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A comparative analysis on the binding characteristics of various mammalian albumins towards a multitherapeutic agent, pinostrobin

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Abstract: The interaction of pinostrobin (PS), a multitherapeutic agent with serum albumins of various mammalian species namely, goat, bovine, human, porcine, rabbit, sheep and dog was investigated using fluorescence quench titration and competitive drug displacement experiments. Analysis of the intrinsic fluorescence quenching data revealed values of the association constant, K_a in the range of $1.49 – 6.12 \times 10^4$ M^{-1}, with 1:1 binding stoichiometry. Based on the PS–albumin binding characteristics, these albumins were grouped into two classes. Ligand displacement studies using warfarin as the site I marker ligand correlated well with the binding data. Albumins from goat and bovine were found to be closely similar to human albumin on the basis of PS binding characteristics.

Key words: animal model, drug–protein interaction, fluorescence spectroscopy, mammalian albumin, pinostrobin

Introduction

Animal models are immensely significant in drug discovery due to their importance in the characterization of disease pathophysiology, mechanism of drug action and identification of new biomarkers and drug targets [3, 23]. Information gathered from such animal studies have proved vital in establishing pharmacodynamic/pharmacokinetic relationships, determining clinical dosing regimens, as well as assessing the toxicity and safety margin of a particular drug [5, 11]. Results of *in vitro* and cell/tissue-based systems are not entirely representative of an *in vivo* response, as the metabolism and disposition properties of a drug are not taken into consideration. Thus, animal testing represents an essential intermediary phase in drug development before a potential drug can be used for human trials.

Flavonoids have emerged as one of the most promising drug candidates in recent times due to their medical significance in a wide range of diseases and ailments [9]. Furthermore, these flavonoids possess a large margin of safety as a result of their low toxicity in animals [14]. Pinostrobin (PS), a flavanone whose molecular structure is shown in Fig. 1, has been successfully tested as a potential anticancer agent based on its apoptotic activities on different cancer cell lines [20, 27, 28, 31]. In addition, PS has also been shown to confer other health benefits such as antiinflammatory [39], antiviral [38], antibacterial [22], antiulcerogenic [1] and antimutagenic [8] properties, to name a few.

As the most abundant plasma protein in the circulatory system, serum albumin functions as the main transport and depot protein for a vast variety of endogenous and exogenous ligands in the body [26]. It plays a critical role in the distribution, metabolism, efficacy and elimination of these molecules as they are significantly

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influenced by the nature and affinity of their interaction with albumin [26]. Thus, investigations into drug–albumin interactions are imperative in assessing the pharmacological potential of new drugs. Furthermore, before drug testing can be performed on human subjects, the safety and toxicity of a drug has to be evaluated using animal models that resemble as closely as possible to the human system in terms of the pharmacological aspects of drug–protein interaction. Species-dependent differences however, have been noticed with regard to the pharmacological response of various drugs/ligands [2, 4, 32, 34].

In the light of the above, we have investigated PS–albumin interaction with serum albumins from seven mammalian species, *i.e.*, goat, bovine, human, porcine, rabbit, sheep and dog. The comparative data obtained from this study will be useful in developing an analogous model that could match certain attributes of the human system for clinical testing of new drugs.

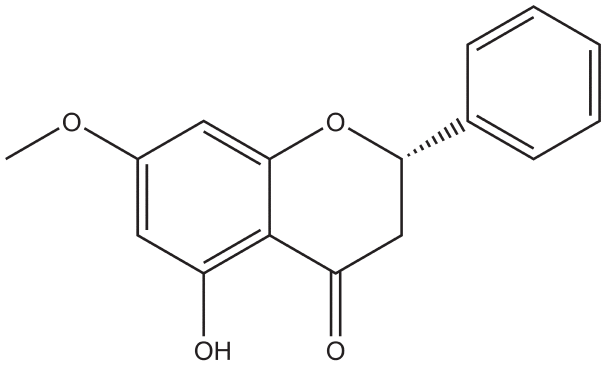

Fig. 1. Molecular structure of pinostrobin.

Materials and Methods

Materials

Essentially fatty acid free serum albumins of goat (GSA), bovine (BSA), human (HSA), porcine (PSA), rabbit (RbSA), sheep (SSA) and dog (DSA); as well as warfarin (WFN) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Pinostrobin (PS) was purified from *Boesenbergia rotunda* rhizomes following the published procedure [12] and its purity was established by NMR. All other chemicals used were of analytical grade purity.

Analytical procedures

Protein stock solutions were prepared in 10 mM sodium phosphate buffer, pH 7.4 and their concentrations except GSA and DSA were determined spectrophotometrically using E_{1cm}^{1M} at 280 nm of 43,827 M^{-1} cm^{-1} (BSA), 35,700 M^{-1} cm^{-1} (HSA), 43,385 M^{-1} cm^{-1} (PSA and RbSA), and 42,925 M^{-1} cm^{-1} (SSA) [16]. The method of Lowry *et al.* [21] was employed to determine the concentrations of GSA and DSA stock solutions.

PS and WFN stock solutions were prepared by dissolving desired quantity of their crystals in 1 ml of ethanol and methanol, respectively; followed by dilution with 10 mM sodium phosphate buffer, pH 7.4 to 100 ml. A molar extinction coefficient, E_{1cm}^{1M} of 13,610 M^{-1} cm^{-1} at 310 nm [36] was used to determine WFN concentration. All absorbance values were recorded on a Shimadzu UV-2450 double beam spectrophotometer (Shimadzu Corp., Kyoto, Japan) using quartz cuvettes of 1 cm path length.

PS–albumin interaction

The interactions between PS and various mammalian albumins were studied using fluorescence quench titration method as described earlier [12]. Increasing concentrations (1.5–24 μM) of PS were added to a fixed protein concentration (3 μM) in a total volume of 3 ml. 10 mM sodium phosphate buffer was used to make up the volume to 3 mL and the incubation mixture was kept for 1 h at 25 °C. Fluorescence measurements were carried out on a Jasco FP-6500 spectrofluorometer (Jasco Corp., Tokyo, Japan) using a 1 cm path length quartz cell. The protein samples were excited at 280 nm and the emission spectra were recorded in the wavelength range, 300–380 nm.

Data analysis

The values of the fluorescence intensity were first corrected for the inner filter effect according to the equation described by Lakowicz [19]:

$$F_{cor} = F_{obs} 10^{(A_{ex} + A_{em})/2)} \quad (1)$$

where F_{cor} and F_{obs} refer to the corrected and the observed fluorescence intensity values; while A_{ex} and A_{em} are the changes in the absorbance of the samples at the excitation and emission wavelengths, respectively, produced by the addition of the ligand.

The corrected fluorescence data were then analyzed according to the following Stern-Volmer equation to determine the Stern-Volmer constant, K_{SV} and the quenching mechanism involved in PS–albumin interaction [19]:

$$F_0 / F = K_{SV}[Q] + 1 = k_q \tau_0 [Q] + 1 \quad (2)$$

where F_0 and F are the fluorescence intensities in the absence and the presence of the quencher, respectively, $[Q]$ is the quencher (PS) concentration and k_q is the bimolecular quenching constant. The value of τ_0, the fluorophore lifetime in the absence of quencher for proteins was taken as 10^{-8} s [19], which is considered as a reasonable estimate of τ_0 for proteins in general and has been used in many previous studies on ligand–albumin interactions [10, 17, 30, 35].

The values of the association constant, K_a and n, the number of binding sites were obtained using the following equation [6]:

$$\log(F_0 - F)/F = n \log K_a - n \log[1/([L_T] - (F_0 - F)[P_T]/F_0)] \quad (3)$$

where $[L_T]$ and $[P_T]$ represent the total ligand concentration and total protein concentration, respectively. The values of K_a, thus obtained, were used to calculate the free energy change of the binding reaction, ΔG at temperature T, with the help of the following relationship:

$$\Delta G = -RT \ln K_a \quad (4)$$

A value of 8.3145 J mol^{-1} K^{-1} was used as the gas constant, R.

Warfarin displacement studies

Competitive displacement experiments using WFN–albumin complexes were performed by recording the fluorescence spectra of equimolar (3 μM) WFN-albumin complexes in the wavelength range, 360–450 nm upon excitation at 335 nm, both in the absence and the presence of increasing PS concentrations (3–24 μM). The WFN-albumin mixtures were incubated for 1 h prior to the addition of PS, followed by additional 1 h incubation before emission spectral measurements.

Results and Discussion

PS-induced fluorescence quenching of serum albumins

Many efforts to characterize the binding of a fluorescent macromolecule to its ligand have relied on fluorescence spectroscopy to shed light on the nature of the interaction. In the case of proteins, the intensity of its fluorescence signal is significantly affected as a result of interaction with other small molecules [19].

Figure 2 shows the fluorescence spectra of seven mammalian serum albumins (3 μM each), namely, GSA, BSA, HSA, PSA, RbSA, SSA and DSA, recorded in the absence and the presence of increasing PS concentrations (1.5–24 μM). The fluorescence properties of various albumins in terms of intensity and emission maximum, in their native form are listed in Table 1. As can be seen, the fluorescence intensity varied greatly among the proteins, following the order: SSA > PSA > BSA > GSA > HSA > DSA > RbSA, whereas the emission maximum was found to lie in the range, 333–342 nm. These results were in line with the published results on the fluorescence characteristics of various serum albumins [16, 33]. It is well known that the main contributors to the fluorescence characteristics of a protein are the aromatic (mainly Trp and Tyr) amino acid residues [19]. Thus,

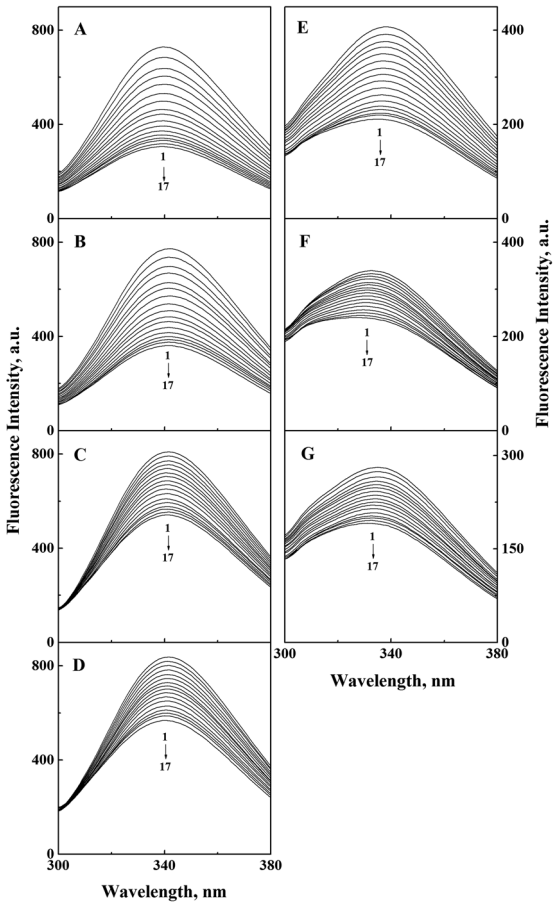

Fig. 2. Fluorescence spectra of different serum albumins with increasing pinostrobin (PS) concentrations. The spectra were obtained in 10 mM sodium phosphate buffer, pH 7.4. [Albumin] = 3 μM, [PS] = 0–24 μM with 1.5 μM intervals (1–17), λ_{ex} = 280 nm, T = 25 °C. (A) GSA, (B) BSA, (C) PSA, (D) SSA, (E) HSA, (F) DSA and (G) RbSA.

Table 1. Fluorescence characteristics of different mammalian albumins in the absence and the presence of pinostrobin (PS)

Albumin	Intensity (a.u.)	Emission maximum (nm)	Trp residues[a]	[PS]:[Albumin] = 8:1 % Quenching[b]
GSA	729	340	Trp-134, Trp-213	58 ± 3.3
BSA	773	342	Trp-134, Trp-213	53 ± 2.9
HSA	407	338	Trp-214	48 ± 2.3
PSA	810	341	Trp-134, Trp-213	33 ± 2.6
RbSA	281	335	Trp-214	32 ± 2.8
SSA	838	341	Trp-134, Trp-213	32 ± 2.9
DSA	339	333	Trp-214	30 ± 2.7

[a]Obtained from the Univesal Protein Resource (Uniprot) database at www.uniprot.org.
[b]Expressed as mean ± SD.

differences in the emission spectra of these albumins can be attributed to the number of Trp and/or Tyr residues and their location in the three-dimensional structure of the protein. However, in class B proteins, major contribution toward protein's fluorescence is due to Trp residues [19]. The details of Trp residues in terms of their number and location in the primary structure of these albumins are given in Table 1. It is interesting to note that location(s) of Trp residues is (are) remarkably well conserved among various animal albumins. As can be seen, proteins with two Trp residues (SSA, PSA, BSA and GSA) produced higher fluorescence intensities along with higher emission maxima compared to those with single Trp residue (Fig. 2 and Table 1).

Addition of increasing PS concentrations to the protein solution produced significant quenching of the protein's intrinsic fluorescence in all albumin species. A gradual decrease in the fluorescence intensity was observed in all albumins with the increase in PS concentration (Fig. 3). However, quantitative differences were noticed among these albumins. As evident from Fig. 3, decrease in the fluorescence intensity at 8:1 PS/albumin molar ratio was more pronounced for GSA (58%), BSA (53%) and HSA (48%) compared to the rest of the albumins, showing ~30% quenching (Table 1). Such quenching in the fluorescence intensity of albumins in the presence of PS was indicative of ligand–protein interaction [18].

PS–albumin interaction: Quenching mechanism and binding parameters

The quenching of protein fluorescence in the presence of a ligand (quencher) is known to involve either collisional or static quenching phenomena [19]. While the former refers to a process where the quencher interacts with the excited state of the fluorophore, the latter describes a mechanism in which the formation of the fluorophore excited state is inhibited by ground state complexation between the ligand and the protein [7].

In order to characterize the mode of quenching involved in the interaction between PS and serum albumins, fluorescence quench titration data were analyzed according to the Stern-Volmer equation (Eq. 2). The Stern-Volmer plots, thus obtained with different PS–albumin systems are shown in Fig. 4A, while the values of the corresponding Stern-Volmer constants, K_{SV} are listed in Table 2. An upward deviation in the Stern-Volmer plots was observed at higher PS concentrations (not shown), as has been reported in several earlier investigations on ligand–protein interactions [13, 24, 25, 29, 37]. Therefore, only those points falling in the initial linear zone were taken into account for regression analysis and K_{SV} value determination. As the gradient of the Stern-Volmer plot, the value of K_{SV} connotes the extent of quenching observed. Higher values of K_{SV} obtained with GSA, BSA and HSA (Table 2) correlated well with the magnitude of quenching observed with these proteins at [PS]:[Albumin] molar ratio of 8:1 (Table 1). Values of K_{SV} were used to determine the bimolecular quenching constant, k_q for different PS–albumin systems according to Eq. 2 and were found to be in the order of 10^{12} M^{-1} s^{-1}. A collisional quenching process typically produces a value of k_q in the region of 10^{10} M^{-1} s^{-1}, whereas the value of k_q larger than this diffusion-controlled limit usually indicates a binding reaction [19]. In view of the higher values of k_q (10^{12} M^{-1} s^{-1}) obtained with different PS–albumin systems, it appears that the quenching process followed the static quenching mechanism, involving ligand–protein complex formation. We have already confirmed the phenomenon of static quenching in PS–HSA system based on inverse temper-

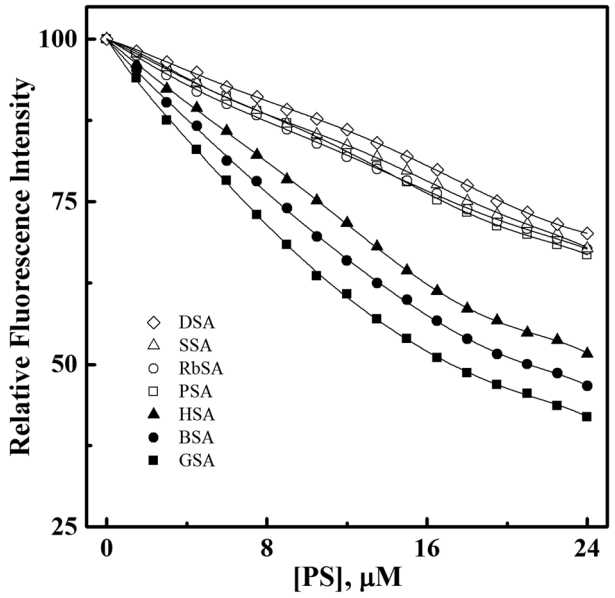

Fig. 3. Fluorescence quenching of albumins by pinostrobin (PS). Different plots show the decrease in the relative fluorescence intensity of different serum albumins at their emission maximum with increasing PS concentrations, as shown in Fig. 2.

ature-dependence in quenching studies [12].

Binding parameters in terms of the association constant, K_a and the binding stoichiometry, n for PS–albumin interaction were obtained after treating the fluorescence quenching data using Eq. 3. The use of this equation is advantageous over other methods of fluorescence data analysis due to the noninvolvement of any assumption with regard to the free and bound ligand concentrations [6]. The linear double logarithmic plots for different PS–albumin systems are shown in Fig. 4B and the values of K_a and n, obtained from these plots are listed in Table 2. Based on the values of the association constant, different serum albumins used in this study can be classified into two distinct groups such as GSA, BSA and HSA with relatively higher PS binding affinity compared to the rest of the proteins, showing relatively lower K_a values. Furthermore, GSA showed highest affinity whereas DSA was found to possess lowest binding affinity (Table 2). Interestingly, the value of n for all PS–albumin complexes was found to be ~1.0, suggesting a 1:1 binding stoichiometry. The calculation of the free energy change (ΔG) of the binding reaction using Eq. 4 also suggested feasibility of the binding process as ΔG was found to vary within the range of −23.8 to −27.3 kJ mol^{-1} (Table 2).

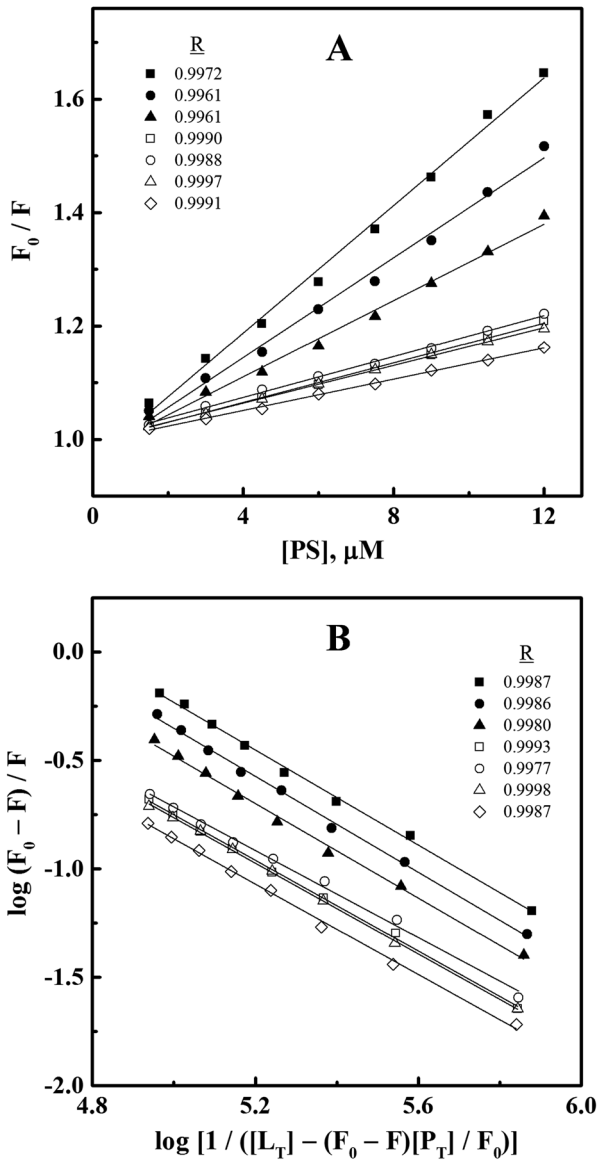

Fig. 4. Analysis of the fluorescence quench titration results. (A) Stern-Volmer plots and (B) double logarithmic plots of log $(F_0 - F) / F$ versus log $[1 / ([L_T] - (F_0 - F)[P_T] / F_0)]$ for the interaction of pinostrobin (PS) to different serum albumins. The symbols used for different albumins are the same as shown in Fig. 3. Values of the correlation coefficient (R) for different linear plots are shown against the symbols.

PS-induced WFN displacement

Recently we have shown Sudlow's site I as the preferred binding site of PS on HSA [12]. In the same investigation, we have clearly demonstrated insignificant binding of PS to Sudlow's site II, the other primary ligand binding site of HSA, even at high PS/HSA molar ratio. In view of the above, we extended our study to

Table 2. Binding parameters for the interaction of pinostrobin (PS) with different mammalian albumins

Albumin	K_{SV} (M^{-1})[a]	K_a (M^{-1})[a]	n	ΔG (kJ mol^{-1})
GSA	$(5.62 \pm 0.35) \times 10^4$	$(6.12 \pm 0.38) \times 10^4$	1.10	−27.3
BSA	$(4.40 \pm 0.31) \times 10^4$	$(4.82 \pm 0.34) \times 10^4$	1.10	−26.7
HSA	$(3.36 \pm 0.24) \times 10^4$	$(3.63 \pm 0.26) \times 10^4$	1.09	−26.0
PSA	$(1.75 \pm 0.22) \times 10^4$	$(1.91 \pm 0.24) \times 10^4$	1.05	−24.4
RbSA	$(1.81 \pm 0.25) \times 10^4$	$(1.95 \pm 0.27) \times 10^4$	1.00	−24.5
SSA	$(1.67 \pm 0.20) \times 10^4$	$(1.86 \pm 0.22) \times 10^4$	1.05	−24.4
DSA	$(1.38 \pm 0.23) \times 10^4$	$(1.49 \pm 0.25) \times 10^4$	1.04	−23.8

[a]Expressed as mean ± SD.

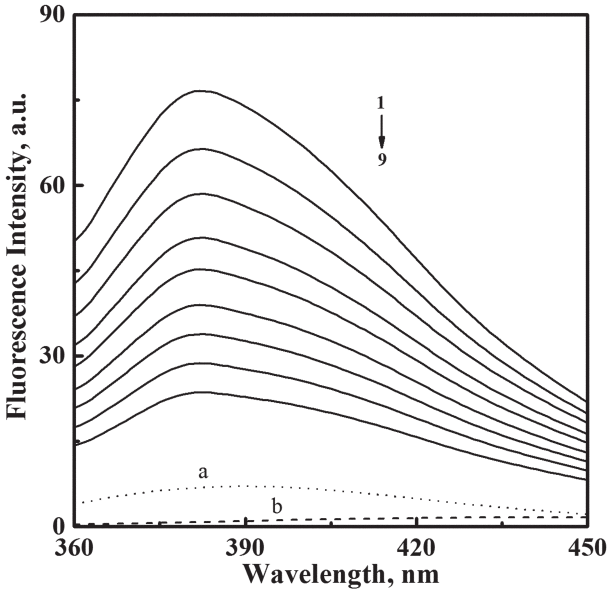

Fig. 5. Displacing effect of pinostrobin (PS) on the fluorescence spectrum of WFN–GSA complex. [GSA] = [WFN] = 3 μM, [PS] = 0–24 μM with 3 μM intervals (1–9), λ_{ex} = 335 nm, T = 25 °C. The spectra marked 'a' and 'b' refer to the fluorescence spectra of 3 μM WFN and 3 μM GSA, respectively.

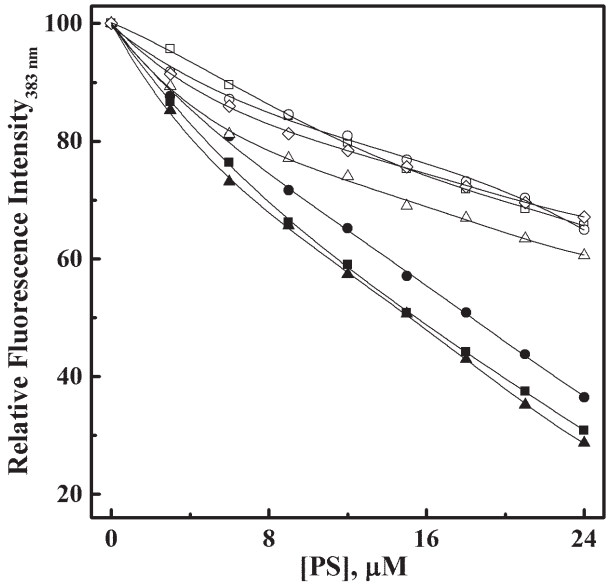

Fig. 6. Quenching of WFN–albumin complex fluorescence by pinostrobin (PS). Plots show the decrease in the relative fluorescence intensity of different WFN–albumin complexes at 383 nm with increasing PS concentrations. The symbols used for different albumins are the same as shown in Fig. 3.

different serum albumins in order to substantiate our binding results, using WFN as a site I marker ligand. PS-induced WFN displacement from different WFN–albumin complexes was studied using fluorescence quenching. Figure 5 shows the effect of increasing PS concentrations on the fluorescence spectra of WFN–GSA complex. Qualitatively similar spectra were also obtained with other albumin complexes (figures are omitted for brevity). As shown in the figure, WFN–GSA complex produced the fluorescence spectrum in the wavelength range, 360–450 nm with an emission maximum at 383 nm, upon excitation at 335 nm. Addition of increasing PS concentrations to WFN–GSA complex, however, led to a progressive decrease in the fluorescence intensity, which was suggestive of the dissociation of WFN from its binding locus (site I) on the protein. It is important to note that both free WFN (spectrum a) and the albumin (spectrum b) solutions produced weak/insignificant fluorescence signals within this range.

These fluorescence data were transformed into relative fluorescence intensity at 383 nm by taking the fluorescence intensity value obtained in the absence of PS as 100 and plotted against PS concentration. Figure 6 depicts the decrease in the relative fluorescence intensity at 383 nm of different WFN–albumin complexes with increasing PS concentrations, while the values of the

Table 3. Pinostrobin (PS)-induced WFN displacement from WFN–albumin complexes

Albumin	[PS]:[Albumin] = 8:1 % Quenching[a]
GSA	69 ± 3.0
BSA	64 ± 3.1
HSA	71 ± 2.7
PSA	34 ± 2.3
RbSA	35 ± 2.6
SSA	39 ± 2.2
DSA	33 ± 2.5

[a]Expressed as mean ± SD.

percentage quenching achieved at the highest PS concentration (8:1 [PS]/[Albumin]) are listed in Table 3. While WFN–albumin complexes of GSA, BSA and HSA exhibited marked quenching (64–71%) at 8:1 PS/albumin molar ratio, lesser quenching (33–39%) was observed with other WFN–albumin complexes (Table 3). Interestingly, a striking positive correlation was observed between the affinity of PS towards albumin and its potential to displace WFN from Sudlow's site I. As shown in Table 2, GSA, BSA and HSA were also found to possess significantly larger K_a values ($3.63 - 6.12 \times 10^4$ M^{-1}) among the albumins studied. The slight variation in the quenching pattern observed within these two groups may be attributed to differences in the microenvironment of ligand binding sites in these albumins. In spite of the high sequence homology among various albumins, slight differences in their drug binding properties have been reported [15]. Thus, it is clear that the degrees of WFN displacement from site I by PS, observed with the various WFN–albumin complexes were largely dependent on the binding affinity of PS to albumins.

In conclusion, a comparison of the WFN displacement results and the binding parameters obtained with different mammalian albumins showed similarity in the PS binding characteristics of GSA and BSA with that of HSA. However, further in depth investigations on PS-albumin interaction are needed before reaching to a conclusion concerning the suitability of the animal model for pharmacological studies.

Acknowledgments

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