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Drug–protein recognition processes investigated by NMR relaxation data
A study on corticosteroid–albumin interactions

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ABSTRACT

In this paper we investigated the interaction processes occurring at the protein–solvent interface for prednisolone–albumin and prednisone–albumin systems, using an approach based on the analysis of proton selective relaxation rate enhancements of the ligand in the presence of the macromolecule. The contribution from the bound ligand fraction to the observed relaxation rate in relation to protein concentration allowed the calculation of *the affinity index* $[A]_L^T$ and *the normalized affinity index* $[A_I^N]_L^T$ which removes the effects of motional anisotropies and different proton densities, and isolates the contribution due to a decrease in the ligand dynamics caused by the binding with the protein. This approach allowed the comparison of the binding ability of prednisolone and prednisone towards albumin.

1. Introduction

Molecular recognition of small molecules (ligands) by proteins is important in many biological processes. In large organized systems, such as cells and organisms, specific classes of biomacromolecules are involved in interactions, uptake and transport processes with small bio-ligands [1]. The recognition step, which is related to the surface properties of the interacting molecules [2–4], is crucial in any biological reaction involving two or more chemical structures.

Interactions with bioactive molecules are of primary interest for defining the biological role of proteins and for the activation of specific chemical processes, since effector molecules such as hormones, drugs, pollutants and synthetic molecules can induce different chemical responses in biopolymers.

Several experimental and theoretical approaches have been developed to study the recognition processes between ligands and receptors [5–11]. Nuclear magnetic resonance has been widely used for studying association equilibria in biological systems [12–18], due to the large number of spectral parameters that can be measured and analyzed (chemical shift [19,20], relaxation rates and linewidth [21], NOE [22,23]), together with NMR methods such as pulsed gradient diffusion [24].

In this paper, we investigate the interaction between two glucocorticoids, prednisone and its active metabolite, prednisolone and bovine serum albumin (BSA).

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Glucorticosteroids (GC) are hormones naturally produced by the adrenal glands which have many important functions, including control of inflammatory responses. They exert their antiinflammatory action through inhibition of lymphocyte proliferation and synthesis of proinflammatory cytokines as well as by down-regulating specific adhesion molecules resulting in redistribution of lymphocyte traffic [25,26].

Prednisolone (11,17,21-tridihydroxy pregna-1,4-diene-3,20-dione) is a synthetic corticosteroid used to decrease inflammation in various different diseases and conditions [27,28]. It works by acting within cells to prevent the release of certain chemicals that are important in the immune system.

Prednisone (17,21-dihydroxy pregna-1,4-diene-3,11,20-trione) is an oral, synthetic corticosteroid used for suppressing the immune system and inflammation [29,30]. It is inactive in the body and, in order to be effective, first must be converted to prednisolone by enzymes in the liver.

The aim of this paper is the characterization of the interaction between albumin and the two glucocorticoids, prednisolone and prednisone, using NMR spectroscopy, which provides the advantage for non-invasivity and non-alteration of the normal bio-functionality of the biomolecules under investigation [31]. Moreover, the system can be analysed in real time under its working conditions so that very important information can be obtained even on very fast interaction processes [32]. NMR investigation is based on the comparison of selective (R_1^{SE}) and non-selective (R_1^{NS}) proton spin–lattice relaxation rate of the ligand in the presence and absence of the macromolecular receptor. The formation of intermolecular adducts affects R_1^{NS} and R_1^{SE} to different extents, depending on the dynamical parameters (i.e. the correlation time τ_c), assuming fast chemical exchange between the bound and the free environments with respect to both chemical shift difference and proton relaxation rate. In particular, the slower ligand dynamics in the ligand–macromolecule complex mostly affects R_1^{SE}. In the presence of well resolved proton resonances, R_1^{SE} can be easily determined in different systems. The contributions arising from the fraction of the ligand bound to the protein allowed the calculation of the "*affinity index*" $[A]_L^T$, a useful parameter to attain fundamental information about the strength of non-specific and/or specific interactions occurring within the systems [33]. The affinity index calculated from proton selective relaxation rate enhancements provides a deeper knowledge of the dynamics of the ligand–macromolecule interaction process. Since it is calculated as the slope of a straight line passing through the origin, it is less affected by intrinsic errors than the intercept calculation. Moreover, its calculation does not require an a priori knowledge of the number of ligand coordination sites present at the macromolecule surface or their specific kinetics constant values. In particular, this methodology can be used in order to compare the strength of the interaction processes involving the same protein and different ligands [34,35].

Since motional anisotropies and different spin densities at ligand proton sites may affect the observed selective relaxation rates, the affinity index has been normalized to the relaxation rate of the free ligand. The new calculated parameter, $[A_I^N]_L^T$, the normalized affinity index, appears to be totally independent from the intrinsic relaxation properties of any proton nuclei and can be proposed as a more suited parameter to compare the recognition processes between a protein and different ligands.

2. Theory

For multispin interaction as it occurs in complex systems of biomolecules, the "non-selective" spin–lattice relaxation rate R_1^{NS} of an i nucleus interacting with neighbouring j nuclei and the selective R_1^{SE} obtained by excitation of the i nucleus, while the j nuclei are at thermal equilibrium [36–39] are as follows:

$$R_1^{NS} = \sum_{i \neq j} \rho_{ij} + \sum_{i \neq j} \sigma_{ij} \tag{1}$$

$$R_1^{SE} = \sum_{i \neq j} \rho_{ij} \tag{2}$$

where ρ_{ij} is the direct self-relaxation rate and σ_{ij} is the cross-relaxation rate, with the approximation of independent pair-wise interactions.

The explicit forms of R_1^{NS} and R_1^{SE} are [38,40,41]:

$$R_1^{NS} = \frac{1}{10} \frac{\gamma_H^4 \hbar^2}{r_{ij}^6} \left[\frac{3\tau_c}{1+\omega_H^2\tau_c^2} + \frac{12\tau_c}{1+4\omega_H^2\tau_c^2} \right] \tag{3}$$

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

Eqs. (3) and (4) indicate the existence of two molecular motion regimes: the fast molecular reorientation typical of the free ligand ($\omega_0\tau_c \ll 1$), in which $R_1^{NS} > R_1^{SE}$, and the slow motion typical of a ligand bound to a macromolecule ($\omega_0\tau_c \gg 1$), where $R_1^{SE} > R_1^{NS}$.

Since the ligand NMR parameter most affected by drastic changes in the molecular dynamics is R_1^{SE}, it appears to be the best experimental parameter for obtaining information about ligand–macromolecule interactions. In conditions of fast chemical exchange between the free and bound environments, R_1^{SE} is expressed by the following equation:

$$R_{1obs}^{SE} = \chi_F R_{1F}^{SE} + \chi_B R_{1B}^{SE} \tag{5}$$

where R_{1obs}^{SE} is the experimentally determined selective relaxation rate, R_{1F}^{SE} and χ_F and R_{1B}^{SE} and χ_B are the selective spin–lattice relaxation rates and the ligand fractions of the free and bound environments, respectively.

If we consider the ligand–macromolecule equilibrium:

$$M + L \leftrightarrows ML \tag{6}$$

with an equilibrium constant $K = [ML]/[M][L]$.

Assuming $[L] \gg [M_0]$, it has been shown that:

$$\Delta R_1^{SE} = \frac{K R_{1B}}{1 + K[L]} [M_0] \tag{7}$$

where $\Delta R_1^{SE} = R_{1obs}^{SE} - R_{1F}^{SE}$, K the thermodynamic equilibrium constant and $[M_0]$ is the initial macromolecule concentration.

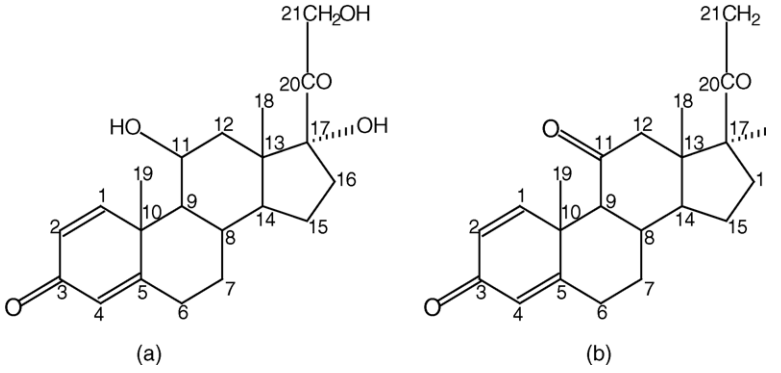


Fig. 1 – Structure and numbering of (a) prednisolone and (b) prednisone.

As suggested by Eq. (7), the plot ΔR_1 versus $[M_0]$ would have a straight line through the origin, with slope:

$$[A]_L^T = \frac{KR_{1B}}{1 + K[L]} \tag{8}$$

which was defined as "*affinity index*" ($l\,mol^{-1}\,s^{-1}$) [33]. 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.

A temperature dependency analysis of R_1^{SE} and R_1^{NS} is also required to test whether $R_1^{SE} > R_1^{NS}$ conditions are really due to a large $\chi_B R_{1B}$ term to R_1^{SE}; in fact, $R_1^{SE} > R_1^{NS}$ could also be the result of a reduction in molecular tumbling due to an increase in viscosity caused by the presence of a macromolecule in the solution. A reduction in both R_1^{SE} and R_1^{NS} with increasing temperature demonstrates that the ligand fast motion condition $\omega_0\tau_c \ll 1$ holds in the solution. This allows the effects on R_1^{SE} to be attributed to the formation of the ligand–macromolecular complex.

In previous studies performed using this methodology, the affinity index was mainly calculated from selective relaxation rate enhancements calculated for a single proton, assuming an isotropic motion for the ligand molecule. However, even for small ligands, there can be differences in the dynamics of different portions of the molecule, leading to effects on the selective relaxation rates and as a consequence, on the affinity index value due to different correlation times modulating the dipolar interactions between protons at different positions. The normalization of $\Delta R_1^{SE} = R_{1obs}^{SE} - R_{1F}^{SE}$ to R_{1F}^{SE} removes the effects of different correlation times and different proton densities and isolates the effects of restricted motions due to the interaction of the ligand with the macromolecule, leading to a normalized affinity index:

$$\Delta R_{1N}^{SE} = \frac{KR_{1B}^{SE}[M_0]}{(1 + K[L])R_{1F}^{SE}} \tag{9}$$

The dependence of the normalized relaxation rate enhancements ΔR_{1N}^{SE} from the concentration of the macromolecule $[M_0]$ is represented by a straight line passing through the origin of the axes with slope:

$$[A_I^{N]}]_L^T = \frac{KR_{1B}^{SE}}{(1 + K[L])R_{1F}^{SE}} \tag{10}$$

$[A_I^{N]}]_L^T$ is still a constant at fixed temperature and ligand concentration and it is defined as "*normalized affinity index*" ($dm^3\,mol^{-1}$) [42].

3. Experimental

3.1. Materials

Prednisone (17,21-dihydroxy pregna-1,4-diene-3,11,20-trione) and prednisolone (11,17,21-tridihydroxy pregna-1,4-diene-3,20-dione) (Fig. 1a and b) and bovine serum albumin (molecular weight 67,000 Da) were purchased from Sigma Chemical Co. and used without any further purification.

4. Methods

4.1. NMR measurements

The solutions for the NMR experiments were obtained by dissolving the appropriate amounts of ligand and protein in DMSO-d_6:D_2O (3:1). The solvent mixture was required due to the low solubility of the two corticosteroids in D_2O. In all the experiments ligand concentration was 4×10^{-2} mol dm^{-3}.

^1H NMR spectra were obtained on a Bruker AC 200 spectrometer, operating at 200.13 MHz. The spin–lattice relaxation rates were measured using the $(180^\circ\text{-}\tau\text{-}90^\circ\text{-}t)_n$ sequence. The τ values used for the selective and non-selective experiments were: 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.8, 1, 1.5, 2, 3, 4, 5, 7 and 20 s, respectively, and the delay time t in this case is 20 s. The 180° selective inversion of the proton spin population was obtained by a selective soft perturbation pulse, generated by the decoupler channel [43]. All the selective and non-selective spin–lattice relaxation rates refer to the H_1, H_2 and H_4 protons of both prednisolone and prednisone. Since in general the recovery of proton longitudinal magnetization after a 180° pulse is not a single exponential, due to the sum of different relaxation terms, 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 affinity index was calculated by linear regression analysis of the experimental data.

Table 1 – Chemical shift values (ppm) calculated for prednisolone protons

Proton	Chemical shift (ppm)	Proton	Chemical shift (ppm)
1	7.35	12α	2.01
2	6.22	12β	1.58
4	5.98	14	1.72
6α	2.32	15α	1.79
6β	2.57	15β	1.43
7α	1.12	16α	1.54
7β	2.10	16β	2.69
8	2.12	18	0.92
9	1.04	19	1.46
11	4.42	21	4.66/4.25

All the spectra were processed using the Bruker Software XWINNMR, Version 2.5 on Silicon Graphics O_2 equipped with RISC R5000 processor, working under the IRIX 6.3 operating system.

5. Results and discussion

Prednisolone proton chemical shift values are summarized in Table 1 as reported in literature [44–46]. As discussed in Section 2, the NMR parameters used in this approach to obtain information about the existance of interaction processes between the ligands and the protein are the non-selective and the selective proton relaxation rates, measured in the absence and presence of the macromolecule. Table 2a and

Table 2 – R_1^{SE} and R_1^{NS} values calculated for H_1, H_2 and H_4 protons of (a) prednisolone (4×10^{-2} mol dm^3) and (b) prednisone (4×10^{-2} mol dm^{-3}) in the presence of variable albumin concentrations at 298 K

Albumin concentration (mg/ml)	Albumin concentration (mol dm^{-3})	R_1^{SE} (s^{-1}) H_1	R_1^{NS} (s^{-1}) H_1	R_1^{SE} (s^{-1}) H_2	R_1^{NS} (s^{-1}) H_2	R_1^{SE} (s^{-1}) H_4	R_1^{NS} (s^{-1}) H_4
(a) Prednisolone							
0	0	2.14 ± 0.05	2.86 ± 0.07	0.79 ± 0.02	0.87 ± 0.02	0.98 ± 0.02	1.09 ± 0.03
2	2.98×10^{-5}	2.57 ± 0.06	2.80 ± 0.07	0.93 ± 0.02	0.90 ± 0.02	1.15 ± 0.03	1.14 ± 0.03
4	5.97×10^{-5}	3.04 ± 0.08	2.84 ± 0.07	1.12 ± 0.03	0.95 ± 0.02	1.45 ± 0.04	1.27 ± 0.03
5	7.46×10^{-5}	3.19 ± 0.08	2.84 ± 0.07	1.21 ± 0.03	1.05 ± 0.03	1.61 ± 0.04	1.35 ± 0.03
8	1.19×10^{-4}	4.00 ± 0.10	2.88 ± 0.07	1.55 ± 0.04	1.15 ± 0.03	1.85 ± 0.05	1.53 ± 0.04
10	1.49×10^{-4}	4.57 ± 0.11	2.90 ± 0.07	1.78 ± 0.04	1.16 ± 0.03	2.10 ± 0.05	1.55 ± 0.04
(b) Prednisone							
0	0	1.10 ± 0.03	1.42 ± 0.04	0.78 ± 0.02	0.84 ± 0.02	0.94 ± 0.02	1.03 ± 0.03
2	2.98×10^{-5}	1.34 ± 0.03	1.45 ± 0.04	1.07 ± 0.03	0.95 ± 0.02	1.35 ± 0.03	1.25 ± 0.03
4	5.97×10^{-5}	1.86 ± 0.05	1.50 ± 0.04	1.43 ± 0.03	1.02 ± 0.03	1.71 ± 0.04	1.41 ± 0.03
5	7.46×10^{-5}	2.10 ± 0.05	1.52 ± 0.04	1.58 ± 0.04	1.04 ± 0.03	1.93 ± 0.05	1.48 ± 0.04
8	1.19×10^{-4}	2.84 ± 0.07	1.52 ± 0.04	1.88 ± 0.05	1.05 ± 0.03	2.31 ± 0.06	1.55 ± 0.04
10	1.49×10^{-4}	3.17 ± 0.08	1.52 ± 0.04	2.09 ± 0.05	1.06 ± 0.03	2.54 ± 0.06	1.58 ± 0.04

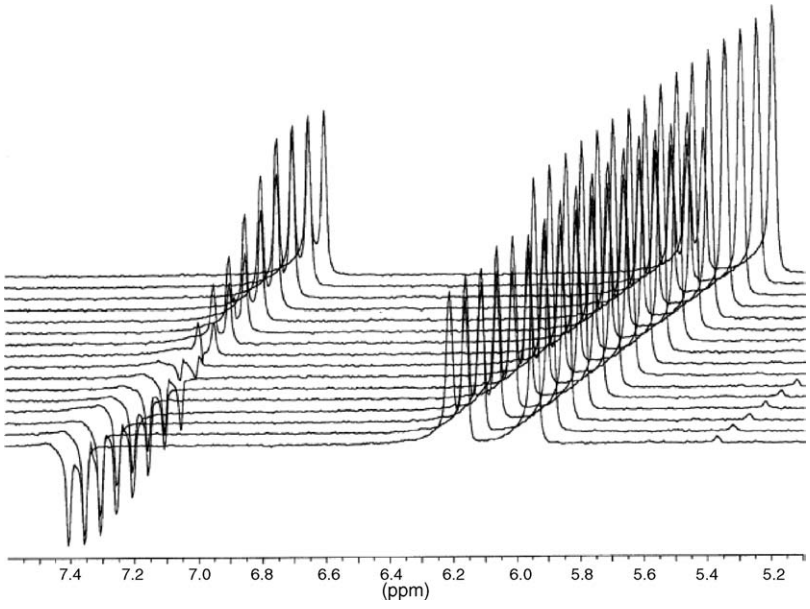


Fig. 2 – Selective partially relaxed aromatic proton spectra of a 4×10^{-2} mol dm^{-3} prednisolone solution. The selective measurements refer to the prednisolone H_1 proton.

b reports the values of R_1^{SE} and R_1^{NS} of H_1, H_2 and H_4 protons of prednisone and prednisolone in relation to albumin concentration. Experimental proton spectra used for R_1^{SE} measurements are shown in Fig. 2. The results show that in the absence of albumin, $R_1^{NS} > R_1^{SE}$ while increasing protein concentration R_1^{SE} becomes greater than R_1^{NS}. This is an important indication about the occurring of interaction processes between the two glucocorticoids and albumin, since the selective relaxation rate enhancements reveal a large contribution from the bound ligand fraction to the observed relaxation rate. Solution containing a relatively high concentration of proteins, as in this case, may be subject to an increase in viscosity, which can lead to a lowering in the ligand dynamics regardless of the existence of interaction processes. In order to clarify this point, a temperature dependence analysis of relaxation rates in the presence of albumin have been carried out. In particular, the effects of temperature changes on R_1^{NS} seem to be diagnostic, since an increase in temperature should cause a decrease in R_1^{NS} if the bulk ligand still experience fast motion conditions. Table 3 reports the experimental values of R_1^{NS} in relation to temperature measured for H_1, H_2 and H_4 protons of prednisone and prednisolone in the presence of 8 mg/ml of albumin. Data show a decrease in R_1^{NS} with increasing temperature, which indicates that the presence of the protein does not affect the dynamics of the free ligand, confirming the existence of ligand–protein interaction between albumin and the two glucocorticoids.

In order to evaluate the strength of the binding processes, the affinity index $[A]_L^T$ for prednisolone–albumin and prednisone–albumin systems were calculated from the slope of the straight line describing the dependence of proton selective relaxation rate enhancements on protein concentration. Fig. 3 shows the plot of ΔR_1^{SE} versus albumin concentration for H_1, H_2 and H_4 protons of prednisone and prednisolone together with the calculated affinity indexes for each proton. The values of $[A]_L^T$ obtained for different nuclei belonging to the same ligand showed appreciable differences for both prednisolone and prednisone. This behaviour can be explained considering that motional anisotropies in the ligand molecule and different spin densities at the observed proton may affect the spin lattice relaxation rates to some extent. In order to remove these effects, $[A]_L^T$ was normalized to the selective spin–lattice relaxation rate of the free ligand and the so called "*normalized affinity index*" $[A^N]_L^T$ was calculated. Fig. 4 clearly shows the effect of the normalization on $[A]_L^T$, leading to very close values of the normalized affinity indexes $[A^N]_L^T$ for all the observed spins of each ligand, as reported in the figure. The averaged values of $[A^N]_L^T$ for prednisone was 12 244 mol^{-1} dm^3, while for prednisolone $[A^N]_L^T$ value was 7694 mol^{-1} dm^3. This appreciable difference in the global affinity of the two corticosteroids towards albumin is in agreement with the literature which reports the evidence of lower blood retention times for prednisolone in respect to prednisone [47].

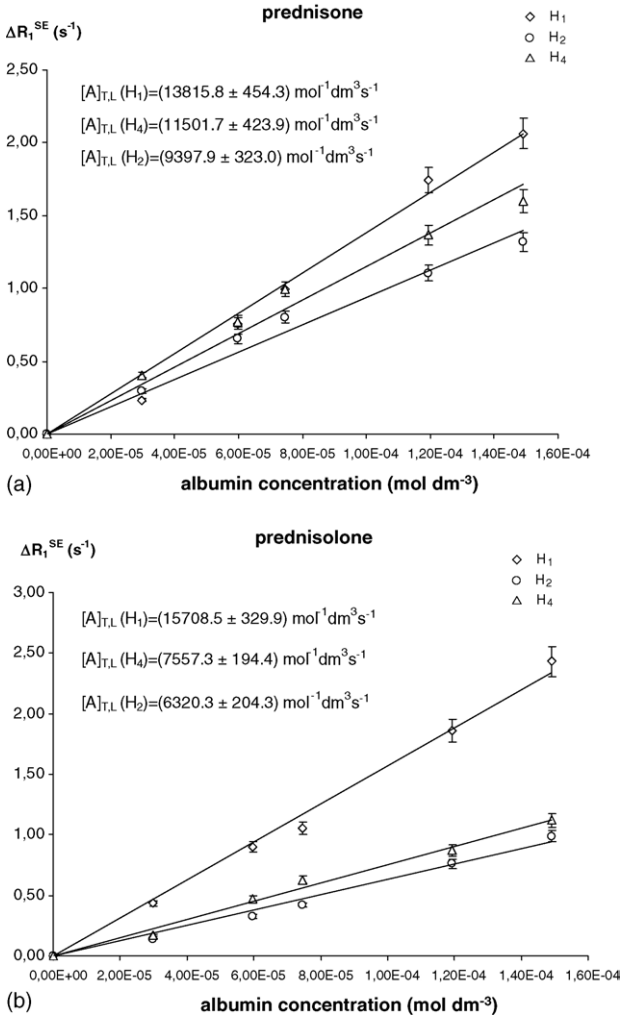
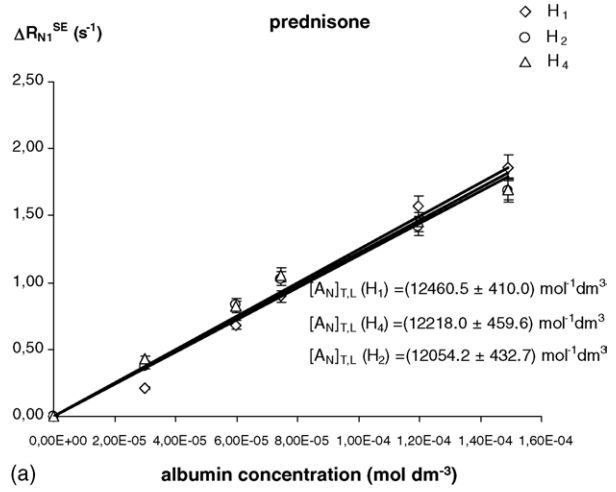


Fig. 3 – Comparison of the linear regression analysis of the H_1, H_2 and H_4 selective relaxation enhancement, ΔR_1^{SE}, as a function of albumin concentration of (a) a solution of prednisone (4×10^{-2} mol dm^{-3} at 298 K); (b) a solution of prednisolone (4×10^{-2} mol dm^{-3} at 298 K). The values of the affinity indexes $[A]_L^T$ are also reported with the corresponding errors.

Table 3 – Experimental R_1^{NS} values in relation to temperature calculated for a 4×10^{-2} M solution of prednisone (a) and prednisolone (b) in the presence of 8 mg/ml of albumin

Temperature (K)	R_1^{NS} (s^{-1}) H_1	R_1^{NS} (s^{-1}) H_2	R_1^{NS} (s^{-1}) H_4
(a) Prednisone			
300	1.55 ± 0.04	1.04 ± 0.03	1.52 ± 0.04
310	1.04 ± 0.03	0.68 ± 0.02	0.95 ± 0.02
320	0.73 ± 0.02	0.47 ± 0.01	0.63 ± 0.02
(b) Prednisolone			
300	2.80 ± 0.07	1.11 ± 0.03	1.47 ± 0.04
308	2.34 ± 0.06	0.83 ± 0.02	1.02 ± 0.03
316	1.76 ± 0.04	0.53 ± 0.01	0.67 ± 0.02
323	1.37 ± 0.03	0.40 ± 0.01	0.50 ± 0.01



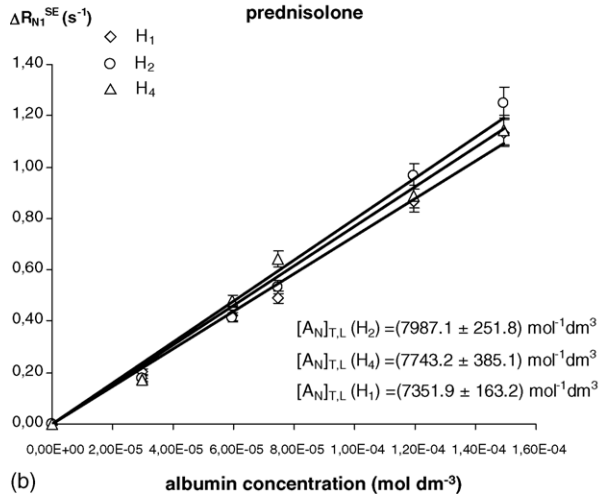


Fig. 4 – Comparison of the linear regression analysis of the H_1, H_2 and H_4 normalized selective relaxation enhancement, ΔR_{N1}^{SE}, as a function of albumin concentration of (a) a solution of prednisone (4×10^{-2} mol dm^{-3} at 298 K); (b) a solution of prednisolone (4×10^{-2} mol dm^{-3} at 298 K). The values of the normalized affinity indexes $[A_N]_L^T$ are also reported with the corresponding errors.

6. Conclusions

The determination of the affinity index from nuclear spin relaxation analysis as a measure of the overall complexing behaviour of different ligands towards macromolecules, constitutes a useful approach in order to evaluate the strength of all specific and non-specific binding phenomena occurring at protein–solvent interface. This parameter, calculated from the slope of the straight line describing the dependence of the proton selective relaxation rate enhancements on protein concentration, can be obtained without the knowledge of the stoichiometry of the interaction, i.e. the number of binding sites. This approach represents a powerful tool to compare the ability of different ligands to interact with a protein.

Moreover, as the contributions of anisotropic dynamics and different proton densities to the nuclear relaxation rates are normalized, the calculated values of $[A_I^N]_L^T$ should be the same when determined for any ligand proton nuclei. In case where the normalized affinity index calculated for different ligand protons still presents different values, these should be attributed to the specificity of the ligand–receptor interactions. The ligand–receptor complexing in favourable cases may re-introduce a difference in the $[A_I^N]_L^T$ values as a consequence of anisotropic contributions of the complex to the ligand proton relaxation properties. The effect could be of interest for the identification of the ligand moiety directly involved in the recognition step.

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