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Journal of Chromatography A, 977 (2002) 185–192

JOURNAL OF CHROMATOGRAPHY A

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Salt effects on the interaction of an amphiphilic model molecule with immobilized phosphatidylcholine monolayers

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Received 5 June 2002; accepted 6 August 2002

Abstract

The retention of hydrocortisone (used as an amphiphilic model solute) on an immobilized artificial membrane (IAM) column was investigated in relation to the mobile phase concentration of three sodium salts (representing different rankings in the Hofmeister series, i.e. perchlorate, chloride and sulfate) in order to provide insight into the nature of the solute interactions with phospholipid monolayers. The influence of the salt series on solute retention was found to follow the Hofmeister series, emphasizing the role of hydrophobic effect in the solute retention mechanism on phospholipid monolayers. Retention models based on the extended Wyman relations (preferential interaction theory) were developed to analyze more quantitatively the salt effects on the hydrocortisone retention factor. This analysis as well as additional thermodynamic study suggested that the hydrocortisone binding to IAM involved both an insertion into the hydrophobic inside governed by hydrophobic effects and contacts with the interfacial region implying interactions such as van der Waals interactions/hydrogen bonds between the solute hydroxyl groups and the polar headgroups of phospholipid monolayers.

Keywords: Salt effects; Immobilized artificial membranes; Retention models; Amphiphilic solute; Membranes; Thermodynamic parameters; Phosphatidylcholine; Hydrocortisone

1. Introduction

Many membrane-like systems have been developed to study the interactions between drugs or biological compounds and lipid layers. The partitioning of bioactive molecules between lipid layers and aqueous solutions has been extensively analyzed using small or large unilamellar vesicles via sedimentation, equilibrium dialysis or ultrafiltration methods [1]. Recently, a number of valuable alternative approaches have been explored using lipid monolayers or bilayers fixed to a support. They include, for example, acoustic techniques, surface plasmon resonance or immobilized artificial membrane chromatography (IAMC). Chromatographic procedures based on IAM columns were introduced firstly by Pidgeon et al. [2]. Monolayers, consisting of phospholipid analogues, were coupled covalently to the silica support of a HPLC column. The immobilized artificial membrane column mimics the lipid environment of fluid cell membranes on a solid

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0021-9673/02/$ – see front matter
PII: S0021-9673(02)01352-3

matrix. IAMC is classically used for membrane protein purification and the prediction of drug transport through cell membranes [3–5]. Several authors have used IAMC as a convenient tool to obtain information about the interaction between phospholipid monolayers and compounds. Studies on temperature dependence of bioactive peptide retention on immobilized phosphatidylcholine monolayers have been performed by Mozsolits et al. [6]. The study of the changes in enthalpy and entropy associated to the transfer of the solutes from the mobile to the stationary phase indicated that peptides interact with the monolayers predominately through a hydrophobic effect. Analysis based on the comparison between several IAM bonded phases with various ligand densities has further demonstrated that the interactions between phenol derivatives or β-blockers and immobilized phospholipid monolayers are mostly based on a partitioning mechanism [7,8]. Another approach for studying the interactions between solutes and phospholipid monolayers has involved the variation of the mobile phase composition. Classically, it has been approached by varying the proportion of the organic modifier or the pH of the mobile phase. A study on the eluent methanol percent dependence on the retention factor has been conducted in order to examine the role of the fluidity of the immobilized lipid monolayers [6]. A non-linear relationship between log k and percent methanol for peptides has been interpreted by the authors as a consequence of changes in both the peptide conformation and the lipid mobility. Additionally, the retention for a varied group of compounds on an IAM column has been investigated for two mobile phases consisting of methanol–water and acetonitrile–water mixtures [9]. It was expected by the authors that the main factors contributing to solute retention were favorable cavity formation and dispersion interactions. Also, the analyses of the influence of the mobile phase pH on the retention of β-adrenolytic drugs and *m*-nitroaniline indicates that the solute interaction with the stationary phase is dominated by a partitioning mechanism [10].

However, only few papers have reported the effects of the variation of the eluent ionic strength on the solute retention in IAMC. Moreover, the knowledge of the salt effect operative in an interacting system could provide a valuable insight into the nature of the binding. Such an approach is of great interest to analyze the relative contributions of driving forces implied in various chromatographic retention processes [11–13]. The choice of the salt is very important. In a hydrophilic/hydrophobic two-phase system, the salts must be chosen for their strong abilities to promote (kosmotropic agent) or inhibit (chaotropic agent) the hydrophobically driven phenomena, according to their position in the Hofmeister series. The anions (keeping sodium as cation) can be arranged in the following order, with regard to their hydrophilicity: $ClO_4^- < SCN^- < I^- < NO_3^- < Br^- < Cl^- < F^- < SO_4^{2-}$ [14]. In a hydrophilic/hydrophobic two-phase system containing the sodium salts, the SO_4^{2-} anion has an affinity for the more hydrophilic phase (kosmotropic character) while the anion ClO_4^- tends to partition to the more hydrophobic phase (chaotropic character). NaCl is usually placed in the center of the Hofmeister series indicating neither chaotropic nor kosmotropic character.

The aim of this paper was to investigate the thermodynamic driving forces for the interaction of hydrocortisone with phosphatidylcholine monolayers by varying salt species and concentration. The use of an amphiphilic molecule such as hydrocortisone as model solute was motivated by the well-known feature that partitioning of several small molecules of biological and medical interest (peptides, drugs or hormones) into lipid bilayers are related to their amphiphilic character [15]. Using the three sodium salts representing different rankings in the Hofmeister series (i.e. sulfate, chloride and perchlorate), the hydrocortisone retention variation with salt concentration was evaluated using interaction models based on the extended Wyman relations. As well, thermodynamic studies were carried out and the enthalpy and entropy variations were discussed relative to salt concentration.

2. Theory

Previous papers have reported that relations derived from the Wyman concept constitute a valuable tool to describe the salt dependence on the analyte retention in hydrophobic [16,17] or electrostatic [12] interaction chromatography and, more recently, in

affinity chromatography [13,18]. The major advantage of the Wyman functions consists in the fact that the variation of the association constant between a ligand and a solute can be described whatever the chemical properties of the mobile phase additives. The theory of linked functions allows to follow the change in the ligand binding when different additives such as kosmotropic or chaotropic agents are added into the medium.

The effects of salt on the equilibrium constant K between a solute (S) and a ligand (L) can be modeled at a thermodynamic level in terms of the direct stoichiometric participation of ions (v_x) and water (v_w) in the association reaction. The stoichiometric representation of the association is as follows:

$$L(v_{Lx}, v_{Lw}) + S(v_{Sx}, v_{Sw}) \rightarrow L.S(v_{LSx}, v_{LSw}) + \Delta v_x + \Delta v_w \quad (1)$$

where

$$\Delta v_x = v_{LSx} - (v_{Lx} + v_{Sx}) \quad (2)$$

and

$$\Delta v_w = v_{LSw} - (v_{Lw} + v_{Sw}) \quad (3)$$

Based on Eq. (1), the dependence of K on the mean ionic activity a_x can be formulated via the linkage Wyman relations modified by Tanford [12,19,20]:

$$\frac{d(\ln K)}{d(\ln a_x)} = (\Delta v_x) - \frac{pm_x}{55.6}(\Delta v_w) \quad (4)$$

where p and m_x are the aggregate valency and the molal concentration of salt, respectively. In assuming that replacing the ionic activity by salt concentration c_x introduces little error over the experimental salt concentration range, an approximate integrated form of Eq. (4) is obtained as previously reported [20]:

$$\ln K \cong \ln K_0 + (\Delta v_x) \ln c_x - \frac{p}{55.6}(\Delta v_w)c_x \quad (5)$$

where K_0 is the binding constant in a hypothetical 1 M salt concentration reference state. In a chromatographic system, the retention factor k is directly proportional to K:

$$k = \frac{t_R - t_0}{t_0} = \phi K \quad (6)$$

where t_R and t_0 are the retention times of a retained and a non-retained solute, respectively, and ϕ is the phase ratio.

Therefore, the following relation can be obtained by combining Eqs. (5) and (6):

$$\ln k \cong \ln k_0 + (\Delta v_x) \ln c_x - \frac{p}{55.6}(\Delta v_w)c_x \quad (7)$$

where k_0 is the retention factor corresponding to K_0.

It can be noted that similar relationships have been developed by Melander et al. [11] for the analysis of the overall salt effects on the retention of proteins in ion-exchange chromatography, combining solvophobic and counterion condensation theories.

3. Experimental and methods

3.1. Apparatus

The HPLC system consisted of an LC Shimadzu pump 10AT (Touzart et Matignon, Courtaboeuf, France), a Rheodyne injection valve model 7125 (Interchim, Montluçon, France) fitted with a 20-μl sample loop, a Shimadzu SPD-10A UV–visible detector. A Regis 30 mm×4.6 mm IAM.PC.MG HPLC column (packed with 12-μm particles) was used with controlled temperature in an Igloocil oven (Interchim).

3.2. Reagents and operating conditions

Hydrocortisone was obtained from Sigma Aldrich (Saint-Quentin, France). Sodium salts (sulfate, chloride and 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. The mobile phase (flow-rate: 0.8 ml/min) consisted of phosphate buffer 5 mM adjusted at pH 7.4 with various concentrations (100 to 600 mM) of sodium salts. To examine the concentration dependencies of solute retention corresponding to the binding capacity of immobilized phospholipids, retention measurements were related to varying amounts of injected solute. Solute samples were prepared at different concentrations in the mobile phase from 5 to 40 mg ml^{-1}. Twenty μl of the solute at a concentration of

20 mg ml^{-1} (where the retention was sample concentration-independent, i.e. in linear elution conditions) were injected in triplicate and the retention times were measured.

3.3. Non-linear regression analysis of retention data

The model equation was fitted to the retention factors of the solutes by a non-linear regression using the software Table curve 2D (SPSS Science Software, Erkrath, Germany).

3.4. Temperature studies

The retention factor was determined at the following temperatures: 25, 30, 35, and 40 °C. The chromatographic system was allowed to equilibrate for at least 1 h prior to each experiment. ΔH and ΔS are, respectively, the standard enthalpy and entropy of transfer of hydrocortisone from the mobile to the stationary phase. These energies can be calculated using the following thermodynamic relationships:

$$\ln k = \frac{-\Delta H}{RT} + \Delta S^* \quad (8)$$

with

$$\Delta S^* = \frac{\Delta S}{R} + \ln \phi \quad (9)$$

where T is the temperature and R the gas constant. For a linear plot of ln k versus $1/T$, the slope and intercept are respectively $-\Delta H/R$ and ΔS^*.

4. Results and discussion

4.1. Analysis of the salt effects on the hydrocortisone affinity to IAM

The retention factors for hydrocortisone were determined at various column temperatures with three sodium salts (sulfate, chloride or perchlorate) as mobile phase additives. The RSDs of the k values were less than 0.7%, indicating a high reproducibility and a good stability for the chromatographic system. Three retention behaviors were observed in relation to the salt type. Fig. 1 shows the ln k–c_x plots at

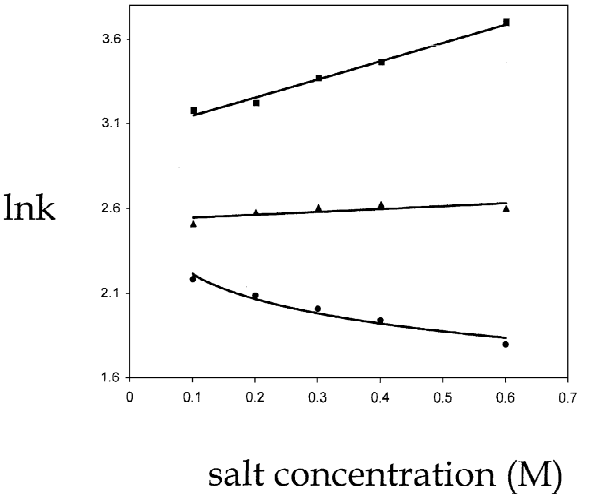

Fig. 1. Plots of ln k against sodium salt (sulfate (■), chloride (▲), perchlorate (●)) concentration for hydrocortisone at T= 25 °C. The theoretical curves (———) are recreated from Eqs. (10) and (11) for sulfate and perchlorate anions. See operating conditions in the Experimental.

25 °C. For a given salt concentration, the solute affinity increases in relation to the salt position in the Hofmeister series, i.e. $k_{ClO_4^-} < k_{Cl^-} < k_{SO_4^{2-}}$. Moreover, the solute retention variation with salt concentration increasing follow the Hofmeister series. The kosmotropic salt (sodium sulfate) tends to increase the hydrocortisone retention on the stationary phase by minimizing the solute contact area exposed to the solvent. NaCl, which is in the center of the Hofmeister series (Na^+ is a small kosmotrope while chloride is more or less chaotropic), has a nil effect on the solute retention over the concentration range. Finally, the chaotropic anion (sodium perchlorate) decreases the solute interaction with IAM due to its ability to interact with the hydrophobic surfaces of the solute and the stationary phase. These results are consistent with a hydrocortisone retention mechanism driven by hydrophobic effects between the solute and the phospholipid monolayers. It involves that the solute penetrates, more or less strongly, into the hydrophobic chain region of the lipids through a partitioning mechanism, as shown previously for various hydrophobic solutes [7–10]. In order to further investigate the solute retention mechanism, a quantitative analysis of the data (for the kosmotropic and chaotropic agents) was carried

out by fitting the model equation to the experimental data.

In the case of sodium sulfate, the use of the following simplified relation was sufficient to fit this experimental data:

$$\ln k \cong \ln k_0 - 0.054(\Delta v_w)c_x \quad (10)$$

In this case, the influence of the salt term was neglected in relation to the water effect. The Δv_w parameter (corresponding to the slope of the ln k–c_x plots) was calculated using Eq. (10) for all the column temperatures. The theoretical curve (at 25 °C) and the regression coefficients R^2 are presented in Fig. 1 and Table 1, respectively. Due to the high degree of hydration of sulfate (preferential hydration) [21], increasing the sulfate concentration decreases the effective concentration of water in the bulk phase and consequently decreases the water activity. Water molecules in contact with non-polar surfaces adopt an ordered organisation. The release of water molecules (negative value of Δv_w) involved in the association of hydrocortisone with immobilized phospholipids results in an increase in entropy contributing to the increase in solute affinity for the stationary phase. Around 20 water molecules are involved in the solute transfer from the aqueous mobile phase to the IAM stationary phase at 25 °C (Table 1). The number of water molecules released per mole of solute associated to a ligand is dependent on the contact surface between the two species [13,16,20,22]. If it is assumed that the hydration of the water-accessible surfaces corresponds to a monolayer of water as reported previously [20,22], and that one water molecule covers ~10 Å^2 of surface [22], then it can be calculated from the hydrocortisone water-accessible surface [23] that a *minimum* of 57 water molecules is released from the water-accessible surfaces in the case of a full solute insertion into the hydrophobic core of IAM. This is significantly higher than the total of 20 water molecules released upon solute binding at 25 °C. This result indicates that the hydrocortisone is not fully embedded in the hydrophobic region of lipid monolayers. It suggests also that some contacts are engaged between the solute and the phospholipid headgroups. Similar conclusions have been made by Rowe et al. [24] for the analysis of partitioning of another type of amphiphilic molecules (i.e. longer-chain alcohols) into phospholipid bilayers. It has been demonstrated by titration calorimetry that while hydrophobic effects play a major role in the partitioning, the solute binding also involves changes in the interfacial region of the phospholipid layers. Okamura and Nakahara [25] have shown by an NMR study that propylbenzene is deeply penetrated into the hydrophobic region of the phospholipid bilayer core while phenol is preferentially trapped in the interfacial region near the carbonyl group of the phospholipids. Additionally, Dimitrov and Lalchev [26] have shown that the capacity of steroid to interact with the phospholipid monolayers is dependent on both the hydrocarbon chain (governing the hydrophobic insertion into the lipid core) and the number of hydroxyl groups which are able to engage hydrogen bonds with both the carbonyl [26] and phosphate [27] groups of the phospholipids. As hydrocortisone contains three hydroxyl groups, one could expect that the solute contacts with the phospholipid headgroups involve, at least in part, hydrogen bonds with the polar interfacial sites of phospholipid monolayers.

Table 1
Determination of the model parameters by fitting Eqs. (10) and (11) to the hydrocortisone retention factors (R^2 is the regression coefficient) for the kosmotropic and the chaotropic salts at various column temperatures

Temperature (°C)	Sodium sulfate Eq. (10)		Sodium perchlorate Eq (11)	
	R^2	Δv_w	R^2	Δv_x
25	0.987	−20	0.953	−0.20
30	0.993	−17	0.941	−0.20
35	0.935	−13	0.992	−0.20
40	0.956	−12	0.993	−0.20

In the case of sodium perchlorate, it was possible to fit the experimental data using the following simplified relation:

$$\ln k \cong \ln k_0 + (\Delta v_x) \ln c_x \quad (11)$$

It corresponded to the case where the influence of the water term was neglected in relation to the direct salt effect. The Δv_x parameter (corresponding to the slope of the ln k–ln c_x plots) was calculated using Eq. (11) for all the column temperatures. The

theoretical curve (at 25 °C) and the regression coefficients R^2 are presented in Fig. 1 and Table 1, respectively. It is well-known that chaotropic anions such as perchlorate or thiocyanate are able to bind to the hydrophobic surfaces due to their low free energy of hydration [21,28,29]. So, the perchlorate concentration dependence on the solute retention can be interpreted as a competitive salt-specific binding to the solute and the stationary phase hydrophobic surfaces. The Δv_x values (Table 1) are in agreement with the values of the salt release for the solute binding to protein or DNA [30,31]. These low values can be interpreted as a consequence of relatively weak interactions of the anion with hydrophobic surfaces of the ligand. Moreover, assuming a lipid per binding site stoichiometry equal to 1:1, Macdonald and Seelig [32] have reported a binding constant of 1.3 M^{-1} for thiocyanate. Kalinin et al. [33] found a value of 21 M^{-1} for the perchlorate binding and 8 M^{-1} for the thiocyanate binding while Clarke and Lupfert [21] determined a value of 9.9 M^{-1} for the perchlorate binding.

4.2. Temperature effects on the solute retention on IAM

In order to gain further information about the mechanistic aspects of the solute binding to phospholipid monolayers, the temperature influence on the solute retention was investigated. The ln k versus $1/T$ plots were obtained for each concentration of the three salts. Linear van't Hoff plots were observed with R^2 higher than 0.937 for the kosmotropic salt (Fig. 2). When the temperature increases, solute binding on immobilized phospholipid monolayers decreases in the three cases. This behaviour is in accordance with the well-known temperature dependence on hydrophobic effects at temperatures higher than 25 °C [34,35]. Moreover, as shown in Table 1, the number of water molecules released upon binding decreases corresponding to a reduction of the surface contact between the solute and the ligand. At $T > 25$ °C, the temperature increase is responsible for the disruption of the ordered water organisation in contact with the non-polar surfaces so that the solute is better accommodated by the aqueous environment [35]. This results in a reduction of the solute attraction into the hydrophobic inside of IAM. From

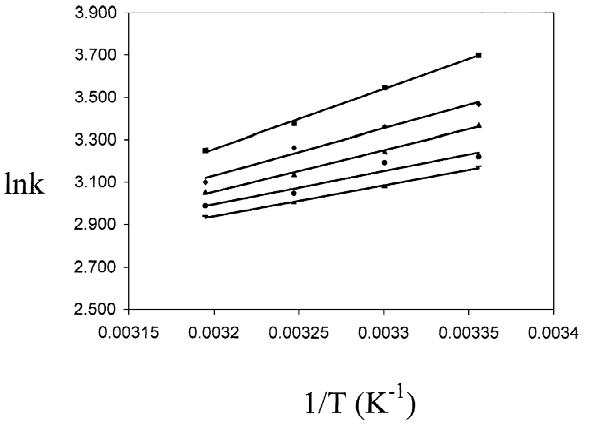

Fig. 2. Van't Hoff plots for hydrocortisone using sodium sulfate as mobile phase additive at concentrations of 0.1 M (–), 0.2 M (●), 0.3 M (▲), 0.4 M (✦) and 0.6 M (■). See operating conditions in the Experimental.

the linear Van't Hoff plots, the slope and intercept were respectively used to determine the enthalpy and entropy change values associated to the solute transfer from the mobile to the stationary phase. Fig. 3 shows the ΔH and ΔS^* variations with salt concentration for the three systems.

The enthalpy and entropy change variations with kosmotropic salt concentration are not consistent with an interaction only due to hydrophobic effects, based on the dehydration of non-polar solute by removal from aqueous environment. Increasing the sulfate concentration increases the mobile phase

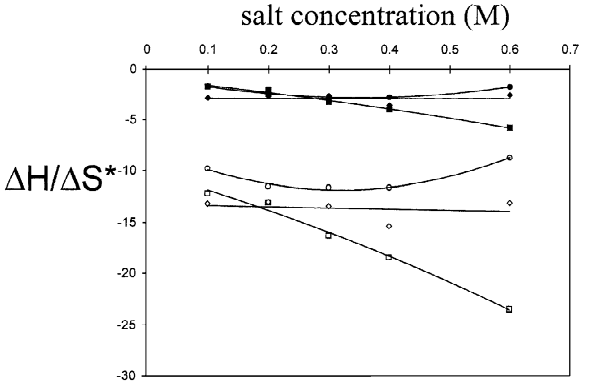

Fig. 3. Plots of ΔH (kJ/mol) in open symbols and ΔS^* in solid symbols against sodium salt (sulfate (□/■), chloride (◇/✦), perchlorate (○/●)) concentration for hydrocortisone. See operating conditions in the Experimental.

hydrophilicity. Consequently, the relative hydrophobicity of solute is enhanced. In the case of a pure hydrophobic effect, i.e. the transfer of non-polar solutes from water to a bulk hydrocarbon phase, it is well-known that the entropy contributions increase with increasing the solute hydrophobicity while the enthalpy contribution is small [36]. In the present case, both enthalpy and entropy changes decrease with kosmotropic salt concentration increasing. This thermodynamic result shows that additional effects are involved in the association of hydrocortisone with IAM as evoked above. It is well-known that solute–ligand association processes governed by van der Waals interactions/hydrogen bond are characterized by strong negative ΔH and ΔS values [37]. This confirms that the hydrocortisone binding to IAM is governed by two processes: (i) the insertion of the solute non-polar surface into the phospholipid core dependent on hydrophobic effects and (ii) additional contacts between solute and the lipid headgroups, involving probably van der Waals interactions/hydrogen bonds between the solute hydroxyl groups and the polar interfacial sites.

For the sodium perchlorate salt, the thermodynamic parameters vary more weakly when the additive concentration increases. A slight increase in the ΔH and ΔS^* values is only observed at high salt concentrations (Fig. 3), confirming that the chaotropic anion has reduced competitive effects for solute binding to the stationary phase hydrophobic surfaces.

4.3. Enthalpy–entropy compensation

Additional analysis was carried out in order to verify if the salt nature influences the solute partitioning mechanism. This was attained by the examination of the enthalpy–entropy compensation. This approach has been previously used in various chromatographic procedures to analyze and compare the solute retention mechanisms in different operating conditions [38–40]. The enthalpy–entropy compensation can be described by the following relation:

$$\Delta H = \beta \Delta S + (\Delta G)_\beta \quad (12)$$

where $(\Delta G)_\beta$ is the Gibbs free energy of a physicochemical interaction at a compensation temperature

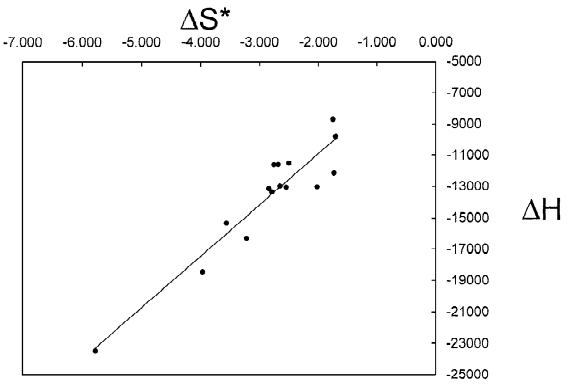

Fig. 4. Plot of ΔH (J/mol) against ΔS^* (enthalpy–entropy compensation) for the three sodium salts (sulfate, chloride, perchlorate) at all the concentrations.

β. In accordance with Eq. (12), when enthalpy–entropy compensation is observed with a compound in a particular chemical interaction, the solute has the same free energy $(\Delta G)_\beta$ at temperature β in the various operating conditions. Therefore, if enthalpy–entropy compensation is observed, solute has the same net retention at the compensation temperature β, although its temperature dependence may differ.

The ΔH versus ΔS^* plots were obtained for each concentration of the three salts as shown in Fig. 4. A linear plot was observed for the two salts with the following linear regression equation:

$$\Delta H = 3287 \Delta S^* - 4330 \quad R^2 = 0.885 \quad (13)$$

This R^2 value may be considered adequate to verify enthalpy–entropy compensation for this chromatographic system [39–41]. This means that the salt nature does not influence the overall solute interaction mechanism with phospholipid monolayers, indicating there is no specific effect of salt anion on the binding mechanism. As well, the compensation temperature β was calculated using Eqs. (12) and (9) and was found to be around 395 K. This is in accordance with the previous values of compensation temperature reported for other hydrophilic/hydrophobic two-phase systems [38,41].

5. Conclusion

On the basis of this work, it appears clearly that

the salt effects on the solute retention follow the Hofmeister series, indicating that hydrophobic effects play an important role in the hydrocortisone partitioning process. An interaction model has been elaborated in order to explore the salt dependence on the solute retention on an IAM stationary phase. The simplified quantitative analysis as well as an additional thermodynamic analysis demonstrate that the solute binding process does not correspond to the classical hydrophobic effect but rather to the combination of hydrophobic effects and other driving forces such as van der Waals interactions/hydrogen bonds between hydroxyl groups of the amphiphilic solute and polar interfacial sites of the phospholipid monolayers.

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