Transient absorption spectroscopy for determining multiple site occupancy in drug-protein conjugates. A comparison between human and bovine serum albumins using flurbiprofen methyl ester as probe

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Abstract

Laser flash photolysis (LFP) has been used to determine the degree of binding of *(S)*- or *(R)*-flurbiprofen methyl ester (FBPMe) to human and bovine serum albumins. Regression analysis of the triplet decay of the drug (λ = 360 nm) in the presence of the proteins led to a satisfactory fitting when considering a set of three lifetimes; the corresponding A_1, A_2 and A_3 preexponential coefficients can be correlated with the presence of FBPMe in the bulk solution and within the two known binding sites. The most remarkable differences between HSA and BSA were found under non-saturating conditions; thus, when the [FBPMe]/[SA] ratio was 1:1, all the drug was bound to HSA, whereas 20-30 % of it remained free in the bulk solution in the presence of BSA. The LFP approach was also applicable to the study of more complex FBPMe/HSA/BSA mixtures; the obtained results were in good agreement with the previous findings in FBPMe/HSA and FBPMe/BSA systems. This suggests the possibility of making use of the transient triplet-triplet absorption for investigating the distribution of a drug between several compartments in different host biomolecules.

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Introduction

Serum albumins (SA) are transport proteins very abundant in blood and plasma; one of their main functions is to carry several endogenous and exogenous agents (i.e. fatty acids, drugs or metabolites) in the bloodstream, to accomplish a selective delivery to specific targets.[1,2] Binding of drugs to SA in biological systems is a key process that can modulate a number of properties of the carried agent, such as increased solubility in plasma, decreased toxicity, protection against oxidation or prolongation of the *in vivo* half-life; hence, binding is essential for understanding biodistribution, metabolism, elimination or pharmacological effect of drugs in the body.

Human (HSA) and bovine (BSA) serum albumins have been widely used as model proteins for diverse studies.[3-5] Both of them have a well known primary structure and a similar folding.[6]

The primary structure of HSA, which is synthesized and secreted by the liver, consists of a single chain of 585 amino acid residues, with 17 disulfide bridges, one tryptophan (Trp) and one free cysteine; the secondary structure is defined by 67% of α helix of six turns, and the three-

dimensional structure can be described in terms of three domains, each of them constituted in turn by two subdomains.[7]

Similarly, BSA is also among the most studied proteins in biochemical research; BSA and HSA have 76% sequence identity, but BSA contains two Trp residues, instead of one.[1]

Binding of small molecules (i.e. drugs or fatty acids) to HSA and BSA has been studied for years through different techniques in order to understand the role of transport proteins and also to establish the structural basis for designing new therapeutic agents. [8-18]

Recent studies have found evidence supporting the existence of a high number of binding sites in HSA.[19,20] However, agreement exists that there are basically two major and structurally selective binding sites for small heterocyclic or aromatic compounds, named site I and site II according to Sudlow's classification.[21-23]

Likewise, numerous studies on the drug-protein binding process have used BSA as model, due to its similarity to HSA.[3-5] However, the binding of a variety of ligands to the bovine protein has demonstrated to be different from that found for the human protein. [24-36] Several techniques have been used for binding studies, including equilibrium dialysis, ultrafiltration, ultracentrifugation, calorimetry, fluorescence quenching, circular dichroism, liquid chromatography, capillary electrophoresis, NMR diffusion measurements and recently also room temperature phosphorescence.[8-18] Thus, the development of new tools for the investigation of drug-protein interactions, as well as for the comparison between binding of drugs to HSA and BSA, seems important to help integration of the existing knowledge.

Arylpropionic acid derivatives are a group of non-steroidal anti-inflammatory drugs (NSAIDs). Currently, they are marketed as racemic mixtures (with few exceptions such as naproxen); however, the pharmacological activity is mainly due to the *(S)*-isomer.[37] These drugs bind preferentially (and often stereoselectively) to site II of HSA by means of hydrogen bonding and electrostatic interactions.[38]

As the properties of the excited states are very sensitive to the experienced microenvironment,[39] they could allow to study drug distribution among the bulk solution and the different protein binding sites. Previously, time resolved techniques have been employed for the study of carprofen-HSA complexes.[40,41] However, in this case fast reaction of the drug triplet state with the Trp unit of site I limits its possibilities to be used as a probe. This problem can be circumvented by choosing "inert" excited states as HSA binding site reporters. Thus, the behavior of *(S)*- and *(R*)- flurbiprofen (FBP) in the presence of HSA or BSA has been recently published.[42] Moreover, a significant stereodifferentiation has been observed in the triplet lifetimes of the methyl esters of *(S)*- and *(R*- FBP (FBPMe) in the presence of HSA (reported as a preliminary communication).[43] Methylation of the carboxy group is not a trivial structural change, as the resulting ester FBPMe binds preferentially to site I, where hydrophobic interactions predominate.[44] In the present work, we wish to report in full the interaction between the excited states of FBPMe (Chart 1) and the binding sites of HSA. These studies have now been extended to BSA, as the binding behavior can be significantly different. Remarkably, the laser flash photolysis (LFP) technique has proven to be useful to determine the distribution of FBPMe between BSA and HSA in mixtures of both proteins, indicating a higher preference for the human albumin. This suggests the possibility of making use of the transient triplet-triplet absorption for investigating the distribution of a drug between several compartments in different host biomolecules.

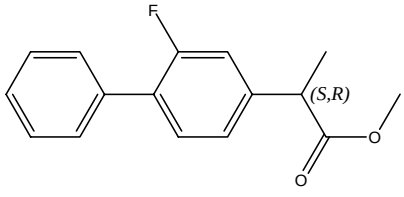

Chart 1

Experimental Section

Materials and solvents

The two proteins HSA and BSA were purchased from Sigma; *(S)*- and *(R)*-FBP were obtained from Aldrich.

(S)-FBPMe was synthesized by treatment of *(S)*- or *(R)*- FBP (200 mg, 0.82 mmol) with $SOCl_2$ (0.82 mmol) and MeOH as solvent. The reaction mixture was maintained under reflux during 2 h. The organic solvent was removed *in vacuo*. The remaining oil was dissolved in methylene chloride (20 mL), washed with saturated $NaHCO_3$ (twice, 10 mL), 1M HCl (twice, 10 mL) and brine (twice, 10 mL). The resulting oil was submitted to silica gel preparative chromatography (methylene chloride as eluent) to afford the desired products in nearly quantitative yield.

Solutions of FBPMe/SA in 0.01 M PBS buffer (pH = 7.4) were prepared from Sigma phosphate buffered saline tablets.

Absorption spectra

Optical spectra in different solvents were measured on a Perkin Elmer Lambda 35 UV/Vis Spectrophotometer.

Laser flash photolysis experiments

Laser flash photolysis experiments were performed by using a Q-switched Nd:YAG laser (Quantel Brilliant, 266 nm or 355 nm for photosensitized experiments, 4 mJ per pulse, 5 ns fwhm) coupled to a mLFP-111 Luzchem miniaturized equipment. All transient spectra were recorded employing 10 x 10 mm^2 quartz cells with 4 mL of solution. The absorbance of FBPMe was *ca*. 0.2 at the laser wavelength. All the experiments were carried out in phosphate buffer (PBS, pH = 7.4, 0.01 M) at room temperature (22 ºC) and under air atmosphere.

For the studies in the presence of SA, a battery of aqueous solutions containing *(S)*- or *(R)*-FBPMe and SA (molar ratios between 10:1 and 0.5:1) were prepared in neutral buffer (0.01 M PBS) and stored overnight at 4 ºC to ensure a complete equilibrium between the drug and the protein. As an example, the experimental procedure to prepare a solution containing FBPMe and SA in 10:1 molar ratio is briefly described: to 20 mL of a 2.5×10^{-5} M solution of *(S)*- or *(R)*-FBPMe in PBS, 25 μL of SA 2×10^{-3} M in PBS were added. The resulting solution (4 mL) was placed in a quartz cuvette and submitted to LFP (10 shots for monitoring decay 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, fresh sample (drug and protein concentration 2.5×10^{-5} M) was submitted to LFP in a regular interval of 10 nm (10 shots per wavelength).

Results and Discussion

Laser flash photolysis of *(S)*- and *(R)*- FBPMe in the presence of HSA

The first series of studies were focused on the behavior of *(S)*-FBPMe in HSA. In the absence of this protein, the transient absorption spectra obtained after excitation at 266 nm (PBS, air) displayed the typical maximum at 360 nm due to the $T_1 \rightarrow T_n$ absorption. The decay at 360 nm followed a first order exponential law with τ_T = 1.5 μs. For the experiments in the presence of HSA, a battery of aqueous solutions containing *(S)*-FBMe and HSA (molar ratios between 5:1 and 0.5:1) were prepared in phosphate buffer and submitted to LFP. Remarkably, the decay at 360 nm in the presence of the protein occurred at longer timescales (Figure 1) and required application of a multiexponential function to achieve a satisfactory fitting.

When the [*(S)*-FBPMe]/[HSA] ratio was between 1:1 and 0.5:1, a double exponential decay was observed with lifetimes of 4.1 and 31.5 μs. The insignificant contribution of the 1.5 μs component indicated that no free *(S)*-FBPMe was present in the bulk solution under these

conditions. The fact that two different τ_T values were necessary to fit the decay in the presence of HSA would agree with the presence of two different types of microenvironments (*i.e.*, two different binding sites) in the protein. The longer triplet lifetimes values of protein-bound *(S)*-FBPMe can be due to a slower deactivation of the excited states inside the HSA binding sites; in such pockets, a particular microenvironment is created, which is able to protect the triplet excited state from the attack by a second *(S)*-FBPMe molecule, oxygen or other reagents.

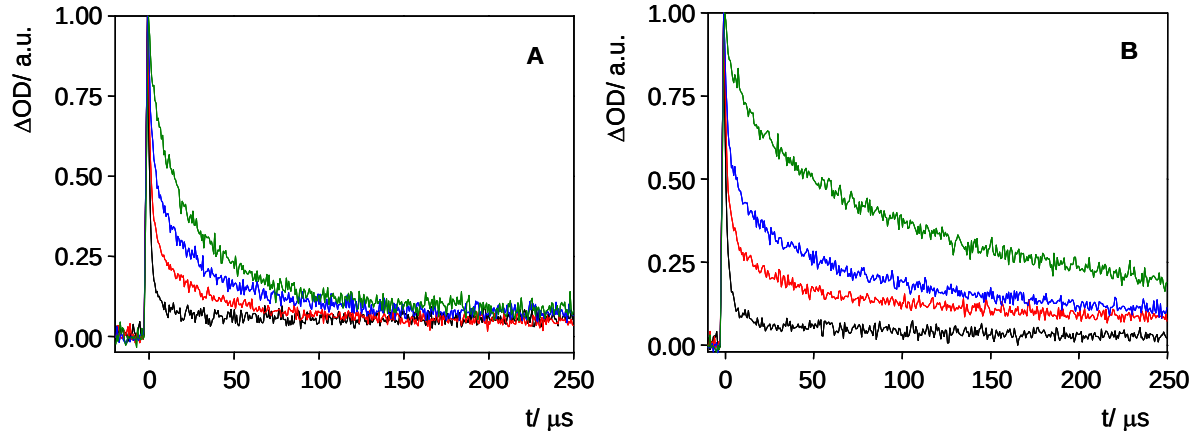

Figure 1. A. Laser flash photolysis (λ_{exc}= 266 nm) of (*S*)-FBPMe (black), (*S*)-FBPMe/HSA 5:1 (red), (*S*)-FBPMe/HSA 2.5:1 (blue) and (*S*)-FBPMe/HSA 1:1 (green) in 0.01 M PBS. **B**. Laser flash photolysis (λ_{exc}= 266 nm) of (*R*)-FBPMe (black), (*R*)-FBPMe/HSA 5:1 (red), (*R*)-FBPMe/HSA 2.5:1 (blue) and (*R*)-FBPMe/HSA 1:1 (green) in 0.01 M PBS. The normalized decays are monitored at 360 nm. The concentration of (*S*)- or *(R)*-FBPMe was 2.5×10^{-5} M in all cases.

At [*(S)*-FBPMe]/[HSA] ratios from 1.25:1 to 5:1, three lifetime values (1.5, 4.1 and 31.5 μs) were necessary to obtain a good fitting of the decay signal (some of these decays are also shown in Figure 1A). This can be explained taking into account the two *(S)*-FBPMe/HSA-bound species, in addition to free *(S)*-FBPMe.

Regression analysis of the decay curves for different [*(S)*-FBPMe]/[HSA] ratios provided the values of the A_1, A_2 and A_3 coefficients associated with the three components of different lifetimes, correlated with free, site I-bound and site II-bound FBPMe. Based on the known fact

that the high affinity site of 2-arylpropionic acids methyl esters is site I,[43] the major component under non-saturating conditions (which was that with the longest τ_T) was assigned to *(S)*-FBPMe within site I; consequently, the minor component (with τ_T = 4.1 μs) was assigned to site II-bound FBPMe. The distribution of *(S)*-FBPMe among the binding sites and the bulk solution (provided by the A_1, A_2 and A_3 coefficients) is represented in Figure 2 (left).

In order to look for a possible sterodifferentiation in the binding behavior of the two FBPMe enantiomers, a similar study was performed on *(R)*-FBPMe in the presence of HSA.

In PBS solution the transient absorption spectra and the triplet lifetimes of the two FBPMe enantiomers did not differ from each other, as expected. By contrast, when the [*(R)*-FBPMe]/[HSA] ratio was between 1:1 and 0.5:1, a double exponential decay was observed with lifetimes values of τ_T 157.6 μs and 16.6 μs were found for *(R)*-FBPMe (Figure 1B). These values were much higher than those obtained for *(S)*-FBPMe, indicating a remarkable stereodifferentiation between the two enantiomers in the protein binding process.

At [*(R)*-FBPMe]/[HSA] ratios from 1.25:1 to 5:1, three lifetime values were again necessary to obtain a good fitting of the decay signal; they corresponded to the two *(R)*-FBMe/HSA-bound species (τ_T = 16.6 and 157.5 μs), in addition to free *(R)*-FBPMe (τ_T = 1.5 μs).

As in the case of *(S)*-FBPMe, the major component under non-saturating conditions (the one with τ_T = 157.6 μs) was assigned to *(R)*-FBPMe within site I and the minor component (with τ_T = 16.6 μs) to site II-bound FBPMe. From the A_1, A_2 and A_3 coefficients, the distribution of *(R)*-FBPMe among the binding sites and the bulk solution was obtained (Figure 2, right).

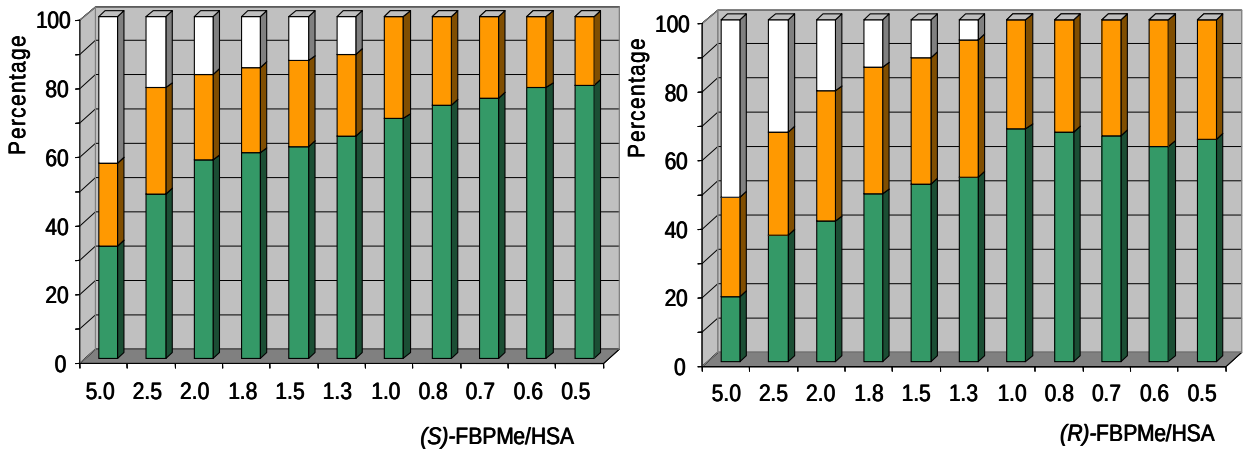

Figure 2. Percentage of FBPMe: free (white), in site I (green) and in site II (orange) of HSA from the A_1, A_2 and A_3 values of the multiexponential decay at 360 nm. **Left**. *(S)*-FBPMe. **Right**. *(R)*-FBPMe.

As a control experiment, in order to ensure that the observed stereodifferentiation in the decay kinetics was real, the behavior of racemic FBPMe in the presence of HSA was compared with that of the pure enantiomers. In agreement with the expectations, the decay obtained at 360 nm for a 1:1 FBPMe/HSA molar ratio was perfectly matched by the semi sum of the decays of *(S)*- and *(R)*-FBPMe with HSA under the same conditions (Figure 3).

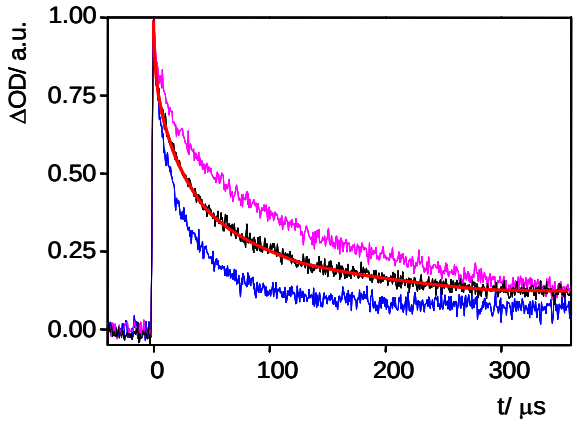

Figure 3. Decay of the 360 nm band obtained by laser flash photolysis (λ_{exc}= 266 nm) of racemic FBPMe/HSA 1:1 (black), *(S)*-FBPMe/HSA 1:1 (blue) and *(R)*-FBPMe/HSA 1:1 (magenta). The ester concentration was 2.5×10^{-5} M in all cases. The red line corresponds to the semi sum of *(S)*-FBPMe and *(R)*-FBPMe.

Laser flash photolysis of *(S)*- and *(R)*- FBPMe in the presence of BSA

To investigate the behavior of *(S)*-FBPMe within the bovine protein, parallel experiments were carried out in the presence of different amounts of BSA. Thus, PBS solutions of *(S)*-FBPMe and BSA (molar ratio in the range from 5:1 to 0.5:1) were submitted to LFP. Again, two components with τ_T = 4.6 and 19.3 μs were obtained for the triplet decay (λ = 360 nm) inside the protein. This is shown in Figure 4A.

Regression analysis of the decay curves for [*(S)*-FBPMe]/[BSA] ratios from 5:1 to 0.5:1 (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 non-saturating conditions (τ_T = 19.3 μs) was assigned to *(S)*-FBPMe in the high affinity binding site (site I), while the minor component (with τ_T = 4.6 μs) was attributed to *(S)*-FBPMe within site II. These results indicate that, again, the triplet excited state of *(S)*-FBPMe is more protected against deactivation (due to attack by a second *(S)*-FBPMe molecule, oxygen, other reagents, etc.) within the microenvironment provided by site I in BSA.

The triplet excited state of site I-bound *(S)*-FBPMe lived longer within BSA than within HSA (*ca*. 1.5 times); however, no significant differences were found in the corresponding values of site II-bound *(S)*-FBPMe (4.6 *vs*. 4.1 μs).

Concerning the site occupancy, as estimated from the A coefficients of the regression analysis curves, Figure 5 (left) shows that the main differences between the behavior of the two proteins were found at low [*(S)*-FBPMe]/[SA] ratios. Thus, when the ratio was 1:1, all the FBPMe was bound to the human protein, whereas more than 30 % of it remained free in the bulk solution in the presence of the bovine protein, under otherwise identical experimental conditions (compare Figs. 2 and 5).

Similar studies were carried out for *(R)*-FBPMe in the presence of BSA. As usually, from the LFP kinetic decays of *(R)*-FBPMe/BSA solutions (Figure 4), two values of τ_T (2.8 and 19.7 μs) were obtained and attributed to site II and site I-bound *(R)*-FBPMe, respectively.

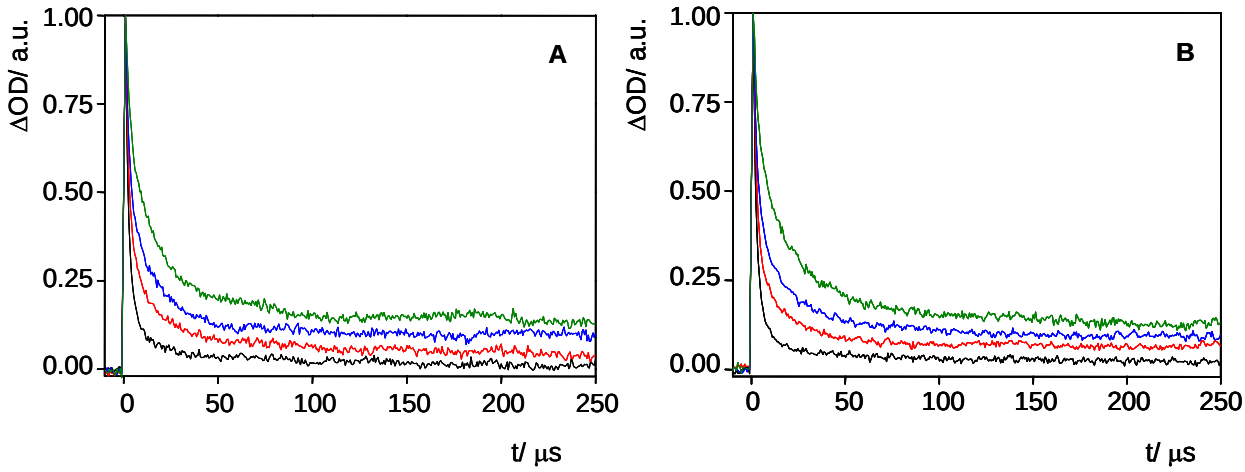

Figure 4. A. Laser flash photolysis (λ_{exc}= 266 nm) of *(S)*-FBPMe (black), *(S)*-FBPMe/BSA 5:1 (red), *(S)*-FBPMe/BSA 2.5:1 (blue) and *(S)*-FBPMe/BSA 1:1 (green) in 0.01 M PBS. **B**. Laser flash photolysis (λ_{exc}= 266 nm) of *(R)*-FBPMe (black), *(R)*-FBPMe/BSA 5:1 (red), *(R)*-FBPMe/BSA 2.5:1 (blue) and *(R)*-FBPMe/HSA 1:1 (green) in 0.01 M PBS. The normalized decays are monitored at 360 nm. The concentration of *(S)*- or *(R)*-FBPMe was $2.5 \cdot 10^{-5}$ M in all cases.

The site occupancy is shown in Figure 5 (right), confirming the trend observed for the *(S)*-enantiomer. The main differences between the behavior of the two proteins were found again at low [*(R)*-FBPMe]/[SA] ratios.

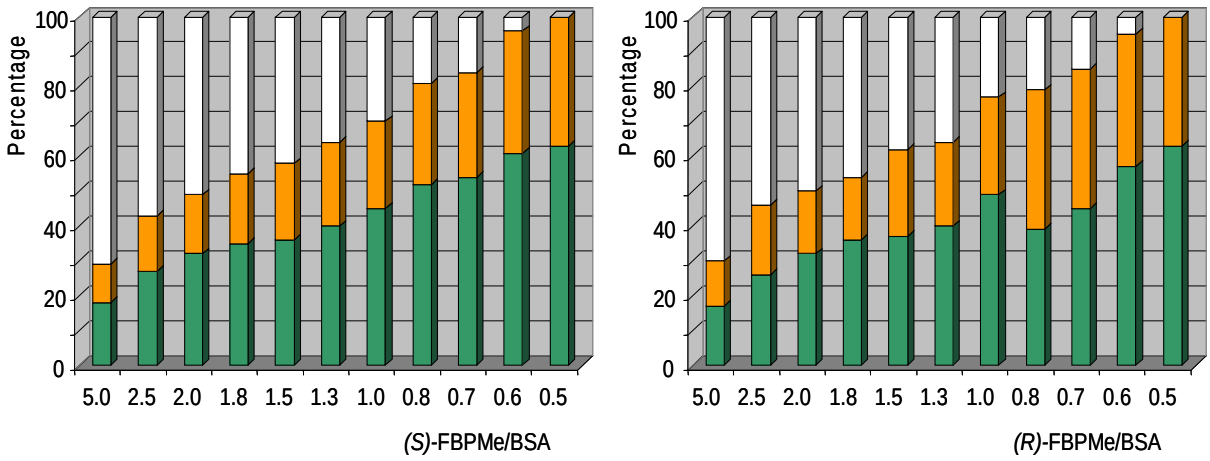

Figure 5. Percentage of FBPMe: free (white), in site I (green) and in site II (orange) of BSA from the A_1, A_2 and A_3 values of the multiexponential decay at 360 nm. **Left**. *(S)*-FBPMe. **Right**. *(R)*-FBPMe.

Competition studies on human versus bovine protein occupancy

As stated above, the most remarkable differences between HSA and BSA as carrier proteins for FBPMe were found under non-saturating conditions. A similar behavior was observed for the two FBPMe enantiomers.

This fact suggested the possibility of making use of the transient triplet-triplet absorption for investigating the distribution of a drug between several compartments in different host biomolecules. To proof the concept, an experiment was designed in which *(R)*-FBPMe was added to a mixture of HSA and BSA in buffered aqueous solution (molar ratio 1.4:1:1). Of course, this is not a real situation, but it could be a good model to check the feasibility of this new methodology.

When the *(R)*-FBPMe/HSA/BSA mixture was flash-photolyzed at 266 nm, the expected transient absorption spectrum was obtained corresponding to the typical biphenyl-like T-T band with λ_{max} = 360 nm. For the regression analysis of the decay at the absorption maxima, four lifetime components (identical to the τ_T values in sites I and II of both albumins) were assumed. Their relative contributions (determined from the preexponential coefficients) led to the

following values: site I of HSA, 55%; site II of HSA, 26%; site I of BSA, 12 %; and site II of BSA, 7 %. These results support that the affinity for the human protein is higher under the non-saturating conditions employed and confirm the preferential occupancy of site I in both albumins.

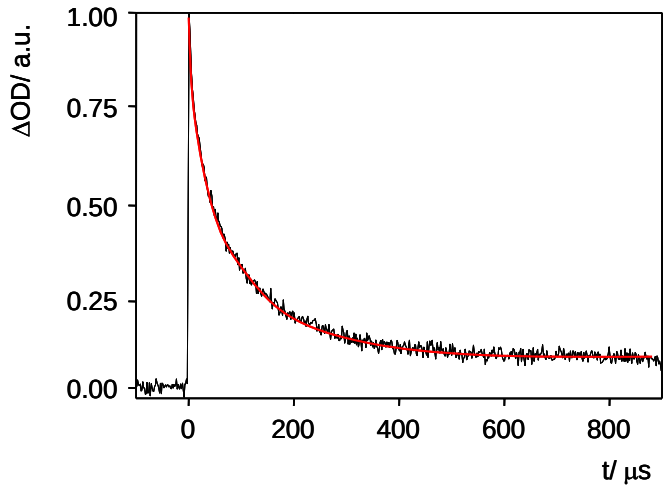

Figure 6. Laser flash photolysis (λ_{exc}= 266 nm) of *(R)*-FBPMe/HSA/BSA at molar ratio 1.4:1.0:1.0. The red trace corresponds to the simulated decay, assuming that the triplet lifetimes are the same as in the experiments without mixing the proteins, and using the following percentages of occupancy: site I of HSA, 55%; site II of HSA, 26%; site I of BSA, 12 %; and site II of BSA, 7 %.

Conclusions

Using flurbiprofen methyl ester as a probe, it has been demonstrated that the laser flash photolysis technique constitutes a valuable tool for the study of drug binding to transport proteins. Regression analysis of the decay kinetics of the transient absorption spectra corresponding to the triplet-triplet absorption provides precise information on the number of binding sites and their degree of occupancy. Triplet lifetimes inside the binding sites are in general much longer than in the bulk aqueous solution and depend on the nature of the sites, on the origin (human or bovine) of the employed serum albumin, and/or on the stereochemistry of the drug. Although similar trends are observed in HSA and BSA, the main differences between

the two proteins are found under non-saturating conditions; thus, when the [FBPMe]/[SA] ratio is 1:1, all the drug is bound to HSA, whereas 20-30 % of it remains free in the bulk solution in the presence of BSA. Finally, the results obtained using mixtures of the two proteins suggest the possibility of making use of the transient triplet-triplet absorption for investigating the distribution of a drug between several compartments in different host biomolecules.

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Suggestion for the table-of-contents entry

Laser flash photolysis for determining multiple site occupancy in drug-protein conjugates. A comparison between human and bovine serum albumins using flurbiprofen methyl ester as probe

Ignacio Vayá, M. Consuelo Jiménez and Miguel A. Miranda**

The figures show the percentage of free (white), site I-bound (green) and site II-bound (orange) *(S)*-FBPMe as obtained from the A_1, A_2 and A_3 values of the multiexponential decay of the triplet-triplet absorption at 360 nm, in the presence of HSA and BSA.