Solvent Effects on Fluorescence Spectroscopic Properties of Benoxaprofen, a Cutaneous Photosensitizer

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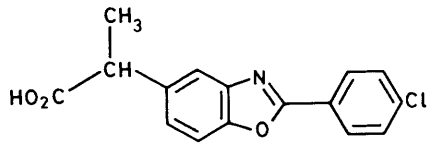
Absorption and fluorescence emission spectra of benoxaprofen (BXP), a cutaneous photosensitizer, have been measured in different solvents. The emission spectrum shifts to longer wavelength as the hydrogen-bonding ability and polarity of the solvent increase, even though the position of the absorption maximum remains similar except for the loss of the spectral structure. The red shift of the emission maximum is exceptionally large in water as compared with non-aqueous solution, indicating that specific solvent relaxation may occur around the excited BXP. In parallel with the red shift of the fluorescence spectrum, the fluorescence quantum yield is markedly decreased in water. The nature of the emitting species of BXP in water and its relationship to the fluorescence quenching are discussed.

Benoxaprofen{2-[2-(4-chlorophenyl)benzoxazol-5-yl]propionic acid} (BXP) is a non-steroidal anti-inflammatory drug and a cutaneous photosensitizer with respect to the hemolysis of cell membranes.[1–3] Its photosensitization involves both oxygen-dependent and oxygen-independent mechanisms.[2] These competitive mechanisms have been partially confirmed by an e.s.r. study of BXP in ethanol.[4] It has shown that BXP generates both superoxide and singlet oxygen as strong oxidizing species in aerated ethanol solution. A recent laser flash photolysis study has also demonstrated that the singlet oxygen can be produced *via* the triplet state of BXP in aqueous solution.[5] However, no superoxide has been detected. This is consistent with the observation that the oxygen-dependent lysis of mast cell membranes is photosensitized by BXP in water but is unaffected by superoxide dismutase.[2] Also the decay of the BXP triplet state in water was observed to only second-order kinetics whereas it follows first-order kinetics in isopropyl alcohol.[5] All the previous results imply that the photochemical properties of BXP are sensitive to solvents.

Furthermore, the photosensitization mechanism of BXP present in the macromolecules such as cell membranes would depend on its own microenvironment as in the case of other photosensitizers.[6] Thus some understanding of the solvent dependence of the photophysical and photochemical properties should be important to gain a knowledge of the possible microenvironmental effects of the BXP photosensitization. However, no systematic investigation has been made to understand the solvent dependence of the photophysical and photochemical properties. We now report the solvent and pH dependence of the absorption and fluorescence spectra of BXP as the first part of work dealing with these fundamental problems. Our results show that the nature of the excited BXP in water is very different from that in non-aqueous solvent.

Experimental

BXP was a generous gift from Lilly Research Laboratories, Indianapolis, and used without further purification. Spectrograde organic solvents were used as obtained from Merck, and triply distilled water was used for the preparation of aqueous solutions. Buffer solutions of known pH were prepared by mixing AnalaR grade acid or base with its salt; KCl–HCl (pH < 3), HAc–NaAc (pH 3—9), NH_4Cl–NH_4OH (pH > 9). Absorption spectra were recorded on a Beckman UV-526 spectrophotometer. Fluorescence measurements were made on a



BXP

scanning SLM-4800 spectrofluorometer which makes it possible to obtain corrected spectra using Rhodamine B as a quantum counter. A narrow excitation slit (less than 5 nm) was used to minimize the photolysis of BXP. Fluorescence quantum yields were determined by comparison with a reference of known quantum yield as described in elsewhere. Quinine sulphate (Fluoroscence Standard grade from J. T. Baker) in 0.5M perchloric acid was used as the reference assuming a quantum yield of 0.55.[7] Solutions of the sample and the standard were made up from degassed solvents. The solutions were accurately diluted to an optical density of not more than 0.1 to eliminate self-absorption. A quadratic correction for refractive index variation was applied.[8] The SLM 4800 spectrofluorometer was also used for the fluorescence lifetime measurements by phase modulation methods. The sample was excited with a sinusoidally modulated light at 30 MHz. The phase and modulation are measured from the amplified difference frequency, increasing the sensitivity with which fluorescence lifetimes of less than 1 nanosecond can be measured with an expected differential precision of 0.05 nanosecond. For the detailed instrumentation, see ref. 9. The pH measurements were made on a Fisher Accumet Model 525 Digital pH meter.

Results and Discussion

Absorption and fluorescence spectra of BXP were observed in different solvents at room temperature as shown in Figures 1 and 2, respectively. A summary of their properties is presented in Table 1. These data show that the wavelength of the absorption maximum (λ^{ab}_{max}) is not significantly affected by changing the solvent polarity, indicating that the lowest excited singlet state is π,π^* in nature. However, the vibrational fine structure of the spectra in non-polar solvents is lost in polar protic solvents. This implies that BXP interacts strongly with the protic solvent *via* hydrogen-bonding.

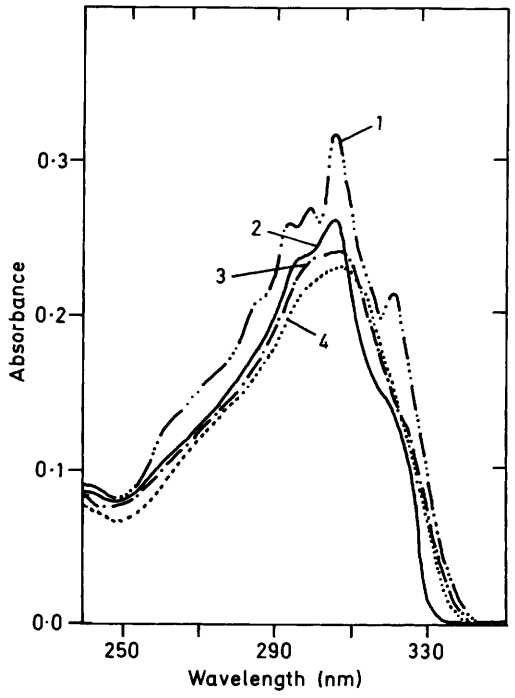


Figure 1. Absorption spectra of 1.0×10^{-5}M benoxaprofen in different solvents. The numbers refer to the following solvents: 1, cyclohexane; 2, acetonitrile; 3, water; 4, ethanol

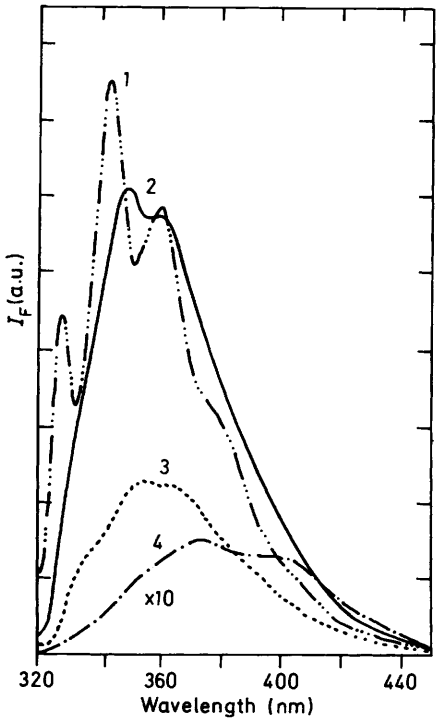


Figure 2. Fluorescence emission spectra of 1.0×10^{-5}M benoxaprofen in different solvents. The numbers refer to the following solvents: 1, cyclohexane; 2, acetonitrile; 3, ethanol; 4, water. λ_{exc} = 313 nm. The sensitivity for aqueous solution is 10 times higher than of that for other solutions

In aqueous solution, the absorption spectra were observed to change but little in the pH range 5—10. But the λ_{max}^{ab} shifts slowly to 296 nm in the pH range 2—5 (see Table 1). Thus it was concluded that the carboxy side-chain deprotonates to form an anionic BXP in water. The pK_a for such a deprotonation was found to be 4.5 which is comparable with that determined by Navaratnam *et al.*, pK_a 4.4.[5] Further proteolytic changes seemed to occur at pH < 2.0, showing the shift of λ_{max}^{ab} to 300 nm. This change is believed to be due to protonation of the nitrogen in the oxazolic ring as observed previously for other benzoxazole derivatives.[10,11] The exact pK_a for this change was not determined because of very poor solubility of the sample at low pH.

Table 1. Absorption and fluorescence characteristics of BXP different solvents[a]

Solvent	λ_{max}^{ab} (log ε)	λ_{max}^{em}	Fluorescence quantum yield, φ_f
Cyclohexane	303 (4.49)	342	0.65
Acetonitrile	303 (4.41)	348	0.57
Dioxane	304 (4.46)	344	0.60
Ethanol	304 (4.36)	350	0.24
D_2O	305	372	0.05
Water	305 (4.34)	372	0.01
pH 1 (Cation)	300	363	0.06
pH 4 (Neutral)	296	355	0.03
pH 10 (Anion)	305 (4.60)	370	0.01

λ_{max}^{ab} and λ_{max}^{em} are in nm; ε is in $dm^3\ mol^{-1}\ cm^{-1}$

Table 2. pK_a and pK_a^* values of BXP[a]

Equilibrium	pK_a	pK_a^*
Cation–Neutral	—	1.2[b]
Neutral–Anion	4.5	2.3

[a] pK_a^* was determined by the Førster method[12] and the position of the 0–0 band was taken to be the mean of the absorption and emission maxima. [b] Only $\Delta pK_a = pK_a^* - pK_a$ is indicated.

In contrast to the weak solvent-dependence of λ_{max}^{ab}, the fluorescence emission maximum (λ_{max}^{em}) is gradually red-shifted with an increase in the polarity of the solvents (Figure 2 and Table 1), indicating a possibility of a change in the character of the electronic state, possibly linked to solvent relaxation in the excited state. This red shift is abruptly large in water, showing a large Stokes shift (~6 000 cm^{-1}). It is also noteworthy that λ_{max}^{em} in dioxane is not highly red-shifted as compared with that in non-polar solvent (Table 1) even though it is practically considered as a polar solvent. These results suggest that for the interaction between water and BXP, intermolecular hydrogen-bonding is strengthened in the excited state with subsequent solvent relaxation.

In order to confirm this, we measured the fluorescence spectra of BXP in aqueous solutions of different pH. The pH dependence was observed to resemble that of the absorption spectra. Thus the assignment of the proteolytic species according to their λ_{max}^{em} is consistent with that based on λ_{max}^{ab} (Table 1). When the data listed in Table 1 are used in the Førster cycle,[12] the differences between the ground-state pK (pK_a) and the excited-state pK (pK_a^*) were determined to be small (Table 2). Nevertheless, it is clear that the pK_a^* for the cation–neutral equilibrium is larger than the pK_a, whereas the pK_a^* for the neutral–anion equilibrium is smaller than the pK_a. This implies that the nitrogen in the oxazolic ring becomes more basic and strengthens the intermolecular hydrogen-bonding upon excitation. This is very similar to the results obtained with other benzoxazole derivatives.[10,11] In contrast, the acidity of the carboxy group is enhanced in the excited state over the ground state.

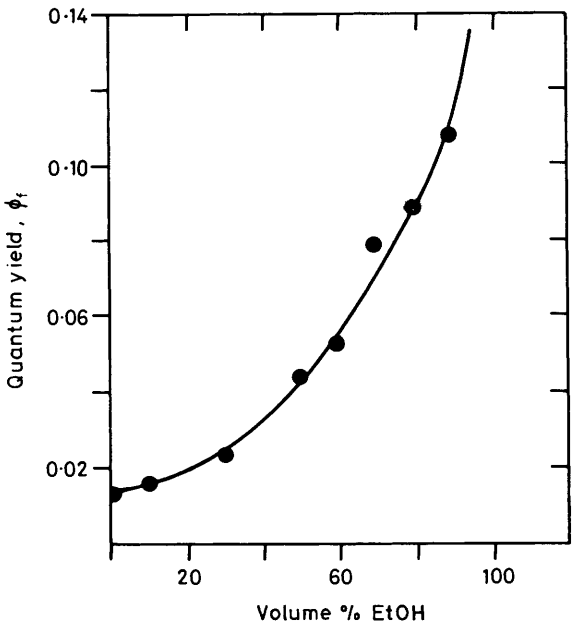


Figure 3. Fluorescence quantum yield of benoxaprofen *versus* %water in water–ethanol

The fluorescence quantum yield of BXP in non-polar solvent is high (0.65) and close to that of the parent 2-phenylbenzoxazole (0.72)[13] in cyclohexane. However, the high quantum yield is markedly decreased in hydrogen-bonding solvent in parallel with the red-shift of λ_{max}^{em} (Table 1). The fluorescence quenching is even more drastic in water as the quantum yield is seen to decrease in a highly non-linear manner as the content of water increases in water–ethanol solvents (Figure 3). In order to explore this aspect further in terms of intramolecular processes, we measured the fluorescence lifetimes of BXP in a series of water–ethanol solvents by phase modulation methods. The fluorescence lifetimes measured by the phase method were determined to be 1.4 ± 0.1 ns, but we had difficulty attaining lifetimes by the modulation method. This indicates that a very short lifetime component exists as well, possibly due to the solvent relaxation around the excited BXP. Thus, at present we cannot make an unambiguous argument for the intramolecular processes with regard to the fluorescence quenching in water, but we suggest possible explanations in the following discussion.

The fact that the fluorescence quantum yield is significantly affected by the hydrogen-bonding ability of the solvent indicates that intermolecular hydrogen-bonding interactions are important in the modification of the non-radiative deactivation pathway. In strongly solvating protic solvents like water, photoionization from the relaxed state of a fluorophore has been considered as the major non-radiative process as for 8,1-ANS [8-(phenylamino)naphthalene-1-sulphonate].[14,15] However, laser flash photolysis of BXP in water has not shown any evidence for the photoionization.[5] Excited-state intermolecular proton transfer (ESIPT) can also be considered as an alternative mechanism for the major deactivation process as observed in many aromatic acids.[16] But the small difference between the acidities of the ground and excited states of BXP ($\Delta pK_a < 2.5$, Table 2) indicate that the contribution of ESIPT to the efficient non-radiative decay is poor.

After all the intersystem crossing or internal conversion, the fluorescence quantum yield seems to be most strongly modified by intermolecular hydrogen-bonding between the excited BXP and water. According to the laser flash photolysis study,[5] the quantum yield of the intersystem crossing to the triplet state (φ_t) of BXP in water is 0.19. Assuming $\varphi_f + \varphi_t + \varphi_{ic} \simeq 1$, the fluorescence quantum yield (φ_f) of BXP in water (0.01) results in a calculated yield of internal conversion (φ_{ic}) of 0.80, indicating that internal conversion is the major non-radiative deactivation process. This is in contrast to the observation that internal conversion is not an important process in the case of the parent 2-phenylbenzoxazole in cyclohexane[13] whose fluorescence quantum yield is as high as BXP in cyclohexane. In fact, it is common that $S_1 \longrightarrow S_0$ internal conversion is negligible relative to the fluorescence or intersystem crossing for aromatic molecules unless the nuclei in one state undergo a rather drastic change in position and momentum as a result of transition.[17] Thus the high φ_{ic} of BXP in water should be attributed to the changes in nuclear geometry of BXP in the excited state. It is consistent with the observation that the mirror image relationship between absorption and fluorescence spectra of BXP is lost in water (Figure 1 and 2) whereas it is fairly maintained in non-aqueous solvents.

In the change of the excited-state geometry, torsional motion such as rotation about the C=N double bond between the benzenic and oxazolic rings is presumably involved as in the case of 2-(2-hydroxyphenyl)benzoxazole and 2-(2-hydroxy-5-methylphenyl)benzotriazole.[18] It is known that intermolecular hydrogen-bonding interactions increase such a torsional motion by increasing the density of low-frequency vibronic states.[18] If this is the case, the fluorescence quantum yield is expected to increase when H_2O is substituted by D_2O.[17] Such isotope effects were significant, the fluorescence yield being enhanced by five-fold in D_2O as compared with that in H_2O (see Table 1). Because of the torsional motion, co-planarity of the two parts of the molecule is not preserved, resulting in an increase in mixing of π,π^* character of the first excited state with n,π^*. This would also contribute to the fluorescence quenching of BXP in water.

Acknowledgement

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