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Seeking to Shed Some Light on the Binding of Fluoroquinolones to Albumins

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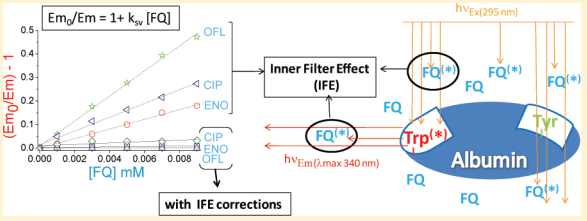
ABSTRACT: Interactions between serum albumins (HSA, human, and BSA, bovine) and fluoroquinolones (FQs), such as enoxacin, norfloxacin, ciprofloxacin, and ofloxacin, have been studied using the laser flash photolysis technique. Lifetimes and quantum yields of FQs triplet excited states (^{3}FQs) are not affected by the presence of albumins, however, the quenching of ^{3}FQs by tryptophan and tyrosine and the subsequent generation of FQs radical anions and tyrosyl or tryptophanyl radicals were detected. These results, besides agreeing with association constants (K_a) for FQs binding to albumins lower than 6×10^2 M^{-1}, are highly relevant to understanding the process of photohapten formation, the first event in the onset of photoallergy. The emission of tryptophan within albumin is not affected by the presence of FQs when the inner filter effects (IFE) of these drugs are taken into account, which explains the discrepancies reported in the literature about K_a of FQs with albumins.



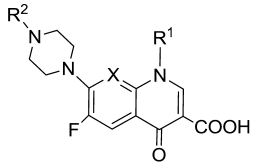
INTRODUCTION

Serum albumins are the proteins most extensively studied because of their abundance, low cost, and stability.[1,2] They are attractive entities with wide application possibilities in photochemistry, from the investigation of fundamental mechanisms[3] to the development of tools for molecular biology[4] or photocatalytic antibodies.[5] Moreover, the photosensitization of biomolecules has recently attracted considerable attention, because a variety of drugs absorbing UV light are able to induce important photobiological damage.[6,7] Besides, the albumins are extremely important from the biopharmacological point of view because they are the major transporter of nonesterified fatty acids, as well as of different drugs and metabolites to the tissues.[2,8] Thus, one of the most relevant properties of these biomolecules is its capability to allow the solubilization of hydrophobic compounds, which produces a homogeneous distribution of them in the body.

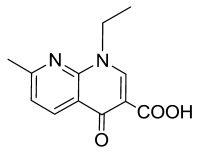
Human serum albumin (HSA) is able to bind an enormous variety of compounds, and display two principal binding sites for aromatic and heterocyclic molecules, the so-called sites I and II, which are located in subdomains IIA and IIIA respectively.[1,8,9] In this context, it is generally assumed to be the same for bovine serum albumin (BSA).[10] The drug−protein binding constant is a physicochemical parameter that help us to understand the absorption, transport, and the target molecules of the drugs at the cellular level.[9] The employed techniques to determine this value are usually based on the separation of the free and the protein-bound fraction of the ligand, the detection of a change in a physicochemical property of the complexed drug, or in a physicochemical behavior of the binding protein.[11−17] During the past few years, continuous progress in analytical methodology has been achieved with a substantial increase in the number of new and more specific methods for ligand−protein assays. Therefore, this type of study has been performed using conventional techniques (dialysis or ultrafiltration), separation methods (chromatography, capillary electrophoresis or microdialysis), and spectroscopic measurements (absorption or fluorescence as well as the laser flash photolysis technique, which enables the calculation of binding parameters, i.e., the number of binding sites and affinity constants).[11−17]

On the other hand, fluoroquinoles (FQs), dyad compounds with a quinolinic main ring and an aminoalkyl substituent (Chart 1), are widely used as antibacterial agents that develop

Chart 1. Structure of Fluoroquinolones (FQs) and Nalidixic Acid (NAL)



FQs	R^1	X	R^2
ENO	CH_2CH_3,	N	H
NOR	CH_2CH_3,	CH	H
CIP	$CH(CH_2CH_2)$,	CH	H
OFL	$CH(CH_3)CH_2OC$		CH_3



NAL

their pharmacological activity through the inhibition of an enzyme involved in the replication and repair of bacterial DNA.[18] However, this family of drugs exhibits important phototoxic side effects. In general, FQs can generate dangerous photoinduced cutaneous reactions, and some of them even

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operate as photomutagenic and photocarcinogenic agents.[19,20] In order to determine the involved mechanisms in these photosensitizing properties, a large number of studies about the photophysical and photochemical properties of FQs have been done during the past few years. Some unexpected features such as photocleavage of the strong C−F bond with alkylating properties or formation of secondary triplet excited species in the presence of phosphate buffer aqueous solutions (PB) have been revealed.[19,21,22] However, despite all efforts made, all behaviors have not been clearly established and/or understood yet. In this context, conflicting reports about the FQs binding to human and bovine serum albumins (HSA and BSA) have been accumulating in the literature.[12,13,23−37]

Studies using methods such as chromatography, capillary electrophoresis, or continuous and discontiuous ultrafiltration are in agreement with low affinities between FQs and human or bovine serum albumin (showing association constants (K_a) from 1 to 5×10^2 M^{-1}).[12,13,23−28] By contrast, values between 5 and 9×10^4 M^{-1} have been described using fluorescence spectroscopy.[29−37] With this background, it was decided to evaluate this discrepancy using the laser flash photolysis (LFP) technique because the detection of transients as experienced reporters within the binding sites of albumins has proven to be a good methodology to obtain information about drug−protein bindings.[15−17,38,39] For this purpose enoxacin (ENO), ciprofloxacin (CIP), norfloxacin (NOR) and ofloxacin (OFL) were selected as the FQs under study (see structures in Chart 1) and nalidixic acid (NAL), a close related compound, was chosen as a reference model compound. The results will show negligible binding levels between FQs triplet excited states and albumins and they will be further support for K_a values below 6×10^2 M^{-1}. Moreover, the analysis of new fluorescence measurements will show a nonsignificant quenching of albumin emission by the presence of FQs when inner filter effect (IFE) corrections are applied.

EXPERIMENTAL SECTION

Materials. Ciprofloxacin (CIP), enoxacin (ENO), human and bovine serum albumin (HSA and BSA essentially free from fatty acid), nalidixic acid (NAL), norfloxacin (NOR), ofloxacin (OFL), tryptophan (Trp), and tyrosine (Tyr) were obtained from Sigma-Aldrich Chemical Co. (St Louis, MO). Phosphate buffer was prepared from reagent-grade products using deionized water. The pH of the solutions was measured through a glass electrode and adjusted with NaOH to pH 7.4.

Laser Flash Photolysis Measurements. A pulsed Nd:YAG SL404G-10 Spectron Laser Systems was used at the excitation wavelength of 355 nm. The single pulses were ∼10 ns in duration and the energy was lower than 10 mJ/pulse. The detecting light source was a pulsed Lo255 Oriel xenon lamp. The laser flash photolysis system consisted of a pulsed laser, a Xe lamp, a 77200 Oriel monochromator, an Oriel photomultiplier tube (PMT) system made up of a 77348 side-on PMT tube, 70680 PMT housing, and a 70705 PMT power supply. The oscilloscope was a TDS-640A Tektronix. The output signal from the oscilloscope was transferred to a personal computer.

Unless otherwise stated, all samples of 10^{-4} M NAL and FQs used for laser flash photolysis were aqueous solutions at pH 7.4 in 4×10^{-3} M PB. Each sample was deaerated by bubbling N_2O to remove residual solvated electron that is mainly generated by a biphotonic pathway in FQ laser 355 nm excitations.[19]

Study of the Interactions between Triplet Excited States of FQs and Albumins. The influence of HSA and BSA on the triplet excite state of NAL and FQs was evaluated performing experiments with 10^{-4} M solutions of each FQ (ENO, NOR, CIP, and OFL) and NAL with and without the presence of 10^{-4} M HSA or BSA under N_2O medium. The samples containing the albumins needed special manipulation because it was not possible to bubble the solutions to remove air. Thus, the N_2O was introduced inside of the sample quartz cells by the flow of gas during 20 min without generating bubbles and by stirring the solution. Transient absorption spectra at different times after the laser pulse were obtained for each sample with and without the presence of the albumins to detect intersystem crossing quantum yield (Φ_{ISC}) changes or the generation of new intermediates. The trace obtained at the absorption maximum of each FQ triplet excited state was also analyzed to determine its lifetime. The Φ_{ISC} of the FQs and NAL in 4×10^{-3} M PB aqueous solutions were taken from bibliography[40−42] but those containing the albumins were determined by the comparative method using the Φ_{ISC} of each FQ in 4×10^{-3} M PB as standard. Thus each Φ_{ISC} was obtained by the application of eq 1

$$\phi_{ISC}(\mathrm{FQ} + \mathrm{H/BSA}) = \phi_{ISC}(\mathrm{FQ})\Delta A(^3\mathrm{FQ} + \mathrm{H/BSA}(\lambda_{max}\mathrm{nm}))/\Delta A(^3\mathrm{FQ}(\lambda_{max}\mathrm{nm}) \quad (1)$$

where $\Delta A(^3$FQ+H/BSA (λ_{max} nm)) and $\Delta A(^3$FQ (λ_{max} nm)) refer to the transient absorbance of each FQ triplet at its λ_{max} nm in the absence (^{3}FQ) or presence of albumins (^{3}FQ+ H/BSA) under anaerobic (N_2O atmosphere) medium.

Study of FQs Triplet Excited State Reactivity with Amino Acids (AA). Determination of the quenching rate constants of FQs triplet excited states (k_q) by amino acids such as tyrosine (Tyr) and tryptophan (Trp) was carried out with each FQ (ENO, NOR, CIP, and OFX) increasing the amount of the amino acids in the solutions (AA, Tyr and Trp, 0.1 to 10×10^{-3} M) checking that no changes in the pH were introduced. To determine FQ quenching rate constants (k_q), the Stern−Volmer equation was applied

$$1/\tau = 1/\tau_0 + k_q[\mathrm{AA}] \quad (2)$$

where τ_0 is the lifetime of the transient species without the quencher (AA).

Determination of Tyrosyl and Tryptophanyl Radical Formation Quantum Yields ($\phi_{AAradical}$). All 4 mM PB aqueous solutions at pH 7.4 of NAL, ENX, NFX, CPX and OFX were set at the absorbance of 0.26 at 355 nm. Thus, tyrosyl and tryptophanyl radical formation quantum yields ($\phi_{AAradical}$) were estimated by application of eq 3 by the comparative method using benzophenone (BPH) in acetonitrile as standard.[16]

$$\phi_{AAradical}(\mathrm{AA}) = \phi_{ISC}(\mathrm{BPH})\,\Delta A_{Total}(\mathrm{AA_{radical}}(\lambda_{max}\mathrm{nm}))\,\varepsilon(^3\mathrm{BPH}(520\mathrm{nm}))/\Delta A(^3\mathrm{BPH}(520\mathrm{nm}))\,\varepsilon(\mathrm{AA_{radical}}(\lambda_{max}\mathrm{nm})) \quad (3)$$

where ΔA_{Total} refers to the absorbance of $AA_{radical}$ at λ_{max} (410 and 520 nm for tyrosyl and tryptophanyl radical, respectively) obtained from this relation; $\Delta A_{Total}(AA_{radical}$ (λ_{max} nm)) = $\Delta A_{maximum\ observed}(AA_{radical}$ (λ_{max} nm))$(k_2/(k_2 - k_1))$,

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$\Delta A_{\text{maximum observed}}$ is the observed maximum absorbance for each AA_{radical} obtained from the reaction of each ^{3}FQ with Tyr and Trp (5×10^{-3} M), k_1 and k_2 are the decay rate constants of ^{3}FQ in the absence and presence of AA (5×10^{-3} M) respectively.

$\Delta A(^3\text{BPH}\ (520\ \text{nm}))$ refers to the absorbance of ^{3}BPH at 520 nm. The BPH triplet molar absorption coefficient and its Φ_{ISC} in acetonitrile were taken to be $\varepsilon(^3\text{BPH}\ (520\ \text{nm})) = 6500\ \text{M}^{-1}\ \text{cm}^{-1}$ and $\phi_{ISC}(\text{BPH}) = 1$ respectively. The Tyr and Trp radical molar absorption coefficients were taken to be ε(Tyr radical (410 nm)) = 2600 $\text{M}^{-1}\ \text{cm}^{-1}$ and ε(Trp radical (520 nm)) = 1960 $\text{M}^{-1}\ \text{cm}^{-1}$.[43]

Emission Measurements. Fluorescence emission spectra were recorded on a Photon Technology International (PTI) LPS-220B fluorimeter. Fluorescence lifetimes were measured with a lifetime spectrometer (TimeMaster fluorescence lifetime spectrometer TM-2/2003) from PTI by means of the stroboscopic technique, which is a variation of the boxcar technique. A hydrogen/nitrogen flash-lamp (1.8 ns pulse width) was used as the excitation source. The measurements were done under aerated conditions at room temperature (25 °C) in cuvettes of 1 cm path length. The emission band centered at 344 nm (Trp fluorescence maximum) was studied using 10^{-5} M HSA and BSA buffered aqueous solutions (4×10^{-3} M PB at pH ~7.4) at 295 nm excitation wavelength. FQs and NAL were the quenchers used (from 10^{-6} to 1.2×10^{-5} M). Before analyzing the data, the so-called inner filter effect (IFE) was applied because FQs and NAL show absorptions at the excitation and emission wavelengths. The IFE corrections were applied using eq 4[44−46]

$$F_{\text{corr}} = F_{\text{obs}} \times 10^{(\text{Aex}+\text{Aem})/2} \quad (4)$$

where F_{corr} and F_{obs} are the corrected and observed fluorescence respectively, and the absorbance values at the excitation and emission wavelength are A_{ex} and A_{em} respectively.

Similar HSA aerated solutions (10^{-5} M) with and without the presence of NAL or FQs (10^{-5} M) were used to determine the fluorescence lifetimes of Trp within HSA. These emission lifetimes were determined from the emission traces obtained at 330 nm using an excitation wavelength of 295 nm. The kinetic traces were fitted by a monoexponential decay function using a reconvolution procedure to separate them from the lamp pulse profile.

RESULTS AND DISCUSSION

1. Study of Interactions between FQs Triplet Excited State and Proteins. The interactions of drugs with albumins usually occur in the major binding regions of this type of protein, namely Sudlow's sites I and II. In this context, several laser flash photolysis (LFP) studies have shown that, when a complexation process between drugs and albumins is produced in these sites, changes in the triplet excited state lifetimes of the drugs or generation of new intermediates can be detected.[15−17,38,39,42] With this background, it was decided to perform the LFP study of FQs with a reference compound such as nalidixic acid (NAL, Chart 1) because it is structurally closely related to FQs and important spectroscopic changes have been observed by the LFP technique when NAL binding to albumins occurs.[42]

LFP experiments were carried out with FQs and NAL (10^{-4} M) in phosphate buffered solutions (4×10^{-4} M PB, at pH ~7.4) under N_2O atmosphere, with and without HSA or BSA (10^{-4} M) using a 355 nm Nd:YAG laser.

Laser excitation of NAL and FQs buffered aqueous solutions showed transient absorption spectra corresponding to their triplet excited states (see absorption maxima in Table 1).[41,42]

Table 1. Properties of FQs and NAL Intermediates Generated in the Presence and in the Absence of Biomolecules

parameters[a]	NAL	ENO	CIP	NOR	OFL
λ_{max} (^{3}X)[b], nm	620	520	610	610	620
τ (^{3}X)[c], μs	8.53	0.26	3.41	3.31	2.45
τ (^{3}X+H/BSA)[c], μs	(0.06/0.05)[d]	0.26	3.41	3.28	2.42
ϕ_{ISC} (X)[e]	≤0.60	0.54	0.52	0.52	0.32
ϕ_{ISC} (X+H/BSA)[e]		0.52	0.50	0.51	0.31
$k_q/10^9$ (Trp)[f], $\text{M}^{-1}\ \text{s}^{-1}$	2.5[g]	2.0	0.9	1.0	0.07
$\phi_{\text{AAradical}}$ ($\text{Trp}^\bullet$)	0.6	0.5	0.5	0.5	0.3
$k_q/10^9$ (Tyr)[f], $\text{M}^{-1}\ \text{s}^{-1}$	1.3[g]	0.8	0.06	0.07	0.02
$\phi_{\text{AAradical}}$ ($\text{Tyr}^\bullet$)	0.6	0.5	0.5	0.5	
λ_{max} ($X^{\bullet-}$)[h], nm	640	670	620	630	550[i]

[a]FQs or NAL (10^{-4} M) in 4×10^{-4} M PB aqueous solutions at pH ca. 7.4 under N_2O atmosphere using a 355 nm laser excitation. [b]Absorption maximum of triplet excited states.[41,42] [c]Triplet excited state lifetimes with and without 10^{-4} M concentrations of serum albumin (H/BSA). [d]Lifetime of NAL radical anion.[42] [e]Intersystem crossing quantum yield (ϕ_{ISC}) of compounds in the absence[40,41] or the presence of 10^{-4} M albumins. [f]Quenching rate constants from Stern−Volmer analysis using Trp and Tyr ($0.1-10 \times 10^{-3}$ M) as quenchers. [g]Values in accordance with the literature.[51] [h]Radical anions absorption maxima. [i]Value in accordance with the literature.[40]

However, while the intersystem crossing quantum yield and lifetime of ^{3}FQs are not affected by adding HSA or BSA, important changes can be observed in the NAL decay traces by the presence of albumins (see Table 1 and Figure 1). In fact, two new intermediates were detected for NAL. One of them shows an absorption maximum at λ_{max} = 640 nm while the other displays a maximum at λ_{max} = 410 nm. These intermediates, according to the literature, can be assigned to nalidixic radical anion ($NAL^{\cdot-}$) and tyrosine radical.[42] A fast electron transfer reaction between NAL excited state and a tyrosine (Tyr) of subdomain IIIA (site II of HSA and BSA) explains the photoreactivity of NAL with albumins. However, this process does not occur in the FQs under study. Three hypotheses could justify the FQs results: (a) FQs binding to proteins are produced in the subdomain IIIA as NAL and/or in subdomain IIA (site I of HSA and BSA) where there is a tryptophan (Trp), but the electron transfer mechanism between ^{1}FQs or ^{3}FQs and Tyr or/and Trp is not thermodynamically allowed (the reduction of FQs excited states by Tyr or Trp would be endothermic processes), (b) FQs binding to proteins takes place but ^{3}FQs are quickly expelled from the cavities of the proteins, or (c) FQs are not into the HSA and BSA cavities.

Interestingly, taking into account the albumin-drug equilibrium 5, the eq 6 and the LFP initial concentrations of drugs and albumins (10^{-4} M), the percentage of complexed drug should be more than 65% if drug-albumin association constants (K_a) were higher than $5 \times 10^4\ \text{M}^{-1}$, which is the minimum K_a value determined for these FQs by fluorescence spectroscopy.[29−37] However, the complexation should be less than 10%

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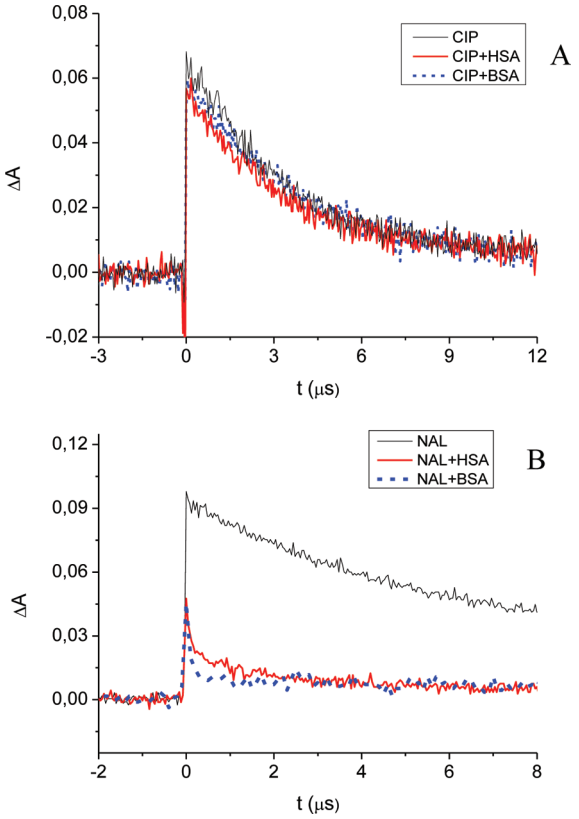

Figure 1. Decay traces obtained at 610 nm upon 355 nm laser excitation of 10^{-4} M CIP or NAL N_2O-purged buffered aqueous solutions (A and B, respectively) with and without the presence of 10^{-4} M HSA or BSA.

for K_a values lower than 6×10^2 M^{-1} (maximum K_a obtained by the other methods).[12,13,23−28]

$$[\mathrm{FQ}] + [\mathrm{Albumin}] \underset{K_d}{\overset{K_a}{\rightleftharpoons}} [\mathrm{FQ-Albumin}] \quad (5)$$

$$K_a = [\mathrm{FQ-Albumin}]/([\mathrm{Albumin}]^*[\mathrm{FQ}]) \quad (6)$$

To analyze the first hypothesis, the photoreactivity of the drugs with the most reactive amino acid residues (Trp and Tyr) of the two major and structurally selective binding sites of HSA and BSA (site I and II) was studied. It is important to note that site I is dominated by the presence of hydrophobic residues, including Trp212 in BSA and Trp214 in HSA, and the most probable candidate for a complex formation at site II is Tyr411 in HSA and Tyr409 in BSA.[47] Therefore, LFP experiments with NAL and FQs aqueous solutions in the presence of increasing amounts of Tyr and Trp were conducted. The quenching rate constants of ^{3}NAL and ^{3}FQs by the amino acids (k_q) were obtained by plotting ^{3}FQ decay rate constants against amino acid concentrations (see Figure 2 and Table 1). It is remarkable that changes in the main skeleton of this type of compound (Chart 1) modify the k_q of ^{3}FQ with Tyr and Trp.

The photoreactivity of ENO and NOR with the amino acids can be correlated very well with their reduction potentials (E_{red}(ENO) ≈ −1.31 V[48] and E_{red}(NOR) ≈ −1.39 V[48] or −1.54 V[49] vs Ag/AgCl). Moreover, CIP behaves similarly to NOR because their reduction potentials should be almost identical (see structures in Chart 1). In this context, the differences obtained between NOR and OFL, which show a

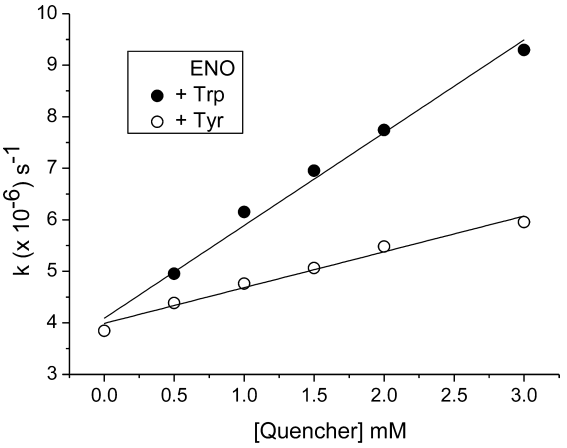

Figure 2. Stern−Volmer plots of ^{3}ENO quenching by tryptophan (Trp) and tyrosine (Tyr), monitored at 500 nm.

similar reduction potential,[48] would be due to the important difference of energy between their excited states (NOR (2.79 eV) and OFL (2.72 eV)).[41] Neglecting Coulombic terms, ΔG(electron transfer) is an exothermic process for all FQs because $E(\mathrm{Tyr}^{\cdot+}/\mathrm{Tyr})$ = 0.930 V and $E(\mathrm{Trp}^{\cdot+}/\mathrm{Trp})$ = 1.01 V at pH 7.[50] However, as described above, the k_q values determined for OFL were lower than 10^8 M^{-1} s^{-1}, which is clearly indicative that its electron transfer reactions with amino acids are endothermic processes. This contradiction can be rationalized in terms of the nature of FQs excited states because a rehybridization accompanied by intramolecular charge transfer (RICT) process occurs when a FQ is excited (Scheme 1).[49] Thus, this geometrical configuration change must be causing a decrease of the capability of FQs to accept an electron from their excited states.

In parallel to the quenching experiments, the analysis of the absorption spectra obtained from aqueous solutions of NAL and FQs in the presence of Trp and Tyr showed the generation of new transient species (see Figure 3).

In the case of NAL, it has been well established that NAL triplet quenching by Tyr and Trp corresponds to an electron transfer process between ^{3}NAL and the amino acids. The new intermediates observed by laser flash photolysis can be assigned to a NAL radical anion ($NAL^{\cdot-}$ with a maximum located at 640 nm) and a tryptophanyl or a tyrosyl radicals (with maxima at 410 and 520 nm, respectively).[43,51] In this context, laser flash photolysis experiments performed with anaerobic solutions of FQs and 3×10^{-3} M Tyr or Trp showed a transient absorption spectrum 3 μs after laser pulse that can be attributed to the result of the overlap between the absorption spectrum of each fluoroquinolone radical anion ($FQ^{\cdot-}$) and the radicals of Trp and Tyr (see Figure 3). A clear evidence of this categorization was found when, performing the same experiments under aerobic conditions, the transient absorption spectrum detected 3 μs after the laser pulse only corresponded to tryptophanyl or tyrosyl radicals (see Figure 4). The well-known low reactivity of molecular oxygen with phenoxy and indolyl radicals (Tyr and Trp radicals respectively) compared to quinolone radical anions (quenched at near diffusion controlled rates) explains the obtained results. Therefore, they confirm that the electron transfer reactions between ^{3}FQs and the amino acids under study are efficiently occurring. In fact, the Tyr and Trp radical formation quantum yields ($\phi_{AAradical}$) resulted to be similar to the corresponding intersystem crossing quantum yield (ϕ_{ISC}) of

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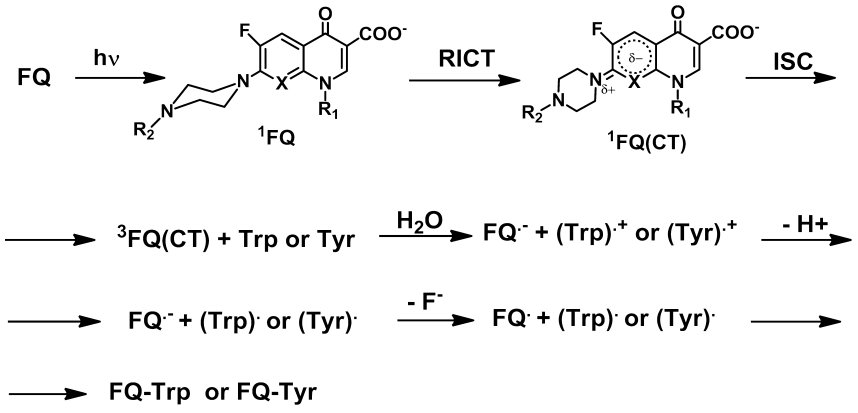
Scheme 1. Photobinding Mechanism between FQs and Tyr or Trp



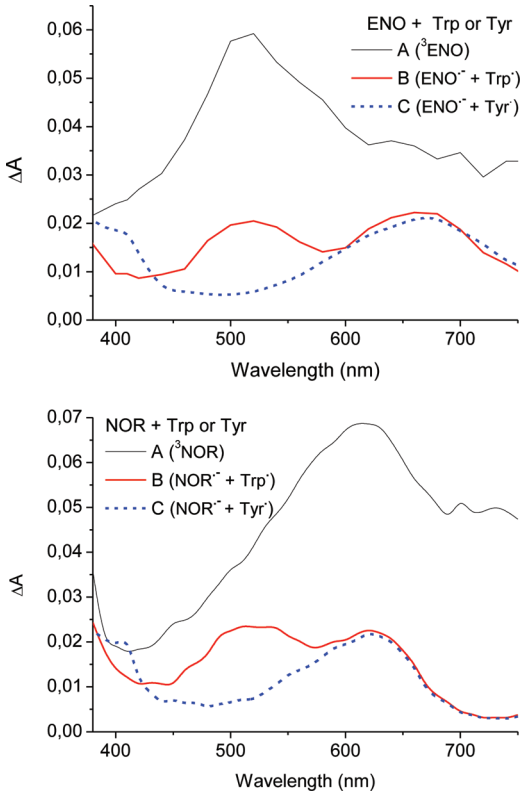


Figure 3. Transient absorption spectra obtained from N_2O-purged buffered aqueous solutions of ENO and NOR (10^{-4} M) containing 5 × 10^{-3} M Tyr or Trp, at different times after a 355 nm laser pulse. Spectra A and B were obtained from FQ solutions with Trp at 0.02 and 3 μs, respectively, and C was obtained from FQ solutions with Tyr at 3 μs.

each FQ (see Table 1). These processes could be the first step of the FQs-albumins photobinding mechanism (Scheme 1). Hence, the observed photoreactivities of FQs with Tyr and Trp rule out the first postulated hypothesis, because if FQs were complexed into site I or II of the albumins, then, as observed in the case of NAL, some changes in the FQs intersystem crossing quantum yield (ϕ_{ISC}) and ^{3}FQs lifetime must be detected. These changes would occur especially for ENO, which shows a photoreactivity with Tyr and Trp similar to those determined for NAL. Moreover, the higher ENO photoreactivity than those determined for the other FQs also explains why ENO appears to be the major photoallergic FQ.[52]

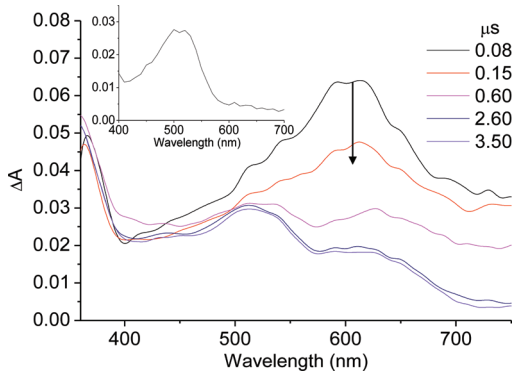


Figure 4. Transient absorption spectra of CIP (10^{-4} M) N_2O-purged buffered aqueous solutions in the presence of Trp (5 × 10^{-3} M) registered at different times after the 355 nm laser pulse. Inset: Transient absorption spectrum obtained 3 μs after the laser pulse under aerobic conditions.

The possibility that ^{3}FQs were quickly expelled from the albumin cavity was also analyzed using the receptor−ligand complex eq 7[53]

$$K_a = k_{on}/k_{off} \quad (7)$$

assuming that the association constant (K_a) for the ground state does not differ much from the expected for ^{3}FQs and that the association rate constant (k_{on}) is a diffusion limit control process in water (5 × 10^9 M^{-1} s^{-1}). Thus, for FQ−albumin K_a values of ca. 5 × 10^4 M^{-1}, the ^{3}FQ−albumin complexation should be more than 70% under our experimental conditions and the dissociation rate constants (k_{off}) of ^{3}FQ−albumin would be ca. 10^5 s^{-1}. This would produce ^{3}FQs lifetime variations due to electron transfer processes between ^{3}FQs and the reactive amino acids of the albumins would occur. The fact that no changes were observed rules out the second hypothesis. Therefore all results are in favor that FQs are predominantly outside of the protein cavities while NAL is mainly inside. In this context, the time profiles of NAL with albumins (Figure 1) show biexponential decays as a result of combined detection of NAL$^{\bullet-}$ (generated from the reaction of ^{3}NAL with the Tyr of the albumins) and the ^{3}NAL that remains outside of the proteins. The important difference between the association constants of FQs binding to albumins and that obtained for NAL reveals the great change produced by the presence of the piperazinyl substituent in the quinolinic compounds. The effect can be attributed to the large dipole moment of the zwitterionic

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forms of FQs (in aqueous solutions, the ionic form of FQs predominates at pH 7, with the carboxyl deprotonated and the piperazinyl substituent protonated).[19]

2. Protein Fluorescence Quenching by the Presence of FQs. Considering the behavior observed for FQs in the presence of albumins and their reactive centers by the LFP technique, a deep study of albumins fluorescence quenching by FQs must be performed to understand why fluorimetry provided K_a values 100 times higher than those determined from all the other techniques. The compounds selected for this purpose were ENO, CIP, NOR, and OFL as FQs and NAL as the reference model compound (see Chart 1). The method used to evaluate drug–protein association constants was the emission quenching of tryptophan within albumin by drugs. This emission band is centered at 344 nm for both albumins (see the emission spectrum of tryptophan within HSA in Figure 5) and is exclusively exhibited by the albumins at a 295 nm

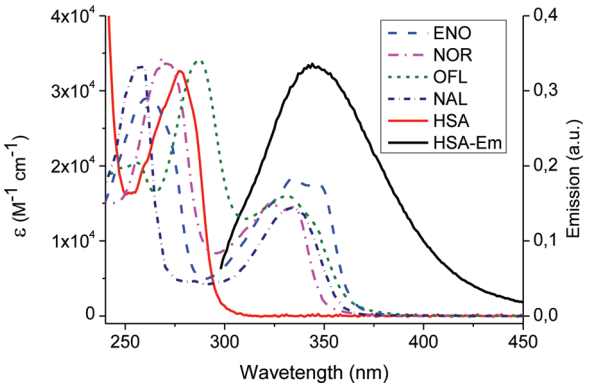

Figure 5. Absorption spectra of FQs, NAL and HSA in 4 mM PB aqueous solutions and emission sprectrum of Trp within HSA obtained using the excitation wavelength of 295 nm.

excitation wavelength.[54] The experiments were performed with 10^{-5} M HSA and BSA buffered aqueous solutions (4×10^{-3} M PB at pH ~7.4) in the presence of FQs and NAL as quenchers (between 10^{-6} and 1.2×10^{-5} M). In this context, as the quenchers show absorption at the excitation (295 nm) and emission wavelengths (Figure 5), inner filter effect (IFE) corrections must be applied for all compounds before the analysis of the data (more details are provided in the experimental section).

Results showed important luminescence quenching of the Trp within albumins by the presence of NAL. By contrast, no significant fluorescence intensity changes were detected after addition of increasing amounts of FQs.

Two equations were selected to determine the drug–protein interactions from fluorescence quenching data[30,55–58]

$$F_0/F = 1 + K_{sv}[Q] \quad (8)$$

$$\log[(F_0 - F)/F] = \log K_b + n \log[Q] \quad (9)$$

where F_0 and F are the fluorescence intensity in the absence and presence of the quencher respectively, [Q] is the quencher concentration, n is the reaction molecularity, K_{sv} is the Stern–Volmer quenching constant, and K_b is the binding constant for small molecules that bind independently to a set of sites in a macromolecule.

In static quenching processes, the K_{sv} values correspond to the total binding constant of quencher to the protein, if the quencher totally suppresses the intrinsic protein fluorescence.[56] Assuming this, as previously suggested,[30] the resulting K_{sv} would be a good approximation for FQ-albumin binding constants.

When the K_{sv} was calculated from the slope of the plot F_0/F versus [Q], values of 1×10^5 M^{-1} for NAL-HSA and 0.9×10^5 M^{-1} for NAL-BSA (Figure 6) were obtained. These data are

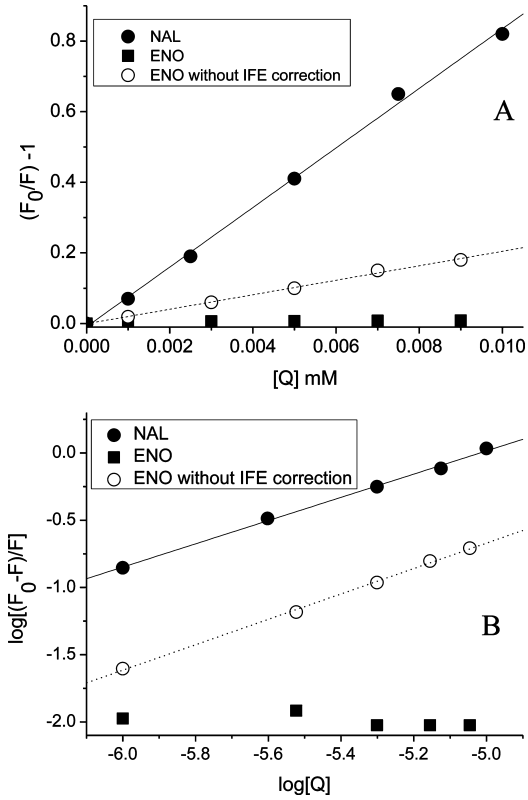

Figure 6. (A) Stern–Volmer plots of BSA fluorescence versus quenchers. (B) $\log[(F_0 - F)/F]$ versus log[quencher] plots of BSA fluorescence. Emission data obtained from NAL (solid circle), ENO (solid square) and ENO without the IFE corrections (open circle).

close to those described in the literature.[42] However, the K_{sv} of FQs could not be determined because the fluorescence quenching data, after the inner filter effect corrections, were practically imperceptible and did not conform to a slope. In this context, similar results were obtained when it was attempted to determine K_b from eq 9, plotting log $[(F_0 - F)/F]$ versus log[Q] (Figure 6).

Moreover, as expected, no changes were detected when FQs fluorescence quenching was analyzed by adding HSA or BSA.

The FQ-albumin association constants (K_a) determined by other methods ($<6 \times 10^2$ M^{-1}) again are in accordance with the results obtained. In fact, using a K_a of 10^3 M^{-1} in eq 6, less than 1% of drug is associated to protein, and consequently it would be virtually impossible to detect any fluorescence quenching. Besides, in agreement with these results, it was observed that the fluorescence lifetime of Trp within HSA decreased from ca. 6 to 3 ns by the presence of 10^{-5} M NAL, while no changes were observed adding 10^{-5} M FQs.

On the other hand, when the fluorescence quenching data of each FQ were analyzed without considering the IFE, the values obtained for K_{sv} were higher than 10^4 M^{-1} (an example is shown in Figure 6), which seems to explain the important

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differences obtained between our results and those described in the literature. This inappropriate use of fluorescence methodology to determine association constants as well as other ligand binding characteristics has appeared in a large number of recent papers, which has generated the discussion of this matter.[57−59]

CONCLUSION

The reactivities of FQs triplet excited states, such as ^{3}ENO, ^{3}NOR, ^{3}CIP, and ^{3}OFL, with the most reactive amino acids (tryptophan, Trp and tyrosine, Tyr) of the two principal albumin binding sites (site I and II) have been determined by laser flash photolysis experiments. The rate quenching constants determined, as well as the detection of FQ radical anions and radicals of Trp and Tyr, can be highly relevant to understand the processes of photohapten formation, which is the primary event in the onset of drug photoallergy. On the other hand, these results, together with the fact that ^{3}FQs lifetimes are not affected by the presence of HSA or BSA, are fully consistent with a low degree of FQs binding to albumins under biological conditions. Thus, laser flash photolysis methodology has confirmed the FQs-albumins association constants (K_a) determined by chromatography, capillary electrophoresis, or continuous and discontinuous ultrafiltration (K_a below 6×10^2 M^{-1}). Moreover, the discrepancy in the literature regarding the values of the FQs-albumins association constants has been explained by the analysis of new measurements of fluorescence quenching of Trp within albumins by FQs. In this context, the conformational changes of HSA induced by FQs, the binding distance between FQs and amino acid residues of HSA, and the thermodynamic parameters described in previous studies (which were obtained from fluorescence measurements) must be revised. Finally, it is important to highlight that omission of the IFE can produce significant errors when values of K_a are below 10^5 M^{-1}.

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Notes

The authors declare no competing financial interest.

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