Bioorganic & Medicinal Chemistry 17 (2009) 1630–1635

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Interaction study of bioactive molecules with fibrinogen and human platelets determined by ^{1}H NMR relaxation experiments

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ARTICLE INFO

Article history:
Received 29 July 2008
Revised 5 December 2008
Accepted 27 December 2008
Available online 3 January 2009

Keywords:
Isoproterenol
Epinephrine
^{1}H spin–lattice relaxation rate
Normalized affinity index

ABSTRACT

In order to investigate the interaction processes between bioactive molecules and macromolecular receptors NMR methodology based on the analysis of selective and non-selective spin–lattice relaxation rate enhancements of ligand protons was used.

The contribution from the bound ligand fraction to the observed relaxation rate in relation to macromolecular target concentration allowed the calculation of *the normalized affinity index* $[A_I^N]_L^T$ in which the effects of motional anisotropies and different proton densities have been removed.

In this paper, we applied this methodology to investigate the affinity of epinephrine and isoproterenol towards two different systems: fibrinogen and platelets.

1. Introduction

Plasma contains a complex mixture of proteins, which can be classified into two major groups, namely albumin, globulins.

Fibrinogen is a glycoprotein (Fbg, MW = 341 kD) made by three pairs of non-identical polypeptide chains forming two identical subunits that represents around 3% of blood plasma proteins.[1] The fibrinogen molecule is formed by three couples of peptide chains, called Aα, Bβ, and γ.[2] Fibrinogen, also known as coagulation factor I, shows a central role in the haemostatic process both as adhesion protein essential to platelet aggregation and as precursor of insoluble fibrin that forms the haemostatic clot.[3,4] Its main role in platelet adhesion involves the secondary formation of aggregates linked to the damaged vessel surface and the increase of the adhesion towards the majority of surfaces due to a preferential adsorption.[5,6]

Fibrinogen is also largely responsible for mediating platelet–surface interactions by serving as a ligand for the αIIbβ3 integrin receptor on the platelet membrane, while Factor XII is involved in contact activation of the intrinsic pathway of the blood coagulation cascade.

Platelet adhesion, activation and fibrinogen-mediated aggregation are primary events in vascular thrombosis and occlusion.[7]

The intravascular coagulation process is induced by agonists such as thrombin and catecholamines,[8] as epinephrine and norepinephrine. Epinephrine strongly stimulate α-adrenergic receptors causing vessel constriction, β1-adrenergic receptors increasing heart frequency, α2 and β2-adrenergic receptors present at articular and bronchial levels which pro-coagulant activity induces platelet activity.[9]

Isoproterenol is a synthetic catecholamine that stimulates both β1 and β2 adrenergic receptors and has no α-receptor capabilities. It is structurally similar to epinephrine but acts for the most part on β receptors. It is used in the treatment of asthma. Isoproterenol's effects on the cardiovascular system are related to its actions on cardiac β_1 receptors and β_2 receptors on skeletal muscle arterioles. It has positive inotropic and chronotropic effects on the heart and in skeletal muscle arterioles and it produces vasodilatation.[10]

Nuclear magnetic resonance (NMR) has been used for studying interactions of small molecules with macromolecules, due to the large number of spectral parameters that can be measured and analysed (chemical shift, relaxation rates and line width, diffusion coefficients or NOE measurements).[11–15]

In this paper, 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} at different extents, depending on the dynamical parameters (i.e., molecular rotational correlation time), assuming fast chemical exchange between the bound and the free environments.

The main purpose of this study is to investigate the interaction processes occurring between isoproterenol and fibrinogen and compare the results to those obtained using epinephrine as a ligand.[16]

The second objective was to extend this approach to more complex biological systems, that is, human platelets, with the aim to

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doi:10.1016/j.bmc.2008.12.063

add a contribution to the comprehension of the role of epinephrine and isoproterenol in the platelet adhesion process.

2. Theory

The explicit forms of non-selective R_1^{NS} and selective R_1^{SE} proton spin–lattice relaxation rates are:[17–22]

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

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

where $\hbar$ is the reduced Plank's constant, γ_H and ω_H are the proton magnetogyric ratio and Larmor frequency, respectively, r_{ij} is the internuclear distance, and τ_c is the effective correlation time, which modulates the $i-j$ magnetic interaction.

R_1^{SE}SE and R_1^{NS} depend on ligand dynamics with different extent: $R_1^{NS} > R_1^{SE}$ in the fast molecular reorientation typical of the free ligand ($\omega_0\tau_c \ll 1$) and $R_1^{SE} > R_1^{NS}$ when the ligand is bound to a macromolecule ($\omega_0\tau_c \gg 1$).

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:

$$R_{1obs}^{SE} = \chi_F R_{1F}^{SE} + \chi_B R_{1B}^{SE} \quad (3)$$

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.

As reported in previous papers,[23] the plot ΔR_1^{SE} ($\Delta R_1^{SE} = R_{1obs}^{SE} - R_{1F}^{SE}$ versus macromolecule concentration ([M_0]) would have a straight line, with slope:

$$[A]_L^T = \left(\frac{KR_{1B}}{1+K[L]}\right) \quad (4)$$

which was defined as «*affinity index*» (l mol^{-1} s^{-1}).[24] In Eq. 1, K is the equilibrium constant. 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.

The affinity index represents the global affinity (specific and non-specific binding sites) between the ligand and the macromolecule and can be calculated without knowledge of the stoichiometry of the interaction, that is, the number of binding sites is not required for the affinity index calculation.

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 normalisation 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:[25]

$$\Delta R_{1N}^{SE} = \frac{KR_{1b}^{SE}[M_0]}{(1+K[L])R_{1f}^{SE}} \quad (5)$$

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:

$$[A_I^N]_L^T = \frac{KR_{1b}^{SE}}{(1+K[L])R_{1f}^{SE}} \quad (6)$$

$[A_I^N]_L^T$ is still a constant at fixed temperature and ligand concentration and it is defined as '*normalized affinity index*' (dm^3 mol^{-1}).

3. Materials and methods

3.1. Materials

Epinephrine and isoprotereonol (Fig. 1a and b) were purchased from SIGMA and used without further purification. Fibrinogen (human plasma, MW = 341 kDa, Fbg) was purchased from CALBIOCHEM and used without further purification.

Pool of platelets was obtained from human blood samples by several centrifugations (Rotofix 32 Hettich). To remove white and red globules from blood sample, it was first centrifugated at 150–200 rpm and 298 K for 15 min and then at 1500 rpm for 15 min. The pellet was suspended in deuterated TRIS-buffered saline solution (2-amino-2-hydroxymethyl-1,3-propanediol, pH 7.4). Four additional washing–centrifuging cycles were performed using deuterated TRIS-buffered saline. The number of platelets was determined by Burke chamber.

3.2. NMR measurements

The solutions for the NMR experiments were obtained by dissolving the appropriate amounts of epinephrine, isoproterenol, fibrinogen and platelets in D_2O and deuterated TRIS-buffered saline, respectively.

^{1}H NMR spectra were obtained on a Bruker DRX 600 spectrometer, operating at 600.13 MHz. The spin–lattice relaxation rates were measured using the $(180°–\tau–90°–t)_n$ 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, 20 s, respectively, and the delay time t in this case was 20 s. All the selective and non-selective spin–lattice relaxation rates refer to the H_6 proton of epinephrine and isoproterenol. The 180° selective inversion of the proton spin population was obtained by a selective soft gaussian perturbation pulse (width: 60 ms, power: 120 dB). 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%. The affinity index was calculated by linear regression analysis of the experimental data.

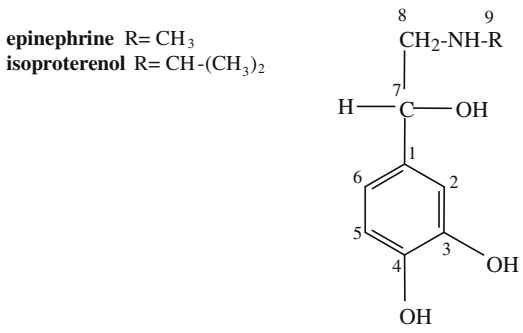

Figure 1. Structure and numbering of epinephrine and isoproterenol.

Table 1
Mean diameters and the 95% confidential limit for size distribution obtained by dynamical light scattering of (a) fibrinogen (in deuterated TRIS-buffered saline at different concentrations) and (b) platelets (in deuterated TRIS-buffered saline at different concentrations)

	Photon counts	Average diameter <*D*> (nm)	95% Confidential limit
(a) Fibrinogen concentration (mg/cm^3)			
12.0	9.0×10^4	65	63–66
6.0	5.0×10^4	66	65–68
3.0	2.5×10^4	66	64–67
1.5	1.0×10^4	70	68–71
(b) Concentration (number of platelets/cm^3)			
2.0×10^6	7.0×10^4	535	505–565
4.0×10^6	1.6×10^5	515	497–543
8.0×10^6	2.0×10^5	516	488–545
1.6×10^7	4.0×10^5	522	493–551
3.2×10^7	4.6×10^5	531	492–550
6.4×10^7	2.0×10^5	530	490–550

A possible concern, when working with solutions of proteins and platelets is that they do not aggregate or oligomerize if their concentration is increased. Here we were able to rule out such possibility by checking the mean size of Fbg and platelets at different concentration with dynamic light scattering. For this purpose, a Coulter Sub-Micrometer Particle Analyzer, Model N4SD (equipped with a 4 mW helium–neon laser and 90° detector) was used. The autocorrelation function of the scattered light was analysed by the cumulant method, assuming a log Gaussian size distribution.[26] Table 1 reports the obtained mean diameters and the 95% confidential limit for size distribution. From these values it is clear that aggregation did not occur either in the case of Fbg or platelets in the concentration range investigated in this work.

4. Results and discussion

4.1. Epinephrine–fibrinogen and isoproterenol–fibrinogen interactions

Figure 2a and b shows the ^{1}H spectra of epinephrine and isoproterenol in D_2O solution at 600 MHz with the resonance assignments. The signal chosen for the selective and non-selective

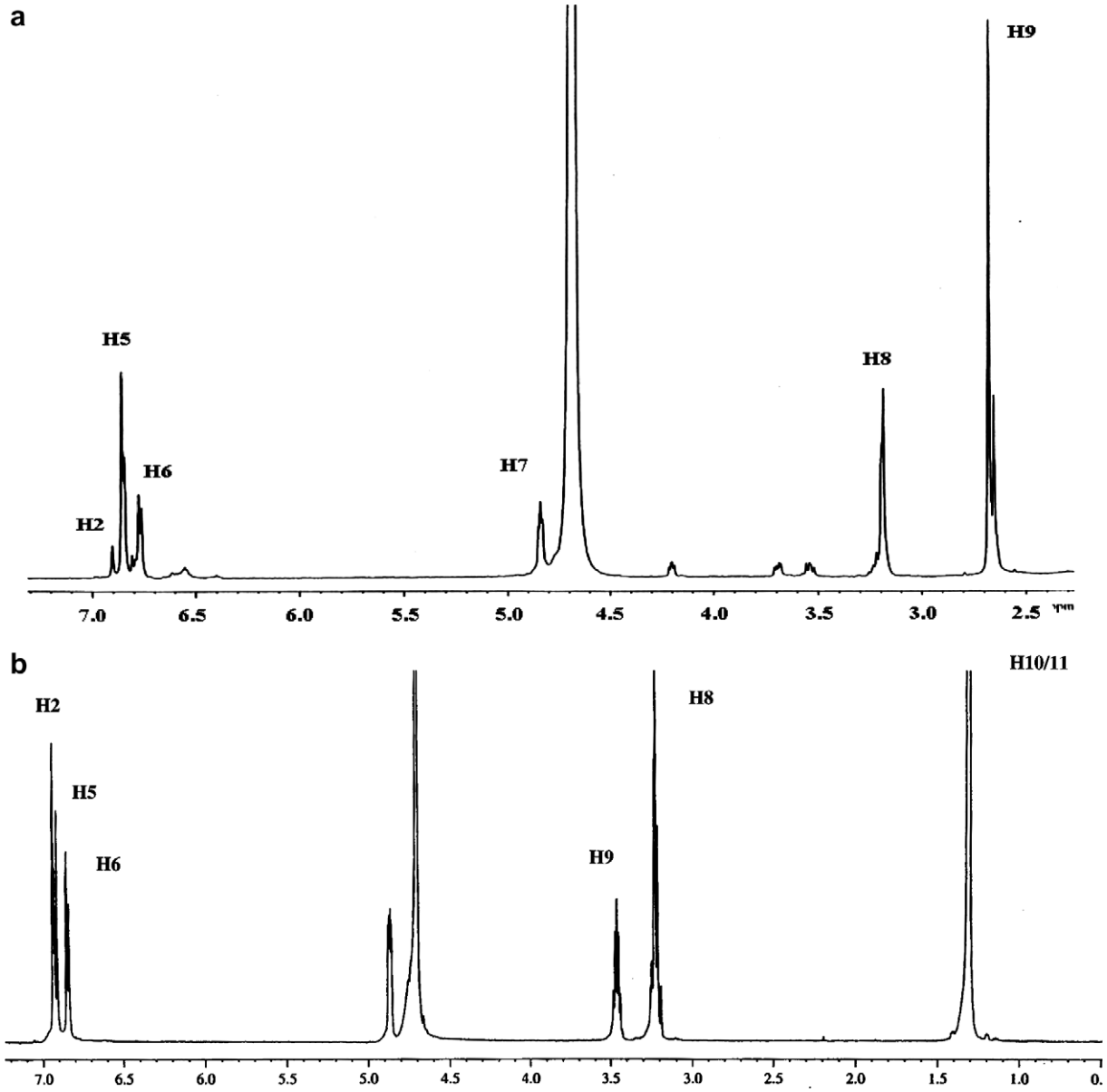

Figure 2. Proton spectra of (a) epinephrine (2×10^{-2} mol dm^{-3}) and (b) isoproterenol (2×10^{-2} mol dm^{-3}) in D_2O solution recorded at 600 MHz and 298 K.

Table 2
Experimental values of non-selective and selective spin lattice relaxation rates of H_6 proton in the presence of variable concentrations of fibrinogen (MW 341KD) at 298K for (a) epinephrine (2×10^{-2} mol dm^{-3} in D_2O) and (b) isoproterenol (2×10^{-2} mol dm^{-3} in D_2O)

Fibrinogen concentration (mol dm^{-3})	R_1^{NS} (s^{-1})	R_1^{SE} (s^{-1})
(a) Epinephrine (this data are published in a previous paper Ref. 16)		
0	0.81	0.76
1.55×10^{-6}	0.63	0.78
2.93×10^{-6}	0.60	0.83
5.87×10^{-6}	0.67	0.90
1.17×10^{-5}	0.65	1.01
2.35×10^{-5}	0.57	1.26
3.52×10^{-5}	0.67	1.53
(b) Isoproterenol		
0	0.66	0.57
2.93×10^{-6}	0.65	0.58
5.87×10^{-6}	0.64	0.59
1.17×10^{-5}	0.60	0.60
2.35×10^{-5}	0.62	0.69
2.93×10^{-5}	0.62	0.74
3.52×10^{-5}	0.65	0.78

Table 3
R_1^{NS} values calculated for H_6 proton of isoproterenol (2×10^{-2} mol dm^{-3}) versus temperature in the presence of fibrinogen 4 mg/cm^3

T (K)	R_1^{NS}
298	0.62
303	0.54
308	0.47
313	0.41

experiments was the H_6 doublet at 6.77 ppm and 6.83 for epinephrine and isoproterenol, respectively.

Table 2 reports the experimental values of R_1^{NS} and R_1^{SE} in relation to Fbg concentration for epinephrine and isoproterenol. The first evidence was that, in the absence of Fbg, for both the two ligands, R_1^{NS} values were bigger than R_1^{SE}. This result permits to confirm that epinephrine and isoproterenol (without protein) show fast reorientational motions. These dynamical conditions allow the interaction processes to be investigated through the analysis of the selective and non-selective spin–lattice relaxation rates of the ligands. The second evidence was that with the increase of Fbg concentration the R_1^{NS} values remained constant while the R_1^{SE} values increased for both the ligands. These results suggest the existence of a strong contribution from the bound ligand fraction to the observed selective relaxation rate, supporting the hypothesis that interaction processes between the two bioactive molecules (epinephrine and isoproterenol) and fibrinogen occurred.

However, solutions containing relatively high concentrations of protein may be subject to an increase in viscosity and this phenomenon may cause a slow down in the dynamics of the ligand even in the absence of an interaction with the protein. In order to investigate this phenomenon, the non-selective spin–lattice relation rate versus temperature was analysed (data for epinephrine was reported in a previous paper[16]). Table 3 reports the experimental R_1^{NS} values for H_6 proton of isoproterenol in the presence of Fbg (4 mg/cm^3) versus temperature. The R_1^{NS} values, which decrease with increasing temperature, indicate that the ligand molecules present fast motion conditions, indicating that the presence of fibrinogen did not affect ligand molecular motion.

In order to evaluate the strength of the interaction between the two ligands, epinephrine and isoproterenol, and Fbg, the *normalized affinity index* $[A_I^{N_1}]_L^T$ was calculated from the slope of the straight line obtained by H_6 normalized selective relaxation enhancements, ΔR_{N1}^{SE}, as a function of protein concentration. As reported in the Theory section, the *normalized affinity index* $[A_I^{N_1}]_L^T$ allows to remove the effects of motional anisotropies along the ligand molecule and differences in the magnetic environment on the experimental spin–lattice relaxation rate values. Figure 3 shows the comparison of linear regression analysis of normalized selective relaxation rate enhancements, ΔR_{N1}^{SE}, as a function of Fbg concentration for epinephrine and isoproterenol, respectively. The $[A^{N_1}]_L^T$ values were found to be 26,926 mol^{-1} dm^3 and 9728 mol^{-1} dm^3 for epinephrine and isoproterenol, respectively.

The comparison of the results obtained for epinephrine–fibrinogen and isoproterenol–fibrinogen systems suggests that the ligands interact with the protein with different strengths,

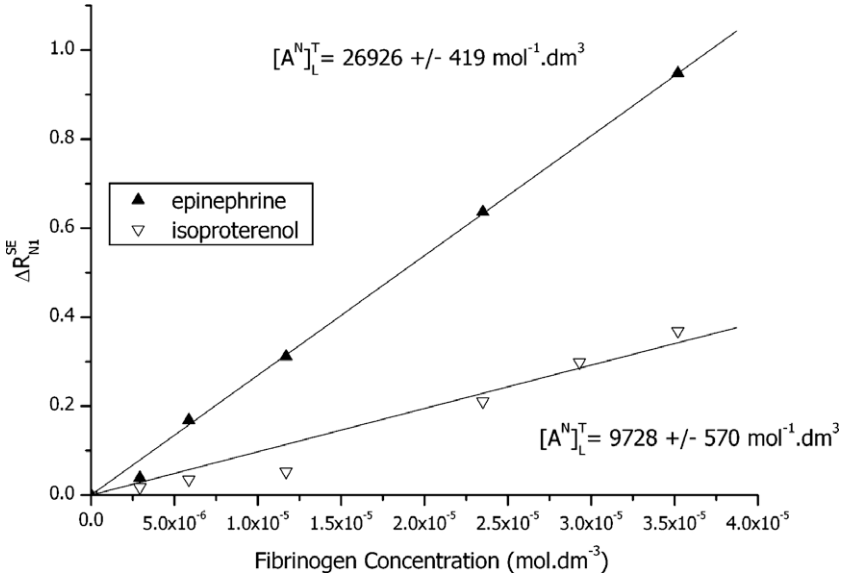

Figure 3. Linear regression analysis of the H_6 normalized selective relaxation enhancement, ΔR_{N1}^{SE}, as a function of fibrinogen concentration of a solution of (a) epinephrine (2×10^{-2} mol dm^{-3}) and (b) isoproterenol (2×10^{-2} mol dm^{-3}). The values of the normalized affinity indexes $[A_I^{N_1}]_L^T$ (mol^{-1} dm^3) are also reported with the corresponding errors.

Table 4
Non-selective and selective spin lattice relaxation rates of H_6 proton of (a) epinephrine (2×10^{-2} mol dm^{-3} in D_2O) and (b) isoproterenol (2×10^{-2} mol dm^{-3} in D_2O) in the presence of variable platelet concentrations

Number of platelets/cm^3	R_1^{NS} (s^{-1})	R_1^{SE} (s^{-1})
(a) Epinephrine		
0	0.80	0.70
$4.00 \times 10 \times 10^6$	0.82	0.76
8.00×10^6	0.76	0.95
1.60×10^7	0.78	0.98
2.00×10^7	0.77	1.15
2.80×10^7	0.76	1.75
(b) Isoproterenol		
0	0.59	0.40
4.00×10^6	0.54	0.45
8.00×10^6	0.55	0.45
1.60×10^7	0.57	0.67
2.00×10^7	0.56	0.70
2.80×10^7	0.55	0.98

indicating a greater affinity of Fbg towards epinephrine with respect to isoproterenol. The affinity index for epinephrine–fibrinogen was three times larger than the isoproterenol–fibrinogen-affinity index. The normalized affinity index found for epinephrine confirms its ability to interact with the protein involved in the coagulation cascade. On the contrary isoproterenol showed a smaller ability to interact with fibrinogen in agreement with its known biological effects of inhibition of the coagulation process.

4.2. Epinephrine–platelet and isoproterenol–platelet interactions

The aim of this approach was to use a spectroscopic method, described in the previous section, in order to evaluate the strength of the interaction between two ligands (epinephrine and isoproterenol) and biological receptors systems as platelet, which are directly involved in the platelet endothelial cell adhesion.

In order to verify the existence of interaction processes between epinephrine and isoproterenol and platelets, H_6 non-selective and selective proton spin–lattice relaxation rates were measured as a function of platelets concentration.

Table 4 reports the non-selective and selective relaxation rate values for epinephrine and isoproterenol in relation to platelet concentration. The results show for both the ligands that in the absence of platelets, $R_1^{NS} > R_1^{SE}$ while increasing platelets concentration R_1^{SE} becomes greater than R_1^{NS}. Also for these systems this behaviour represents the main indication of the existence of interaction processes between the epinephrine and platelets and isoproterenol and platelets. In fact, selective relaxation rate enhancements reflect a large contribution from the bound ligand fraction to the experimentally calculated relaxation rate.

To confirm this results, as reported in the previous section, the analysis of the behaviour of the non-selective relaxation rates with changing temperature in the presence of the receptors systems has been carried out. The experimental data show that in the presence of platelets, the two ligands experience fast motion conditions (data not shown). Therefore, this provides the evidence that the increase observed in R_1^{SE} values was due to the formation of epinephrine–platelets and isoproterenol–platelets complexes.

In order to evaluate the strength of the binding processes, the *normalized affinity index*, $[A_I^N]_L^T$, for epinephrine–platelets and isoproterenol–platelets systems was calculated from the slope of the straight line describing the dependence of normalized proton selective relaxation rate enhancements ΔR_{N1}^{SE} on platelet numbers (Fig. 4). The normalized affinity indexes for the epinephrine–platelet and isoproterenol–platelet were found to be $4.66.10^{-8} \pm 3.39.10^{-9}$ and $4.47.10^{-8} \pm 3.95.10^{-9}$ n.platelets^{-1} cm^3, respectively. These values suggest that the ligands interact with the receptor system with the same strength. The biological behaviour of epinephrine and isoproterenol in the presence of platelets are mediated by α and β-adrenergic receptors and both these receptors are present in the platelet surface. These results may be of interest in order to understand the mechanism of activation and inhibition of platelet aggregation. In particular, the distribution of adrenergic receptors on platelet surface allows to confirm that the biological effects of drugs are driven by surface interaction processes, confirming the potentiality of this experimental approach for studying recognition processes occurring in complex biological systems.

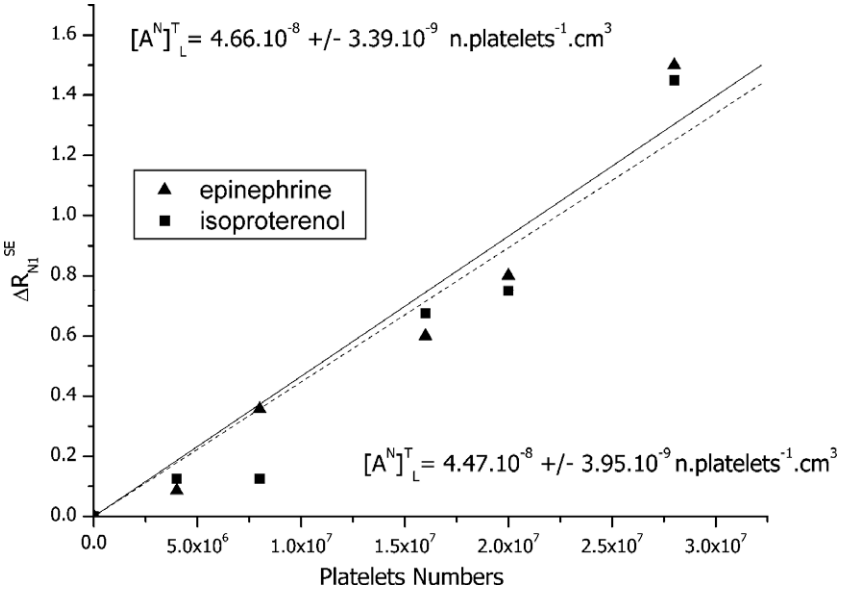

Figure 4. Linear regression analysis of the H_6 normalized selective relaxation enhancement, ΔR_{N1}^{SE}, as a function of platelets concentration for epinephrine (fill line) and isoproterenol (dash line). The values of the normalized affinity indexes $[A_I^N]_L^T$ are 4.66×10^{-8} and 4.47×10^{-8} n.platelets^{-1} cm^3 for epinephrine and isoproterenol, respectively.

5. Conclusions

The analysis of selective proton spin–lattice relaxation enhancements of small ligands due to the occurrence of recognition processes with the plasma proteins and cells, provides a useful tool to investigate the interactions between bioactive compounds and macromolecular receptors. In particular this approach allows to compare the strength of the interaction processes involving biological receptors systems, as platelets, and different ligands.

Acknowledgements

The authors would like to thank the University of Siena for financial support and Dr. Sandra Ristori for providing the dynamical light scattering measurements.

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