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Solute complexation degree with human serum albumin: biochromatographic approach

Y.C. Guillaume[a,*], J. Millet[b], L. Nicod[c], T. Truong-Thanh[a], C. Guinchard[a], A. Xicluna[d], M. Thomassin[a]

[a]*Laboratoire de Chimie Analytique, Faculté de Médecine et de Pharmacie, Place Saint-Jacques, 25030 Besançon Cedex, France*
[b]*Laboratoire de Galénique, Faculté de Médecine et de Pharmacie, Place Saint-Jacques, 25030 Besançon Cedex, France*
[c]*Laboratoire de Biologie Cellulaire, Faculté de Médecine et de Pharmacie, Place Saint-Jacques, 25030 Besançon Cedex, France*
[d]*Laboratoire de Chimie Thérapeutique, Faculté de Médecine et de Pharmacie, Place Saint-Jacques, 25030 Besançon Cedex, France*

Abstract

A mathematical model was developed for the study of the D,L-dansylamino acid retention mechanism in reversed-phase liquid chromatography using a C_{18} column as a stationary phase and human serum albumin (HSA) as an eluent modifier. The solute retention factor is dependent on the HSA concentration in the eluent as well as the binding constant of the guest–HSA complex. A determination of the degree of complexation n_c (the percent of the complexed guest) could be carried out. Different Van 't Hoff plot shapes of the degree of complexation were observed with different eluent pH, confirming a change in the solute complexation mechanism for physiological pH (between 7–7.5). Enthalpy–entropy compensation was also analysed in relation to this mathematical model to confirm the solute complexation behavior with HSA. These results finally confirmed that at physiological pH and temperature (~35°C) values the HSA was in a favorable structural conformation for its binding with a great majority of drugs. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Enthalpy–entropy compensation; Complexation degree; Human serum albumin; Dansyl amino acids

1. Introduction

Several chromatographic or electrophoretic separations based on a host–guest complexation have been described in the literature. These methods implied the use of cyclodextrins [1–3], crown-ether [4–7], calixarenes [8,9] or proteins [10–17] as a stationary phase or mobile phase additives. These techniques are very convenient to assess the apparent association constant of the complex formed when the host molecule is added to the mobile phase or the electrolyte. Studies concerning host–guest complex between the solute molecule and proteins added to the running buffer, i.e., bovine serum albumin (BSA) and human serum albumin (HSA) have been performed in capillary electrophoresis [18,19]. HSA was the model ligand used in a great number of these studies. The main advantage of using HSA is that data are available for its interaction with a wide range of organic and inorganic compounds [20]. These interactions occurred principally at two sites I and II, which are also called the warfarin binding site and benzodiazepine binding site [21–23]. Together,

*Corresponding author. Tel.: +33-3-8166-5546; fax: +33-3-8166-5655.
E-mail address: yves.guillaume@univ-fcomte.fr (Y.C. Guillaume).

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these two sites account for the majority of compound interactions with HSA [21].

The analytes tested in this work were a series of D,L-dansylamino acids. One attractive feature of these test compounds is that they are all believed to have a single site on HSA, i.e., site II [14,17]. In addition, both of the major HSA binding regions, the indole and warfarin sites are represented by the interactions of these analytes. The aim of this paper was to study the D,L-dansylamino acid retention on a C_{18} column using HSA as mobile phase additive in such a way that the percentage of the guest that contributes to the formation of the complex (degree of complexation n_c) was determined at different pH values of the mobile phase. A novel model, based on multiple equilibria between HSA, the D,L-dansylamino acid molecule and both the stationary and the mobile phases, was built and applied to the compound behavior in this chromatographic system. Thermodynamic data were determined from Van't Hoff plots and the enthalpy–entropy compensation was studied in relation to this model.

2. Theory

High-performance liquid chromatography (HPLC) is a very convenient method of studying the equilibria taking place in solutions, especially in the mixtures of water and organic modifier. The equilibrium of the species M with the stationary phase S is a follows:

$$\mathrm{M} + \mathrm{S} \leftrightarrows \mathrm{M{-}S} \quad (1)$$

The following reaction can be used to describe the binding of the analyte M to the single binding site on HSA, i.e., site II, in the mobile phase:

$$\mathrm{M} + \mathrm{HSA} \leftrightarrows \mathrm{M.HSA} \quad (2)$$

It is important to note, that with the three pH values of the mobile phase used in this study (7.0, 7.5 and 8), the HSA molecule was negatively charged (at pH 8 albumin charge = −26) [24]. The HSA protein was assimilated so that it was a multivalent spherical particle with negative charges uniformly distributed on its surface. Therefore, the hydrophobic alkylstationary phase modification with HSA which causes a slow resistance to mass transfer [25–28] can be neglected due to the fast desorption of the negatively charged HSA. This fact was corroborated by its very low retention time ~1.10 min which is approximately the same as the column dead time. The equilibrium of the species M.HSA with the stationary phase S is:

$$\mathrm{M.HSA} + \mathrm{S} \leftrightarrows \mathrm{M.HSA{-}S} \quad (3)$$

The equilibrium constant for Eq. (2) is:

$$K = [\mathrm{M.HSA}]/[\mathrm{M}] \cdot [\mathrm{HSA}] \quad (4)$$

If all equilibria are fast, the retention factor of M is a linear combination of its retention factor in a free solution (without HSA), k'_{M}, and its retention factor when it is totally complexed, $k'_{\mathrm{M.HSA}}$:

$$k' = x_{\mathrm{M}} k'_{\mathrm{M}} + x_{\mathrm{M.HSA}} k'_{\mathrm{M.HSA}} \quad (5)$$

where x_{M} and $x_{\mathrm{M.HSA}}$ are the molar fractions of the free and complexed forms:

$$x_{\mathrm{M}} = [\mathrm{M}]/([\mathrm{M}] + [\mathrm{M.HSA}]) \quad (6)$$

and:

$$x_{\mathrm{M.HSA}} = [\mathrm{M.HSA}]/([\mathrm{M}] + [\mathrm{M.HSA}]) \quad (7)$$

Using Eqs. (4)–(7), k' can be given by the following equation:

$$k' = (k'_{\mathrm{M}} + k'_{\mathrm{M.HSA}} K[\mathrm{HSA}])/(1 + K[\mathrm{HSA}]) \quad (8)$$

The free (unbound) HSA concentration is:

$$[\mathrm{HSA}] = [\mathrm{HSA_t}] - [\mathrm{HSA_b}] \quad (9)$$

where $[\mathrm{HSA_t}]$ and $[\mathrm{HSA_b}]$ are, respectively, the total HSA and total bound HSA concentrations in the mobile phase. The average number n_c of HSA molecules bound per molecule is:

$$n_c = [\mathrm{HSA_b}]/[\mathrm{M_t}] \quad (10)$$

where $[\mathrm{M_t}]$ is the total solute concentration in the mobile phase obtained from total amount of solute loaded on the column and volume of the mobile phase. Therefore, combining Eqs. (8), (9) and (10) gives:

$$k' = \{k'_{\mathrm{M}} + k'_{\mathrm{M.HSA}} K([\mathrm{HAS_t}] - n_c[\mathrm{M_t}])\}/\{1 + K([\mathrm{HSA_t}] - n_c[\mathrm{M_t}])\} \quad (11)$$

In this equation k'_M, $k'_{M.HSA}$, K and n_c are constant. The proposed non linear model involves no approximation of the concentration of the two species M and HSA and correlates their concentrations with the effective retention factors of the free k'_M and complexed $k'_{M.HSA}$ solute. Enthalpy and entropy of transfer of the solute molecule to the alkyl stationary phase S (ΔH^0 and ΔS^0, respectively) are determined by plotting the logarithm of k' in relation to the temperature reciprocal:

$$\ln k' = -\Delta H^0/RT + \Delta S^0/R + \ln \phi \qquad (12)$$

For a linear plot, the slope and intercept are $-\Delta H^0/R$ and $\Delta S^0/R + \ln \phi$. R is the gas constant and ϕ the column phase ratio (volume of the stationary phase divided by the volume of the mobile phase). Usually, ΔS^0 is not provided due to the ambiguity in the calculation of the phase ratio for commercial columns. Nevertheless, ΔS^0 and $\Delta S^{0*} = \Delta S^0/R + \ln \phi$ are identical variations.

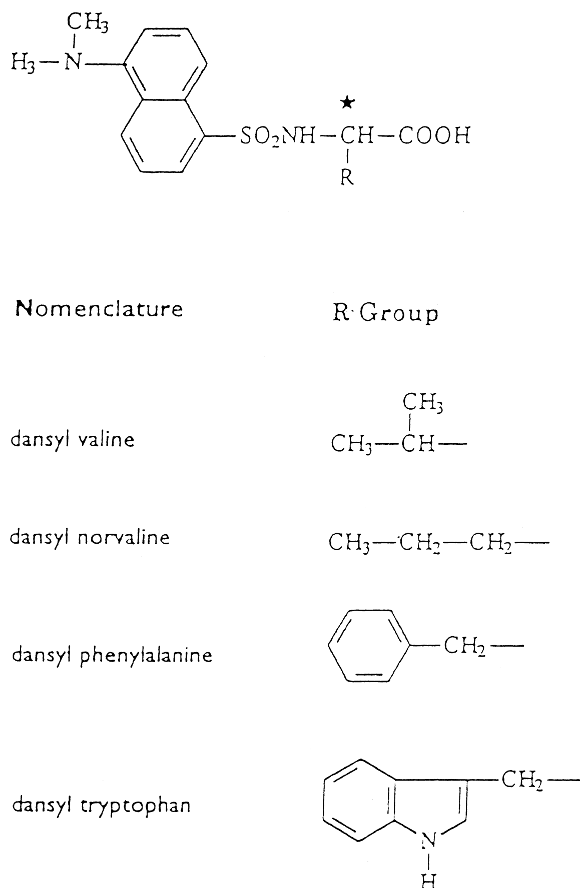

Fig. 1. Dansylamino acid structures.

3. Experimental

3.1. Apparatus

The HPLC system consisted of a HPLC Waters pump 501 (Saint Quentin, Yvelines, France), an Interchim Rheodyne injection valve Model 7125 (Montluçon, France) fitted with a 20-μl sample loop and a Merck 2500 diode array detector (Nogent-sur-Marne, France). An Interchim 125 mm×4 mm I.D. RP 18 column (5 μm, particle size) was used with a controlled temperature [in an Interchim oven, TM No. 701 for high temperatures and an OSI Julabo FT 200 cryoimmerser (Elancourt, France) for low temperatures]. The mobile phase was fixed at 1 ml/min and the wavelength at 254 nm.

3.2. Reagents

All the D,L-dansylamino acids were obtained from Sigma–Aldrich (Saint-Quentin, France). The chemical structures of these compounds are given in Fig. 1. Fresh samples were prepared daily in acetonitrile at a concentration varying from 0.1 to 1 m*M*. Sodium nitrate (Merck) was used as a dead time marker. Sodium hydrogenphosphate and sodium dihydrogenphosphate were supplied by Prolabo (Paris, France). HPLC-grade acetonitrile (Merck, Paris, France) was used without further purification. Water was obtained from an Elgastat option water purification system (Odil, Talant, France) fitted with a reverse osmosis cartridge. The mobile phase consisted of $2 \cdot 10^{-4}$ *M* sodium phosphate buffer–acetonitrile (ACN) mixture. In a previous paper [29], it was demonstrated that the dansylamino acid solvation by ACN was minimal for low ACN fraction ($\cong 0.12$) for which the electrostatic interactions between HSA binding cavity and dansylamino acid were maximal [29]. In addition, whatever the chosen ACN fraction value in the range 0–0.12 (v/v) the geometrical characteristics of the cavity were the same. Thus, in this study, the ACN fraction in the phosphate buffer was fixed

at 0.12 (v/v). The buffer pH were adjusted to values equal to 7, 7.5 and 8. The mobile phase contained 0.005 *M* of triethylamine to reduce the silanophilic effect. The mobile phases, at all pH values were stored for 1, 2 and 4 h at ambient room temperature to study the accuracy of their pH values. No fluctuations were observed; the maximum relative difference of the pH values of the different mobile phases was always 0.4%. The variation range in the mobile phase of the HSA (Aldrich, Paris, France) was 1.50–4.50 μ*M*. A 20-μl volume of each solute was injected in triplicate and the retention times were measured.

3.3. Temperature studies

Compound retention factors were determined over the temperature range −2 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 compound retention time of the D-dansylvaline was measured every hour for 7 h and again after 22, 23 and 24 h. The maximum relative difference of the retention time of this compound was 0.6% making the chromatographic system sufficiently equilibrated for use after 1 h.

4. Results and discussion

4.1. Validation of the retention model

To obtain the constant k'_{M}, $k'_{M.HSA}$, K and n_c, at 25°C and pH 8, the retention factors of all the solutes were determined for a wide range of concentrations of HSA and M. All the experiments were repeated three times. The variation coefficients of the k' values were less than 1% in most cases indicating a high reproducibility and good stability for the chromatographic system. Using a non linear regression procedure (NLRP) as described in a previous paper [30–32], for various chromatographic studies the data were fitted to Eq. (11). After the NLRP, the previous constants of Eq. (11) were determined and the correlation between the predicted by Eq. (11) and experimental k' values can be obtained and exhibited a slope equal to 0.997 (ideal is 1.000) and an $r^2 = 0.998$ (Fig. 2). This correlation between the theoretical and experimental values was considered adequate to verify the model. Table 1 contains a complete list of k'_{M}, and $k'_{M.HSA}$ values for the D enantiomer, for example, at pH 8 and 25°C. The weak value of $k'_{M.HSA}$ showed that the solute–HSA complex retention was negligible. A similar conclusion was obtained for L enantiomers. The Van't Hoff

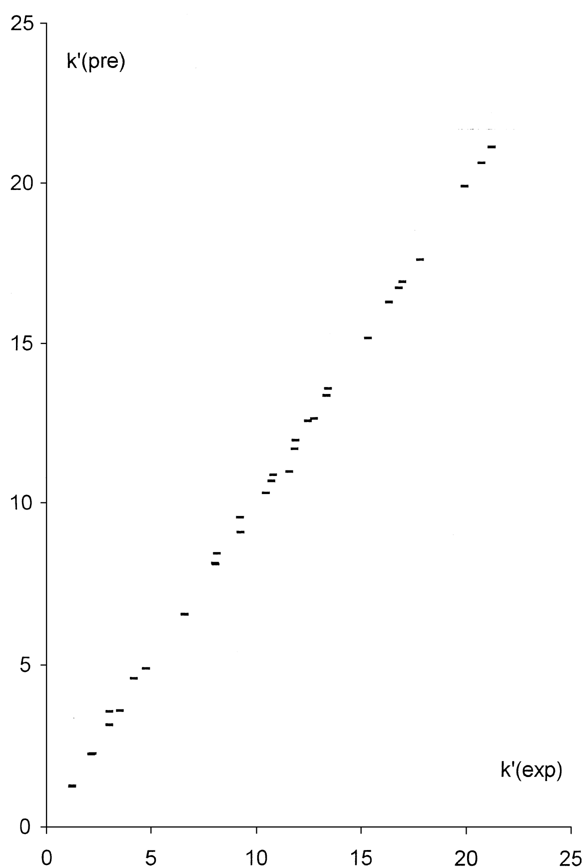

Fig. 2. Correlation between the predicted (Eq. (11)) and the experimental retention factor for the D,L-dansylamino acid derivatives. The slope is 0.997 with a correlation coefficient of 0.998, as determined by linear regression.

Table 1
k'_{M}, and $k'_{M.HSA}$ values for D enantiomers at pH 8 and at a column temperature equal to 25°C

	k'_{M}	$k'_{M.HSA}$
Dns valine	5.32	0.31
Dns norvaline	7.14	0.32
Dns phenylalanine	15.25	0.52
Dns tryptophan	22.56	0.56

plots (Eq. (12)) analysed at pH 8 were all linear for the solute molecules at all the $[HAS_t]$ and $[M_t]$ values. The correlation coefficients for the linear fits were in excess of 0.97. These linear behaviors were thermodynamically what were expected when there was no change in the transfer mechanism of the solute molecule from the mobile to the stationary phase in relation to temperature [30,33]. All ΔH^0 and ΔS^{0*} values were negative which showed that this retention mechanism was enthalpically controlled. This was consistent with results reported in the literature for various chromatographic systems [30,34]. In order to gain further insight into the validity of the interaction model, the enthalpy–entropy compensation was examined. This approach has been previously used in chromatographic procedures to analyse and compare the retention mechanism for a group of compounds [30,35–37]. The enthalpy–entropy compensation can be described by the following equation [37]:

$$\Delta H^0 = \beta \Delta S^0 + \Delta G^0_\beta \tag{13}$$

where ΔG^0_β is the Gibbs free energy of a physicochemical interaction at a β compensation temperature. Eq. (13) shows that if a plot of ΔH^0 against ΔS^0 is linear, then the solutes are retained by an essentially identical interaction mechanism. A $\Delta H^0 - \Delta S^0$ plot determined at the different values of $[HSA_t]$, $[M_t]$ was drawn at pH 8 for the D and L enantiomers. The correlation coefficient for the linear fit was equal to 0.990. Fig. 3 shows ΔH^0 values plotted in relation to ΔS^{0*}. This degree of correlation can be considered to be adequate to verify enthalpy–entropy compensation, and indicating that the interaction mechanism was independent of (i) the HSA and M concentrations in the mobile phase (ii) the R chain structure (iii) the asymmetric carbon configuration (D or L). This result is in accordance with the fact that D and L enantiomers bind on the same site on HSA, i.e., site II [14,17]. This also confirmed that the complex M. HSA was principally formed in the mobile phase and, thus, the eventual adsorption of the HSA on the alkylstationary phase and the possible complexation of enantiomer with the coated HSA seemed to be negligible as expected in our model. Table 2 contains a complete list of n_c values for D and L enantiomers at pH 8 and 25°C. In

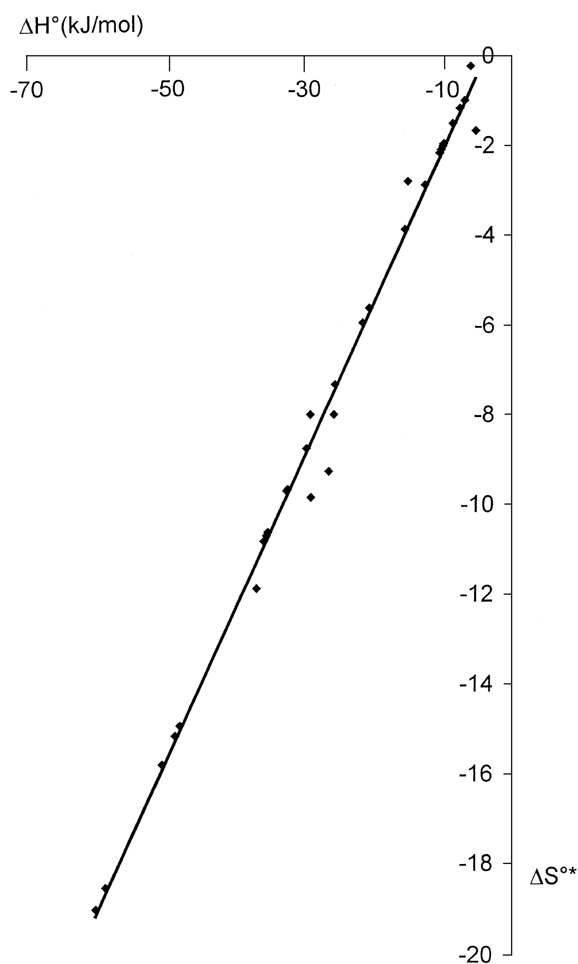

Fig. 3. Enthalpy–entropy compensation represented by the $\Delta H^0 - \Delta S^{0*}$ plots at pH 8 and at the different values of $[M_t]$, and $[HAS_t]$ for all the dansylamino acid derivatives.

all cases $n_{c,D} > n_{c,L}$ which corroborated the fact that the D enantiomer interacted more favorably with the binding cavity than the L enantiomer [14,17]. The dansyl tryptophan had the highest n_c value and

Table 2
Degree of complexation for the D and L enantiomers ($n_{c,D}$ and $n_{c,L}$) of the dansylamino acids at pH 8 and a column temperature equal to 25°C

	$n_{c,D}$	$n_{c,L}$
Dns valine	0.67	0.62
Dns norvaline	0.69	0.64
Dns phenylalanine	0.86	0.76
Dns tryptophan	0.91	0.81

dansyl valine the lowest. This fact which showed that the binding cavity on HSA interacted more favorably for the more hydrophobic species, was in accordance with previous results [14,17]. The model parameters corresponding to Eq. (11) were also determined at pH 7 and 7.5 and in the temperature range −2 to 35°C for each D and L enantiomer. The solute complexation isotherms (ln n_c versus $1/T$) were drawn. In all cases, n_c values decreased with increasing temperature. Ln n_c versus $1/T$ plots were linear at pH 8 (Fig. 4) for each enantiomer, but presented a break for pH 7 or 7.5 (Fig. 4) at $T \sim T_c \sim 4$°C. It has been demonstrated by differential scanning colorimetry (DSC) [17] that when the pH are near the physiological pH (between pH 7 and 7.5) the HSA protein structure is in equilibrium between a disordered and an ordered solid like state [17,38]. For pH 7 and 7.5, at $T > T_c$ the binding cavity was in a more ordered state than for $T < T_c$ and the enantiomer molecule interacted more easily [17]. This was corroborated by the magnitude of the curve slope (ln n_c vs. $1/T$) which was higher for $T < T_c$ than for $T > T_c$ (Table 3 and Fig. 5). For pH 8, the HSA binding cavity was always in an ordered

Table 3
Temperature contribution to the solute complexation degree changes $\partial \ln n_c / \partial(1/T)$ (K) for the D enantiomers at pH 7.5 (for $T < T_c$ and $T > T_c$) and pH 8

	pH 8	pH 7.5	
		$T < T_c$	$T > T_c$
Dns valine	567	1400	635
Dns norvaline	598	1640	680
Dns phenylalanine	727	1700	810
Dns tryptophan	780	1885	920

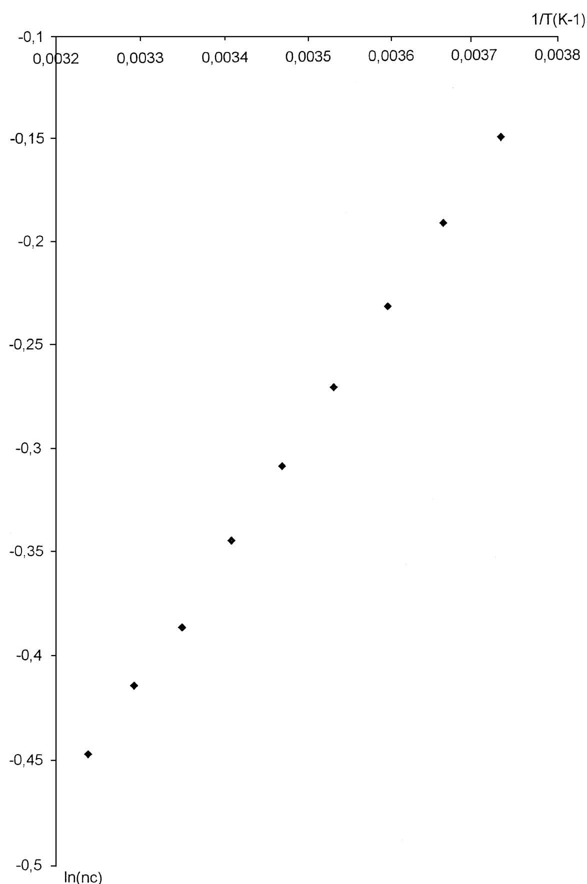

Fig. 4. Ln n_c versus $1/T$ plot for D-dansylvaline at pH 8.0.

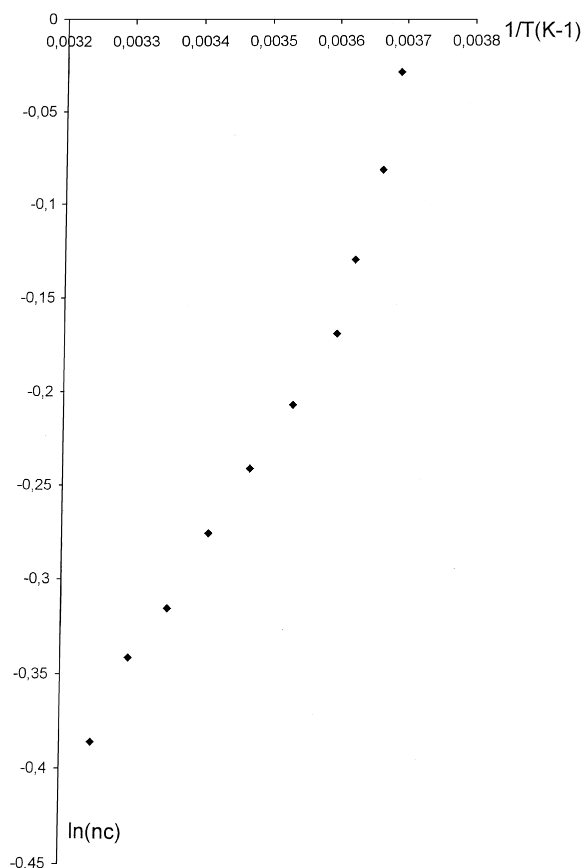

Fig. 5. Ln n_c versus $1/T$ plot for D-dansylvaline at pH 7.5.

solid like state [17]. Thus, the solute complexation process was the same as for the physiological pH (7 and 7.5) at $T > T_c$. In summary, this paper described a model that could determine the contribution of HSA to the D,L-dansylamino acid retention on a alkylstationary phase at different pH values. Using this treatment it was possible to describe the different equilibria which were implied when the solute molecule was transferred from the mobile to the stationary phase. As this model requires only the initial concentrations of the two species (HSA and M), without any limitations and approximations, experimental and theoretical shortcomings are avoided. This model was validated by both a non linear regression procedure and an enthalpy–entropy compensation. With this approach the solute complexation isotherms could be calculated at different pH values. These data confirmed the previous DSC measurements, i.e., the existence of a phase transition in the HSA binding cavity near the physiological pH. At these pH values (7 and 7.5) and at a physiological temperature ~35°C, the HSA (site II) was in a favorable conformation (ordered solid-like state) to be able to bind with a large number of drugs.

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