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Short communication

Reanalysis of the testosterone displacement from its HSA binding site by DHEA using competitive Langmuir isotherm

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Abstract

In a previous manuscript [C. Andre, A. Berthelot, J.F. Robert, M. Thomassin, Y.C. Guillaume, J. Pharm. Bio. Med., in press], the Mg^{2+} effect on the testosterone displacement equilibrium from its human serum albumin (HSA) binding site by DHEA was investigated using a thermodynamic approach. In this paper, a novel concept based on the competitive Langmuir distribution isotherms was proposed to calculate the association constant of the HSA–hormone binding and to confirm the Mg^{2+} role on the testosterone displacement equilibrium from its HSA binding site by DHEA. Thus, both the HSA–hormone binding and the displacement equilibrium processes were reanalysed. The results obtained confirmed that:

– DHEA and testosterone bound on the same HSA site;
– DHEA displaced well testosterone to its HSA binding site;
– Mg^{2+} cation led a change on both the HSA–hormone binding and the displacement equilibrium at a critical Mg^{2+} concentration x_c around 1 mM.

Keywords: Testosterone; HSA–hormone; Human serum albumin

1. Introduction

Protein binding in blood is a significant factor in the transport and release of many drugs and hormone. These interactions can influence the biological distribution of these compounds as well as their excretion, therapeutic activity, and toxicity [1]. One protein that is involved in such binding processes is human serum albumin (HSA). It is also known that competition between hormones or drugs to interact on the same HSA site exist and contribute to modify the activity and the toxicity of drugs. For example, Guillaume's group have been previously reported that DHEA displaced testosterone from its HSA binding site [2]. It was thus confirmed Morley's hypothesis, i.e. DHEA supplementation can increase the testosterone rate [3]. The role of the Mg^{2+} on the bio-available testosterone rate (not bound to HSA) was also clearly visualised [2]. It was observed that in the biological concentration range (0.75–1 mM), increasing Mg^{2+} concentration was favorable to the testosterone displacement from its HSA binding site by DHEA and consequently, the free testosterone concentration increased. This result can be useful to better treat by DHEA old men who suffer from andropause [3]. Numerous analytical techniques are used for HSA binding studies and they are continuously being added to, along with extending knowledge about the complex mechanisms involved in

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the drug–HSA binding process. The advantages and limitations of various methods are discussed by Oravkova et al. [4]. In recent years, high performance liquid affinity chromatography (HPLAC) appeared to be a powerful tool for the study of bio-molecular interactions. Different mathematical methods allowed to elucidate the drug–HSA interaction [5–17]. In this paper, the HSA–hormone (DHEA and testosterone) binding and the displacement equilibrium processes were reanalysed using competitive Langmuir isotherm concept at human temperature ($T = 37\,^{\circ}C$). Moreover, the association constants of the HSA–hormone (DHEA and testosterone) binding were determined.

2. Theory

Numerous models have been suggested to describe the competitive equilibria involved between the compounds A, B of a binary mixture and the adsorbent, yielding various competitive isotherm equations [18]. The often-used competitive Langmuir isotherm can be expressed as:

$$C_{sA} = \frac{\alpha K_A C_{mA}}{1 + K_A C_{mA} + K_B C_{mB}} \quad (1)$$

$$C_{sB} = \frac{\alpha K_B C_{mB}}{1 + K_A C_{mA} + K_B C_{mB}} \quad (2)$$

where C_{sA} and C_{sB} are ,respectively, the A and B compound concentrations in the stationary phase, K_A and K_B the adsorption constants between the HSA and respectively the solute A and B, α the column saturation factors of the compound A and B, C_{mA}, and C_{mB}, respectively the A and B compound concentrations in the mobile phase. The competitive Langmuir isotherm approach was allowed only if the column has the same saturation capacities for the two compounds [18]. The column used for the determination of the isotherms is first equilibrated with a solution containing a mixture of the compounds A and B dissolved in a non-adsorbable solvent. Then a sample volume containing different concentrations 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 [18,19]. When such a wave reaches the column outlet, a peak is registered by the detector. By this method, two peaks were observed (i.e. peak one and two). Heber et al. have previously used an equation allowing to determine the expected elution times of the two disturbances, t_{R1calc} and t_{R2calc} [18]:

$$t_{R1cacl} = t_0 \left[1 + \phi \left(\frac{\partial C_{sA}}{\partial C_{mA}} + \frac{\partial C_{sA}}{\partial C_{mB}} \left(\frac{dC_{mB}}{dC_{mA}}\right)_1\right)\right] \quad (3)$$

$$t_{Récacl} = t_0 \left[1 + \phi \left(\frac{\partial C_{sA}}{\partial C_{mA}} + \frac{\partial C_{sA}}{\partial C_{mB}} \left(\frac{dC_{mB}}{dC_{mA}}\right)_2\right)\right] \quad (4)$$

where t_0 is the column hold-up time, and f the column phase ratio (volume of the stationary phase divided by the volume of the mobile phase). Analogous equations are obtained with respect to the concentrations of the hormone. For the two-component competitive Langmuir isotherms, the following equation are obtained [18–19]:

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

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

Analogous equations was obtained for $\delta C_{sB}/\delta C_{mB}$ and $\delta C_{sB}/\delta C_{mA}$. 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 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_{R1cal} and t_{R2calc} can be set equal to the single-component Langmuir or linear isotherm coefficient [18].

Moreover, the adsorption constants of the compounds A and B with the HSA (K_A and K_B) can be visualised by the following equilibria:

$$A + HSA \leftrightarrow A\text{-}HSA \quad (8)$$

$$B + HSA \leftrightarrow B\text{-}HSA \quad (9)$$

where A-HSA and B-HSA were respectively the A and B hormones bound to HSA.

For the A displacement from its HSA binding site by B, the displacement equilibrium can be written as:

$$B + A\text{-}HSA \leftrightarrow A + B\text{-}HSA \quad (10)$$

with an equilibrium constant given by the following equation:

$$\tilde{K} = \frac{K_B}{K_A} \quad (11)$$

3. Experimental

3.1. Apparatus

The high performance liquid chromatography (HPLC) 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 injection, and a Merck L4500 diode array detector. 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).

3.2. Reagents and operating conditions

DHEA and testosterone were obtained from Sigma–Aldrich (Saint-Quentin, France). The chemical structure of these two steroid hormones was given in Fig. 1. Methanol HPLC grade, sodium hydrogenphosphate and sodium dihydrogenphosphate were supplied by Prolabo (Paris, France). $MgCl_2$ was obtained from Sigma–Aldrich (Saint-Quentin, France). Water was obtained from an Elgastat option water purification system (Odils, Talant, France) fitted with a reverse osmotic cartridge. The mobile phase consisted of a 70/30 (v/v) methanol–sodium phosphate buffer (7×10^{-4} M) at pH = 7.00 with different $MgCl_2$ concentrations, x, varying from 0 to 1.7 mM. Its flow-rate was maintained constant and equal to 0.6 mL min^{-1}. Nine x values were included in this range, i.e., 0, 0.6, 0.7, 0.9, 1.0, 1.1, 1.3, 1.5, 1.7 mM. UV detection was used at a wavelength of 256 nm. The equilibration of the column was carried out with 20 different concentrations of DHEA and testosterone (0–4 mM) in the mobile phase used to obtain a stable detection.

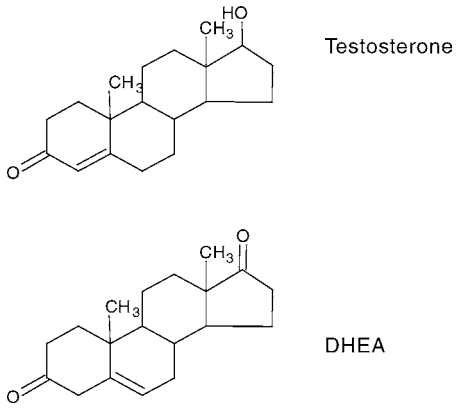

Fig. 1. Chemical structures of DHEA and testosterone.

4. Result and discussion

4.1. Hormone–HSA binding and testosterone displacement equilibrium to its HSA binding site by DHEA processes when the Mg^{2+} concentration (x) in the mobile phase was nil

In order to verify that the column has the same saturation capacities for all sample compounds, the single-component (i.e. DHEA or testosterone) isotherm parameters was determined using the well known equation, Langmuir equation [18,19]

$$k = \frac{\phi \alpha K}{(1 + KC_m)^2} \quad (12)$$

where the α is the column saturation factor of the solute (DHEA or testosterone) and ϕ the column phase ratio (volume of the stationary phase divided by the volume of the mobile phase). C_m is the steroid hormone concentration in the mobile phase and K the solute–HSA adsorption constant. The difference of the column saturation factor for the two compounds (DHEA and testosterone) was always lower than 0.04%, justifying the use of the competitive Langmuir isotherm equation for this study.

A first series of experiments was carried out without Mg^{2+} and with various DHEA and testosterone concentrations in the bulk solvent at T = 37 °C. For each pair of DHEA and testosterone concentrations in the bulk solvent, the most concentrated DHEA and testosterone samples were injected and their retention factors were determined. 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 isotherm, the iterative Marquadt approach was used to fit the best isotherm coefficients' values as shown in the Section 2. Theoretical represented surface of the variation of the K_{DHEA} values versus DHEA and testosterone concentrations in the bulk solvent was given in Fig. 2. There is a good agreement between theoretical and experimental data also confirmed by the low standard de-

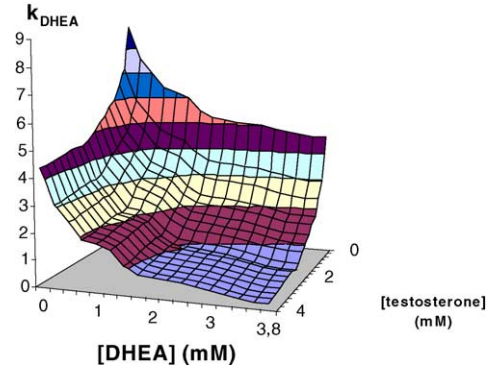

Fig. 2. Theoretical representative of the variation of the surface of the K_{DHEA} vs. DHEA and testosterone concentration; mobile phase, x = 0 mM; phosphate buffer, pH = 7.0; HSA stationary phase; column temperature 37 °C.

viation for all total isotherm derivative (ε, Table 1). These results indicated the importance of the competitive effect between testosterone and DHEA to bound on the same HSA site. The existence of an identical binding site on HSA for DHEA and testosterone was previously observed by Guillaume's group by an enthalpy–entropy compensation investigation [2]. $K_{testosterone}$ value ($=20.31 \times 10^3$) was lower than the one of K_{DHEA} ($=32.09 \times 10^3$) confirming that DHEA can well displace testosterone from its HSA binding site [2]. The $\tilde{K}$ values were calculated from Eq. (11) (Table 1). At 37 °C when the magnesium concentration in the bulk solvent was nil, the $\tilde{K}$ value was equal to 1.58. This value was similar to the one obtained with the thermodynamic approach [2]. The fact that the $\tilde{K}$ value was >1 confirmed our previous results and Morley's hypothesis, i.e. a DHEA supplementation can increase the bio-available testosterone (free testosterone, not bound to HSA) [2,3].

4.2. Role of the Mg^{2+} on both the HSA–hormone binding and the displacement equilibrium

A second series of experiments were carried out with both various DHEA, testosterone, and Mg^{2+} concentrations (x) in the bulk solvent. For each pair of testosterone and DHEA concentrations in the mobile phase, the most concentrated testosterone and DHEA samples were injected and their retention factors were determined at each magnesium concentration in the mobile phase. There is a good agreement between theoretical and experimental data also confirmed by the low standard deviation for all total isotherm derivatives (ε, Table 1). For each x values, the corresponding K values of DHEA and testosterone were determined at human temperature (Table 1). The K values of each steroid hormone were plotted against the magnesium chloride concentration x in the mobile phase. For example, Fig. 3 reports the curve K_{DHEA} versus x. Similar variation was observed for testosterone compound. Under a critical x_c value around 1 mM (region I), the K values enhanced with x whereas above x_c (region II) the K values decreased significantly with salt concentration increasing (Fig. 3). This break on the curve confirmed our previous results, i.e. a change in the DHEA (or testosterone)-HSA

Table 1
K_{DHEA}, $K_{testosterone}$, α, ε (standard deviation between the expected and the experimental values), $\tilde{K}$ values; mobile phase; phosphate buffer, pH = 7.0; HSA stationary phase; column temperature 37 °C

x (mM)	K_{DHEA} (10^3)	$K_{testosterone}$ (10^3)	α	ε	$\tilde{K}$
0	32.09	20.31	110	1.35	1.58
0.6	37.79	23.33	122	1.42	1.62
0.7	47.11	28.21	112	1.81	1.67
0.9	59.17	33.43	124	1.99	1.77
1	69.16	38.21	110	2.12	1.81
1.1	53.81	30.11	112	1.95	1.79
1.3	45.91	26.23	111	1.82	1.75
1.5	30.17	19.22	116	1.78	1.57
1.7	22.49	15.10	119	1.71	1.49

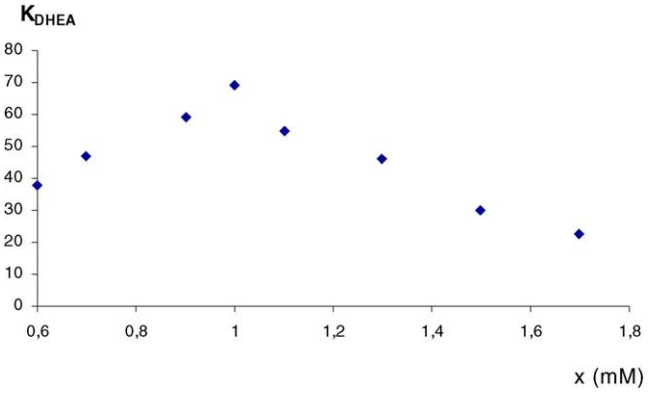

Fig. 3. K_{DHEA} vs. the magnesium concentration (x); mobile phase; phosphate buffer, pH = 7.0; HSA stationary phase; column temperature 37 °C.

binding processes around x_c [2]. As previously explained, whereas the $K_{DHEA\ or\ testosterone}$ value enhancement can be classically attributed to the Mg^{2+} chaotropic character, the decrease of the HSA–hormone (DHEA or testosterone) binding constant was due to the ability of HSA to bind divalent inorganic cation such as Mg^{2+} [20–22]. The equilibrium constant of the testosterone displacement from its HSA binding site by DHEA, $\tilde{K}$ was also determinated for all the x values (Table 1) and the plot $\tilde{K}$ values versus $MgCl_2$ concentrations was drawn at $T = 37$ °C (Fig. 4). Fig. 4 shows that there was a change in the testosterone displacement from its HSA binding site by DHEA mechanism with x. As explained in a previous paper [2], this change was due to a competition effect between hormone and Mg^{2+} cation to bind to HSA in region II ($x > x_c$) which implied an unfavourable contribution of the testosterone displacement from its HSA binding site by DHEA (i.e. the free testosterone concentration decreased). In the biological concentration range (0.75–1 mM), the $\tilde{K}$ values increased with x and consequently the bio-available testosterone rate increased. This last results confirmed that a magnesium supplementation during DHEA treatment for old men who suffer from andropause (i.e. partial testosterone deficient) can be useful [2].

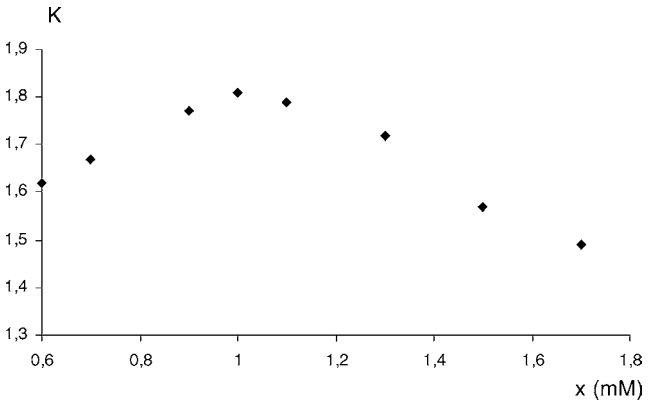

Fig. 4. $\tilde{K}$ vs. the magnesium concentration (x); mobile phase; phosphate buffer, pH = 7.0; HSA stationary phase; column temperature 37 °C.

5. Conclusion

In this paper, the concept of Langmuir isotherms was applied to verify our previous conclusion on the Mg^{2+} effect on the testosterone displacement from its HSA binding site by DHEA. Using an extended Langmuir equation, both the HSA–hormone (i.e. DHEA and testosterone) binding and the testosterone displacement equilibrium from its HSA binding site processes were reanalysed. Thus, the constant of the HSA–hormone association was determined. Results confirmed that DHEA and testosterone bound on the same HSA site. Moreover, 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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