In Situ Transient Spectroscopy for the Study of Glucuronidase Activity within Serum Albumin

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Laser flash photolysis (LFP) has shown to be an efficient technique for in situ determination of the glucuronidase activity of human serum albumin (HSA). After incubation of the steroisomeric flurbiprofen glucuronides (FBPGluc) during regular time intervals at the selected temperatures, in the presence of protein, regression analysis was applied to the triplet decay at $\lambda = 360$ nm. This led to a satisfactory fitting when considering a set of four lifetimes; the corresponding preexponential coefficients A_I^{FBP}, A_{II}^{FBP}, $A_F^{FBPGluc}$, and $A_B^{FBPGluc}$ can be correlated with the presence of flurbiprofen (FBP) within the two known binding sites (I and II), together with FBPGluc free in solution (F) and bound (B) to the protein. The new methodology based on LFP of glucuronides in the presence of HSA is fast, experimentally straightforward, and does not involve any workup. This suggests the possibility of making use of the transient triplet−triplet absorption for investigating the enzymatic-like activity of different host biomolecules and at the same time determining the distribution of the generated drug between several compartments in the protein.

Introduction

Arylpropionic acids are an important class of pharmacologically active substances with antiinflammatory properties.[1−3] They possess a chiral center and contain a carboxylic group in their chemical structure.[4] Conjugation with D-glucuronic acid during the phase II metabolic pathway leads to the corresponding β-anomers of 1-O-acyl-D-glucuronides.[5−8] This crucial biological process, which is catalyzed by the uridine diphosphoglucuronosyltransferases (UGTs) family of membrane-bound enzymes,[9−12] operates as detoxication pathway by enhancing the hydrophilicity of the parent drug, thus allowing its final elimination into urine or bile.[13,14]

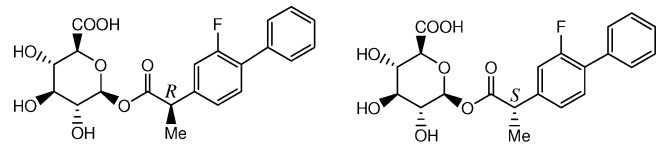
The 1-β-O-acyl glucuronide metabolites are intrinsically reactive molecules (both in vivo and in vitro) that can undergo a number of reactions; by far, hydrolysis back to the parent aglycon is the predominating process.[15−17] This reaction can be catalyzed by several agents (β-glucuronidases, esterases, etc.) resulting in a futile cycle, since plasma clearance is the result of competition between conjugation of the parent drug, deconjugation of the glucuronide, and renal clearance.[13,14]

Although arylpropionic acids are commonly used as racemates, different degrees in the enzymatic biotransformation or in the transport protein binding of the enantiomers may result in an enantioselective pharmacokinetic behavior.[3,17]

Some effort has been devoted to investigate the ability of serum albumins to act as catalysts for deconjugation of acyl glucuronides, mainly using liquid chromatography (LC, eventually coupled to NMR or MS).[7,18−21] However, chromatographic separation is time-consuming, and the required workup may lead to artifacts.

Flurbiprofen (FBP) is an arylpropionic acid widely used for the treatment of several processes, as rheumatoid arthritis, osteoarthritis, etc. It is readily absorbed after oral administration, completely bound to plasma proteins at therapeutic concentrations, and excreted essentially as the corresponding acyl glucuronide (FBPGluc) in urine.[22]

CHART 1: Structures of the 1β-O-acyl Glucuronides of the Two Flurbiprofen Enantiomers



(2*R*)-FBPGluc (2*S*)-FBPGluc

We have recently made use of the laser flash photolysis (LFP) technique for the study of (*S*)- and (*R*)-FBP binding to human and bovine serum albumin (HSA and BSA);[23−25] the triplet excited state (^{3}FBP*) was found to be a reporter for the microenvironments experienced within the protein. Fitting of the triplet decays was satisfactorily correlated with the distribution of the drug among the two albumin binding sites and its presence as the noncomplexed form in the bulk solution.

In the present work, the glucuronidase activity of HSA toward (2*R*)- and (2*S*)-FBPGluc (Chart 1) has been investigated by means of a new methodology making use of in situ transient absorption spectroscopy. It is based on LFP of both glucuronides in the presence of HSA, with ^{3}FBP* as reporter.

Experimental Section

Materials and Solvents. Human serum albumin, Lipase AS Amano (LAS), Porcine Liver Esterase (PLE) were purchased from Sigma; (*S*)-FBP, (*R*)-FBP, and 3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate were obtained from Aldrich. Phosphate buffered saline solution (PBS, pH = 7.4, 0.01 M) was prepared by dissolving Sigma tablets in the appropriate amount of deionized water. All the solvents were HPLC quality.

Experimental Procedure for the Synthesis of (2*S*)- and (2*R*)-FBPGluc. The synthesis of (2*S*)- and (2*R*)-FBPGluc was performed in three steps by using a previously reported

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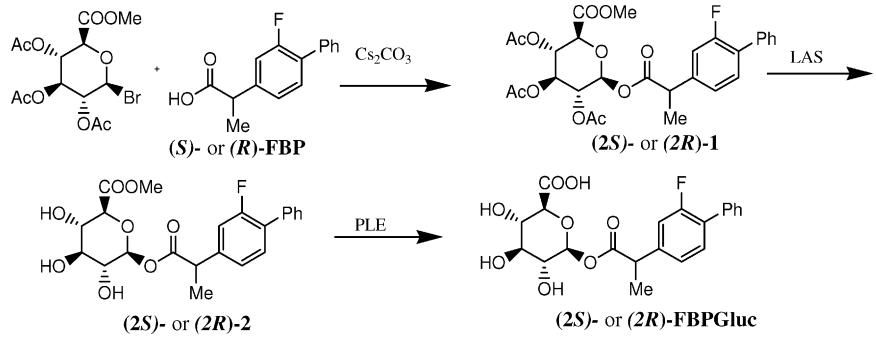

Figure 1. Synthesis of (2*S*)- and (2*R*)-FBPGluc. Abbreviations: LAS, Lipase AS Amano; PLE, Porcine Liver Esterase.

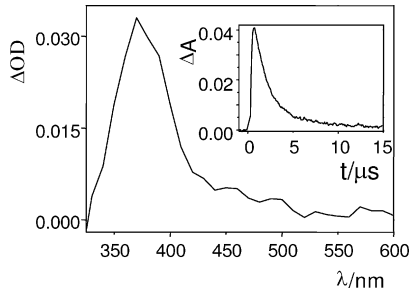

Figure 2. Laser flash photolysis (λ_{exc} = 266 nm, PBS, pH = 7.4, air) of (2*R*)-FBPGluc obtained 0.5 μs after the laser pulse. Inset: Decay monitored at 360 nm.

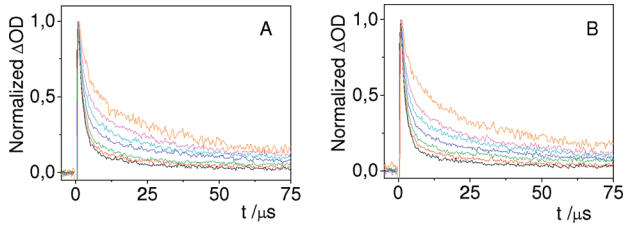

Figure 3. Laser flash photolysis (λ_{exc} = 266 nm) of FBPGluc/HSA at different molar ratios. Normalized decays monitored at 360 nm. (A) (2*R*)-FBPGluc. (B) (2*S*)-FBPGluc. Molar ratios 1:0.25 (black), 1:0.33 (red), 1:0.50 (green), 1:1.00 (dark blue), 1:1.43 (pale blue), 1:2.00 (pink), 1:3.33 (orange).

procedure for other arylpropionic acid derivatives[26,27] with some modifications (see Figure 1). Briefly, a solution of methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-α-D-glucopyranuronate (397 mg, 1 mmol) and the cesium salt of (*S*)- or (*R*)-FBP (244 mg, 1 mmol) in DMSO (8 mL) were stirred at 30 °C for 3 h. The reaction mixtures were diluted with EtOAc (100 mL) and then washed with water (3 × 50 mL), saturated aqueous $NaHCO_3$ (3 × 50 mL), and saturated aqueous NaCl (3 × 50 mL). The organic phase was dried over Na_2SO_4 and the solvent evaporated in vacuo to afford a viscous oil. Products (2*R*)-1 and (2*S*)-1 were purified by HPLC, using $CH_3CN/H_2O/CH_3COOH$ (v/v/v 70:30:1) as eluent. The yields were 40 and 41%, respectively. A solution of (2*R*)-1 or (2*S*)-1 (155 mg, 0.28 mmol) in 2-methoxyethanol (126 mL) was added to a solution of LAS (15 mg/mL of incubation mixture) in 30 mM phosphate buffer (pH 5.2) and incubated at 40 °C for 2 h. The reaction mixture was then extracted with EtOAc (3 × 50 mL) and dried over Na_2SO_4. The organic solvent was rotavaporated, and the crude product was purified by HPLC, using $CH_3CN/H_2O/CH_3COOH$ (v/v/v 70:30:1) as eluent to afford (2*R*)-2 and (2*S*)-2 (yields 20 and 16%, respectively). The methyl esters (2*R*)-2 and (2*S*)-2 (50 mg) were dissolved in DMSO (18 mL), and the solution was poured into a solution of PLE (0.4 mg/mL of incubation mixture) in 30 mM phosphate buffer (pH 5.2) and incubated at 40 °C for 3 h. The crude was extracted with EtOAc (3 × 50 mL), dried over Na_2SO_4, and the solvent evaporated in vacuo. After purification by HPLC, using $CH_3CN/H_2O/CH_3COOH$ (v/v/v 95:5:0.1) as eluent, (2*R*)-FBPGluc (16%) and (2*S*)-FBPGluc (30%) were obtained as final products.

TABLE 1: Initial Percentage of Free and HSA Bound (2S)- and (2R)-FBPGluc at Different Molar Ratios[a]

	free		protein-bound	
[FBPGluc]/[HSA][b]	(2*R*)-	(2*S*)-	(2*R*)-	(2*S*)-
1:3.33	66	63	34	37
1:2.00	73	73	27	27
1:1.43	76	78	24	22
1:1.00	81	82	19	18
1:0.50	89	88	11	12
1:0.33	91	91	9	9
1:0.25	93	93	7	7

[a] Relative errors were lower than 5% of the stated values. [b] Conditions: [FBPGluc] = 1.5 × 10^{-5} M, PBS 0.01 M, T = 22 °C.

Laser Flash Photolysis. Laser flash photolysis (LFP) experiments were performed by using a Q-switched Nd:YAG laser (Quantel Brilliant, 266 nm, 10 mJ per pulse, 5 ns fwhm) coupled to a mLFP-111 Luzchem miniaturized equipment. This transient absorption spectrometer includes a ceramic xenon light source, 125 mm monochromator, Tektronix 9-bit digitizer TDS-3000 series with 300 MHz bandwidth, compact photomultiplier and power supply, cell holder and fiber optic connectors, fiber optic sensor for laser-sensing pretrigger signal, computer interfaces and a software package developed in the LabVIEW environment from National Instruments. The LFP equipment supplies 5 V trigger pulses with programmable frequency and delay. The risetime of the detector/digitizer is ~3 ns up to 300 MHz (2.5 GHz sampling). The monitoring beam is provided by a ceramic xenon lamp and delivered through fiber optic cables. The laser pulse is probed by a fiber that synchronizes the LFP system with the digitizer operating in the pretrigger mode.

All transient spectra were recorded employing 10 × 10 mm^2 quartz cells with 4 mL capacity; the spot diameter in the cuvette was ca. 4 mm. The absorbance of FBPGluc was ca. 0.2 at the laser wavelength, corresponding to a concentration of 1.5 × 10^{-5} M; concentration of HSA was determined using the molar absorption coefficient at 280 nm of 35 700 M^{-1} cm^{-1}. All the experiments were carried out in PBS (pH = 7.4, 0.01 M) at 22 or 37 °C and under air atmosphere.

Experimental Procedure to Determine the Glucuronidase Activity of HSA. A battery of aqueous solutions containing (2*S*)- or (2*R*)-FBPGluc and HSA (molar ratio 1:3.33) were prepared in neutral buffer (0.01 M PBS) and maintained at 22 or 37 °C in a thermostatted water bath. As an example, 0.25 mL of HSA 2 × 10^{-3} M in PBS was added to 9.75 mL of a

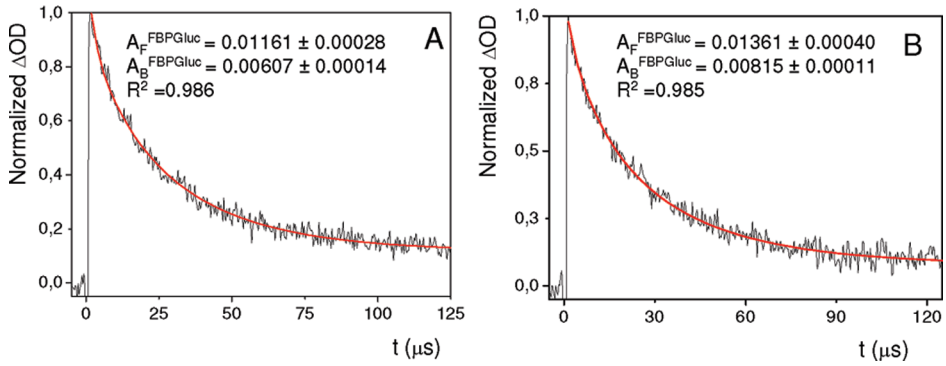

Figure 4. Laser flash photolysis (λ_{exc} = 266 nm) of FBPGluc/HSA at 1:3.33 molar ratio (black line) together with the corresponding fit (red line). (A) (2*R*)-FBPGluc. (B) (2*S*)-FBPGluc. The parameters indicated correspond to the red fit and eq 1.

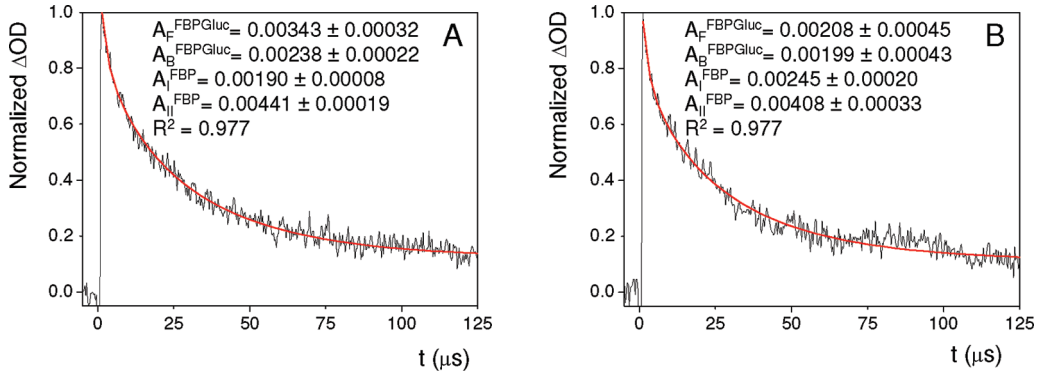

Figure 5. Laser flash photolysis (λ_{exc} = 266 nm) of FBPGluc/HSA at 1:3.33 molar ratio (black line) after 6.5 h at 37 °C, together with the corresponding fit (red line). (A) (2*R*)-FBPGluc. (B) (2*S*)-FBPGluc. The parameters indicated correspond to the red fit and eq 2.

1.54×10^{-5} M solution of (2*S*)- or (2*R*)-FBPGluc in PBS. After several reaction times at the selected temperatures (22 or 37 °C), aliquots were taken, placed in a quartz cuvette, and submitted to LFP (5 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.

Spectroscopic Data of the Synthetic Intermediates. The ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) spectra of (*R*)- and (*S*)-1 were recorded in $CDCl_3$ and those of (*R*)- and (*S*)-2 in CD_3OD; the chemical shifts are reported in ppm downfield from TMS.

(2S)-1. ^{1}H NMR: 1.56 (d, J = 7.3 Hz, 3H), 1.93 (s, 3H), 2.01 (s, 3H), 2.02 (s, 3H), 3.71 (s, 3H), 3.82 (q, J = 7.3 Hz, 1H), 4.17 (d, J = 9.4 Hz, 1H), 5.24 (m, 3H), 5.77 (d, J = 7.8 Hz, 1H), 7.02−7.15 (m, 2H), 7.35−7.46 (m, 4H), 7.48−7.54 (m, 2H). ^{13}C NMR: 18.0 (CH_3), 20.3 (CH_3), 20.4 (CH_3), 20.5 (CH_3), 44.8 (CH), 52.9 (CH_3), 68.9 (CH), 69.9 (CH), 71.8 (CH), 72.9 (CH), 91.7 (CH), 115.1−140.1 (aromatic C), 166.6 (C), 168.9 (C), 169.3 (C), 169.8 (C), 171.8 (C).

(2R)-1. ^{1}H NMR: 1.53 (d, J = 7.1 Hz, 3H), 1.61 (s, 3H), 1.98 (s, 3H), 2.03 (s, 3H), 3.76 (s, 3H), 3.80 (q, J = 7.1 Hz, 1H), 4.17 (d, J = 9.4 Hz, 1H), 5.08 (t, J = 8.5 Hz, 1H), 5.16−5.30 (m, 2H), 5.71 (d, J = 8.1 Hz, 1H), 7.02−7.15 (m, 2H), 7.35−7.54 (m, 6H). ^{13}C NMR: 17.3 (CH_3), 19.8 (CH_3), 20.3 (CH_3), 20.4 (CH_3), 44.4 (CH), 52.9 (CH_3), 68.9 (CH), 69.5 (CH), 71.6 (CH), 72.9 (CH), 91.4 (CH), 114.8−135.1 (aromatic C), 166.6 (C), 168.5 (C), 169.3 (C), 169.7 (C), 171.8 (C).

(2S)-2. ^{1}H NMR: 1.53 (d, J = 7.1 Hz, 3H), 3.37−3.58 (m, 3*H*), 3.74 (s, 3H), 3.87−3.99 (m, 2H), 5.53 (d, J = 7.8 Hz, 1H), 7.12−7.22 (m, 2H), 7.32−7.46 (m, 4H), 7.50−7.55 (m, 2H). ^{13}C NMR: 19.0 (CH_3), 46.0 (CH), 53.0 (CH_3), 72.9 (CH), 73.6 (CH), 77.3 (CH), 77.4 (CH), 96.0 (CH), 115.0−136.9 (aromatic C), 170.8 (C), 174.2 (C).

(2R)-2. ^{1}H NMR: 1.54 (d, J = 7.2 Hz, 3H), 3.35−3.50 (m, 3H), 3.77 (s, 3H), 3.89−3.99 (m, 2H), 5.52 (d, J = 7.8 Hz, 1H), 7.12−7.24 (m, 2H), 7.31−7.46 (m, 4H), 7.49−7.54 (m, 2H). ^{13}C NMR: 19.0 (CH_3), 46.0 (CH), 53.0 (CH_3), 72.9 (CH), 73.6 (CH), 77.3 (CH), 77.4 (CH), 96.0 (CH), 115.0−136.9 (aromatic C), 170.8 (C), 174.3 (C).

Results and Discussion

Synthesis of the glucuronides was achieved starting from the cesium salt of (*S*)- or (*R*)-FBP and methyl 2,3,4-tri-*O*-acetyl-

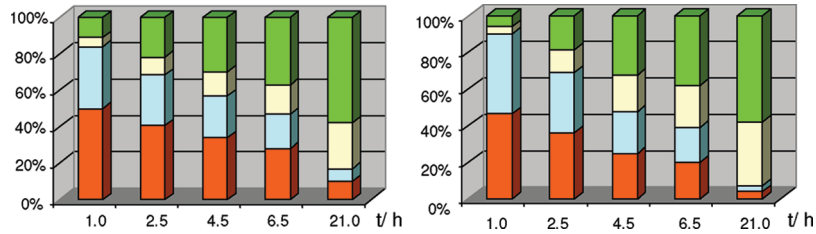

Figure 6. Percentages of free FBPGluc (orange), HSA-bound FBPGluc (blue), FBP in site I (yellow) and FPB in site II (green) for an initial mixture of FBPGluc/HSA 1:3.33 at different times at 37 °C. Left: (2*R*)-FBPGluc. Right: (2*S*)-FBPGluc.

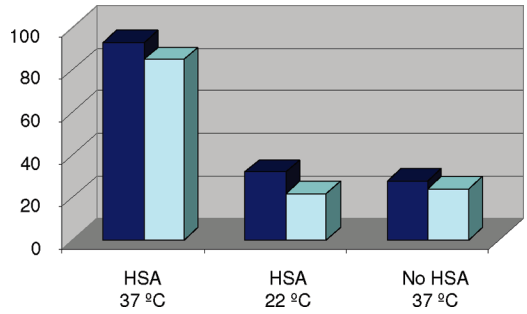

Figure 7. Percentages of FBP formed by hydrolysis of a FBPGluc/HSA 1:3.33 mixture after reaction during 21 h at 37 °C and at 22 °C, as well as for FBPGluc alone after 21 h at 37 °C. (2*S*)-FBPGluc (dark blue) and (2*R*)-FBPGluc (pale blue).

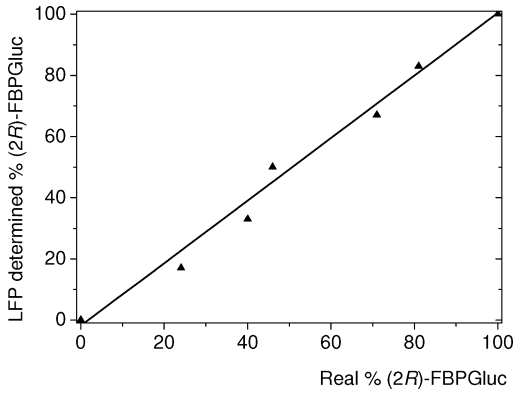

Figure 8. LFP-determined against known real percentages of (2*R*)-FBPGluc for several mixtures in the presence of HSA, together with the linear fit of the experimental points.

1-bromo-1-deoxy-α-D-glucopyranuronate, as described in the literature for analogous drugs.[26,27] The procedure is depicted in Figure 1.

Upon LFP of (2*S*)- or (2*R*)-FBPGluc (1.5×10^{-5} M, PBS 0.01 M, pH = 7.4, λ_{exc} = 266 nm, air) a transient spectrum with maximum at 360 nm was observed, corresponding to the T-T absorption of the FBP chromophore[28] (see Figure 2).

Under these conditions, the lifetime (τ_T) was found to be 1.8 μs for both stereoisomers (inset of Figure 2). In the presence of different amounts of HSA, LFP of (2*R*)-FBPGluc also gave rise to the characteristic ^{3}FBP* band. Unlike the behavior in the absence of protein, the decays at 360 nm (Figure 3A) did not follow a simple monoexponential law and took place at longer time scales.

To analyze the decays at λ_{max} = 360 nm, we proceeded as follows: for the case of (2*R*)-FBPGluc in HSA (molar ratio 1:3.33) at zero time, only two transient species are present and then the fitting law (eq 1) contains two monoexponential terms

$$\Delta OD = \Delta OD_0 + A_F^{FBPGluc} e^{(-t/\tau_F^{FBPGluc})} + A_B^{FBPGluc} e^{(-t/\tau_B^{FBPGluc})} \quad (1)$$

where $\tau_F^{FBPGluc}$ (1.8 μs) and $\tau_B^{FBPGluc}$ (25.0 μs) correspond to (2*R*)-FBPGluc free in solution and bound to the protein, respectively. From the corresponding pre-exponential factors $A_F^{FBPGluc}$ and $A_B^{FBPGluc}$ obtained after the fit, the percentage of (2*R*)-FBPGluc in each environment was obtained (see Table 1 entry 1). Parallel experiments were conducted on (2*S*)-FBPGluc, and a similar trend was observed (Figure 3B). The two triplet lifetimes found in the presence of serum albumin were identical to those found for (2*R*)-FBPGluc within the error margins. The distribution between free and bound glucuronide, obtained again from $A_F^{FBPGluc}$ and $A_B^{FBPGluc}$, was very close to the values determined for the (2*R*)-isomer (Table 1).

Then, the triplet lifetimes were fixed at 1.8 and 25.0 μs within the entire concentration range to apply eq 1; nonetheless, very similar results (τ_T, *A*) were obtained without fixing the τ_T values. An important contribution of the short-lived component was always found, indicating that free glucuronide was present in the bulk solution; however the longer lived component, attributed to the transient species inside the protein cavities, was detectable even at the highest FBPGluc/HSA values. Again, regression analysis of the decay curves (Figure 3A,B) allowed us to obtain the values of the $A_F^{FBPGluc}$ and $A_B^{FBPGluc}$ coefficients and hence the percentages of the two (free and bound) species with different lifetimes (Table 1). The fitting curves and the parameters derived therefrom are shown for two representative cases in Figure 4.

Among the above results, several observations deserve special comments for their relevant biological implications. First, even at low FBPGluc/HSA molar ratios, a high amount of glucuronide remains unbound to the protein. This is in good agreement with the enhanced hydrosolubility of the glucuronide in respect to the parent drug, which facilitates its elimination; moreover, no stereodifferentiation took place in the protein-binding process, since no significant differences were observed between the binding degree of (2*S*)- and (2*R*)-FBPGluc determined from $A_F^{FBPGluc}$ and $A_B^{FBPGluc}$. In addition, it is worth mentioning that only a type of microenvironment is provided by the protein for bound (2*S*)- and (2*R*)-FBPGluc, unlike the case of the parent drug FBP, where two different triplet lifetimes were found and assigned to different binding sites.[23] Displacement experiments were carried out with a specific site II probe (ibuprofen).[29] In the presence of this probe, at 1:3:3 [FBPGluc]/[HSA]/[ibuprofen] molar ratio, the contribution of the τ_T = 25 μs component dropped by ca. 20%.

After establishing that the methodology is suitable to discriminate between the drug and its metabolite, LFP was applied to investigate in situ the glucuronidase activity of albumin at physiological temperature (37 °C). Since the amount of complexed glucuronide was higher in the presence of excess HSA, the 1:3.33 ratio was chosen to monitor the reaction. Aliquots of the solution were taken at different times and submitted to LFP. Again, a signal centered at 360 nm was observed, but now four fixed τ_T values were used for fitting the decay. In the case of (2*R*)- or (2*S*)-FBPGluc, the two values reported above at zero time (1.8 and 25 μs), plus the two ones previously ascribed to (*R*)-FBP (10.2 and 39.0 μs) or (*S*)-FBP (11.2 and 35.9 μs) within the protein binding sites were used.[23] The law containing four monoexponential terms needed to fit the decay of the signal at λ = 360 nm, takes then the form of eq 2

$$\Delta OD = \Delta OD_0 + A_I^{FBP} e^{(-t/\tau_I^{FBP})} + A_{II}^{FBP} e^{(-t/\tau_{II}^{FBP})} + A_F^{FBPGluc} e^{(-t/\tau_F^{FBPGluc})} + A_B^{FBPGluc} e^{(-t/\tau_B^{FBPGluc})} \quad (2)$$

where τ_I^{FBP} and τ_{II}^{FBP} are the triplet lifetimes of FBP (formed by hydrolysis of FBPGluc within the two protein binding sites), while $\tau_F^{FBPGluc}$ and $\tau_B^{FBPGluc}$ correspond again to FBPGluc free in solution and bound to the protein. Accordingly, the preexponential factors A_I^{FBP} and A_{II}^{FBP} are due to FBP in site I and site II of HSA, whereas $A_F^{FBPGluc}$ and $A_B^{FBPGluc}$ correspond to free and bound glucuronide. The four characteristic triplet lifetimes were exploited to determine the percentage of compounds in each sample after different reaction times. No (*R*)-FBP or (*S*)-FBP was expected to be present free in solution under these conditions, since inclusion within the protein is

known to be complete even at higher molar ratios. Assuming that A_I^{FBP}/A_{II}^{FBP} remains constant (and equal to 30/70 for (*R*)-FBP or 40/60 for (*S*)-FBP),[23] the preexponential factors and consequently the percentages of FBP and FBPGluc can be obtained. As an example, the decay traces and the preexponential factors for FBPGluc/HSA mixtures incubated 6.5 h are shown in Figure 5.

The procedure was repeated for aliquots taken at given reaction times. The course of the reaction, expressed as the percentages of the various species at different times, is represented in Figure 6.

Similar studies were conducted at 22 °C, and a much slower process was observed to take place. The results obtained after 21 h under these conditions are shown in Figure 7. As a control experiment, hydrolysis of the glucuronides was also attempted in the absence of HSA at 37 °C. Aliquots were taken at different times, HSA was added, and the mixture was allowed to equilibrate at 4 °C. Now the role of the protein was to act as selector, providing discrimination between the FBP and FBP-Gluc triplet lifetimes in order to follow the progress of the reaction. The results clearly showed that in the absence of HSA hydrolysis occurs to a much lower extent (Figure 7).

The use of four lifetimes to follow the glucuronidase activity of HSA was based on the results of independent experiments performed with FBP/HSA and FBPGluc/HSA systems. However, it appeared interesting to check whether a "blind" fitting of the decays to just two lifetime components could also be useful for monitoring the deglucuronidation process. Here, advantage could be taken from the fact that the short-lived (1.8 μs) component can be safely and exclusively assigned to free glucuronide in the bulk solution. This lifetime is different enough from those of all bound species (10.2−39.0 μs) and could provide an easy way to make rough estimations. Thus, the experimental data were also treated using a fitting equation with only two monoexponential terms (see Supporting Information). In this case, the two lifetimes values were fixed at 1.8 μs (for free FBPGluc) and 25 μs (as an average value for all bound species, namely FBPGluc and FBP in sites I and II). Interestingly, the results were similar to those obtained with the four monoexponential fitting, although the errors associated with the half-life of the reaction kinetics were much larger (see pages S4 and S5 in Supporting Information).

To check the reliability of the percentages obtained from the decay fitting, solutions containing (*R*)-FBP and (2*R*)-FBPGluc (at known ratios) in the presence of HSA were prepared and submitted to LFP. Indeed, a satisfactory correlation was found between the LFP-determined and the real values (Figure 8).

Conclusions

In conclusion, in situ transient absorption spectroscopy is a novel and convenient method to investigate the glucuronidase activity of serum albumins. It is based on LFP detection of triplet excited states, whose lifetimes are highly sensitive to the microenvironment. This is a fast and simple tool, very useful for the purpose. In addition, it avoids a complicated workup and time-consuming chromatographic separation.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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