Characterisation by fluorescence of human and bovine serum albumins in interaction with eosin Y

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Human and bovine serum albumins (HSA and BSA) are blood proteins involved in interactions with various types of molecules (e.g. hormones, fatty acids, drugs). This paper is approaching the albumin interactions with eosin Y, a fluorescent red dye usually used to stain cytoplasm, collagen and muscle fibres. Our experiments were focussed on spectroscopic and thermodynamic characterisation of albumins-eosin Y interactions. The experiments were performed both in Hepes and Tris-HCl buffers, at two temperatures (26 °C and 36 °C) and at pH 7.4. It was observed that the Stern-Volmer constants have larger values in Hepes than in Tris-HCl buffer presenting changes with temperature. The strength of the interactions was characterised by the affinity constants (K_A). The affinity constants are one order of magnitude greater for eosin Y interactions with HSA in Hepes than those in Tris-HCl buffer. The stoichiometry shows that there are two binding sites on BSA and one on HSA. The thermodynamic parameters (ΔG, ΔH, ΔS and ΔC_p) show that both proteins are involved in spontaneous interactions with the dye, these processes being driven by entropy. The caloric capacity variation is greater for the BSA-eosin Y interaction than for the case of HSA-eosin Y interaction.

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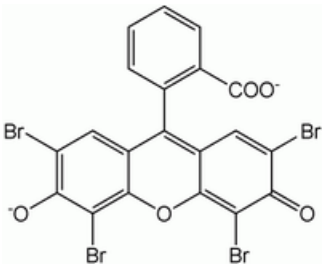
1. Introduction

The interest in biological functions of serum proteins has grown enormously in the recent years. Serum albumins are important carrier proteins for numerous compounds [1]. Understanding the behaviour of these molecules in their specific interactions with different ligands is an important step to albumin use in various domains from food industry to medicine. There is hypothesis that albumins manifest antioxidant activities that are revealed by their multiple-binding sites and free radical-trapping [2].

The most studied albumin is the bovine serum albumin (BSA), with two Trp (Trp-212 and Trp-134). Thus the primary structure of BSA is quite similar with that of the human serum albumin (HSA) the last one contains one Trp residue (Trp-214). Among the binding sites of these albumins there are those for yellow (Y) and brown (B) eosins. Eosin Y is a fluorescent red dye, with a relatively weak fluorescence, usually used to stain cytoplasm, collagen and muscle fibres, but also used for protein determination in solution [3]. The chemical structure of the deprotonated form of this fluorescent red dye is presented below:



There are studies that revealed the fluorescent characteristics of the BSA and HSA complexes for many years [4]. The complex characterization is important because the eosin can be used as site marker in the interaction of proteins with different other molecules [5] or in the protein assay for the mechanism of binding elucidation [6]. The albumin-eosin Y complexes are pH-dependent [7], due to the pH influence on the albumin space conformations [8]. In this study, the binding of the BSA and HSA in complex with eosin Y was characterized, at pH 7.4 and at different temperatures.

2. Materials and methods

Proteins. BSA and HSA (purity over 98 %) were purchased form SIGMA. Protein concentrations were measured using UV absorption spectra and the standard molar absorption coefficients: ε_{280} = 44,000 M^{-1} cm^{-1} for BSA and ε_{280} = 36,000 M^{-1} cm^{-1} for HSA.

Ligand. Eosin Y ($C_{20}H_6Br_4Na_2O_5$, with molecular weight of 691.85 g mol^{-1}) was obtained from Aldrich.

Buffer solutions. All experiments were performed both in 50 mM Hepes and 50 mM Tris-HCl buffers, at pH 7.4.

Fluorescence. All fluorescence spectra were recorded with a *Perkin Elmer MS 55* steady state spectrofluorimeter. The measurements were performed in 10 mm × 10 mm quartz cuvettes. Protein concentrations were kept constant at 2 μM. BSA and HSA were excitated at 290 nm and the fluorescence emission was recorded in the spectral range, 300-450 nm.

3. Results and discussions

3.1 Analysis of the fluorescence quenching of BSA and HSA by the eosin Y

Buffer solutions, which increase protein stability or reduce their aggregation, may be useful in the understanding of protein functions. For this reason, both BSA and HSA samples were dissolved in Hepes and Tris-HCl buffers and the results were compared. Fluorescence emission spectra of BSA (2 μM) in interaction with eosin Y (0 - 6 mM) and HSA (2 μM) in interaction with eosin Y (0-10 mM) were recorded at 290 nm excitation wavelength. Fluorescence of the BSA and HSA solutions is mainly due to Trp residues. We have noticed that eosin Y acts as a fluorescence quencher (Figure 1 A and B). The nature of the binding mechanism (dynamic or static quenching) was investigated by analyzing the fluorescence data, at 26 and 36 °C, and comparing the Stern-Volmer constants, K_{SV}, for these temperatures. Experimental data are fitted with the Stern-Volmer equation [9]:

$$F_0 / F = 1 + K_{SV}[Q] \quad (1)$$

where F and F_o are the fluorescence in the presence and respectively, absence of the quencher. [Q] is the quencher concentration and K_{SV} is the dynamic quenching constant, also named Stern-Volmer constant.

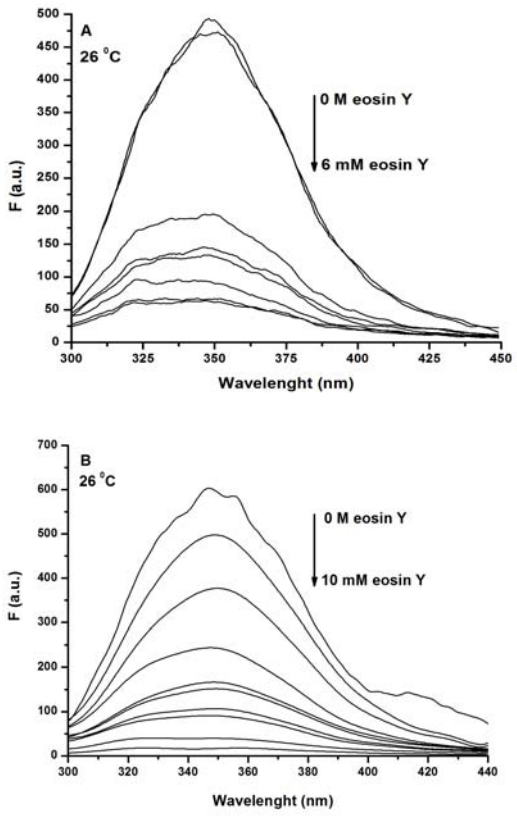


Fig. 1. The fluorescence quenching of BSA (A) and HSA (B) in the presence of eosin Y, at 26 °C, in Hepes buffer, at pH 7.4

The Stern-Volmer plots at 26 and 36 °C for the BSA and HSA-eosin Y complexes, in Hepes and Tris-HCl buffer, are shown in Figure 2 A, B, C, and D, respectively. The quencher penetration towards the hydrophobic cavities of the albumin molecules [10] is not a very easy process, because the Trp residues are located in regions with different spatial conformations preventing, more or less, the accessibility of the quencher. Thus, the eosin Y binding sites of the BSA and HSA may be more or less accessible. For the BSA-eosin Y complex, $K_{SV} = 1.70 \times 10^6$ M^{-1} at 26 °C and 1.29×10^6 M^{-1} at 36 °C, in Hepes buffer. For the HSA-eosin Y complex, $K_{SV} = 1.06 \times 10^6$ M^{-1} at 26 °C and 1.01×10^6 M^{-1} at 36 °C, in Hepes buffer. The K_{SV} value at 36 °C is lower than at 26 °C both for the BSA-eosin Y and HSA-eosin Y binding (Table 1), indicating that the binding is temperature dependent. The same complexes were obtained in Tris-HCl buffer and the Stern-Volmer constants were calculated and listed in Table 1. The values of the Stern-Volmer constants are smaller for the samples in Tris-HCl than in Hepes buffer.

Table 1. Stern-Volmer constants for BSA-eosin Y and HSA-eosin Y complexes at 26 and 36 °C, in Tris-HCl and Hepes buffers, at pH 7.4

BSA-eosin Y complex		
Buffer solution	T (^{0}C)	$K_{SV} \times 10^{-6}$ (M^{-1})
Tris-HCl	26	0.91
	36	0.78
Hepes	26	1.70
	36	1.29
HSA-eosin Y complex		
Buffer solution	T (^{0}C)	$K_{SV} \times 10^{-6}$ (M^{-1})
Tris-HCl	26	0.74
	36	0.41
Hepes	26	1.06
	36	1.01

The correlations between the slope of the plot and the temperature suggest that the albumin-eosin Y interactions, in both buffers, are static quenching processes (the slope decreases with temperature increase).

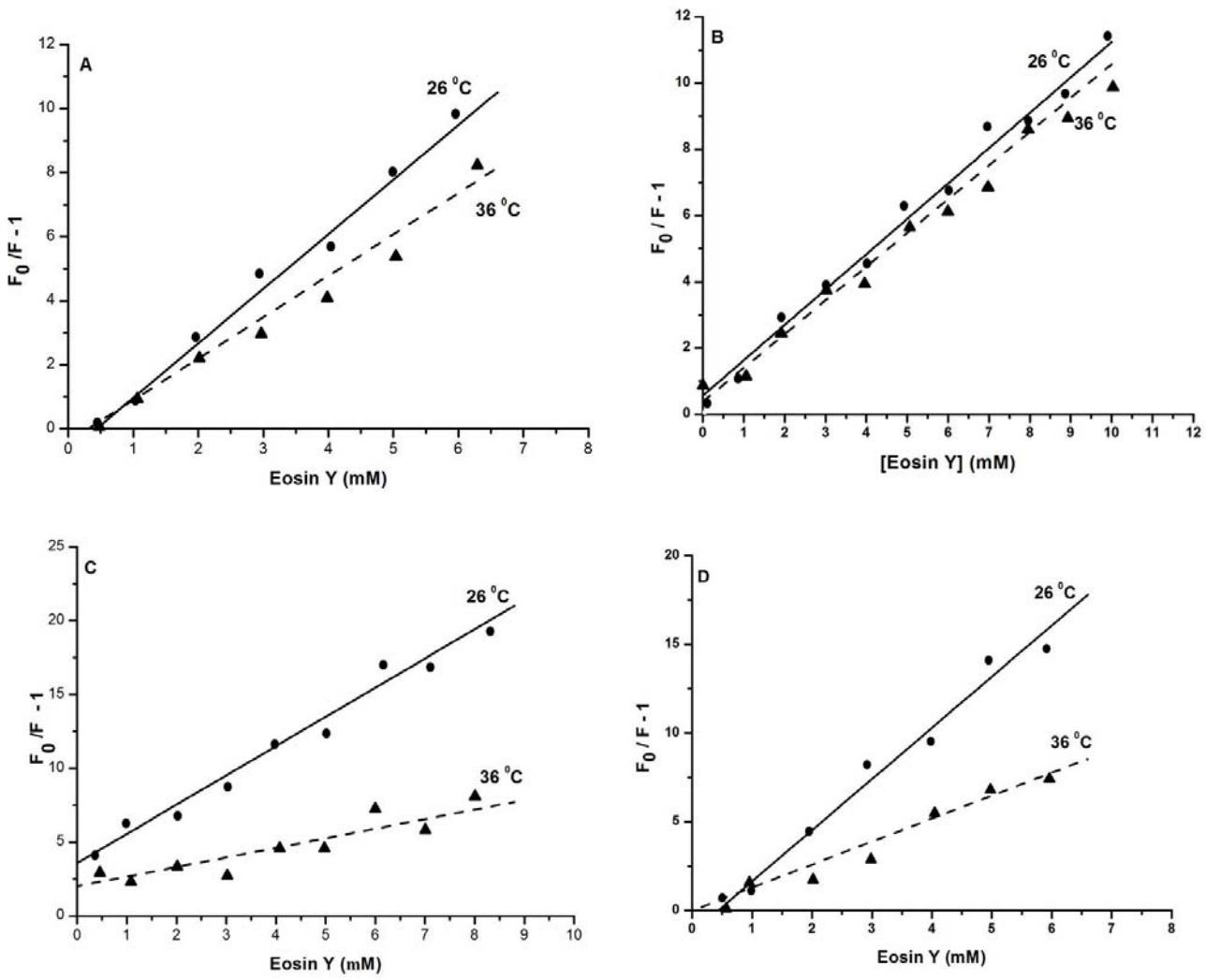


Fig. 2. Stern-Volmer plots for the BSA-eosin Y (A) and HSA-eosin Y (B) complexes, in Hepes buffer and respectively, for the BSA-eosin Y (C) and HSA-eosin Y (D) complexes in Tris-HCl, at 26 and 36 °C, at pH 7.4

3.2 Determination of the binding mode between eosin Y and serum albumins

In the case of the static quenching, a non-fluorescent ground state complex between the fluorophore and quencher appears [9]. Considering the formation of a non-fluorescent 1:*n* complex, the dependence of the fluorescence intensity on quencher concentration is:

$$F_0 / F = 1 + K_A [Q]^n \quad (2)$$

where K_A is the affinity constant and n is the number of binding sites.

3.2.1. The number of the binding sites and the affinity constant

To analyse quantitatively the mechanism of the binding of eosin Y to BSA and HSA, the Scatchard plots were used for the two experiment sets.

The Scatchard plots (in the double logarithmic scales) for the BSA-eosin Y (Fig. 3) and HSA-eosin Y (Fig. 4) complexes in Hepes buffer, at 26 °C and 36 °C are shown. The similar experiments were done in Tris-HCl buffer and the results are listed in the Table 2. The linearity of the Scatchard plots allows the calculation of the number of binding sites, n, and the affinity constants, K_A. For the BSA-eosin Y interaction, $K_A = 1.09 \times 10^6$ M^{-1} at 26 °C and $K_A = 0.98 \times 10^6$ M^{-1} at 36 °C, in Hepes buffer. For the HSA-eosin Y binding, $K_A = 2.70 \times 10^6$ M^{-1} at 26 °C and $K_A = 2.45 \times 10^6$ M^{-1} at 36 °C, in Hepes buffer too. The affinity constants are smaller in Tris-HCl buffer (see Table 2), suggesting that Hepes buffer is a more favourable medium for albumin-eosin Y interactions.

Table 2. The stoichiometry and the binding constants for the BSA-eosin Y and HSA-eosin Y complexes at 26 and 36 °C, in Tris-HCl and Hepes buffer, at pH 7.4

BSA-eosin Y complex			
Buffer solution	T (^{0}C)	n	$K_A \times 10^{-6}$ (M^{-1})
Tris-HCl	26	1.59	0.50
	36	1.76	0.41
Hepes	26	1.46	1.09
	36	1.34	0.98
HSA-eosin Y complex			
Buffer solution	T (^{0}C)	n	$K_A \times 10^{-6}$ (M^{-1})
Tris-HCl	26	1.19	0.31
	36	1.30	0.29
Hepes	26	1.07	2.70
	36	1.09	2.45

The number of the binding sites of BSA for eosin Y in Tris-HCl ($1.59 \leq n \leq 1.76$, see Table 2) seems to be 2, one binding site being completely occupied, due to its possible exposure to the solvent. In the case of interaction in Hepes buffer, the masking of the second site is even more effective ($1.34 \leq n \leq 1.46$, see Table 2) [11]. This hypothesis is in accord with the fact that the Trp-134 can be more exposed to solvent [12]. The other site, less accessible to solvent, is located near Trp-212 being thought to be in a hydrophobic micro-environment, as a single Trp-214 in HSA [12].

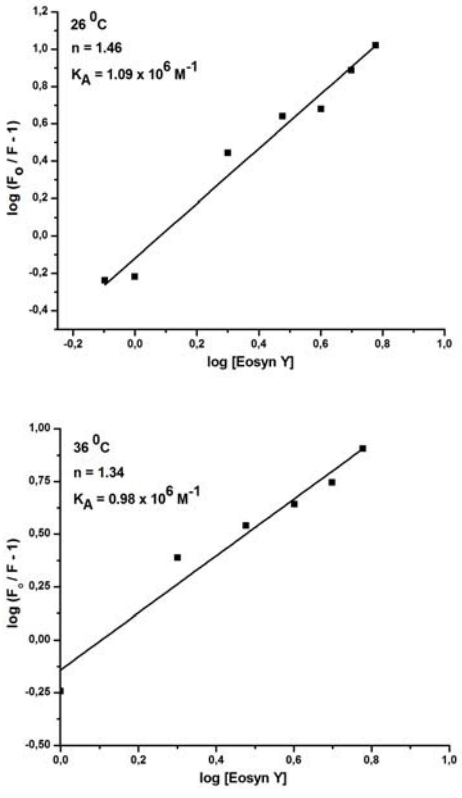


Fig. 3. The Scatchard plot for the BSA-eosin Y complexes at 26 and 36 °C, in Hepes buffer, at pH 7.4

The stoichiometry values for the BSA-eosin Y complexes suggest that the two sites are dependent, the binding to one site decreasing the affinity of the second site. For the HSA-eosin Y complexes, the stoichiometry indicate only a binding site. One may assume that the binding sites are associated with Trp residues (two on BSA chain and one on HSA chain).

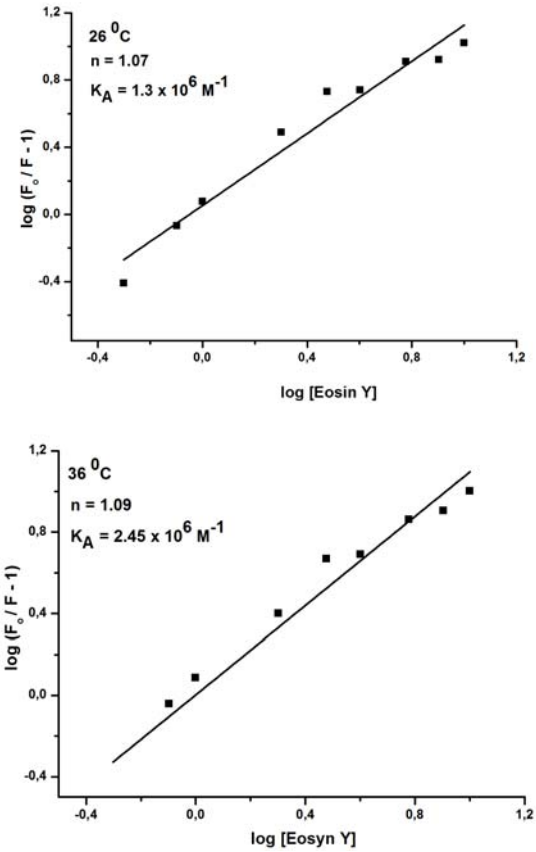


Fig. 4. The Scatchard plot for the HSA-eosin Y complexes at 26 and 36 °C, in Hepes buffer, at pH 7.4

Affinity constant values, for the occupied binding site, show that the strength of the interaction between albumins and eosin Y is moderate, in both buffer solutions, the effect of temperature being not significant. Table 2 shows a slight decrease of K_A with the temperature increase, both in Hepes and Tris-HCl buffers. This is an indication that the interaction strength of the albumins-eosin Y complexes slightly decreases with temperature increase.

3.2.2. The thermodynamic parameters of the binding process

The thermodynamic parameters, ΔG, ΔH, ΔS and ΔC_p are the main evidence to confirm the binding mode for an interaction between a macromolecule and its ligand. The thermodynamic analysis of the BSA-eosin Y and HSA-eosin Y complexes was performed starting from the fluorescence emission measurements. Experiments were

performed at two temperature values, 26 °C and 36 °C, in Tris-HCl and Hepes buffer, at pH 7.4.

The relation between the variation of the Gibbs free energy and the affinity constant is given by the well known equation:

$$\Delta G = - RT \ln K_A \quad (3)$$

where R is the universal constant of gases.

The ΔG_1 (at 26 °C) and ΔG_2 (at 36 °C) values were determined for both complexes, in both buffers, at pH 7.4. The variation of the enthalpy, ΔH can be obtained from equation [7]:

$$\ln K_{A2}/K_{A1} = (1/T_1 - 1/T_2)\, \Delta H / R \quad (4)$$

where K_{A1} and K_{A2} are the binding constants at temperature T_1 and T_2, respectively.

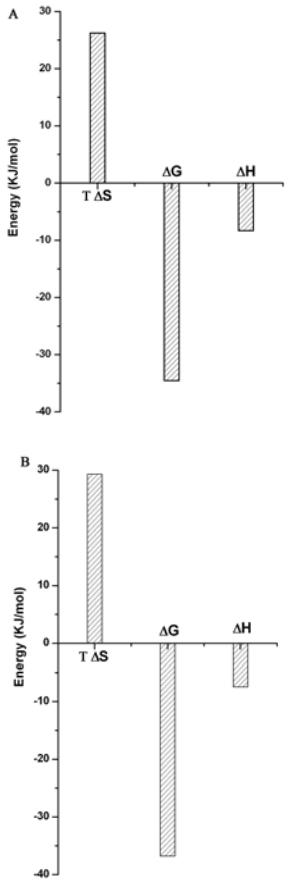


Fig. 5. The values of thermodynamic parameters, ΔG, ΔH, ΔS, for the BSA-eosin Y (A) and HSA-eosin Y (B) complex formation, at 26 °C, in Hepes buffer, at pH 7.4

The ΔH values of the albumins-eosin Y complexes were calculated according with this equation (Table 3).

The variation of the entropic factor, $T\Delta S$, for the BSA-eosin Y and HSA-eosin Y interaction was calculated from the equation (5) and the values for this parameter were listed in Table 3:

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

BSA-eosin Y interaction (Fig. 4A) in Hepes buffer is characterized by a negative variation of the enthalpy (ΔH = -8.32 kJ mol^{-1}), negative variations of the free Gibbs energy (ΔG = -34.55 kJ mol^{-1} at 26°C and ΔG = -35.43 kJ mol^{-1} at 36°C, respectively) and positive entropic factors ($T\Delta S$ = 26.23 kJ mol^{-1} at 26°C and $T\Delta S$ = 27.11 kJ mol^{-1} at 36°C, respectively).

For the HSA-eosin Y interaction (Fig. 4B) in Hepes buffer we found a negative variation of the enthalpy (ΔH = -7.46 kJ mol^{-1}), negative variations of the free Gibbs energy (ΔG = -36.81 kJ mol^{-1} at 26°C and ΔG = -37.79 kJ mol^{-1} at 36°C, respectively) and positive entropic factors ($T\Delta S$ = 29.35 kJ mol^{-1} at 26°C and $T\Delta S$ = 30.33 kJ mol^{-1} at 36°C, respectively). One can see the enthalpic-entropic compensation. For the same binding processes of the two serum albumins, in Tris-HCl buffer, the results are presented in Table 3.

The values of the thermodynamic parameters show that the interaction between the serum albumins and the ligand, eosin Y, are exothermic processes ($\Delta H < 0$). The entropic term is, in absolute value, greater than the enthalpic one, so all these binding processes are driven by entropy (Figure 5), both in Tris-HCl and Hepes buffer solution, at pH 7.4.

The variation of the caloric capacity, ΔC_p, was calculated from the equation (6) and its values are shown in the Table 3, for both BSA and HSA interactions:

$$\Delta C_p = d(\Delta H)/dT \cong \Delta(\Delta H)/\Delta T \quad (6)$$

where $\Delta T = T_2 - T_1$. The value for ΔC_p is greater for the BSA-eosin Y interaction than for HSA-eosin Y complex, suggesting a more pronounced ionic interaction in the case of BSA-eosin Y.

The thermodynamic parameters (ΔG, ΔH, ΔS and ΔC_p) show that both proteins are involved in spontaneous interactions with the dye, these processes being entropically driven.

Table 3. The thermodynamic parameters for the BSA-eosin Y complex formation at 26 and 36 °C, in Tris-HCl and Hepes buffer, at pH 7.4

BSA-eosin Y complex					
Buffer solution	T (^{0}C)	ΔG (kJ mol^{-1})	ΔH (kJ mol^{-1})	TΔS (kJ mol^{-1})	ΔC_p (kJ mol^{-1} K^{-1})
Tris-HCl	26	-32.58	-14.84	17.74	-1.48
	36	-33.16		18.32	
Hepes	26	-34.55	-8.32	26.23	-0.82
	36	-35.43		27.11	
HSA-eosin Y complex					
Buffer solution	T (^{0}C)	ΔG (kJ mol^{-1})	ΔH (kJ mol^{-1})	TΔS (kJ mol^{-1})	ΔC_p (kJ mol^{-1} K^{-1})
Tris-HCl	26	-31.40	-4.87	26.53	-0.48
	36	-32.28		27.41	
Hepes	26	-36.81	-7.46	29.35	-0.74
	36	-37.79		30.33	

4. Conclusions

The analysis of the fluorescence quenching of BSA and HSA by the eosin Y dye shows that the albumin-eosin Y interactions are static quenching processes, quite evident both for BSA-eosin Y and HSA-eosin Y complexes, both in Hepes and Tris-HCl buffers.

The Scatchard plots analysis for the BSA-eosin Y and HSA-eosin Y complexes in Hepes and Tris-HCl buffers, at 26 °C and 36 °C showed that Hepes buffer is a more favourable medium for albumin-eosin Y interactions than Tris-HCl buffer.

The affinity constants indicate that the interaction between BSA/HSA with eosin Y is moderate, depending on the temperature.

BSA-eosin Y complex seems to present two binding sites, the binding to one site being influenced by the affinity of the second one. For the HSA-eosin Y complex, the stoichiometry indicates only a binding site.

Both BSA and HSA proteins are involved in entropically driven interactions with eosin Y, as it is shown by the thermodynamic parameters. The HSA-eosin Y complex formation is characterised by a more pronounced ionic interaction than the BSA-eosin Y complex formation.

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