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Acid–base equilibrium of drugs in time-resolved fluorescence measurements: Theoretical aspects and expressions for apparent pK_a shifts

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ABSTRACT

Spectroscopic properties of molecules which undergo acid–base equilibrium are greatly influenced by the local pH. Their acid–base equilibrium constants can be experimentally obtained under different environments by measuring spectroscopic parameters such as absorbance, fluorescence intensity and fluorescence lifetimes as a function of pH. The pK_a values are obtained fitting the data with the well known Henderson–Hasselbalch equation. Decay curves obtained with time-resolved pulse fluorometry are usually analyzed in terms of multi-exponential functions characterized by the pre-exponential factors or by the integrated fractional fluorescence intensities associated with each lifetime, this last being analogous to the steady state fluorescence contributions. In this paper we show that under normal conditions for many fluorescent drugs, a two-exponential analysis is adequate. We develop expressions for the pH dependence of pre-exponential factors and fractional fluorescence intensities in time-resolved pulse fluorometry of a fluorophore undergoing acid–base transition. We show that the obtained pK_a values are apparently shifted, and give the expressions for the pK-shifts. The expressions were tested using the fluorescence decay properties of two compounds: the well characterized local anesthetic dibucaine and the antibiotic levofloxacin.

1. Introduction

Acid–base equilibrium of various substrates, drugs and indicators are often investigated in homogeneous and heterogeneous solutions [1–14]. Spectroscopic properties are greatly affected by the ionization state of molecules, which can serve as indicators for local pH [1,2,14–19], and local electric potential [2,3,6,20]. Drugs activity and toxicity also frequently depend on the ionization state. Evaluation of equilibrium constants is therefore important for analytical applications, and for the investigation of their pharmacological properties [1,2].

During the past decades time resolved fluorescence experiments has been increasingly used in multidisciplinary science [2]. Fluorescent drugs and dyes are known to be useful molecular probes for examination of interfacial regions and binding reactions [4,6–12]. Many of them undergo acid–base transitions with environment-dependent equilibrium constants pK_a. Their spectroscopic parameters provide important means to observe the acid–base equilibrium. The most important characteristics of a fluorescent drug are the fluorescence quantum yields and lifetimes. The quantum yields are assessed by steady state measurements. The lifetimes, which determine the time that the excited drug has to interact or diffuse in the environment before returning to the ground state, is measured using phase-modulation or pulse fluorometry.

In general, when a fluorescent drug undergoes an acid–base transition, the fluorescence properties of the two species are different. Both steady state and time-resolved fluorometry provide important parameters that allow obtaining the apparent pK_a under different conditions. In the case of steady state fluorescence, the expression for the fluorescence intensity at a single wavelength, or for the intensity ratio at two different wavelengths, as a function of pH is well known and appears in Chapter 10, Appendix A of [1]. On the other hand, the acid–base properties of a molecule that absorbs light can change when the molecule is in the excited state. The excited state equilibrium constant, K_a^*, can be different from that of the ground state, K_a. Several methods have been described to obtain K_a^* using both steady-state and time resolved fluorescence [1,21].

Valeur treats the case of a selective excitation of the acidic form in Chapter 4 of [1]. In general, it is not possible to selectively excite one form because many drugs present only minor differences between the acidic and basic UV–visible absorption spectra. Expressions based on the Henderson–Hasselbalch equation are no

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longer valid either for the pre-exponential factors or the fractional intensities associated with each lifetime.

In order to analyze the time-correlated single photon counting (TCSPC) fluorescence data associated with pH transitions of fluorescent drugs, we developed theoretical expressions for the pre-exponential factors and fractional intensities associated with each characteristic lifetime. We demonstrated that the titration curves obtained with these parameters give shifted pK_a values. The apparent pK_a shifts were found as functions of the quantum yields and lifetimes of the involved species. The expressions were tested using the fluorescence of the local anesthetic dibucaine and of the fluoroquinolone antibiotic levofloxacin. The fluorescence decay curves were obtained by TCSPC pulse fluorometry. Pre-exponential factors and fractional contributions were obtained using global analysis. Apparent pK_a shifts obtained from the experimental titration plots were compared with the calculated values.

2. Theory

Let us consider a molecule undergoing a protonation–deprotonation reaction in aqueous solution. The usual equation for analyzing pH titration curves of a single ionization equilibrium in the ground state (Scheme 1) is the well known Henderson–Hasselbalch Eq. (1), where r is the molar fraction of the basic form, $r = [A]/([A]+[AH])$, and K_a is the apparent acid–base equilibrium constant, $K_a = [A].[H]/[AH]$ (electric charges were omitted).

$$AH \quad \xleftrightarrow{K_a} \quad A^- + H^+ \qquad \text{(Scheme 1)}$$

$$\mathrm{pH} = \mathrm{p}K_a + \log\frac{r}{1-r} \tag{1}$$

In general, the molar fraction r can be expressed as a function of spectroscopic parameters of the acidic and basic forms. The acidic and basic forms of a drug are differently charged. The different charge distributions lead to different physico-chemical characteristics. Considering a spectroscopic parameter P,

$$P = (1-r)\,P^{\mathrm{AH}} + r\,P^{\mathrm{A}} \tag{2}$$

with P^{AH} and P^{A} being the values of P when all the molecules are in the acidic and basic forms, respectively, Eq. (1) becomes:

$$\mathrm{pH} = \mathrm{p}K_a + \log\frac{P - P^{\mathrm{AH}}}{P^{\mathrm{A}} - P} \tag{3}$$

Parameter P is obtained from Eq. (3) as a function of the pH:

$$P = \frac{P^{\mathrm{AH}}10^{\mathrm{p}K_a} + P^{\mathrm{A}}\,10^{\mathrm{pH}}}{10^{\mathrm{p}K_a} + 10^{\mathrm{pH}}} \tag{4}$$

In steady state fluorescence, for example, parameter P is the fluorescence intensity at a given wavelength, I_λ [1].

The excited state equilibrium constant K_a^* can be different from that of the ground state. The events following the excitation of both the acidic and basic forms of a molecule are described by (Scheme 2). τ_o^{AH} and τ_o^{A} are the excited state lifetimes of the acidic (AH^*) and basic (A^*) forms (electric charge omitted), respectively, and k_1^* and k_{-1}^* are the rate constants for the excited state deprotonation and reprotonation, respectively. The excited state equilibrium constant is $K_a^* = k_1^*/k_{-1}^*$. Valeur treats the case of a selective excitation of the acidic form in Chapter 4 of [1], but in general this is not possible because many drugs present only minor differences between the acidic and basic UV–visible absorption spectra.

$$\begin{array}{ccc} AH^* & \xleftrightarrow[k_{-1}^*]{k_1^*} & A^{*-} + H^+ \\ \uparrow\downarrow \ 1/\tau_o^{AH} & & \uparrow\downarrow \ 1/\tau_o^{A} \\ AH & \xleftrightarrow{K_a} & A^- + H^+ \end{array} \qquad \text{(Scheme 2)}$$

Taking into account that H^+ is in fact H_3O^+, the differential equations expressing the evolution of the species after a δ-pulse excitation of both AH and A are written according to (Scheme 2):

$$\begin{aligned} \frac{d[AH^*]}{dt} &= -(k_1^* + 1/\tau_o^{\mathrm{AH}})[AH^*] + k_{-1}^*[\mathrm{H_3O}][A^*] \\ \frac{d[A^*]}{dt} &= k_1^*[AH^*] - (k_{-1}^*[\mathrm{H_3O}] + 1/\tau_o^{A})[A^*] \end{aligned} \tag{5}$$

In general the excited state back-protonation reaction needs to be taken into account only if pH ≤ 2 or 3, because this reaction is diffusion-controlled ($k_{-1}^* \approx 5\times10^{10}$ L mol^{-1} s^{-1}) and at pH ≈ 3 the pH-dependent rate is $k_{-1}^*[\mathrm{H_3O}] \approx 5\times10^{7}$ s^{-1}. The reciprocal of this value is much greater than the excited-state lifetimes of most organic bases [1]. Eq. (5) become:

$$\begin{aligned} \frac{d[AH^*]}{dt} &= -(k_1^* + 1/\tau_0^{\mathrm{AH}})[AH^*] \\ \frac{d[A^*]}{dt} &= k_1^*[AH^*] - (1/\tau_0^{\mathrm{A}})[A^*] \end{aligned} \tag{6}$$

The solutions of Eq. (6) when both AH and A are excited, under the initial conditions that at $t=0$, $[AH^*] = [AH^*]_0$; $[A^*] = [A^*]_0$; $d[AH^*]/dt = -k_1^* + 1/\tau_o^{\mathrm{AH}}[AH^*]_0$; and $d[A^*]/dt = k_1^*[AH^*]_0 - 1/\tau_o^{A}[A^*]_0$, leads to the following expressions for the fluorescence intensities after the δ-pulse:

$$\begin{aligned} i_{AH^*}(t) &= k_{\mathrm{r}}^{\mathrm{AH}}[AH^*](t) = k_r^{\mathrm{AH}}[AH^*]_0 e^{-\beta_1 t} \\ i_{A^*}(t) &= k_{\mathrm{r}}^{\mathrm{A}}[A^*](t) = -k_{\mathrm{r}}^{\mathrm{A}}\frac{k_1^*}{\beta_1-\beta_2}[AH^*]_0 e^{-\beta_1 t} \\ &\quad + k_{\mathrm{r}}^{\mathrm{A}}\left(\frac{k_1^*}{\beta_1-\beta_2}[AH^*]_0 + [A^*]_0\right)e^{-\beta_2 t} \end{aligned} \tag{7}$$

In Eq. (7), $k_{\mathrm{r}}^{\mathrm{AH}}$ and $k_{\mathrm{r}}^{\mathrm{A}}$ are the rate constant for radiative de-excitation of AH^* and A^*, respectively, and $\beta_1 = k_1^* + 1/\tau_0^{\mathrm{AH}}$ and $\beta_2 = 1/\tau_0^{\mathrm{A}}$. The total fluorescence intensity is the sum of the contributions in Eq. (7), having in mind that $k_{\mathrm{r}}^{\mathrm{AH}}$ and $k_{\mathrm{r}}^{\mathrm{A}}$ must be the radiative rate constants at a given emission wavelength and bandwidth.

$$\begin{aligned} i(t) &= \left(k_{\mathrm{r}}^{\mathrm{AH}} - k_{\mathrm{r}}^{\mathrm{A}}\frac{k_1^*}{\beta_1-\beta_2}\right)[AH^*]_0 e^{-\beta_1 t} \\ &\quad + k_{\mathrm{r}}^{\mathrm{A}}\left(\frac{k_1^*}{\beta_1-\beta_2}[AH^*]_0 + [A^*]_0\right)e^{-\beta_2 t} \end{aligned} \tag{8}$$

Eq. (8) tells that the decay is frequently biexponential, with decay rates given by β_1 and β_2. In the case that $k_1^* << 1/\tau_0^{\mathrm{AH}}$, the decay rates correspond to those of the acidic and basic species: $\beta_1 = 1/\tau_0^{\mathrm{AH}}$ and $\beta_2 = 1/\tau_0^{\mathrm{A}}$.

The decay of the two species will be independent and Eq. (8) will reduce to Eq. (9) in the case of the additional restriction $k_1^* << (1/\tau_0^{\mathrm{AH}} - 1/\tau_0^{\mathrm{A}})$.

$$i(t) = k_{\mathrm{r}}^{\mathrm{AH}}\,[AH^*]_0\,e^{-\beta_1 t} + k_{\mathrm{r}}^{\mathrm{A}}[A^*]_0\,e^{-\beta_2 t} \tag{9}$$

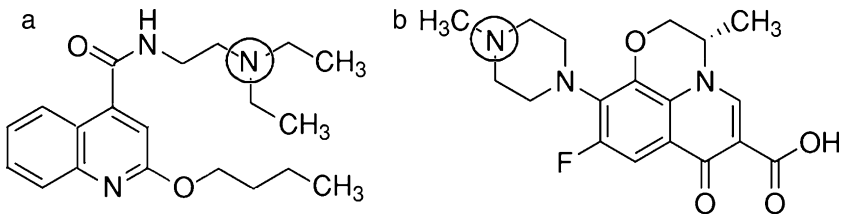

Fig. 1. Chemical structures of dibucaine (a) and levofloxacin (b). The pK_a of circled groups are 8.8 and 8.2, respectively.

In order to analyze when this will occur, it is worth recalling that Eq. (8) is valid only for diffusion-controlled reprotonation, $k^*_{-1} \approx 5 \times 10^{10}$ L mol^{-1} s^{-1}. Therefore, it is possible to estimate $k^*_1 = K^*_a k^*_{-1}$ from the values of pK^*_a. For example, a lifetime $\tau^{AH}_o \approx 2 \times 10^{-9}$ s gives $k^*_1 \tau^{AH}_o < 0.1$ for p$K^*_a > 3$. In fact, many fluorescent molecules satisfy this condition. Intensity decays are typically fit to the multi-exponential model:

$$I_\lambda(t) = C + \sum_i \alpha_i \exp \frac{-t}{\tau_i} \tag{10}$$

where C is a constant associated with the noise, τ_i are the lifetimes of the individual species present in the sample and α_i are the pre-exponential factors. Each lifetime contributes with a fraction f_i to the total number of emitted photons, f_i being proportional to $\alpha_i \tau_i$, the area under the decay curve for each decay time. It is demonstrated [1] that the fractional contributions f_i represent the steady state fractional intensities of each fluorophore at the observation wavelength.

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \tag{11}$$

When Eq. (9) is valid, there will be no excited-state proton transfer during the excited-state lifetime, and the deconvoluted fluorescence decay $I_\lambda(t)$ in pulse fluorometry will be fitted by a double exponential function and the time-resolved spectroscopic parameters will reflect the presence of the two species.

The proportionality constant between Eqs. (9) and (10) depends only on the instrumental setup. The initial concentration of excited molecules in Eq. (9), on the other hand, depends on the ground state concentrations [AH] and [A], and on the molar absorption coefficients ε^{AH} and ε^A at the excitation wavelength. Then, the pre-exponential factors in Eq. (9) are proportional to:

$$\alpha^{AH} \propto [AH]\varepsilon^{AH} k^{AH}_r \quad \text{and} \quad \alpha^A \propto [A]\varepsilon^A k^A_r \tag{12}$$

The ratio between expressions in Eq. (12) gives:

$$\frac{r}{1-r} = \frac{[A]}{[AH]} = \frac{\alpha^A}{\alpha^{AH}} \frac{\varepsilon^{AH}}{\varepsilon^A} \frac{k^{AH}_r}{k^A_r} \tag{13}$$

which can be used in Eq. (1) to express the Henderson–Hasselbalch equation in terms of the pre-exponential factors α^{AH} and α^A.

$$\text{pH} = \text{p}K_a + \log \frac{\varepsilon^{AH} k^{AH}_r}{\varepsilon^A k^A_r} + \log \frac{\alpha^A}{\alpha^{AH}} \tag{14}$$

Eq. (14) indicates that, when using the pre-exponential factors as spectroscopic parameters in a pH titration experiment, the pK_a will be apparently shifted to pK'_a, with

$$\text{p}K'_a = \text{p}K_a + \log \frac{\varepsilon^{AH} k^{AH}_r}{\varepsilon^A k^A_r} \tag{15}$$

The radiative rate constants K^{AH}_r and k^A_r can be written in terms of the fluorescence quantum yields, ϕ^{AH}_f and ϕ^A_f, and lifetimes τ^{AH} and τ^A.

$$k^{AH}_r = \frac{\phi^{AH}_f}{\tau^{AH}} \quad \text{and} \quad k^A_r = \frac{\phi^A_f}{\tau^A} \tag{16}$$

where ϕ^{AH}_f and ϕ^A_f are the quantum yields computed only for the measured part of the emission band. Using expressions Eq. (16) in Eq. (15), the pK_a shift of becomes:

$$\Delta \text{p}K^\alpha_a = \text{p}K'_a - \text{p}K_a = \log \frac{\varepsilon^{AH} \phi^{AH}_f \tau^A}{\varepsilon^A \phi^A_f \tau^{AH}} \tag{17}$$

The normalized pre-exponential factors as a function of pH are obtained from Eq. (14):

$$\frac{\alpha^{AH}}{(\alpha^A + \alpha^{AH})} = \frac{10^{\text{p}K'_a}}{10^{\text{pH}} + 10^{\text{p}K'_a}} \quad \text{and} \quad \frac{\alpha^A}{(\alpha^A + \alpha^{AH})} = \frac{10^{\text{pH}}}{10^{\text{pH}} + 10^{\text{p}K'_a}} \tag{18}$$

These equations are equal to Eq. (4), with parameter P given by the normalized pre-exponential factors and $P^{AH} = 1$, $P^A = 0$ and $P^{AH} = 0$ and $P^A = 1$, respectively. The shifted pK'_a given by Eq. (17) are obtained when fitting the normalized pre-exponential factors as a function of pH with the Henderson–Hasselbalch Eq. (4).

The time-resolved parameters associated with the contributions of each species in a steady-state fluorescence experiment are the fractional intensities f_i (Eq. (11)) [1,2]. Equation 13 can be expressed as a function of the fractional intensities using Eq. (16):

$$\frac{r}{1-r} = \frac{[A]}{[AH]} = \frac{f^A}{f^{AH}} \frac{\varepsilon^{AH} \phi^{AH}}{\varepsilon^A \phi^A} \tag{19}$$

Hence, the Henderson–Hasselbalch Eq. (4) in terms of the fractional intensities becomes:

$$pH = pK_a + \log \frac{\varepsilon^{AH} \phi^{AH}}{\varepsilon^A \phi^A} + \log \frac{f^A}{f^{AH}} \tag{20}$$

Eq. (20) indicates that, when the fractional intensities are used as spectroscopic parameters in a pH titration experiment, there will be an apparent pK_a shift, given by:

$$\Delta \text{p}K^f_a = pK''_a - pK_a = \log \frac{\varepsilon^{AH} \phi^{AH}}{\varepsilon^A \phi^A} \tag{21}$$

The equations used to fit the fractional intensities versus pH obtained from Eq. (20) are:

$$\frac{f^{AH}}{f^{AH} + f^A} = \frac{10^{\text{p}K''}}{10^{\text{pH}} + 10^{\text{p}K''}} \quad \text{and} \quad \frac{f^A}{f^{AH} + f^A} = \frac{10^{\text{pH}}}{10^{\text{pH}} + 10^{\text{p}K''}} \tag{22}$$

Eq. (22) are equal to Eq. (4), with parameter P given by the fractional intensities and with $P^{AH} = 1$, $P^A = 0$ and $P^{AH} = 0$, $P^A = 1$, respectively.

It is worth noting that, although the fractional intensities in a TCSPC experiment are equivalent to the steady state fluorescence intensities, they give shifted pK_a values, according to Eq. (21). This may look strange, but it can be explained by the fact that in the steady-state experiment the fluorescence intensity I_λ is the contribution of both species during a fixed pre-determined time interval,

while in a TCSPC experiment the total acquisition time is not fixed. The acquisition time is different for each pH, and the fractional intensities are normalized separately.

3. Experimental

3.1. Materials

High purity dibucaine hydrochloride and levofloxacin (Fig. 1) were purchased from Sigma and used without further purification. Concentrated stock solutions (1 mM) were prepared in ethanol. Analytical grade chemicals were used for the preparation of buffers and for the adjustment of pH. Milli-Q water quality was used for all the preparations.

3.2. Methods

Dibucaine and levofloxacin solutions (10 μM) at different pH were prepared in buffers. Appropriate amounts of the 1 mM ethanol stock solutions were partially dried and diluted in the buffer solutions so that the final ethanol concentrations were less than 0.1%. The buffers stock solution contained 33 mM citric acid, 50 mM phosphoric acid, 50 mM boric acid and 330 mM NaOH. After a 1:15 dilution (20 mM Na) and dissolution of the drug, the pH was adjusted with small amounts of concentrated solutions of HCl and measured using a Cole-Parmer Chemcadet 5986–25 pH meter with an Ag/AgCl semimicro combination electrode. pH measurements were performed immediately before and after the fluorescence measurements. Spectrophotometric measurements were carried out at ambient temperature (24–26 °C). Optical absorption spectra in the range of 220–800 nm were obtained with a HP 8452A Diode Array spectrophotometer. Fluorescence measurements were performed on a PTI–QM1 Fluorescence System equipped with a temperature controller and magnetic stirrer. Lifetime measurements were performed on a Horiba – Jobin Ivon – IBH TCSPC fluorometer. The light source used for the excitation of dibucaine was a 330 nm nanoLED N-16, 1.0 ns nominal pulse duration, 1 MHz repetition rate. Computer programs supplied by Horiba Jobin Ivon IBH were employed in the processing of the time resolved fluorescence data. Fluorescence intensity decay curves were fitted using two- or three-exponential expressions, with a global analysis procedure.

4. Results and discussion

The fluorescence of two drugs, e.g. the local anesthetic dibucaine and the antibiotic levofloxacin, are used to test the expressions obtained in the section "Theoretical Survey". Steady-state and time resolved fluorescence measurements were performed for dibucaine (three pH titration series) of for levofloxacin (two pH titration series). The titration series for each drug gave similar results with differences in pK_a values of the order of 0.1, at most. The errors presented below are the standard errors given by the least squares fitting procedure.

Steady state fluorescence spectra of dibucaine at different pH are presented in Fig. 2. The inset depicts the fluorescence at 405 nm (peak at pH 7) as a function of the pH. The data was fitted using Eq. (4), with a pK_a = 8.78 ± 0.02.

TCSPC fluorescence decay curves of dibucaine were obtained for each pH. Fig. 3 shows typical results. For each titration sequence, all the curves at different pH were submitted to a global analysis using the multi-exponential expression of Eq. (10).

Since the data was not conveniently fitted using a two-exponential function, a three-exponential expression was used. Using global analysis, it was possible to find a set of three lifetimes (τ_1 = 3.14 ns, τ_2 = 0.67 ns and τ_3 = 0.23 ns) to fit all the fluorescence

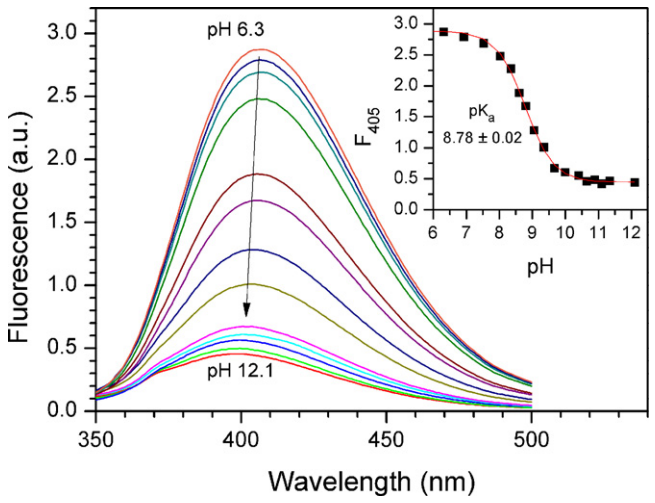

Fig. 2. Steady state fluorescence spectra of dibucaine (excitation at 330 nm) at different pH (6.2, 6.9, 7.5, 8.0, 8.6, 8.8, 9.0, 9.3, 9.7, 10.0, 10.4, 10.9, 12.1, from top to bottom as indicated by the arrow). The inset shows the fluorescence at 405 nm as a function of pH; the solid line is the least-squares fit obtained using Eq. (4), with P^{AH}, P^{A} and pK_a as fitting parameters. The pK_a was 8.78 ± 0.02.

decay curves in the alkaline pH range, from 7.3 to 12.1. The global χ^2 value was ~1.2 and the set of lifetimes was consistent with the individual analysis initially performed.

The fractional contributions f_1, f_2 and f_3, calculated from the pre-exponential factors α_1,α_2 and α_3 and corresponding lifetimes according to Eq. (11), appear in the inset of Fig. 3 as a function of pH. It can be observed that the largest lifetime τ_1 = 3.14 ns is due to protonated dibucaine, since both α_1 and f_1 decrease with increasing pH. The lifetime τ_2 = 0.67 ns is due to the neutral, unprotonated drug, and the small lifetime, τ_3, is usually introduced in a multi-exponential analysis to account for some scattering. Since f_3 displayed the same pattern as the fractional intensity f_2 due to unprotonated dibucaine, it was attributed to aggregation of neutral dibucaine molecules, developed in the absence of electrostatic repulsion. According to this interpretation, f_2 plus f_3 in the inset of Fig. 3 is the total contribution of neutral molecules.

The fractional intensities f_1, and f_2 + f_3 fitted using Eq. (4) gave a pK_a of 9.8 ± 0.1, which gives a positive ΔpK_a of 1.0 relative to the

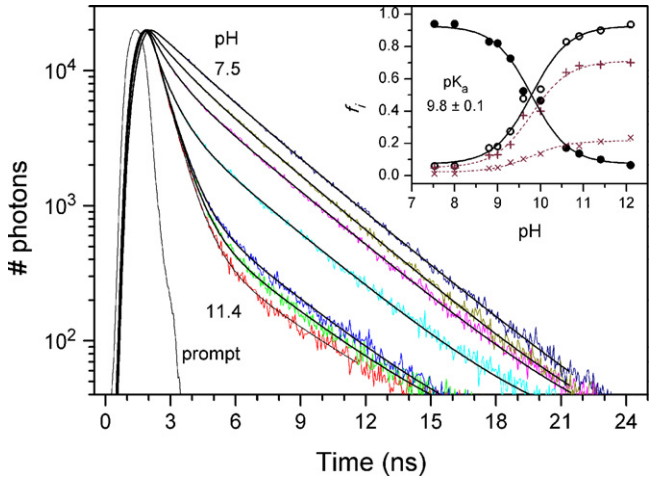

Fig. 3. Dibucaine fluorescence decay curves at different pH (7.5, 8.8, 9.4, 10.0, 10.6, 10.9, 11.4, from top to bottom). Excitation at 330 nm, emission at 405 nm. The solid lines represent the 3-exponential fits obtained from the global analysis with τ_1 = 3.14 ns, τ_2 = 0.67 ns and τ_3 = 0.23 ns. The inset shows the corresponding fractional intensities: (●) f_1, attributed to protonated dibucaine, (+) f_2; (×) f_3, and (○) f_2 + f_3, attributed to neutral dibucaine.

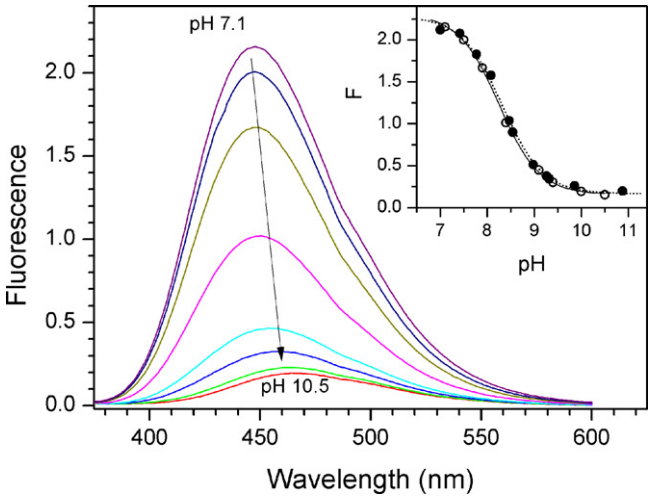

Fig. 4. Typical steady state fluorescence spectra of levofloxacin (excitation at 330 nm) at different pH (7.1, 7.5, 7.9, 8.4, 9.1, 9.4, 10.0, 10.6, from top to bottom as indicated by the arrow). The inset shows the fluorescence intensity as a function of pH for two different series of measurements: (●) at 457 nm, and (○) at 448 nm. The solid and dotted lines are the least-squares fits using Eq. (4), with P^{AH}, P^{A} and pK_a as fitting parameters. The obtained pK_a values were 8.25 ± 0.02 (○) and 8.32 ± 0.03 (●).

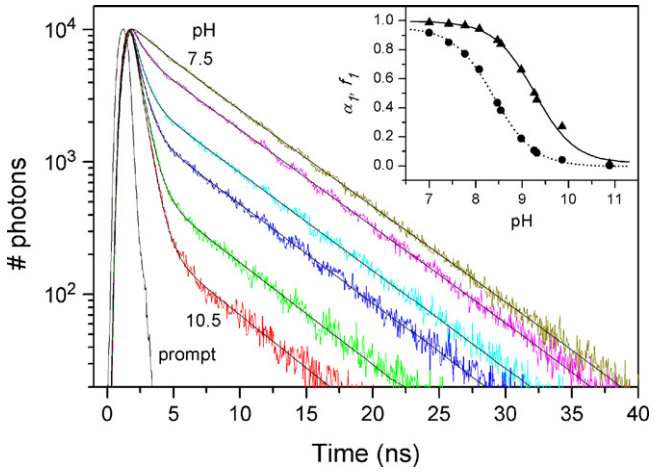

Fig. 5. Typical fluorescence decay curves of levofloxacin at different pH (7.5, 8.8, 9.4, 10.0, 10.6, 10.9, 11.4, from top to bottom). Excitation at 330 nm, emission at 448 nm. The solid lines represent the 2-exponential fits obtained from the global analysis with $\tau_1 = 5.4$ ns (zwiterionic) and $\tau_2 = 0.6$ ns (anionic). The inset shows the normalized pre-exponential factor α_1 (●) and the fractional intensity f_1 (▲) of zwiterionic levofloxacin as a function of the pH. The dotted and solid lines are the least-squares fits using Eq. (4), with pK_a values 8.39 ± 0.02 and 9.26 ± 0.03, respectively.

steady state fluorescence. The normalized pre-exponential factor $\alpha_1/(\alpha_1 + \alpha_2 + \alpha_3)$ plotted as a function of the pH (results not shown) gave a pK_a of 9.0 ± 0.1, with $\Delta pK_a = 0.2$ relative to that obtained by steady state fluorescence.

For dibucaine, the molar absorption coefficients for the neutral and protonated species are similar ($\varepsilon^{AH} = \varepsilon^{A}$); the quantum yields are $\Phi^{A} = 0.04$ and $\Phi^{AH} = 0.27$ [2] and the lifetimes $\tau^{A} = 0.67$ ns and $\tau^{AH} = 3.14$ ns were found in this work. Using these values in Eq. (21) and Eq. (17), the expected apparent pK shifts are: $\Delta pK_a^f = 0.83$ and $\Delta pK_a^\alpha = 0.16$, for the fractional intensity and pre-exponential factor, which are very similar to the values obtained from the fits of the experimental results.

Vanderkooi [3] also obtained experimental pK_a shifts for dibucaine in plots of the apparent phase and modulation lifetimes as a function of pH obtained from phase-modulation fluorometry, and performed theoretical computations for the different curves found upon using the lifetimes τ_P^{app} and τ_M^{app}, obtained from phase delays and relative modulations, respectively.

In the case of levofloxacin the experimental pK_a shifts obtained using the fluorescence decay parameters were also explained by Eqs. (17) and (21). Typical steady state fluorescence spectra of levofloxacin at different pH are presented in Fig. 4. The inset presents the fluorescence intensity (at 457 nm, mean wavelength between peaks at pH 7 and 11, for one of the experimental series, and at 448 nm, peak at pH 7, for the other series) as a function of the pH. The least squares fit of the data using Eq. (4) gave a $pK_a = 8.32 \pm 0.03$ and 8.25 ± 0.02. The difference between the two values shows that the experimental errors are small.

Typical fluorescence decay curves of levofloxacin are presented in Fig. 5 for several pH values. For each titration sequence, the curves at different pH were submitted to a global analysis using a two-exponential expression (Eq. (10), with $i = 1, 2$). For one titration sequence, the two lifetimes that fitted the fluorescence decay curves in the alkaline pH range were $\tau_1 = 6.1$ ns (zwiterionic levofloxacin) and $\tau_2 = 0.7$ ns (anionic levofloxacin). These values were different for the other titration sequence ($\tau_1 = 5.4$ ns and $\tau_2 = 0.6$ ns), probably because of the different ambient temperatures during the measurements. Nevertheless, the obtained pK_a were very similar for both titration sequences.

The inset of Fig. 5 shows the plots of the normalized pre-exponential factor $\alpha_1/(\alpha_1 + \alpha_2)$ and of the fractional contributions f_1 as a function of pH. The fractional intensity f_1 fitted using Eq. (4) gave a pK_a of 9.26 ± 0.03, which gives a positive ΔpK_a of ~1.0 relative to that obtained with the steady state fluorescence. The plot of the normalized pre-exponential factor gave a pK_a of 8.39 ± 0.02, with almost no pK shift relative to the steady state fluorescence.

The pK shifts predicted by Eqs. (17) and (21) are calculated as follows: for levofloxacin, the molar absorption coefficients for the anionic and zwitterionic species are similar ($\varepsilon^{AH} = \varepsilon^{A}$); the ratio of the quantum yields are obtained from the steady state fluorescence results $\Phi^{AH}/\Phi^{A} = 11$, and the lifetimes $\tau^{A} = 0.7$ ns and $\tau^{AH} = 6.1$ ns were obtained. Using these values in Eqs. (21) and (17), the expected apparent pK shifts are: $\Delta pK_a^f = 1.0$ and $\Delta pK_a^\alpha = 0.1$. They are also very similar to the obtained experimental values.

It is worth calling attention to the fact that, in general, the pre-exponential factors give smaller pK_a shifts than the fractional intensities. In conclusion, this work concerns to corrections that have to be made when using time-resolved fluorometry parameters in analytical applications. Expressions to correct the acid–base equilibrium constants obtained with pre-exponential factors and fractional intensities were obtained, and their limits of validity were discussed. The expressions were shown to explain the experimentally obtained pK_a shifts of two different drugs.

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