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Use of Triplet Excited States for the Study of Drug Binding to Human and Bovine Serum Albumins

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The triplet excited states of (S)- and (R)-flurbiprofen (FBP) have been used as reporters for the microenvironments experienced within the binding sites of human and bovine serum albumins. Regression analysis of triplet decay provides valuable information on the degree of protection that these excited states are afforded from attack by a second FBP molecule, oxygen, or other reagents. The multiexponential fitting of these decays can be satisfactorily correlated with the distribution of the drug among the two binding sites and its presence as the noncomplexed form in the bulk solution. This assignment has been confirmed by using (S)-ibuprofen or capric acid as selective site II replacement probes. Triplet lifetimes and site occupancy are sensitive to the type of serum albumin employed (human versus bovine). Finally, the binding behaviour of (S)- and (R)-FBP exhibits little stereoselectivity.

Introduction

Serum albumins (SA) are very abundant proteins in blood and plasma. One of their most important physiological roles is to carry a variety of agents such as fatty acids, drugs, or metabolites in the bloodstream to deliver them selectively to specific targets.[1,2] The binding of drugs to SA in biological systems is a key process, as it is relevant to the modulation of a number of properties, including the drug's solubility in plasma, toxicity, susceptibility to oxidation, and in vivo half-life.

Human (HSA) and bovine (BSA) serum albumins have been used as model proteins for diverse studies.[3–5] They have a well-known primary structure, and a similar higher-order structure. The binding of small molecules (i.e. drugs or fatty acids) to HSA and BSA has been studied for years with different techniques in order to understand the functions of this unique carrier, and to disclose the structural basis for designing new therapeutic agents.[6]

Thus, it is known that HSA is synthesised and secreted by the liver. Its primary structure consists of a single chain of 585 amino acid residues. Further, it contains 17 disulfide bridges, one tryptophan, and one free cysteine; 67% of the secondary structure is formed by an α helix of six turns, whereas the three-dimensional structure can be described in terms of three domains, each of them constituted by two subdomains.[7] Usually, drugs bind primarily to the high-affinity sites, with typical association constants in the range of 10^4–$10^6\ M^{-1}$. In addition to the primary sites, lower-affinity sites are often populated. The pioneering work of Sudlow and co-workers, which was based on the displacement of fluorescence probes, revealed that a great number of drugs bind with high affinity to one or two sites, called site I (warfarin binding site) and site II (indole–benzodiazepine binding site).[8]

BSA is among the most studied and commonly used proteins in biochemical research. BSA and HSA present 76% sequence similarity, but the former contains two Trp residues instead of one. In general, the structural differences observed between both albumins are conservative in nature. For example, hydrophobic amino acids are replaced by other hydrophobic amino acids rather than by polar residues.

Owing to its similarity to HSA, many studies on the drug–protein binding process have used BSA as a model.[3–5] However, the binding strength of several ligands (naproxen, carprofen, ibuprofen, and others) to the bovine protein has proven to be different from that found for the human protein.[9–21] Therefore, the development of new tools for the investigation of drug–protein interactions, as well as for the comparison between the binding of drugs to HSA and BSA, seems to be important for the integration of existing knowledge.

In the past, a number of techniques including equilibrium dialysis–HPLC, ultrafiltration, spectrophotometry, fluorimetry, calorimetry, circular dichroism, capillary electrophoresis, and NMR diffusion measurements have been used for drug–protein binding studies.[22–31] Each of these methods has its own advantages and limitations; most of the limitations are related to sensitivity, interference, diffusion problems or lack of reproducibility arising from a complicated workup.

A possible alternative to these methodologies could be based on the detection of transients by laser flash photolysis (LFP). Because their dynamic properties can be very medium-dependent, triplet excited states have been shown to be ex-

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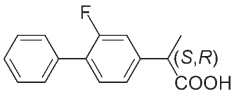
tremely sensitive to their microenvironment.[32] Hence, these transient species can be regarded as potential reporters for the binding of drugs to serum albumins. From the multiexponential decay kinetics, it could even be possible to analyse the drug distribution among the bulk solution and the different protein binding sites. More interestingly, triplet lifetime measurements would depend on the nature of the drug–HSA complexes, such as the strength of the interaction, conformational restrictions, stereochemical requirements, inhibition of self-quenching or triplet–triplet annihilation, and protection from attack by oxygen or other reagents.

In a recent preliminary communication, we have reported on the suitability of the triplet excited states of (*R*)- and (*S*)-flurbiprofen methyl ester (FBPMe) to obtain relevant information about the binding of this compound to HSA.[33] The triplet lifetimes (τ_T) of (*R*)- and (*S*)-FBPMe were dramatically enhanced within the HSA microenvironment. Moreover, two different τ_T values were found for the protein-included FBPMe, which were associated with the presence of the drug in two different binding sites. Based on these values, the populations of the two binding sites at different FBPMe/HSA ratios were determined by regression analysis of the triplet decay traces.

In our previous work, FPBMe was used for convenience, as its hydrophobic character favours inclusion within the protein. However, the analogous information obtained from the triplet state of flurbiprofen (FBP), which is the form usually prescribed to patients, would be more relevant for pharmacokinetic purposes.

The nonsteroidal anti-inflammatory drug FBP is a 2-arylpropionic acid. It is prescribed for the relief of mild to moderately



FBP

severe pain and inflammation (rheumatoid arthritis, osteoarthritis, tendonitis, bursitis), and also for ophthalmic disorders. Although the pharmacological effect of FBP is mainly due to the *S* enantiomer, it is usually sold as a racemic mixture.[34] Recently, it has been reported that FBP selectively inhibits the secretion of β-amyloid 42 (Aβ42), which is the most toxic component of the senile plaques present in the brain of Alzheimer patients.[35] Furthermore, it has also been previously established that FBP binds preferentially to site II in HSA, although the lower-affinity binding site I is also populated to some extent.[36, 37]

In the present work, we have undertaken a systematic LFP study on both (*S*)- and (*R*)-FBP in the presence of different amounts of HSA or BSA, by using the well-characterised triplet–triplet absorption of FBP as reporter.[38] It will be shown that the most significant differences between both albumins are related to dynamic ranges found for the FBP excited state lifetimes within the proteins and to the relative occupancy of the two binding sites.

Results and Discussion

(*S*)-flurbiprofen–SA systems

The behaviour of the *S* enantiomer of FBP was examined first. For the studies in the presence of HSA, a battery of aqueous solutions containing (*S*)-FBP and HSA (molar ratios between 10:1 and 0.3:1) were prepared in neutral buffer (0.01 M PBS) and submitted to LFP. In all cases, the transient absorption spectra obtained after laser excitation ($\lambda_{ex} = 266$ nm) displayed a maximum at 360 nm, which was assigned to the FBP first triplet excited state.[38] Remarkably, whereas the decay at 360 nm in the absence of protein followed a first order exponential law with a lifetime of 1.5 μs, in the presence of HSA it required multiexponential fitting and occurred on longer timescales (Figure 1).

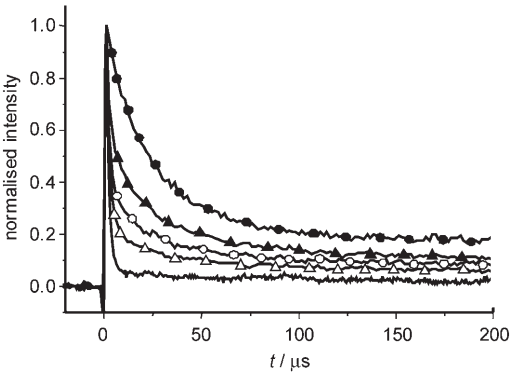


Figure 1. Laser flash photolysis ($\lambda_{ex} = 266$ nm) of (*S*)-FBP (——) and (*S*)-FBP–HSA at molar ratios of 7.5:1 (△), 5.0:1 (○), 2.5:1 (▲), and 0.7:1 (●). Normalised decays were monitored at 360 nm. The concentration of (*S*)-FBP was 2.5×10^{-5} M in all cases.

Thus, when the (*S*)-FBP/HSA ratio was between 0.7:1 and 0.5:1, a double exponential decay was observed with lifetimes of 11.2 and 35.9 μs. The negligible contribution of the 1.5 μs component indicated the absence of free (*S*)-FBP in solution under these conditions. The fact that two different τ_T values were obtained in the presence of HSA correlates well with the existence of two different types of microenvironments (i.e., two different binding sites) in the protein. It is worth mentioning that the protein-bound (*S*)-FBP has considerably longer lifetimes (up to 24-fold) than the noncomplexed form. This can be attributed to a slower deactivation of the excited states inside the HSA binding sites, where an exceptional microenvironment protects the triplet excited state from attack by a second (*S*)-FBP molecule, oxygen, and other reagents. To check this hypothesis, parallel experiments were performed in N_2- and O_2-purged solutions of (*S*)-FBP in PBS, both in the absence and in the presence of HSA. In fact, the triplet decay rate increased with increasing oxygen concentration. The decay traces are shown in the Supporting Information (see Figure S5 and S6, pp. S11 and S12), and the rate constants are given in Table 1. Clearly, the quenching process occurs within the protein microenvironment much more slowly than in the bulk so-

Table 1. Rate constants of triplet quenching by oxygen for FBP free, in site I, and site II.

	Microenvironment	k_q (O_2) [$M^{-1}s^{-1}$]
(*S*)-FBP	solution	1.0×10^9
(*S*)-FBP/HSA[a]	site I	2.1×10^8
	site II	9.1×10^7
(*R*)-FBP/HSA[a]	site I	2.0×10^8
	site II	3.9×10^7
(*S*)-FBP/BSA[a]	site I	4.0×10^8
	site II	3.4×10^7
(*R*)-FBP/BSA[a]	site I	1.9×10^8
	site II	4.6×10^7

[a] Ratio = 0.7:1.

Table 2. Percentage of free, site-I-, and site-II-bound (*S*)-FBP or (*R*)-FBP under various conditions.

	Free [%]	Site-I-bound [%]	Site-II-bound [%]
(*S*)-FBP	100	–	–
(*S*)-FBP/HSA[a]	0	40	60
(*S*)-FBP/HSA/(*S*)-IBP[b]	27	65	8
(*S*)-FBP/HSA/CA[b]	29	59	12
(*S*)-FBP/BSA[a]	0	39	61
(*S*)-FBP/BSA/(*S*)-IBP[b]	8	58	34
(*S*)-FBP/BSA/CA[b]	5	57	38
(*R*)-FBP/HSA[a]	0	30	70
(*R*)-FBP/HSA/(*S*)-IBP[b]	22	59	19
(*R*)-FBP/HSA/CA[b]	26	61	13
(*R*)-FBP/BSA[a]	0	33	67
(*R*)-FBP/BSA/(*S*)-IBP[b]	0	56	44
(*R*)-FBP/BSA/CA[b]	0	50	50

[a] Ratio = 0.7:1. [b] Ratio = 0.7:1:1.

lution. Moreover, protection from oxygen attack seems to be more effective within the high affinity site II.

At higher (*S*)-FBP/HSA ratios (from 0.8:1 to 10:1), three lifetime values (1.5, 11.2 and 35.9 μs) were necessary to obtain a good fitting of the decay signal (also shown in Figure 1). This could be associated with the presence of the two (*S*)-FBP–HSA species (τ_T = 11.2 and 35.9 μs), in addition to free (*S*)-FBP (τ_T = 1.5 μs).

A regression analysis of the decay curves for (*S*)-FBP/HSA ratios from 10:1 to 0.8:1 allowed us to determine the values of the A_1, A_2, and A_3 coefficients (corresponding to the three components with different lifetimes, correlated with free, site-I-bound, and site-II-bound FBP). Based on the fact that the high affinity site of FBP is site II,[36,37] the major component under nonsaturating conditions (associated with the longest τ_T) was assigned to FBP within site II. Consequently, the minor component (with τ_T = 11.2 μs) was assigned to site-I-bound FBP. Moreover, to obtain independent evidence supporting this assignment, additional LFP experiments were performed in the presence of (*S*)-ibuprofen ((*S*)-IBP) and capric acid (CA), whose affinity for binding in site II is known to be very high.[39,40] In good agreement with the initial assumptions, the addition of IBP or CA to the (*S*)-FBP–HSA solutions resulted in a decreased contribution of the longest lifetime component (site-II-bound FBP), with a concomitant enhancement of the components assigned to free-, and site-I-bound FBP. The percentages of free-, site-I-, and site-II-bound FBP in the presence of HSA (and eventually (*S*)-IBP or CA) are shown in Table 2.

To investigate the behaviour of (*S*)-FBP in the bovine protein, parallel experiments were carried out in the presence of increasing amounts of BSA. Thus, PBS solutions of (*S*)-FBP and BSA (molar ratio in the range from 10:1 to 0.3:1) were submitted to LFP. Again, two components with τ_T = 10.8 and 86.5 μs were obtained for the triplet decay (λ = 360 nm) inside the protein. This is shown in Figure 2.

A regression analysis of the decay curves for (*S*)-FBP/BSA ratios from 10:1 to 1:1 (where some free drug is present) allowed us to obtain the values of the A_1, A_2, and A_3 coefficients. The major component under nonsaturating conditions (τ_T = 86.5 μs) was assigned to (*S*)-FBP in the high affinity binding site (site II), while the minor component (with τ_T = 10.8 μs) was attributed to (*S*)-FBP within site I. Experiments in the presence of (*S*)-IBP or CA supported this assumption. Relevant data are shown in Table 2.

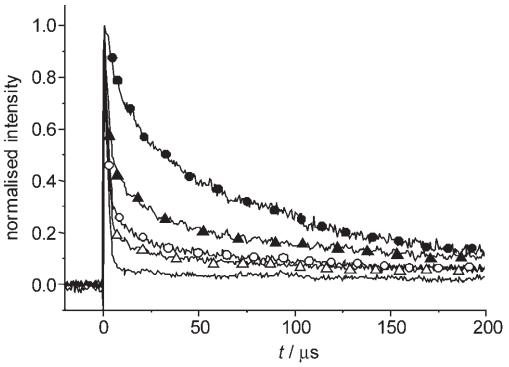


Figure 2. Laser flash photolysis (λ_{ex} = 266 nm) of (*S*)-FBP (——) and (*S*)-FBP–BSA at molar ratios of 7.5:1 (△), 5.0:1 (○), 2.5:1 (▲), and 0.7:1 (●). Normalised decays were monitored at 360 nm. The concentration of (*S*)-FBP was 2.5×10^{-5} M in all cases.

It is remarkable that the triplet excited state of site-II-bound (*S*)-FBP lived much longer (2.4 times) within BSA than within HSA. However, no significant differences were found in the corresponding values of the site-I-bound (*S*)-FBP. The results indicate that the triplet excited state of (*S*)-FBP is more protected from deactivation (due to attack by a second (*S*)-FBP molecule, oxygen, or other reagents) within the microenvironment provided by site II in BSA. Furthermore, this site appears to bind (*S*)-FBP with the highest affinity, as supported by the fact that replacement of (*S*)-FBP is more difficult here than in the other SA microenvironments. Accordingly, triplet quenching by oxygen was slower inside the protein, especially in site II (see Table 1 and supporting information, Figure S5 and S7, pp. S11 and S13).

Further, the *A* coefficients of the regression analysis curves indicate that the main differences between the behaviour of the two proteins were found at low (*S*)-FBP/SA ratios. This is shown in Figure 3; further plots illustrating the binding behav-

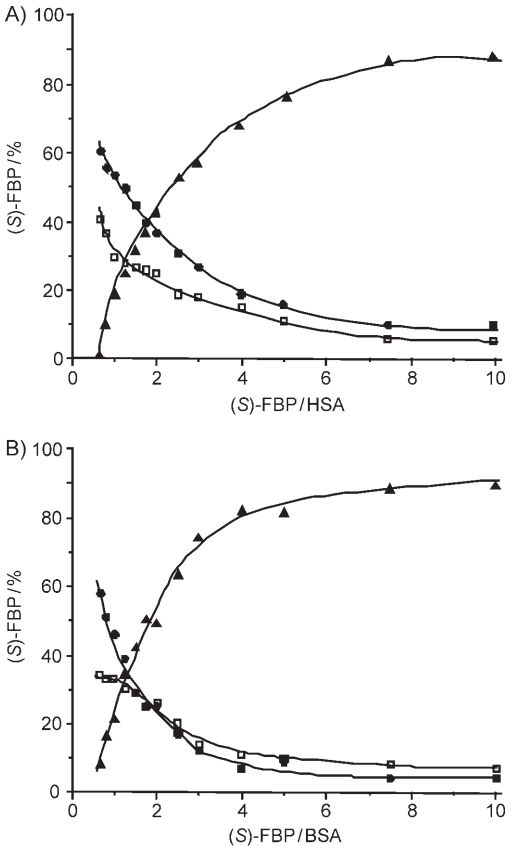


Figure 3. A) Percentage of free (▲), site-I- (□), and site-II-bound (●) (*S*)-FBP in the presence of HSA at different (*S*)-FBP/HSA ratios. B) Percentage of free (▲), site-I- (□), and site-II-bound (●) (*S*)-FBP in the presence of BSA at different (*S*)-FBP/BSA ratios.

iour can be found in the supporting information, Figure S10 and S11, pp. S16 and S17.

(*R*)-flurbiprofen/SA systems

As stated above, the pharmacological effect of flurbiprofen is mainly attributed to the *S* enantiomer, although the drug is sold for therapeutic purposes as a racemic mixture. To check whether significant differences in the binding behaviour were observable for the two enantiomers, a similar study was performed on (*R*)-FBP in the presence of both serum albumins.

As expected, in PBS solution the transient absorption spectra and the triplet lifetimes of the two FBP enantiomers did not differ from each other. When included within HSA, two components with τ_T 10.2 μs and 39.0 μs were found for (*R*)-FBP. These values were only slightly different from those obtained for (*S*)-FBP, which indicates very modest stereoselectivity in the HSA binding process. Representative decays of the signal, which was monitored at 360 nm, are shown in Figure 4.

As in the case of (*S*)-FBP, LFP experiments for the (*R*)-FBP/HSA systems in the presence of (*S*)-IBP or CA supported the initial site I and site II assignments. Thus, addition of (*S*)-IBP or CP to the (*R*)-FBP/HSA solutions resulted in a dramatic decrease in the amount of site-II-bound (*R*)-FBP (up to 5.8 times lower),

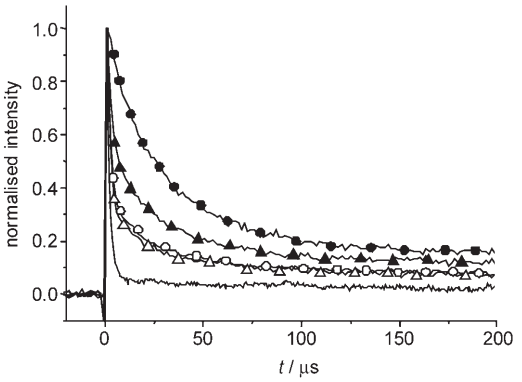


Figure 4. Laser flash photolysis (λ_{ex} = 266 nm) of (*R*)-FBP (——) and (*R*)-FBP–HSA at molar ratios of 7.5:1 (△), 5.0:1 (○), 2.5:1 (▲), and 0.7:1 (●). Normalised decays were monitored at 360 nm. The concentration of (*R*)-FBP was 2.5×10^{-5} M in all cases.

with a parallel enhancement of the free and site-I-bound species (significant data are shown in Table 2).

Finally, similar studies were carried out for (*R*)-FBP in the presence of the bovine protein. From the LFP kinetic decays of the (*R*)-FBP–BSA solutions (Figure 5), two values of τ_T (6.6 and 58.6 μs) were obtained and attributed to site-I-bound and site-II-bound (*R*)-FBP, respectively. Oxygen quenching experiments led to results similar to those obtained with (*S*)-FBP (Table 1 and supporting information, Figure S8 and S9, pp. S14 and S15).

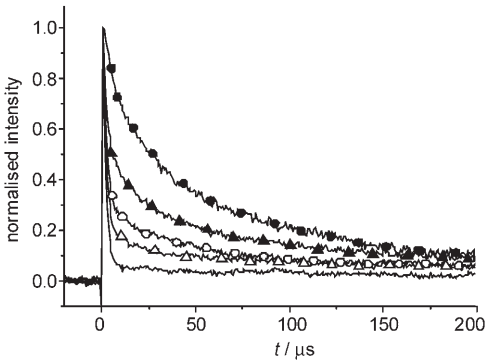


Figure 5. Laser flash photolysis (λ_{ex} = 266 nm) of (*R*)-FBP (——) and (*R*)-FBP–BSA at molar ratios of 7.5:1 (△), 5.0:1 (○), 2.5:1 (▲), and 0.7:1 (●). Normalised decays were monitored at 360 nm. The concentration of (*R*)-FBP was 2.5×10^{-5} M in all cases.

On the other hand, the addition of (*S*)-IBP or CA to the (*R*)-FBP–BSA solutions resulted in a redistribution of the bound drug (Table 2), which partially moved from site II to site I. Interestingly, no detectable amounts of free drug were found under these conditions.

The site occupancy, which was estimated from the *A* coefficients of the regression analysis curves, are shown in Figure 6. The main differences between the behaviour of the two proteins were found again at low (*S*)-FBP/SA ratios.

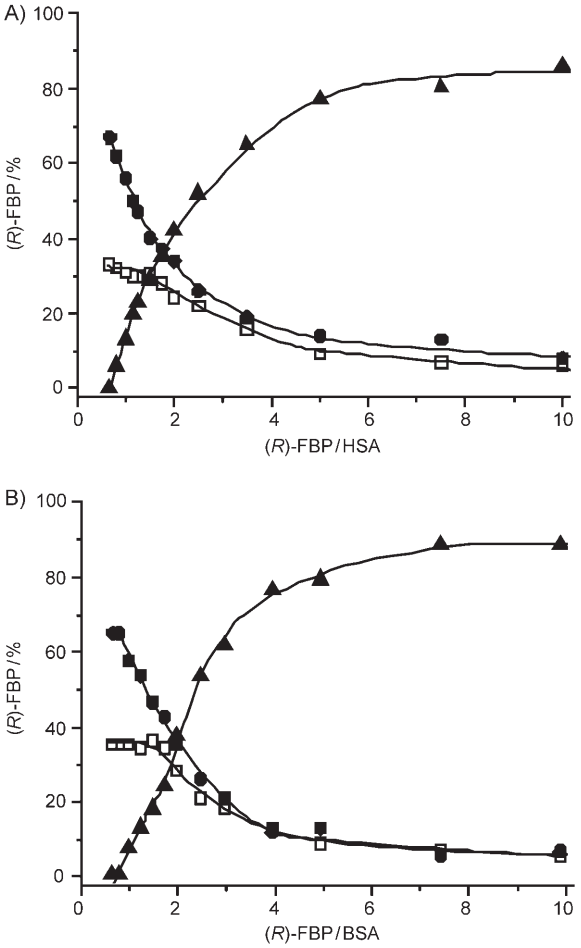


Figure 6. A) Percentage of free (▲), site-I- (□), and site-II-bound (●) (*R*)-FBP in the presence of HSA at different (*R*)-FBP/HSA ratios. B) Percentage of free (▲), site-I- (□), and site-II-bound (●) (*R*)-FBP in the presence of BSA at different (*R*)-FBP/BSA ratios.

A comparison between the decays monitored at 360 nm for (*S*)-FBP–HSA, (*S*)-FBP–BSA, (*R*)-FBP–HSA, and (*R*)-FBP–BSA is shown in Figure 7. It clearly shows that the binding behaviour of FBP is different in the two proteins. Moreover, some stereoselectivity occurs in BSA, whereas no significant differences between the two FBP enantiomers were observed within HSA.

Conclusions

The results obtained in the present work confirm that the triplet drug excited states can be used as reporters for the microenvironments experienced within the binding sites of transport proteins. Regression analysis of triplet decays can provide valuable information on the degree of protection from attack by a second drug molecule, oxygen or other reagents, which is afforded these excited states by the protein. The multiexponential fitting of these decays can be satisfactorily correlated with the distribution of the drug among the two binding sites and its presence as the noncomplexed form in the bulk solution. This assignment has been confirmed by using selective site II replacement probes. Both triplet lifetimes and site occupancy

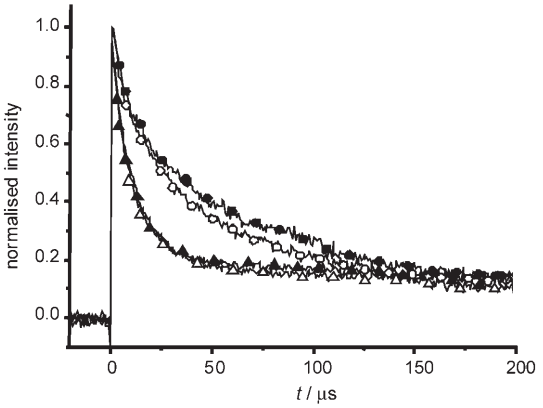


Figure 7. Decays monitored at 360 nm which were obtained upon laser flash photolysis ($\lambda_{ex} = 266$ nm; 0.01 M PBS, air) of (*S*)-FBP/BSA (●), (*R*)-FBP/BSA (○), (*S*)-FBP/HSA (▲), and (*R*)-FBP/HSA (△). The drug/SA ratio was kept constant at 0.7:1.

are sensitive to the type of serum albumin employed (human vs. bovine). Finally, less stereoselectivity has been found in the binding behaviour of (*S*)- and (*R*)-FBP than in the case of their methyl esters.

Experimental Section

Materials: HSA and BSA were purchased from Sigma. (*S*)- and (*R*)-FBP were obtained from Aldrich. The absorbance of the solutions was measured in a Perkin–Elmer Lambda 35 UV/Vis spectrometer.

Laser flash photolysis experiments: Laser flash photolysis experiments were performed by using a Q-switched Nd:YAG laser (Quantel Brilliant, 266 nm, 14 mJ pulse^{-1}, 5 ns fwhm) coupled to a mLFP-111 Luzchem miniaturised equipment. All transient spectra were recorded employing 10×10 mm^2 quartz cells with 4 mL capacity. The absorbance of FBP was found to be ≈ 0.2 at the laser wavelength, which corresponds to a concentration of 2.5×10^{-5} M. The concentration of SA was determined with the molar absorption coefficients at 280 nm of 35700 M^{-1}cm^{-1} for HSA, and 44720 M^{-1}cm^{-1} for BSA. All of the experiments were carried out in PBS (pH 7.4, 0.01 M) at room temperature (22 °C) and under an air atmosphere.

For the studies in the presence of SA, a battery of aqueous solutions containing (*S*)- or (*R*)-FBP and SA (molar ratios between 10:1 and 0.3:1) were prepared in neutral buffer (0.01 M PBS) and stored for 15 h at 4 °C to ensure a complete equilibrium between the drug and the protein. As an example, the experimental procedure to prepare a solution containing FBP and SA in 10:1 molar ratio is briefly described. To a 2.5×10^{-5} M solution of (*S*)- or (*R*)-FBP (20 mL) in PBS was added 2×10^{-3} M SA (25 μL) in PBS. The resulting solution (4 mL) was placed in a quartz cuvette and submitted to LFP (10 shots for monitoring at 360 nm). To obtain an accurate decay trace, this experiment was repeated at least three times with fresh sample. Triplet lifetimes and fittings of the decay traces were coincident within the experimental error margins. To obtain the transient absorption spectra from 700 to 290 nm, a fresh sample (drug and protein concentration 2.5×10^{-5} M) was submitted to LFP at a regular interval of 10 nm (10 shots per wavelength). A total of 41 decays for each spectrum acquisition was measured.

Acknowledgements

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Keywords: binding sites · flurbiprofen · laser flash photolysis · serum albumins

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