Journal of Inclusion Phenomena and Macrocyclic Chemistry
https://doi.org/10.1007/s10847-018-0874-1

ORIGINAL ARTICLE

Inclusion complex of Tramadol in β-cyclodextrin enhances fluorescence by preventing self-quenching

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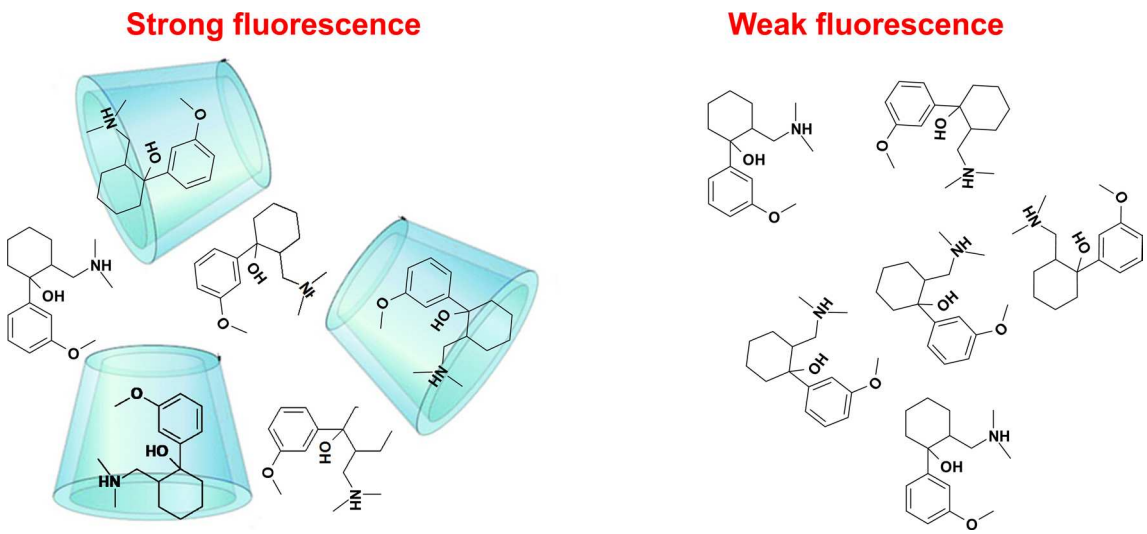
Received: 14 June 2018 / Accepted: 12 December 2018

Abstract

Fluorescence self-quenching occurs at high concentration. Inhibition of self-quenching by inclusion of fluorescence emitters inside the hydrophobic cavity of β-cyclodextrin (β-CD) has been addressed taking the example of the fluorescence behavior of Tramadol hydrochloride. Indeed complexation by β-CD enhanced fluorescence emission of Tramadol under conditions where self-quenching was operative. A quantitative account of self-quenching and its inhibition by β-CD was done through determination of complexation equilibrium by ^{1}H NMR experiments and a detailed study of absorption and fluorescence properties. Tramadol and β-CD associate as a complex of 1:1 stoichiometry with a formation constant $K_{11} = 260$. Complexation of Tramadol by β-CD does not cause modification of its absorbance and fluorescence spectra. Fluorescence self-quenching of Tramadol above ~ 1 mmol·L^{-1} was characterized by a Stern–Volmer constant $K = 810$ L·mol^{-1}. Inhibition of self-quenching by formation of an inclusion complex was manifested by lower Stern–Volmer constants in the presence of β-CD. Such study required a correct account of Inner Filter Effects on fluorescence, which is mandatory in all physicochemical studies using fluorescence where concentrations are rather high.

Graphical abstract



Keywords Fluorescence · β-Cyclodextrin · Tramadol · Quenching

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10847-018-0874-1) contains supplementary material, which is available to authorized users.

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Published online: 15 December 2018

Introduction

Enhancement of fluorescence emission is often observed for dyes in confined media, either immobilized in their solid state or bound as complex species with polymers, proteins or cyclodextrins (CDs). Fluorescence enhancement by CDs with respect to that of free dye can be related to several physicochemical phenomena, namely polarity of solubilization site that changes the absorption spectrum, restricted mobility that prevents non-radiative decay of fluorescence, inhibition of quenching by restricted accessibility of the quenchers [1–3]. It is generally difficult to figure out which phenomenon is responsible for enhanced emission. Such complex issues call for quantitative investigations of each phenomenon. The present work aims at a quantitative study self-quenching and its inhibition by complexation by β-CD.

High fluorescence enhancements have been disclosed when the local environment of the dye restricts its mobility. Aggregation-induced emission is the most powerful mechanism, especially when intramolecular rotations cause fluorescence decay [4]. Crystallization causes a strong fluorescence enhancement by factor of hundreds is such cases. Binding to polymers and proteins also causes similar effects [5, 6]. Formation of inclusion complex with cyclodextrins yields similar fluorescence enhancement when the fluorophore is involved in complexation. As example, Xu et al. reported a 30-fold enhancement for a pseudorotaxane of a salicylaldehyde azine bolaform surfactant with γ-cyclodextrin [7]. Fluorescence enhancement is weaker when the complexation site with CD is taken apart from the fluorescent part of the dye. As example, complexation of a four-arms pentaphenylmethylsilole by β-CD led to a twofold increase of fluorescence because complexation took place on the poly(propylene oxide) arms instead of the dye itself [8]. Apart from the fluorescence enhancement driven by restricted mobility, another mechanism is inhibition of quenching. Thus, complexation of the fluorescent species by β-CD makes a steric barrier against close proximity to quenchers [9–14]. A related phenomenon that did not receive attention so far is self-quenching that takes place at high concentrations of fluorescent molecules. The present work addresses this latter point using Tramadol hydrochloride as a fluorescent dye.

Tramadol is a synthetic analgesic opioid [15, 16] classified at level two of painkillers category; it can be used in case of acute pains (e.g. after surgery) and chronic pains (as in back injury or cancer). Tramadol, (2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol), is a racemic mixture of two enantiomers (Fig. 1), enantiomer (1*R*,2*R*)-(+) being the most active [17]. Tramadol has a low affinity for μ receptors and almost negligible affinity

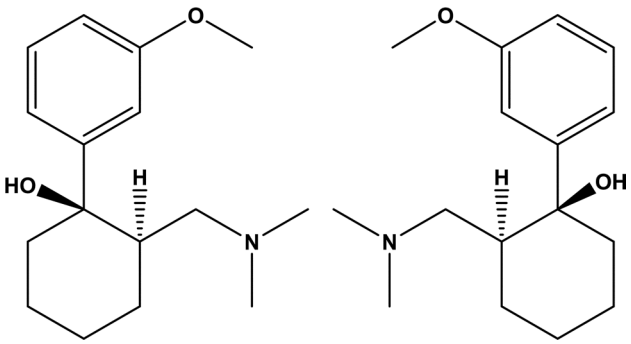

Fig. 1 Structure of enantiomers (1*R*,2*R*)-Tramadol (left) and (1*S*,2*S*)-Tramadol (right)

for other receptors of endogenous opioids (Kappa, Delta) because of the presence of a methyl radical on the phenyl ring [18]. Its main mechanism of analgesic activity is inhibition of re-uptake of serotonin and noradrelaline in the dorsal raphe nucleus [17, 19]. The main metabolite of its biotransformation is *O*-desmethyltramadol (*O*-DSMT) of analgesic potential 2 to 4 times higher than Tramadol; the affinity of this latter compound for μ receptors is 200 times stronger than Tramadol [17].

Cyclodextrins form inclusion complexes with a large variety of organic substances, either in solution or in solid state [20, 21]. Drug solubility enhancement by formation of inclusion complex makes CDs quite useful as solubilizing agents in pharmaceutical dosage forms [20]. Besides, complexation inside CD cavity allows modulating drug bioavailability [22]; CDs are useful excipients in formulations [23, 24]. It is worth noticing that the concentration range of the present work is different of therapeutic doses, so that pharmaceutical relevance is low; the present purpose is to investigate photochemistry. β-CD does form inclusion complexes with Tramadol [25]. Owing to the p*K*a of Tramadol hydrochloride of 9.488 [26], it is under its cationic form when it is in aqueous solution with no buffer.

The present work aims at investigating fluorescent properties of Tramadol undergoing inclusion complexation with β-CD so as to reach a quantitative account of them.. For that purpose, the complexation of Tramadol by β-CD was first studied by means of ^{1}H NMR experiments in aqueous solution; UV–vis absorption and fluorescent properties of Tramadol were studied in a second part; lastly, the influence of inclusion complexation by β-CD was addressed in dilute solutions where no significant quenching occurred and under conditions of higher concentration where self-quenching actually took place. Fluorescence at high concentration requires a correction for absorbance known as Inner Filter Effects that was implemented in the theoretical account of quenching inhibition. This part provides a methodology for studying fluorescence phenomena at high

concentrations where high absorbance weakens fluorescence emission. The paper is constructed as follows: (i) Determination of complexation constant of Tramadol with β-CD; (ii) Evidence of fluorescence self-quenching of Tramadol at high concentrations; (iii) Inhibition of self-quenching by inclusion complexation with β-CD.

Materials and methods

Materials

Tramadol hydrochloride of purity > 99% was purchased from Sigma–Aldrich and used as received. β-Cyclodextrin was provided by Roquette Frères (Lestrem, France) as Kleptose™ and used as received. Deionized water of 18 MΩ cm resistivity was used for the preparation of all solutions. Inclusion complexes were prepared by simple mixing aqueous solutions of Tramadol and β-CD; they were used for experiments after equilibration overnight.

Methods

All experiments were performed at 20 °C which was the regulated temperature of rooms where instruments were installed.

UV absorbance measurements were carried out in Hellma quartz cells of 10 mm and 1 mm optical path with a Varian spectrophotometer Cary®50 in the wavelength range 200–400 nm. The spectrum of pure water was subtracted as background.

Fluorescence measurements were performed using an Agilent Cary Eclipse Spectrophotometer. Excitation wavelength was at maximum absorbance $\lambda = 271$ nm of Tramadol. Fluorescence emission was recorded in the 280–500 nm range. Excitation and emission slit widths giving a resolution of 2.5 nm allowed measurements over the whole concentration range. 10×10 mm^2 square quartz fluorescence cuvettes were used for all measurements. Because of the high absorbance of concentrated solutions, strong Inner Filter Effect was expected and it has to be accounted for. Detailed presentation of IFE, its calculation, and an experimental validation are given in Supplementary Material file. The normalization factor for different recording conditions was measured from the Rayleigh scattering peak of the Raman O–H stretching mode of pure water appearing at 301 nm for an excitation at 271 nm. It is noted that the Raman scattering line of water was small with respect to the fluorescence of Tramadol, even for the most dilute samples. The fluorescence intensity was nevertheless corrected for this Raman O–H band; and it was normalized to it as $I(\text{normalized}) = [I(\text{sample}) - I(\text{H}_2\text{O})]/I(\text{H}_2\text{O})$. Fluorescence intensities are given in units of the Raman scattering line of a 1 cm thick sample of pure water thereafter called "H_2O units".

^{1}H NMR spectra of solutions in D_2O were recorded with a Bruker DRX500 spectrometer operating at 500 MHz Larmor frequency. The chemical shift reference was the NMR line of HDO at 4.70 ppm. The assignment of the ^{1}H NMR lines of Tramadol was achieved using a special 2D-NMR method as given in Supplementary Material file.

Results and discussion

Formation of inclusion complexes of Tramadol and β-CD

^{1}H NMR spectra were recorded for several mixed solutions of Tramadol and β-CD in D_2O by varying the concentration of β-CD at constant concentration of Tramadol. Chemical shifts variations of both Tramadol and β-CD lines with respect to the concentration of β-CD were analyzed for the stoichiometry of the complex and the affinity constant.

The assignment of ^{1}H NMR lines of β-CD is well-known [27]. Lines of Tramadol have been partially assigned by Smyj et al. [28]; their full assignment was done with the help of HSQC-TOCSY experiments given in Supplementary Material. Spectral assignments given in Fig. 2 and Table S1 are in agreement with those reported by Smyj et al. [28]; supplementary assignments of protons of the cyclohexyl ring are specified.

A series of ^{1}H NMR spectra of Tramadol/β-CD mixtures were recorded in D_2O solution at constant concentration of Tramadol hydrochloride (1 mmol·L^{-1}). β-CD concentrations varied from the 1/10 to the 10/1 β-CD/Tramadol mole ratios. All NMR lines shifted as a function of the concentration of β-CD. Tramadol is a racemic mixture of two optical isomers that could bind differently to β-CD. There was no indication for enantiomeric separation of Tramadol optical isomer by β-CD in NMR spectra. NMR lines of both Tramadol and β-CD showing the largest shifts were selected for analysis of complexation equilibrium. The lines of the β-CD protons H3 and H5 shifted the most because these protons are located inside the cyclodextrin hydrophobic cavity where invited molecules enter. A larger shift of protons H3 and H5 is considered as a strong indication of formation of an inclusion complex [29, 30]. The well-resolved lines of Tramadol showing largest shifts were in decreasing order, NCH_3 and NCH_2b in the vicinity of the tertiary ammonio group, protons 3a of the cyclohexyl ring, OCH_3 attached to the aromatic ring, protons H6′ and H5′ of the aromatic ring, protons 2 and 4a of the cyclohexyl ring, and protons H2′ and H4′ of the aromatic ring. Typical spectra shown in Fig. 3 give an illustration of spectral shifts.

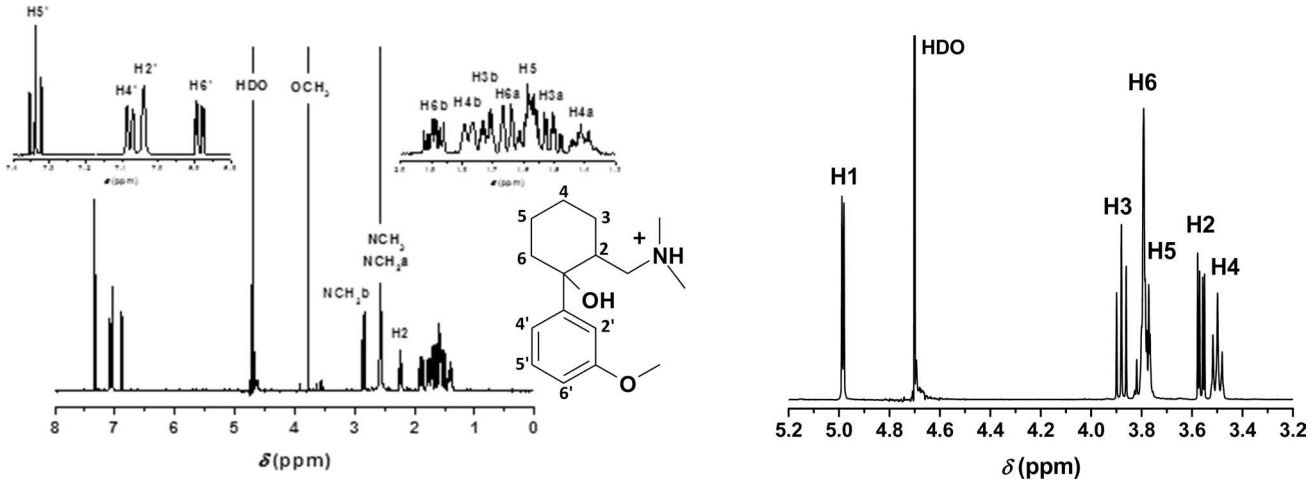

Fig. 2 ^{1}H NMR spectra and assignment of NMR lines of Tramadol and β-cyclodextrin

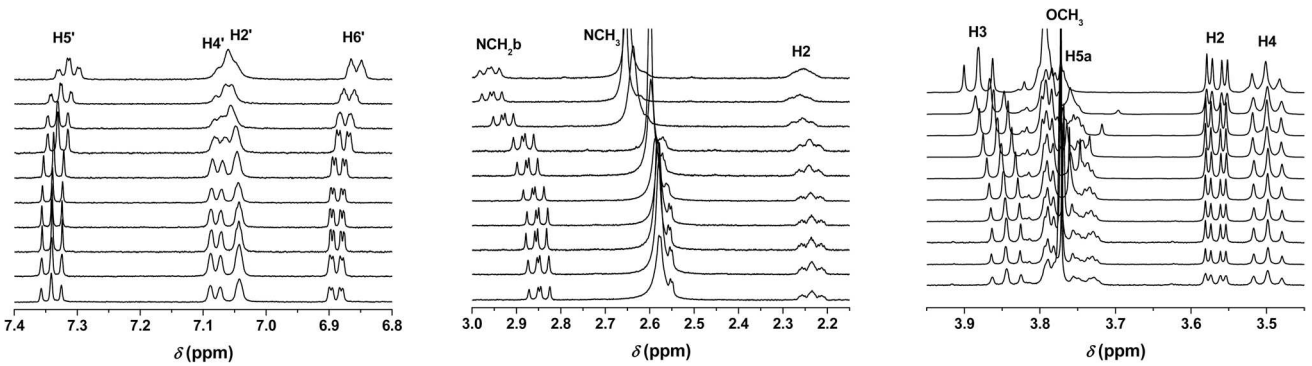

Fig. 3 Variation of ^{1}H NMR spectra with respect to the β-CD/Tramadol mole ratio increasing from bottom to top between 1/10 and 10/1. The spectrum at the bottom is that of pure Tramadol on the left and central series of stacked spectra; the spectrum at the top is that of pure β-CD on the right series of stacked spectra

A simple classical model of complexation with a 1:1 stoichiometry could account for the ^{1}H NMR data. Therefore it did not make sense to consider more elaborated models of complexation equilibria including formation of a β-CD–Tramadol complex of other stoichiometry. The present NMR measurements did not confirm the claim by Anton Smith et al. [25] for a complex of 2:1 stoichiometry inferred on the basis of fluorescence experiments.

The chemical equilibrium and corresponding mass action law of the model are given by Eq. 1 that takes concentrations in place of activities by assuming ideal behavior:

$$\mathrm{A} + \mathrm{CD} \rightleftarrows \mathrm{A\text{-}CD} \quad K_{11} = \frac{[\mathrm{A\text{-}CD}]}{[\mathrm{A}][\mathrm{CD}]} \tag{1}$$

where A stands for Tramadol. The total concentrations of Tramadol and β-CD are:

$$[\mathrm{A}]_0 = [\mathrm{A}] + [\mathrm{A\text{-}CD}] \tag{2}$$

$$[\mathrm{CD}]_0 = [\mathrm{CD}] + [\mathrm{A\text{-}CD}] \tag{3}$$

The concentrations of free Tramadol, free β-CD, and Tramadol-CD complex were calculated from the values of K_{11}, $[\mathrm{A}]_0$ and $[\mathrm{CD}]_0$ by solving Eqs. 1–3 for [A], [CD], and [A-CD], yielding

$$[\mathrm{A\text{-}CD}] = \frac{\left([\mathrm{A}]_0 + [\mathrm{CD}]_0 + \frac{1}{K_{11}}\right) - \sqrt{\left([\mathrm{A}]_0 + [\mathrm{CD}]_0 + \frac{1}{K_{11}}\right)^2 - 4[\mathrm{A}]_0[\mathrm{CD}]_0}}{2} \tag{4}$$

The ^{1}H NMR chemical shifts of Tramadol and β-CD lines were calculated in the fast exchange limit from the concentrations and chemical shifts of each species as:

$$\text{For Tramadol lines: } \delta = \delta\,(\text{free Tramadol})\frac{[\text{A}]}{[\text{A}]_0} + \delta(\text{A-CD})\frac{[\text{A-CD}]}{[\text{A}]_0} \quad (5)$$

$$\text{For } \beta\text{-CD lines: } \delta = \delta\,(\text{free } \beta\text{-CD})\frac{[\text{CD}]}{[\text{CD}]_0} + \delta(\text{ACD})\frac{[\text{A-CD}]}{[\text{CD}]_0} \quad (6)$$

The chemical shifts of free Tramadol and β-CD were measured with pure compounds. Predictions of chemical shifts were compared to experimental data for Tramadol/β-CD mixtures. The best fit of the model to experimental data was searched for by adjusting unknown parameters K_{11} and δ(A-CD) for each NMR line (Fig. 4) through minimizing the sum of the root mean square deviations of all NMR lines of Tramadol and β-CD: The minimizing function accounting at the same time for all NMR lines ($i = 1$ to N_i) and sample concentrations ($j = 1$ to N_j) reads as.

$$\sqrt{\frac{1}{N_i N_j}\sum_{i=1}^{N_i}\sum_{j=1}^{N_j}\left(\delta_{\text{exp},i,j} - \delta_{\text{calc},i,j}\right)^2} \quad (7)$$

The good fit to experimental data for all NMR lines over the whole concentration range showed that the complex was of 1:1 stoichiometry with no significant contribution of other complex species. The equilibrium constant is $K_{11} = 260 \pm 10$ at 20 °C ($\text{Log}K_{11} = 2.42$) where concentrations are expressed in mol·L^{-1} units. All parameters are given in Table 1.

Owing to such a value of K_{11} and the moderate solubility of β-CD in water (18.5 g L^{-1} = 15 mmol·L^{-1}), complexation of Tramadol cannot reach completion, even in the presence of a maximum concentration of β-CD (saturated solution). Indeed the concentration ratio of complexed to free forms of Tramadol tends to $\frac{[\text{A-CD}]}{[\text{A}]} = K_{11}[\text{CD}]_0$ as the

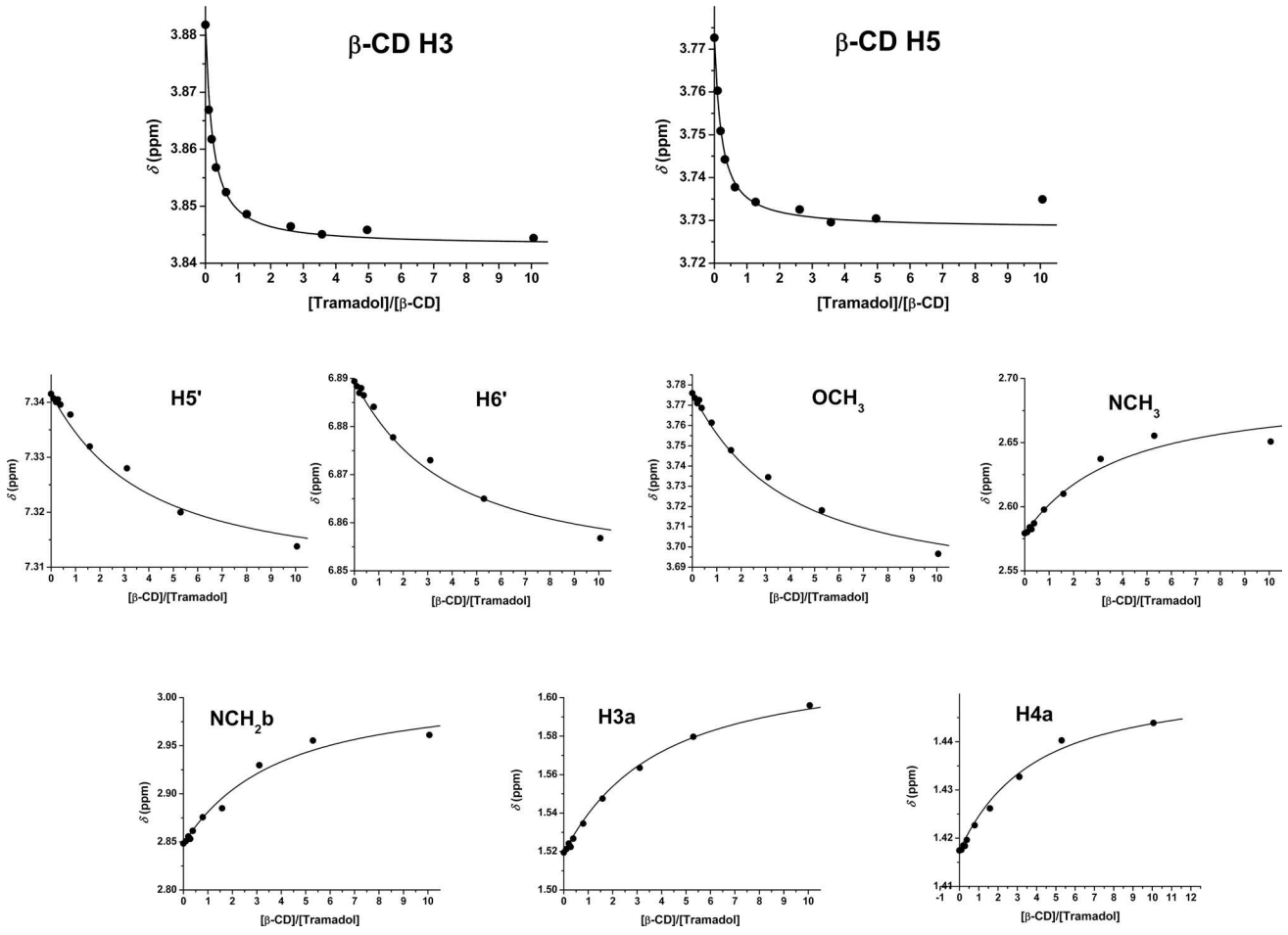

Fig. 4 Variation of ^{1}H NMR chemical shifts with respect to the relative concentrations of Tramadol and β-CD. Solid lines are the best fits of the theory to experimental data for complex formation of 1:1 stoichiometry with the parameters given in Table 1

Table 1 Equilibrium constant K_{11} of the 1:1 complex and values of the ^{1}H NMR chemical shifts of Tramadol and β-CD under their free and complexed forms

	Stoichiometry 1:1 Equilibrium constant $K_{11}=260$ Log(K_{11})=2.42											
	Aromatic protons of Tramadol					Other protons of Tramadol					β-CD	
NMR line	H2′	H4′	H5′	H6′	OCH_3	NCH_2b	NCH_3	H2	H4a	H5a	H3	H5
δ(free) (ppm)	7.043	7.081	7.342	6.889	3.776	2.848	2.579	2.235	1.417	1.519	3.882	3.773
δ(complex) (ppm)	7.068	7.064	7.307	6.848	3.676	3.010	2.690	2.276	1.453	1.619	3.721	3.588

total concentration of β-CD is in large excess with respect to that of Tramadol. Therefore Eq. 1 reduces to

$$\frac{[\text{A-CD}]}{[\text{A}]+[\text{A-CD}]}=\frac{K_{11}[\text{CD}]_0}{1+K_{11}[\text{CD}]_0} \quad (8)$$

Accordingly, the maximum fraction of complexed Tramadol is 0.78 (78%).

Absorbance and fluorescence properties of Tramadol

The absorption spectrum of Tramadol hydrochloride in aqueous solution shows two well-defined bands with maxima at 216 nm and 271 nm wavelength corresponding to π–π* electronic transitions (Fig. 5). The absorbances at maxima increase linearly with respect to the Tramadol concentration according to the Lambert–Beer law and there is no shift of spectra upon changing the concentration. The molar absorptivity (extinction) coefficients are $\varepsilon=7130$ L·mol^{-1}·cm^{-1} at 216 nm and $\varepsilon=1790$ L·mol^{-1}·cm^{-1} at 271 nm, in agreement with previous reports [25, 28].

Fluorescence spectra of Tramadol showed an emission peak with maximum at 297 nm whatever the excitation wavelength. All experiments have been performed for excitation at 271 nm. Excitation at 216 nm yielded the same fluorescence spectrum; its intensity was higher just because absorbance at 216 nm was higher than at 271 nm (Fig. 6). The shape of fluorescence spectra did not change upon varying the concentration of Tramadol. Fluorescence spectra in water and in de-aerated water were identical, showing that

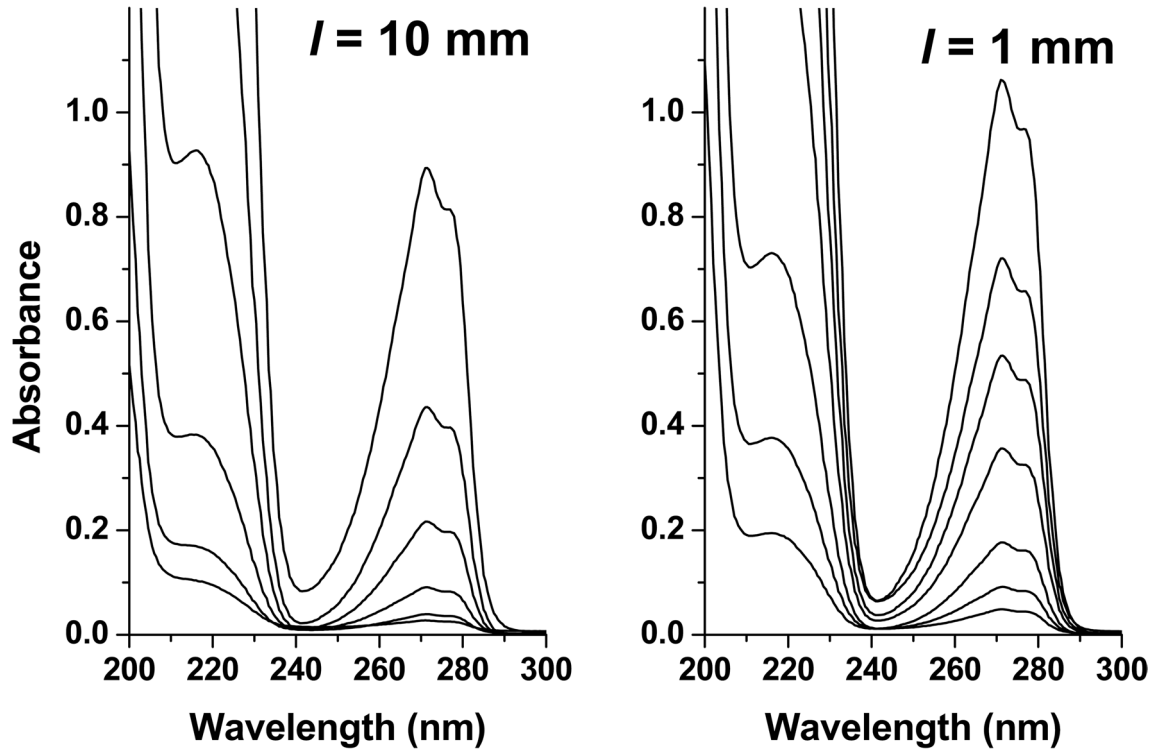

Fig. 5 Absorption spectra of Tramadol at several concentrations. Left: Low concentrations in $l=10$ mm optical path cuvettes; concentrations are in order of increasing absorbance 0.01, 0.02, 0.05, 0.1, 0.25, and 0.5 mmol·L^{-1}. Right: Low concentrations in $l=1$ mm optical path cuvettes; concentrations are in order of increasing absorbance 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, and 6.0 mmol·L^{-1}

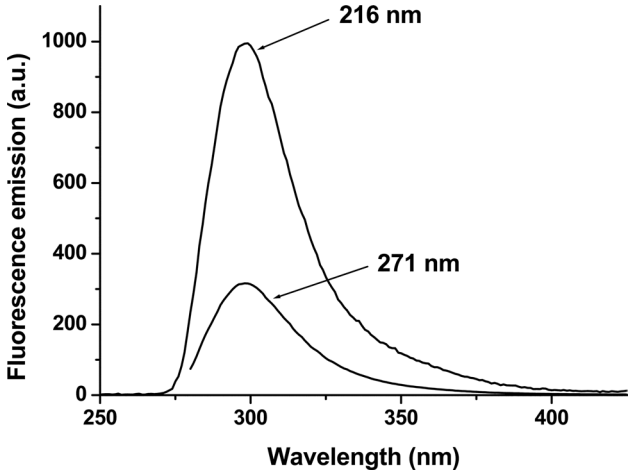

Fig. 6 Fluorescence spectra of Tramadol 0.01 mmol·L^{-1} for excitation wavelengths of 216 nm and 271 nm

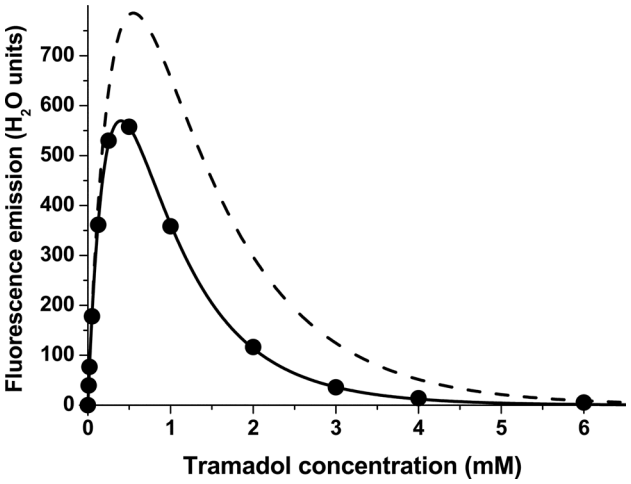

Fig. 7 Fluorescence emission of Tramadol solutions of various concentrations and best fit of Eqs. 10 and 11 to the experimental data using the parameters given in the text (solid line). The dashed line shows the prediction with no self-quenching ($K=0$)

Tramadol fluorescence was not significantly quenched by oxygen.

Fluorescence intensity did not vary linearly with respect to Tramadol concentration (Fig. 7). Fluorescence intensity increased linearly with respect to the Tramadol concentration up to 0.02 mM. Deviation from linearity towards lower emission was observed beyond this concentration and the fluorescence intensity decreased with respect to concentration above 0.5 mM. Indeed this is not expected that fluorescence varies in a linear manner with respect to concentration. If the fluorescence quantum yield does not depend on concentration, fluorescence intensity is proportional to absorbed intensity of excitation light. As absorbance at excitation wavelength, $Abs(\lambda_{ex})$, is proportional to concentration through the Beer–Lambert relationship, absorbed light along the optical path l and total fluorescence intensity at emission wavelength, $Fluo(\lambda_{em})$, are exponential functions of concentration, C, as [31].

$$Fluo(\lambda_{em}) \propto (1 - 10^{-Abs(\lambda_{ex})}) = (1 - 10^{\varepsilon_{ex} l C}) \tag{9}$$

where the prefactor is related to the fluorescence quantum yield and geometrical factors coming from the optical design of the spectrophotometer. Equation 9 reduces to linear behavior in the low concentration limit.

Another cause of deviation from the simple relationship between fluorescence emission and absorption is the attenuation of excitation light by absorption. This effect known as Inner Filter Effect (IFE) is strong for concentrated solutions of high absorbance. As self-quenching takes place for fairly concentrated solutions, fluorescence emission has to be corrected for IFE [32]. The emission beam is attenuated by absorption along its optical path from the entry optical face to the dye emitting fluorescence. This leads to a correction called "primary IFE" (*PIFE*). Emitted light is also attenuated by absorbance at emission wavelength along the optical path from the emitting dye to the exit optical face. This latter correction called "secondary IFE" (*SIFE*) is small because of absorbance at emission wavelength is weak. The fluorescence emission corrected for IFE is given by an approximate equation from by MacDonald et al. [33] (Eq. 10).

$$Fluo(\lambda_e m) = aC \times PIFE \times SIFE = aC \times \frac{10^{-\varepsilon_{ex} C \frac{l}{2}} \left(10^{\varepsilon_{ex} C \frac{\Delta x}{2}} - 10^{-\varepsilon_{ex} C \frac{\Delta x}{2}}\right)}{\ln(10)\varepsilon_{ex} C \Delta x} \times \frac{10^{-\varepsilon_{em} C \frac{l}{2}} \left(10^{\varepsilon_{em} C \frac{\Delta y}{2}} - 10^{-\varepsilon_{em} C \frac{\Delta y}{2}}\right)}{\ln(10)\varepsilon_{em} C \Delta y} \tag{10}$$

where ε_{ex} and ε_{em} are the experimental values of extinction coefficients at excitation and emission wavelengths ($\varepsilon_{ex} = 1790$ L·mol^{-1}·cm^{-1}, $\varepsilon_{em} = 14$ L·mol^{-1}·cm^{-1}), $l = 1$ cm is the full optical path of the square cuvette, and parameters Δx and Δy are slit widths of the excitation monochromator and detector respectively. The prefactor a depends on fluorescence quantum yield and geometrical parameters of the experimental setup. The detailed presentation of the model

for IFE correction together with its domain of validity and an experimental validation are given in the Supplementary Material file.

Evidence of self-quenching

Such effects could not account for the full concentration dependence of fluorescence, so that a supplementary attenuation phenomenon necessarily took place, namely self-quenching. Self-quenching was put forward since larger deviation from calculated fluorescence with respect to experimental data occurred at high concentrations of Tramadol. Possible quenching by chloride counter-ions was discarded since addition of sodium chloride up to 100 mM did not decrease fluorescence emission. Self-quenching could occur by means of static and/or dynamic quenching [34]. Static quenching involves the formation of complex species between fluorescent emitter and quencher that should have been revealed by spectral changes as a function of concentration. Static quenching was discarded because both absorbance and fluorescence spectra did not vary in shape over the whole Tramadol concentration range. Dynamic self-quenching takes place by collisions between Tramadol molecules. It was accounted for by a single Stern–Volmer term characterized by a self-quenching constant K. Such modification of Eq. 10 yields Eq. 11.

$$Fluo\left(\lambda_{\mathrm{em}}\right) = aC \times PIFE \times SIFE \, \frac{1}{1+KC} \tag{11}$$

The best fit to experimental fluorescence data was achieved with $a = 4.17 \times 10^6$ H_2O units·L·mol^{-1}, $\Delta x = 5.8$ mm, $\Delta y = 5.8$ mm, and $K = 812$ L·mol^{-1} (Fig. 7). As expected, the value of a corresponded to the slope at low concentrations. The precise value of Δy did not matter because this parameter had a weak influence on the *SIFE* correction; so Δy was set equal to Δx. As a whole, only Δx and K were adjustable parameters. Removing the Stern–Volmer term in Eq. 11 ($K = 0$) gave the prediction with no self-quenching that was much larger than the experimental data. Self-quenching operating at large Tramadol concentrations is obvious from this analysis of fluorescence measurements.

Influence of complexation by β-CD

Addition of β-CD caused the formation of inclusion complexes as shown by ^{1}H NMR experiments. Whatever the concentration of Tramadol, its UV absorption spectrum did not significantly change upon the formation of their inclusion complex (Fig. 8). Variations of UV absorption were

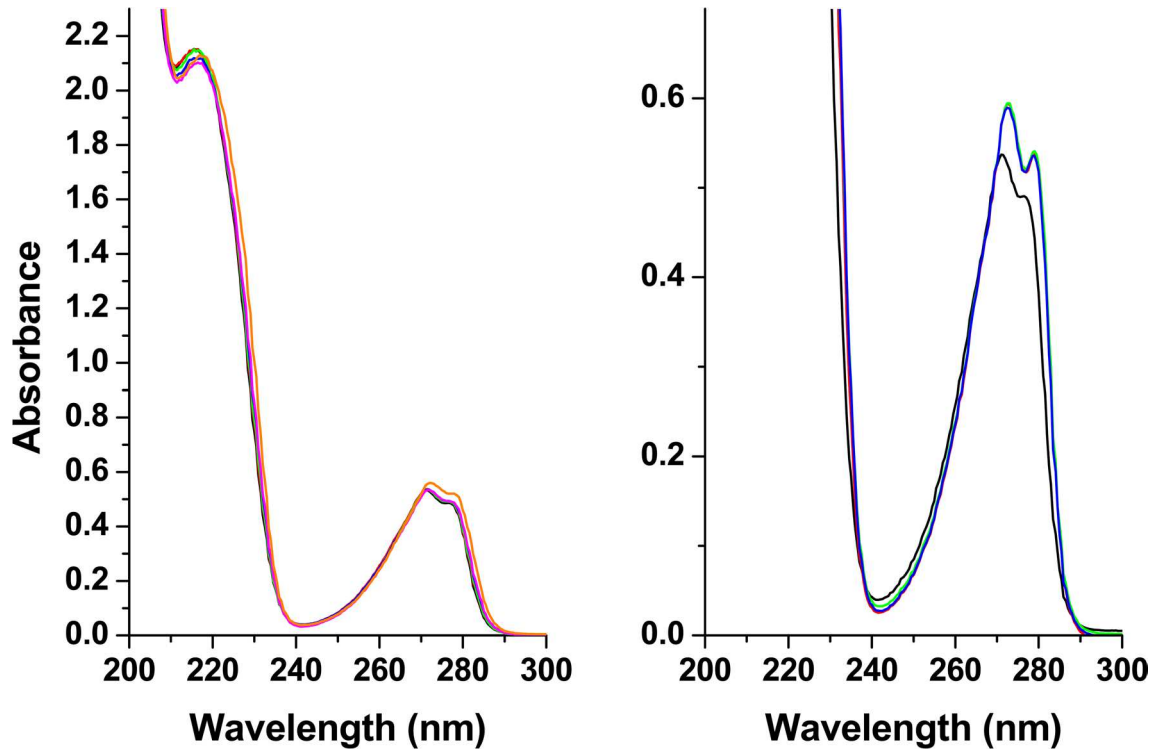

Fig. 8 Left: Absorption spectra of 3.0 mmol·L^{-1} Tramadol solutions in the presence of β-CD at increasing mole ratios [β-CD]/[Tramadol]: 0 (black), 0.25 (red), 0.5 (green), 1.0 (blue), 1.5 (cyan), 2.5 (magenta), 5.0 (orange). Right: Absorption spectra of 3.0 mmol·L^{-1} Tramadol solutions in various solvents: water (black), methanol (red), ethanol (green), and 2-propanol (blue). (Color figure online)

often observed upon complexation by cyclodextrins; they were ascribed to polarity differences between β-CD cavity and water. In the present case, the spectra of free Tramadol and complexed Tramadol were identical for no overall effect of the presence of β-CD was observed. Indeed the UV spectra for the same concentration of Tramadol in water and in organic solvents of various polarities were quite similar in shape and absorbance (Fig. 8). Slight spectral differences were visible: the spectra in methanol, ethanol and 2-propanol were all identical and only differed from that in water by a slight red-shift by 1.5 nm. Therefore, UV absorbance spectra cannot be used for studying complexation of Tramadol by β-CD.

Fluorescence emission spectra of Tramadol showed definite variations in the presence of β-CD. Such enhanced fluorescence was not caused by a higher absorbance of the complex at excitation wavelength since measured absorbance did not vary upon complexation. There was no change of the shape of the fluorescence spectra but the emission intensity did increase and fluorescence enhancement depended on Tramadol concentration. In the dilute regime where self-quenching was negligible, formation of an inclusion complex required a very large excess of β-CD (a solution close to its saturation limit). As example, for a Tramadol concentration of 0.01 mM and a 10-fold excess of β-CD, only 3% of Tramadol is involved in an inclusion complex with β-CD. As previously inferred in section "Formation of inclusion complexes of Tramadol and β-CD", higher concentration of β-CD close to its solubility led to the maximum formation of inclusion complexes up to 78%. Figure 9 shows the fluorescence emission of Tramadol in the presence of a large concentration of β-CD (12 mM) compared to that of pure Tramadol. The fraction of complexed Tramadol was the same along the whole series since the concentration of β-CD was constant and in excess with respect to Tramadol. For [β-CD] = 12 mM, it was 76% (Eq. 8). Inclusion of Tramadol inside the cavity of β-CD increased its emission intensity in the concentrated regime only. Because enhanced fluorescence was observed for Tramadol concentrations high enough for fluorescence self-quenching significantly took place, it was concluded that inclusion in the β-CD cavity did not cause significant change of fluorescence quantum yield, but prevented self-quenching by taking excited Tramadol molecules apart from each other.

The same analysis of fluorescence data as for pure Tramadol was done for Tramadol solution in the presence of β-CD that contains two species emitting fluorescence: free Tramadol (A) and complexed Tramadol (A-CD). A global model was devised where both free and complexed Tramadol experienced quenching by free and complexed Tramadol. The concentrations of free and complexed Tramadol, [A] and [A-CD], were calculated from Eq. 4 using the equilibrium constant $K_{11} = 260$ as determined by NMR experiments. The variation of fluorescence emission can be calculated

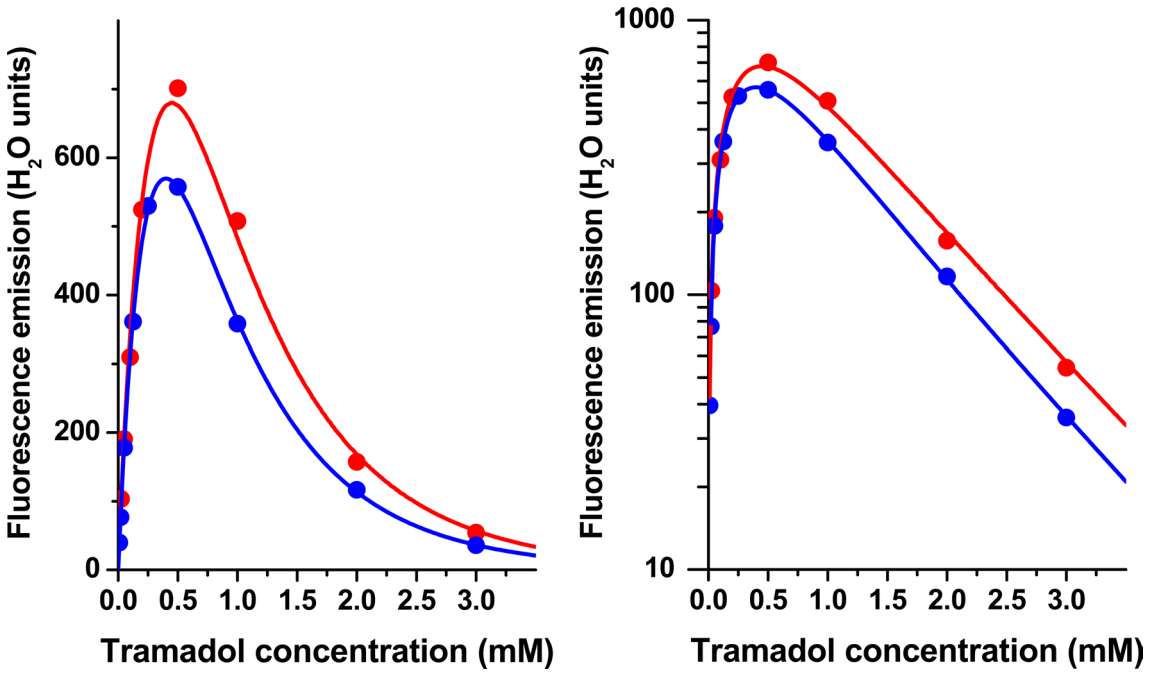

Fig. 9 Fluorescence emission at 297 nm as a function of Tramadol concentration for a constant concentration of β-CD = 12 mM (red dots) compared to pure Tramadol (blue dots). The red solid line is the prediction of Eq. 12 using parameters given in the text. The same data is shown with linear scale (left side) and log scale (right side) as a way to expand low fluorescence intensities. (Color figure online)

from the concentrations, a parameters and Stern–Volmer constants of the two forms A and A-CD quenched by A and A-CD as

$$Fluo(\lambda_{em}) = \left\{ \frac{a_A[A]}{1 + K_{A/A}[A] + K_{A/A\text{-}CD}[A\text{-}CD]} + \frac{a_{A\text{-}CD}[A\text{-}CD]}{1 + K_{A\text{-}CD/A}[A] + K_{A\text{-}CD/A\text{-}CD}[A\text{-}CD]} \right\} \times PIFE \times SIFE \quad (12)$$

where the symbols $K_{A/B}$ stand for the Stern–Volmer constants of quenched emitter A by quencher B. The parameters a_A and $K_{A/A}$ have already been determined from experiments on pure Tramadol. The IFE corrections were the same in the presence and in the absence of β-CD since complexed Tramadol has the same absorbance as free Tramadol. The prefactor $a_{A\text{-}CD} = a_A$ because the fluorescence intensity at low Tramadol concentration was not changed by the presence of a large concentration of β-CD. This meant that fluorescence quantum yields of free and complexed Tramadol were the same. Complexation did not cause variation (enhancement) of quantum yield, which was at variance with many literature reports on different fluorescent dyes [7, 8]. Only the three Stern–Volmer constants $K_{A/A\text{-}CD}$, $K_{A\text{-}CD/A}$ and $K_{A\text{-}CD/A\text{-}CD}$ remained as adjustable parameters, giving satisfactory fit of Eq. 12 to the experimental data (Fig. 9). If $a_{A\text{-}CD}$ is included in the set of adjustable parameters, best fit was achieved with $a_{A\text{-}CD} = a_A$ within experimental significance. The sensitivity of the fit to the relative values of Stern–Volmer constants $K_{A/A\text{-}CD}$, $K_{A\text{-}CD/A}$ and $K_{A\text{-}CD/A\text{-}CD}$ was poor however. It was definitely required that all of them were lower than $K_{A/A}$. The solid line in Fig. 9 is an example of good fit for $K_{A/A} = 812$ L·mol^{-1} (self-quenching of pure Tramadol) and $K_{A/A\text{-}CD} = K_{A\text{-}CD/A} = K_{A\text{-}CD/A\text{-}CD} = 400$ L·mol^{-1}. Increasing one of these parameters while decreasing another makes them compensate each other such that a good fit to experimental data was retained. The exact values of Stern–Volmer constants cannot be determined from the present experiments; but they are definitely smaller than $K_{A/A}$. As conclusion, quenching phenomena were weaker when Tramadol was protected inside an inclusion complex with β-CD.

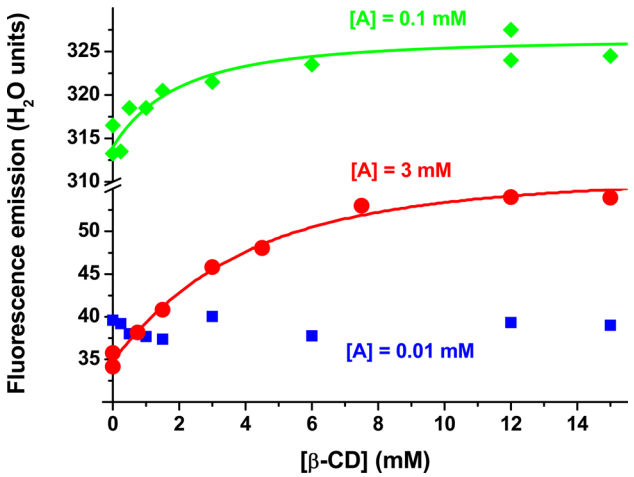

Fig. 10 Fluorescence emission of Tramadol at 297 nm as a function of β-CD concentration. Green diamonds: [Tramadol] = 0.01 mM; Blue squares: [Tramadol] = 0.1 mM; Red circles: [Tramadol] = 3.0 mM. The solid line is the prediction of Eq. 12 using parameters obtained for fitting it to the whole data as a function of Tramadol concentration. (Color figure online)

In another series of experiments, concentration of β-CD was varied at constant concentration of Tramadol. The fraction of complexed Tramadol varied from 0% ([β-CD] = 0) to 78% ([β-CD] = 15 mM). As shown in Fig. 10, the fluorescence of 0.01 mM Tramadol at did not vary upon addition of β-CD up to a 1000-fold excess with respect to stoichiometry. Complexation by β-CD enhanced fluorescence emission at 0.1 mM and 3 mM of Tramadol. Successful prediction of Eq. 12 using the same parameters as for the previous experiments (Fig. 9) is given as a solid line in Fig. 10. The conclusion is the same as for the previous experiment; this supplementary experiment just provides a confirmation using a different experimental protocol.

The present effect of the complexation by cyclodextrins on self-quenching is different of all those reported so far [2]. Rather similar effects leading to inhibition of fluorescence quenching have been reported however [2, 3]. Enhancement of fluorescence by formation of an inclusion complex that prevents quenching of the complexed fluorophore by an external quencher has been reported in several instances [9–14]. Fluorescence quenching by intramolecular excimer formation could also be prevented by complexation of the chromophore inside the CD cavity [35]. In the present case, self-quenching has not been fully eliminated, so that fluorescence enhancement remained of moderate magnitude. The decrease of Stern–Volmer constant upon complexation by β-CD is by a factor of two only. The origin is probably the weak sensitivity to intermolecular distance of fluorescence quenching by a non-radiative Förster energy transfer mechanism. Thus, several quenching mechanisms might be considered as operative [36, 37]. Only non-radiative ones are of relevance since the shape of the fluorescence spectrum was not affected by changing the concentration of Tramadol. Quenching by intersystem crossing and photoinduced electron transfer cannot operate self-quenching. Actually, radiative transfer from excited molecule to another at its ground state is possible since absorbance and fluorescence spectra of Tramadol overlap a bit; but the net balance of such a transfer is nil because it keeps one Tramadol molecule in

its excited state. Finally, Förster and Dexter non-radiative energy transfer can be responsible for the present quenching [37]. Förster energy transfer is caused by long range dipole–dipole interactions between an excited molecule and another in its ground state. This energy transfer operates over long distances owing to the long-range feature of such interactions. Dexter energy transfer involves overlapping molecular orbitals of the emitter and the quencher; this is a very short range mechanism. Since protection against self-quenching by β-CD was weak, it was presumed that Förster energy transfer was the predominant self-quenching mechanism.

Conclusion

Complexation of Tramadol hydrochloride by β-cyclodextrin enhanced its fluorescence emission intensity. The origin of such effect was the inclusion of Tramadol molecules inside the hydrophobic cavity of β-cyclodextrin that prevented self-quenching. Although both absorbance and fluorescence properties of Tramadol in classical concentration ranges did not vary upon complexation by β-cyclodextrin, experiments at high Tramadol concentrations revealed a variation of fluorescence emission. A detailed investigation of fluorescence properties together with thermodynamic appraisal of complexation equilibrium allows quantitative accounts of self-quenching and influence of complexation by cyclodextrin through Stern–Volmer quenching constants.

The methodology requires a correct implementation of the effect of absorbance at high concentration known as Inner Filter Effects. The same methodology can be used in other physicochemical studies using fluorescence at high concentration such as studies of association equilibrium. It is indeed worth noticing that working at high dilution for avoiding Inner Filter Effects is unsuccessful because dilution causes associated structures to dissociate.

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