Water-Protein and Ligand-Protein Interactions as Determined by Selective NMR Relaxation Studies

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Summary: Water-macromolecules and ligand-macromolecules interactions were investigated considering the effects induced by the presence of a macromolecule on both the water and the ligand NMR selective (R_1^{SE}) and non-selective (R_1^{NS}) spin-lattice relaxation rates. The results obtained from the solvent studies were used to describe the solvent dynamics at the macromolecule-solvent interface. On the other hand, ligand R_1^{SE} and R_1^{NS} analysis allowed the definition of the "affinity index", $[A]_L^T$, an index related to the extent of the macromolecule-ligand recognition process.

Keywords: affinity index; NMR; relaxation; solvent-macromolecule interactions; water behaviour

Introduction

Water-macromolecule and ligand-macromolecule interactions are different phenomena and have different chemical and biological importance. However both solvent-macromolecule and ligand-macromolecule interaction can be studied using similar approaches based on selective NMR relaxation experiments. Macromolecules in solution induce solvent-solute interactions. The extent of interaction can be studied by checking the solvent parameters mostly affected by the presence of a large, slowly reorienting biomacromolecule. Water proton relaxation rates have been used to investigate different systems and phenomena, and theoretical interpretations of the experimental results have been proposed.[1-10]

In this paper both the water proton spin-lattice relaxation rates, R_1^{NS} and R_1^{SE}, are analyzed considering all possible sources of dipolar contributions. On the basis of the discussed equations, the average effective rotational water correlation time was calculated. The result shows that this approach could be used as a parameter to monitor the extent of solute-solvent

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interaction. Selective R_1^{SE} and non-selective R_1^{NS} spin-lattice relaxation rates were also used to investigate ligand-macromolecules interactions. Several experimental and theoretical approaches have been developed to study the recognition processes between ligands and receptors,[11-17] including methods based on Nuclear Magnetic Resonance (NMR) analysis of the solution behaviour of the ligand in the presence of receptors.[18-21] In this case the analysis is based on the comparison of selective (R_1^{SE}) and non-selective (R_1^{NS}) spin-lattice relaxation rate investigations of the ligand in the presence and absence of macromolecular receptors. From these studies, the ligand-receptor binding strength was defined on the basis of the *"affinity index"* $[A]_L^T$. An index derived on the basis of specific consideration about the macromolecule-ligand equilibrium kinetics.

Theory

Water-macromolecule Interactions

Dipolar non-selective R_1^{NS} and selective R_1^{SE} spin-lattice relaxation rates have the following expressions:

$$R_1^{NS} = \sum \rho_{ij} + \sum \sigma_{ij} \tag{1}$$

$$R_1^{SE} = \sum \rho_{ij} \tag{2}$$

For any i, j dipolar coupling R_1^{NS} and R_1^{SE} assume the explicit form:

$$R_1^{NS} = \frac{3}{10}\frac{\gamma_H^4\hbar^2}{r_{ij}^6}\left[\frac{4\tau_c}{1+4\omega_H^2\tau_c^2} + \frac{\tau_c}{1+\omega_H^2\tau_c^2}\right] \tag{3}$$

$$R_1^{SE} = \frac{1}{10}\frac{\gamma_H^4\hbar^2}{r_{ij}^6}\left[\frac{3\tau_c}{1+\omega_H^2\tau_c^2} + \frac{6\tau_c}{1+4\omega_H^2\tau_c^2} + \tau_c\right] \tag{4}$$

and their dependence as well as the dependence of the water R_1^{NS}/R_1^{SE} on τ_c are shown in Figure 1.

In pure water, the water non-selective wR_1^{NS} and selective wR_1^{SE} spin-lattice relaxation rates are:

$$wR_1^{NS} = \sum \rho_{wi} + \sum \sigma_{wi} + \sum \rho_{ww} + \sum \sigma_{ww} \quad (5)$$

$$wR_1^{SE} = \sum \rho_{wi} + \sum \sigma_{wi} + \sum \rho_{ww} + \sum \sigma_{ww} \quad (6)$$

where ρ_{wi} e σ_{wi} are the intramolecular water direct and cross-relaxation rate contributions and ρ_{ww} and σ_{ww} the water intermolecular terms. In conditions that deuteron is largely predominant on proton ([D]>90%), equations (5) and (6) become:

$$wR_1^{NS} = \sum \rho_{ww} + \sum \sigma_{ww} \quad (7)$$

$$wR_1^{SE} = \sum \rho_{ww} + \sum \sigma_{ww} \quad (8)$$

and only the intermolecular contributions (*ww*) affect the water selective and non-selective spin-lattice relaxation rates.

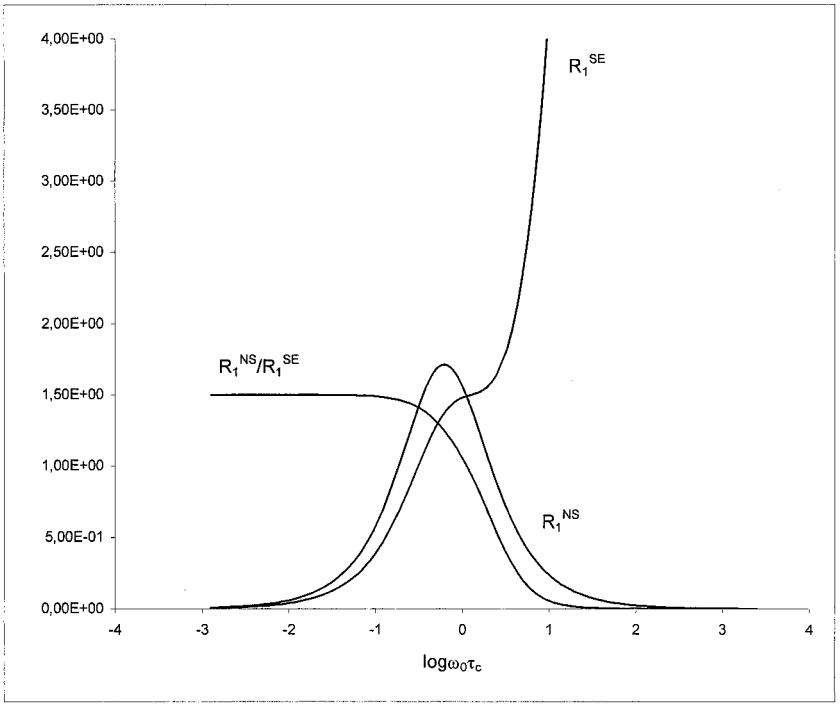

Fig. 1. R_1^{NS} and R_1^{SE} and the R_1^{NS}/R_1^{SE} ratio of a proton pair in relation to $\omega_0\tau_c$.

Moreover in pure water both wR_1^{NS} and wR_1^{SE} assume the same value as the cross-relaxation term σ_{ww} affects the selective and non-selective measurements equally. In binary systems (water-protein), the water molecules are treated as ligands. Under fast chemical exchange conditions between the free and bound state, the changes observed in water spin-lattice relaxation rates with respect to the bulk water reflect the presence of water molecules in environments characterised by different dynamical properties. In these conditions non-selective (wR_1^{NS}) and selective (wR_1^{SE}) water spin-lattice relaxation rates are defined as:

$$wR_{1\exp} = \chi_b R_{1b} + \chi_f R_{1f} \tag{9}$$

where $wR_{1\exp}$ is the experimental relaxation rate of water in the presence of the protein, R_{1b} and R_{1f} the water relaxation rates of the pure bound and free environments and χ_b and χ_f the molar fraction of water in bound and bulk conditions.

Considering the following equilibrium:

$$M(H_2O)_m^A(H_2O)_n^B + n(H_2O)^C \rightleftharpoons M(H_2O)_m^A(H_2O)_n^C + n(H_2O)^B \tag{10}$$

where M is the macromolecule, A are the non-exchangeable and B and C are the exchangeable water molecules respectively. χ_b, the bound water molar fraction assumes the form:

$$\chi_b = \frac{n\left[M(H_2O)_m^A(H_2O)_n^B\right]}{[H_2O]+\left[M(H_2O)_m^A(H_2O)_n^B\right]} \approx \frac{n\left[M(H_2O)_m^A(H_2O)_n^B\right]}{[H_2O]} \tag{11}$$

Considering that $[H_2O] >> \left[M(H_2O)_m^A(H_2O)_n^B\right]$, χ_f of the free water molar fraction is assumed to be: $\chi_f = 1 - \chi_b \cong 1$

Under these conditions $wR_{1\exp}^{NS}$ and $wR_{1\exp}^{SE}$ are described by the following equations:

$$wR_{1\exp}^{NS} = \sum \rho_{ww}^f + \sum \sigma_{ww}^f + \chi_b \sum \rho_{ww}^b + \chi_b \sum \sigma_{ww}^b + \chi_b \sum \rho_{wp}^b + \chi_b \sum \sigma_{wp}^b \tag{12}$$

$$wR_{1\exp}^{SE} = \sum \rho_{ww}^f + \sum \sigma_{ww}^f + \chi_b \sum \rho_{ww}^b + \chi_b \sum \sigma_{ww}^b + \chi_b \sum \rho_{wp}^b \tag{13}$$

where ρ_{wp} and σ_{wp} represent the water-protein ^{1}H-^{1}H direct and cross-relaxation terms respectively and the indexes *f* and *b* consider the free and bound water.

At the bound site, in the presence of [D]>90%, the dipolar interactions between water protons have a sufficient low frequency to be neglected and the equations (12) and (13) result:

$$wR_{1\exp}^{NS} = \sum \rho_{ww}^{f} + \sum \sigma_{ww}^{f} + \chi_b \sum \rho_{wp}^{b} + \chi_b \sum \sigma_{wp}^{b} \tag{14}$$

$$wR_{1\exp}^{SE} = \sum \rho_{ww}^{f} + \sum \sigma_{ww}^{f} + \chi_b \sum \rho_{wp}^{b} \tag{15}$$

From equations (7),(8) and (14),(15) the protein contribution to the water relaxation rates (ΔR_1) can be calculated as:

$$\Delta R_1^{NS} = wR_{1\exp}^{NS} - wR_1^{NS} = \chi_b \left(\sum \rho_{wp} + \sum \sigma_{wp}\right) = \chi_b R_{1b}^{NS} \tag{16}$$

$$\Delta R_1^{SE} = wR_{1\exp}^{SE} - wR_1^{SE} = \chi_b \left(\sum \rho_{wp}\right) = \chi_b R_{1b}^{SE} \tag{17}$$

where R_{1b}^{NS} and R_{1b}^{SE} are the relaxation rates of the water molecules present in the bound conditions.

Considering the dependence of the R_1^{NS}/R_1^{SE} ratio on τ_c (see Figure 1), the ratios $\Delta R_1^{NS}/\Delta R_1^{SE}$ allows the calculation of the water rotational correlation time of the bound site. In fact:

$$\Delta R_1^{NS}/\Delta R_1^{SE} = \chi_b R_{1b}^{NS}/\chi_b R_{1b}^{SE} = R_{1b}^{NS}/R_{1b}^{SE} = \frac{\dfrac{12\tau_c}{1+4\omega_H^2\tau_c^2} + \dfrac{3\tau_c}{1+\omega_H^2\tau_c^2}}{\dfrac{6\tau_c}{1+4\omega_H^2\tau_c^2} + \dfrac{3\tau_c}{1+\omega_H^2\tau_c^2} + \tau_c} \tag{18}$$

and the correlation time τ_c of the bound water can be calculated. This value represents the average τ_c value between all the water molecules who experience restricted motion and are in fast chemical exchange with the bulk water.

Ligand-macromolecule Interactions

The spin-lattice relaxation rate of a ligand under conditions of fast chemical exchange between the free and bound states is described by:

$$R_{1\exp} = \chi_B R_{1B} + \chi_F R_{1F} \tag{19}$$

where $R_{1\exp}$ is the relaxation rate of the ligand in the presence of the macromolecule, R_{1B} and R_{1F} the relaxation rates of the pure bound and free environments and χ_B and χ_F the molar fraction of the ligand in the bound and free conditions. If we consider ligand-macromolecule equilibrium:

$$\mathrm{M} + \mathrm{L} \rightleftharpoons \mathrm{ML} \tag{20}$$

assuming [L]>>[M_0], it has been shown that:[17]

$$\Delta R_1 = \frac{KR_{1B}}{1 + K[L]}[M_0] \tag{21}$$

where $\Delta R = R_{1\exp} - R_{1F}$, K is the thermodynamic equilibrium constant, and [M_0] the initial macromolecule concentration. As suggested by equation (21) a plot of ΔR vs [M_0] would be a straight line through the origin, with slope:

$$[A]_L^T = \left(\frac{KR_{1B}}{1 + K[L]}\right) \tag{22}$$

which was defined *«affinity index»* ($mol^{-1}s^{-1}l$).[17] The affinity index is a constant if temperature and ligand concentration are specified, as suggested by the T and L subscripts in the affinity index symbol. In the case of n binding sites of equal strength,

$$\mathrm{M} + \mathrm{nL} \rightleftharpoons \mathrm{ML_n} \tag{23}$$

an equation similar to (21) can be derived:

$$\Delta R_1 = \frac{KR_{1B}[L]^{n-1}}{1 + K[L]^n}[M_0] \tag{24}$$

In this case a linear relations between ΔR_1 and $[M_0]$ is expected so that the affinity index takes the form:

$$[A]_L^T = \left(\frac{K\ R_{1B}[L]^{n-1}}{1+K[L]^n} \right) \tag{25}$$

In any situation (equations (21) and (24)) the relation between ΔR_1 and $[M_0]$ is always linear. This enables us to write the general equation:

$$\Delta R = [A]_L^T [M_0] \tag{26}$$

where $[A]_L^T$ accounts for all specific and non-specific interactions and all possible stoichiometries.

Experimental

^{1}H-NMR spectra were obtained on a Bruker AC 200 spectrometer operating at 200.13 MHz. Spin-lattice relaxation rates were measured using the $(180°\text{-}\tau\text{-}90°\text{-}t)_n$ sequence. The 180° selective inversion of the proton spin population was obtained with a selective perturbation pulse, generated by the decoupler channel. The selective spin-lattice relaxation rates were calculated using the initial slope approximation and subsequent three parameter exponential regression analysis of the longitudinal recovery curves. The maximum experimental error in the relaxation rate measurements was 5%. Lamotrigine was donated by Wellcome. Ovine albumin (molecular weight 66200 Dalton) was purchased from Sigma Chemical Co. Solutions were obtained by dissolving the appropriate amounts of ligand and protein in DMSO-d_6:D_2O (3:1). The solvent mixture was required because of the low solubility of lamotrigine in D_2O.

Results and Discussion

Water-macromolecule Investigation

The results of the water selective and non-selective spin-lattice relaxation rates as a function of ovine albumin molecular concentration are reported in Figure 2. These data show that wR_1^{SE} and wR_1^{NS} have the same value in the absence of protein. On addition of ovine albumin, the water molecules of the protein hydration sphere, in fast chemical exchange with

the bulk water molecules, contribute to the observed water spin-lattice relaxation rates. The water molecules of the two compartments (free and bound) differ mainly in correlation time which was short for bulk water and long for macromolecule-bound water. On addition of protein, wR_1^{SE} and wR_1^{NS} got contributions from bound water molecules. In slow motion conditions ($\omega_0\tau_c>>1$), these contributions, however, are different: large to wR_1^{SE} and negligible to wR_1^{NS}. Whenever this occurs it is possible to observe that wR_1^{SE} becomes larger than wR_1^{NS} as showed in Figure 2.

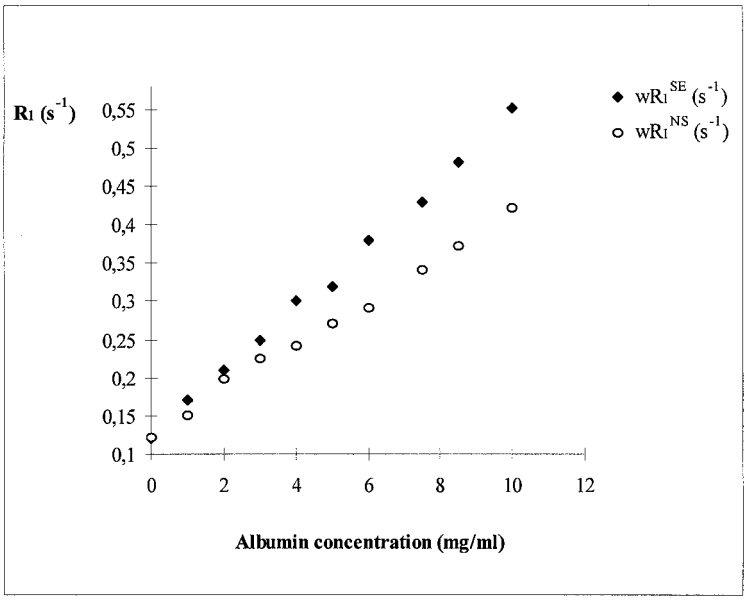

Fig. 2. Proton water selective and non-selective spin-lattice relaxation rates in relation to albumin concentration at 300K.

Figure 3 shows the ΔR_1^{NS} and ΔR_1^{SE} behaviour as a function of albumin concentration and their linear fitting. From the $\Delta R_1^{NS}/\Delta R_1^{SE}$ ratio (equal to 0.7) using equation (18) the correlation time value of $1.5\text{x}10^{-9}$ s was calculated.

As expected this value is three orders of magnitude larger than the rotational bulk water correlation time, but roughly one order of magnitude lower than the protein rotational

correlation time. This value represents the average rotational correlation time of the ensemble of exchangeable water molecule at the protein surface which experience restricted motion.

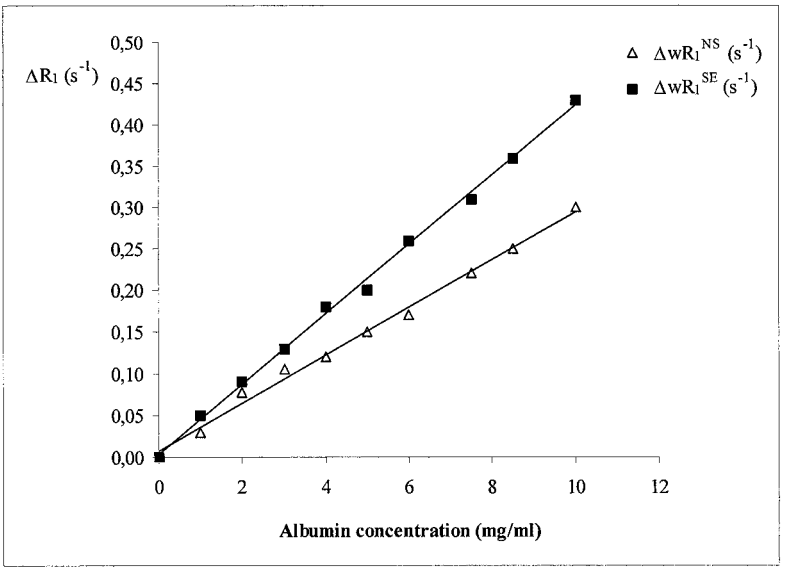

Fig. 3. Experimental values of ΔwR_1^{NS} and ΔwR_1^{SE} vs albumin concentration.

On the basis of previous investigations obtained mainly by relaxometry[15] and considering our results some considerations about the water relaxation behaviour in the presence of macromolecular surfaces appear to be important.

The results obtained by water relaxometry of diluted solutions of proteins and other macromolecules show dispersion curves with an unique inflection point. This behaviour was explained considering that only the long-lived buried water molecules bound to the protein are responsible for the dispersion profile.

This hypothesis is sustained by the fact that the calculated water correlation time has the value found for the macromolecular reorientation tumbling.

The results obtained in this paper show that a significant contribution to the bulk water molecules appears in the presence of a macromolecule. This contribution contains two terms: the first arises from the long-lived buried water molecules surrounded by the macromolecular constituents, the second is due to water molecules present in the hydration shell around the

macromolecular surface which are affected by some extent in their dynamical properties by the presence of the protein. The water exchange rates between the buried water molecules, the surface water molecules and the bulk water molecules could be suggested as:

$$\text{buried water molecules} \underset{\text{exchange}}{\overset{\text{slow}}{\rightleftharpoons}} \text{surface water molecules} \underset{\text{exchange}}{\overset{\text{fast}}{\rightleftharpoons}} \text{bulk water molecules}$$

Assuming this model and considering the theoretical background on which this paper is based, the previous discussed results could elucidate the dynamical properties of water molecules present at the interface with the macromolecule. This makes the two approaches exactly complementary. By relaxometric investigation the behaviour of buried water molecules was studied, by selective relaxation studies the contribution of slowly reoriented water molecules present on the macromolecular surface to the water relaxation.

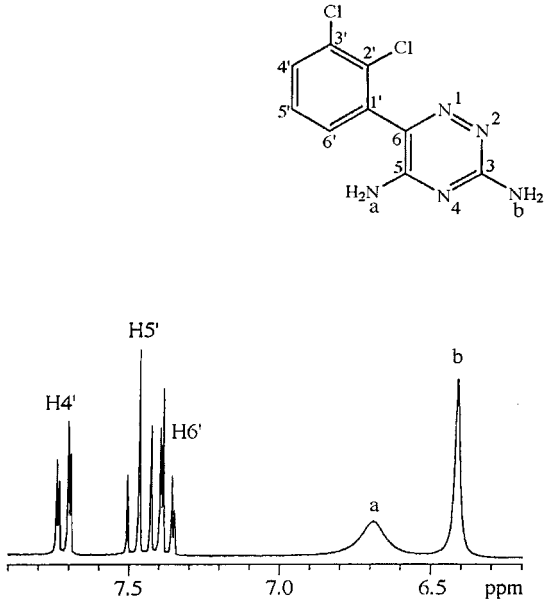

Fig. 4. ^{1}H NMR spectrum of $7.5x10^{-2}$ mol dm^{-3} lamotrigine in DMSO-d_6. The structure and numbering of lamotrigine are also shown.

Ligand-macromolecule Investigation

The affinity index between a ligand, lamotrigine and several ovine proteins (i.e., albumine, α-globulin, β-globulin, and γ-globulin) were calculated. Figure 4 shows the assigned ^{1}H-NMR spectrum of lamotrigine with the molecular structure and numbering. All the experimental results refer to selective (R_1^{SE}) and non-selective (R_1^{NS}) spin-lattice relaxation rate measurements for $H_{4'}$ proton of lamotrigine and assume fast chemical exchange conditions between the free and bound forms of the ligand with respect to its relaxation rates.

The calculation of the affinity index is based on measured selective spin-lattice relaxation rates. Nevertheless both R_1^{SE} and R_1^{NS} are required for a complete investigation of the interaction process.

Table 1. Selective (R_1^{SE}) and non-selective (R_1^{NS}) experimental spin-lattice relaxation rates (s^{-1}) determined for the $H_{4'}$ proton of lamotrigine in the presence of different concentrations of four ovine plasma proteins at 298K. The concentration of lamotrigine was $7.5x10^{-2}$ mol.dm^{-3}.

	albumine		α-globulin		β-globulin		γ-globulin	
Protein Conc. (mg/ml)	$\mathbf{R_1^{SE}}$	$\mathbf{R_1^{NS}}$	$\mathbf{R_1^{SE}}$	$\mathbf{R_1^{NS}}$	$\mathbf{R_1^{SE}}$	$\mathbf{R_1^{NS}}$	$\mathbf{R_1^{SE}}$	$\mathbf{R_1^{NS}}$
0	0.53	0.71	0.53	0.71	0.53	0.70	0.53	0.71
0.1	0.60	0.71	0.56	0.72	0.71	0.73	0.55	0.71
0.5	0.66	0.72	0.68	0.77	0.75	0.77	0.72	0.72
1	0.82	0.76	0.82	0.80	1.05	0.79	0.85	0.76
2	1.05	0.81	1.01	0.91	1.21	1.04	0.98	0.83
5	2.57	0.95	1.80	1.02	2.82	1.15	1.59	0.96
10	4.20	1.19	2.77	1.23	4.90	1.30	2.88	1.30

The free ligand is in fast motion conditions ($\omega_0\tau_c<<1$) and $R_1^{NS} > R_1^{SE}$. In the presence of macromolecule, contributions from the bound ligand appear to both R_1^{SE} and R_1^{NS} and $R_1^{SE} > R_1^{NS}$ if ligand-macromolecule interactions occur. In fact in slow motion conditions ($\omega_0\tau_c>>1$), contributions to R_1^{SE} are more relevant than to R_1^{NS}, due to the different relationships between R_1^{SE} and R_1^{NS} and $\omega_0\tau_c$ (see Figure 1). Table 1 shows the selective

(R_1^{SE}) and non-selective (R_1^{NS}) spin-lattice relaxation rates of lamotrigine in the presence of different plasma proteins and at different macromolecule concentrations.

Figure 5 shows the linear relation between ΔR_1^{SE} and concentrations of the four ovine plasma proteins.

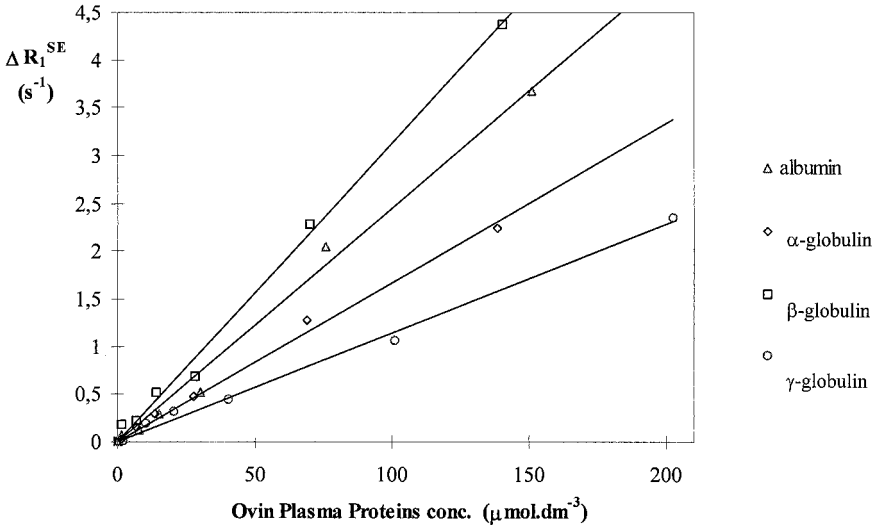

Fig. 5. Linear relationship between $H_{4'}$ selective relaxation enhancements, ΔR_1^{SE} and ovine plasma protein concentrations.

The affinity indexes and R^2 values are reported in Table 2.

Table 2. Affinity Index determined at 298 K for a lamotrigine concentration of 7.5×10^{-2} mol.dm^{-3}.

Ovine Plasma Protein	Affinity Index $[A]_L^T$ x10^{-4} (l.mol^{-1}.s^{-1})	R^2
albumin	2.48	0.994
α-globulin	1.70	0.982
β-globulin	3.13	0.997
γ-globulin	1.14	0.996

These results confirm the validity of the proposed approach which suggests that the *«affinity index»* can be used to measure the strength of all the specific and non-specific interactions that occur at the ligand-macromolecule interface. Moreover the linear relation between ΔR_1^{SE} and $[M_0]$ detected for all systems indicates that equation (26) derived in the theoretical section is formally correct.

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