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Chromatographic study of magnesium and calcium binding to immobilized human serum albumin

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Abstract

The use of immobilized human serum albumin (HSA) as a stationary phase in affinity chromatography has been shown to be useful in resolving optical antipodes or to investigate interactions between drugs and protein. However, to our knowledge, no inorganic ion binding has been studied on this immobilized protein type. To do this, the human serum albumin stationary phase was assimilated to a weak cation-exchanger by working with a mobile phase pH equal to 6.5. A study of the eluent ionic strength effect on ion retention was carried out by varying the buffer concentrations and the column temperatures. The thermodynamic parameters for magnesium and calcium transfer from the mobile to the stationary phase were determined from linear van't Hoff plots. An enthalpy–entropy compensation study revealed that the type of interaction was independent of the mobile phase composition. A simple model based on the Gouy–Chapman theory was considered in order to describe the retention behavior of the test cations with the mobile phase ionic strength. From this theoretical approach, the relative charge densities of the human serum albumin surface implied in the binding process were estimated at different column temperatures.

Keywords: Human serum albumin; Magnesium; Calcium

1. Introduction

Human serum albumin (HSA) is the most abundant protein in blood plasma and possesses a capability of reversible binding of a great number of substances including bilirubin, hormones, drugs and ions. Affinity chromatography with HSA immobilized on the support is specially suited to the study of drug–protein interactions. A number of recent reports have examined the mechanisms of organic compound binding on the HSA stationary phase. The association constants of many ligands have been determined by zonal elution [1] or frontal analysis [2]. The thermodynamic processes involved in the binding and separation of warfarin and thyroxine enantiomers have been characterized by Hage and co-workers [3,4]. Peyrin et al. [5–8] have studied the interactions implied in the binding of negatively charged test molecules, i.e. dansyl amino acids, on the HSA site II cavity.

Many previous investigations of inorganic ion binding to the HSA have been reported in the literature. These studies have been performed using

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experimental methods such as equilibrium dialysis, nuclear magnetic resonance or potentiomety with selective electrodes. Kragh-Hansen and co-workers [9] used radioactive metal cations and equilibrium dialysis to investigate the binding of Ni^{2+}, Zn^{2+} and Ca^{2+} to natural mutants of HSA and proalbumin and the relative importance of the three domains of human serum albumin for Ca^{2+} binding. Harmsen et al. [10] and more recently Bos et al. [11] have observed by acid/base titration and proton nuclear magnetic resonance that the neutral-to-base transition affected calcium binding and vice versa, and that the presence of calcium ions greatly influenced this pH-dependent conformational change in the protein. Gunther et al. [12] measured the concentration of free Ca^{2+} and Mg^{2+} by ion-selective electrodes to explore the influence of fatty acids on the binding of these ions to HSA. Aguanno et al. [13] compared equilibrium dialysis and the potentiometric method to study calcium binding.

Immobilized metal ion affinity chromatography constitutes another approach to examine the interactions between HSA and metal ions. The interaction mode between protein and transition metal ions (Cu(II) or Ni(II)) immobilized on a silica support involves lone pair coordination binding. Using this technique, Finette et al. [14] have analyzed the effect of buffer ionic strength and temperature on the HSA-immobilized ligand interactions. Recently, a new chromatographic study based on a large-zone Hummel and Dreyer method was applied to the examination of copper-protein (bovine serum albumin) complexes [15]. However, to our knowledge, no chromatographic study using immobilized HSA has been applied to the investigation of the interaction between an inorganic cation and HSA. This study examined the magnesium and calcium binding to an HSA stationary phase at a mobile phase pH equal to 6.5. The retention mechanism was investigated over a range of eluent ionic strengths I and column temperatures T. A theoretical approach was presented to explain the retention variations of the test cations with I. This was based on the assumption that the interaction between magnesium and calcium ions and the surface of HSA stationary phase can be treated as electrostatic interaction described by the Gouy–Chapman theory. The charge densities of the protein stationary phase surface implied in the cation binding process was determined from the present theory at all column temperatures. The thermodynamic parameters involved in the ion transfer from the mobile to the stationary phases were calculated from van't Hoff plots. Enthalpy–entropy compensation was also applied to the chromatographic system to evaluate the interaction mechanism at the different mobile phases.

2. Theory

A general phenomenon found in early studies on HSA is its ability to bind divalent inorganic cations. In contrast with the great majority of cationic ligands such as Cu^{2+}, Zn^{2+}, Ni^{2+} or Cd^{2+} which are bound specifically to albumin with the binding mode involving the formation of multiple chelate rings [16], Ca^{2+} and Mg^{2+} interact preferentially with several sites which differ only slightly in their affinity towards the ligand [17]. This ionic binding on the protein is strongly influenced by changes in the medium pH. This dependence on pH is the signature of electrostatically driven processes. For example, the calcium binding to HSA increases with increasing pH, especially above pH=6.5 [18]. This fact corresponds to an enhancement of the electrostatic interaction between the positively charged ion and the albumin, which becomes more and more negatively charged when pH is above 5.4. For example, there is no calcium ion binding an albumin molecule between pH=4.5 and pH=5.0 (albumin charge $\approx+10$) whereas approximately two calcium ions are bound per albumin molecule at pH=8.8 (albumin charge ≈-26) [18].

On the basis of this major «non specific» binding mode of magnesium and calcium to HSA [17], it is proposed that the retention of the cations is dominated by the electrostatic interactions between a charged species and the oppositely charged surface of HSA. The protein is treated as a multivalent spherical particle with its charges uniformly distributed at the surface. The stoichiometric relations, used to study the system behavior when interactions between the ligand and HSA are short ranged, i.e. for Cu^{2+} or Ni^{2+}, are inadequate to describe the electrostatic interactions. Thus, the Gibbs free energy ΔG° incurred during the transfer of cation from the

mobile to the stationary phase can be related to the HSA surface potential φ, the charge of the anion z and the Faraday constant F by the following equation:

$$\Delta G^\circ = zF\varphi \quad (1)$$

The application of the Gouy–Chapman theory gave the calculations for the HSA surface charge densities and its dependence upon the ionic strength of the medium. This theory has been previously used in a chromatographic system to establish a generally charged solute–HSA binding model and to investigate the relative contribution of different interactions implied in the solute transfer [19]. The Gouy–Chapman theoretical approach relates the surface charge potential φ to the surface charge density σ [20]:

$$\sinh(zF\varphi/2RT) = \sigma/(8RT\epsilon_0\epsilon I)^{1/2} \quad (2)$$

This relation accounts for a mobile phase (the bulk solution) of the dielectric constant ϵ and ionic strength I. ϵ_0 is the permittivity of free space and R the gas constant. As $\sinh x \approx x$ under typical chromatographic conditions [21], combining Eqs. (1) and (2) gives:

$$\Delta G^\circ/RT = 2\sigma/(8RT\epsilon_0\epsilon I)^{1/2} \quad (3)$$

Species retention is usually expressed in terms of the retention factor k' which is proportional to the equilibrium constant K and can be written:

$$k' = \chi K \quad (4)$$

Here, χ is a constant assimilated to the effective concentration of protein.

ΔG° is related to the equilibrium constant by the following equation:

$$\Delta G^\circ/RT = -\ln K \quad (5)$$

Combining Eqs. (3), (4) and (5), the logarithmic retention factor can be expressed by the following equation:

$$\ln k' = [-2\sigma/(8RT\epsilon_0\epsilon)^{1/2}]\,(1/I^{1/2}) + \ln \chi \quad (6)$$

This last equation predicts that the logarithm of the retention factor is proportional to the inverse square root of the mobile phase ionic strength. A charge density estimation of the HSA stationary phase surface implied in the cation binding can be obtained from the slope of this $\ln k'$ vs. $1/I^{1/2}$ plot. In this approach, the σ value should be constant regardless of the kind of test ions. However, the surface charge density value does not take into account all the charges on HSA but only the negative charge with which each cation can interact on the basis of their respective affinity for the protein. In other words, our model takes into account only the dominant electrostatic effect by neglecting the weak short range specific forces such as those due to steric hindrance or chelate interaction. So, the magnitude of the retention, i.e. the binding affinity, can be quantified by the number of cation accessible sites per HSA molecule. This type of treatment is, of course, limited to the case of divalent ions interacting preferentially with several roughly identical low-affinity sites such as magnesium or calcium.

3. Experimental section

3.1. Apparatus

The HPLC system consisted of a Merck Hitachi pump L7100 (Nogent-sur-Marne, France), an Interchim Rheodyne injection value model 7125 (Montluçon, France) fitted with a 20 μl sample loop and a Waters conductimetric detector (Saint Quentin en Yvelines, France). An HSA protein chiral Shandon column (150 mm×4.6 mm) was used with controlled temperature in an Interchim Crococil oven TM N° 701 (Montluçon, France). After each utilization, the column was stored at 4°C until further use.

3.2. Solvents and samples

Sodium hydrogen phosphate and sodium dihydrogen phosphate were supplied by Prolabo (Paris, France). Water was obtained from an Elgastat option water purification system (Odil, Talant, France) fitted with a reverse osmosis cartridge. $MgCl_2$, $CaCl_2$, NaCl and KCl were obtained from Sigma Aldrich (Saint Quentin, France) and were made fresh daily at a concentration of 5.25×10^{-3} M for $MgCl_2$ and $CaCl_2$ and 1.05×10^{-2} M for NaCl and KCl in water. The mobile phase consisted of a sodium phosphate buffer at pH=6.5 with salt concentrations varying from 8.25×10^{-4} to 25.25×10^{-4}

M. The mobile phase rate was kept at 1ml min^{-1}. Twenty μl of $MgCl_2$, $CaCl_2$, NaCl and KCl samples were injected at different buffer concentrations. It has been known for many years that monovalent cations such as sodium or potassium are not able to bind to HSA [18,22]. Therefore, NaCl and KCl samples were injected in the HSA column to examine the eventual interference of the chloride ion in the detection of the peak of magnesium and calcium. Contrary to the $MgCl_2$ and $CaCl_2$ sample injection, no detectable peak different from the blank peak corresponding to the injection of a phosphate buffer sample was detected at various mobile phases. This result demonstrated the specificity of the assay using $MgCl_2$ and $CaCl_2$ salts as suppliers of magnesium and calcium respectively.

3.3. Temperature studies

Retention factors of magnesium and calcium were determined over the temperature range 15° to 35°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 magnesium and calcium was measured every h for five h and again after 23 and 24 h. The maximum relative difference of the retention time of these compounds was always 0.7%, making the chromatographic system sufficiently equilibrated for use after 1 h. The ions were injected three times at each temperature and phosphate buffer 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.

4. Results and discussion

4.1. Van't Hoff plots

The free energy ΔG° from Eq. (1) can be broken down into enthalpic and entropic terms to give the van't Hoff equation:

$$\ln k' = (-\Delta H^\circ/RT) + \Delta S^\circ* \quad (7)$$

$$\Delta S^\circ* = (\Delta S^\circ/R) + \ln \chi \quad (8)$$

If the cation binds to the HSA stationary phase with a constant enthalpy of association, then a plot of ln k' vs. $1/T$ should be linear with a slope of $-\Delta H^\circ/R$ and an intercept of $\Delta S^\circ*$. The retention factor of magnesium and calcium was calculated for the range of ionic strength at all column temperatures. From these retention factors, the plots of ln k' in relation to 1/T were determined for different phosphate buffer concentrations. The van't Hoff plots were all linear. The correlation coefficients for the linear fits were over 0.96. Fig. 1 shows the van't Hoff plots for magnesium at all mobile phase ionic strength values. These linear behaviors were thermodynamically what was expected when there was no change in the interaction mechanism in relation to temperature. Table 1 contains a complete list of ΔH° and $\Delta S^\circ*$ values at all phosphate buffer concentrations for magnesium and calcium.

4.2. Enthalpy–entropy compensation study

Investigation of the enthalpy–entropy compensation temperature is a further thermodynamic approach to the analysis of physicochemical data. It has been previously applied to chromatographic systems to evaluate the retention mechanism [23]. Mathematically, enthalpy–entropy compensation can be expressed by the formula:

$$\Delta H^\circ = \beta \Delta S^\circ + \Delta G_\beta{}^\circ \quad (9)$$

where $\Delta G_\beta{}^\circ$ 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. Rewriting Eq. (9) using Eq. (7) gives:

$$\ln k'_T = \ln k'_\beta - \frac{\Delta H^\circ}{R}\left(\frac{1}{T} - \frac{1}{\beta}\right) \quad (10)$$

where

$$\ln k'_\beta = -\frac{\Delta G_\beta{}^\circ}{R\beta} + \ln \chi \quad (11)$$

Eq. (10) shows that, if a plot of ln k'_T against $-\Delta H^\circ$ is linear, then the solute is retained by an essentially identical interaction mechanism. A plot of ln k'_T ($T = 308$ K) calculated for the magnesium ion against

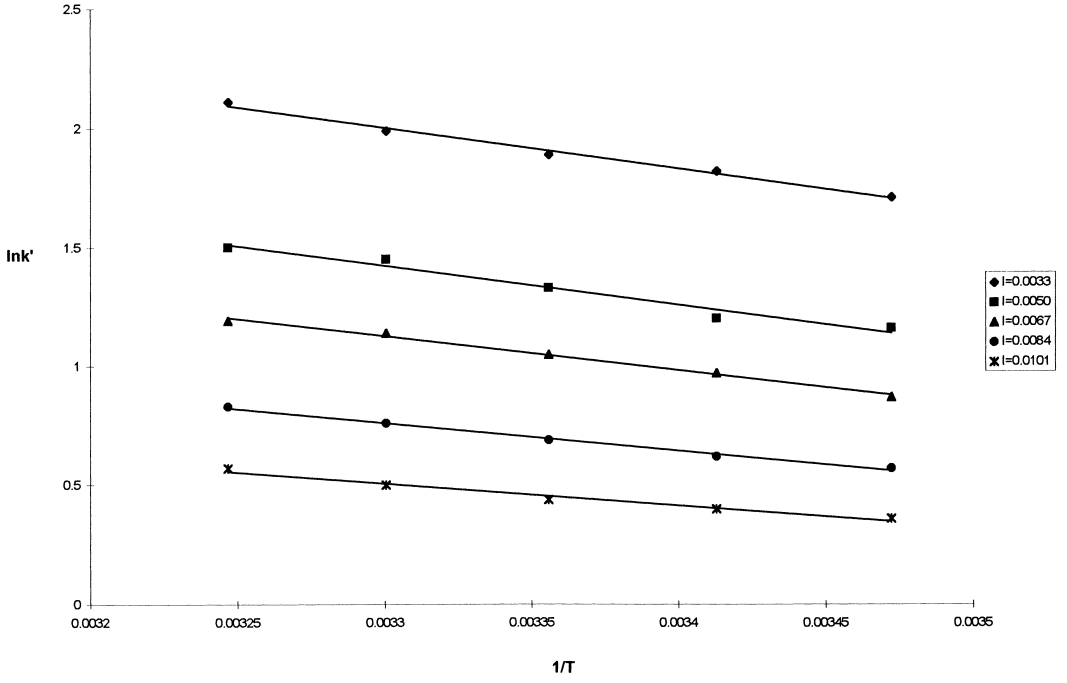

Fig. 1. Van't Hoff plots for a magnesium ion at each mobile phase ionic strength *I*.

$-\Delta H^\circ$ determined at the different values of ionic strength was drawn. The correlation coefficient for the linear fit was equal to 0.97. Fig. 2 shows ln k'_T values plotted in relation to $-\Delta H^\circ$ for magnesium. This degree of correlation can be considered to be adequate to verify enthalpy–entropy compensation [24], thus indicating that the interaction mechanism was independent of the ionic strength value chosen.

Table 1
Thermodynamic parameters ΔH° (kJ mol^{-1}) and $\Delta S^{\circ *}$ with standard deviations (in parentheses) for magnesium (Mg) and calcium (Ca) at different ionic strength (*I*)

I	ΔH°		$\Delta S^{\circ *}$	
	Mg	Ca	Mg	Ca
0.0033	15.1 (0.2)	17.3(0.1)	8.0 (0.1)	10.5 (0.2)
0.0050	13.1 (0.1)	15.0 (0.1)	6.6 (0.1)	9.1 (0.1)
0.0067	11.8 (0.1)	12.9 (0.3)	5.5 (0.1)	7.2 (0.1)
0.0084	9.7 (0.2)	11.1 (0.1)	4.6 (0.1)	6.1 (0.1)
0.0101	7.9 (0.1)	9.2 (0.2)	3.0 (0.1)	5.2 (0.2)

4.3. Ionic strength effect on retention factor

From the retention factor, the plots of ln k' in relation to the reciprocal square root of the ionic strength were determined at each temperature for the two charged species. These plots exhibited a good linearity in all cases. The correlation coefficients of the fits were over 0.96. Fig. 3 represents the ln k' vs. $1/I^{1/2}$ plots of magnesium for *T* equal to 15–20–25–30–35°C. These results were consistent with the proposed theoretical approach, i.e. a retention behavior controlled by electrostatic forces between cations and the oppositely charged surface of HSA. According to Eq. (6), the HSA surface charge densities can be calculated from the slope of the ln k' vs. $1/I^{1/2}$ plots. The corresponding σ/F value was found to be equal to around 3.6×10^{-3} μmol m^{-2} and 7.4×10^{-3} μmol m^{-2} for magnesium and calcium respectively (Table 2). The relative difference in these values obtained for all *T* values was inferior to 10%, thus indicating that the surface charge densities implied in the binding process were in-

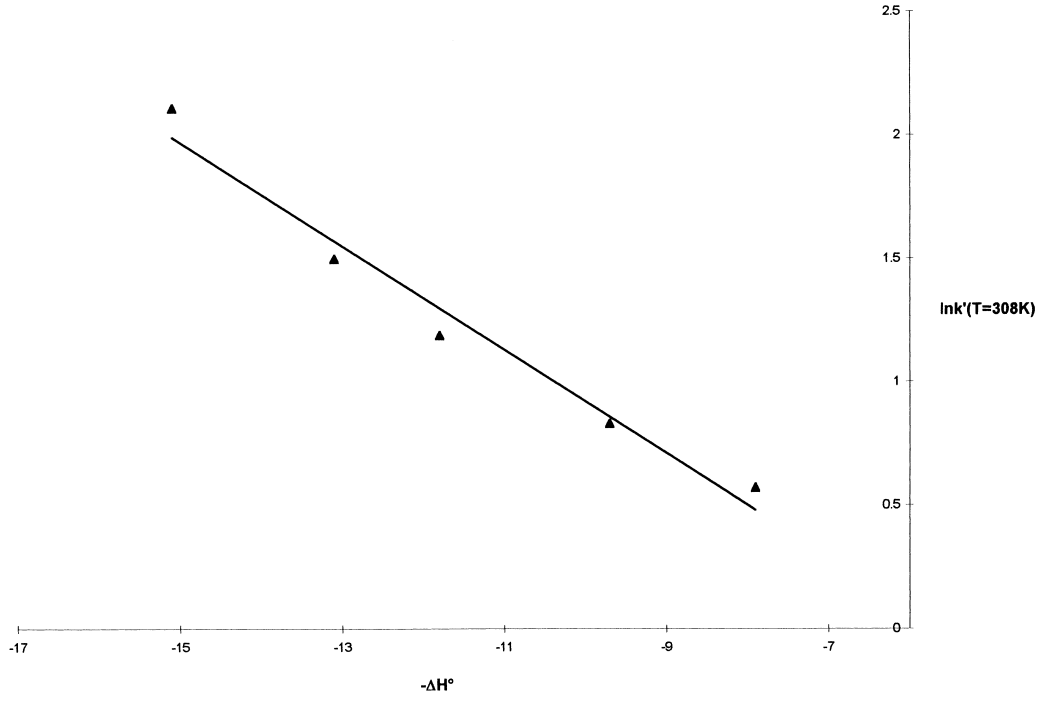

Fig. 2. Plots of ln k'_T (T=308 K) against $-\Delta H^\circ$ (kJ mol^{-1}) for enthalpy–entropy compensation study (Eq. (11)).

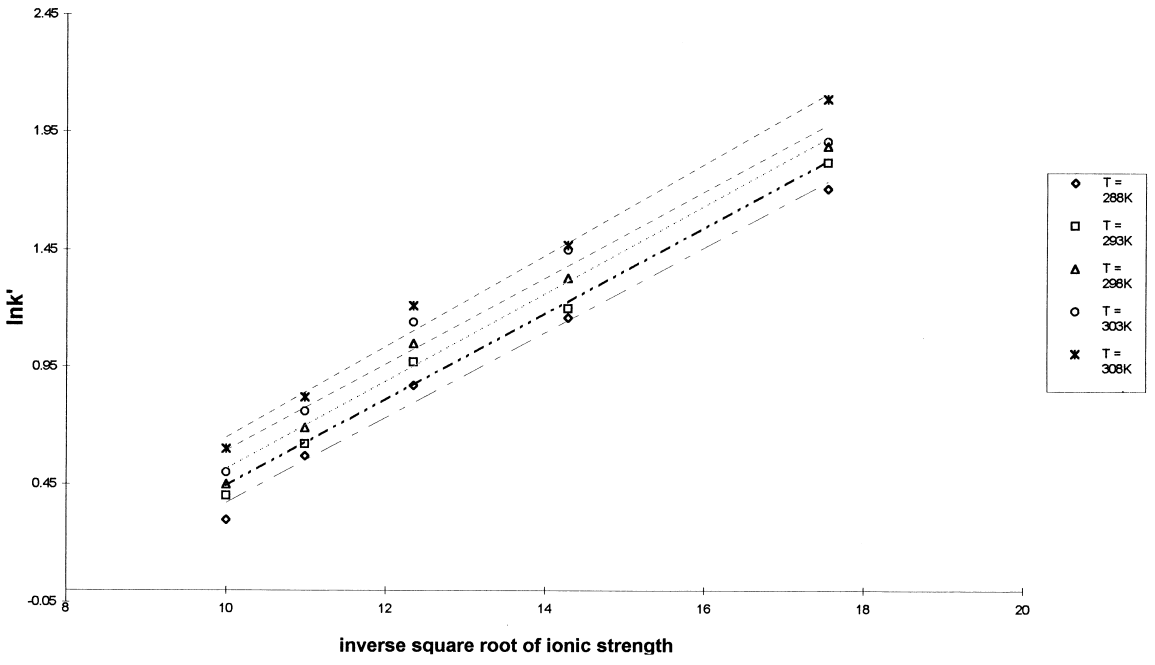

Fig. 3. Plots of ln k' against $1/I^{1/2}$ for a magnesium cation at all column temperatures (Eq. (6)).

Table 2
Relative charge densities of the HSA stationary phase surface σ/F (μmol m^{-2}) related to magnesium (Mg) and calcium (Ca) binding and calculated from Eq. (6) at different column temperatures (T)

T (°C)	σ/F ($\times 10^{-3}$)	
	Mg binding	Ca binding
15	3.5	7.4
20	3.5	7.3
25	3.6	7.6
30	3.5	7.4
35	3.7	7.4

dependent of the temperature. This is in agreement with the linear van't Hoff plots which demonstrated no change in the interaction mechanism over the column temperature range (Fig. 1). By determining the surface area of the HSA molecule from its molecular data [25], it was possible to calculate using the surface charge density values, the number of charges per protein molecule which interacted with the test cations at pH = 6.5. Values of 2.2 and 4.5 charges per albumin molecule interacting with magnesium and calcium respectively were obtained. This approached the values found in an earlier studies by Copeland et al. [26] for magnesium (≈one principal site/HSA) and Fogh-Andersen [27] for calcium (≈four principal sites/HSA). The divergence between these values can be explained by the fact that the charge distribution over the protein surface is inhomogeneous, resulting in domains of higher charge density and thus, in stronger interactions than assumed in the model.

ΔH° and $\Delta S^{\circ *}$ values of the transfer of magnesium and calcium from the mobile to the stationary phase were always positive (Table 1) whatever the ionic strength of the mobile phase. These results indicate that the retention mechanism is entropically driven for all eluent compositions. It has been known for several years that the interactions between ionic species in aqueous solution are characterized by small positive enthalpy changes and positive entropy changes [28,29]. Accordingly, the present thermodynamic behavior corresponded well to the model describing the electrostatic attraction that occurs between the negatively charged non specific regions of HSA and the positively charged test ions.

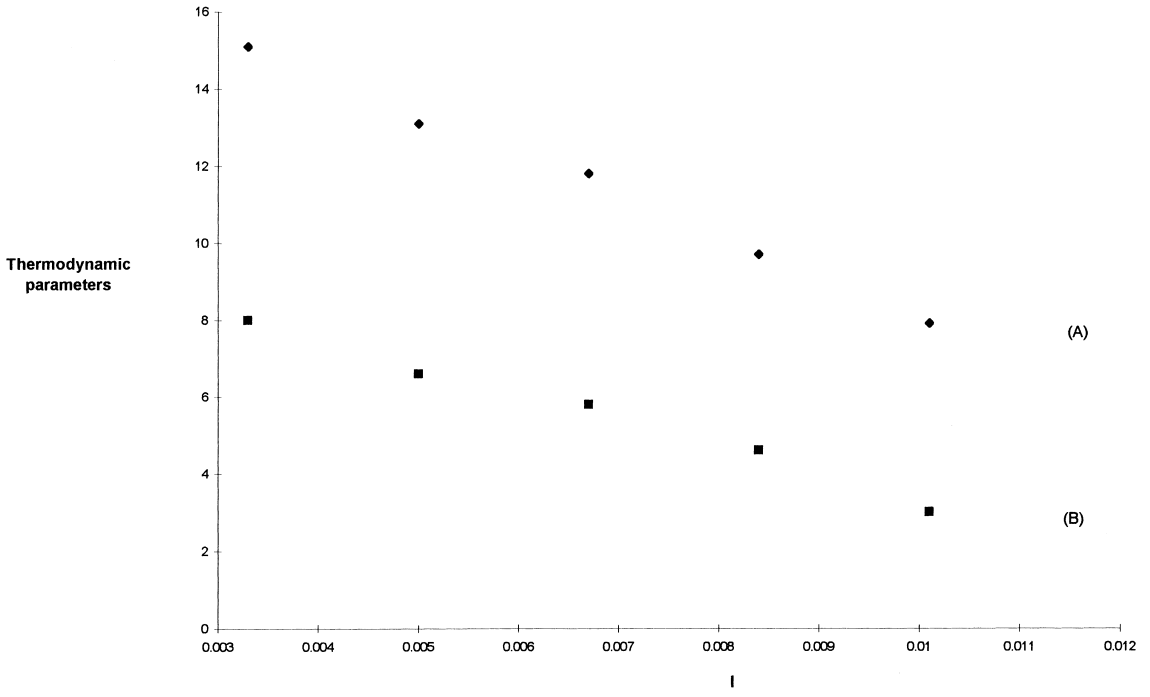

Fig. 4. Plots of ΔH° (kJ mol^{-1}) (A) and $\Delta S^{\circ *}$ (B) against mobile phase ionic strength I for a magnesium cation.

The thermodynamic terms ΔH° and $\Delta S^{\circ *}$ were plotted against the ionic strength of the mobile phase. Fig. 4 represents these two plots for magnesium. When I increased, both ΔH° and $\Delta S^{\circ *}$ decreased, becoming less positive. In this ionic strength range, the ionic double layer of charged species was thick with a high Debye length. The phosphate buffer concentration increase was responsible for a Debye length reduction by affecting the electrostatic shielding, which governed an ionic attraction decrease. The decrease in the test cation binding was accompanied by a reduction in enthalpy and entropy changes for the transfer of magnesium and calcium from the mobile to the stationary phase. Thus, the ΔH° and $\Delta S^{\circ *}$ values became progressively less positive corresponding to a weaker retention.

5. Conclusion

In this paper, a theoretical model was presented to investigate the retention behavior of magnesium and calcium on the HSA stationary phase. At pH = 6.5, the protein was assumed to be a weak cation-exchanger which exhibited negative charges uniformly distributed at the surface. The application of the Gouy–Chapman theory accurately described the cation binding dependence on the ionic strength of the eluent. An estimation of the charge densities of the HSA surface implied in the retention of the two test ions was determined using this theoretical approach. As well, the thermodynamic parameter trends with the mobile phase ionic strength for retention of cations supported the fact that the binding was controlled by an entropically driven mechanism corresponding to its electrostatic attraction for different «non specific» areas of serum albumin.

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