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A time resolved and steady-state fluorescence quenching study on naproxen and its cyclodextrin complexes in water

N. Sadlej-Sosnowska [a,*], A. Siemiarczuk [b]

[a] *Drug Institute, 30/34 Chełmska Street, 00-725 Warsaw, Poland*

[b] *Photon Technology International (Canada) Inc., 347 Consortium Court, London, Ont., Canada N6E 2S8*

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Abstract

Steady-state and time-domain fluorescence quenching studies of naproxen in water were carried out in solutions in the absence or presence of a cyclodextrin (addition of β-cyclodextrin or methyl-β-cyclodextrin was investigated). KI and acrylamide were used as quenchers. From the steady-state quenching experiments the quenching constants in the Stern–Volmer equation were found and compared for the free and the complexed molecules. The decay curves were analyzed by utilizing either a discrete one to four exponential fit or the lifetime distributions (exponential series method). The lifetimes of complexes, their bimolecular quenching constants and quenching efficiencies were determined and compared with the corresponding values for free naproxen. All measurements were carried out separately for the naproxen anion at higher pH and for naproxen acid at lower pH.

Keywords: Naproxen; Fluorescence; Time resolved fluorescence quenching; Complex; β-cyclodextrin; Methyl-β-cyclodextrin

1. Introduction

Collisional quenching of fluorescence has been thoroughly studied during the last two decades because it is of considerable interest for physical chemistry as well as for biochemistry and biophysics where quenching is frequently used in protein, membrane and nucleic acid research to elucidate structural and functional features of these systems. A new area of sensing technology for medical and industrial applications based on fluorescence and luminescence quenching has been rapidly developing. In this report we examine quenching of naproxen by iodide and acrylamide in aqueous solutions. Naproxen and its complexes with two cyclodextrins (CDs) were selected because their association constants have recently been measured fluorometrically [1] and this helped us calculate separately the quenching parameters for the free naproxen molecule and for its complexes, in spite of the fact that in aqueous solutions containing naproxen and a CD, there coexist free and bound naproxen molecules together. To our knowledge, quenching parameters for the free and bound forms coexisting in the same solution were determined previously only for indole with beta-cyclodextrin (β-CD) by Örstan and Ross [2].

Naproxen (6-methoxy-α-methyl-2-naphthalene acetic acid) is a weak acid ($pK_a = 4.2$ [3]). In the aqueous unbuffered solutions with neither acid nor base added, it exists as a mixture of dissociated and non-dissociated (anionic) forms and the pH of the solution is about 4.2 (at a concentration of 5×10^{-5} M). After addition of a CD, there exist four species in the solution, viz. free dissociated and non-dissociated naproxen and two different complexes produced with either form. In order to deal with a more simple system, we have carried quenching experiments in the solutions of naproxen acid in 0.1 M HCl (pH = 1) or in the solutions of naproxen sodium salt in a diluted NaOH (1.25×10^{-4} M, pH = 9). In each of the two solutions, naproxen was supposed to exist in the acidic or in the anionic form, respectively. The association constants of naproxen acid and its sodium salt with cyclodextrins (CDs) were measured in the solutions having an identical pH [1].

2. Experimental

2.1. Fluorescence quenching measurements

Steady-state fluorescence spectra were measured with a Shimadzu RF-5000 spectrofluorometer equipped with a thermostatically controlled cell compartment. Solution

* Corresponding author. Fax: +48-22-8410652.
E-mail address: sadlej@il.waw.pl (N. Sadlej-Sosnowska).

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temperature was held at 35°C by means of a circulating thermobath. The excitation wavelength was 329.6 nm. At this wavelength, the molar absorption coefficients of naproxen acid and naproxen anion were about 1520 and 1600 M^{-1}, respectively. The maximum value at 356.4 nm was taken as a measure of fluorescence intensity.

For fluorescence titration, 2 ml of naproxen acid or salt solution ((4–6) × 10^{-5} M), without or with an addition of β-CD or methyl-beta-cyclodextrin (Me-β-CD), at a concentration of about 3 mM, were placed in a fluorescence cell equipped with a Teflon stopper and a magnetic stirrer. The solution was titrated with successive additions of a quencher dissolved in the same naproxen or naproxen/CD solution at a concentration of about 0.6 M (KI) or 0.4 M (acrylamide). The final concentrations of the quenchers ranged from 0 to 150 mM (KI) and from 0 to 100 mM (acrylamide). After each titration, the fluorescence spectrum as well as the intensity at the maximum wavelength was recorded as a function of quencher concentration.

2.2. Time resolved fluorescence experiments

Fluorescence decays were measured with a PTI Model C-71 TimeMaster fluorescence lifetime spectrometer. The instrument utilizes a nanosecond flash lamp as an excitation source and a stroboscopic detection system. Temperature was kept at 35°C. The decay curves were analyzed with a PTI TimeMaster Pro-software package by utilizing either a discrete 1-to-4 exponential fit or the exponential series (ESM) lifetime distribution analysis [4]. Reduced chi-square values and weighed residuals were used as the goodness-of-fit criteria.

3. Results

Quenching of naproxen fluorescence resulted in decreased intensity, but did not result in any spectral shift in the quenchers concentration range used. Both CDs protected the fluorescing molecules against quenching. A plot of the fluorescence intensity of naproxen acid, free or with the addition of β-CD, as a function of acrylamide concentration, is shown in Fig. 1.

As a first step, the results were represented as Stern–Volmer plots. In the solutions of naproxen without any CD the plots show slight positive deviations, the phenomenon was observed many times for the fluorescence quenching by acrylamide and KI [2,5–7] and other quenchers [8–12]. This deviation was smaller or even absent after addition of a CD to the solution. This finding is illustrated in Fig. 2, which shows the Stern–Volmer plots for quenching of fluorescence of naproxen-anion with KI in solutions either with or without a CD added.

The non-linear (in the Stern–Volmer simple representation) quenching curves were frequently analyzed by using the Stern–Volmer equation modified to include static

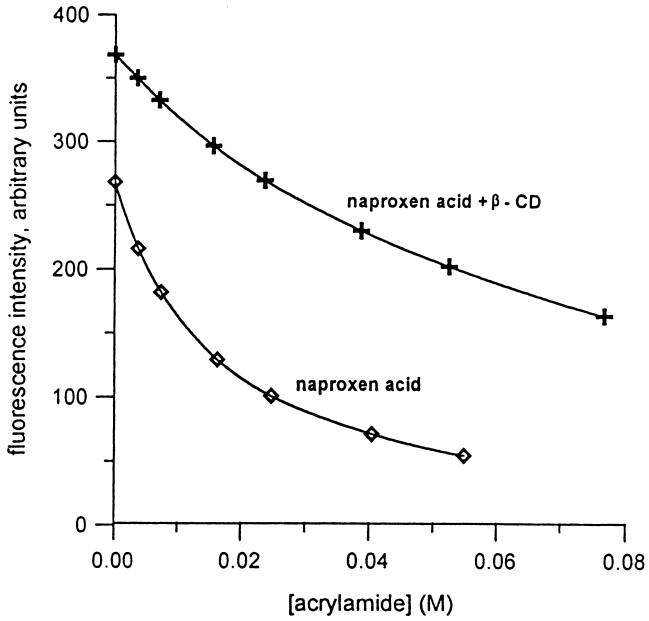

Fig. 1. Quenching of fluorescence intensity of naproxen acid (4×10^{-5} M) by acrylamide in water at 35°C, with and without 3 mM β-CD.

quenching [2–13]:

$$\frac{F_0}{F\,e^{(V[Q])}} = 1 + K[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of a quencher, respectively, and [Q] is the quencher concentration. V can be considered to be a volume element surrounding the fluorophore within which quenching is instantaneous and K is a collisional quenching

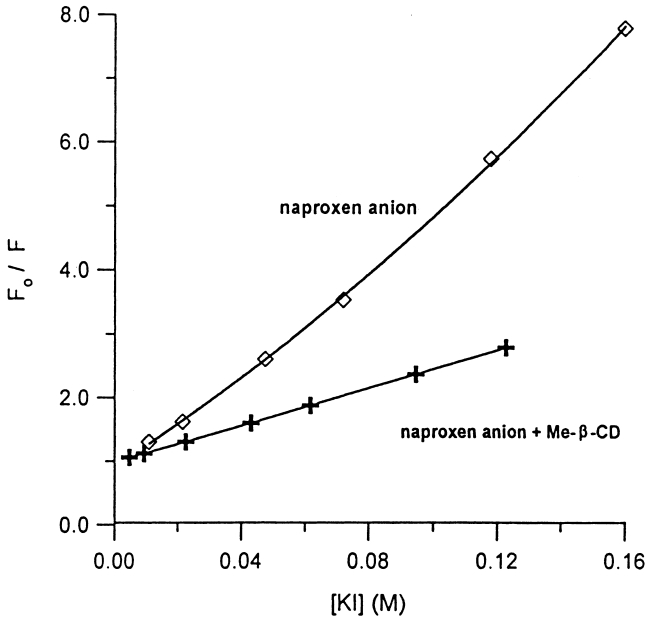

Fig. 2. Stern–Volmer plots for quenching of naproxen anion (6×10^{-5} M) by KI in water at 35°C, with and without 3 mM Me-β-CD.

constant. Both V and K were treated as floating parameters and were determined by the non-linear regression method included in the STATGRAPHICS package [14].

In the solutions with an addition of a CD we have two fluorescing species: free and complexed naproxen molecules. In the solutions containing 3 mM β-CD, the concentrations of naproxen complexed was 65% for the anion and 85% for the acid, in solutions containing Me-β-CD the corresponding values were 68 and 95% (the values were calculated from the known association constants of the two naproxen forms with the CDs [1]). The quenching parameters of the complex (bound form) were calculated from data obtained for the solutions containing naproxen and a CD, by using the following form of the Stern–Volmer equation [2]:

$$\frac{F}{F_0} = \frac{f_f}{(1 + K_f[Q])\, e^{V_f[Q]}} + \frac{f_b}{(1 + K_b[Q])\, e^{V_b[Q]}} \quad (2)$$

where f_f and f_b are the fractional contributions of free and bound naproxen, respectively, to the total fluorescence in the absence of quencher; K_b and V_b are the Stern–Volmer quenching constant and volume element, respectively, for bound naproxen. The values f_f and f_b were estimated from the relative emission intensities of the solutions used for quenching experiments and K_f and V_f were obtained from Eq. (1). The remaining unknown, K_b and V_b, were determined by fitting the quenching data to Eq. (2) by using a non-linear least-squares procedure.

The results of the calculations of quenching parameters for free naproxen in acidic and anionic forms and their complexes with the two CDs are given in Table 1. It can be seen that the values of quenching constants K are for acrylamide several times larger than those for KI, whereas in the case of indole, the analogous constants are very close (33 and 31 M^{-1}, respectively [7]). Quenching constants for the acidic form of naproxen with KI could not be measured because KI is readily oxidized in the acidic medium. Acrylamide is more effective in the steady-state fluorescence quenching of naproxen than of indole.

The parameter V is smaller for the complexed naproxen than for its free form. This result is consistent with the fact that the quenching curves for the free compound analyzed in terms of Eq. (1) show the greater deviation from linearity than that of the corresponding curves for the mixture of both forms (Fig. 2). For complexes the S–V plots (Eq. (1)), are in fact rectilinear. Parameter V is proportional to a "sphere of action", v: $V = Nv/1000$, where N is Avogadro's number. These quencher molecules which are contained within volume v around a fluorescing molecule, quench it immediately. The reasons why V is equal or close to zero are not quite clear. Perhaps, for the quenching process to be effected, a fluorophore should be in a closer contact with the quencher when the excited fluorophore wave function is shielded inside a cyclodextrin cavity. For example, values of V equal to 3.8, 2.6, 1.7 and 1 M^{-1} correspond to the sphere-of-action radii 11.4, 10.1, 8.8 and 7.3Å, respectively. In the paper of Örstan and Ross [2] on the quenching of indole fluorescence, the V value for indole bound to β-CD is also smaller than the value for free indole, 1.7 and 2.5 M^{-1}, respectively. It is also possible that V has more a mathematical than a physical meaning, serving as a convenient parameter to decribe the functional dependence of fluorescence on quencher concentration.

Table 1
Parameters of modified Stern–Volmer equation for quenching of free naproxen and for its cyclodextrins complexes with acrylamide and KI (values for complexes calculated by using Eq. (2))

	Acrylamide		KI	
	V (M^{-1})	K (M^{-1})	V (M^{-1})	K (M^{-1})
Anion	1.7	55	3.8	23
Anion-β-CD	1	13	2.6	6.5
Anion-Me-β-CD	0	8.9	1.1	9.1
Acid	1.7	63		
Acid-β-CD	1	14		
Acid-Me-β-CD	0	7.4		

In order to get more insight into the quenching processes, we measured the fluorescence decay curves for the solutions containing: (A) naproxen acid and naproxen anion in water; (B) naproxen acid and naproxen anion with the additions of 3 mM of β-CD or Me-β-CD; (C) as in (A) with the addition of 0.1 M KI or 0.056 M of acrylamide; (D) as in (B) with the addition of 0.1 M KI or 0.056 M of acrylamide. The decay curves for naproxen acid itself and naproxen acid containing 0.056 M acrylamide (solutions A and C) are shown in Fig. 3a, whereas the decay curves for the same solutions containing β-CD (solutions B and D) are shown in Fig. 3b.

For naproxen acid and naproxen anion in water in the absence of cyclodextrins and in the presence and absence of quenchers (solutions A and C) the decays could be fitted with a single exponential function adequately fulfilling the fitting criteria. On the other hand, all fluorescence decays with cyclodextrins without the quencher added (solution B) required a risetime component for adequate fits to be obtained. The risetime amplitude was always much lower than that for the decay time. After adding the quencher to naproxen solutions with cyclodextrins (solutions D), the risetime component practically disappeared and the decays could be fitted with the single exponential function (except for naproxen acid with Me-β-CD and acrylamide).

The decay curves in solutions containing quenchers, reported previously for a number of substances, could not be represented as single-exponential. They were interpreted as two-exponential decays or in some cases, e.g. quenching of indole with iodide, a more complex than a one-exponential decay behavior was attributed to transient effects, which according to Smoluchowski are of the form $\exp(-t/\tau - 2bt^{1/2})$ [7].

The decay curves for all naproxen solutions were also analyzed for lifetime distributions by using the exponential series method (ESM). The results of ESM lifetime distribution analysis are shown in Fig. 4a and b. These solutions

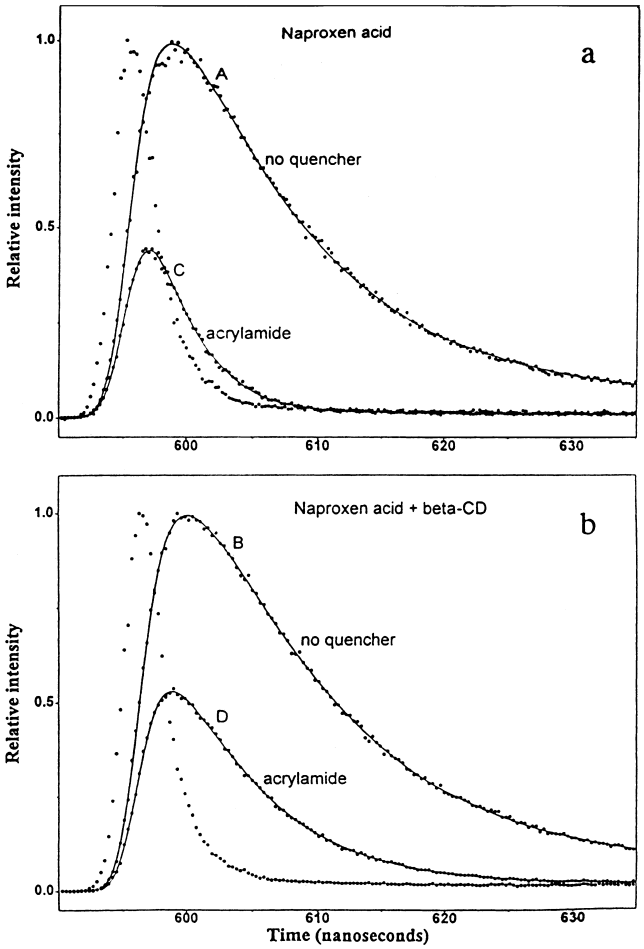

Fig. 3. Fluorescence decays of naproxen acid in water (a) and in the presence of 3 mM β-CD (b). Curves A and B measured in the absence of quencher and C and D in the presence of 0.56 M acrylamide.

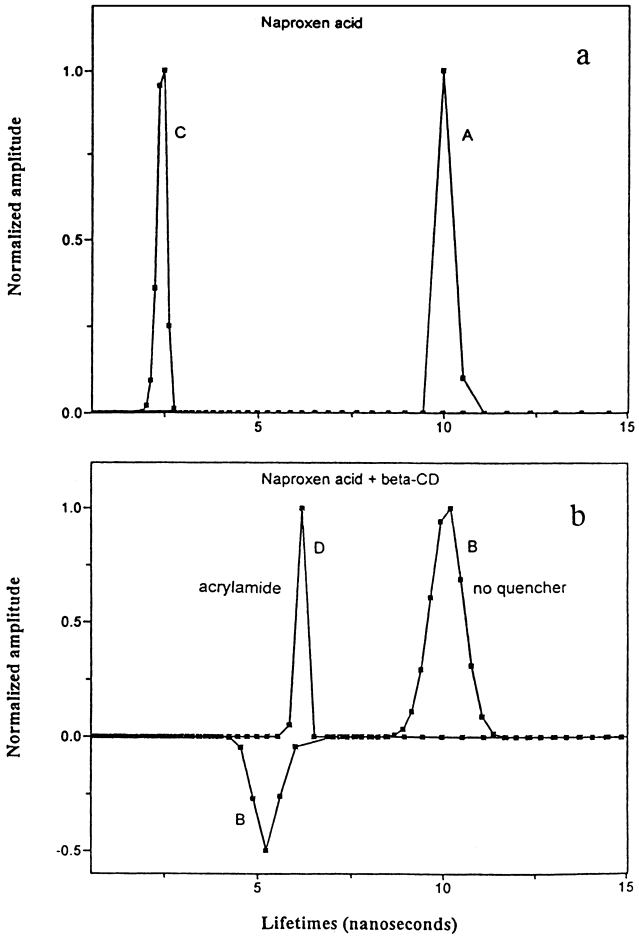

Fig. 4. Fluorescence lifetime distributions recovered by the ESM from the decays depicted in Fig. 3: (a) naproxen acid in water in the absence (curve A) and presence (curve C) of 0.056 M acrylamide; (b) naproxen acid in water with 3 mM β-CD in the absence (curve B) and presence (curve D) of 0.056 M acrylamide.

were the same as in Fig. 3a and b. For naproxen acid (solution A, Fig. 4a), naproxen acid with acrylamide (solution C, Fig. 4a) and naproxen acid with β-CD and acrylamide (solution D, Fig. 4b), there are single distribution peaks, whereas for naproxen acid with β-CD (solution B) a negative peak corresponding to the risetime component is evident. The ESM lifetime distribution analysis with no a priori model assumptions has resulted in distributions which are in agreement with the results employing discrete fitting functions, i.e. single distribution peaks are observed for all solutions A, C and D and double distributions with one negative peak have been obtained for solutions B. The origin of the fractional risetimes in the 1-to-4 exponetial fit and of negative peaks in the ESM lifetimes distribution analysis is not quite clear. One possibility is that they reflect a change in the equilibrium constant for the naproxen–cyclodextrin complex in the excited state. Further work is currently underway to elucidate this phenomenon.

In order to investigate the influence of complexation with β-CD and Me-β-CD on the fluorescence decay lifetimes of naproxen anion and naproxen acid, the results for solutions A and B are compared in Table 2. It can be seen that the lifetimes of both naproxen forms changed only little after addition of either of the CD to the solution. We believe that in the solutions containing cyclodextrins the measured lifetime

Table 2
Influence of naproxen complexation with β-CD and Me-β-CD on its fluorescence lifetime[a]

	τ_f	τ_{f+b}	τ_b (calculated)
Anion	9.1 (9.2)		
Anion + β-CD		10.3 (10.0)	10.6
Anion + Me-β-CD		10.2 (10.0)	10.5
Acid	10.0 (10.0)		
Acid + β-CD		10.3 (10.1)	10.3
Acid + Me-β-CD		11.6 (10.9)	11.7

[a] Concentrations of both cyclodextrins were 3 mM. τ_f: lifetime of a free molecule; τ_b: lifetime of the molecule complexed; τ_{f+b}: lifetime measured in solution containing both forms. All lifetimes in nanoseconds. Left values denote the results of 1-to-4 exponential fit; values in parentheses denote the results of ESM lifetime distribution analysis.

Table 3
Fluorescence lifetimes of naproxen, free and complexed, in the presence of two quenchers, acrylamide and KI[a]

	Acrylamide		KI	
	τ_f	τ_b	τ_f	τ_b
Anion	2.26 (2.3)		2.25 (2.3)	
Anion-β-CD		6.3 (6.3)		5.5 (5.4)
Anion-Me-β-CD		7.7 (7.7)		6.4 (6.4)
Acid	2.39 (2.41)			
Acid-β-CD		6.1 (6.1)		
Acid-Me-β-CD		8.1 (7.8)		

[a] τ_f: lifetime of a free molecule; τ_b: lifetime of the molecule complexed. All lifetimes in nanoseconds. Left values denote the results of 1-to-4 exponential fit; values in parentheses denote the results of ESM lifetime distribution analysis.

τ_{f+b} is a weighted average of both component lifetimes, $\tau_{f+b} = \langle\tau\rangle = f_f\tau_f + f_b\tau_b$, where τ_f is a free molecule lifetime, τ_b is the lifetime of the molecule bound to CD and f_f and f_b are the respective fractions of free and bound naproxen which are known from the previous work [1]. Apparently, the lifetimes were too close to be resolved as two distinct components. However, the lifetime of CD-bound naproxen can be calculated from the above expression for the average lifetime. The calculated lifetimes for complexed naproxen, τ_b, are given in the last column of Table 2. As can be seen, the ratio of bound and free naproxen lifetimes does not exceed 1.2, whereas for indole with β-CD it was 1.49 [2].

The corresponding lifetime results for solutions C and D are given in Table 3. Here it was assumed that the lifetimes found for the decays in solutions D represent the lifetimes of quenched complexes and that the shorter lifetimes of free naproxen in the presence of a quencher are masked by fluorescence risetimes, which were found in solutions B. Data of Tables 2 and 3 were used for the calculation of bimolecular quenching constants [13]. If the lifetimes of a species with and without a quencher (τ_q and τ_0) are known, the bimolecular quenching constant can be calculated from the expression $k_q = (1/\tau_q - 1/\tau_0)/[Q]$. As τ_0, the values from the first and third columns of Table 2 were extracted, whereas values from Table 3 were used as τ_q. The results are given in Table 4 as k_q (I).

In the next column of Table 4, values of the bimolecular quenching constant obtained from the slopes of modified Stern–Volmer plots, $K = k_q\tau_0$, are presented as k_q (II). The agreement of the two values of k_q is considered satisfactory and the suggestion that the single lifetimes observed for the naproxen solution with CDs and acrylamide or iodide are the lifetimes of the complex, seems to be validated.

Theoretical values of bimolecular quenching of naproxen anion and acid and their complexes are also given in Table 4. They were evaluated with the use of the equation given by Ware and Novros [15], accounting for non-stationary diffusion. In the low quencher concentration regime the quenching constant can be expressed as follows:

$$k_q = 4\pi RD\left[1 + \frac{R}{\sqrt{D\tau_0}}\right] \quad (3)$$

where D is the sum of the diffusion coefficients of two colliding molecules and R is the collision radius. The radius of naproxen, 3.85 Å, was calculated from the molecule volume (238 Å^3) obtained by semiempirical modeling [16]. The radius of β-CD was calculated as if it were a sphere of volume 1450 Å^3, which was estimated from the molecule dimensions [17]. The volume of the complex V_c was arbitrary taken as 1500 Å^3. The ratio of radii of β-CD-naproxen complex and the free molecule was taken as $(V_c/V)^{1/3} = 1.8$. The corresponding value for the naproxen-Me-β-CD complex was calculated as 1.95. Using Edward's theory [18], Joshi et al. [19] obtained 2.8 and 2.6 Å for the respective radii of iodide ion and acrylamide and a value of 1×10^{-5} cm^2 s^{-1} for the difussion coefficients of both quenchers (at 25°C). These values suggest that the sum of the diffusion coefficients of naproxen and one of the quenchers is approximately 1.8×10^{-5} cm^2 s^{-1}, because diffusion coefficient of naproxen, calculated from the Stokes–Einstein equation is equal to 0.9×10^{-5} cm^2 s^{-1}. Diffusion coefficients of the complexes of naproxen with β-CD and Me-β-CD were calculated (from

Table 4
Bimolecular quenching constants of naproxen and its complexes with acrylamide and KI[a]

	τ_0 ($\times10^{-9}$ s)	τ_q ($\times10^{-9}$ s)	k_q (I) ($\times10^9$ M^{-1} s^{-1})	k_q (II) ($\times10^9$ M^{-1} s^{-1})	k_q (calculated) ($\times10^9$ M^{-1} s^{-1})	η
Acrylamide						
Anion	9.1	2.26	5.9	6.0	10.2	0.6
Anion-β-CD	10.6	6.3	1.2	1.2	13.2	0.09
Anion-Me-β-CD	10.5	7.7	0.62	0.85	13.9	0.04
Acid	10.0	2.39	5.7	6.3	10	0.6
Acid-β-CD	10.3	6.1	1.2	1.4	13	0.09
Acid-Me-β-CD	11.6	8.1	0.68	0.63	13.7	0.05
KI						
Anion	9.1	2.25	3.3	2.5	10.5	0.3
Anion-β-CD	10.6	5.5	0.88	0.61	13.3	0.07
Anion-Me-β-CD	10.5	6.4	0.61	0.87	13.9	0.04

[a] k_q (I) calculated from the lifetimes without (τ_0) and with the quencher (τ_q); k_q (II) determined from parameter K (from Table 1) of the Stern–Volmer plot (Eq. (1)); k_q (calculated) was calculated from Eq. (3). η is quenching efficiency.

Stokes–Einstein equation) as 0.45×10^{-5} cm^2 s^{-1} and 0.42×10^{-5} cm^2 s^{-1}, respectively. So D, the sum of diffusion coefficients of the complexes and one of the quenchers is equal to 1.45×10^{-5} cm^2 s^{-1} and 1.42×10^{-5} cm^2 s^{-1}. Collision radii R for naproxen, naproxen-β-CD and naproxen-Me-β-CD with acrylamide are 6.45 (3.85 + 2.6), 9.53 (6.93 + 2.6) and 10.1 (7.5 + 2.6) Å, respectively. The corresponding collision radii with KI are larger by 0.2 Å.

Efficiences η were obtained by dividing k_q (I) by the theoretical values and are given in the last column of Table 4. It can be seen that efficiencies of quenching of naproxen acid and its complexes are actually the same as of naproxen anion and its complexes. The quenching efficiency of free naproxen in acidic and anionic form with acrylamide is 0.6, whereas with KI it is 0.3. The efficiency of quenching of naproxen anion by iodide is lower than that of quenching by acrylamide. The result appears to be just as expected because naproxen anion and iodide have the same electrostatic charge and the resulting electrostatic repulsion is not accounted for by Eq. (3). For the complexes of naproxen anion with β-CD and Me-β-CD, this electrostatic repulsion has less influence on η. It can also be seen that naproxen in the complex with Me-β-CD, either in the anionic or in the acidic form, is better protected against quenching than in the complex with β-CD.

Complexation with CDs does not necessarily result in the fluorophores protection against quenching. It was found that the quenching of naphthalene fluorescence by trimethylaniline was more efficient in the solution containing β-CD [20], β- and γ-CD accelerated the fluorescence quenching of naphthalene and pyrene by aliphatic amines because of the formation of the three-component complexes of fluorophore, quencher and cyclodextrin [21].

4. Conclusions

The steady-state quenching experiments were interpreted with the use of the Stern–Volmer equation. β- and Me-β-cyclodextrins protected naproxen in the acidic and anionic forms against quenching by acrylamide and KI. For solutions containing cyclodextrins the modified S–V equation with two terms: one for the free molecule and one for the molecule complexed with a cyclodextrin, was used and S–V parameters were calculated separately for both species. Fluorescence decay constants obtained from a discrete 1-to-4 exponential fit and those determined with the use of the exponential series lifetime distribution analysis were actually the same. Both methods revealed the presence of fluorescence risetimes in the solutions of naproxen with CDs. The processes responsible for the risetimes will be examined in the forthcoming study. Bimolecular quenching rate constants were determined for the free and complexed molecules; the values obtained from the S–V equation were consistent with those determined from the lifetimes in the presence and absence of the quenchers. Quenching efficiencies were obtained by comparing these experimental values with those calculated by means of Eq. (3). For the complexed molecules they were considerably lower than for free naproxen in the solution. The quenching efficiencies revealed that the accessibility of the complexed molecules to solvent/quenchers is lower than that of the free molecules.

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