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On the Interaction of Dipyridamole Derivatives with Bovine Serum Albumin: a Fluorescence Study

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A interação de derivados do vasodilatador dipiridamol (DIP) com a proteína transportadora albumina de soro bovino foi estudada utilizando-se espectroscopia de fluorescência. A análise dos espectros de emissão e da intensidade de fluorescência dos derivados de DIP em solução permite a determinação de parâmetros de ligação e da estequiometria. Foram estudados os derivados RA14, RA39, RA25 e RA47, que têm em comum o anel pirimido[5,4-d]pirimidina e possuem diferentes substituintes. As medidas foram realizadas em pH 7,0 e pH 5,0 para analisar o efeito da protonação das moléculas de derivados de DIP na ligação. As constantes de associação, K_a, obtidas por titulação de intensidade de emissão estão no intervalo de 2,0 x 10^2 a 10 x 10^3 M^{-1} e o número de sítios de droga por proteína é próximo de um. Também utilizou-se anisotropia estática de fluorescência para monitorar a interação. Os dados obtidos foram ajustados com um modelo simples de ligação e os valores obtidos para r e K_a mostram uma boa concordância com os parâmetros obtidos através das medidas de intensidade de fluorescência. DIP, RA14 e RA39 interagem mais fortemente com BSA; observou-se um aumento de anisotropia maior do que o observado para RA25 e RA47, sugerindo que esses dois últimos derivados estão ligados mais fracamente na superfície da proteína. A ordem observada para os valores das constantes de associação é RA25 < RA47 < DIP = RA14, o que está de acordo com os dados de atividade biológica. Medidas de supressão de fluorescência utilizando-se acrilamida e iodeto permitiram estimar a localização da droga na molécula de proteína. A pH 7,0 os derivados estão mais protegidos no interior da proteína, enquanto a pH 5,0 eles tornam-se mais expostos e susceptíveis a uma supressão mais eficiente, especialmente por iodeto.

Interaction of several derivatives of the coronary vasodilator dipyridamole (DIP) with the transport protein bovine serum albumin (BSA) has been studied using fluorescence spectroscopy. Analysis of emission spectra and fluorescence intensity of DIP derivatives in solution allowed the determination of binding parameters and stoichiometry. The derivatives studied were RA14, RA39, RA25 and RA47. They have the same pyrimido-pyrimidine structure and different substituents, so our aim is to obtain information on chemical structure-biological activity relationship. Measurements were performed at pH 7.0 and pH 5.0 in order to analyze the effect of protonation of the DIP molecule on binding. The association constants, K_a, obtained from emission intensity titration, were in the range (0.2-10.0) x 10^3 M^{-1} and the number of binding sites was close to one. Static anisotropy of fluorescence was also used to monitor the binding. In aqueous solution the anisotropy is essentially zero; binding to the protein leads to an increase in the values of r_0 to 0.2-0.3. Data were fitted to a simple binding model and maximal values of r and K_a were obtained showing a good agreement with the parameters obtained from emission intensity measurements. DIP, RA14 and RA39 are tightly bound to the protein displaying a larger anisotropy than RA47 and RA25, suggesting that these last two derivatives form a weak binding in the surface of the protein. The order for the values of association constants is RA25 < RA47 < DIP = RA14, which is in accordance with the reported biological activity. Fluorescence quenching using acrylamide and iodide allowed further localization of the drug in the protein molecule. At pH 7.0 the derivatives are more protected in the interior of the protein, while at pH 5.0 they become more exposed and susceptible to a more efficient quenching, especially by iodide.

keywords: *dipyridamole, fluorescence, interaction with protein, albumin*

Introduction

Dipyridamole (DIP), 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido-[5,4-d]pyrimidine is a drug widely used in clinics as a coronary vasodilator[1,2]. Besides, it has recently been show that DIP has a role as co-activator in the effect of a number of antitumor compounds[3,4]. This compound has quite interesting spectroscopic properties presenting an absorption band in the visible region and an intense fluorescence. In previous work[5,6,7] we have characterized the spectroscopic properties of DIP and derivatives in homogeneous solutions and demonstrated its interaction both with micelles and protein. The biological activity of DIP is dependent upon the nature of the substituents at the different positions of the molecule[8], and derivatives have been synthesized that show increased activity[9]. This effect is certainly related to the influence of these substituents on the binding of DIP derivatives to different microheterogeneous biological structures (membranes, macromolecules).

Aiming to contribute to the elucidation of the correlation between the structure of the drug and its binding properties and biological activity in the present work the interaction of several derivatives of DIP with the transport protein bovine serum albumin (BSA) was investigated using the analysis of the spectra and intensity of the fluorescence of these compounds. The derivatives of DIP are presented in Fig. 1. They have substantially different effects on the transport of both adenosine and phosphate ions in red blood cells[8], this being the reason of their choice. The fluorescence quenching induced by acrylamide and iodide was also used in order to obtain information on the localization of the derivatives of DIP molecules on the protein. Finally, the anisotropy of the fluorescence is used to confirm the interaction and to assess the localization of the drug in the protein molecule.

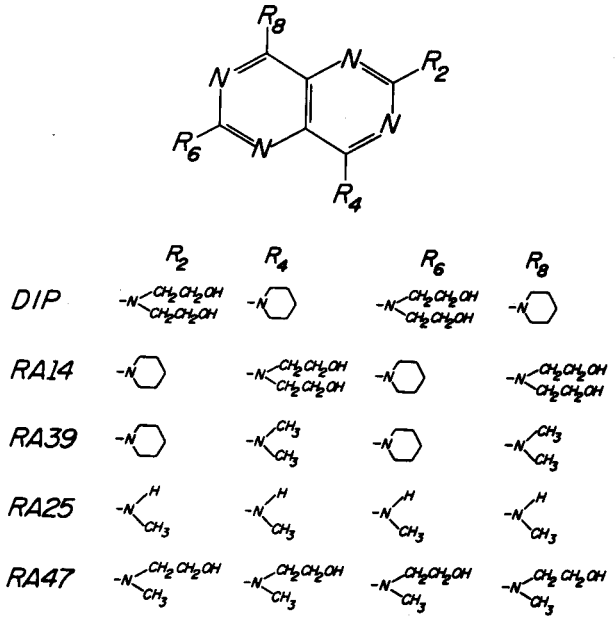

Figure 1. Structural formulae of dipyridamole and derivatives.

Materials and Methods

Dipyridamole (DIP) was obtained from Sigma Chem. Co. The other DIP derivatives, RA39, RA14, RA25 and RA47 were a kind gift from Dr. Karl Thomae GmbH, Biberach/Riß. Solutions of the drugs were prepared in acetate buffer 0.02 M, pH 5.0, and phosphate buffer 0.02 M, pH 7.0. Concentrations were in general in the range of 10^{-5} M, consistent with an optical density in the lower energy absorption band[7] below 0.1. Bovine serum albumin (BSA) was obtained from Sigma Chem. Co. Stock solutions of 1 mM of protein were prepared in distilled, deionized water or in the same buffer as the drugs and used for protein titration experiments at fixed drug concentration. Small aliquots of stock protein solution were added to a solution of the drug directly in the fluorescence cuvette[6] and the emission intensity was monitored at the appropriate wavelength (excitation of DIP, RA39, RA14 and RA47 was at 405 nm and for RA25, at 370 nm). Emission was monitored at the wavelength where maximal changes were observed in the presence of protein. Data from titrations were analyzed both as described by Tabak[6] as double reciprocal plots of fluorescence change and protein concentration, and also, as described by Favilla[10] as Scatchard-like plots, which allowed to estimate both the dissociation constant and the number of binding sites for the drug on the protein. All fluorescence measurements were performed on a JASCO FP-777 spectrofluorimeter at room temperature (20 °C). Absorption of the samples was measured in a Shimadzu UV-180 spectrophotometer. Measurements of static anisotropy were performed with a Perkin-Elmer MPF44 spectrofluorimeter coupled to an Apple computer which allowed semi-automatic measurements and analysis as reported by Mazzini[12]. The polarizer position was set manually and the measurement and averaging of data and calculation of the anisotropy was done on the computer. Each intensity was measured in a cycle of 100 times and an average value is used both for the calculation of the correction factor G and the anisotropy. Each point in the titration is measured 3-4 times and the average value is used. The static anisotropy was obtained from the expression

$$r_o = (I_{vv} - G*I_{vh})/(I_{vv} + 2*G*I_{vh})$$

where the first and second indexes refer to the orientation of the excitation and emission polarizers. The factor G is a correction for both partial light polarization and unequal transmission of excitation through the sample. The error of the anisotropy is in general around 0.002. The titration of solutions of the drugs with protein solution as described above allowed to obtain the anisotropy of fluorescence as

a function of protein concentration. Since the anisotropy of the free drug in solutions is negligible the increase in anisotropy is due to the drug bound to the protein. In this way the association constants for the binding can be evaluated from anisotropy measurements[11].

Experiments were also performed of fluorescence quenching of the drugs in the presence of BSA using acrylamide and potassium iodide. This was performed as a titration of the drug-BSA solution directly in the fluorescence cuvette by addition of small aliquots of concentrated solutions of the quenchers. The data was treated as Stern-Volmer plots[6,11]. Fluorescence lifetimes of the DIP derivatives in the presence of BSA were measured using single photon counting technique as described by Borges[7]. Decays were fitted to both mono- and bi-exponential models. Criteria for the goodness of the fit were the values of Chi^2 and the randomness of the autocorrelation function. This allowed us to estimate the bimolecular quenching constants from the relation $K_{sv} = k_q * \tau$, where K_{sv} is the slope of Stern-Volmer plot, k_q the bimolecular quenching constant and τ the fluorescence lifetime in absence of quencher[11].

Results and Discussion

Spectroscopic and kinetic properties of DIP derivatives in homogeneous solutions

The electronic absorption spectra of DIP derivatives are characterized in the range 250-500 nm by two broad bands with absorptivities typical of $\pi \rightarrow \pi^*$ transitions: for DIP at pH 7.0 they are $\varepsilon_{280} = 2.0 \times 10^4\ M^{-1}\ cm^{-1}$ and $\varepsilon_{415} = 5.4 \times 10^3\ M^{-1}\ cm^{-1}$. The quantum yield of fluorescence depends on the pH and excitation at the maximum of the longer wavelength band leads to an emission centered around 500 nm. In the case of RA25 both the absorption and the emission are shifted to shorter wavelengths. The fluorescence decay is biexponential due to the equilibrium between the protonated and neutral species in the range of pH 4-7[7]. In Table 1 are presented data of spectroscopic (maximum emission wavelength and quantum yield) and kinetic (fluorescence lifetimes) parameters for DIP derivatives. Data for lifetimes are the average values obtained from the expression $\langle\tau\rangle = (\alpha_1 \tau_1^2 + \alpha_2 \tau_2^2)/(\alpha_1 \tau_1 + \alpha_2 \tau_2)$

Interaction with bovine serum albumin

To determine the wavelengths convenient for monitoring changes in fluorescence in the presence of BSA, experiments were performed where relatively high concentrations of protein (in excess to drug) were added to the solution of the drug, and the differential emission spectra were measured. In Fig. 2 the result of such experiment is presented for RA14. It can be seen that at pH 7.0 the differential emission spectrum has a maximum around 460 nm and a minimum around 520 nm. This suggests a blue shift of λ_{max} in the presence of BSA. At pH 5.0 (Fig. 2b) only the maximum is observed and the blue shift is much smaller than that at pH 7.0. Similar results were obtained for DIP and RA39. Results of this experiment for the DIP derivatives studied in this work are shown in Table 2. The percentage change in fluorescence emission upon

Table 1. Spectroscopic and kinetic parameters of DIP derivatives.

compound	λ^{fl}_{max} (nm)	ϕ_{fl} (%)	τ (ns)
DIP pH 7	495	99 ± 5	17.7 ± 0.9
DIP pH 5	496	16 ± 1	8.6 ± 0.4
DIP pH 7 + BSA	487		15.6 ± 0.7
DIP pH 5 + BSA	490		11.4 ± 0.5
RA14 pH 7	513	79 ± 5	19.1
RA14 pH 5	515	15 ± 2	14.4 ± 0.7
RA14 pH 7 + BSA	506		18.5 ± 0.9
RA14 pH 5 + BSA	507		17.2 ± 0.8
RA39 pH 7	488	100	
RA39 pH 5	487	16	
RA39 pH 7 + BSA	484		
RA39 pH 5 + BSA	483		
RA25 pH 7	451	100	15.8 ± 0.8
RA25 pH 5	451	81 ± 5	13.4 ± 0.6
RA25 pH 7 + BSA	451		16.1 ± 0.8
RA25 pH 5 + BSA	451		14.9 ± 0.7
RA47 pH 7	495	73	
RA47 pH 5	496	10	
RA47 pH 7 + BSA	492		
RA47 pH 5 + BSA	493		

Table 2. Parameters used in measurement of the interaction of DIP derivatives with BSA.

compound	λ^{df}_{max} (nm)	λ^{meas} (nm)	ΔF_{max}	F_{max}/F_0
DIP pH 7	463	460	188.7	2.15
DIP pH 5	478	480	1695	2.36
RA14 pH 7	477	480	103.2	2.38
RA14 pH 5	493	500	1492.5	4.50
RA39 pH 7	453	460	315.4	1.62
RA39 pH 5	472	480	952.4	1.84
RA25 pH 7	460	460	1370	1.55
RA25 pH 5	450	450	1724	2.49
RA47 pH 7	448	450	370.4	4.88
RA47 pH 5	471	470		

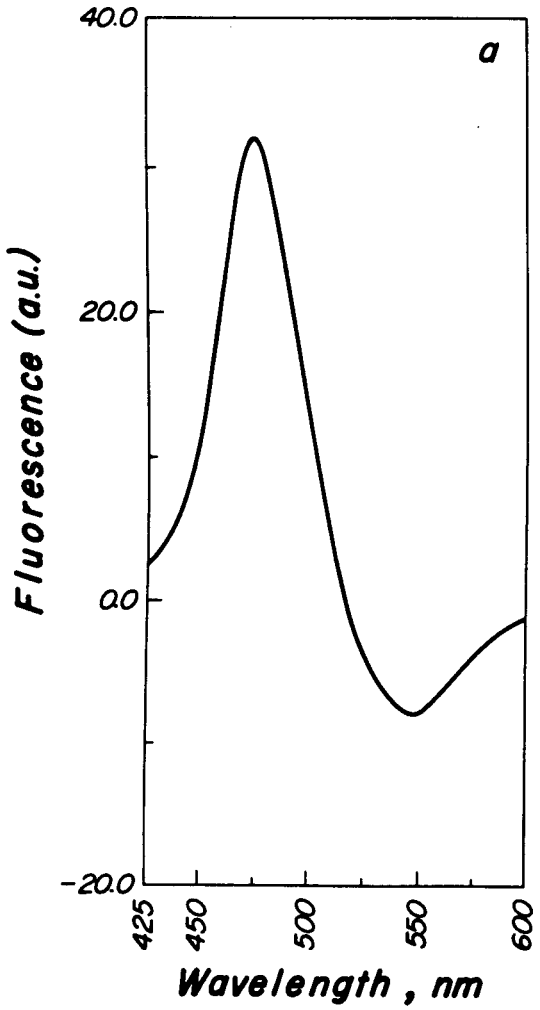

Figure 2a. Differential emission spectra for solutions of RA14 1.6 x 10^{-6} M in buffer and in the presence of 2 x 10^{-4} M BSA in phosphate buffer pH 7.0.

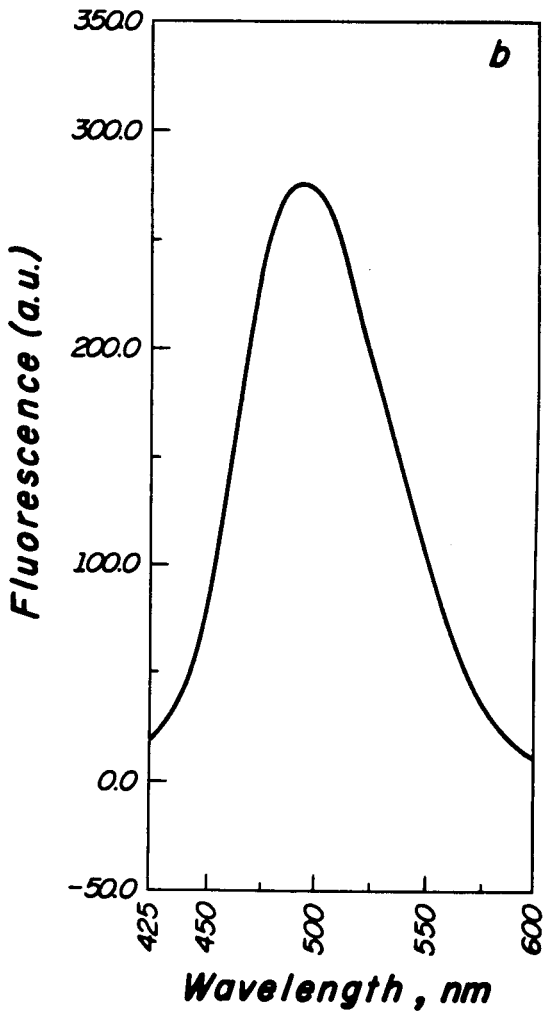

Figure 2b. Differential emission spectra for solutions of RA14 5.4 x 10^{-6} M in buffer and in the presence of 2 x 10^{-4} M BSA in acetate buffer pH 5.0.

addition of protein was 20% for DIP, 30% for RA14 and 20% for RA39. Those figures are lower limits since the concentration of protein added was below the total saturation level. In the case of RA47 the changes were quite small due to smaller binding constants. This was especially critical at pH 5.0.

The above experiments allowed to decide the optimal wavelengths (corresponding to maximal changes) to measure the interaction of DIP derivatives with BSA. Table 2 shows the maximum wavelengths obtained from the differential spectra as well as the wavelengths used in the measurements. Protein titrations of drug solutions at a fixed concentration by addition of aliquots of stock protein, were performed as described in the Methods Section. In Fig. 3 the results for RA14 at pH 7.0 are presented. Data were analyzed as double reciprocal plots of fluorescence intensity change and protein concentration. This allows the extrapolation of the maximal fluorescence change, ΔF_{max}, and the determination of the association constant for the

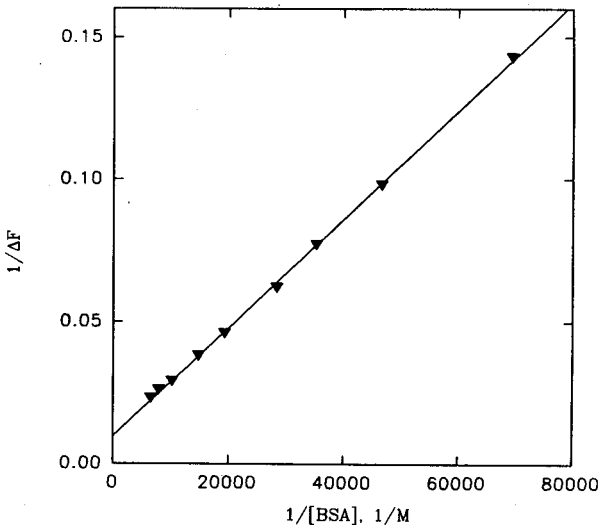

Figure 3. Reciprocal plot of the dependence of the fluorescence intensity change of RA14 on the concentration of BSA, at pH 7.0 in phosphate buffer 0.02 M. Concentration of RA14 3.23 x 10^{-6} M. Concentration of BSA in the range 1.44 x 10^{-5} M - 1.52 x 10^{-4} M.

binding. In Table 2 the values for ΔF_{max} and F_{max}/F_o are included for the DIP derivatives. F_{max}/F_o corresponds to the intensity increase at the wavelength used in the protein titration. The values of the association constants are presented in Table 3 for the various derivatives. Values obtained both from the double reciprocal plots and direct fitting of the law of mass action are presented. In Fig. 4 results for the Scatchard-like treatment[10] of data for RA14 are presented. From these plots both the number of binding sites of the drug in the protein and the dissociation constants can be evaluated. They are included for all compounds in Table 3. Notice that the dissociation constants, which are obtained directly from the plots, were transformed into association constants.

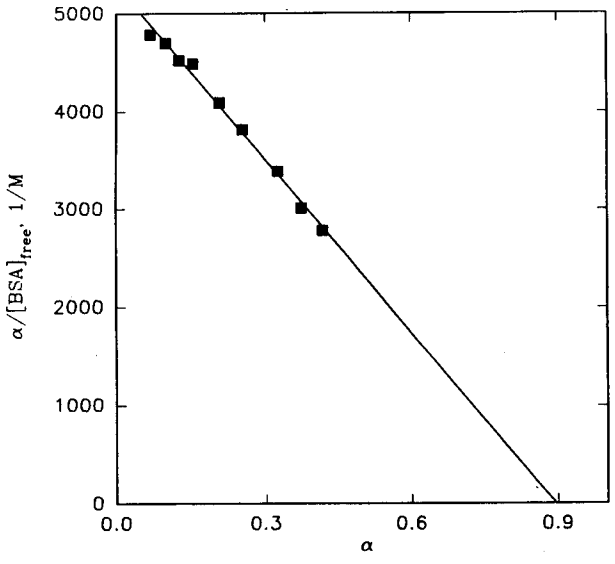

Figure 4. Scatchard-like plot for data for RA14 titration with BSA in phosphate buffer 0.02 M pH 7.0. Concentrations the same as in Fig. 3.

Static anisotropy

Static fluorescence anisotropy was measured for the drugs in the same conditions as used for the fluorescence measurements described above. Excitation and emission were monitored at 405 nm and 500 nm for DIP, RA14, RA47 and 370 nm and 450 nm for RA25. The anisotropies as a function of BSA concentration are presented in Fig. 5 for DIP at pH 7.0 and 5.0. Full lines correspond to the fitting of data using the mass action law from which the following relation between the static anisotropy r and the protein concentration [P][11] can be obtained:

$$(r-r_F)/(r_B-r_F) = [P]/(K_d+[P])$$

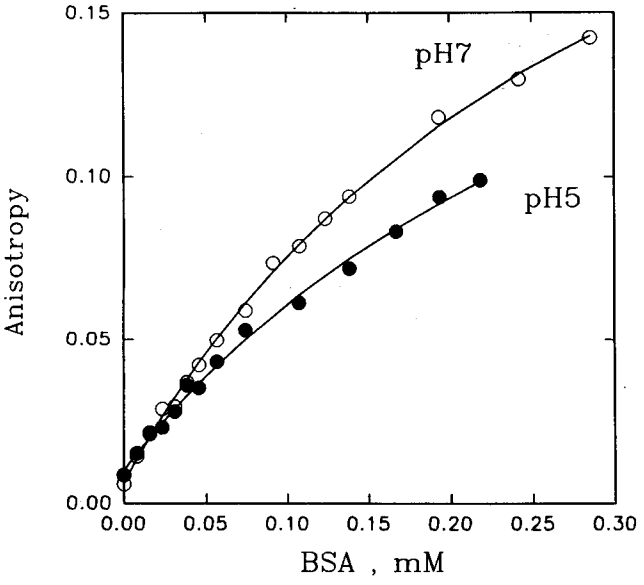

Figure 5. Static anisotropy of DIP as a function of BSA concentration at pH 7.0 phosphate buffer and pH 5.0 acetate buffer. Full lines are the best fit for the law of mass action.

Table 3. Association constants and number of binding sites for DIP derivatives in the protein.

	$K_1 \times 10^{-3}$ (M^{-1})	$K_2 \times 10^{-3}$ (M^{-1})	n° sites	$K^* \times 10^{-3}$ (M^{-1})
DIP pH 7	9.5 ± 0.3	8.8 ± 0.2	1.0	10.5
DIP pH 5	4.0 ± 0.8	2.8 ± 0.5	1.1	3.9
RA14 pH 7	5.1 ± 0.2	6.0 ± 0.2	0.9	5.9
RA14 pH 5	1.7 ± 0.3	1.5 ± 0.3	1.1	1.5
RA39 pH 7	5.2 ± 0.2		1.1	5.0
RA39 pH 5	4.5 ± 0.9		1.2	3.8
RA25 pH 7	4.0 ± 0.1	2.7 ± 0.3	1.0	4.3
RA25 pH 5	0.26 ± 0.05		0.7	0.4
RA47 pH 7	1.1 ± 0.1		0.9	1.3

K_1 were obtained by double reciprocal plots[6], K_2 obtained through the fitting of data using the mass action law and K^* from the Scatchard-like plots.

where r_B and r_F are the static anisotropies for bound and free drug and K_d is the dissociation constant. The fit allows to determine the anisotropy in the absence of protein (r_F), the dissociation constant for the drug-protein complex and the maximal anisotropy for infinite protein concentration (r_B). The results for all compounds are summarized in Table 4. Our data show that both DIP and RA14 bind tightly to BSA, having anisotropies which increase from very low values near zero to around 0.2. In the case of RA47 and RA25 at pH 7.0 the anisotropies are lower than for the other compounds; this could be explained if the RA25 and RA47 molecules, which are significantly more soluble in water, were located in the surface of the protein, having a greater degree of mobility in comparison to the other compounds which are more deeply located in the protein having a more restricted motion and a larger anisotropy and tighter binding. It is worth noticing that both for RA25 and RA47 at pH 5.0 the errors in r_B and K are large suggesting that these parameters are very inaccurate.

Table 4. Fluorescence anisotropy parameters for DIP derivatives in the presence of BSA.

	r_F	r_B	$K(M^{-1}) \times 10^{-3}$
DIP pH 7	0.006 (1)	0.28 (1)	3.3 (2)
DIP pH 5	0.010 (1)	0.26 (3)	2.5 (5)
RA14 pH 7	-0.009 (1)	0.194 (4)	9.7(6)
RA14 pH 5	0.018 (2)	0.218 (8)	11.0 (1)
RA25 pH 7	0.004 (1)	0.11 (4)	1.6 (5)
RA25 pH 5	0.0054 (8)	0.2 (2)	0.7 (7)
RA47 pH 7	0.0076 (5)	0.10 (2)	1.6 (5)
RA47 pH 5	0.0292 (6)	0.3 (2)	0.8 (8)

Numbers in parenthesis are the errors for the fitted values of parameters and correspond to the last significant number.

Fluorescence quenching

Quenching experiments were performed of the drugs in the presence and absence of BSA using different types of quenchers: acrylamide and iodide, which are commonly used in protein studies being, respectively, a neutral and a charged molecule. The Stern-Volmer plots of the quenching data were linear for practically all experiments, allowing the Stern-Volmer quenching constants to be determined from the plots as the slopes. They are presented in Table 5. Using the fluorescence lifetimes for the drugs in the presence and absence of BSA given in Table 1, the bimolecular quenching constants were calculated for DIP, RA14 and RA25 and are shown in parenthesis in Table 5. Two concentrations of BSA were used in our quenching studies in order to be sure that the change in K_{sv} follows a trend, but k_q was obtained only for the higher BSA concentration, where the lifetimes were measured. The values of the bimolecular constants allow some comparisons to be made: for the neutral quencher acrylamide it is seen that both at pH 7.0 and pH 5.0 the constants are very close for RA14 and DIP; binding to the protein reduces the constant by a factor of 1.9-2.1 for DIP and 1.6-1.7 for RA14. In the case of RA25 there is a marked difference in quenching constants at pH 7.0 and pH 5.0 being a factor of three larger at pH 7.0; in the protein there is a reduction of k_q at pH 7.0 but practically no change at pH 5.0. The effect of iodide, which is charged, is rather different: the values of k_q are low at pH 7.0 being practically unchanged in the presence of BSA for DIP and RA25 and reducing twice for RA14; at pH 5.0 there is a considerable increase in the bimolecular constants which is significantly reduced upon binding to the protein. This could be partly explained by static quenching due to an ionic pair between I^- and $DIPH^+$. Binding to the protein reduces the quenching compared to the effect in the buffer (pH 5.0), but the bimolecular constant remains larger than that at

Table 5. Parameters for quenching of the fluorescence of DIP derivatives

	Acrylamide			Iodide		
BSA (mM)	0.0	0.038	0.138	0.0	0.038	0.138
DIP pH 7	30.0 (1.69)	22.5	14.0 (0.90)	1.6 (0.09)	2.3	1.6 (0.10)
DIP pH 5	15.0 (1.53)	9.6	8.0 (0.70)	7.8 (0.79)	8.6	4.5 (0.39)
RA14 pH 7	17.0 (0.89)	11.0	10.0 (0.54)	3.3 (0.17)	1.9	1.6 (0.09)
RA14 pH 5	12.0 (0.77)	10.5	7.7 (0.45)	10.0 (0.64)	7.5	3.7 (0.21)
RA25 pH 7	56.0 (3.5)	49.0	43.0 (2.67)	1.6 (0.10)	1.75	1.8 (0.11)
RA25 pH 5	15.0 (1.08)	16.0	15.5 (1.04)	40.0 (2.88)	32.0	27.7 (1.86)
RA47 pH 7	7.0	6.7	7.0	12.0	9.8	7.8
RA47 pH 5	32.0	30.5	24.5		5.3	4.4

Numbers in parenthesis correspond to the bimolecular quenching constants, k_q, in $M^{-1}s^{-1} \times 10^{-9}$, calculated from $K_{SV} = \tau * k_q$.

pH 7.0. This result suggests that the drug is bound to the surface of the protein. Considering the values of k_q for iodide at pH 5.0 it can be seen that binding to the protein reduces the quenching in the following order: RA14, DIP and RA25 suggesting that this is the order of localization deeper in the protein.

General Comments and Conclusions

The aim of this work was to assess the binding of different DIP derivatives to serum albumin in order to obtain a correlation of their chemical structure with biological activity as studied in a model system. Our previous work with DIP[6] has shown that this drug is able to bind to BSA and the values of association constants, 4×10^3 M^{-1} at pH 5.0 and 1.2×10^4 M^{-1} at pH 7.0, are in agreement with the values in this work.

Our results demonstrate that the DIP derivatives used in this work bind to BSA with association constants in the range $1\text{-}10 \times 10^3$ M^{-1}. Besides that a single binding site per protein molecule is observed for all derivatives. Our data showed also that RA25 and RA47 bind less tightly than RA14 and DIP in agreement with data for the inhibitory effect on transport functions. The inhibitory effect of DIP derivatives upon transport of adenosine and phosphate ions across red blood cells increased in the order RA25, RA47, RA14 and DIP[8]. In the case of RA25 and RA47 the association constants from fluorimetric titrations and anisotropy measurements agree quite well. Both RA25 and RA47 seem to be superficially bound, especially at pH 5.0 where the association constants are the smallest and the anisotropy increases to a smaller extent (the error is quite great). DIP and RA14 have the highest constant in agreement with a significant increase in static anisotropy and reduced accessibility for the quenchers when bound to the protein. The sensitivity to pH was observed from fluorescence intensity titrations, while from the anisotropy measurements this sensitivity is much lower. Our values of association constants are of the same order of magnitude as those reported for the binding of tryptophan and several other ligands to BSA, and are lower than the values for binding of fatty acids, bilirubin and hematin[13]. It has recently been reported that the albumin molecule has three types of binding sites in the whole structure and evidences were reported for the binding of different drugs to these sites[14]. We believe that DIP and derivatives are probably bound to sites in the region named III in the protein.

The results of quenching experiments with iodide support this view that the interaction of DIP with BSA is essentially hydrophobic in nature since, in spite of excess of negative charge on the protein, quenching at pH 7.0 in the presence or absence of the protein is essentially the same (k_q is the same) with the exception of RA14 for which the quenching is reduced. This suggests that the binding of the drug does not alter the charge in the protein and that RA14 is deeper inside the protein. The fact that at pH 5.0 in buffered solutions the quenching by iodide increases considerably could be explained by the electrostatic attractive effect of protonated DIP molecule to iodide. Binding to the protein at pH 5.0 still leaves k_q greater than at pH 7.0, but a reduction is observed probably due to the fact that at pH 5.0 the protein still has an excess of negative charge which will avoid iodide ions and that the binding protects the DIP molecule from the solvent. The protection occurs in the increasing order RA25, DIP and RA14.

Our results suggest then that the DIP derivatives bind mostly due to hydrophobic interactions and the localization is relatively superficial in the protein, which is especially observed for RA25 and RA47.

A preliminary experiment of binding of DIP to human serum albumin (HSA) suggests that the association constants are in the same range as for BSA, namely 1.48×10^3 M^{-1} at pH 5.0 and 1.36×10^4 M^{-1} at pH 7.0. Since the typical plasma concentration of albumin is around 0.6 mM, similar to the one that gives saturating effect for binding of DIP, and since upon administration of therapeutic doses of DIP its concentration in plasma is close to 3.5×10^{-6} M, our results could indicate that the primary target of DIP in clinical use is circulating serum albumin. In this way the binding described in our work could be of physiological relevance showing a good correlation with the inhibitory effect in the transport of adenosine and phosphate ions in red blood cells[8].

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