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Retention behavior of D,L-dansyl-amino acids on a human serum albumin chiral stationary phase: effect of a mobile phase modifier

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Abstract

The effect of perchlorate anion as a mobile phase modifier on the retention of dansyl norvaline and dansyl tryptophan enantiomers on a human serum albumin (HSA) column was studied by varying the chaotropic agent concentrations. The thermodynamic parameters for the transfer of a solute from the mobile to the HSA stationary phases were determined from linear van't Hoff plots. An enthalpy–entropy compensation study revealed that the type of interaction between the solute and HSA was independent of the molecular structure. The parabolic variations observed with the enthalpic and entropic terms of dansyl amino acid transfer in relation to the concentration of perchlorate anion were considered to be the result of the change from reversed to normal-phase conditions for this chromatographic system. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

The observation that the chirality of a drug or pro-drug can influence one or all of the processes of absorption, distribution, metabolism or excretion has led to the search for suitable methods for separating the individual drug enantiomers. Liquid and gas chromatography are the most powerful techniques in enantiomeric separation. Many chiral stationary phases for liquid chromatography have been developed, but one approach involves the use of immobilized proteins: Commercially available protein-bonded phase chiral columns have been made with human α_1-acid glycoprotein (AGP) [1], ovomucoid (OV) [2], bovine serum albumin (BSA) [3] and human serum albumin (HSA) [4]. A number of recent reports have examined the thermodynamic properties and binding mechanisms involved in the interactions of some model compounds with HSA. Soltes et al. [5] have investigated the stereoselectivity of the reversible binding interactions between the D- and L- tryptophan enantiomers and fragments of HSA by applying three novel high-performance liquid chromatographic arrangements. The thermodynamic and kinetic processes involved in the binding and separation of warfarin enantiomers on an HSA column have been characterized using frontal analysis by Loun et al. [6]. Tittelbach et al. [7] have studied the influence of subtle changes in the protein structure on the specific binding and chromatographic selectivity for differently substituted L-tryptophans. As well, earlier studies investigated the

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role of various mobile phase parameters on the compound-HSA binding. Binding and its influence on the support's chromatographic performance have been examined in relation to the ionic strength and the addition of small amounts of organic solvents [8–10]. Lloyd et al. [11] have compared the retention factors of benzoin enantiomers measured on liquid chromatography and capillary electrophoresis using differing concentrations of organic modifiers. The effects of changing the pH of the mobile phase and the column temperatures on the binding properties of HSA for a series of dansyl amino acids have been examined by Peyrin et al. [12].

Sudlow et al. [13] have characterized two specific binding sites on HSA which are designated site I (or the warfarin site) and site II (or the benzodiazepine site). Recently, the three dimensional structure of HSA has been determined cristallographically [14]. Binding sites are located respectively in hydrophobic cavities in subdomains IIA and IIIA which exhibit similar chemistry. The non polar residues are sequestered into the hydrophobic cavities inside the protein core and the polar residues onto the surface. It has been shown that L-dansyl norvaline and L-dansyl tryptophan have a single binding region on HSA that is known to be located at site II [15]. In a previous study, it was demonstrated that the D-enantiomers of these dansyl amino acids interact on the HSA with a stronger affinity in the same location that L- enantiomers [16]. A simple two step model of dansyl amino acid-HSA interaction has been proposed [16]. The guest molecule approaches the cavity by mutual penetration of the hydration layers (this step is driven by the hydrophobic effect). In the second step, which is responsible for chiral recognition, the most hydrophobic group (dimethyl amino naphtyl) of dansyl amino acid occupied the interior of the cavity and the carboxylate and sulfonylamido groups interacted with polar residues on the cavity rim by forming electrostatic and hydrogen bonds [16]. In an effort to extend our investigation to the dansyl amino acid-HSA interaction, this study examined how the change in mobile phase chaotropicity effected the solute binding on site II cavity. It was known that chaotropic agents such as perchlorate anion increased the water solubility of weak polar solute and that this was due to a decrease in hydrophobic interaction [17]. Thus, it was thought useful to evaluate the effect of this mobile phase modifier on the retention behavior of dansyl amino acid on HSA column. Van't Hoff plots were used to determine the thermodynamic parameters involved in the transfer of the dansyl amino acids from the mobile to the stationary phases. Enthalpy–entropy compensation was applied to the chromatographic system to evaluate the type of interaction for all dansyl amino acids. The variations in the enthalpic and entropic terms of solute binding to the immobilized HSA column are explained on the basis of the chemical properties of perchlorate anion.

2. Experimental

2.1. Apparatus

The HPLC system consisted of a 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, France). An HSA protein chiral Shandon column (150 mm×4.6 mm) was used with controlled temperature in an Interchim Crococil oven TM No 701 (Montluçon, France). 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

HPLC grade acetonitrile (Merck) was used without further purification. Sodium hydrogen phosphate, sodium dihydrogen phosphate and lithium perchlorate 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. (1) L- and D,L-dansyl norvaline, (2) L- and D,L-dansyl tryptophan, were obtained from Sigma Aldrich (Saint Quentin, France) and were made fresh daily at a concentration of 20 mg l^{-1} in acetonitrile. Sodium nitrate was used as a dead time marker (Merck). The mobile phase consisted of a 0.05 *M* sodium phosphate buffer–acetonitrile (90–10, *v*/*v*) at pH 6.0 with lithium perchlorate concentrations varying from 0 to 0.05 *M*. Twenty μl

of each solute and a mixture of these was injected and the retention times were measured.

2.3. Temperature studies

Compound retention factors were determined over the temperature range 0°C to 40°C. The chromatographic system was allowed to equilibrate at each temperature for at least one hour prior to each experiment. To study this equilibration, the compound retention time of dansyl norvaline was measured after, 22, 23 and 24 h. The maximum relative difference of the retention time of this compound was always 0.9%, making the chromatographic system sufficiently equilibrated for use after 1 h. All the solutes were injected three times at each temperature and lithium perchlorate concentration. Once the measurements were completed at the maximum temperature, the column was immediately cooled to ambient condition to minimize any denaturation of the immobilized HSA.

3. Results and discussion

3.1. Retention factors and van't Hoff plots

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

$$k' = \phi K \tag{1}$$

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:

$$\Delta G^\circ = -RT \ln K \tag{2}$$

where

$$\Delta G^\circ = \Delta H^\circ - T\,\Delta S^\circ \tag{3}$$

ΔH° (respectively ΔS°) is the enthalpy (respectively entropy) of transfer of the solute from the mobile to the stationary phase, T is the temperature and R the gas constant. Combining Eq. (1) and Eq. (3), the capacity factor can be expressed by the equation:

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

Eq. (4) is a van't Hoff plot. From the slope and the intercept, ΔH° and $(\Delta S^\circ/R)+\ln \phi$ can be respectively calculated. The van't Hoff plots were all linear for L- and D-dansyl amino acids. The correlation coefficients (r) for the fits were at least equal to 0.968. The relative typical standard deviations of the slope and the intercept calculated from three replicates were less than 0.008 and 0.04 respectively. Fig. 1 shows the van't Hoff plots for the dansyl norvaline and dansyl tryptophan enantiomers at a perchlorate anion concentration equal to 0.02 M. These linear behaviors were thermodynamically what was expected when there was no change in the retention mechanism in relation to temperature. This is consistent with a solute binding which occurs at a fixed number of specific sites with a constant negative enthalpy of association. Tables 1 and 2 contain a complete list of ΔH° and $(\Delta S^\circ/R)+\ln \phi$ values for all solutes at all perchlorate anion concentrations. As can be seen from Fig. 1, dansyl tryptophan was retained more on the HSA column than dansyl norvaline. This fact was explained by the difference in the hydrophobic character of these solutes. The hydrophobic effect was dominated by three factors (i) the cavitation energy (ii) the interaction energy associated with dispersion forces and (iii) loss of solvent entropy in the first solute hydratation shell due to a change in the water structure [18]. The solute hydrophobicity is proportional to this solvent accessible surface area. The apolar groups of dansyl amino acids acted as factors governing the degree of solvation of the analyte in the bulk mobile phase. When the apolar group surface area increased, the tendency of the analyte to be excluded from the bulk mobile phase and go towards the vicinity of site II cavity on the HSA increased. Thus, D- and L-dansyl tryptophan were retained more on the HSA column than D- and L-dansyl norvaline (Fig. 1) because the hydrophobicity of the indolyl methyl group was greater than that of the propyl group [19].

3.2. Enthalpy–entropy compensation

Enthalpy–entropy compensation is a term used to describe a compensation temperature which is system independent for a class of similar experimental systems. It has been applied to chromatographic systems to evaluate the retention mechanism [20].

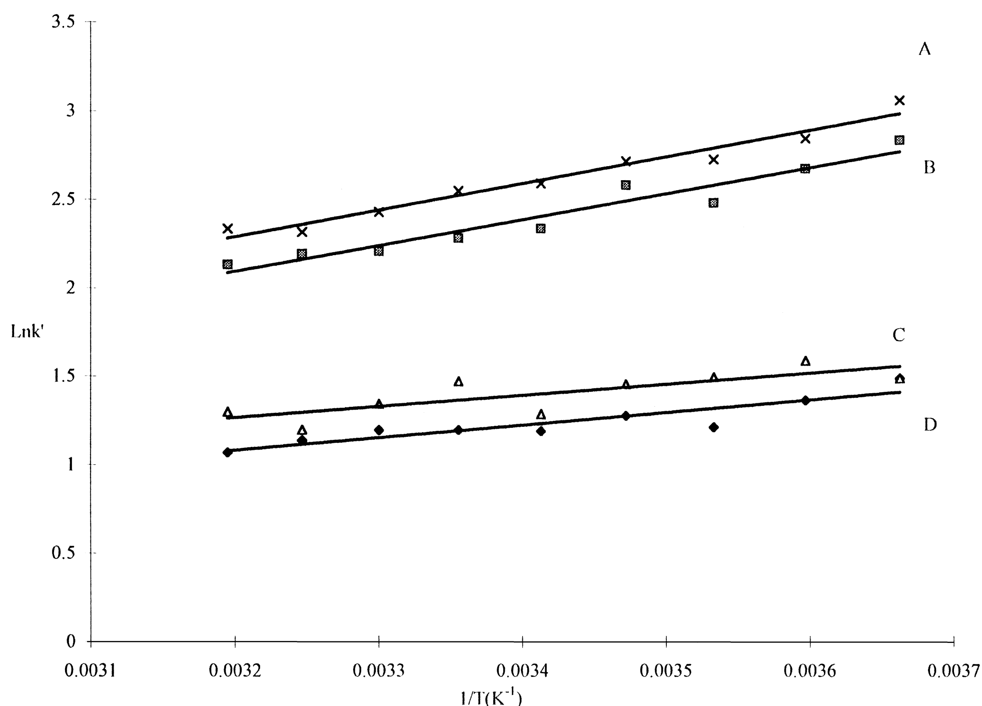

Fig. 1. Van't Hoff plots at a perchlorate anion concentration equal to 0.02 *M* for D- (A) and L-dansyl tryptophan (B) and D- (C) and L-dansyl norvaline (D).

The enthalpy–entropy compensation can be expressed by the formula:

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

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. 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 $\Delta G_{\beta}{}^{\circ}$ at temperature β. If therefore, enthalpy–entropy compensation is observed for the D,L-dansyl amino acids, all of them will have the same net retention at the compensation temperature

Table 1
Thermodynamic parameter ΔH° (kJ mol^{-1}) with standard deviations (in parentheses) at different lithium perchlorate concentrations for the dansyl amino acid transfer from the bulk solvent to the HSA stationary phase

	$LiClO_4$ (*M*)					
Compounds	0	0.01	0.02	0.03	0.04	0.05
L-Dansyl norvaline	−8.3 (0.1)	−5.8 (0.1)	−5.2 (0.1)	−5.4 (0.1)	−6.4 (0.1)	−8.0 (0.2)
D-Dansyl norvaline	−8.1 (0.1)	−5.8 (0.2)	−5.3 (0.1)	−4.4 (0.1)	−6.6 (0.2)	−7.0 (0.1)
L-Dansyl tryptophan	−14.5 (0.2)	−13.2 (0.2)	−12.2 (0.1)	−11.9 (0.1)	−13.2 (0.2)	−13.9 (0.1)
D-Dansyl tryptophan	−17.4 (0.2)	−14.0 (0.3)	−12.5 (0.1)	−12.2 (0.2)	−14.0 (0.1)	−15.7 (0.3)

Table 2
Thermodynamic parameter $(\Delta S°/R)+\ln \phi$ with standard deviations (in parentheses) at different lithium perchlorate concentrations for the D,L-dansyl amino acid transfer from the bulk solvent to the HSA stationary phase

	$LiClO_4$ (M)					
Compounds	0	0.01	0.02	0.03	0.04	0.05
L-Dansyl norvaline	−12.1 (0.1)	−8.7 (0.1)	−7.2 (0.1)	−8.8 (0.1)	−13.7 (0.1)	−19.7 (0.2)
D-Dansyl norvaline	−8.9 (0.1)	−6.9 (0.2)	−6.3 (0.1)	−4.5 (0.1)	−12.3 (0.2)	−14.5 (0.1)
L-Dansyl tryptophan	−23.9 (0.2)	−23.8 (0.1)	−21.5 (0.4)	−21.8 (0.2)	−26.5 (0.2)	−29.5 (0.1)
D-Dansyl tryptophan	−32.2 (0.4)	−25.3 (0.1)	−20.9 (0.3)	−20.9 (0.2)	−27.7 (0.1)	−33.7 (0.3)

β, although their temperature dependencies may differ. Combining Eqs. (4) and (5), the following equation is obtained:

$$\ln k'_T = \ln k'_\beta - \Delta H°/R(1/T - 1/\beta) \quad (6)$$

Eq. (6) shows that, if a plot of ln k'_T against $-\Delta H°$ is linear, then the D,L-dansyl amino acids are retained by an essentially identical interaction mechanism.

A plot of ln k'_T (for $T=308$ K) calculated for each enantiomer of the dansyl amino acids against $-\Delta H°$ determined at each perchlorate anion concentration was drawn. The correlation coefficients for the linear fits were over 0.992. Fig. 2 shows ln k'_T values plotted as a function of $-\Delta H°$ for the perchlorate anion concentrations equal to 0.02 and 0.04 M. The high degree of correlation can be considered to be adequate to verify enthalpy–entropy compensation

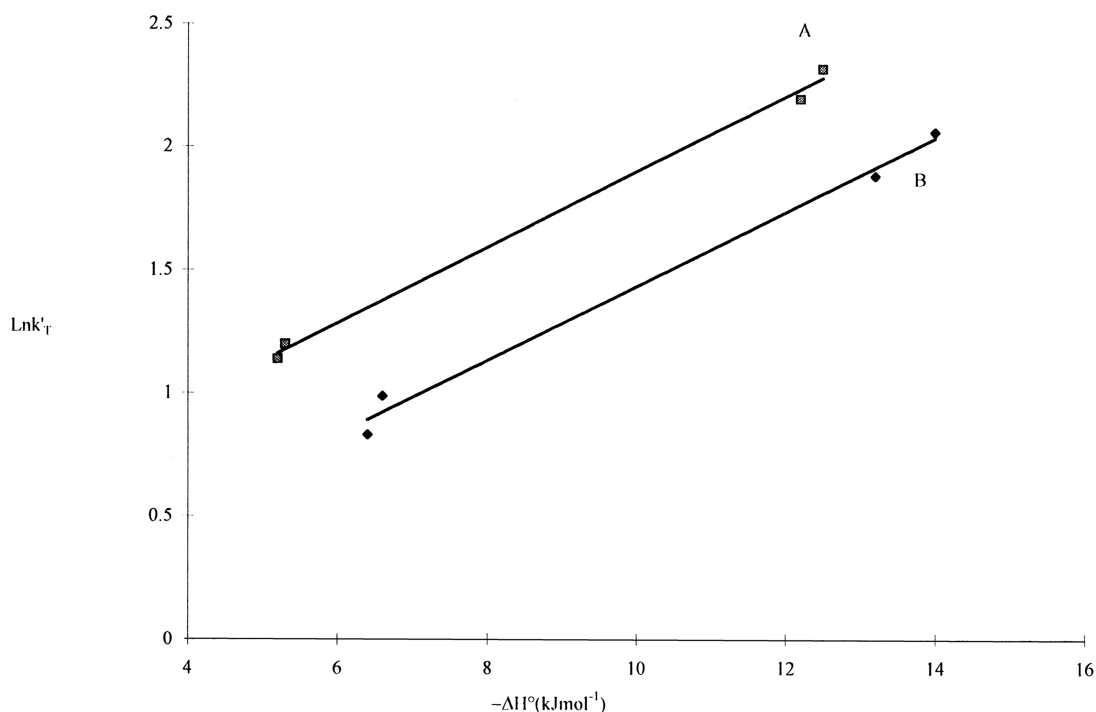

Fig. 2. Plots of ln k'_T against $-\Delta H°$ (kJ mol^{-1}) for perchlorate anion concentrations equal to 0.02 (A) and 0.04 M (B).

[21]. This is in agreement with previous work in which the type of interaction was found to be the same for various dansyl amino acids using a similar chromatographic system and enthalpy–entropy compensation [12]. Using Eq. (6), the β values were calculated from the slopes and went from approximately 460 K to 520 K over the perchlorate anion concentration range.

3.3. Effect of perchlorate anion concentration

ΔH° and $(\Delta S^\circ/R)+\ln\phi$ values versus lithium perchlorate concentrations C showed a parabolic curve for all compounds. Figs. 3 and 4 represent these thermodynamic parameters in relation to C for dansyl norvaline and dansyl tryptophan enantiomers. When C was less than or equal to a critical value C_c varying from 0.02 M to 0.03 M in relation to the compound, ΔH° and $(\Delta S^\circ/R)+\ln\phi$ values increased with C. In this concentration range, a large decrease in the ln k' of solute was observed. For example, see Fig. 5 for D- and L-dansyl tryptophan at $T=283$ K. Above C_c, enthalpic and entropic terms of transfer quickly decreased in relation to C when there was a weakened or nil variation in ln k' (Fig. 5).

To explain the trends of ΔH° and $(\Delta S^\circ/R)+\ln\phi$ with C, the chaotropic properties of the perchlorate anion were applied to study the interaction of dansyl amino acids with both the bulk mobile and stationary phases. Breslow [22] has recently demonstrated that a perchlorate anion acts by a direct solvation interaction of a solute. This direct solute solvation effect counterbalances the negative contribution of the perchlorate anion on the cavity formation in water [22]. The chaotropic agents are predicted to cause unfolding of proteins by solvating non polar groups better than water does. However, for the perchlorate concentration and temperature range studied in this paper, the unfolding process was not observed on the extremely good cyclic reproducibility of the van't Hoff curves. This can be explained by the fact that the HSA used in HPLC system probably differed greatly from a free protein in solution due to changes induced during immobilization, although some com-

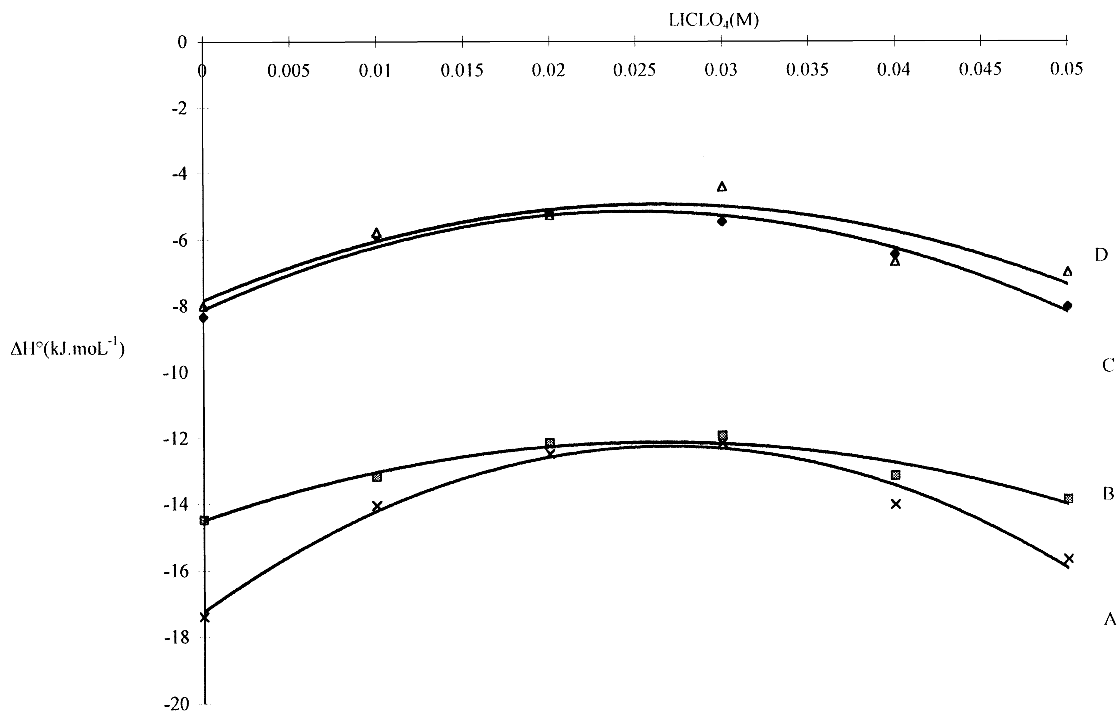

Fig. 3. Plots of ΔH° (kJ mol^{-1}) vs perchlorate anion concentration for D- (A) and L-dansyl tryptophan (B) and D- (D) and L-dansyl norvaline (C).

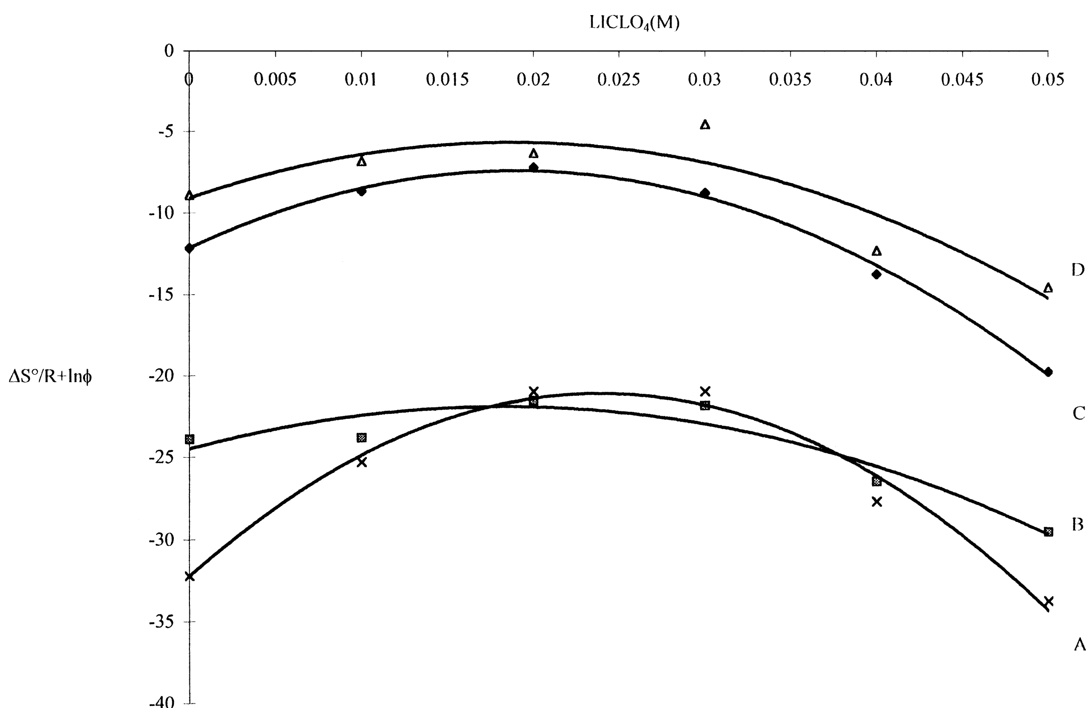

Fig. 4. Plots of $(\Delta S°/R)+\ln\ \phi$ vs perchlorate anion concentration for D- (A) and L-dansyl tryptophan (B) and D- (D) and L-dansyl norvaline (C).

mercially prepared columns have been shown to conserve the protein's native binding properties [23]. As the binding cavity corresponds to a solid like state [24], the solute molar enthalpy associated with

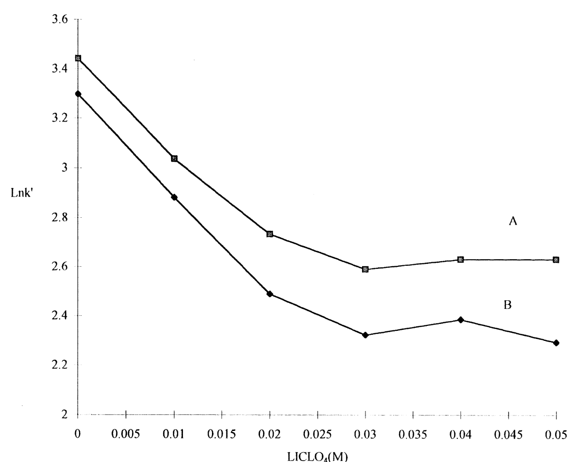

Fig. 5. Influence of perchlorate anion concentration on ln k' of D- (A) and L-dansyl tryptophan (B) at $T=283$ K.

the stationary phase could be expected to be lower than the solute molar enthalpy associated with the mobile phase because of the formation in the strong HSA interactions between solute and tightly packed residues in the course of the solute transfer process. As well, these interactions promote a lower entropy state (high order) of solute in the stationary than the mobile phase by a large immobilization in the cavity. Thus, $\Delta H°$ and $(\Delta S°/R)+\ln\ \phi$ of the solute transfer were always negative values for all chaotropic salt concentrations (Tables 1 and 2).

When lithium perchlorate concentrations varied from 0 to 0.05 M, the following explanations can be given:

– Below C_c, the plots of ln k' vs C were mostly linear (Fig. 5), suggesting a reversed-phase mode for the interaction between the solute and stationary phase. Similar behavior has been reported in an earlier report examining the effect of 1-propanol on HSA-® warfarin interaction [6]. Also, the chaotropic agent behaves as a typical organic modifier by contributing to solute solvation in the mobile phase

by a direct interaction. Perchlorate anions enter the solvation sphere of dansyl amino acid and act as bridges between the solute and water molecules. The hydrophobic interactions between the site II cavity and solute decreased with C due to a strong affinity of dansyl amino acid for the bulk mobile phase. Thus, the solute molar enthalpy associated with the mobile phase in the solute transfer became more negative and ΔH° increased. The observed trend of $(\Delta S^\circ/R)+\ln \phi$ to positively increase when C increases can be explained by a low entropy state in the mobile phase due to a well structured solvation shield around the dansyl amino acid.

– Above C_c, a weak or nil increase in $\ln k'$ in the relation to the mobile phase composition was observed (Fig. 5). Such behavior suggests that the compound was likely to interact with polar residues on the cavity rim through a normal-phase retention. This probably reflects the ability of perchlorate anion to strongly compete for the non polar residues that take place in binding to dansyl amino acid at site II of HSA. Also, at a high perchlorate anion concentration, the solute was partially excluded from the hydrophobic cavity core, its retention being mostly due to polar interactions with the residues at the periphery of the cavity site II. Similar observations were observed by Chang et al. [25] who used a γ-cyclodextrin column in reversed-phase mode, 2-propanol as the organic solvent, and nitrophenols as the substrates. These investigators reported the occurrence of minima in plots of $\ln k'$ vs percent organic modifier and considered this unusual behavior to be the result of solute–solvent competition for the interaction with the hydrophobic cavity [25]. When the dansyl amino acid was transferred from the mobile to the stationary phase, stronger H-bonding and/or van der Waals interactions replaced the solute–solvent interactions and ΔH° decreased. The greater immobilization effect following the binding process explained the decrease in $(\Delta S^\circ/R)+\ln \phi$ values.

4. Conclusion

This work investigated the role of a mobile phase modifier such as perchlorate anion in controlling the type of interaction involved in the dansyl amino acid binding on HSA. Reversed-phase behavior occurred only when the cavity was more hydrophobic than the bulk mobile phase, i.e below a perchlorate anion concentration equal to 0.02–0.03 M. As the amount of mobile phase modifier increased, the competition of perchlorate anion for the non polar residues of site II caused dansyl amino acid to interact preferentially with polar residues at binding cavity through a normal-phase mode.

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