Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 175–180

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Magnesium effect on testosterone–SHBG association studied by a novel molecular chromatography approach

L. Excoffon, Y.C. Guillaume*, M.C. Woronoff-Lemsi, C. André

Equipe des Sciences Séparatives Biologiques et Pharmaceutiques (2SBP/EA-4267), Laboratoire de Chimie Analytique, CHU-Jean Minjoz, Faculté de Médecine Pharmacie, Université de Franche-Comté, Place Saint Jacques, 25030 Besançon Cedex, France

ARTICLE INFO

Article history:
Received 27 June 2008
Received in revised form 20 October 2008
Accepted 21 October 2008
Available online 5 November 2008

Keywords:
SHBG
Testosterone
Magnesium
Binding

ABSTRACT

A biochromatographic approach is developed to measure for the first time thermodynamic data and magnesium (Mg^{2+}) effect for the binding of testosterone (TT) to sex hormone-binding globulin (SHBG) in a wide temperature range. For this, the SHBG was immobilized on a chromatographic support. It was established that this novel SHBG column was stable during an extended period of time. The affinity of TT to SHBG is high and changes slightly with the Mg^{2+} concentration because the number of Mg^{2+} linked to binding is low. The determination of the testosterone retention with the steroid hormone at different Mg^{2+} concentrations and temperatures demonstrated that the Mg^{2+} binding heat effect associated with this Mg^{2+} release or uptake during this binding was in magnitude around 17 kJ/mol corresponding to the model describing the electrostatic attraction that occurs between the negatively charged non specific areas of SHBG and the positively charged of magnesium. At all the magnesium concentrations studied, the ΔH values were negative due to van der Waals interactions and hydrogen bonding which are engaged at the complex interface confirming strong TT–SHBG hydrogen bond networks. As well, the ΔS values were all positive due to hydrophobic forces in the testosterone–SHBG complex formation. In addition our results suggest that adaptive conformational transitions contribute to the specific testosterone–SHBG complex formation. As well, in the biological Mg^{2+} concentration domain, it was clearly demonstrated that there was an uncompetitive inhibition of Mg^{2+} on TT–SHBG binding which led an enhancement of bioavailable TT. Our work indicated that our biochromatographic approach could soon become very attractive for study other SHBG–steroid (or phytoestrogen) binding.

1. Introduction

In men, ageing is associated with a progressive decrease in testosterone (TT) levels [1]. This hormonal change has been related to a complex clinical syndrome [2] and [3], which includes physical and psychological symptoms, and has received several names: andropause, androgen deficiency of the aging male (ADAM), partial androgen deficiency of the aging male (PADAM), late onset hypogonadism (LOH) [4] or, more recently "testosterone deficiency syndrome" [5].

The potential etiologies responsible for the decrease in serum TT include a decrease in testicular function with age (primary testicular failure), a decrease in hypothalamic-pituitary axis function with a corresponding decrease in circulating gonadotropins (secondary testicular failure), and an increase in the production of sex hormone-binding globulin (SHBG) with subsequent increased binding of TT. A mixed etiology has also been described [6]. The signs and symptoms of low TT in adult men include diminished libido, erectile dysfunction, decreased muscle mass and muscle strength, and decreased bone mass. Other symptoms may include decreased cognitive function and memory, depression, irritability, sleep disturbance, fatigue and hot flashes. The presence of both a compatible medical profile and a biochemical failure (defined as a serum androgen deficiency) is necessary to diagnose and treat this syndrome [4].

Most of the circulating plasma TT is protein bound with approximately 2–3% available as a free form. In men, circulating TT is 44–65% bound to SHBG and 33–54% is bound to albumin. Albumin has a high capacity for binding to sex steroids but binds to TT with low affinity, and as a result, the TT is loosely bound and physiologically active [7]. Bioavailable testosterone (BTT) is the bioactive fraction of circulating TT that is not tightly bound to SHBG and is thought to more accurately reflect the clinical androgen state of the patient (versus serum TT levels)[7]. Recently, it was shown that BTT levels would be significantly lower among middle-aged (age 40–65 years), untreated depressed men when compared to a matched non-depressed control sample [8]. The activity of TT depends on its binding with SHBG. Human SHBG transports sex steroids in the

* Corresponding author. Tel.: +33 3 81 66 55 44; fax: +33 3 81 66 56 55.
E-mail address: yves.guillaume@univ-fcomte.fr (Y.C. Guillaume).

0731-7085/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2008.10.041

blood and regulates their access to target cells. SHBG is a homodimer, and each monomer comprises 373 amino acid residues [9,10] and consists of a tandem repeat of laminin G-like domains. Only the N-terminal G domain (residues 1–194) in SHBG is needed for steroid-binding and the formation of a homodimer [11]. The steroid ligand 5α-DHT intercalates into the hydrophobic core of the SHBG N-terminal G domain in between two β-sheets. Its oxygen atom at C3 of ring A points into the interior of the protein and is anchored to Ser42, which is strictly conserved among species [9]. Only 2% of the 5α–DHT surface is accessible to solvent in the complex. The partially accessible atoms are C12 and C17 from rings C and D of the steroid, respectively, while rings A and B are completely buried. The steroid-binding pocket is lined by residues contributed from eight β–strands, and is predominantly hydrophobic. The main binding contributions are from Phe67, Met107 and Met139. Two additional hydrophilic contacts (hydrogenal bond) are formed between the hydroxyl group at C17 and the strictly conserved residues Asp65 and Asn82. The conservation of Gly58 also appears to be important because any side chain at this position would generate steric clashes with atoms C6 and C7 of the steroid. The conformation of 5α–DHT does not change when bound to SHBG [12]. Studies of crystal structure of human SHBG, have indicated that each monomer of SHBG contains three metal-binding sites. One calcium-binding site and two zinc-binding sites [13,14]. There are both divalent cations such as Mg^{2+}.

It has been demonstrated that, the serum levels of Mg^{2+} in young men directly and significantly related to serum levels of progesterone. The data are compatible with the possibility that serum Mg^{2+} contributes to the regulation of circulating progesterone in humans [15]. Moreover Mg^{2+} has shown efficacy in treating depression and some related mental disorders [16], and depression is a symptom of low TT in adult men.

It is important to investigate the mechanism of TT binding to SHBG and the Mg^{2+} effect on this binding. The main experimental approach employs fluorescence spectroscopy, a simple and non-invasive method for studying TT binding to SHBG [17,18]. However, ambiguities exist in resolving the dominant mode of quenching and in quantifying adsorption of incident radiation by SHBG (i.e., the inner-filter effect) [19]. As well, equilibrium dialysis is very often difficult to apply because of the technical difficulties such as undesirable drug adsorption into the membrane and linkage of bound drug through the membrane due to a high affinity of hydrophobic contaminant for SHBG. In recent years, high performance liquid chromatography (HPLC) appeared to be powerful tool for study of bio-molecular interactions. The association constants of many ligands have been determined by zonal elution [20] or frontal analysis [21]. The thermodynamic process involved in the binding have been already studied [22,23]. Moreover Guillaume's group focused on the TT binding to human serum albumin (HSA) using HPLC. With this method, they investigated the mechanism of TT binding to HSA and demonstrated the role of Mg^{2+} cation on the TT–HSA binding process [22,23]. A novel high-performance liquid chromatography column containing SHBG immobilized on silica was thus developed by our group. In addition, this technique consumes very small sample volume, does not suffer from drug adsorption onto the membrane, and allows one to obtain more information such as the thermodynamic data on the binding process.

2. Experimental

2.1. Reagents

Water was obtained from an Elgastat option water purification system (Odil, Talant, France) fitted with a reverse osmosis cartridge. SHBG and TT were obtained from Sigma Aldrich (Saint Quentin, France). Acetonitrile was analytical grade and supplied by Carlo Erba (Val de Reuil, France). Magnesium chloride, potassium dihydrogen phosphate and dipotassium hydrogen phosphate used for the preparation of the mobile phases were of analytical grade and purchased from Merck (Darmstadt, Germany).

2.2. Apparatus

The HPLC system for these measurements consisted of a Merck Hitachi Pump L-7100 (Nogent sur Marne, France), a Rheodyne injection valve with a 20 μL sample loop (Montluçon, France) and a Merck L-4500 diode array detector (Nogent sur Marne, France). The C1 reversed phase support (50 mm × 4.6 mm column size) was purchased from Interchim (Montluçon, France). The SHBG column prepared via the in situ technique was given below. This SHBG column temperature was controlled with a cryoimmerser (Elancourt, France) for low temperature and an Interchim oven, TM no. 701 for high temperature.

2.3. SHBG immobilization process

The SHBG solution was prepared by adding 1 μmol of SHBG into 30 mL of phosphate buffer 1 mM, at pH 5.30 with NaCl (0.5 M). The C1 reversed-phase support in the column was first equilibrated with a 0.20/0.80 (v/v) acetonitrile/sodium phosphate buffer (1 mM) mixture at pH 5.35 (isoelectric point of SHBG) at a flow rate of 0.6 mL/min. Then, the SHBG solution was pumped through the column at a flow rate of 0.3 mL/min until SHBG breakthrough was detected. From this point, the amount of SHBG coupled to the chromatographic support was calculated. Therefore the column was washed with the sodium phosphate buffer at pH 5.3. When not used, the column was stored at 4 °C in this buffer.

2.4. Chromatographic operating conditions and column stability

The mobile phase consisted of a 0.20/0.80 (v/v) acetonitrile/phosphate buffer (1 mM, pH 6.0) with different concentrations of magnesium x from 0.5 to 2 mmol L^{-1}. The experiments were carried out over the temperature range 15–40 °C and at a 215 nm detection wavelength. Throughout the study the flow rate was maintained constant and equal to 0.6 mL/min. In order to confirm the binding properties of the immobilized SHBG on the chromatographic support, the study of the estradiol (an other well known steroid which is known to bind on the same active site than testosterone) displacement of its SHBG binding site by testosterone was investigated using the Langmuir approach [24–28]. For this, single and multi-component isotherms were determined using the perturbation technique [24]. This method was described previously for the analysis of the progesterone displacement of its human binding site by β-estradiol [29]. Briefly, single component isotherms of testosterone and estradiol (each in the concentration range 0.01–0.1. mol L^{-1}) and two-component isotherms of a mixture of testosterone and estradiol (at a constant concentration ratio 0.01:0.01 to 0.1:0.1 mol L^{-1}) were measured in the phosphate buffer (1 mM; pH 7.4) at 20 °C. Each isotherm data point was measured in 11 subsequent steps after equilibration of the SHBG column with a solution containing a single compound (testosterone or 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})) or a mixture of testosterone and estradiol ($C_{testosterone} + C_{estradiol}$ = 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 times were measured.

3. Results and discussion

3.1. Column binding properties and stability

The Langmuir approach was found to describe adequately the experimental data (non-linear coefficients of the models were always higher than 0.997). It was found that the column saturation factor for the two compounds was identical ($\alpha = 97.20$) (the difference for the two steroid hormones was always lower than 0.01%) justifying the use of the competitive Langmuir isotherm equation for this study [29]. For the evaluation of the coefficients of the two-components competitive bi-Langmuir isotherms, the iterative Marquadt approach was used to fit the best isotherm coefficients values as shown previously [25,26,29]. There is a good agreement between the theoretical and experimental data also confirmed by the low standard deviation ($\varepsilon = 1.11$) for all total isotherm derivatives. These results confirmed the importance of the competitive effect between testosterone and estradiol to bind on the same active binding site. For example, the corresponding equilibrium affinity constant K calculated for testosterone was $1.10^9 \pm 3.10^8$ M^{-1} in the same order magnitude as the one obtained by previous authors [30,31]. This confirmed that bonded SHBG on silicea do not modify the structure of the active site and the binding properties. In order to further investigate the SHBG column binding capacity for testosterone, the concentration dependencies of the testosterone retention factor were measured at 25 °C. The retention factor varied linearly ($r^2 \geq 0.98$) from 20.35 at 10 mM of injected solute solution to 17.45 at 20 mM of injected solute solution. This variation confirms that testosterone retention is governed mainly by interactions with specific sites [32] which are characterized by a high interaction energy and a low saturation capacity. A high binding affinity of dihydrotestosterone derivatives to homodimeric SHBG was also clearly demonstrated using a surface plasma resonance biosensor [33]. As well, the number of testosterone binding active sites in the column was evaluated. Under moderate non-linear conditions, Snyder and co-workers [34,35] have established that the solute retention factor k' can be described by the following equation

$$k'/k'_{low} = f\left\{\left[\frac{k'_{low}}{(k'_{low}+1)}\right]\frac{N_{low}^{1/2}Q_S}{m_L}\right\} = f(\omega_k) \quad (1)$$

where k'_{low} and N_{low} are respectively the retention factor and the number of theoretical plate (at the lowest solute concentration used), m_L the number of active sites in the column, Q_S the amount of testosterone injected and $\omega_{k'}$ is the loading function. From the overloading experiments, the m_L can be approached using the empirical dependence k'/k'_{low} versus the loading function $\omega_{k'}$ (table IV of [35]). Using a polynomial fitting function as described [34], the best fit testosterone m_L value obtained for the data of the concentration dependencies of the testosterone retention was found to be around 0.97 μmol. As shown by Eble et al. [35], the binding capacity estimated by this approach reflects the number of high-energy/low-capacity sites in the column for a heterogeneous surface. Thus in our chromatographic system such value represents roughly the number of specific sites for testosterone.

To evaluate the column to column reproducibility, three SHBG columns were prepared under identical conditions. The results showed that the technique was reliable and reproducible. As well typical reproducibility of these columns in retention time measured as relative standard deviation was <0.4%. After half a year and more than 60 times injections, the decrease for the values of the retention factor on the three columns was <1%.

3.2. Testosterone–SHBG binding mechanism and magnesium effect

To study the mechanism into the TT–SHBG association, the experiments were carried out at five temperatures (i.e., 15, 20, 25, 30, 35 °C). The testosterone solutions were prepared in the bulk solvent at a concentration of 7 and 20 μM was injected at least three times. If ΔG°, ΔH°, and ΔS° are the Gibbs free energy, enthalpy, and entropy, respectively, for the TT–SHBG association, the van't Hoff plot equations are as follows.

$$\ln K = \frac{-\Delta G^\circ}{RT} \quad (2)$$

with

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (3)$$

where K is the association constant between testosterone and SHBG, T is the temperature and R the gas constant. As K can be linked with the testosterone retention factor (k') by $K = k'/\Phi$, where Φ is equal to the ratio of the active binding site number in the column (m_L) over the void volume of the chromatographic column (V_M), k' represents the TT/SHBG binding intensity. Then combining these the above equations gives:

$$\ln k' = \frac{-\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln \Phi \quad (4)$$

As well, the testosterone retention on the SHBG stationary phase can be evaluated using the well known equation:

$$k' = \frac{t - t_o}{t_o} \quad (5)$$

where t is the retention time of testosterone and t_o is the column void time. To obtain the thermodynamic retention time, i.e., the accurate measure of testosterone retention, t was determined by calculating the first moment of the peak as previously described [36]. The void time was determined using the mobile phase peak. The retentions time and column void time were corrected for the extracolumn void time. It was assessed by injections of solute onto the chromatographic system when no column was present.

The plots $\ln k'$ versus $1/T$ (van't Hoff plots) were determined for the different concentrations of magnesium in the bulk solvent. Linear fits were obtained at all the Mg^{2+} concentrations with correlation coefficients $r > 0.98$. This linear behaviour demonstrated that, in the studied temperature range, there was no change in the interaction mechanism in relation to temperature [37]. In order to evaluate a possible change in the testosterone binding capacity with increasing temperature, the concentration dependencies of the testosterone retention factor k' were measured for all the column temperatures. In order to compare the retention data, the normalized parameter $100(k'/k'_{low})$ was used. For the column temperature range 15–35 °C the normalized parameter value was constant for testosterone and around 99.7. This behaviour is in accordance with no change in the number of binding sites when the temperature varied [35]. According to Eq. (4), these linear van't Hoff plots provided a conventional way of calculating the thermodynamic parameters ΔH° and ΔS°. For the determination of ΔS°, the number of mole of immobilized SHBG (m_L) was used, assuming that the SHBG immobilized on the column was available for an interaction with testosterone. However, it is not always verified, and the number of active sites in an affinity protein based column can be lower than the number of moles of ligand effectively immobilized. This is due to various factors such as steric hindrance, denaturation, or inefficient orientation [38–40]. For example, it has been shown that from around 10% to around 80% of the β blocker sites [38], benzoin sites

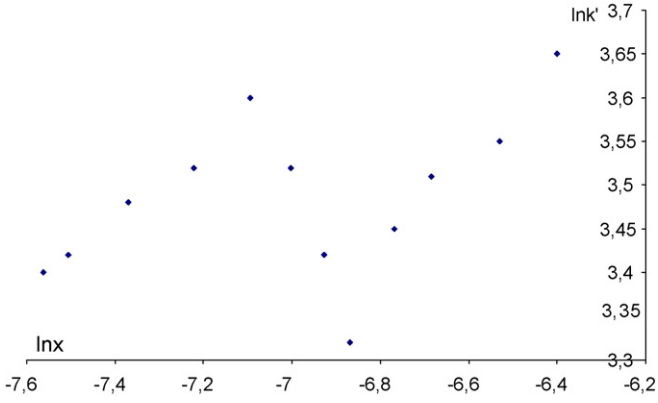

Fig. 1. $\ln k'$ versus $\ln x$ at 20 °C.

[39] and warfarin sites [40] are active in various protein-based column. Therefore, ΔS values were also determined using an m_L value arbitrarily fixed to a value representing around 50% of the number of moles of testosterone effectively immobilized. Very low changes in ΔS values are observed. The entropy change was only 0.6% higher than the ΔS value obtained using the number of moles of testosterone effectively immobilized. The maximum relative difference observed of the ΔS values between these different measurements was always 0.7%. Therefore, neglecting these effects has no serious consequences on the interpretation of the thermodynamics. The enthalpic values obtained were negative (i.e., exothermic) at all the magnesium concentrations studied and consequently favourable for the TT–SHBG association. For example, for a magnesium concentration equal to 0.8 mM, $\Delta H = -7.90$ kJ/mol. The transfer of the testosterone molecule from the bulk solvent to the testosterone binding cavity was enthalpically driven and can be described as the replacement of weak TT–bulk solvent interactions by strong TT–SHBG surface interaction. This indicates that enthalpic factors associated with hydrogen bonding and van der Waals interactions (characterized by negative enthalpy values at these temperatures) [41] of the TT with SHBG dominate the binding. As well, the positive entropy values (for example, for a magnesium concentration equal to 0.8 mM, $\Delta S = +3.39$ J/mol/K) obtained at all the magnesium concentrations can be justified by the hydrophobic effect [42], which is defined as the tendency of hydrophobic surfaces "sticking together" in water under the exclusion of solvent molecules [43,44]. The testosterone/SHBG binding is thus also entropically driven, indicating a contribution from hydrophobic effect due to the release of water molecules when TT and SHBG associated. These results confirmed by Grishkovkaya et al. study which demonstrated that the steroid ligand intercalates into the hydrophobic core of the SHBG (entropically driven) and hydrogen bonds are formed between TT and SHBG (enthalpically driven) [12]. The logarithm of retention factor $\ln k'$ of the testosterone molecule with SHBG was also plotted against the logarithm of the magnesium concentration x in the bulk solvent and the same profile plot was observed at all the studied temperatures. An example of plot at 20 °C is presented in Fig. 1. To assess if this retention factor change with increasing x was due to a variation in the binding capacity of the column, the concentration dependencies of the testosterone retention were measured at different x values [35]. As reported above for the temperature experiments, the normalized parameter value was constant around 99.6. Then the binding capacity of the SHBG column was invariant when x changed and thus the magnesium concentration did not alter the number of binding sites of the immobilized SHBG [35]. For all temperatures, the plot showed a favourable TT association affinity for SHBG (domain 1, $x < x_{c1} = 0.8$ mM) followed by a decrease (domain 2, $x_{c1} < x < x_{c2} = 1.04$ mM) and an increase (domain 3, $x > x_{c2}$) of the TT–SHBG binding. When the Mg^{2+} concentration in the bulk solvent changed, a fuller description is essential, which explicitly maintains conservation of mass of each species and takes into account binding of Mg^{2+} to TT, SHBG, and the complex TT–SHBG:

$$TT(Mg^{2+})_A + SHBG(Mg^{2+})_B + nMg^{2+} \rightleftarrows TT\text{–}SHBG(Mg^{2+})_C$$

where $n = C - (A + B)$ is the number of Mg^{2+} linked to this SHBG binding reaction of TT. The association constant of this equilibrium was given by:

$$K = [TT\text{–}SHBG]/([TT][SHBG][Mg^{2+}]^n) \quad (6)$$

Eq. (6) can be rewritten as:

$$K = K_0/[Mg^{2+}]^n \quad (7)$$

where K_0 is the K values for $x = 1$ M. Taking the logarithm of Eq. (7) gives:

$$\ln K = \ln K_0 - n\ln[Mg^{2+}] \quad (8)$$

after derivation of Eq. (8), the following equation was obtained [41]:

$$\partial \ln K/\partial \ln[Mg^{2+}] = -n \quad (9)$$

as k' was proportionnal to K ($K = k'/\Phi$), Eq. (9) was rewritten as:

$$\frac{\partial \ln k'}{\partial \ln[Mg^{2+}]} = -n \quad (10)$$

where n is linked to the Mg^{2+} molecule release or uptake at the TT–SHBG interface.

The Mg^{2+} binding heat effect associated with this Mg^{2+} release or uptake can be described by the following single function [42,43]:

$$\Delta H^*_{(Mg^{2+})} = -2.3\,RT^2\left(\frac{\partial N}{\partial T}\right)_{[Mg^{2+}]} \quad (11)$$

The n values were determined from the slope of the plot $\ln k'$ versus $\ln x$ and at 25 °C were, respectively equal to −0.40; +1.20 and −0.50 in domain 1, 2 and 3, respectively. In the three domains, the plots n versus T were determined. Linear fits were obtained with correlation coefficients r higher than 0.97. Using Eq. (11), $\Delta H^*_{(Mg^{2+})}$ values were calculated and a value in magnitude around 17.0 (0.9) kJ/mol was obtained in the three domains at 25 °C. This value was in the same order as the one obtained for HSA–Mg^{2+} binding [45]. Solubility modifiers such as magnesium chloride can affect (i) the energy required to produce a solvent cavity (cavitation process) into which the testosterone can go and (ii) the energy of solute medium solvation interaction [46]. Thus in domain 1 and 3, the magnesium cation increased the TT–SHBG association (Fig. 1) by electrostriction that squeezed out free space, made cavity creation harder and increased surface tension [46]. In these two domains, contrary to the classical results (i.e., the salting-out addition led to an increase of the thermodynamic data) [47], the enthalpic and entropic values of this association mechanism decreased with the Mg^{2+} concentration in the bulk solvent (Figs. 2 and 3). This thermodynamic trend can be explained by the fact that different interactions (van der Waals interactions and hydrogen bounds between the TT and the SHBG molecule) were implied in the TT association on the SHBG molecule. As well, ion-induced effects on surface tension, in addition to affecting hydrogen bonding [48,49], can also affect hydrophobic interactions within the SHBG protein [50], resulting in the burial of certain amino acid residues into the protein hydrophobic core and slowing down hydrogen exchange rates. Such conformational changes would result in a more compact and less flexible structure [51–54]. Therefore, the change of the net number of ions released or bound upon TT–SHBG complex formation when the salt concentration increased demonstrated

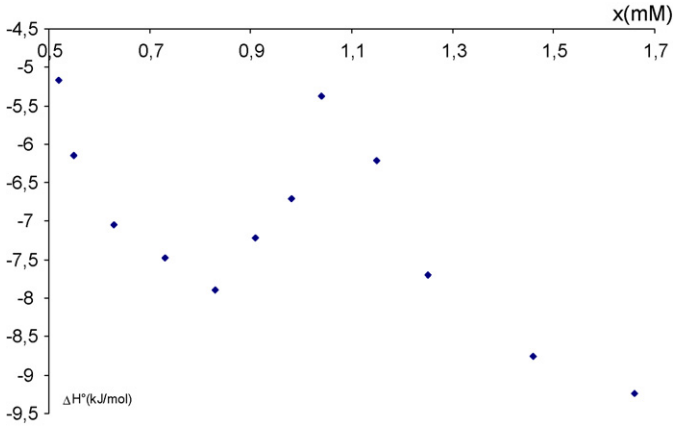

Fig. 2. ΔH° (kJ/mol) values versus x (mM).

changes in the SHBG conformation. For $x_{c1} < x < x_{c2}$ the SHBG protein adopted a less flexible structure, its binding site was thus less accessible to testosterone and the TT–SHBG association decreased slightly (Fig. 1). This confirmed the importance of the SHBG protein conformation for the interaction between SHBG and testosterone and the conformation change on SHBG immobilized on silica. As well, in order to confirm this change, the enthalpy entropy compensation (EEC) phenomenon was used. The enthalpy entropy compensation temperature is a useful thermodynamic approach to the analysis of physicochemical data [55–57]. Mathematically, the enthalpy entropy compensation can be described by the following equation [55,56]:

$$\ln k'_{\mathrm{T}} = -\frac{\Delta H^{\circ}}{R}\left(\frac{1}{T} - \frac{1}{\beta}\right) - \frac{\Delta G^{\circ}_{\beta}}{R\beta} + \ln \Phi \qquad (12)$$

where ΔG°_{β} is the corresponding Gibbs free energy variation at the compensation temperature β. According to this last equation, when enthalpy entropy compensation is observed; when the magnesium concentration x varied in the bulk solvent, testosterone molecule has the same free energy ΔG°_{β} at the temperature β for all the x values. The plots $\ln k'$ versus ΔH° (kJ/mol) (Eq. (12)) determined in domain 1 ($x < x_{c1} = 0.8$ mM), domain 2 ($x_{c1} < x < x_{c2} = 1.04$ mM) and domain 3 ($x > x_{c2}$) were drawn. The regression lines for the three domains were:

$$x < x_{c1} \quad \ln k' = -0.062\ \Delta H^{\circ} + 3.061 \quad r^2 = 0.96 \qquad (13)$$

$$x_{c1} < x < x_{c2} \quad \ln k' = -0.120\ \Delta H^{\circ} + 2.762 \quad r^2 = 0.97 \qquad (14)$$

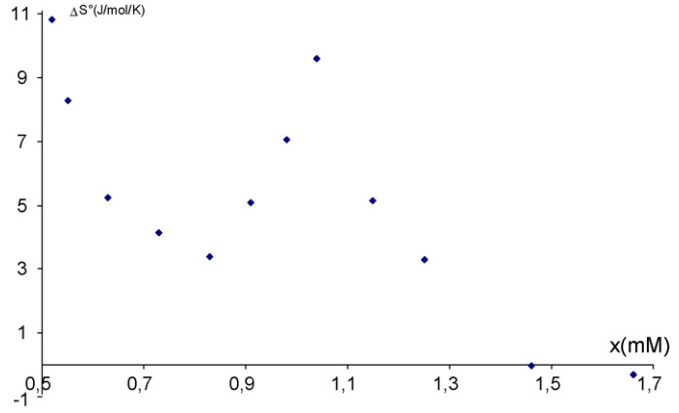

Fig. 3. ΔS° (J/mol/K) values versus x (mM).

$$x > x_{c2} \quad \ln k' = -0.071\ \Delta H^{\circ} + 2.920 \quad r^2 = 0.97 \qquad (15)$$

It appeared that the slopes were different confirming well a change in the TT–SHBG binding mechanism between the different x domains and thus a change of the SHBG conformation. The SHBG immobilization on the chromatographic support did not alter its conformation change capacity. As well, our result confirmed that adaptive conformational transitions are associated with the testosterone–SHBG complex formation where both components are able to adjust their recognition surfaces in order to maximize complementarities through tightly packed contacts involving hydrogen bonding [9,12]. As well, in domain 2, the enthalpic values progressively became less negative and were accompanied by a conventional increasing variation of entropic values attributed to a weaker adsorption process of the TT molecule with its binding site (Figs. 2 and 3). A general phenomenon found in early studies on SHBG is its ability to bind divalent inorganic cations (Zn^{2+}, Ca^{2+}) [13,14]. The determination of the Mg^{2+} binding heat effect associated with Mg^{2+} release (or uptake) on the SHBG was in magnitude approximately 17(0.9) kJ/mol and in the same order as the one obtained for HAS–Mg^{2+} binding [45]. This confirmed that in domain 2, the Mg^{2+} bound to SHBG by electrostatic interactions between its positively charge and the oppositively charged surface of SHBG (SHBG at pH 7 was negatively charged) for different 'non specific' areas of SHBG. Then the non-specific binding mode of Mg^{2+} with SHBG led an uncompetitive inhibition between the steroid hormone and this divalent cation to bind to SHBG and consequently a decrease of TT–SHBG affinity, because SHBG adopted a less flexible structure. In this Mg^{2+} concentration domain (domain.2), the decrease of the interactions between the TT and SHBG cavity due to the uncompetitive inhibition additive to the classical salt effect on the surface tension in the bulk solvent (i.e., water activity) led an increase of the thermodynamic data (Figs. 2 and 3). Moreover, the positive values of n obtained in domain 2 (i.e., related to the decrease of the TT–SHBG affinity) demonstrated the inhibition effect between the magnesium cation and the steroid hormone to bind to SHBG. In addition, the results presented here provide evidence for an Mg^{2+}-mediated variation of the testosterone–SHBG association, suggesting that an increase of the Mg^{2+} concentration inside the biological concentration range (0.75–1.0 mM) could lead an enhancement of the bioavailable testosterone.

4. Conclusion

For the first time, the binding TT–SHBG was analyzed in a wide temperature range from 15 to 40 °C using a novel biochromatographic approach developed in our laboratory. This novel SHBG column was stable during a long period of time and allowed us the determination of the thermodynamic data of this association. The affinity of TT to SHBG was high and changed slightly with the Mg^{2+}concentration. The binding is accompanied by a Mg^{2+} release (or uptake) with a corresponding heat effect around in magnitude 17 kJ/mol. For a magnesium concentration in the biological range, the TT–SHBG affinity decreased and could thus lead an enhancement of the bioavailable testosterone. As well, the results obtained in the presence of salt ions could be presented in a coherent plot in which the concentration of salt was expressed by the number of salt ions (n) displaced or bound in forming the TT–SHBG complex. Further experiments are now in progress in our laboratory in order to demonstrate the role of other divalent cation (Zn^{2+}, Ca^{2+}) on testosterone–SHBG binding. As well, the binding of a series of phytoestrogens (genistein, diadzein, genistin and daidzin), estrogen like compounds, with SHBG will be analyzed using this novel SHBG column.

Acknowledgments

This work was supported by grants 2007 from Direction Genérale de la Recherche from Programme National de Biotechnologie, Ministère de la Recherche, France.

References

[1] A. Gray, H.A. Feldman, J.B. McKinlay, C. Longcope, J. Clin. Endocrinol. Metab. 73 (1991) 1016–1025.
[2] A. Morales, J.P. Heaton, C.C. Carson, J. Urol. 163 (2000) 705–712.
[3] J.E. Morley, H.M. Perry, Med. Clin. North Am. 83 (1999) 1279–1289.
[4] A. Morales, B. Lunenfeld, Aging Male 5 (2002) 74–86.
[5] A. Morales, C.C. Schulman, J. Tostain, F.C.W. Wu, Eur. Urol. 50 (2006) 407–409.
[6] M.T. Haren, M.J. Kim, S.H. Tariq, G.A. Wittert, J.E. Morley, Med. Clin. North Am. 90 (2006) 1005–1023.
[7] P. Emadi-Konjin, J. Bain, I.L. Bromberg, Clin. Biochem. 36 (2003) 591–596.
[8] R.S. McIntyre, D. Mancini, B.S. Eisfeld, J.K. Soczynska, L. Grupp, J.Z. Konarski, S.H. Kennedy, Psychoneuroendocrinoly 31 (2006) 1029–1035.
[9] G.L. Hammond, in: M. Parker (Ed.), Extracellular Steroid-Binding Proteins, Steroid Hormone Action, 1993, pp. 1–25.
[10] K.A. Walsh, K. Titani, K. Takio, S. Kumar, R. Hayes, P.H. Petra, Biochemistry 25 (1986) 7584–7590.
[11] C. Hildebrand, W.P. Bocchinfuso, D. Dales, G.L. Hammond, Biochemistry 34 (1995) 3231–3238.
[12] I. Grishkovskaya, G.V. Avvakumov, G. Sklenar, D. Dales, G.L. Hammond, Y.A. Muller, Embo J. 19 (2000) 504–512.
[13] G.V. Avvakumov, I. Grishkovskaya, Y.A. Muller, G.L. Hammond, J. Biol. Chem. 277 (2002) 45219–45225.
[14] G.V. Avvakumov, Y.A. Muller, G.L. Hammond, J. Biol. Chem. 275 (2000) 25920–25925.
[15] O. Muneyyirci-Delate, V.L. Nacharaju, M. Dalloul, B.M. Altura, B.T. Altura, Fertil. Steril. 71 (1999) 869–872.
[16] G.A. Eby, K.L. Eby, Medi. Hypotheses 67 (2006) 362–370.
[17] T.D. Gauthier, E.C. Shane, W.E. Guerin, W.R. Seitz, C.L. Grant, Environ. Sci. Technol. 20 (1986) 1162–1166.
[18] K.G. Karthikeyan, J. Chorover, J. Environ. Sci. Technol. 34 (2000) 2939–2946.
[19] M.J. Morra, M.O. Corapcioglu, R.M.A. von Wandruszka, D.B. Marshall, K. Topper, Soil Sci. Soc. Am. 54 (1990) 1283–1289.
[20] C. Vidal-Madjar, A. Jaulnes, M. Racine, B. Sebille, J. Chromatogr. 458 (1998) 13–18.
[21] N.I. Nakano, Y. Shimamori, S. Yagamuchi, J. Chromatogr. 188 (1980) 347–351.
[22] C. André, A. Berthelot, J.F. Robert, M. Thomassin, Y.C. Guillaume, J. Pharm. Biomed. Anal. 33 (2003) 911–921.
[23] C. Andre, A. Xicluna, J.F. Robert, M. Thomassin, Y.C. Guillaume, Talanta 65 (2005) 814–818.
[24] M.J. De Barro, Sharon. B. McGregor, C.J. Glover, Ind. Eng. Chem. Res. 27 (1988) 1066–1073.
[25] C. Heuer, E. Küsters, T. Plattner, Seidel A. Morgenstern, J. Chromatogr. A 827 (1998) 175–179.
[26] C. Blumel, P. Hugo, Seidel A. Morgenstern, J. Chromatogr. A 865 (1999) 51–71.
[27] P. Jandera, S. Berncekova, K. Mihlbachler, G. Guiochon, V. Backovska, J. Planeta, J. Chromatogr. A 925 (2001) 19–29.
[28] C. André, Y.C. Guillaume, Chromatographia 58 (2003) 193–200.
[29] C. André, Y. Jacquot, T.T. Truong, M. Thomassin, J.F. Robert, Y.C. Guillaume, J. Chromatogr. B 796 (2003) 267–281.
[30] F. Gitton, J. Fiet, J. Guechot, F. Ibrahim, F. Bronsard, D. Chopin, J.P. Raynaud, Clin. Chem. 52 (2006) 474–481.
[31] R. Södergard, T. Bäckstrom, V. Shanbtrag, H. Carstensen, J. Steroïd Biochem. 16 (2006) 801–810.
[32] V. Tittelbach, R.K. Gilpin, Anal. Chem. 67 (1995) 44–49.
[33] J. Metzger, A. Schnitzbauer, M. Meyer, M. Söder, C.Y. Cuilleron, H. Peter, B. Luppa, Biochemistry 42 (2003) 13735–13745.
[34] J.E. Eble, R.L. Grob, P.E. Antle, L.R. Snyder, J. Chromatogr. 384 (1987) 25–31.
[35] J.E. Eble, R.L. Grob, P.E. Antle, L.R. Snyder, J. Chromatogr. 384 (1987) 45–52.
[36] M. Goedert, G. Guiochon, Chromatographia 6 (1973) 39–45.
[37] W. Melander, D.E. Campbell, C. Horvath, J. Chromatogr. 158 (1978) 215–225.
[38] G. Gotmar, N.R. Albareda, T. Fornstedt, Anal. Chem. 74 (2002) 2950–2959.
[39] K.L. LLyod, A. Ahmed, F. Pastore, J. Chromtogr. 18 (1997) 958–964.
[40] B. Loun, D.S. Hage, J. Chromatogr. 579 (1992) 225–235.
[41] P.D. Ross, S. Subramanian, Biochemistry 20 (1981) 3096–3102.
[42] R. Lakshminarayanan, D. Fan, C. Du, J. Moradian-Oldak, Biophys. J. 93 (2007) 3664–3674.
[43] C. Tanford, The Hydrophobic Effect: Formation Of Micelles And Biological Membranes, 2nd ed., John Wiley and Sons, New York, 1980.
[44] J.R. Livingstone, R.S. Spolar, M.T. Record, Biochemistry 30 (1991) 4237–4244.
[45] Y.C. Guillaume, C. Guinchard, A. Berthelot, Talanta 53 (2000) 561–569.
[46] M.R.J. Dack, Chem. Soc. Rev. 4 (1975) 211–229.
[47] C. Tanford, J. Mol. Biol. 39 (1969) 539–544.
[48] T. Arakawa, S.N. Timasheff, Biochemistry 23 (1984) 5912–5916.
[49] E. Goormaghtigh, V. Raussens, J.M. Ruysschaert, Biochem. Biophys. Acta 1422 (1999) 105–109.
[50] N.K.D. Kella, J.E. Kinsella, Int. J. Pept. Protein Res. 32 (1988) 396–400.
[51] C. Nishimura, V.N. Uversky, A.L. Fink, Biochemistry 40 (2001) 2113–2117.
[52] S.E. Harding, Prog. Biophys. Mol. Biol. 68 (1997) 207–211.
[53] D. Lairez, E. Pauthe, J. Pelta, Biophys. J. 84 (2003) 3904–3907.
[54] C. Pace, R. Alston, K. Shaw, Protein Sci. 9 (2000) 1396–1400.
[55] C. André, Y.C. Guillaume, Chromatographia 59 (2004) 429–433.
[56] A. Peter, G. Torok, D.W. Armastrong, G. Toth, D. Tourvé, J. Chromatogr. A 828 (1998) 177–182.
[57] J. Li, P.W. Carr, J. Chromatogr. A 670 (1994) 105–109.