (Fig. 1) and (ii) the testosterone displacement of its binding HSA cavity by DHEA (Fig. 2) was investigated by affinity chromatography. The thermodynamic data corresponding to these physico-chemical processes were calculated. In a first approach, the role of Na^+ cation as a physico-chemical marker of these binding processes was clearly visualized. As well, enthalpy–entropy compensation was investigated to evaluate the main parameter controlling the binding mechanism. In a second approach, the effect of Mg^{2+} on these two binding mechanisms was investigated.

2. Experimental

2.1. Apparatus

The high performance liquid chromatography (HPLC) system consisted of a Merck-Hitachi pump L7100 (Nogent-sur-Marne, France), an interchim Rheodyne injection valve Model 7125 (Montluçon, France) fitted with a 20 μl sample loop and a Merck L 4500 diode array detector (Nogent-sur-Marne, France). An HSA protein chiral Shandon column (Montluçon, France) (150 × 4.6 mm) was used with controlled temperature in a Interchim Crocodil oven TMN° 701 (Montluçon, France). The HSA was grafted on the chromatographic support in means that 98.5% of the stationary support was coated by HSA (commercial data). Throughout the study, the flow rate was maintained constant and equal to 0.7 ml min^{-1}.

2.2. Solvent and samples

Sodium hydrogenphosphate and sodium dihydrogenphosphate were supplied by Prolabo (Paris, France). NaCl and $MgCl_2$ was obtained from Sigma-Aldrich (Saint-Quentin, France). Water was obtained from an Elgastat option water purification system (Odil, Talant, France) fitted with a reverse osmotic cartridge. DHEA and testosterone were obtained from Sigma (Saint-Quentin Fallavier, France). Sodium nitrate was used as a dead time marker (Merck) [13]. The bulk solvent consisted of a sodium phosphate buffer (7×10^{-4}) at pH 7.3 (pH of the plasma). To examine the concentration dependency of the hormone retention, corresponding to the binding to HSA, retention measurements were related to varying amounts of injected solute. Hormone samples were prepared at different concentrations in the mobile phase: 5–25 μg ml^{-1}. 20 μl of each hormone were injected in triplicate and retention times measured. The plots of retention factor exhibited a plateau at sample concentrations < 15 μg ml^{-1} followed by a small decrease at higher hormone concentrations. Therefore, each solute was injected at a concentration of 15 μg ml^{-1} when the hormone–HSA binding was sample concentration independent, i.e. in linear elution conditions.

2.3. Temperature study

Retention factors of each hormone were determined at six temperatures 20, 25, 20, 35, 40, 45 °C. The chromatographic system was allowed to equilibrate at each temperature for at least 1 h prior to each experiment. To study this equilibration, the retention time of DHEA 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 lower than 0.5%, making the chromatographic system sufficiently equilibrated for use after 1 h. The steroid hormones were injected three times at each temperature and salt concentration (Na^+ and Mg^{2+} concentration). Once the measurements were completed at the maximum temperature, the column was immediately cooled to ambient conditions to minimize the possibility of any unfolding of the immobilized HSA.

2.4. HSA binding character study

The DHEA association constants with both the immobilized HSA (HSA stationary phase) and the free HSA (HSA on the mobile phase) were determined at 37 °C and when the salt concentration in the mobile phase was nil. The maximum relative difference of the association constant of this compound was always lower than 0.7% showing that immobilization of HSA on a chromatographic support not altered the HSA binding character.

3. Results and discussion

3.1. Confirmation of the testosterone displacement to its HSA binding site by DHEA using the Na^+ cation as a retention marker

Much information on the DHEA (or testosterone)/HSA binding mechanism (Fig. 1) may be gained by examining the temperature dependence of hormone elution. The Gibbs free energy of the

Table 1
Values of ΔH°, ΔS° and $\Delta G^\circ_{37.5\ ^\circ C}$ for DHEA (D) and testosterone (T) at all the natrium cation concentration studied

$[Na]^+$ (mmol l^{-1})	ΔH°_D (kJ mol^{-1})	ΔS°_D (J mol^{-1} K^{-1})	$\Delta G^\circ_{D,\ 37.5\ ^\circ C}$ (kJ mol^{-1})	ΔH°_T (kJ mol^{-1})	ΔS°_T (J mol^{-1} K^{-1})	$\Delta G^\circ_{T,\ 37.5\ ^\circ C}$ (kJ mol^{-1})
0	−23.4	−62.6	−4.0	−16.3	−43.3	−3.0
20.2	−23.4	−62.3	−4.0	−16.3	−43.2	−2.9
50	−23.4	−62.2	−4.0	−16.3	−43.1	−2.9
85.5	−23.4	−62.2	−4.0	−16.3	−43.1	−2.9
102.6	−22.1	−61.0	−3.1	−16.2	−43.1	−2.8
119.7	−20.2	−56.8	−2.5	−15.5	−40.6	−2.8
136.8	−19.4	−53.9	−2.4	−13.4	−36.3	−1.9
153.9	−18.3	−50.9	−2.3	−11.8	−32.1	−1.8
200.1	−15.7	−45.8	−1.5	−10.6	−30.9	−1.0

S.D. < 0.05.

hormone transfer ΔG° from the bulk solvent to the HSA cavity could be linked to its equilibrium constant K with the following equation [17]:

$$\ln K = -\Delta G^\circ / RT \quad (1)$$

where R is the gas constant and T is the absolute temperature. As K can be linked with the retention factor (k') by $K = k'/\phi$ where ϕ is the phase ratio of the HSA column (volume of the mobile phase divided by the volume of the stationary phase), k' represents the hormone/HSA binding intensity. k' can also be expressed by the well known equation [18]:

$$\ln k' = -\Delta H^\circ / RT + \Delta S^\circ / R - \ln \phi \quad (2)$$

where ΔH° and ΔS° are, respectively, the enthalpy and entropy of the hormone–HSA binding mechanism. With an invariant binding mechanism over the temperature range being studied, the enthalpy of hormone–HSA binding (ΔH°) remained constant and a plot of $\ln k'$ in relation to $1/T$ (called van't Hoff plot) led to a straight line with an enthalpic slope and entropic origin [18].

The experimental k' values were calculated for both DHEA and testosterone when the Na^+ cation concentration in the bulk solvent was nil. Each experiment was repeated three times. The relative standard deviations of the k' values were usually less than 0.5%, indicating high reproducibility and good stability for the chromatographic system. The plots of $\ln k'$ in relation to $1/T$ were determined for the two steroid hormones. The van't Hoff plots were all linear. The correlation coefficients for the linear fits were in excess of 0.97. These linear behaviors were thermodynamically what was expected when there was no change in the binding mechanism in relation to temperature [19]. The thermodynamic values (ΔH° and ΔS°) were given in Table 1. ΔH° always higher than $T\Delta S^\circ$ values for both DHEA and testosterone indicated that hormone–HSA binding was enthalpically controlled. Both ΔH° and ΔS° were negative, as was usually the case for several pharmacomolecule–HSA association [20]. The solute molar enthalpy associated with the HSA was as expected lower than the solute molar enthalpy associated with the bulk solvent due to the formation of strong interactions (van der Walls interactions) between the steroid hormone and the HSA cavity. In addition these interactions promote a lower entropy (high order) of steroid hormone in the HSA cavity than in the bulk solvent by a large immobilization of hormone in the HSA cavity. Moreover, the ΔH° and ΔS° values were smaller for DHEA than for testosterone (Table 1). This result was confirmed by the fact that testosterone was eluted before DHEA. Thus, the HSA (H)–DHEA (D) binding (H–D) was more stabilized and more ordered than the HSA (H)–testosterone (T) association (H–T). This result tends to confirm Morley hypothesis [6]: "DHEA supplementation can increased the bioavailable testosterone" (also known as "free testosterone", not bound to HSA) by a competition effect between DHEA and testosterone to bind to HSA cavity. This competition effect could

Table 2
$\tilde{K}$ values at six temperatures when the salt concentrations in the bulk solvent were nil

Temperature (°C)	$\tilde{K}$
20	1.51
25	1.47
30	1.43
35	1.41
40	1.39
45	1.36

S.D. < 0.02.

be expressed by the following displacement equilibrium (Fig. 2):

$$H\text{–}T + D \leftrightarrow H\text{–}D + T \quad (3)$$

The constant $\tilde{K}$ of this displacement equilibrium could be expressed by the following equation [17,18]:

$$\tilde{K} = K_D/K_T = k'_D/k'_T \quad (4)$$

where k'_D and k'_T represent, respectively, the binding intensity of HSA–DHEA (H–D) and HSA–testosterone (H–T). Table 2 gave the $\tilde{K}$ values obtained at six temperatures when the Na^+ concentration in the mobile phase was nil. These values were in accordance the one obtained recently with Langmuir theory (i.e. chromatographic methods which allowed to determine the retention factors under a competitive binding condition) [8], confirming well that the $\tilde{K}$ value can be considered as an index proportional to a competitive binding character. Combining Equations 2 and 4, the following equation was obtained:

$$\ln \tilde{K} = -\Delta\tilde{H}^\circ/RT + \Delta\tilde{S}^\circ/R \quad (5)$$

where $\Delta\tilde{H}^\circ$ and $\Delta\tilde{S}^\circ$ are, respectively, the enthalpy and entropy of the displacement equilibrium (Equation 3, Fig. 2).

The plot of $\ln \tilde{K}$ versus $1/T$ (van't Hoff plot) when the sodium cation concentration in the bulk solvent was nil was drawn. The correlation coefficient for the linear fit was equal to 0.97. $\Delta\tilde{H}^\circ = -8.9$ kJ mol^{-1} and $\Delta\tilde{S}^\circ = -19.3$ J mol^{-1} K^{-1} (Table 3) were determined from the slope and intercept, respectively (Equation 5). The Gibbs free dissolution energy $\Delta\tilde{G}^\circ$ of this equilibrium (Equation 3) were determined at different Na^+ concentration using the well known equation:

$$\Delta\tilde{G}^\circ = \Delta\tilde{H}^\circ - T\Delta\tilde{S}^\circ \quad (6)$$

$\Delta\tilde{G}^\circ$ was always negative (for example at human temperature 37.5 °C when the NaCl concentration was nil, $\Delta\tilde{G}^\circ = -2.09$ kJ mol^{-1}) indicated that DHEA well displaced testosterone of its HSA binding site. This competition effect was also confirmed by a mathematical model based on Langmuir isotherms [21]. Moreover $\Delta\tilde{H}^\circ$ was always higher than $T\Delta\tilde{S}^\circ$ value indicating that the mechanism of the testosterone displacement of its HSA binding cavity by DHEA was enthalpically controlled [17,18]. This result confirmed that DHEA could increase the free testosterone by a competition effect. Consequently, a DHEA supplementation for old men who suffer from andropause could be useful in order to increase the bioavailable testosterone.

Table 3
Values of $\Delta\tilde{H}^\circ$ (kJ mol^{-1}), $\Delta\tilde{S}^\circ$(J mol^{-1} K^{-1}) and $\Delta\tilde{G}^\circ_{37.5\,°C}$ (kJ mol^{-1}) at all the sodium cation concentrations in the bulk solvent

$[Na]^+$ (mmol l^{-1})	$\Delta\tilde{H}^\circ$ (kJ mol^{-1})	$\Delta\tilde{S}^\circ$ (J mol^{-1} K^{-1})	$\Delta\tilde{G}^\circ_{37.5\,°C}$ (kJ mol^{-1})
0	−8.9	−19.3	−2.9
20.2	−8.1	−19.2	−2.1
50.0	−7.4	−19.1	−1.5
85.5	−6.0	−18.0	−0.4
102.6	−5.0	−15.6	−0.2
119.7	−4.3	−13.0	−0.2
136.8	−4.0	−12.0	−0.2
153.9	−3.7	−11.8	−0.1
200.1	−3.0	−9.7	−0.1

S.D. < 0.06.

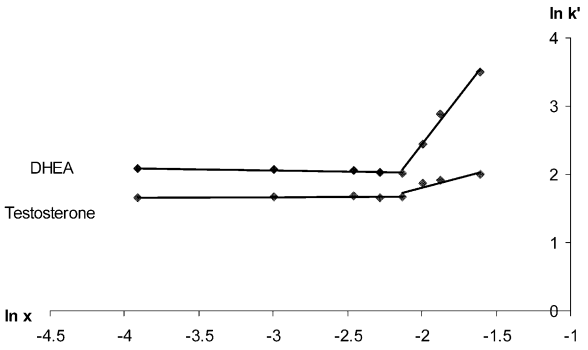

Fig. 3. $\ln k'$ vs. $\ln x$ at $T = 20\ ^{\circ}C$.

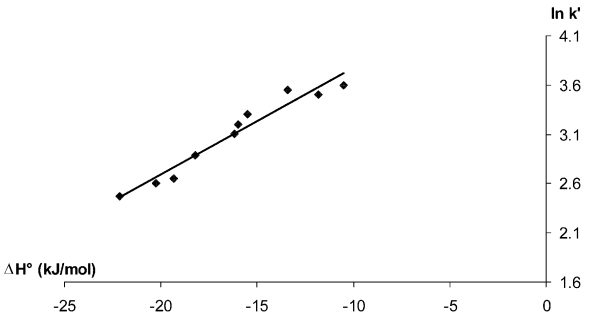

Fig. 4. Enthalpy–entropy compensation represented by a $\Delta H^{\circ} - \ln k'$ plot for DHEA and testosterone at all the Na^+ concentrations of the domain 2 ($x > 0.1$ M).

In order to gain further insight into the mechanism of DHEA and testosterone binding to HSA (Fig. 1), the influence of Na^+ cation was studied. The plots $\ln k'$ versus x were drawn for the two steroid hormones and for a large variation range of Na^+ concentration ($0 < x < 0.2$ M). Fig. 3 reports the curve obtained for DHEA and testosterone at $T = 25\ ^{\circ}C$. It has been known for several years that increasing the ionic strength of the bulk solvent increased its surface tension. Therefore, if the Na^+ concentration increased, the surface tension of the bulk solvent increased [22,23]. The thermodynamic data of the hormone–HSA binding can be expressed as [23,24]:

$$\Delta H^{\circ} = H^{\circ}_{HSA} - H^{\circ}_{m} \quad (7)$$

$$\Delta S^{\circ} = S^{\circ}_{HSA} - S^{\circ}_{m} \quad (8)$$

Where H°_{HSA}, H°_{m}, S°_{HSA}, S°_{m}, are, respectively, enthalpy and entropy of the solute associated with the HSA and the bulk solvent.

Below Na^+ concentration equal to 0.1 M (domain 1), the thermodynamic data were roughly constant (Table 1) and there was no or weak variation in the hormone–HSA binding intensity (Fig. 3). For weak Na^+ concentration, no significant change on the surface tension was observed, consequently H°_{m} and S°_{m} kept constant leading to no ΔH° and ΔS° variation (Table 1, Equations 7, 8).

Above Na^+ concentration equal to 0.1 M (domain 2), the hormone–HSA binding (k') would be enhanced when x increased (Fig. 3). In this Na^+ concentration domain, the sodium ion was predicted to increase the surface tension of the bulk solvent. Consequently both the hormone molar enthalpy and entropy associated to the bulk solvent decreased (H_m and S_m), leading an increase of the thermodynamic data (Table 1, Equations 7, 8). In order to gain further insight into the validity of this binding mechanism, the enthalpy–entropy compensation was examined in this Na^+ cation concentration domain. Enthalpy–entropy compensation is a term used to describe a compensation temperature which is a system independent for a class of similar experimental systems [25–28]. It has been applied to chromatographic system to evaluate the binding mechanism. The enthalpy–entropy compensation can be expressed by the formula:

$$\Delta G^{\circ}_{\beta} = \Delta H^{\circ} - \beta \Delta S^{\circ} \quad (9)$$

where ΔG°_{β} is the Gibbs free energy of a physicochemical interaction at a compensation temperature β. ΔH° and ΔS° are, respectively, the corresponding standard enthalpy and entropy. According to Equation 9, when enthalpy–entropy compensation is observed with a group of compounds in a particular chemical interaction, all the compounds have the same free energy ΔG°_{β} at temperature β. If therefore, enthalpy–entropy compensation is observed for the two steroid hormones, all of them will have the same net retention at the temperature β, although their temperature dependencies may differ [26–28]. Combining Equation (2) and (9), the following equation is obtained:

Table 4
Δn_{Na^+} values at six temperatures

Temperature (°C)	$\Delta n_{DHEA,\ Na^+}$	$\Delta n_{testosterone,\ Na^+}$
20	−3.02	−1.02
25	−2.88	−0.98
30	−2.76	−0.97
35	−2.75	−0.95
40	−2.72	−0.93
45	−2.68	−0.89

S.D. < 0.06.

$$\ln k' = -\Delta H^\circ/R(1/T - 1/\beta) - \Delta G^\circ_\beta/(R\beta) - \ln \phi \quad (10)$$

Equation 10 shows that, if a plot of $\ln k'_T$ against ΔH° is linear, then the hormones are retained by an essentially identical interaction mechanism. The plot of $\ln k'$ versus ΔH° determined at $T = 25$ °C and at all the Na^+ cation concentrations in the bulk solvent ($x > 0.1$ M) was drawn for the two steroid hormones (Fig. 4). The correlation coefficient for the linear fit was equal to 0.96. This degree of correlation can be considered adequate to verify enthalpy–entropy compensation, indicating that (i) DHEA and testosterone bound effectively on the same HSA cavity, and (ii) the binding mechanism was independent on both the hormone structure and the Na^+ concentration in the bulk solvent. Moreover, this confirmed that the eventual adsorption of the Na^+ cation on HSA seems to be negligible as expected in the model [17,26]. If addition of Na^+ disturbs the surface tension, its concentration in the surface layer of the HSA cavity and hormone must differ from its concentration in the medium. Considering n as the excess of Na^+ cation at the hormone–HSA interface implied in the binding process, k' can be linked to the change in salt concentration, x, using the following equation [26–30]:

$$\left(\frac{d \ln k'}{d \ln x}\right)_T = -\Delta n \quad (11)$$

Integrating Equation 11 gives:

$$\ln k' = \gamma - (\Delta n) \ln x \quad (12)$$

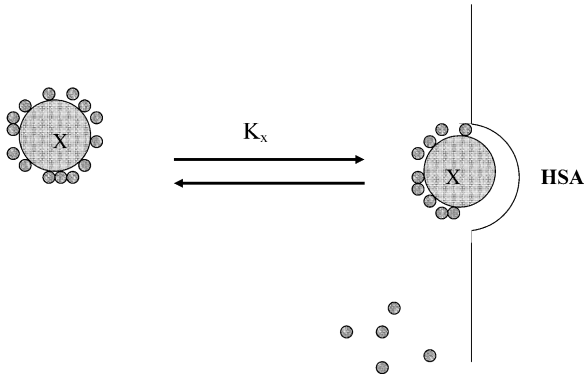

Fig. 5. Exclusion of sodium (or magnesium) cation when hormone (X = DHEA or testosterone) bound to HSA surface.

where γ is a constant. The Δn_{Na^+} values, for both DHEA (D) and testosterone (T) were determined from the slope of the plot $\ln k'$ versus $\ln x$ in the domain 2 ($x > 0.1$ M). The Δn_{Na^+} obtained values were reported in Table 4. The negative values of Δn reflected the exclusion of sodium cation when hormone bound to HSA cavity (Fig. 5). It must be pointed out that the Δn values varied similarly to the elution order: $\Delta n_{D,\ Na^+}$ was higher than $\Delta n_{T,\ Na^+}$. Moreover, the Δn values of DHEA and testosterone were higher than the Δn values obtained with the binding of tryptophan to HSA [28]. As the main parameter controlling the binding of solute to HSA cavity was the hydrophobic interactions [11,12], this result could be explain by the higher hydrophobicity of the two hormones than tryptophan. Consequently, Δn_{Na^+} reflects well the hormone–HSA binding mechanism and can be considered as an affinity marker of steroid hormone for the HSA cavity.

In order to study, the influence of Na^+ concentration on the displacement equilibrium (Equation 4, Fig. 2), $\Delta\tilde{H}^\circ$ versus x (sodium concentration) was drawn. The thermodynamic data of the testosterone displacement of its HSA binding site (Fig. 2) can be expressed:

$$\Delta\tilde{H}^\circ = \tilde{H}^\circ_{HSA(D\to T)} - \tilde{H}^\circ_{m(D\to T)} \quad (13)$$

$$\Delta\tilde{S}^\circ = \tilde{S}^\circ_{HSA(D\to T)} - \tilde{S}^\circ_{m(D\to T)} \quad (14)$$

where $\tilde{H}^\circ_{HSA\ (D\to T)}$, $\tilde{H}^\circ_{m\ (D\to T)}$ and $\tilde{S}^\circ_{HSA\ (D\to T)}$, $\tilde{S}^\circ_{m\ (D\to T)}$ are, respectively, the enthalpy and

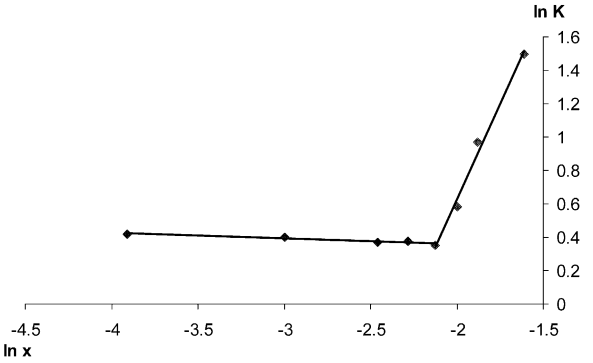

Fig. 6. ln $\tilde{K}$vs. sodium cation concentration in the bulk solvent (x) at $T = 20\ ^{\circ}C$.

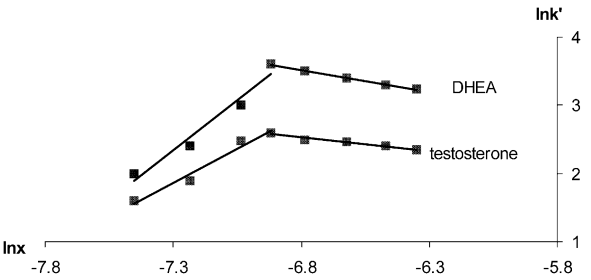

Fig. 7. ln k' vs. the magnesium concentration (x') for DHEA and testosterone at $T = 20\ ^{\circ}C$.

entropy difference between the DHEA (D) and the testosterone (T) when they are associated with HSA and the bulk solvent. As for the HSA–DHEA and HSA–testosterone binding mechanisms, the plot can be divided into two domains (Fig. 6). For $x < x_c$ ($x_c = 0.1$ M), the Na^+ concentration was too weak to have an influence on the surface tension. Above x_c, when x increased, the Na^+ cation effect on the surface tension of the bulk solvent increased, then $\tilde{H}^{\circ}_{m\ (D\rightarrow T)}$ and $\tilde{S}^{\circ}_{m\ (D\rightarrow T)}$ decreased, leading to an increase in the $\Delta\tilde{H}^{\circ}$, $\Delta\tilde{S}^{\circ}$ values (Table 3, Equations 13, 14). The plot ln $\tilde{K}$ versus ln x was also drawn at all the temperature studied. All the plots were linear (with $r >$ 0.96) for the total range of Na^+ cation concentration. For the total range of Na^+ concentration (x), the $\tilde{K}$ values increased when x increased. This result demonstrated that the testosterone displacement to its HSA binding cavity by DHEA was more favorable when the surface tension in the bulk solvent increased. Then, when the Na^+ concentration increased, the bioavailable testosterone concentration enhanced and consequently, more testosterone could interact with receptor organs.

3.2. Effect of Mg^{2+} on these binding processes

3.2.1. Role of the Mg^{2+} cation on the HSA–hormone binding process

Linear van't Hoff plots were obtained with correlation coefficients r higher than 0.97 for all fits for all the magnesium concentration studied. Table 5 reports a complete list of ΔH° and ΔS° values at different Mg^{2+} concentration (x') in the bulk solvent. DHEA and testosterone exhibited a similar variation for the thermodynamic data whatever the Mg^{2+} cation concentration (Table 5). In order to elucidate the effect of Mg^{2+} concentration in the bulk solvent on the steroid

Table 5
Thermodynamic data for DHEA and testosterone at all the magnesium concentrations

$[Mg^{2+}]$ (mmol l^{-1})	ΔH°_D (kJ mol^{-1})	ΔS°_D (J mol^{-1} K^{-1})	ΔH°_T (kJ mol^{-1})	ΔS°_T (J mol^{-1} K^{-1})
0	−23.3	−62.5	−16.2	−43.3
0.58	−22.4	−61.7	−15.8	−42.1
0.72	−21.1	−60.1	−15.2	−41.1
0.88	−20.2	−59.2	−14.8	−40.6
1.01	−18.7	−57.2	−11.5	−38.4
1.13	−16.4	−54.6	−9.8	−36.2
1.33	−13.1	−49.1	−7.9	−34.5
1.55	−11.1	−43.1	−6.5	−32.1
1.75	−9.6	−41.5	−5.2	−30.2

S.D. < 0.06.

Table 6
$\Delta n_{1,\ Mg^{2+}}$ and $\Delta n_{2,\ Mg^{2+}}$ values at all the temperature studied

Temperature (°C)	$\Delta n_{1\ DHEA,\ Mg^{2+}}$	$\Delta n_{1\ testosterone\ Mg^{2+}}$	$\Delta n_{2\ DHEA\ Mg^{2+}}$	$\Delta n_{2\ testosterone\ Mg^{2+}}$
20	−2.97	−1.59	0.92	0.56
25	−2.81	−1.51	0.89	0.54
30	−2.78	−1.49	0.82	0.51
35	−2.71	−1.42	0.79	0.49
40	−2.68	−1.37	0.72	0.48
45	−2.61	−1.31	0.66	0.46

S.D. < 0.06.

HSA–hormone binding, $\ln k'$ were plotted against $\ln x'$ for DHEA and testosterone and for a variation range of salt concentration (0–1.75 mM). Fig. 7 reported the curves obtained for DHEA and testosterone at $T = 20$ °C. The dependence of $\ln k'$ on $\ln x'$ was similar for the two steroid hormones. These plots showed a curvature around a critical x'_c value around 1.0 mM. At low additive concentration (i) an increase in the HSA–hormone binding was observed followed by (ii) a small decrease of HSA–hormone affinity for high Mg^{2+} concentration in the bulk solvent. As previously described with the role of Na^+ on the HSA–testosterone (or DHEA) binding, the direct salt effect was predicted to increase the surface tension of the bulk solvent and thus HSA–hormone binding would be enhanced when x increased. Nevertheless the non-linearity of the plots $\ln k'$ versus $\ln x'$ showed that theories based only on the change of the surface tension effect (i.e. water activity) can not reflect the negative salt influence on the HSA–hormone association at high Mg^{2+} cation additive [29,31]. This trends of the binding intensity with Mg^{2+} concentration can be explained by (i) a change in the water activity classically attributed to salt and (ii) the possible interaction of Mg^{2+} with HSA [31–33]. Then for a variation range of Mg^{2+} from 0 to 1.75 mM.

For $x' < 1.0$ mM, the effect (i) was dominant. Thus the change in the water activity led to a favorable HSA–hormone binding. The increase of the hydrophobic interactions between the HSA and the steroid hormone led an increase of the thermodynamic data (Table 5). 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 (Equation 12). Then, the $\Delta n_{1,\ Mg^{2+}}$ values, for both DHEA (D) and testosterone (T) were determined from the slope of the plot $\ln k'$ versus $\ln x'$ ($x' < x'_c$, Fig. 7) and are reported in Table 6. It appeared that the number of the magnesium ions excluded when hormone bound to HSA (Table 6, Fig. 7) was similar as the one obtained with Na^+ (Table 4).

For $x' > 1.0$ mM contrary to the result obtained with Na^+, the HSA–hormone binding decreased when the Mg^{2+} concentration increased and the thermodynamic data of these binding mechanism decreased strongly. A general phenomenon found in early studies on HSA is its ability to bind divalent inorganic cations (Mg^{2+}, Ca^{2+}) [30–33]. Thus in this Mg^{2+} concentration domain, the Mg^{2+} bound to HSA by electrostatic interactions between its charge and the oppositely charged surface of HSA (HSA at pH 7 was negatively charged) [12]. Then the non-specific binding mode of Mg^{2+} led a competition effect between the steroid hormones and this divalent cation to bind to HSA and consequently a decrease of HSA–hormone affinity. 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 a strong increased of the thermodynamic data (Table 5).

Moreover, using Equation 12, the $\Delta n_{2,\ Mg^{2+}}$ parameters (i.e. related to the decrease of the hormone–HSA affinity) were calculated. These values are shown in Table 6. The positive values of

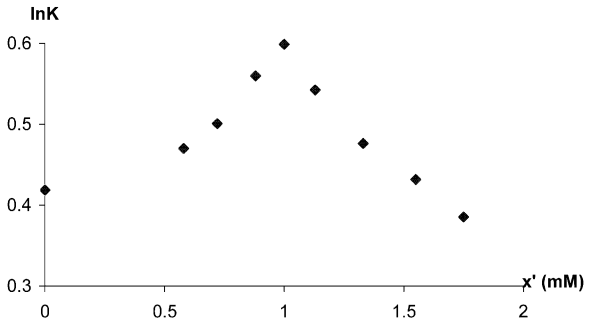

Fig. 8. $\ln \tilde{K}$ vs. $\ln x'$ at $T = 20$ °C.

$\Delta n_{2,\ Mg^{2+}}$ well proved the competition effect between the magnesium cation and the steroid hormones to bind to HSA [29].

3.2.2. Role of Mg^{2+} on the testosterone displacement of its HSA binding cavity by DHEA

The existence of the testosterone displacement to its HSA binding site was previously proved by both a thermodynamic and Langmuir approach [8]. In order to show the Mg^{2+} influence on this displacement, the values of $\tilde{K}$ were determined at all the temperatures and different Mg^{2+} concentrations (Equation 2). The van't Hoff ($\ln \tilde{K}$ vs. $1/T$) were drawn for the high Mg^{2+} concentrations. All the plots were linear. From the slope and the intercept, respectively, of these plots the thermodynamic data of this displacement equilibrium $\Delta \tilde{H}^\circ$ and $\Delta \tilde{S}^\circ$ were, respectively, determined. In order to determine the role of Mg^{2+} cation on the displacement equilibrium, the plots $\ln \tilde{K}$ versus $\ln x'$ were drawn at all the temperatures. Fig. 8 represents the curve obtained at $T = 20$ °C. All the plots present similar variation. For $x' < x'_c$ ($x'_c = 1.0$ mM), the increase of the surface tension in the bulk solvent (i.e. the water activity) was favorable to the testosterone displacement of its binding cavity by DHEA. The enhancement of the hydrophobic interactions led an increase of $\Delta \tilde{H}^\circ$ and $\Delta \tilde{S}^\circ$ values (Fig. 9). Thus, in this Mg^{2+} concentration domain, when the Mg^{2+} concentration increased, the free testosterone (not bound to HSA) concentration increased.

Above x'_c, the DHEA displaced less the testosterone to its HSA binding cavity. In this Mg^{2+} concentration domain, the thermodynamic data of this displacement equilibrium increased strongly (Fig. 9) due to the decrease of the interaction between the HSA and hormone (i.e. competition phenomena, effect (ii)). Then in high Mg^{2+} concentration ($x' > x'_c$), the bioavailable testosterone (not bound to HSA) decreased.

It was important to note that in the biological Mg^{2+} concentration (0.75–0.10 mM), an increase of Mg^{2+} led an enhancement of the testosterone displacement of its HSA binding cavity and consequently an increase of bioavalaible testosterone. Then, this study tends to show that for old men who suffer from andropause, an Mg^{2+} supplementation during treatment with DHEA can increase the free testosterone concentration.

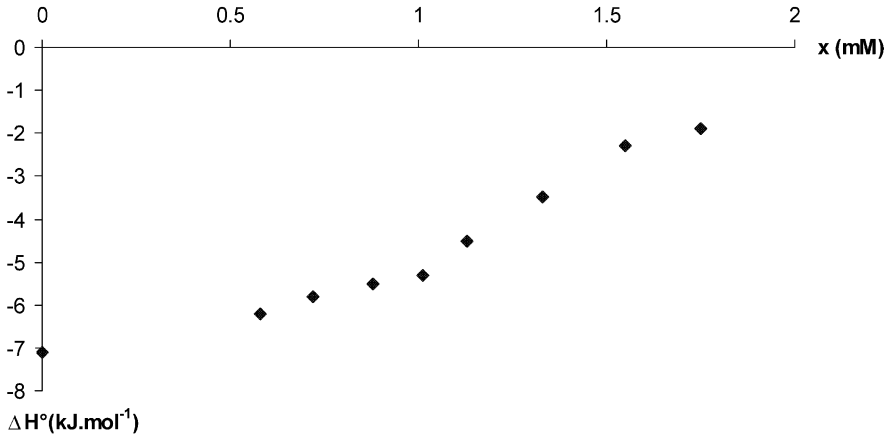

Fig. 9. $\Delta \tilde{H}^\circ$ vs. magnesium cation concentration.

4. Conclusion

In this manuscript, the mechanism of the (i) testosterone and DHEA binding to the HSA cavity and (ii) testosterone displacement to its HSA binding cavity by DHEA were investigated by biochromatography. The thermodynamic results of these processes confirmed Morley's hypothesis: "DHEA can increased the free testosterone (bioavailable testosterone) by a competition effect between testosterone and DHEA to bind on the same HSA cavity". The role of the Na^+ cation, as a marker of these physico-chemical processes was investigated. It appeared that the surface tension increase led to a better liberation of the testosterone. Moreover, the effect of Mg^{2+} on these binding mechanisms was investigated. This study demonstrated that it seems to be interesting to test in vivo, the magnesium supplementation during DHEA treatment for old men who suffer from andropause.

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