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A biochromatographic framework to evaluate the calcium effect on the antihypertensive molecule-human serum albumin binding

Claire André, Mireille Thomassin, Catherine Guyon, Tong-Thanh Truong, Yves-Claude Guillaume *

Equipe des Sciences Séparatives et Biopharmaceutiques (2SB), Laboratoire de Chimie Analytique, Faculté Médecine Pharmacie, Univ. de Franche-Comte, Place Saint-Jacques, Besançon 25030, Cedex, France

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Abstract

The Ca^{2+} cation effect on the antihypertensive molecule binding on human serum albumin (HSA) was studied by biochromatography. The thermodynamic parameters corresponding to this binding were determined for a wide range of Ca^{2+} concentration (x). For the two antihypertensive molecules under study, their binding to HSA can be divided into two Ca^{2+} cation concentration regions due to a HSA phase transition. This result was confirmed by an enthalpy-entropy investigation. For a low x value (below $x_c = 1.6$ mmol l^{-1}), the HSA cavity was in an ordered solid-like state leading to an increase in the interactions between the antihypertensive drugs and the HSA cavity and consequently, a solute–HSA affinity increase. For x above x_c, the HSA cavity was in a disordered solid-like state, implying a decrease in the antihypertensive drug–HSA binding.

Keywords: Calcium; Human serum albumin; Antihypertensive molecule; Transition; Thermodynamic

1. Introduction

From birth to death, calcium is an essential element for human beings. Besides its presence in bones and teeth, the mineralized tissues that contain 99% of the body's calcium, it is present in ionized form in the blood, extracellular fluids and within the cells of soft tissues, such as muscles. It is necessary for the release of energy in muscular contraction [1], for nerve transmission [2] and the regulation of heart beat [3,4], it must be present for blood to clot and influences the transport function of cell membranes [2]. As the calcium reabsorption

* Corresponding author. Tel.: +33-381-665-544; fax: +33-381-665-655.
E-mail address: yves.guillaume@univ-fcomte.fr (Y.-C. Guillaume).

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is linked to sodium and fluid reabsorption in the proximal renal tubules, calcium trouble can lead repercussions on the heart beat [3,4]. In some hypertensive patients, a calcium deficit must be controlled and may sometimes behave as a cofactor of constitutional or acquired hypertensive factors [3,4]. This seems to be particularly the case with people who suffer from rachitism or hypothyroidism [5]. The calcium channel blockers are one of several classes of medications used in the treatment of hypertension (high blood pressure). The molecules most known in this pharmaceutical drug group are verapamil and diltiazem. The calcium channel blockers work to decrease the blood pressure by interfering with the normal role of calcium in the heart and the blood vessels [6,7]. By blocking calcium's entry into the heart muscle and blood vessel, verapamil and diltiazem cause blood vessels to dilate and contract to slow and thus blood pressure is lower [6,7]. HSA is the most abundant protein in blood and can reversibly bind a large number of pharmacological substances, such as antihypertensive molecules. HSA was the model ligand used in a great number of studies [8]. The main advantage of using HSA is the data are available for its interaction with a wide range of organic and inorganic compounds [9]. Affinity chromatography with HSA immobilized on the support (HSA was immobilized on porous spherical silica particle) is specially suited to the study of drug–protein interactions. The association constants of many ligands have been determined by zonal elution [10] or frontal analysis [11]. The thermodynamic process involved in the binding have been already been studied [12–17]. This paper describes the effect of both the Ca^{2+} concentration (x) (in the plasma biological concentration range) in the bulk solvent (i.e. the mobile phase) and the column temperature T on the binding process of two well-known antihypertensive drugs (i.e. diltiazem and verapamil) with human serum albumin (HSA). The shapes of the van't Hoff plots were used to assess the effects of temperature and Ca^{2+} concentration changes on the antihypertensive drugs–HSA binding process. Enthalpy-entropy compensation was also applied to evaluate this binding mechanism.

2. Experimental

2.1. Apparatus

The HLPC system consisted of Merck Hitachi pump L 7100 (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). A Shandon HSA column (Montluçon) (150 × 4.6 mm) was used with controlled temperature in an Interchim Crocodil oven TM No. 701 (Montluçon). After each utilization, the column was stored at 4 °C until further use in a phosphate buffer (0.05 M) at pH 7.0. The mobile phase rate was kept at 1 ml min^{-1}.

2.2. Solvents and samples

Sodium hydrogenphosphate and sodium dihydrogenphosphate were supplied by Prolabo (Paris, France). $CaCl_2$ 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 osmosis cartridge. Verapamil and diltiazem, the two antihypertensive drugs, were obtained from RBI (Natick, USA) and were made fresh daily at a concentration of 20 mg l^{-1}. The chemical structures are given in Fig. 1. It has been known that monovalent cations, such as sodium or potassium, are unable to bind to HSA [16,17]. Thus, sodium nitrate as a dead time marker (Merck) [18,19] and a 7.10^{-4} M sodium phosphate buffer at pH 7.3 (pH of the human plasma) as mobile phase can be used. The $CaCl_2$ concentration (x) in the mobile phase varied from 0.3 to 3.0 mmol l^{-1}. Fourteen x values were included in this range (i.e. 0.3, 0.4, 0.6, 0.8; 1, 1.2, 1.4, 1.6; 1.8; 2, 2.4, 2.6, 2.8, 3.0 mmol l^{-1}).

2.3. Temperature study

Retention factors of each antihypertensive drugs were determined at five temperatures 25, 30, 35, 40 and 45 °C. The chromatographic system was allowed to equilibrate at each temperature for at least 1 h prior to each experiment. To study this

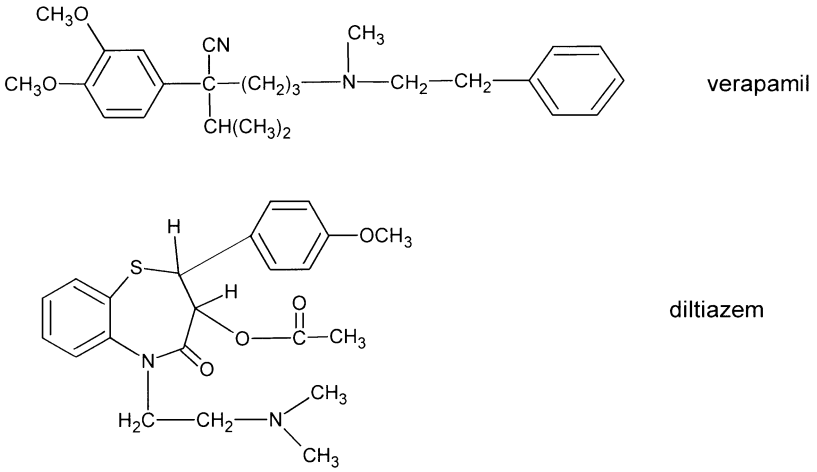

Fig. 1. The two antihypertensive drug structures.

equilibration, the retention time of verapamil was measured every hour for 5 h and again after 23 and 24 h. The maximal 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 antihypertensive molecules were injected three times at each temperature and Ca^{2+} concentration (x). Once the measurements were completed at the maximum temperature, the column was immediately cooled to ambient conditions to minimize the possibility of any denaturation of the immobilized HSA.

To examine the concentration dependency of the solute retention, corresponding to the binding capacity of HSA, retention measurements were related to varying amounts of injected solute. Solute samples were prepared at different concentrations in the mobile phase: 10–50 μg ml^{-1}. Each solute (20 μl) were injected in triplicate and retention times measured. The plots of retention factor exhibited a plateau at sample concentrations < 35 μg ml^{-1} followed by a small decreased at higher solute concentrations. Therefore, each solute was injected at a concentration of 20 μg ml^{-1} when the retention was sample concentration independent, i.e. in linear elution conditions.

3. Results and discussion

Solute retention is usually expressed in terms of the retention factor k' which is proportional to the association constant K between the antihypertensive drugs and HSA and can be written:

$$k' = \phi K \quad (1)$$

where ϕ is the phase ratio (volume of the stationary phase divided by the volume of the mobile phase).

Gibbs free energy ΔG° is related to the equilibrium constant by the equation [20]:

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

where

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

ΔH° (respectively ΔS°) is the enthalpy (respectively entropy) of the transfer of the antihypertensive drug from the mobile phase to the HSA phase (stationary phase), T is the temperature and R, the gas constant. Combining Eqs. (1) and (3), the retention factor can also be expressed by the equation [21]:

$$\ln k' = -(\Delta H^\circ/RT) + (\Delta S^\circ/R) + \ln \phi \quad (4)$$

Eq. (4) is a van't Hoff plot [21]. From the slope

Table 1
ΔH° (kJ mol^{-1}) values for the two antihypertensive molecules at all Ca^{2+} concentrations

$[Ca]^{2+}$ (mmol l^{-1})	$\Delta H^{\circ}_{Diltiazem}$ (kJ mol^{-1})	$\Delta H^{\circ}_{verapamil}$ (kJ mol^{-1})
0.3	−4.9	−6.6
0.4	−5.7	−6.3
0.6	−10.4	−11.6
0.8	−15.3	−17.3
1.0	−18.8	−20.3
1.2	−24.6	−26.7
1.4	−29.8	−31.8
1.6	−40.5	−41.6
1.8	−32.6	−35.4
2.0	−27.9	−29.6
2.2	−23.4	−25.5
2.4	−14.9	−17.7
2.6	−10.5	−12.3
2.8	−6.9	−9.0
3.0	−4.6	−6.3

S.D. <0.1.

and intercept, ΔH° and $(\Delta S^{\circ}/R)+\ln\phi=\Delta S^{\circ}*$ can be, respectively, calculated. Usually, ΔS° is not available because of the ambiguity of the calculation of the phase ratio for commercial columns. Nevertheless, $\Delta S^{\circ}*$ varied identically to ΔS°. The van't Hoff plots both for verapamil and diltiazem were linear. The correlation coefficients r for the fits were at least equal to 0.97. The relative typical S.D. of the slope and the intercept calculated from the three replicates were <0.006 and 0.03, respectively. Fig. 2 shows the van't Hoff plots for verapamil and diltiazem at a Ca^{2+} concentration equal to 1.6 mmol l^{-1}. These linear behaviours were thermodynamically what were

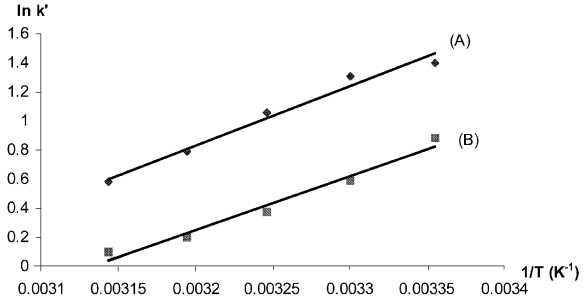

Fig. 2. The van't Hoff plots for verapamil (A) and diltiazem (B) at a Ca^{2+} concentration equal to 1.6 mmol l^{-1}.

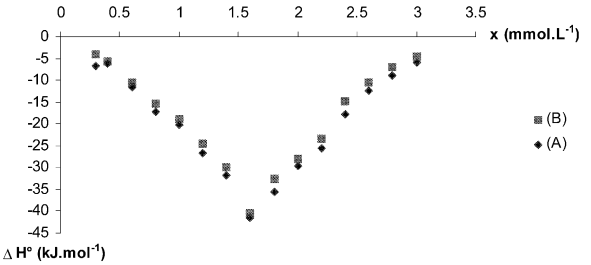

Fig. 3. Plot of ΔH° (kJ mol^{-1}) against calcium concentration (x) for verapamil (A) and diltiazem (B).

expected when there was no change in the retention mechanism in relation to temperature [22]. This was also consistent with a solute binding, which occurs at a fixed number of specific sites with a constant negative enthalpy of association [8]. Table 1 contains a complete list of ΔH° values for the two antihypertensive molecules at all Ca^{2+} concentrations. For both verapamil and diltiazem, the negative ΔH° values (Table 1, Fig. 3) indicate that it is energetically favourable for the antihypertensive molecules to bind to HSA. The entropy values ($\Delta S^{\circ}*$) were also negatives (Fig. 4) for the two antihypertensive drugs indicating the lower dof of solutes bound with HSA. Moreover, as previously reported [8], verapamil was more retained on HSA than diltiazem ($k'_{verapamil} > k'_{diltiazem}$). Thus, HSA was more ordered and more energetically stabilized with verapamil than for diltiazem (i.e. ΔH° and $\Delta S^{\circ}*$ were smaller for verapamil than for diltiazem).

In order to see the calcium cation effect on this binding mechanism, Fig. 3 show the variation in ΔH° with calcium cation concentration (x) for the two antihypertensive drugs. It appeared that for

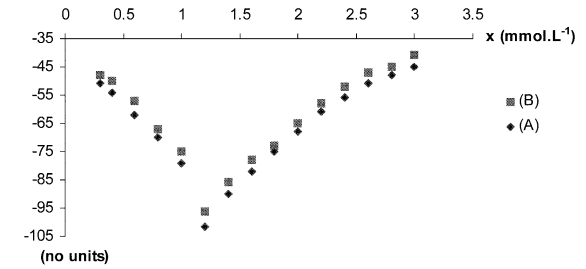

Fig. 4. Plot of $\Delta S^{\circ}*$ (no units) against calcium concentration (x) for verapamil (A) and diltiazem (B).

verapamil and diltiazem, a critical calcium concentration was observed ($x_c = 1.6$ mmol l^{-1}). The curve ΔS°* versus x was similar (Fig. 4). When x was less than or equal to this critical values x_c (region I), ΔH° and ΔS°* values decreased with x. In this cation calcium concentration range, a large increase in the $\ln k'$ of solute was observed. For example, see Fig. 5 for verapamil and diltiazem at $T = 25$ °C. Above x_c (region II), enthalpic and entropic terms of transfer increased in relation to x when there was a decrease in $\ln k'$ (Figs. 3–5). Similar results were previously observed by Bender et al. who have shown a change in the pilpophene and diphenyldramine binding on HSA with Ca^{2+} cation due to an allosteric transition [23]. This transition was applied to explain the trends of ΔH° and ΔS°* with x. When Ca^{2+} concentration varied from 0 to 3 mmol l^{-1}, the following explanations can be given:

- below x_c, as x increased ΔH°, ΔS°* became progressively more negative (Figs. 3 and 4) corresponding to solute–HSA binding increase (i.e. k' increase, Fig. 5). In this Ca^{2+} region (region I), when x increased, the HSA cavity was in a more and more ordered solid-like state [23–25]. Thus, when x increased, the antihypertensive drug came into more and more contact with the HSA cavity and consequently, the solute–HSA binding increase. The decrease in solute–bulk solvent interactions and the increase in the solute–HSA cavity interactions would explain that ΔH° values decreased when calcium cation concentration in the mobile phase increased. As well, these strong drug–HSA interactions promote a low entropy state (high order) of the solute in the HSA by its large immobilization in the HSA cavity (i.e. ΔS°* decreased).
- above x_c, as x increased, ΔH° and ΔS°* became progressively less negative (Figs. 3 and 4) corresponding to solute–HSA binding decrease (i.e. k' decrease, Fig. 5). According to our theory, the HSA cavity was in a disordered solid-like state [23–25]. In this HSA conformation, the interaction between antihypertensive drugs and the human serum albumin decreased. The decrease in the solute–HSA cavity interactions would explain that ΔH° values increased when x increased. Moreover, the decrease of the antihypertensive drugs–HSA interactions led to a gain of freedom for the antihypertensive drug binding to HSA, inducing an increase of ΔS°*.

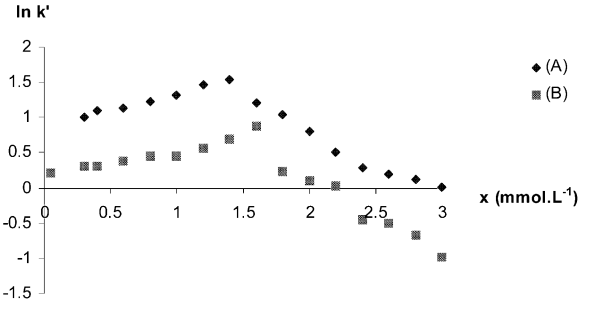

Fig. 5. Plot of $\ln k'$ against x for verapamil and diltiazem at a column temperature equal to 25 °C: (A) verapamil, (B) diltiazem.

In order to gain further insight into the validity of this binding mechanism, the enthalpy-entropy compensation was examined. Enthalpy-entropy compensation is a term used to describe a compensation temperature, which is a system independent for a class of similar experimental systems [24,26–28]. It has been applied to chromatographic system to evaluate the retention mechanism. The enthalpy-entropy compensation can be expressed by the formula:

$$\Delta G^{\circ}_{\beta} = \Delta H^{\circ} - \beta \Delta S^{\circ} \quad (5)$$

where $\Delta G^{\circ}{}_{\beta}$ 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 Eq. (5), when enthalpy-entropy compensation is observed with a group of compounds in a particular chemical interaction, all the compounds have the same free energy $\Delta G^{\circ}{}_{\beta}$ at temperature β. If therefore, enthalpy-entropy is observed for the two antihypertensive molecules, all of two will have the same net retention at the temperature β, although their temperature dependencies may differ [26–28]. Combining Eq. (3) and Eq. (5), the following equation is obtained:

$$\ln k'_T = -\Delta H^\circ/R(1/T - 1/\beta) - \Delta G^\circ_\beta/(R\beta) + \ln \phi \quad (6)$$

Eq. (6) shows that, if a plots of $\ln k'_T$ against ΔH° is linear, then antihypertensive molecules are retained by an essentially identical interaction mechanism. However, the results obtained with this method can be misleading due to the cumulative errors associated with the determination of enthalpy [29,30]. According to Krug et al. analysis [31], similar mechanisms could be mapped through thermodynamic studies if the correlation between $\ln k'$ and ΔH° was used at the harmonic temperature T_{hm} [32].

Ln $k'_{T\mathrm{hm}}$ versus ΔH° determined in region I ($x < x_c$) and II ($x > x_c$) were drawn for the two antihypertensive drugs (Figs. 6 and 7). The correlation coefficient for the four linear fits was higher than 0.97, showing that the retention mechanism was in each region independent of the calcium concentration. The regression lines for both the two antihypertensive drugs and the two regions were:

- at low Ca^{2+} concentration ($x < x_c$, i.e. region I)
  Verapamil: $\ln k'_{T\mathrm{hm}} = -0.0142\ \Delta H^\circ + 1.0094$
  Diltiazem: $\ln k'_{T\mathrm{hm}} = -0.0149\ \Delta H^\circ + 0.239$
- at high Ca^{2+} concentration ($x > x_c$, i.e. region II)
  Verapamil: $\ln k'_{T\mathrm{hm}} = -0.0510\ \Delta H^\circ - 1.0868$
  Diltiazem: $\ln k'_{T\mathrm{hm}} = -0.0453\ \Delta H^\circ - 1.0869$

According to these regression analyses, the following conclusions can be drawn:

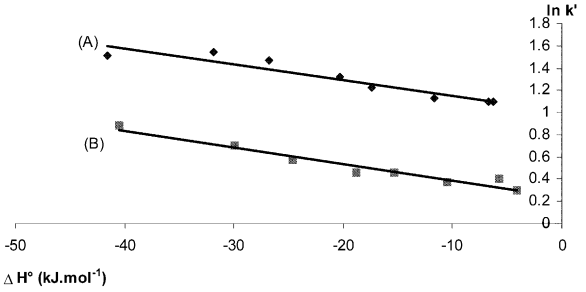

Fig. 6. Enthalpy-entropy compensation for the region I represented by a $\Delta H^\circ - \ln k'$ plot at different values of Ca^{2+} concentration ($x < x_c$) for (A) verapamil and (B) diltiazem.

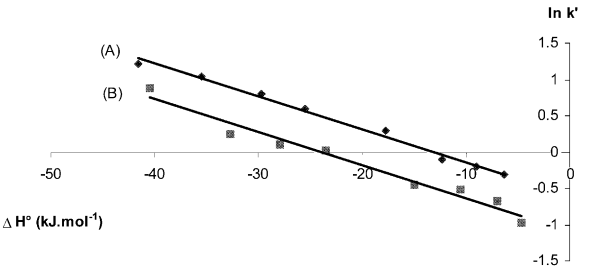

Fig. 7. Enthalpy-entropy compensation for the region II represented by a $\Delta H^\circ - \ln k'$ plot at different values of Ca^{2+} concentration ($x > x_c$) for (A) verapamil and (B) diltiazem.

- In each region, the slopes of the linear plots were the same for the two solutes ($\beta_{\text{verapamil, region I}} \approx \beta_{\text{diltiazem, region I}} \approx 280$ K and $\beta_{\text{verapamil, region II}} \approx \beta_{\text{diltiazem, region II}} \approx 360$ K) indicating that the binding mechanism were identical for the two antihypertensive drugs [8,26–28] and confirmed that they bound at the same localisation on HSA [8].
- However, for each antihypertensive drugs, the slopes of the linear plots were different ($\beta_{\text{verapamil, region I}} \neq \beta_{\text{verapamil, region II}}$) confirming a change in the antihypertensive drug–HSA binding mechanism in this two regions (I and II).

4. Conclusion

The effect of the calcium concentration on the binding on HSA of verapamil and diltiazem, two antihypertensive drugs, was examined. The dependence of retention data on temperature was investigated and trends in thermodynamic parameters were determined. It appeared that a critical calcium concentration was observed ($x_c = 1.6$ mmol l^{-1}) where a change in the antihypertensive drugs–HSA binding was observed. Below x_c, the HSA was in an ordered solid like state leading an increase in the interactions between the solute and the HSA and then an increase in the antihypertensive HSA affinity. Above x_c, the HSA cavity was in a disordered solid like state. Then, for this Ca^{2+} concentration region, the interactions between these antihypertensive drugs and the HSA mole-

cule were unfavourable and consequently, the antihypertensive drug–HSA binding decreased.

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