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Effect of pH on the Encapsulation of the Salicylic Acid / Salicylate System by Hydroxypropyl-β-Cyclodextrin at 25 °C. A Fluorescence Enhancement Study in Aqueous Solutions

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Abstract. The encapsulation of the acid/base conjugated system salicylic acid (HSA)/salicylate (SA^-) by hydroxypropyl-β-cyclodextrin (HPBCD) has been studied through fluorescence emission enhancement measurements in aqueous solutions at 25 °C. With the aim of analyzing the crucial importance of a proper and cautious choice of the pH of the medium (i.e. choice of the buffer), the study has been carried out at pH = 1, 2, 4 and 7. Since the pK_a of the HSA/SA^- system is 2.95 at 25 °C, the presence of the protonated (HSA) and non-protonated (SA^-) forms suitable for inclusion by cyclodextrin vary appreciably within the different pH conditions: 99% HSA, 1% SA^- at pH = 1, 90% HSA, 10% SA^- at pH = 2, 10% HSA, 90% SA^- at pH = 4, and ≈0% HSA, ≈100% SA^- at pH =7. The association constants $K_{CD:HSA}$ and $K_{CD:SA-}$ have been determined in all cases by using a nonlinear regression analysis of the experimental data at three different λ^{em}. The effect of the pH of the medium on all the equilibria involved as well as in the K values is fully discussed. The 8-anilino-1-naphthalene sulfonate (ANS) + β-cyclodextrin (β-CD) system, widely reported in literature, has initially been studied to check the experimental protocol and the numerical method.

Key words: Cyclodextrin, fluorescence, inclusion complex, molecular encapsulation, pH effect, salicylic acid.

1. Introduction

Organized media have often been used to examine many important photophysical processes [1–7]. Specifically, cyclodextrins, which are well known host molecules with an apolar cavity in which different types of guest molecules can be partially or totally included, have been employed in the investigation of excimer formation and energy transfer analysis of the twisted intramolecular charge transfer state (TICT) [3–7]. Aqueous solutions of cyclodextrins have also been widely used to enhance the luminescence properties of different compounds [8-12]. The intensification of luminescent processes of lumiphores partially or totally encapsulated by the

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CD cavity is a result of the better protection from quenching and other processes ocurring in the bulk solvent. The CD cavity behaves similarly to an organic solvent; it affords an apolar environment and a non-hydrated state for the included probe. The maximum fluorescence intensity is obtained from a molecule which is totally encapsulated inside the cavity, and the more a molecule is subjected to an aqueous media, the lower is its fluorescence intensity.

The observation of steady-state emission can be used as an indication of complexation when the fluorescence spectrum of a fluorophore in an aqueous environment changes markedly on addition of cyclodextrin to the aqueous solution. A great number or references in the literature [8–18] use this change in the spectroscopic properties of the inclusate (S) as encapsulation by the cyclodextrin (CD) occurs, to study the binding process through the association constant $K_{CD:S}$, and in many of these references the inclusate is an acid/base conjugated system [13–18]. Several general precautions must be taken when determining these associations constants, and in particular when the included substrate is not a unique species in the medium but itself participates in an equilibrium with other species. It is very important to take this fact into consideration since in such a case there is more than one association equilibrium, with the formation of more than one inclusion complex. The proportion of the complexes depends on the equilibrium concentrations of the different free substrates, the equilibrium constant which relates them, and, obviously, the binding constants of the different inclusion complexes. In the case of an acid/base system this situation is very dependent on the pH of the bulk solution and the pK_a of the HA/A^- pair, since these parameters will 'decide' the ratio between the species to be encapsulated by the CD. Unfortunately, some of the K values reported in the literature have been determined using buffered solutions at a pH close to the pK_a of the system [19–21], or, even worse, using unbuffered solutions [22,23]. In either case, the reported K values are not real since they consist of different contributions of both CD : HA and CD : A^- inclusion complexes.

Another cause of inaccuracy and inconsistency in these reported K values is the numerical method used to determine them. Unfortunately, different linearization methods are frequently used, assuming some premises and/or approximations to be valid that are usually wrong. Nowdays, the necessity of a careful nonlinear regression analysis (NLR methods) of the experimental data is well established, whatever technique may be used, when accurate and meaningful binding constants are required. Moreover, the choice of the experimental conditions must be cautious as well, since they should guarantee that at least 80% of the saturation curve is covered (the saturation degree, f = [complex]/[substrate]$_{TOTAL}$, must range from 0.2 to 0.8). In the case of spectroscopic techniques, another indication of accurate K results is their independence of the wavelength λ at which the experimental data are fitted. It is advisable to evaluate the association constant at least at three different emission wavelengths (in the case of fluorescence experiments), and the results must be consistent within the experimental and fitting errors. This caution is not considered in most of the literature references.

The aim of this work is to set up a method to obtain the binding constants of acid/base pair + CD systems from fluorescence measurements, bearing in mind all the premises mentioned above. Initially, and in order to check the experimental protocol and the NLR numerical method, the β-cyclodextrin (β-CD) + 8-anilino-1-naphthalene sodium sulphonate (ANS) system, which has been widely studied in the literature by different types of experimental techniques [12,24–31], has been analyzed at 25 °C. Since the pK_a of the substrate is 1.3 at 25 °C, a phosphate buffer at pH = 6.966 guaranteed that only the anionic form of the pair was present, matching the experimental conditions of the reported experiments.

Once the method has been checked, the encapsulation of the salicylic acid (HSA)/salicylate (SA^-) pair by hydroxypropyl-β-cyclodextrin (HPBCD) at 25 °C was also studied using fluorescence measurements. Salicylic acid, the active metabolite of aspirin, is a weak hydroxyacid with an acid/base dissociation characterized by a pK_a of 2.95 at 25 °C (determined by us from pH measurements). It is a crystalline powder that melts at 157–159 °C, being moderately soluble in water and very soluble in organic solvents. On the other hand, hydroxypropyl-β-cyclodextrin is characterized by an apolar cavity (7 Å width and 8 Å depth), similar to that of β-CD, a markedly higher solubility (50 g/100 mL at 25 °C), and the compound generally induces the largest fluorescence enhancements factors on binding a fluorophore [32].

In this work, the encapsulation of the salicylic system by HPBCD has been carried out at pH = 1, 2, 4 and 7. As can be seen, considering the value of 2.95 for the pK_a of the substrate at 25 °C, at pH = 1 and pH = 7 the protonated (HSA) and the ionized (SA^-) species are respectively predominant in the medium, with a negligible contribution of the conjugated species. But at pH = 2 and pH = 4, which differs from pK_a by one unit, both species are present in the medium (in different proportions) and both form the corresponding inclusion complexes when HPBCD is added. Consequently, the results obtained in this study and the final conclusions are expected to be indicative of the importance of a proper and cautious choice of the pH conditions when analyzing the binding of these systems to cyclodextrins.

2. Experimental

2.1. MATERIALS

8-Anilino-1-naphthalene-sulfonic acid (ANS), β-cyclodextrin (β-CD) and salicylic acid (HSA) were from Aldrich Co., while hydroxypropyl-β-cyclodextrin (HPBCD), containing an average of 0.4 hydroxypropyl groups per glucopyranose unit, was from Janssen Biotech. All were used without further purification. β-CD and HPBCD have water contents of 13.6 and 3.4 wt.-%, respectively, which was considered when calculating solute concentrations. Doubly distilled water was deionized using a Super Q Millipore system before final degassing with a vacuum pump prior to the preparation of the solutions. The following buffered solutions were prepared to fix the different experimental pH conditions: (a) two KCl/HCl

solutions with I = 0.2 m and I = 0.06 m for pH = 1 and pH = 2 respectively; (b) an AcNa/AcH solution with I = 0.1 m for pH = 4; and (c) an NaH_2PO_4/Na_2HPO_4 solution with I = 0.2 m for pH = 7.

2.2. FLUORESCENCE MEASUREMENTS

Steady-state fluorescence experiments were carried out with a Perkin-Elmer LS-50B Luminescence Spectrometer. The equipment is connected to a PC-386 SX computer via an RS-232C interface. Data acquisition and analysis of fluorescence spectra were performed with the Fluorescence Data Manager Software supported by Perkin Elmer. A 10 mm stoppered rectangular silica cell was placed in a stirred cuvette holder whose temperature was kept constant at 25.00 $\pm$ 0.01 °C with a recirculating water circuit (at 25.000 $\pm$ 0.001 °C). In the ANS + β-CD titration, excitation and emission slits were fixed at 5 and 2.5 nm, respectively, the excitation wavelength was set at 365 nm and the emission spectra were collected from 380 to 650 nm. In the HSA + HPBCD titrations, however, both the excitation and emission slits were fixed at 5 nm, the excitation wavelength was 330 nm and the spectra were collected from 340 to 600 nm. In all cases the scan rate was selected as 240 nm/min.

2.3. METHODS

In all the titrations made, the luminescent species ANS and HSA were kept at constant concentrations while CD concentrations varied from 0 to a certain value, in such a way that the degree of saturation (f) ranged from 0.2 to 0.7–0.8. In fluorescence intensity enhancement studies care must be taken with the sample preparation procedure [33], which must ensure that the concentration of the fluorophore is the same both in the presence and in the absence of cyclodextrin. In order to match this condition, the following protocol was used for the preparation of solutions. Two equal aliquots of a probe stock solution were placed in flasks 1 and 2, and two equal aliquots of a CD stock solution were placed in flasks 2 and 3. The total volume of the probe and CD aliquots in flask 2 was matched in flasks 1 and 3 by adding the necessary volume of buffered solution. Working in this way, flasks 1 and 2 contain the same concentration of luminescence probe, while flasks 2 and 3 contain the same concentration of CD. An initial volume of 1.5 mL of solution 1 was placed in the fluorescence cell and titrated with different volumes of solution 2 up to a final volume of 2.7 mL. The correction due to the buffer was made with a blank titration, where the conditions of the first titration were reproduced but with 1.5 mL of buffer titrated with the same volumes of solution 3. In this way, in the first titration the probe concentration was kept constant and its fluorescence monitored as a function of CD concentration, while in the second titration (blank titration), where no probe was present, the effect of the same CD concentrations in the buffer were followed. The corresponding difference spectra

showed the influence of the inclusion of the probe in the CD cavity, if any, on the spectrofluorometric properties of the probe initially in the buffered aqueous solution. Following this protocol, [ANS] was kept constant at 1.5×10^{-4} M, while [HSA] was fixed at 5×10^{-4} M at pH = 1 and 2, and at 10^{-5} M at pH = 4 and 7. The β-CD concentration was varied from 0 (pure probe) to 8 mm in the ANS + β-CD titration, while [HPBCD] ranged from 0 to 6 mm (at pH = 1), from 0 to 14 mm (at pH = 2), and from 0 to 32 mm (at pH = 4 and 7) for the HSA + HPBCD titrations.

3. Results and Discussion

3.1. Determination of binding constants

As mentioned before, an enhancement of fluorescence intensity in the presence of cyclodextrins compared to water was observed because, due to the organizing ability of CD media, cyclodextrin cavities offer a protective, more constrained microenvironment in which an electronically excited lumiphore can be isolated from the surrounding environment, the excited states being shielded from extinction processes.

If the luminescent substrate is an acid/base conjugated pair HA/A$^-$, the equilibria involved, assuming a 1 : 1 stoichiometry [17,34,35] for the inclusion complexes, are the following:

$$\begin{array}{ccc} \mathrm{HA} & \overset{K_\mathrm{a}}{\rightleftharpoons} & \mathrm{H^+ + A^-} \\ K_{\mathrm{CD:HA}} \uparrow\downarrow +\mathrm{CD} & & K_{\mathrm{CD:A^-}} \uparrow\downarrow +\mathrm{CD} \\ \mathrm{CD:HA} & \overset{K'_\mathrm{a}}{\rightleftharpoons} & \mathrm{CD:A^- + H^+} \end{array}$$

where

$$K_\mathrm{a} = (a_{\mathrm{H^+}} a_{\mathrm{A^-}})/(a_{\mathrm{HA}}) \tag{1}$$

$$K_{\mathrm{CD:HA}} = (a_{\mathrm{CD:HA}})/(a_{\mathrm{CD}} a_{\mathrm{HA}}) \tag{2}$$

$$K_{\mathrm{CD:A^-}} = (a_{\mathrm{CD:A^-}})/(a_{\mathrm{CD}} a_{\mathrm{A^-}}) \tag{3}$$

$$K'_\mathrm{a} = (a_{\mathrm{CD:A^-}} a_{\mathrm{H^+}})/(a_{\mathrm{CD:HA}}) \tag{4}$$

The constants K_a and K'_a are the acid/base dissociation constants of HA in the free and complexed form, respectively, while $K_{\mathrm{CD:HA}}$ and $K_{\mathrm{CD:A^-}}$ are the binding constants of the inclusion complexes formed by cyclodextrin and the protonated and non-protonated form of HA, respectively. Since the concentrations in these experiments are very low, the activity coefficients of ionic species can be assumed to be close to unity, and Equations (1)–(4) can be expressed in terms of concentrations rather than activities.

There are three possibilities for studying the association of these species with CD: (i) fix the pH of the medium with a buffer in such a way that only one of the two species is in the solution (protonated HA or nonprotonated A^-) with a negligible contribution from the other one, following the change in a physicochemical property, i.e. steady-state fluorescence intensity, as a function of CD concentration; (ii) buffer the solution at a pH close to the pK_a of the HA/A^- system, so that both species contribute appreciably to the fluorescence characteristics of the medium, also following the variation of this fluorescence as a function of CD concentration; and (iii) perform this titration with the HA in aqueous unbuffered solution, monitoring the pH of the medium as a faithful indication of the shifting of the equilibria 1–4, as long as the two complexes CD : HA and CD : A^- are formed depending on the magnitude of all the implied equilibrium constants.

A quantitative treatment of fluorescence binding data starts with the premise that the intensity is a sum of contributions, as follows [36]:

$$I = k_S[S] + k_{CD:S}[CD:S] + k_{CD}[CD] \tag{5}$$

where S represents the predominant substrate at each pH condition (HA or A^-).

Considering Equation (5), the mass balance on substrate, and that cyclodextrins do not fluoresce ($k_{CD} = 0$), the binding isotherm finally results:

$$\frac{I}{I_0} = \frac{I + (k_{CD:S}/k_S)[CD]}{1 + K_{CD:S}[CD]} \tag{6}$$

where I_0 is the fluorescence intensity in the absence of ligand (CD), but in the presence of the same total substrate concentration $[S]_{tot}$ ($I_0 = k_S\,[S]_{tot}$).

The values of $K_{CD:S}$ and $k_{CD:S}/k_S$ are obtained as fitting parameters of a nonlinear regression (NLR) analysis of the experimental I/I_0 values as a function of [CD]. The NLR procedure is based on a McQuardt algorithm, which we have written as a TURBO C program.

In this work, where option (i) has been chosen, we try to demonstrate how crucial an adequate choice of the working pH is. Thus, the relative proportion between the protonated and nonprotonated species, given by the expression $[HA/[A^-] = 10^{pK_a - pH}$, varies depending on the difference between the pK_a of the acid/base conjugate system and the pH of the medium. It is of great importance, when applying method (i), to ensure that just one species (the protonated or the nonprotonated one) is present in the medium, because only in such a case can just one of the equilibria 1–4 be considered, since there is only one substrate, one ligand and one complex. It is also important to ensure that none of the buffer species are capable of being encapsulated by the CD, because this fact could interfere in the final results since more equilibria should be considered. Only when all these premises are considered does the association constant obtained actually correspond either to the complexes CD : HA or CD : A^-. Otherwise, if the fixed pH is not much higher or much lower than the pK_a (option ii), such that both HA and A^- species are

involved in the inclusion process with the CD, the four equilibria (1–4) have to be considered. In such a case, the NLR analysis is much more complicated since the number of fitting parameters becomes so high that the physical meaning and the uncertainty of the association constant $K_{\mathrm{CD:S}}$ thus obtained are questionable. The application of Equation (6) to such a situation, as is frequently done in the literature [19–23], derives binding constants which are in fact apparent constants, an average of all those equilibrium constants involved in the processes.

3.2. ANS + β-CD SYSTEM

In order to check the model proposed in this work for obtaining the binding constants, as well as the numerical method, we have chosen the system ANS + β-CD, which is well documented in the literature [12,24–31]. The fluorescence emission spectra of ANS in a phosphate buffer (pH = 6.966) has been recorded both in the absence and in the presence of β-CD at different concentrations ranging from 0.1 to 7.7 mM. ANS is known to be a fluorescent probe for exploring hydrophobic regions in complex molecules [37]. The fluorescence of this agent is quenched in water, but in apolar environments it is substantially enhanced with shifts of the emission toward shorter wavelengths. It has been reported that the fluorescence emitted by ANS increases in the presence of cyclodextrins such as β-CD and γ-CD [12,31], due to the encapsulation of the fluorophore in the apolar CD cavity. ANS presents a maximun on the fluorescence intensity ($I_{\max}$ = 10 u.a.) at $\lambda_{\max}^{\mathrm{em}}$ = 517 nm, in the absence of β-CD. When β-CD is added, this maximun is shifted toward shorter wavelengths, as documented in the literature, and the intensity (I) experiences an enhancement up to 7.8 times the intensity of pure ANS (I_0). These I/I_0 data have been fitted as a function of cyclodextrin concentration following Equation (6), and the values of $K_{\beta\text{-CD:ANS}}$ and $k_{\beta\text{-CD}}/k_{\mathrm{ANS}}$ have been obtained with the NLR analysis. This fitting procedure has been done at three different emission wavelengths, $\lambda_{\max}^{\mathrm{em}} \pm \delta\lambda^{\mathrm{em}}$, in order to check the independence of the binding constant $K_{\beta\text{-CD:ANS}}$ from the wavelength at which the intensity enhancement is measured. The resulting values, reported in Table I, are in very good agreement with the literature data [12,24,26,28–30] reported under similar experimental conditions.

3.3. HSA/SA$^-$ + HPBCD SYSTEM

The encapsulation of salicylic acid (HSA) by hydroxypropyl-β-cyclodextrin (HPB-CD) has been studied by following its fluorescence behaviour. In order to demonstrate the importance of the pH at which the experiment is conducted, four titrations were performed at four different pH conditions: (i) pH = 1 and 2, where the protonated species (HSA) is predominant with a contribution of the salicylate (SA$^-$) of around 1% and 10% respectively; and (ii) pH = 4 and 7, where the predominant species is salicylate with a contribution of around 10% and $\cong$0% of salicylic acid respectively. Figures 1–4 show the fluorescence emission spectra of

Table I. Values of the association constants $K_{CD:S}$ and proportionality constant ratio $k_{CD:S}/k_S$ of Equation (6) under different pH conditions and at different λ^{em}, for the systems ANS + β-CD and HSA + HPBCD at 25 °C.

System	pH	λ^{em} (nm)	$K_{CD:S}$ (M^{-1})	$k_{CD:S}/k_S$	10^2 St.Dv.
ANS + β-CD[a]	7	480	89 ± 13	25 ± 4	13.5
		520	90 ± 13	14 ± 2	8.4
		540	89 ± 13	11 ± 2	6.4
HSA + HPBCD[b]	1	420	1220 ± 180	7 ± 1	13
		440	1190 ± 180	7 ± 1	12
		480	1250 ± 190	7 ± 1	11
	7	390	270 ± 40	1.6 ± 0.3	2.4
		410	220 ± 33	1.6 ± 0.3	2.3
		430	250 ± 38	1.5 ± 0.3	2.5

[a] λ_{exc} = 365 nm, λ^{em} ranges from 380 to 650 nm, ex/em slitwidths = 5/2.5 nm, scan rate = 240 nm/min, [ANS] = 1.5×10^{-4} M, [β-CD] ranges from 0 to 7.7 mM, buffer: a NaH_2PO_4/Na_2HPO_4 solution at pH = 6.966.
[b] λ_{exc} = 330 nm, λ^{em} ranges from 340 to 600 nm, ex/em slitwidths = 5/5 nm, scan rate = 240 nm/min, [HSA] = 5×10^{-4} m at pH = 1 and [HSA] = 10^{-5} m at pH = 7, [HPBCD] ranges from 0 to 6 mm at pH =1 and from 0 to 32 mm at pH = 7, buffers: a KCl/HCl solution at pH = 1.013 and a NaH_2PO_4/Na_2HPO_4 solution at pH = 6.987.

salicylic acid in the absence and in the presence of different HPBCD concentrations at pH = 1, 2, 4 and 7 respectively. The total [HSA] is kept constant and the CD concentration is varied in all the titrations.

Several features can be analyzed in these figures. First of all, it can be clearly seen in the figures that only at pH = 1 (Figure 1) and 7 (Figure 4), where the presence of the salicylate or salicylic forms are respectively almost negligible in the medium, does the addition of HPBCD result in the formation of the corresponding inclusion complexes HPBCD:HSA (pH = 1) or HPBCD : SA^- (pH = 7), with an emission intensity enhancement and basically no shift to shorter or longer wavelengths. From the spectrum of the pure substrate in Figures 1 and 4 it can be deduced that the protonated form (HSA) fluoresces with a maximum intensity (122 a.u.) at 441 nm for a total [HSA] = 5.04×10^{-4} m, while in the case of the ionized form (SA^-) λ^{em}_{max} = 409 nm and I_{max} = 380 a.u. for a total [HSA] = 1.01×10^{-5} M. These values reveal that the salicylate is a much more fluorescent species than its protonated counterpart, its emission being more energetic ($\Delta\lambda^{em}_{max} = \lambda^{em}_{max}$ (HSA) $- \lambda^{em}_{max}$ (SA^-) = 32 nm). In fact, it was not possible to run these spectra at the same concentration conditions because at total [HSA] $> 5 \times 10^{-5}$ M, the emission of SA^- is absolutely saturated, and at total [HSA] $< 10^{-4}$ M the protonated form HSA barely fluoresces. The enhancement of fluorescence intensity due to the inclusion of the salicylic or salicylate species in the cyclodextrin cavity is sharper, however, in the former case, as can be seen in Figure 1, where the addition of CD up to a

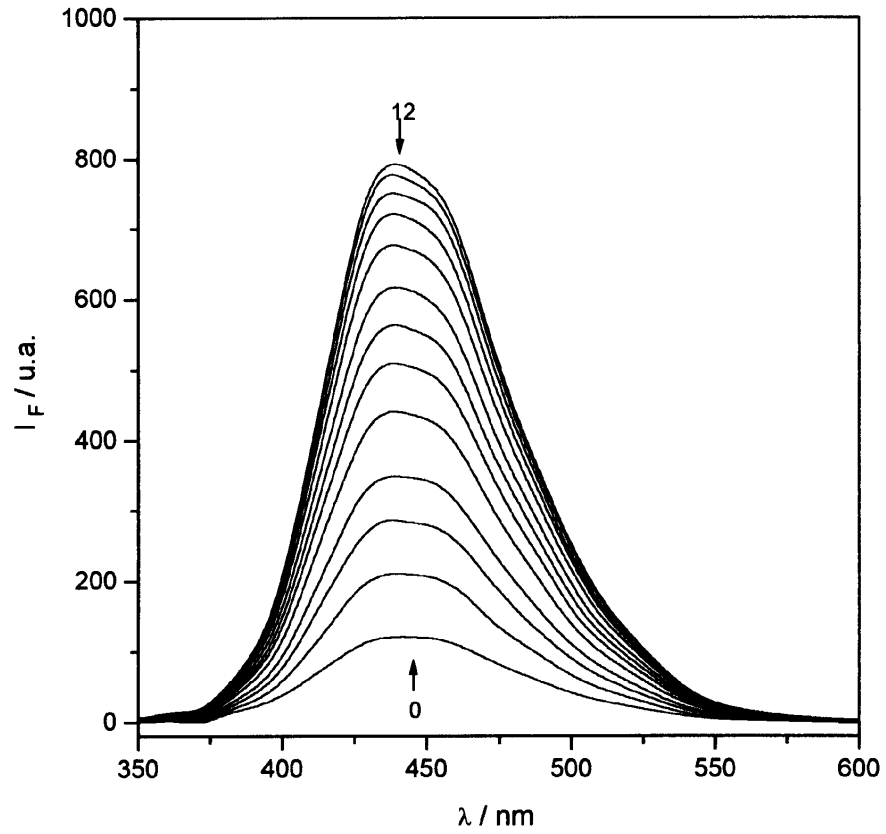

Figure 1. Fluorescence emission spectra of HSA ([HSA] = 5.04 × 10^{-4} M) at pH = 1 at different HPBCD concentrations: **0**, 0.00 mM; **1**, 0.16 mM; **2**, 0.32 mM; **3**, 0.48 mM; **4**, 0.77 mM; **5**, 1.12 mM; **6**, 1.61 mM; **7**, 2.20 mM; **8**, 2.97 mM; **9**, 3.73 mM; **10**, 4.47 mM; **11**, 5.36 mM; **12**, 6.07 mM. λ_{exc} = 330 nm, ex/em slitwidths = 5/5 nm, scan rate = 240 nm/min, buffer: a KCl/HCl solution at pH = 1.013, T = 25.00 ± 0.01 °C.

6 mM concentration results in an increase of the I/I_0 ratio of around 6.5 at the three λ^{em} values studied. At pH = 7, with a final CD concentration of 32 mM, the I/I_0 ratio only increases up to 1.6. These parameters, as well as the other fitting parameters, have been calculated at the three different λ^{em} values by fitting the experimental I/I_0 values vs. [CD] (see Figure 5 for pH = 1, as an example) to Equation (6) using the NLR method, and they are reported in Table I. Again, the independence of not only the K values but also the k_{CD}/k_S values with the λ^{em} at which the I/I_0 ratio are fitted reveals the satisfactory nature of the method and the accuracy of the results. The data in Table I indicate that $\bar{K}_{CD:HSA}$ (= 1200 ± 180 M^{-1}) > $\bar{K}_{CD:SA^-}$ (= 240 ± 35 M^{-1}), which corresponds to the normal behaviour of carboxylic acids on binding cyclodextrins, in contrast with other pairs such as *p*-nitrophenol/*p*-nitrophenolate. It has been reported [15,17,18,34,38–

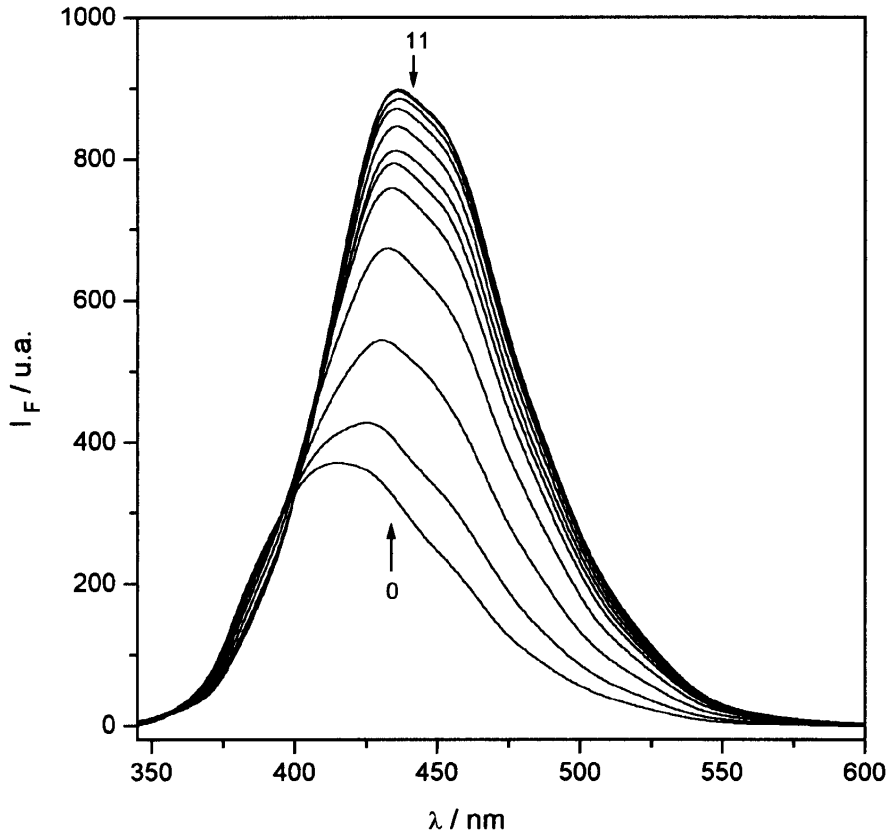

Figure 2. Fluorescence emission spectra of HSA ([HSA] = 4.97×10^{-4} M) at pH = 2 at different HPBCD concentrations: **0**, 0.00 mM; **1**, 0.20 mM; **2**, 0.60 mM; **3**, 1.18 mM; **4**, 1.91 mM; **5**, 2.78 mM; **6**, 4.36 mM; **7**, 6.43 mM; **8**, 8.73 mM; **9**, 10.63 mM; **10**, 12.22 mM; **11**, 13.58 mM. λ_{exc} = 330 nm, ex/em slitwidths = 5/5 nm, scan rate = 240 nm/min, buffer: a KCl/HCl solution at pH = 1.910, $T = 25.00 \pm 0.01$ °C.

40] that *p*-nitrophenolate and *p*-nitrophenol related substrates penetrate the CD cavity through the wider entrance, nitro end first. The preferential binding of the anionic form in such cases has been attributed to the existence of a dispersion interaction between the CD cavity and the delocalized charge of the anion. It is logical that, if cyclodextrin-substrate association is controlled by an induced dipole-dipole mechanism, the ionized form of the substrate should bind more tightly than its neutral partner. However, some evidence has been found [15,17,18,34,35] to support the idea that, when the substrate is a carboxylic acid derivative, it penetrates the cavity through the wider ring as well, but carboxylic group end first. Thus, while the ionizable group remains in contact with the bulk aqueous solution in the case of *p*-nitrophenolate, it is included in the cavity in the case of carboxylate substrates. In the latter cases, the neutral forms of the ligands are

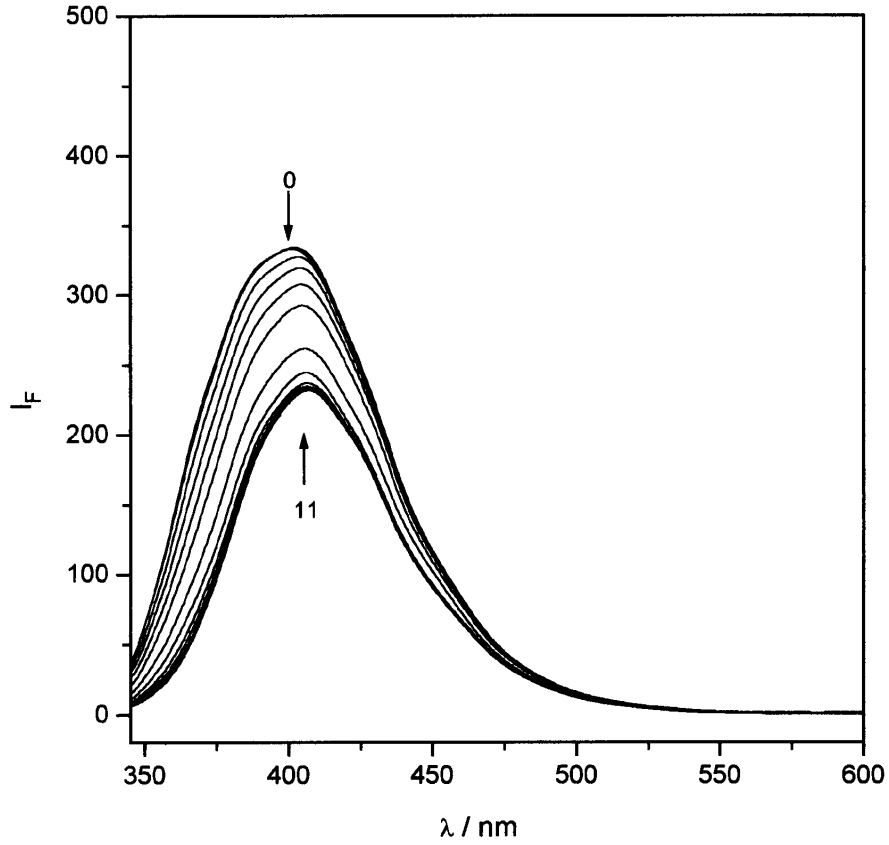

Figure 3. Fluorescence emission spectra of HSA ([HSA] = 1.01×10^{-5} M) at pH = 4 at different HPBCD concentrations: **0**, 0.00 mM; **1**, 0.47 mM; **2**, 1.40 mM; **3**, 2.74 mM; **4**, 4.46 mM; **5**, 6.48 mM; **6**, 10.19 mM; **7**, 15.01 mM; **8**, 20.37 mM; **9**, 24.80 mM; **10**, 28.52 mM; **11**, 31.69 mM. λ_{exc} = 330 nm, ex/em slitwidths = 5/5 nm, scan rate = 240 nm/min, buffer: a CH_3COOH/CH_3COONa solution at pH = 4.012, $T = 25.00 \pm 0.01$ °C.

found to bind tighter to CDs than the ionic forms. Some authors [34] attribute this binding preference, which is opposite to that for p-nitrophenol, to the fact that carboxylate species, although they may experience some charge delocalization, penetrate the cavity more randomly than their neutral partners and without the concurrence of any additional dispersion interaction. Besides, it is believed [34] that these species would not be completely stripped of their water of solvation when they are encapsulated by CDs, thus lowering the insertion interaction. In fact, the energy required to transfer charged species from the bulk with a high dielectric constant to a medium of low dielectric constant seems to play an important role in governing the affinity of these associations. Whatever the possible explanations might be – the random character of the inclusion or the energetic balance of transferring the charged species from the bulk to the CD cavity – the behaviour

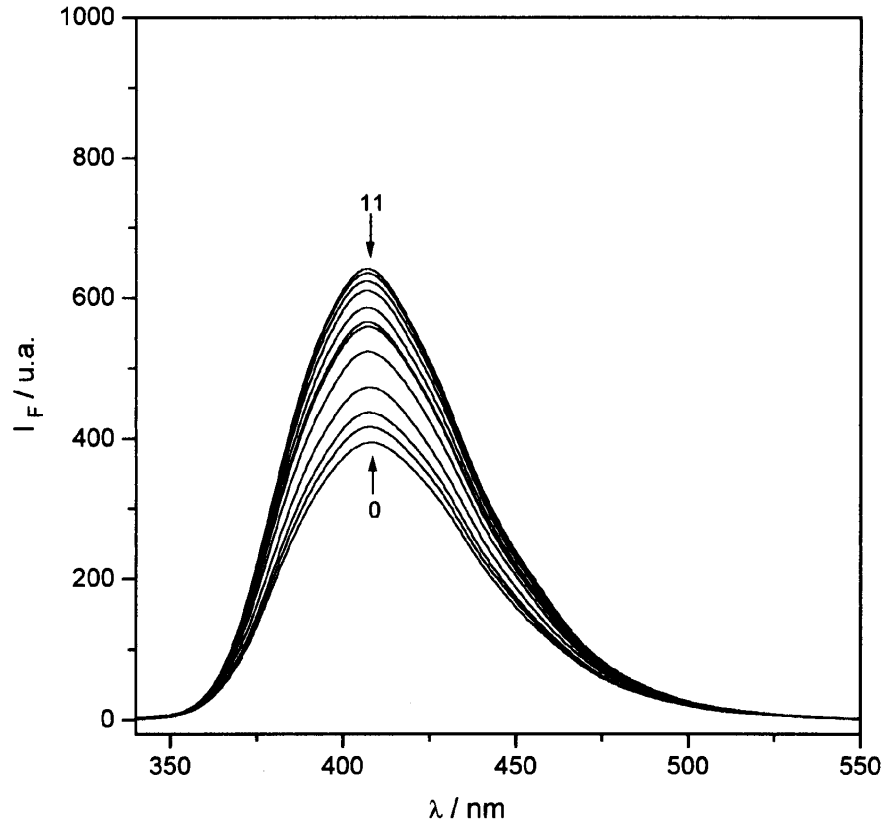

Figure 4. Fluorescence emission spectra of HSA ([HSA] = 1.01 × 10^{-5} M) at pH = 7 at different HPBCD concentrations: **0**, 0.00 mM; **1**, 0.48 mM; **2**, 0.95 mM; **3**, 1.87 mM; **4**, 3.65 mM; **5**, 6.56 mM; **6**, 10.31 mM; **7**, 15.19 mM; **8**, 20.61 mM; **9**, 25.10 mM; **10**, 28.86 mM; **11**, 32.07 mM. λ_{exc} = 330 nm, ex/em slitwidths = 5/5 nm, scan rate = 240 nm/min scan rate = 240 nm/min, buffer: a NaH_2PO_4/Na_2HPO_4 solution at pH = 6.987, T = 25.00 ± 0.01 °C.

of our system corresponds just to the previous findings of the literature, i.e. the protonated species binds to HPBCD around five times tighter than the salicylate. Figure 6 clearly shows this result. When the protonated form is the substrate, a final [CD] of 6 mM is sufficient to cover almost 90% of the saturation curve (f = 0.9), while in the case of salicylate a final [CD] around five times higher (32 mM) is necessary to reach this saturation, even for a substrate concentration 50 times lower.

The behaviour of the fluorescence emission spectra when both salicylic and salicylate forms are simultaneously present in the medium, and consequently both CD : HSA and CD : SA^- complexes are formed is shown in Figures 2 and 3, where the emission spectra are reported for pH = 2 and 4, respectively. The excitation wavelength, the excitation and emission slit widths and scan rate values are the same as those for the experiments at pH = 1 and 7. Again, $[HSA]_{TOT}$ is kept constant

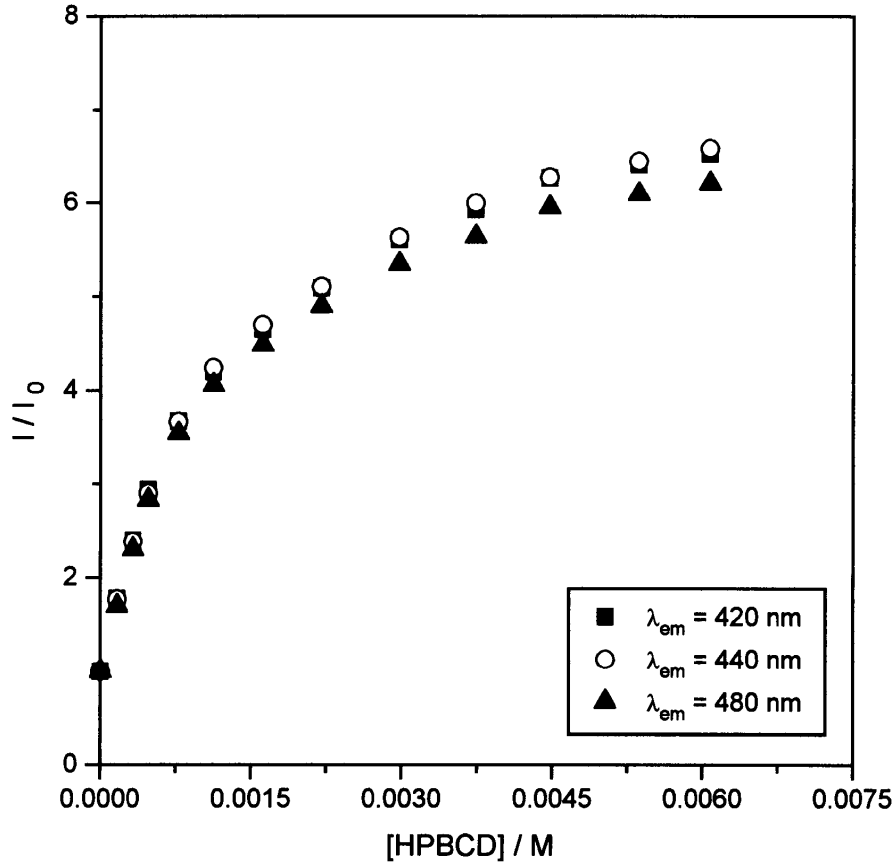

Figure 5. Values of the fluorescence intensity enhancement (I/I_0) due to the encapsulation by the CD cavity of HSA ([HSA] = 5.04 $\times$ 10^{-4} M) at pH = 1, as a function of HPBCD concentration at three different emission wavelengths (λ^{em}).

at 4.97 $\times$ 10^{-4} M at pH = 2 and at 1.01 $\times$ 10^{-5} M at pH = 4, while the HPBCD concentration is varied. There are two features clearly apparent when analyzing these figures: (i) when the CD is added, the emission spectra experiences a red shift in both cases, with respect to the λ^{em}_{max} of the pure substrate, and (ii) at pH = 4, the addition of CD results in a decrease of the fluorescence intensity, in contrast with the behaviour found at the other pH conditions. All these features can be explained if one realizes that at pH = 2 and 4, the situation is not as 'simple' as in the previous cases (at pH = 1 and 7), since the competition between the four equilibria (1–4) plays an important role.

Comparing the spectrum for the pure substrate at pH = 2 with respect to that at pH = 1, where [HSA] is also around 5 $\times$ 10^{-4} M, it can be observed that the maximum intensity at pH = 2 is higher by a factor of 3 than that at pH = 1, while the λ^{em}_{max} is shorter at pH = 2 than at pH = 1. This feature can be explained

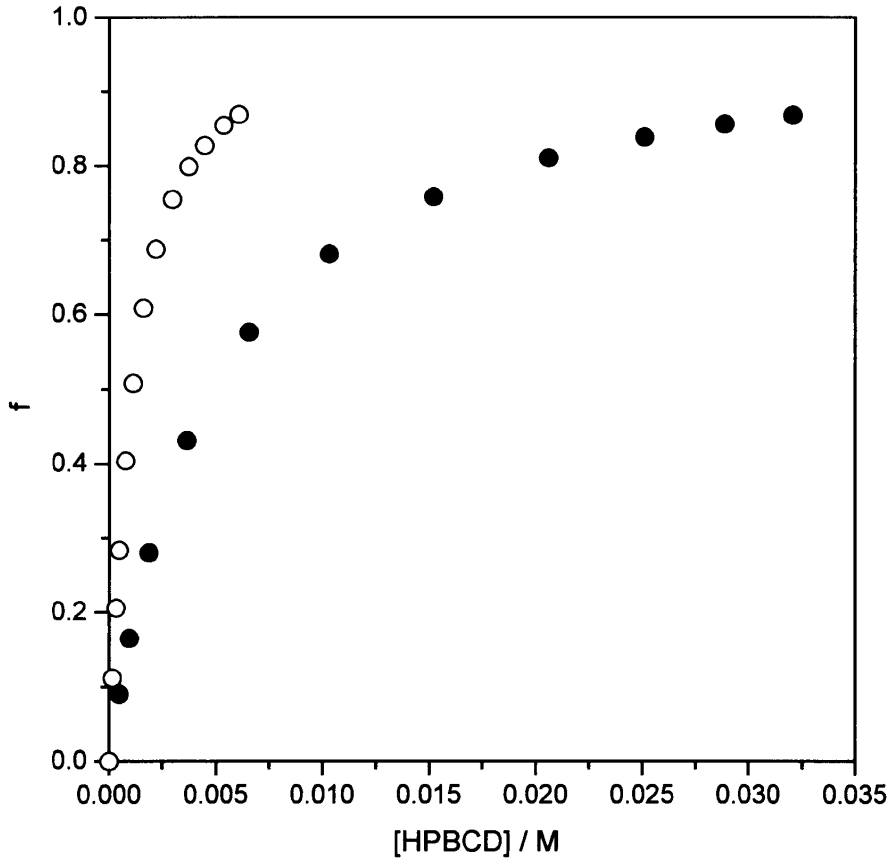

Figure 6. Plot of the saturation degree f as a function of HPBCD concentration at: ○, pH = 1, and ●, pH = 7, considering the association constants $K_{\mathrm{HPBCD:HSA}}$ (pH = 1) and $K_{\mathrm{HPBCD:SA^-}}$ (pH = 7) reported in Table I.

by considering that the presence of 10% of salicylate ion at pH = 2, which is much more fluorescent than salicylic acid and which has a less energetic emission, enhances the intensity of the emission and shifts it towards longer wavelengths.

For the same reason, the spectrum of the pure substrate at pH = 4 shows an $I_{\max}$ that is slightly lower and a very similar but shorter $\lambda^{\mathrm{em}}_{\max}$ than at pH = 7. It seems that, in this case, 10% of the HSA form has a low contribution to the emission parameters of the salicylate form. When the CD is added, equilibria 1–4 must be considered, always keeping in mind that the protonated species (HSA) forms a tighter complex with the HPBCD ($K_{\mathrm{CD:HSA}} \approx 5\ K_{\mathrm{CD:SA^-}}$; $K_{\mathrm{a}} \approx 5\ K'_{\mathrm{a}}$). At pH = 2, the acidic form HSA is predominant, but 10% of the ionized form (SA^-) is present. The CD forms inclusion complexes with both species, although, since the complex with salicylic acid is the stronger of the two, a greater fraction of HSA is complexed. Simple application of Le Châtelier's principle to the equilibria

Table II. Values of the apparent association constants $K_{CD:S}$ and apparent proportionality constant ratio $k_{CD:S}/k_S$ of Equation (6) at pH = 2 and 4 and at different λ^{em}, for the system HSA + HPBCD at 25 °C[a].

System	pH	λ^{em} (nm)	$K_{CD:S}$ (M^{-1})	$k_{CD:S}/k_S$	10^2 St.Dv.
HSA + HPBCD[b]	2	420	1830	2.0	2.5
		440	1470	3.1	3.9
		480	1430	5.5	5.5
	4	380	71	0.18	2.2
		400	58	0.46	2.3
		420	47	0.53	2.3

[a] These values result from a NLR analysis of the experimental data to Equation (6), but with the contribution of equilibria 1–4 (see text for further discussion).
[b] λ_{ex} = 330 nm, λ^{em} ranges from 340 to 600 nm, ex/em slitwidths = 5/5 nm, scan rate = 240 nm/min, [HSA] = 5×10^{-4} M at pH = 2 and [HSA] = 10^{-5} M at pH = 4, [HPBCD] ranges from 0 to 14 mM at pH = 2 and from 0 to 32 mM at pH = 4, buffers: a KCl/HCl solution at pH = 1.910 and a CH_3COOH/CH_3COONa solution at pH = 4.012.

indicates that the dissociation equilibrium of the salicylic is shifted toward the acidic form. As a result, what we observe in Figure 2 is that, as long as CD is added, the spectra are shifted toward longer wavelengths and are enhanced toward higher intensities, corresponding to the behaviour of an increasing concentration of the CD : HSA complex.

Analogously, at pH = 4, salicylate species is clearly predominat, but the affinity of the 10% of the salicylic form for the CD is so much higher than that of the predominant salicylate, that all the equilibria are again shifted toward the formation of the CD : HSA complex. Since the CD : HSA complex is characterized by a longer λ^{em}_{max} and a much lower intensity for a given concentration with respect to the CD : SA$^-$ complex, what we observe in Figure 3 is a decrease of the intensity and a red shift of the spectra, as long as HPBCD is added and [CD : HSA] increases. It seems that, in the absence of cyclodextrin the salicylate 'governs' the salicylic form since it is much more fluorescent at the same concentration, while in the presence of CD the salicylic form 'controls' the situation due to its tighter binding with the cyclodextrin. Generally speaking, the magnitude of these changes at pH $\approx$ pK_a of any other acid/base conjugate system depends on the binding constants of both CD : HA and CD : A$^-$ complexes, the pH of the solution, the fluorescence maximum intensity and the λ^{em}_{max} of the different species, and their concentrations.

The quantitative treatment of the experimental data in Figures 2 and 3 is problematic, as we have commented above. If we try to proceed in this case, as we have done with the two other titrations, fitting the I/I_0 values vs. [CD] to Equation (6) through the NLR method at three different wavelengths (see Figures 7 for pH = 2, as an example), we obtain the results reported in Table II. It can be observed in

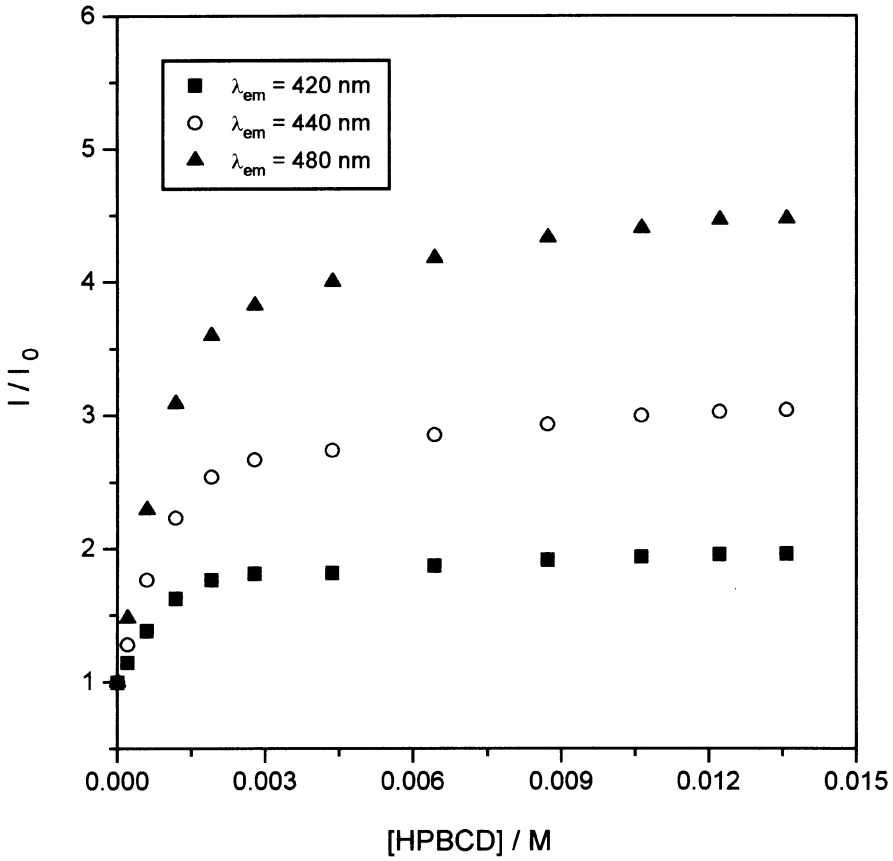

Figure 7. Values of the fluorescence intensity enhancement (I/I_0) due to the encapsulation by the CD cavity of HSA ([HSA] = 4.97 $\times$ 10^{-4} M) at pH = 2, as a function of HPBCD concentration at three different emission wavelengths (λ^{em}).

this table that neither the $K_{CD:S}$ values nor the $k_{CD:S}/k_S$ results are independent of λ. Besides, the values obtained are unacceptable compared to those previously independently obtained for the HSA and SA^- complexes. The point is that it is absolutely wrong to apply Equation (6) to a situation in which there is more than one equilibrium involved. In such a case, Equation (6) should include the terms related to all the species involved. But if an NLR method is used for a system of equations with six or seven fitting parameters, i.e. the equilibrium constants ($K_{CD:HSA}$, $K_{CD:SA^-}$, K_a and K'_a, although K_a can be previously known and K'_a is related with the others through Equation (4)) and the proportionality constants ($k_{CD:HSA}$, $k_{CD:SA^-}$, k_{HSA} and k_{A^-}), the physical meaning and the accuracy of the parameters obtained is almost a utopia. In this connection, our group is developing a method, based on that of Gelb and coworkers [18], to obtain these association

constants while avoiding the use of buffered solutions, measuring just the pH of the solution as long as the four equilibria are shifted when the cyclodextrin is added.

4. Conclusions

Several conclusions result from this work: (a) the study of the encapsulation by CDs of substrates which can be in different proportions in solution depending on an equilibrium, such as the case of an acid/base conjugate system, must be carried out with a careful choice of the pH at which the measurements are made. This pH value must differ by at least two units from the pK_a value of the system. If this premise is not followed, the association constant is not real and reflects the contributions of different inclusion processes; (b) as a consequence, one must be very cautious when comparing the results with the literature. The comparison must be done always under the same pH conditions, and those K values obtained at pH values close to pK_a must be suspect; (c) we insist that linearization methods must be absolutely avoided when accurate and reproducible binding constants are required. NLR methods should be the method of choice. Besides, when spectroscopic techniques are used to carry out these studies, the calculation must be done at different wavelengths to check the independence of K on λ; (d) when for any reason, it is not possible to work at pH values differing by at least two units from pK_a, the method reported in this paper must be avoided even if the four equilibria (1–4) are considered, due to the large number of fitting parameters; (e) in such cases, the method of choice would be to work with unbuffered solutions, just measuring how the pH of the medium varies as the CD is added (option iii).

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