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Thermodynamic analysis of binding between drugs and glycosaminoglycans by isothermal titration calorimetry and fluorescence spectroscopy

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ABSTRACT

The thermodynamics of the interaction of positively charged drug molecules with negatively charged glycosaminoglycans (GAGs) is investigated by isothermal titration calorimetry (ITC) and fluorescence spectroscopy. The drugs considered are propranolol hydrochloride, tacrine, and aminacrine, and the polymers used as model GAGs are dextran sulfate, chondroitin sulfate, and hyaluronic acid. The ITC results show that the interaction between drugs and GAGs is via direct binding and that GAGs bind to drugs at one set of sites. Large negative values of heat capacity change (ΔC_p) are observed upon binding of GAGs to drugs. Such negative ΔC_p is not expected for purely electrostatic interactions and suggests that hydrophobic and other interactions may be also involved in the binding process. These results are corroborated by fluorescence spectroscopy measurements, which show that specific drug/GAG complex formation is accompanied by a clear enhancement of the fluorescence intensity. The results highlight the importance of the formation of drug/GAG complexes as a primary step for the drug delivery process into cell membranes. It is concluded that the interactions are dependent on the nature of both GAG and drug and this is a fact to be taken into account when new drugs are designed.

1. Introduction

A wide variety of molecules of pharmacological interest have surface-active properties due to their amphiphilic nature. To achieve their target in the intracellular medium, their hydrophilic and hydrophobic moieties firstly interact with the surface of the cellular membrane and its components. This interaction plays a fundamental role for the biological phenomena.

Amphiphilic drugs may adopt different structures which are dependent, for example, on the pH, temperature, ionic strength, and concentration, as well as the molecular shape and the hydrophilic–hydrophobic balance (Schreier et al., 2000). Many surface-active drugs associate and bind to membranes in a detergent-like manner (Schreier et al., 2000). Among others, non-steroidal anti-inflammatory drugs (Rades, 1997), anticancer drugs (King et al., 1989), analgesics (Attwood et al., 1997), anticholinergics (Yokoyama et al., 1994), and

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β-blockers (Ruso et al., 1999) are amphiphilic drugs which contain one or more aromatic nuclei. Propranolol hydrochloride (propranolol), 9-amino-1,2,3,4,-tetrahydroacridine (tacrine), and 9-aminoacridine hydrochloride (aminacrine) belong to this class of drugs. Propranolol is a synthetic non-selective B-adrenergic receptor blocking agent, therapeutically referred to as a membrane stabilizer due to its hydrophobicity (Attwood and Lemmer, 1979) tacrine is an acetylcholinesterase inhibitor used in the treatment of Alzheimer's disease; and aminacrine is a highly fluorescent therapeutic anti-infective dye used clinically as an antiseptic and experimentally, as a mutagen and also as an intracellular pH indicator. These drugs have a very similar chemical structure and their pK_a are 9.2, 9.8, and 9.99 for propranolol, tacrine, and aminacrine, respectively (Drayton, 1990).

Amphiphilic drug molecules have been extensively studied in relation to drug-model cell membrane (lipids/liposomes) or to drug–protein interactions. Glycosaminoglycans (GAGs) are highly negatively charged biomolecules (like sulfated polysaccharides) that are found in the extracellular matrixes of many tissues, and on the surface of cells (Schlessinger et al., 1995). They are involved in diverse biological processes such as neural development, gene delivery, and have also been linked to the pathology of the Alzheimer's disease (Laabs et al., 2005; Capila and Linhardt, 2002; Ruponen et al., 1999; Büow and Hobert, 2004). Recently, we have investigated the interaction of this kind of drugs with phospholipid modified-monolayers at a liquid–liquid interface in the presence of dextran sulfate (DS), which is used as model for GAGs (Santos et al., 2005a,b, 2007a,b). In general, these results have qualitatively shown that the presence of DS affects the drug transfer through the lipid monolayer.

Calorimetric techniques are very powerful for the study and understanding of biological processes at molecular level. Isothermal titration calorimetry (ITC) can detect the small changes of heat during reaction. It allows for the determination of the thermodynamic parameters such as enthalpy (ΔH), entropy (ΔS), Gibbs free energy (ΔG), heat capacity (ΔC_p), binding constant (K_b), and effective number of binding sites (N) in biological reactions (Bäuerle and Seelig, 1991; Milhaud et al., 1996; Rowe et al., 1998; Ladbury, 2001).

The interactions between drug molecules and GAGs are of great importance for optimizing drug formulations. In this context, and continuing our previous work, ITC is used to determine the thermodynamic parameters of the binding of amphiphilic drugs (propranolol, tacrine, and aminacrine) with model, surface-membrane GAGs such as DS, chondroitin 6-sulfate (CS), and hyaluronic acid (HA) at pH 5, 7.4, and 9, and at two temperatures, 298 and 313 K.

Fluorescence spectroscopy is also used to study of interactions between GAGs and drugs. This complementary technique is based on the fact that the fluorescence of a number of molecules (fluorescent probes) is very sensitive to the characteristics of their immediate surroundings.

Finally, the paper highlights the fact that drug/GAG interactions can be interpreted in a similar way as protein–protein or carbohydrate–protein interactions. To the best of our knowledge, this is the first time that drug/GAG interactions have been explored in this way.

2. Experimental

2.1. Chemicals

The drugs used were aminacrine (Sigma, minimum 98%), tacrine (Sigma, reagent grade), and propranolol (Sigma, reagent grade). The model GAGs used were DS sodium salt from Leuconostoc ssp (DS 500, Fluka, 500 kDa), CS sodium salt from shark cartilage (Sigma, 50–60 kDa), and HA sodium salt from human umbilical cord (Fluka, 3000–5800 kDa) (see Fig. 1). DS contains ~17% sulfur, which is about 2.3 sulfate groups per glucosyl residue, and CS ~7%. The aqueous solutions were prepared in 15–150 mM NaCl (Merck, p.a.) and buffered to the desired pH using 2–20 mM HEPES (Sigma, minimum 99.5% titration) or 2–60 mM phosphate buffer (J.T. Baker B.V., "Baker Analysed"®), NaOH (Merck, p.a.), and HCl (Merck, p.a.). All the chemicals were used as received without further purification. Millipore water (resistivity > 18 Mω cm) was used to prepare all the aqueous solutions and for rinsing.

2.2. Calorimetry

The heat flow resulting from the binding between GAGs and drugs was measured using a high-sensitivity MicroCal isothermal titration calorimeter (VP-ITC, Northampton, MA) in a reaction cell (volume 1.4413 mL) at a stirring speed of 300–450 rpm. Prior to the measurements all the aqueous solutions were degassed under vacuum about 10–15 min for elimination of any air bubbles. In each titration the reaction cell was loaded with GAG solution and sequences of 29–59 successive 10–15 μL aliquot injections were performed using a 250 μL auto-syringe filled with either drug solutions, at 4–6 min intervals between each injection. To correct for the heat effects of dilution and mixing, control experiments were performed at the same concentration of drugs and GAGs. The calorimetric data were analyzed and converted into enthalpy change using MicroCal Origin 5.0 software provided with the instrument. The enthalpy change for each injection was calculated by integrating the area under the peaks of the recorded time and then corrected with the control titrations. The experimental data were fitted to a binding model using a non-linear least squares method with N, ΔH, and K_b as adjustable parameters. The experiments were performed at 298 and 313 K, and at pH 5, 7.4, and 9.

The titrations were carried out in HEPES and phosphate buffers described as follows: GAG solution in the syringe (typically 8.80–2.24 mM), drug (referred as ligand) solution in the reaction cell (typically 2.60–0.33 mM). At the studied pHs and temperatures, the data fitted well to a one set of sites for GAG-into-drug (further fitting details can be found in the supporting information). The molar concentrations in the case of GAGs refer to the concentrations of the monomer (in the case of DS) or dimer units (in the cases of CS and HA) shown in Fig. 1.

2.3. Fluorescence spectroscopy

The intrinsic fluorescence of the interactions between drugs and GAGs were studied using a Perkin-Elmer LB-50 spectrofluorimeter with a 3 mL quartz cell of 1 cm path length. Drug

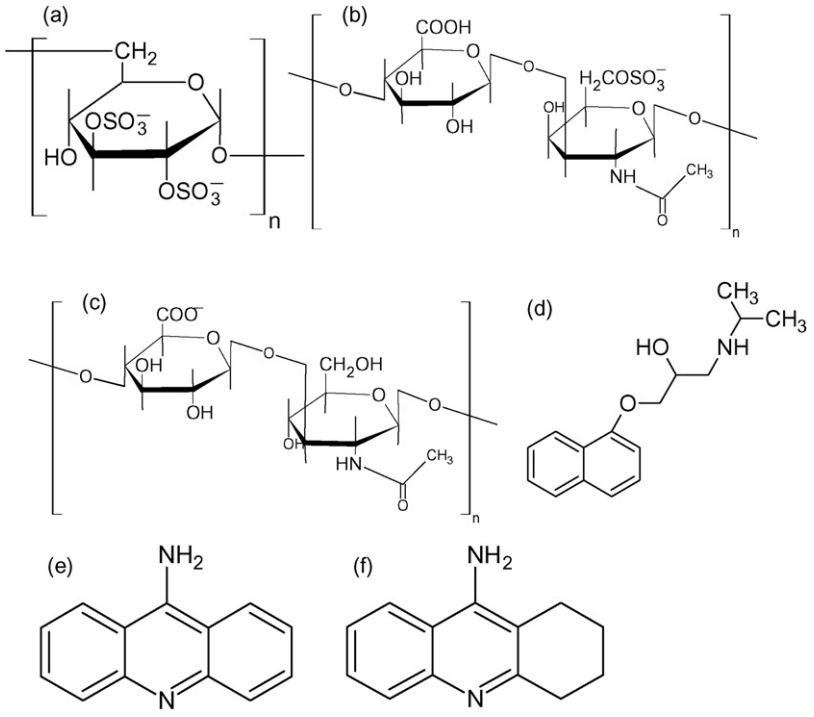

Fig. 1 – Chemical structures of GAGs: DS (a), CS (b), and HA (c); and the drugs: propranolol (d), aminacrine (e), and tacrine (f) in their neutral form.

and GAG concentrations were similar to those used in the ITC measurements. Typically, the measurements were performed by successive 20 μL aliquot additions of GAGs into the drug solutions. The excitation and emission slit widths were fixed at 5–10 nm. Excitation wavelengths of 290, 325, and 400 nm were selected for propranolol (Hunt and Ansell, 2006), tacrine (Vetkin et al., 2004), and aminacrine (Murza et al., 1998), respectively. The emission spectra were monitored in the range 310–600 nm. All the experiments were performed at room temperature (~298 K). The blanks were subtracted from each spectrum, and an average of at least three accumulated scans was recorded.

3. Results and discussion

In this study, and within the pH range considered, the amphiphilic drugs are positively charged and the GAGs are negatively charged. It is known that the electrostatic interactions imply positive contributions to the binding entropy and to the heat capacity change (Sturtevant, 1977).

The drugs contain several hydrophobic aromatic rings, and therefore hydrophobic contributions due to the drug/GAG interaction and/or from aromatic ring stacking (mediated by GAGs) are also expected to be significant. When some hydrophobic groups are buried (i.e. when the water-accessible hydrophobic surface area is reduced), solvation water molecules are released (Banerjee et al., 2005; Gonçalves et al., 2006). The hydrophobic interactions give rise to positive contributions to the binding entropy and to negative contributions to the heat capacity change (Sturtevant, 1977).

Finally, drug binding to GAGs is also expected to affect the possible conformational changes of GAGs in such a way that the internal degrees of freedom of the GAGs are decreased. This implies another negative contribution to the binding heat capacity. Moreover, since the heat capacity associated to these degrees of freedom is large, the corresponding change is also expected to be large (Banerjee et al., 2005; Ortiz-Salmerón et al., 1998).

3.1. *Isothermal titration calorimetry*

Fig. 2 shows the calorimetric titrations of DS and CS into tacrine at 298 K and at various pH. In general, the titration patterns are exothermic. A monotonic decrease in the heat evolved when increasing amounts of GAG are added suggests the drug molecules display only one type of binding sites. However, a different trend in the isotherms for DS and for CS can be observed when changing the pH. For DS in the first few injections, tacrine is much in excess over the added DS, and an almost constant heat release is observed. The plateau region reveals that the added DS is bound completely to tacrine. As the titration continues (the concentration of DS increases), the free drug concentration in the reaction cell decreases, and the heat flow also decreases. Due to an increase in the endothermic contribution, the peaks suddenly become smaller, and at higher DS concentrations, no heat of interaction is observed (i.e. the heat value is constant and around zero). This means that after injection of a certain amount of DS no significant interaction between tacrine and DS takes place, and thus, the DS-into-drug titration leads to a complete binding of all drug contained in the reaction cell. Furthermore, to get the full

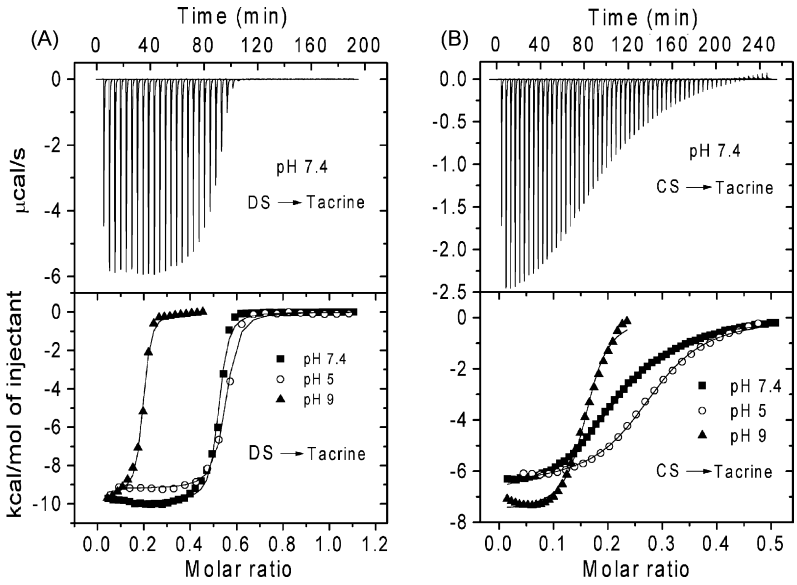

Fig. 2 – Calorimetric titrations of DS (A) and CS (B) into tacrine at 298 K and various pH. The concentration ranges of DS and CS in the syringe were 3.91–3.25 and 2.76–2.31 mM, respectively. The concentrations of tacrine in the reaction cell for the pH of 7.4 (■), 5 (○), and 9 (▲) were: (A) 0.50, 0.51, and 1.28 mM and (B) 1.07, 1.07, and 2.60 mM, respectively. All the solution concentrations were prepared in HEPES buffer. The upper panels show the raw power data obtained from 29 (A) and 59 (B) injections (10 and 5 μL each, respectively) at pH 7.4, and the lower panels show the plots of the total energy exchanged as a function of the molar ratio of GAGs to tacrine. The solid lines correspond to the best fit using a binding model with one set of sites.

binding isotherm an increase in the concentration of tacrine is needed as the pH is increased up to 9.

In the case of CS the titration isotherms show slight differences relative to the DS one. The initial heat release is low and in average about −7 kcal/mol, while in the case of DS the initial heat release is about −10 kcal/mol. Moreover, for the same amount of CS and DS, twice as much GAG is needed to reach the isotherm saturation in the case of CS.

Fig. 3 shows the calorimetric titrations of DS and CS into drugs at 313 K for pH 7.4 and for pH 5. At pH 5 the binding of DS and CS to drugs is initially exothermic and the heat drops to zero when the binding is complete. However, at pH 7.4 the binding is also initially exothermic, but in contrast to Figs. 2 and 3B and D, it becomes endothermic after addition of a certain amount of GAG. If the titration is performed with more diluted GAG and drug solutions, the binding is always exothermic.

The similar chemical structure of tacrine and aminacrine led to similar binding isotherms when DS is added, whereas marked differences are observed when CS is added. As it can be seen from Fig. 3C and D (lower panels), that when CS is added to propranolol and tacrine there is a clear decrease in the heat change and the isotherm features vary significantly compared to that observed for aminacrine. It should be mentioned that in order to reproduce the main features in the binding isotherms, different drug concentrations were used for the different experiments.

3.1.1. *DS binding to drugs*

The results of the analysis of the binding isotherms of DS to drugs (Fig. 2) are summarized in Table 1. Although the binding process is enthalpically favoured ($\Delta H < 0$), it is mainly entropically-driven since the contribution $-T\Delta S$ to ΔG is often larger in magnitude than ΔH. For all the titrations ΔH and ΔS showed clear temperature dependence, both decreasing as the temperature increases. That is, the binding process becomes more exothermic and less entropically favoured as the temperature increases. Thus, the binding weakens upon increase in temperature, as indicated by the smaller values of the binding constant K_b at 313 K. From this temperature dependence, the heat capacity changes ΔC_p are evaluated and large negative values are obtained. These are not expected for a purely electrostatic interaction and, therefore, they indicate that hydrophobic and other interactions are also involved in the binding process (Banerjee et al., 2005; Gonçalves et al., 2006). This is consistent with the presence of very hydrophobic groups in the structure of the drug molecules.

These results for ΔH and ΔS are in contrast to those observed for carbohydrate–protein (Gupta et al., 1996) and drug–protein (Banerjee et al., 2005) interactions, where the enthalpic and entropic contributions to the binding free energy compensate each other and show similar temperature dependence. In carbohydrate–protein interactions (Gupta et al., 1996; Sharma et al., 1998; Williams et al., 1992), enthalpy–entropy compensation has been attributed to solvent reorganization and it has also been associated to weak intramolecular interactions. This compensation tends to occur in systems with $\Delta C_p \neq 0$ (Swaminathan et al., 1999), and for $\Delta C_p \gg \Delta S$ (Banerjee et al., 2005; Williams et al., 1992; Lee, 1994). Furthermore, negative ΔC_p has been observed for protein folding and protein–ligand

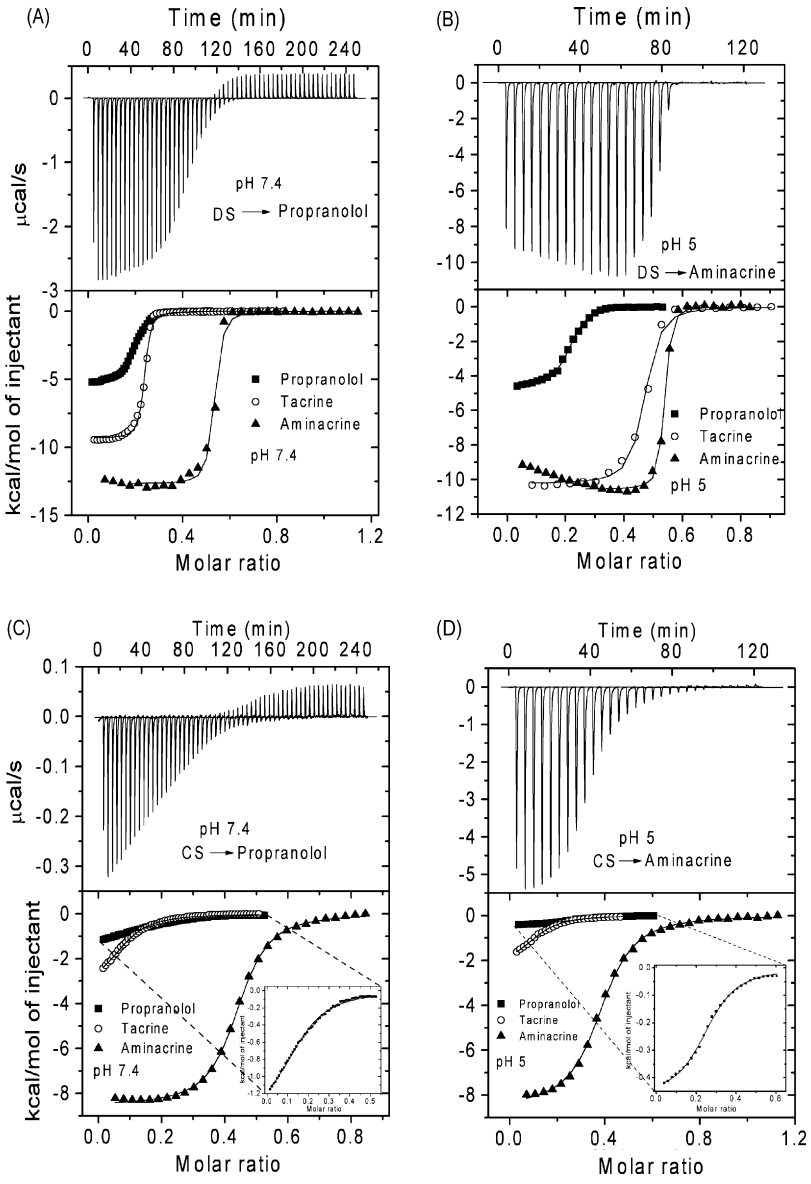

Fig. 3 – Calorimetric titrations of DS (A and B) and CS (C and D) into drugs at 313 K and pH 7.4 (A and C) and 5 (B and D). The concentration ranges of DS and CS in the syringe were 3.91–3.25 and 2.40–2.24 mM, respectively. The concentrations of propranolol (■), tacrine (○), and aminacrine (▲) in the reaction cell were: (A) 1.37, 0.50, and 0.33 mM, respectively; (B) 1.70, 0.55, and 0.87 mM, respectively; (C) 0.96, 1.07, and 0.63 mM, respectively; and (D) 0.84, 1.10, and 0.45 mM, respectively. All the solution concentrations were prepared in HEPES buffer. The upper panels show the raw power data obtained from 29 (A and C) and 59 (B and D) injections (10 and 5 μL each, respectively) of GAGs into drugs (A and C for propranolol, and B and D for aminacrine). The lower panels show the plots of the total energy exchanged as a function of the ratio of the GAGs to the drugs. The solid lines correspond to the best fit using a binding model with one set of sites.

associations (Banerjee et al., 2005; Ortiz-Salmerón et al., 1998) due to the contribution of hydrophobic effects. The ΔC_p values in Table 1 are relatively smaller than those found in binding processes involving proteins (Sturtevant, 1977).

In relation to the drug nature, the ΔH values show that DS binds more strongly to tacrine and aminacrine than to propranolol, that is, the sulfate groups of DS bind more strongly to the amino groups of the first two drugs than to the charged group of propranolol. Similarly, the fact that the positive ΔS is larger for propranolol than for tacrine and aminacrine seems to be related to the presence of more hydrophobic groups in the latter drugs, which might also partially restrict the internal degrees of freedom of DS and lead to a less favourable entropy change. The ITC analysis has also revealed remarkable differences in ΔC_p. The negative ΔC_p values are larger in magnitude for the binding of DS to aminacrine than to propranolol and tacrine; except for the case of tacrine at pH 9 where the largest negative ΔC_p was observed ($-97\,\mathrm{cal\,mol^{-1}\,K^{-1}}$). This might indicate

Table 1 – Thermodynamic parameters for the binding of DS to different drugs at 298 and 313 K, and pH 5, 7.4, and 9, in 2 mM HEPES buffer

T (K)	N	K_b ($\times 10^6$ M^{-1})	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	ΔG (kcal mol^{-1})	ΔC_p (cal mol^{-1} K^{-1})
DS → propranolol (pH 5)						
298	4.54 ± 0.03	0.044 ± 0.003	−0.756 ± 0.006	18.7	−6.3	−15
313	4.74 ± 0.03	0.037 ± 0.003	−0.977 ± 0.008	17.8	−6.5	
DS → tacrine (pH 5)						
298	1.882 ± 0.007	0.85 ± 0.1	−4.89 ± 0.04	11.6	−8.3	−28
313	2.21 ± 0.01	0.57 ± 0.8	−5.33 ± 0.04	10.7	−8.7	
DS → aminacrine (pH 5)						
298	1.837 ± 0.005	6.8 ± 0.2	−4.96 ± 0.04	14.6	−9.3	−40
313	1.904 ± 0.004	3.3 ± 0.6	−5.56 ± 0.03	12.0	−9.3	
DS → propranolol (pH 7.4)						
298	3.63 ± 0.01	0.059 ± 0.003	−1.033 ± 0.004	18.4	−6.5	∼0
313	4.09 ± 0.02	0.036 ± 0.002	−1.039 ± 0.005	17.5	−6.5	
DS → tacrine (pH 7.4)						
298	1.950 ± 0.006	1.3 ± 0.2	−5.06 ± 0.03	10.9	−8.3	−8
313	3.28 ± 0.01	0.30 ± 0.03	−5.179 ± 0.012	18.0	−10.8	
DS → aminacrine (pH 7.4)						
298	1.902 ± 0.004	4.3 ± 0.7	−5.63 ± 0.03	11.5	−9.0	−63
313	1.926 ± 0.008	3.2 ± 0.8	−6.58 ± 0.07	8.6	−9.3	
DS → tacrine (pH 9)						
298	5.33 ± 0.04	0.10 ± 0.01	−1.78 ± 0.02	16.9	−6.8	−97
313	4.60 ± 0.05	0.14 ± 0.03	−3.23 ± 0.05	13.2	−7.4	

stronger hydrophobic interactions and/or a more significant conformational change in DS upon binding to aminacrine (in comparison with the binding to the other drugs) (Bains et al., 1992).

The binding constants K_b of the drugs are in the order $K_{b,propranolol} < K_{b,tacrine} < K_{b,aminacrine}$. The effective numbers of binding sites per DS monomer are in the order $N_{propranolol} > N_{tacrine} > N_{aminacrine}$, and increase as the temperature increases (except for tacrine at pH 9). The stoichiometry for the binding of a DS monomer to propranolol is ca. 5:1 (pH 5) and 4:1 (pH 7.4), and for binding to tacrine and aminacrine is ca. 2:1 (at pH 5 and 7.4). The stoichiometry of tacrine with DS at pH 9 is 5:1. These values of *N* seem to be rather high and deserve further comment. First of all, it should be stressed that all the results are based on three independent measurements. A possible reason for these values of *N* is that the DS long chain might fold in such a way that its monomers are close enough to allow drugs to bind both electrostatically and through H bonding. Stacking and steric effects of the drug molecules might also play some role, since two drug molecules may not bind/stack if they are charged but could do if the negative charge in the DS compensates for the charge on one drug molecule.

Both *N* and the binding constants are also very dependent on pH. For example, in the case of tacrine *N* is about 2 at pH 5 and 7.4, and about 5 at pH 9 for 298 K; K_b is highest at pH 7.4 and lowest at pH 9. Similarly, the ΔC_p values show a complex pH dependence; at lower pH they decrease for aminacrine (less negative ΔC_p), and increase (more negative ΔC_p) for tacrine and propranolol. The $\Delta C_p \sim 0$ observed for propranolol at pH 7.4 may arise from a compensation of opposite sign contributions.

3.1.2. *Hydrophobic and vibrational contribution to ΔC_p and ΔS for DS–drug interactions*

We have analyzed the data in Table 1 using the empirical method of Sturtevant (Sturtevant, 1977) (further details can be found in the supporting information) in order to calculate the hydrophobic and intramolecular vibrational contributions to ΔC_p and ΔS. The results obtained from this analysis cannot be considered as rigorous as those obtained directly from ITC because they are affected by the hypothesis that hydrophobic and vibrational interactions are the two main contributors to ΔC_p and ΔS. Thus, contributions to these magnitudes from the electrostatic interaction are not evaluated. Similarly, any possible additional contribution due to hydrogen bonding or the displacement of counterions (Hileman et al., 1998; Lohman and Mascotti, 1992) are considered to be negligible.

It is found (Table S1, supporting information) that the ΔC_p (hydro) is more negative than ΔC_p (vib), which supports the conclusion that the negative ΔC_p is due to a favourable hydrophobic contribution. On the other hand, ΔS (hydro) is more positive than ΔS (vib) for tacrine (pH 5 and 9) and aminacrine, and ΔS (hydro/vib) slightly decrease as the temperature increases. For propranolol (pH 5 and 7.4) and tacrine (pH 7.4), ΔS (hydro) < ΔS (vib). This might indicate an increase in the excitability of internal vibrational modes of DS upon binding to these drugs.

3.1.3. *CS and HA binding to drugs*

For CS binding to drugs a different behaviour is observed in terms of *N* and K_b (Table 2). Similarly to the case of DS binding, the binding process is enthalpically and entropically favoured, with a dominant entropic contribution to ΔG quite likely due to the hydrophobic interaction. The enthalpic con-

Table 2 – Thermodynamic parameters for the binding of CS and HA to drugs at 298 and 313 K, and pH 5, 7.4, and 9, in 2 mM HEPES buffer

T (K)	N	K_b ($\times 10^3$ M^{-1})	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	ΔG (kcal mol^{-1})	ΔC_p (cal mol^{-1} K^{-1})
CS → propranolol (pH 5)						
298	4.0 ± 0.1	3.3 ± 0.4	−0.379 ± 0.008	14.8	−4.8	+17
313	3.74 ± 0.02	12.8 ± 0.6	−0.125 ± 0.007	18.4	−5.9	
CS → tacrine (pH 5)						
298	3.60 ± 0.01	25.4 ± 0.1	−1.818 ± 0.009	14.1	−6.0	+103
313	4.2 ± 0.1	3.2 ± 0.2	−0.273 ± 0.002	15.2	−5.0	
CS → aminacrine (pH 5)						
298	1.926 ± 0.007	253 ± 19	−3.79 ± 0.02	12.0	−7.7	+37
313	2.64 ± 0.01	62 ± 3	−3.23 ± 0.016	11.6	−6.9	
CS → propranolol (pH 7.4)						
298	4.66 ± 0.08	3.4 ± 0.2	−0.326 ± 0.004	15.0	−4.8	+5
313	5.84 ± 0.07	4.0 ± 0.2	−0.253 ± 0.002	15.5	−5.1	
CS → tacrine (pH 7.4)						
298	4.50 ± 0.02	11.7 ± 0.4	−1.576 ± 0.007	13.3	−5.5	+85
313	5.2 ± 0.2	28 ± 2	−0.302 ± 0.003	14.8	−4.9	
CS → aminacrine (pH 7.4)						
298	2.306 ± 0.006	125 ± 5	−3.702 ± 0.012	11.5	−7.1	−27
313	1.827 ± 0.003	388 ± 16	−4.109 ± 0.013	11.8	−7.8	
CS → tacrine (pH 9)						
298	6.27 ± 0.04	24 ± 2	−1.206 ± 0.010	16.0	−6.0	+54
313	7.9 ± 0.5	22 ± 3	−0.399 ± 0.009	14.0	−4.8	
HA → tacrine (pH 7.4)						
298	3.4 ± 0.1	0.93 ± 0.08	−0.222 ± 0.004	12.8	−4.0	
HA → aminacrine (pH 7.4)						
298	5.70 ± 0.05	70 ± 1	−0.630 ± 0.008	20.1	−6.6	

tribution is less exothermic in the case of CS than in the case of DS. The binding free energy ΔG shows no clear temperature dependence within the range considered. Interestingly, the entropy generally increases as the temperature increases, in contrast to that observed for DS. This increase may be related with some conformational change of CS, due to increased CS–solvent interactions, and thus leading to positive ΔC_p.

It is noteworthy that the differences observed in the heat capacity for the binding of CS to drugs suggest corresponding differences in energetics and binding mechanism. The negative ΔC_p observed for aminacrine at pH 7.4 (−27 cal mol^{-1} K^{-1}) suggests that hydrophobic interactions are involved in the binding process (although, weaker than in the case of DS). In contrast, the positive ΔC_p observed for the binding of tacrine and propranolol suggests ionization/charge neutralization reactions, i.e. binding of CS to drugs is dominated by electrostatic interactions, which seem to be stronger in the case of tacrine. In general, other factors contributing to a positive ΔC_p might be the following: (i) solvent–drug interactions; (ii) pH-dependent, solvent interactions with CS–drug binding sites; and (iii) changes in the CS (and more unlikely in the drug) conformation as result of binding. However, factor (i) is not likely explanation because all the drugs were in aqueous solution.

Since aminacrine and tacrine are structurally similar, the larger $-\Delta H$ values observed for aminacrine than for tacrine might be due to the presence of three aromatic rings in the aminacrine structure, which indicates a stronger binding to GAGs due to hydrophobic interactions. On the other hand, the binding ΔS is more favourable for tacrine than for aminacrine.

In general, the binding constants K_b are smaller for CS than for DS (note the different units in Tables 1 and 3), while the values of N do not show a significant difference. The stoichiometry for the binding of CS to propranolol/tacrine is ca. 4:1 (pH 5) and 5:1 (pH 7.4), while for binding of CS to aminacrine is 2:1 (at pH 5 and 7.4). The binding stoichiometry of tacrine with CS dimers at pH 9 is 7:1. The temperature dependence of N and K_b is not clear, for these magnitudes increase with T in some cases and decrease in others.

Although HA is structurally similar to CS, its binding behaviour is different to that of CS, likely because it has carboxylate groups instead of sulfate groups. When binding to tacrine, HA shows a lower value of N and K_b than CS, while when binding to aminacrine, N is higher and K_b is lower than in the case of CS. The stoichiometry for the binding of HA dimers to tacrine and aminacrine is ca. 3:1 and 6:1, respectively. Furthermore, the binding of HA to drugs is enthalpically less favourable than that of CS under otherwise similar conditions. It should be noted that for pH of 5 and 9 no binding between HA and the drug molecules was observed. This indicates that HA binding is more restricted than DS and CS, and only under physiological conditions is it suitable to interact with these drug molecules.

3.1.4. *Hydrophobic and vibrational contributions to ΔC_p and ΔS for CS and HA binding to drugs*

We have also calculated the hydrophobic and intramolecular vibrational contributions to ΔC_p and ΔS (Table S2, support-

Table 3 – Thermodynamic parameters for the binding of CS to tacrine at 298 and 313 K, and pH 5, 7.4, and 9, in 2 mM phosphate buffer

T (K)	N	K_b ($\times 10^3$ M^{-1})	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})	ΔG (kcal mol^{-1})	ΔC_p (cal mol^{-1} K^{-1})
CS → tacrine (pH 5)						
298	3.84 ± 0.01	33 ± 2	−1.931 ± 0.01	14.2	−6.2	+47
313	6.07 ± 0.05	12 ± 1	−1.22 ± 0.01	14.7	−5.8	
CS → tacrine (pH 7.4)						
298	3.44 ± 0.01	35 ± 2	−2.30 ± 0.01	13.0	−6.2	+28
313	4.23 ± 0.01	22 ± 1	−1.876 ± 0.009	13.9	−6.2	
CS → tacrine (pH 9)						
298	3.72 ± 0.01	32 ± 0.1	−2.231 ± 0.009	13.1	−6.1	+40
313	5.45 ± 0.03	13.4 ± 0.8	−1.64 ± 0.01	13.7	−5.9	

ing information). For the titrations of CS to propranolol it is observed that ΔC_p (hydro) < ΔC_p (vib), and ΔS (hydro) < ΔS (vib), while for titrations of CS to tacrine (pH 5, 7.4, and 9) and aminacrine (pH 5) it is observed that ΔC_p (hydro) > ΔC_p (vib). This indicates that the excitability of internal vibrational modes of CS upon drug binding has a major contribution relative to the hydrophobic effects, in contrast to that observed for DS. For titrations of CS to aminacrine (pH 7.4), ΔC_p (hydro) and ΔS (hydro) are slightly greater than ΔC_p (vib) and ΔS (vib), respectively, and these results confirm that the negative ΔC_p may primarily result from a favourable hydrophobic contribution.

3.1.5. *Effect of ionic strength and protonation/deprotonation on the binding enthalpy*

To understand the role of polar interactions, the effect of ionic strength on the binding of GAGs to tacrine was studied by performing the titrations with 150 mM NaCl + 20 mM HEPES buffer. For that purpose, we have considered the titration of DS or CS into tacrine at pH 7.4 and 298 K. In the case of DS, the following values for N, K_b, and ΔH, it were observed: 2.468 ± 0.006, (2.41 ± 0.02) × 10^6 M^{-1}, and −2.630 ± 0.009 kcal/mol. Comparison between these results and those of the Table 1, show that the increase of K_b with the rise in the ionic strength is a result of the hydrophobic interactions in the binding. On the other hand, the titration of CS to tacrine have yielded values for N, K_b, and ΔH of 7.8 ± 0.4, (1.2 ± 0.2) × 10^3 M^{-1}, and −0.179 ± 0.006 kcal/mol, respectively. The obvious effect of increasing the ionic strength in the case of CS is a fall in the value of K_b, which indicates that polar interactions may also play a significant role in the interaction between CS and tacrine. In this last case, we have also further studied the binding of CS to tacrine in 15 mM NaCl + 60 mM phosphate buffer (supporting information, Tables S3 and S4) and a similar trend as those described above was also observed for this system. All these results were qualitatively in good agreement with the previous discussion, supporting the data presented here.

The observed enthalpy changes of the studied interactions are a global property of the whole system, representing the sum of all processes happening in solution, such as protons transfer between the interacting species and the buffer solution. In order to validate the described ITC results, and taking into account that the amino-drugs or the carboxylates groups of CS or HA may change during binding at one or more of the studied pHs, we have also evaluated the effect of the protonation/deprotonation of the binding enthalpy, by repeating the titrations of CS into tacrine at different pHs and temperatures using a phosphate buffer with a different heat of ionisation. The results are shown in Table 3.

These results (see also supporting information Table S5) are qualitatively independent of the buffer used. However, in the case of phosphate buffer, the increase in the pH of the solution did not induce significant changes in K_b. Although some variation was observed for the enthalpy of binding, a binding reaction coupled to protonation/deprotonation may not be totally excluded from the binding process and may occur to some extent. However, proton transfer seems to play a minor role in the present study and does not qualitatively affect the interactions described.

As a result of drug/GAG binding process some information on drug delivery effects (facilitating or inhibiting) can be given. For example, if the cell surface carries only a few GAGs, drug binding is limited to a few receptor molecules. In contrast, for a cell surface with a broad variety of GAGs, drug binding is nonselective. Adding a sufficient amount of drug, a neutral drug/GAG complex can be formed which, in turn, can be adsorbed to the membrane surface, facilitating either consecutive reactions steps or drug diffusion through the cell membrane. On the other hand, adding relatively small amounts of drug, the complex will have an excess of negative charge, and hence the complex is repelled from the membrane surface inhibiting the drug transfer. Consequently, the formation of drug/GAG complexes seems to have an important role as a primary step for the drug delivery process into cell membranes, and should be taken into account when new drugs are designed.

3.2. *Fluorescence spectroscopy*

The fluorescence emission spectrum of a fluorophore is sensitive to its environment, and therefore it may be affected in the presence of GAGs. We have been able to follow the GAG–drug binding at room temperature (~298 K) using the change in intrinsic fluorescence of the drugs upon binding at different pH values. Similar results were also obtained at 313 K (results not shown).

The analysis of the spectra obtained from the fluorescence measurements (Fig. 4) shows a dramatic increase in the fluorescence intensity of tacrine as the GAG concentrations were increased. This indicates that the binding is not purely electrostatic and that a hydrophobic contact of the drug molecule with the DS long chain is also important, as confirmed by ITC measurements. These results do not imply penetration

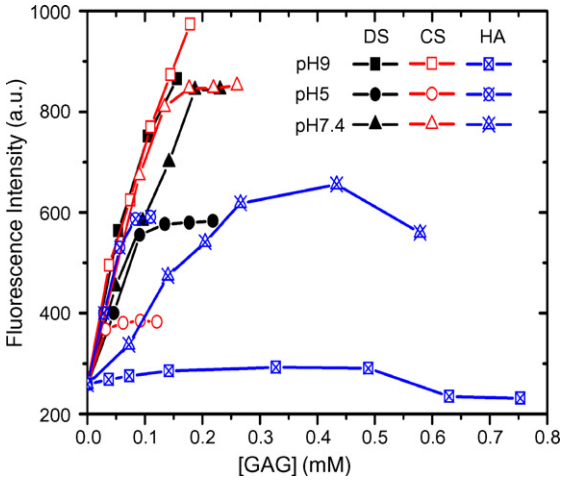

Fig. 4 – Fluorescence intensity of tacrine as a function of the added DS concentration at different pHs.

of the drug molecule into the DS chain since this would apparently cause quenching rather than enhancement of the fluorescence intensity. An alternative explanation could be a conformational change in DS, shielding the drug molecule and simultaneously exposing binding sites. The events may not be mutually exclusive.

A possible scenario explaining our data could be as follows. Binding of the drug molecule to a specific site of the DS chain involves the electrostatic interaction of the positively charged groups of the drug molecule with the negatively charged groups of DS. This also implies that the hydrophobic groups of the drug molecule get closer to the sulfate groups and are shielded from solvent influence, and as a consequence an increase in fluorescence intensity is observed. Therefore, the negative charge of GAGs is important to induce the approach and concentration of drug molecules in the vicinity of the biomolecules, allowing the interaction and binding between them.

Furthermore, the fluorescence intensity of the drug molecules seems to increase with the GAG concentration until a certain point (see Fig. 4). After this point the fluorescence stays rather constant, and further addition of GAG leads to a decrease of the fluorescence intensity due to complex aggregation and possible precipitation. The fluorescence intensity is stronger for DS than for CS and HA, and tends to be higher for aminacrine and tacrine than for propranolol (results not shown). These results also are in good agreement with the ITC experiments where a rather strong interaction between DS and both aminacrine and tacrine was also observed.

The increase of the fluorescence intensity is rather high at pH 9 and reaches a constant value at higher added concentrations of DS. The intensity decreases at pH 7.4 and is reduced to about half at pH 5 (see Fig. 4). Similar results were also observed for CS, although the pH change (from basic to acidic) was even more drastic in this case. In the case of HA, the fluorescence intensity is first saturated at acidic pH, then at neutral pH, and it is almost constant at pH 9. The results show that the drug/GAG interactions are also pH dependent to some extent. Similar results were also obtained with aminacrine and propranolol (results not shown).

4. Conclusions

The interaction between drug molecules and GAGs has been studied by ITC and fluorescence. The ITC results have shown large negative values of ΔC_p in binding of GAGs. These negative values are not expected for a purely electrostatic interaction and suggest that hydrophobic and other interactions may be also involved in the binding process, in a similar way as in protein–protein or carbohydrate–protein interactions. Further investigation using ITC on the binding of drugs to GAGs have shown a variation on ΔC_p and on the other thermodynamic parameters, which was thought to be related to three main factors: (i) electrostatic forces, (ii) hydrophobic interaction between the apolar moieties, and (iii) possible GAG conformational changes. ITC has also shown that GAGs bind to drugs at one set of sites. Fluorescence spectra of drug/GAG complexes have shown that the electrostatic interaction between positively charged drugs and negatively charged groups existing in GAGs might play an important role in the formation of drug/GAG complexes as a possible first step in the establishment of a hydrophobic interaction via aromatic rings stacking. The drug/GAG complex formation is accompanied by an enhancement of the fluorescence intensity.

Furthermore, the interaction between drugs and GAGs is via direct binding, and therefore it is very dependent on the nature of both GAG and the drug. These facts should be taken into account when new drugs are designed. For example, the thermodynamic parameters determined by using ITC may provide qualitative readout of the thermodynamic effects of altering lead compound structures and may increase the success rate in structure-based drug design and/or optimisation. Finally, it is also demonstrated for the studied systems that the thermodynamic parameters can be changed in some extent by changing the medium conditions. This feature can be further used to regulate and improve the drug transport through the cell membrane.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejps.2007.06.003.

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