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The Determination of the Stoichiometry of Cyclodextrin Inclusion Complexes by Spectral Methods: Possibilities and Limitations

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1. Introduction

The inclusion complexes of many organic ligands, drugs or metal ions, in cyclodextrins (CDs) represent a class of the simplest supramolecular systems widely studied for the last several decades. The increasing interest for their investigation arises from both a theoretical and an applicative points of view. Considering the first one, their study contributes to the understanding of the molecular recognition and molecular interactions, emphasizing the role of different structural factors. From a more practical purpose, the encapsulation of different drugs in the CD cavity produces an increase in the solubility (Brewster & Loftsson, 2007) and allows for a more controlled oral, parental, ocular, nasal or rectal drug release (Challa et al., 2005). A special interest resides in the CDs potential as alternatives for conventional anti-obesity medications (Grunberger et al., 2007). The same goal is also realized using the supramolecular systems, such as hydrogels, obtained by the self-assembling of CDs with some polymers (Li, 2010; Zhang & Ma, 2010). One of the CDs applications that deserves both an experimental and theoretical focus is based on their interaction with the cellular or model membranes, resulting in cholesterol extraction (Abi-Mosleh et al., 2009). It was shown that the cholesterol removal occurs via inclusion complex formation, for which the stoichiometry plays an important role. Thus, it was stated on both experimental grounds and molecular dynamics calculations that the efficient stoichiometry for the extraction is 1:2 guest:host, requiring, for a good effect, the presence of CD dimers oriented in an appropriate way on the membrane layer (Lopez et al., 2011). Besides these, a remarkable amount of studies were devoted to CDs inclusion complexes, covering an extended basis for the discussion of their properties and applications (Arunkumar et al., 2005; Challa et al., 2005; Li & Loh, 2008; Loftsson et al., 2004; Loftsson & Duchene, 2007; Martin Del Valle, 2004; Sjetli, 1982; Vyas et al., 2008).

Continuing our studies on the CDs inclusion process (Oana et al., 2002; Matei et al., 2007; Tablet & Hillebrand, 2008, Tintaru et al., 2003), we report here some cases encountered in the study of some potentially bioactive compounds like coumarin and phenoxathiin derivatives, and of two drugs, atenolol and indapamide, presenting some peculiar structural factors which make their characterization more difficult. We will focus on the possibilities and limitations of some spectroscopic methods for the estimation of the stoichiometry and

association constants for both cases, i.e. when the procedure is straightforward and when some problems were encountered. The guest molecules were chosen to elucidate some difficulties occurring during the analysis of the experimental data in establishing the real stoichiometry of the inclusion complexes.

This paper starts with a short description of the main features of CDs and their inclusion complexes. After a summary of the main mathematical models used for the estimation of the inclusion complexes properties, we will discuss our results on the specific aforementioned cases.

1.1 Cyclodextrins and the characterization of their inclusion complexes

Native CDs are macrocyclic oligosaccharides formed by the α -1,4-linkage of 6–8 glucose residues and named α -, β - and γ -CD, respectively. They have a toroidal structure and, based on molecular recognition principles, are able to include in their cavity different organic molecules and ions. Therefore, they will be hereafter named as Host, H, while the ligands are generally labelled as Guests, G. Differing from the hydrophobic character of the cavity, the presence of the primary and secondary OH groups ensures an hydrophilic exterior. Besides these three CDs, a large number of modified CDs were synthesized and used for experimental studies, aiming to a better understanding of the driving forces of the inclusion process. Furthermore there is an increasing interest on CDs, due to their multiple applications, especially in pharmacology and biotechnology. The characterization of a CD inclusion complex can be performed using different experimental methods and implicates several factors briefly summarized below.

1.1.1 Methodology

The spectral methods are based on monitoring the guest corresponding spectra, at a constant concentration, when increasing amounts of CDs are added. In absorption and fluorescence spectroscopy, the main effects in the guest spectra are changes in the intensity and/or band shifts. They may be accompanied by the appearance of isosbestic/isoemissive points, which indicate an equilibrium between the free and the complexed guest. Differently, the absence of isosbestic points can be indicative of the occurrence of high order associations in solution (Hamai et al., 1992). In the spectral range of the common guest molecules, there is no interference from the absorption bands of the CDs, located in the far UV region. Using NMR spectroscopy, the experimental data for characterizing the complexes are the variation in the chemical shifts of both guest and host protons (Tintaru et al., 2003). IR and Raman spectroscopies can be used as well for the characterization of the inclusion complexes.

A spectral method which brings about interesting information is the circular dichroism spectroscopy. Circular dichroism is essentially an absorption phenomenon occurring when an optically active molecule absorbs to different degrees the right and left components of a circularly polarized light beam. The magnitude of the dichroic signal depends on the difference between the molar extinction coefficients of the molecule for the right- and left-handed components. The changes that inclusion to CDs, chiral environments themselves, may produce in the circular dichroism spectrum of a guest molecule are of two types, depending on the nature of the guest: a) for a chiral guest, CD inclusion may lead to changes

in its intrinsic dichroic signal; b) for an achiral guest, CD inclusion may lead to the appearance of an induced dichroic signal of the guest, by chirality transfer from the CD. As the circular dichroism technique offers data on the steric match between the two interacting partners, i.e. on the “geometry” of the interaction, the dimensions of the CD cavity mainly determine the changes observed in the spectrum of the guest upon CD incorporation. The dichroic band position is the same as the absorption one corresponding to the chromophore involved in the electronic transition.

The sign of the dichroic band of a guest molecule included in the CD cavity depends on the angle between the electronic transition moment and the symmetry axis of the CD. The rules of Harata and Kodaka state that the dichroic signal is positive for an axial inclusion (parallel orientation of the transition moment with respect to the CD axis) and negative for an equatorial inclusion (perpendicular orientation) (Harata & Uedaira, 1975; Kodaka, 1993). Thus, it is possible to obtain valuable information on the stoichiometry and the orientation of the guest molecule in the complex. Moreover, the intensity of the dichroic signal gives an indication on the magnitude of the interaction, namely on the induced asymmetry.

1.1.2 The stoichiometry

The stoichiometry of the complex is given by the number of G and H molecules contained in the supramolecular complex, the general notation being G_nH_m ; the most common stoichiometry is 1:1 (GH), implying the inclusion of a single guest molecule, but other stoichiometries like G_1H_2 , G_2H_1 , G_2H_2 , G_1H_3 , G_3H_1 , etc., can be encountered as well (Baglolle et al., 2005; De Azevedo et al., 2000; Ge et al., 2011; Sancho et al., 2011; Shen et al., 1998) and some of them will be further discussed from a structural point of view. As the formation of the G_1H_2 complex can be the result of two successive equilibriums, the simultaneous presence of 1:1 and 1:2 complexes is also frequently mentioned.

Self-assembled, stable supramolecular systems can also be formed, containing a large number of H and G molecules. Such systems include the rotaxanes, catenanes and nanotubes (Li et al., 2011; Qu et al. 2005; Yui et al., 2009). In some cases, the obtained complexes form extended linear aggregates, revealed mostly from the unexpectedly large value of the fluorescence anisotropy, e.g. 2,5-diphenyl-oxazole with γ -CD (Agbaria & Gill, 1988), 2,5-diphenyl-1,3,4-oxadiazole, 2-phenyl-5-(4-biphenyl)-1,3,4-oxadiazole and 2,5-biphenyl-1,3,4-oxadiazole with γ -CD (Agbaria & Gill, 1994), 2,5-bis-(4-methylphenyl)-oxazole with β -CD and γ -CD (Yarabe et al., 2002), 1,4-diphenyl-1,3-butadiene with β -CD and γ -CD (De La Pena et al., 1997), trans-1,6-diphenyl-1,3,5-hexatriene with β -CD and γ -CD (Li & McGown, 1994), 4,4'-bis(2-benzoxazolyl) stilbene with β -CD and γ -CD (Wu et al., 2006) and coumarin 153 with γ -CD (Mandal et al., 2011).

1.1.3 The association constant

The quantitative measure of the guest–host interaction at a given temperature is represented by the value of the equilibrium constant governing the formation of the inclusion complex. It is also referred to as binding or association constant. The mathematical models for processing the experimental data in order to estimate the association constants will be discussed further for some of the most common cases.

1.1.4 The thermodynamic parameters

Performing the experiments at different temperatures and using the estimated associated constants in a Van't Hoff treatment allow for the determination of the thermodynamic parameters of the inclusion process, ΔH and ΔS (Tablet & Hillebrand, 2008). The sign and the absolute relative values of these quantities are further used in the discussion of the main forces involved in the inclusion process, i.e. electrostatic, hydrophobic, hydrogen bonds, etc.

1.1.5 Structural aspects

The structure of an inclusion complex in solution is difficult to be established. The guest can penetrate the CD cavity in several ways, through the wide or the narrow rim, in an axial or equatorial position with respect to the cavity long axis. Although for obtaining the previously mentioned features all the spectral methods (absorption, fluorescence, circular dichroism and NMR spectroscopy) can be used, information on the structure of the complexes can be experimentally obtained only from 2D-NMR and induced circular dichroism (ICD) spectra. The presence of the asymmetric environment of the cavity can induce a dichroic signal even for the achiral guests. According to the Harata-Kodaka rules, the positive/negative sign of the dichroic band indicates the axial/equatorial inclusion of the guest in respect to the cavity main axis. This information can be used as starting point to elaborate structural models that will be further optimized at different theoretical levels (molecular mechanics, semiempirical methods, DFT). The best test for judging the consistency of the model, although difficult to reach, is the agreement between the simulated ICD spectrum of the G_nH_m system and the experimental one.

1.2 Determination of the stoichiometry. Job's method

One of the first methods used for the determination of the stoichiometry of inclusion complexes was Job's method, also known as the continuous variation method (Job, 1928). The experiments use stock solutions with equimolecular concentrations of H and G components. The samples are prepared by mixing different volumes of these two solutions in such a way that the total concentration $[H]+[G]$ remains constant and the molar fraction of the guest, X_G varies in the range 0–1. The variation of the experimental measured property, ΔP , in presence of the host in respect with the value for the free guest is plotted vs. X_G or X_H . The value of X_G for which the plot presents the maximum deviation gives the stoichiometry of the inclusion complex ($X_G = 0.5$ for 1:1 or 2:2 G:H complexes; $X_H = 0.33$ for 1:2 G:H complexes). Although, in most cases, in a Job plot ΔP represents the change of the absorbance of the guest during addition of the host, ΔA , (Rajaram et al., 2011), other properties correlated with the concentration of the complex, like the change in the NMR chemical shifts ($\Delta\delta$) or the enthalpy changes (ΔH) can be used as well (Chadha et al., 2011; Kacso et al., 2010; Thi et al., 2011).

Two typical schematic Job's plots for 1:1 and 1:2 inclusion complexes are given in Fig. 1, considering ΔP as the absorbance change, ΔA , for the HG complexes and the NMR proton shifts ($\Delta\delta$) for the HG_2 complexes. It can be seen that in the case of 1:1 complexes, the maximum deviation is obtained for $X_H = 0.5$, while for the second type of complexes the maximum is reached for $X_H \sim 0.37$. Literature data offers a lot of examples for the application of Job's method (Ge et al., 2011; Jadhav et al., 2007; Liu et al., 2001; Sainz-Rozas et al., 2005).

A development of Job's method was described by Landy *et al.* (Landy *et al.*, 2007) for the determination of the stoichiometry of CD inclusion complexes and named Competitive Continuous Variation Plot. In fact, this new approach represents a coupling of Job's method with the competitive experiments, spectral displacements, well known in the study of biopolymer-ligand interactions. The basic idea was to monitor the changes of a given experimental property and to build a Job plot when a competitor ligand, for which the features of the inclusion complex were previously determined, is introduced in the system. The method is recommended for the cases in which either the low solubility prevents the usual experimental determinations or the spectral properties of the guest are not in the experimental accessible range.

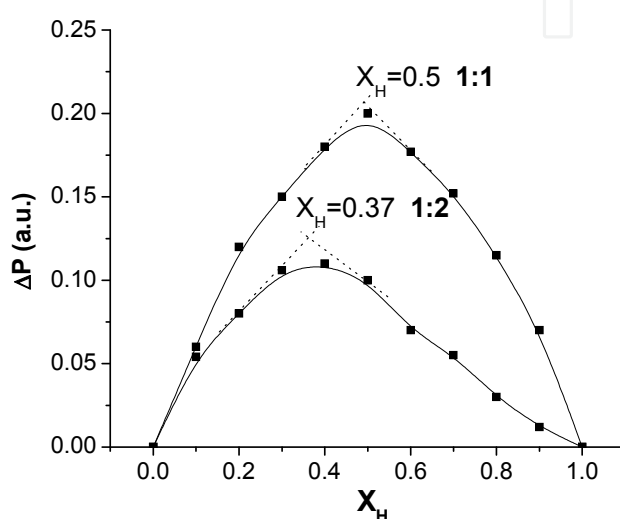


Fig. 1. Typical Job's plots for 1:1 and 1:2 complexes.

1.3 Determination of both the stoichiometry and the association constant

The determination of the stoichiometry in the host-guest interaction is strongly correlated with the estimation of the association constant. Excepting Job's method which gives indications only on the stoichiometry of the inclusion complexes, for all other methods the following procedure is applied. Several stoichiometries are assumed and the experimental data are fitted to the corresponding linear or nonlinear models. The description of all these models and their different applications are given in very well known books and reviews (Connors, 1987; Singh *et al.*, 2010). Therefore, we will further present in Table 1 the main formula used in the analysis of the spectral experimental data, without the corresponding deductions. Starting with the equations of the assumed chemical equilibria, the general idea is to monitor the changes of an experimental property (P_{obs}) directly correlated with the concentration of the former or the new-formed species, at gradual host addition. The function $P_{\text{obs}} = f(C_i, \text{parameters})$, where C_i represents the equilibrium concentration of the species i , is called the binding isotherm. As for Job's plot, this property can be the absorbance (A), fluorescence quantum yield or fluorescence emission (Φ or F), ellipticity (θ) or NMR chemical shift ($\Delta\delta$). Starting from the binding isotherm, the equations corresponding to some widely used linear (eqs. 1, 2, 5) (Benesi & Hildebrand, 1949; Scott, 1956) and nonlinear (eqs. 3, 4, 6-9) (Liu *et al.*, 2001; Park *et al.*, 2002) models are given in Table 1.

| Stoichiometry | Equations |
|---------------|--|
| 1:1 | $G + H \rightleftharpoons GH$ |
| | $\frac{1}{\Delta P_{obs}} = \frac{1}{(P_{GH} - P_{G_0})K_{11}[H]} + \frac{1}{(P_{GH} - P_{G_0})} \quad (1)$ |
| | $\frac{[H][G]_0}{\Delta P_{obs}} = \frac{[G]_0}{P_{GH}} + \frac{1}{K \cdot P_{GH}} \quad (2)$ |
| | $P_{obs} = \frac{P_{G_0} + P_{GH}K_{11}[H]}{1 + K_{11}[H]} \quad (3)$ |
| | $\Delta P_{obs} = \frac{1}{2}([H] + [G] + \frac{1}{K_{11}}) - \sqrt{([H] + [G] + \frac{1}{K_{11}})^2 - 4[H][G]} \quad (4)$ |
| 1:2 | $G + 2H \rightleftharpoons GH_2$ |
| | $\frac{1}{\Delta P_{obs}} = \frac{1}{(P_{GH} - P_{G_0})K_{12}[H]^2} + \frac{1}{(P_{GH} - P_{G_0})} \quad (5)$ |
| | $P_{obs} = \frac{P_{G_0} + P_{GH_2}K_{12}[H]^2}{1 + K_{12}[H]^2} \quad (6)$ |
| 1:1+1:2 | $G + H \rightleftharpoons GH; \quad GH + H \rightleftharpoons GH_2$ |
| | $P_{obs} = \frac{P_{G_0} + P_{GH}K_{11}[H] + P_{GH_2}K_{11}K_{12}[H]^2}{1 + K_{11}[H] + K_{11}K_{12}[H]^2} \quad (7)$ |
| 2:1 | $G + H \rightleftharpoons GH; \quad GH + G \rightleftharpoons G_2H$ |
| | $P = P_{G_2H} \frac{1}{2}([G]_0 - [G] - K_{11}[H][G]) \quad (8)$ where $[G] = \frac{-(K_{11}[H] + 1) + \sqrt{(K_{11}[H] + 1)^2 + 8K_{11}K_{21}[H][G]_0}}{4K_{11}K_{21}[H]}$ |
| 2:2 | $G + H \rightleftharpoons GH; \quad GH + GH \rightleftharpoons G_2H_2$ |
| | $P = P_{G_2H_2} \frac{1}{2}([G]_0 - [G] - K_{11}[H][G]) \quad (9)$ where $[G] = \frac{-(K_{11}[H] + 1) + \sqrt{(K_{11}[H] + 1)^2 + 8K_{11}^2K_{22}[H]^2[G]_0}}{4K_{11}^2K_{22}[H]^2}$ |

Table 1. Nonlinear and linear fitting models for the determination of the stoichiometry and association constant of a CD inclusion complex.

An interesting case is the formation of inclusion complexes in which the guest is included as a dimer. There are two types of stoichiometry for such complexes: 2:1, obtained by the

inclusion of a second guest molecule in a 1:1 complex already formed, or 2:2, resulted from the association of two 1:1 complexes. Over the time, Hamai has reported extensive studies on this regard (Hamai, 1990, 1999, 2005, 2010). Fluorescence is an excellent tool to reveal the formation of these complexes because the dimer, called excimer in excited state, has a distinct band localized at longer wavelength than the monomer. Monitoring the dependence of its fluorescence intensity *vs.* its concentration we can distinguish between the 2:1 and 2:2 stoichiometries using the eqs. 8 and 9 from Table 1. There are two ways to find the K_{21} and K_{22} values. The first one is to drive the experiment at small guest concentrations, when the dimer does not exist, and thus to obtain the K_{11} value. Then, with this value, we can simulate curves according to eqs. 8 and 9 for various values of K_{21} and K_{22} . The best fit gives the value of the association constant. The second one is to fit the data directly with the said equations and to obtain K_{11} and K_{21} or K_{22} .

When several complex types, i.e. complexes with different stoichiometry, are present in the system, the necessity to introduce several fitting parameters reduces the reliability of the fits. Therefore, for spectral measurements, one recommended method (Davies & Deary, 1999; Sainz-Rozas et al., 2005) is to work with sets of data read at different wavelengths and to perform a multivariable analysis of the whole set of data, imposing the condition that the association constants are the same, independent on the wavelength.

A special case when it is impossible to unambiguously differentiate by fitting procedures between the 1:1 and the mixture of 1:1+1:2 complexes was discussed by Pistolis and Malliaris (Pistolis & Malliaris, 1999). They found that this happens when the ratio of the corresponding equilibrium constants K_{11}/K_{12} depends on the extinction coefficients of the species present in the system, the free guest, the 1:1 complex and the 1:2 complex, according to eq. 10, written in respect to the extinction coefficients:

$$\frac{K_{11}}{K_{12}} = \frac{(\epsilon_{GH_2} - \epsilon_G)^2}{(\epsilon_{GH} - \epsilon_G)(\epsilon_{GH_2} - \epsilon_{GH})} \quad (10)$$

The same relationship holds also for the analysis of fluorescence data, with the fluorescence quantum yields of the three species, Φ_G , Φ_{GH} , Φ_{GH_2} , replacing the extinction coefficients. The authors recommend that in such cases additional experiments must be carried out, for example fluorescence time-resolved or anisotropy experiments.

The most used equations are the Benesi-Hildebrand linear or double reciprocal equations. However, their reliability was the subject of many discussions, especially to differentiate the formation of 1:1 and 1:2 complexes. Besides the usual approximations included in the deduction of the Benesi-Hildebrand equation, Wang (Wang & Yu, 2007) used computer simulations to establish some required experimental conditions for obtaining consistent results with the Benesi-Hildebrand equations, rules that can also be applied for the guest-CD systems.

Shortly, they stated that the CD concentration should be at least an order of magnitude larger than the guest's, the minimum concentration ratio of the two partners should be sufficiently large and the ratio $1/(K[G])$ should be large, around 20. Analyzing the electronic absorption data, they also emphasized the role of the differences in the extinction coefficients for the 1:1 and 1:2 complexes compared to the free guest.

A slight different methodology for the association constants estimation considers the time-resolved fluorescence experiments. The study of the decay fluorescence curves allows for the estimation of the stoichiometry, and, consequently of the association constants. In the case of the 1:1 complexes, the decay fit by two exponentials is expected, corresponding to the presence of two species in the system, namely the complexed and uncomplexed guest. Considering the ratio of the two pre-exponential coefficients (B), the following relation is obtained:

$$\frac{B_{bound}}{B_{free}} = \frac{C_{bound}kr_{bound}\epsilon_{bound}}{C_{free}kr_{free}\epsilon_{free}} \quad (11)$$

where C, kr and ϵ represent the concentration, the radiative rate constant and the extinction coefficient, respectively, for the bound and free species (Monti et al., 1993).

Since kr is constant and we can consider $\epsilon_{bound} = \epsilon_{free}$, in excess of CD eq. 11 becomes:

$$\frac{B_{bound}}{B_{free}} = K[CD] \quad (12)$$

The linearity of the plot of B_{bound}/B_{free} vs. the CD concentration confirms the formation of a complex with 1:1 stoichiometry and allows for the estimation of the association constant (Rajaram et al., 2011).

Some examples, emphasizing different situations encountered in the CD inclusion complexes study, starting with a guest for which the stoichiometry and the association constants were unambiguously established and continuing with cases of increasing complexity, are reported.

2. Experimental methods and computational details

α -, β -, 2-HP- β and γ -CDs (Aldrich) were used as received. 2-[2'-quinoxaliny]-phenoxathiin was synthesised as described in Ref. (Nicolae et al., 1998). Indapamide was purchased from Helcor (Romania). Two samples of atenolol were used, one purchased from Helcor and the other from IPCA (India). The guest-CD samples were prepared from stock solution of both components, keeping in all cases the guest concentration constant (10^{-6} - 10^{-5} M for fluorescence measurements and 10^{-5} - 10^{-4} M for absorption and circular dichroism measurements). The spectra were recorded on a Jasco FP-6300 spectrofluorimeter, Jasco J-815 CD spectropolarimeter and Jasco V-560 UV-VIS spectrophotometer.

All the necessary computational steps to find the most stable complexes in vacuo and in water were detailed in a previous paper (Matei et al., 2009).

3. Some particular cases encountered in the estimation of the stoichiometry of guest-cyclodextrin inclusion complexes

3.1 2-[2'-quinoxaliny]-phenoxathiin (QP)

An unequivocal example for the determination of the stoichiometry is given by the inclusion complex formed by QP with 2-HP- β -CD. QP is a fluorescence probe sensitive to the medium

polarity and ability to form hydrogen bonds. In presence of 2-HP- β -CD, a decrease in the emission from QP is observed (Fig. 2A). The changes in the fluorescence intensity of guests upon CD inclusion are usually rationalized by comparing the emission properties of the complexed guest with those of the free guest in different solvents (Wagner et al., 2003). The fluorescence quantum yield of QP in propanol, solvent of polarity similar to that of the CD cavity, is smaller than in dimethylformamide–aqueous solution, the medium in which the experiment was performed. On this basis, the experimentally observed fluorescence decrease was ascribed as due to the lower polarity experienced by QP inside the CD cavity, as compared to aqueous solution.

In this case, the fluorescence–concentration dependence reflects reliable experimental data; the entire binding isotherm was obtained even without using the maximum possible host concentration. Fitting the data with eq. 3 evidenced the 1:1 stoichiometry of the complex (Fig. 2B) and the value of its association constant (inset of Fig. 2B). The results obtained for the 2-HP- β -CD complex of QP are in accordance with our results previously obtained for its β -CD complex (Matei et al, 2007).

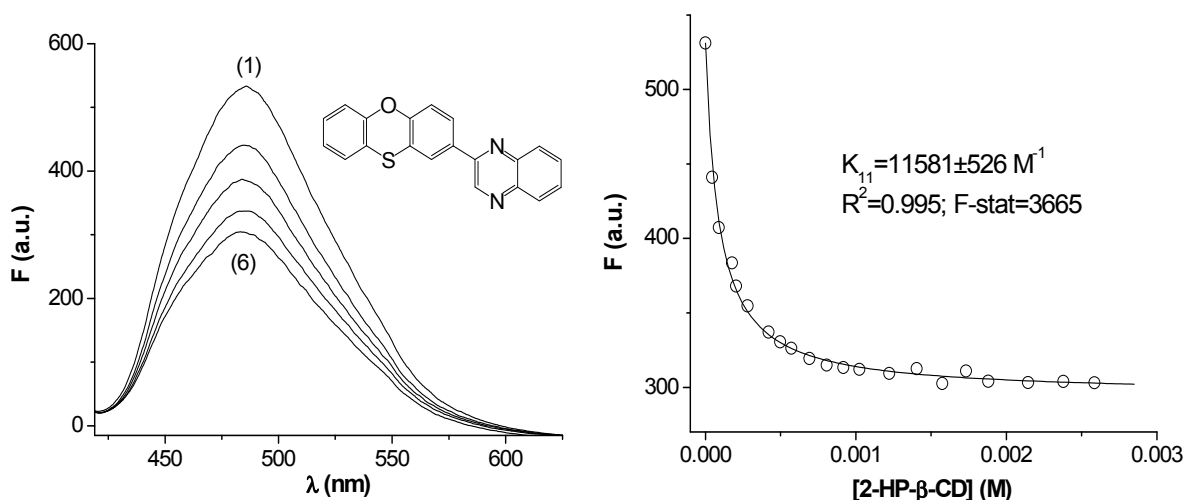


Fig. 2. (A) The fluorescence spectrum of QP (10^{-5} M in dimethylformamide:water 1:9 v:v) in absence (1) and presence (2–6) of increasing amounts of 2-HP- β -CD (up to 3×10^{-3} M). (B) Determination of the 1:1 stoichiometry of the complex by means of eq. 3. Inset: parameters of the fit.

3.2 7-Diethylamino-3-carboxycoumarin (DEAC)

The CD inclusion complexes were often considered as a starting point for choosing good fluorophores to be used in protein studies (Abou-Zied & Al-Hinai, 2006). The changes in the photophysical properties of the guests upon inclusion in the CD cavity can be correlated to their sensitivity on the local polarity, possibility to be involved in hydrogen bonds, etc. Our previous studies on 3-carboxy-coumarin derivatives (Varlan & Hillebrand, 2011) showed that this class of compounds, present in aqueous solution of pH = 7.4 as the corresponding carboxylate ions, are suitable for protein studies. Among the carboxycoumarin derivatives, DEAC represents an interesting guest to be studied in the presence of CD, due to the existence of a second substituent with donor character (the diethylamino group), (Jung et al., 2009; Zhang, et al., 2008).

The fluorescence spectrum of DEAC in the presence of γ -CD, the largest native cyclodextrin, is given in Fig. 3. The increase of the γ -CD concentration induces an enhancement of the emission intensity accompanied by a blue shift of the band (~ 7 nm), without the appearance of an isoemissive point. All this data clearly indicate that the DEAC molecule has passed from the bulk solution to the less polar cavity of the CD, where it has a higher quantum yield and emits at shorter wavelength, as stated by Ramakrishna and Ghosh (Ramakrishna & Ghosh, 2002).

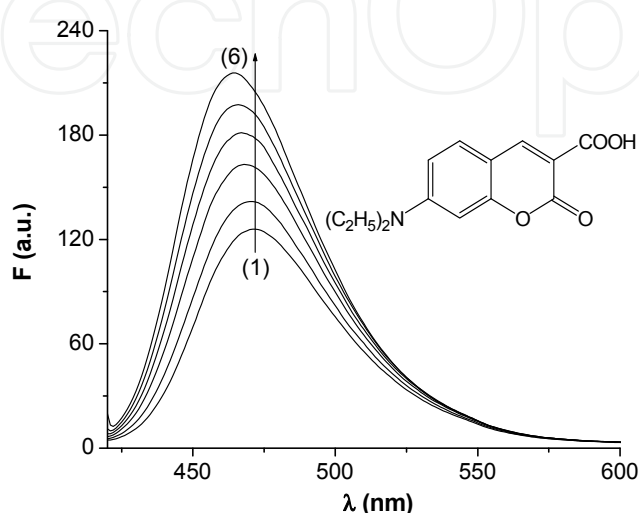


Fig. 3. The fluorescence spectrum of DEAC (2×10^{-6} M) in the presence of increasing amounts of γ -CD. $\lambda_{ex} = 410$ nm.

In order to find the stoichiometry of the complexes, the dependence of the fluorescence intensity *vs.* the γ -CD concentration was analyzed. The experimental points could be fitted with good statistical parameters to both eqs. 3 and 7 corresponding to the formation of 1:1 and of a mixture of 1:1+1:2 complexes (Fig. 4), the parameters being given in Table 2. As it can be seen from the R^2 values, the better fit corresponds to the mixture of 1:1+1:2 complexes. This is also supported by the lower and more random distribution of the fits residuals, included as insets in Fig. 4. Another point to be stressed is that when the ratio of intensities of the bound and free guest in a 1:1 complex is near 1, as in the case of DEAC ($F_{11}/F_0=1.19$), it is very important to have experimental data in the first domain of concentration, otherwise the complexation process will be misinterpreted as a solely 1:1 complex formation.

| Stoichiometry | K_{11} (M^{-1}) | K_{12} (M^{-1}) | F_{11}/F_0 | F_{12}/F_0 | R^2 | F-stat |
|---------------|-----------------------|-----------------------|-----------------|-----------------|-------|--------|
| 1:1 | 1256 ± 112 | - | 1.64 ± 0.01 | - | 0.971 | 831 |
| 1:1+1:2 | 15450 ± 2987 | 421 ± 36 | 1.19 ± 0.01 | 1.72 ± 0.08 | 0.998 | 5721 |

Table 2. Fitted parameters for the DEAC- γ -CD interaction: association constants (K), normalized fluorescence of the complex (F/F_0); R^2 - correlation coefficient; F-stat - Fisher statistic coefficient.

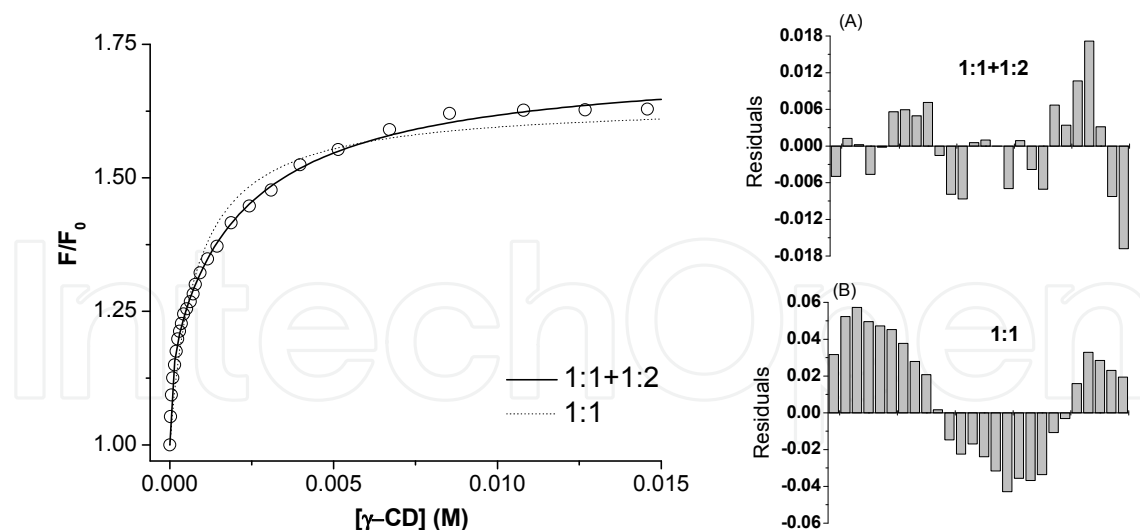


Fig. 4. Normalized experimental data fits with eqs. 7 (solid line) and 3 (dotted line) for the determination of the stoichiometry of the DEAC- γ -CD complex. Inset: The distribution of the residuals for the (A) 1:1+1:2 and (B) 1:1 fits.

3.3 Indapamide

Indapamide is an antihypertensive, diuretic drug of the sulphonamide class (Ciborowski et al., 2004; Ghugare et al., 2010; Radi & Eissa, 2011). We have studied its complexation with α -, β -, 2-HP- β - and γ -CD, by means of absorption spectroscopy. The isolated guest presents two absorption bands, located at 240 nm and 280 nm. In presence of CD, the absorbance of these bands increases, without any band shift or appearance of isosbestic points (Fig. 5). Absorbance increase by complexation has been reported by several authors (El-Kemary, 2002) and considered as evidence for the formation of an inclusion complex. The shape of the spectra of the complexes is similar, irrespective of the CD type, but the magnitude of the absorbance increase depends on the CD type.

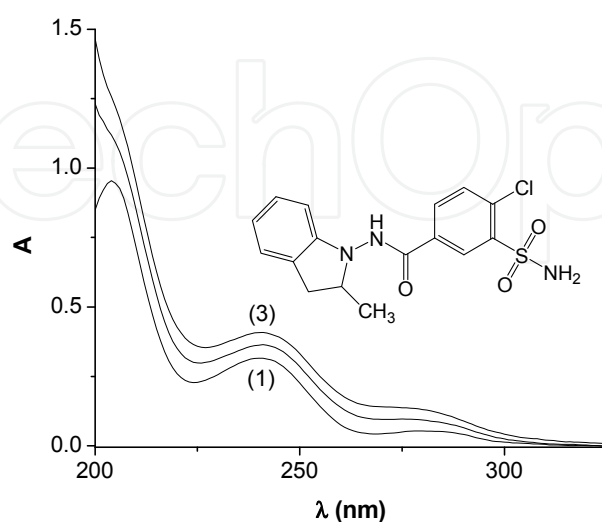


Fig. 5. The absorption spectrum of indapamide (10^{-5} M in methanol:water 1:9 v:v) in absence (1) and presence (2, 3) of increasing concentrations of 2-HP- β -CD (up to 2×10^{-2} M).

In the first attempt to determine the stoichiometry of the complexes, the Benesi-Hildebrand model was applied. As previously discussed, a linear dependency of the type $1/(A-A_0)$ vs. $1/[\text{CD}]^n$, with $n = 1$ or 2 , indicates the presence of complexes of 1:1 or 1:2 stoichiometry, respectively. For indapamide, the plot $1/(A-A_0)$ vs. $1/[\text{CD}]$ shows a positive deviation, irrespective of the CD type (inset of Fig. 6A), which eliminates the hypothesis of the formation of solely 1:1 complexes. This is in accordance with the lack of isobestic points. The plot $1/(A-A_0)$ vs. $1/[\text{CD}]^2$, although characterized by a good correlation coefficient ($R^2 = 0.990$), has unacceptably high standard errors and standard deviations of the fits (Fig. 6A). The Benesi-Hildebrand model cannot offer conclusive data on the presence of the 1:2 stoichiometry.

Applying the nonlinear model describing the formation of a 1:2 complex (eq. 6, dotted line), we obtained good results for all CDs, with small errors (<15%), good correlation coefficients ($R^2 > 0.990$) and Fisher statistic coefficients ($F \sim 1500-7000$) (Fig. 6B). The predicted association constants are: $K_{\alpha\text{-CD}} = 2447 \pm 89 \text{ M}^{-1}$, $K_{\beta\text{-CD}} = 25550 \pm 783 \text{ M}^{-1}$, $K_{2\text{-HP-}\beta\text{-CD}} = 3330 \pm 370 \text{ M}^{-1}$ and $K_{\gamma\text{-CD}} = 3897 \pm 481 \text{ M}^{-1}$.

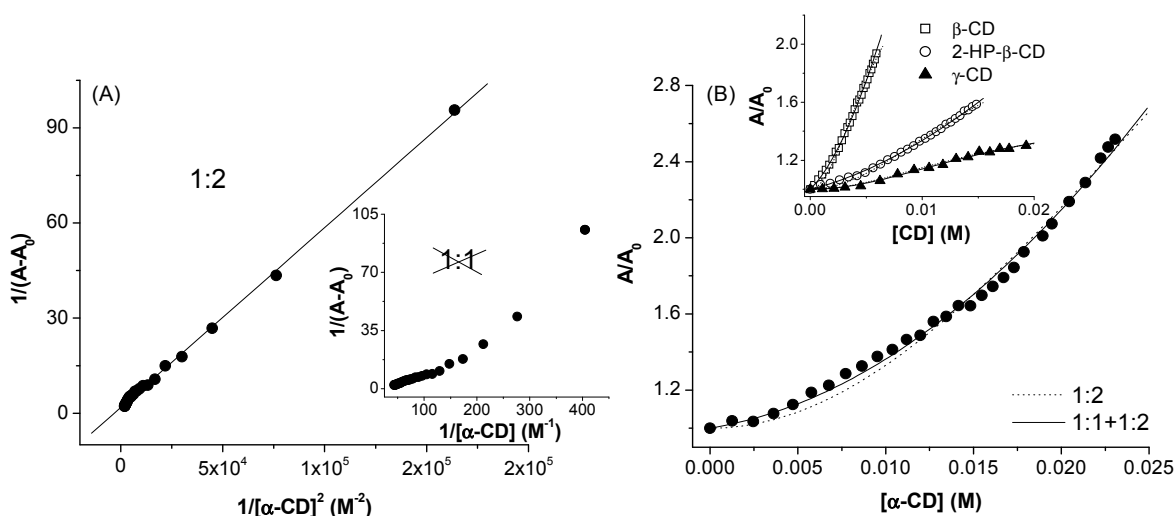


Fig. 6. (A) Benesi-Hildebrand plots for the investigation of the formation of indapamide- α -CD complexes of 1:2 and 1:1 (inset) stoichiometry. (B) The dependence of the normalized absorption of indapamide on the CD concentration. Fits with eqs. 6 and 7.

However, the examination of the first segment of the curves, corresponding to low CD concentrations, reveals some deviations of the experimental points, reflected in the shape of the fit residuals. This prompted us to check for the presence of a mixture of 1:1+1:2 complexes (eq. 7, solid line). These fits are characterized by higher correlation coefficients ($R^2 > 0.995$), as well as smaller and more randomly distributed residuals for the first segment of the curve. Still, in this case the standard errors have high values for K_{12} and A_{12} , due to the uncertainty introduced in the fit by the lack of experimental points on the last segment of the curve, at high CD concentrations.

In the attempt to discern between the two cases, i.e. the 1:2 stoichiometry and the mixture of 1:1+1:2 complexes, we have generated fitting curves on a broader range of CD concentrations, using the values of the association constants and absorbances of the complexes obtained with eqs. 6 and 7, respectively (Fig. 7A). This technique has been

employed before, for the stoichiometries determination of some simvastatin-CD complexes (Matei et al., 2009). From Fig. 7A we can observe that up to a CD concentration of $\sim 2.5 \times 10^{-2}$ M, the two curves are identical, the difference between them appearing only at high CD concentrations. Therefore, in the absence of experimental points in this range, it is impossible to assess without a doubt the stoichiometry of the inclusion complexes. As these experimental data are of crucial importance for the determination of the stoichiometry, the working conditions must be selected in such a way to be as close as possible to the CD solubility limit, i.e. α : 1.5×10^{-1} M, β : 1.6×10^{-2} M, 2-HP- β : 3.3×10^{-1} M, γ : 1.8×10^{-1} M (Connors, 1987).

We performed such an experiment, using indapamide and high concentrations of α -CD (Fig. 7B). This experiment was revelatory and allowed us to discard the formation of mixtures of 1:1+1:2 complexes. Thus, when the experimental points at high CD concentrations were added to the previous experimental data, they aligned on the generated curve corresponding to the formation of 1:2 complexes, revealing that this is the real stoichiometry.

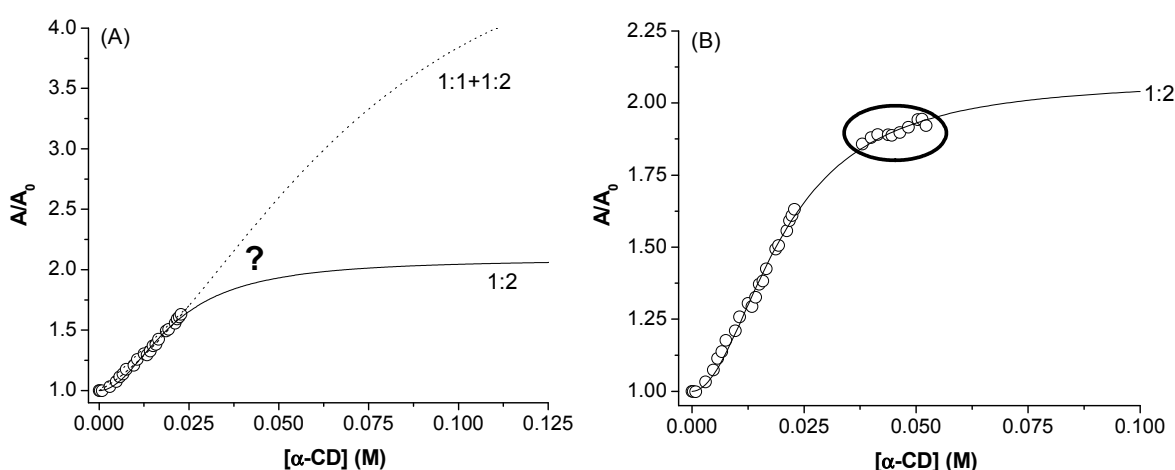


Fig. 7. Generation of the curves describing the formation of 1:2 (eq. 6) and 1:1+1:2 (eq. 7) complexes on a greater domain of CD concentrations than the experimental one (experimental data as open circles), in the absence (A) and presence (B) of experimental points at high CD concentrations.

3.4 Atenolol

Atenolol (inset of Fig. 8), a beta-blocker drug used in the treatment of cardiovascular diseases (Bontchev et al., 2000; Castro et al., 1998; Esteves de Castro et al., 2007; Moloney et al., 1998; Pandeewaran & Elango, 1009; Pulgarin et al., 1998; Ranta et al., 2002), is a very flexible molecule, consisting of fragments with different features: an amido-substituted aromatic ring, a flexible three-carbon chain (Siquintuya et al., 2007) and a dimethyl-substituted amino group.

3.4.1 Photophysical properties of atenolol

The photophysical properties of atenolol, measured prior to the study of its CD inclusion complexes, showed very interesting features. The absorption spectra of atenolol in water, methanol and acetonitrile are presented in Fig. 8A. An intense band ($\epsilon \sim 10^4$ M $^{-1}$ cm $^{-1}$) is recorded at 225 nm and assigned to a π - π^* transition correlated to the presence of the aromatic ring (Pillot et al., 1997). In the range of 265–290 nm, a second band is observed,

characterized by a lower ϵ value ($\sim 10^3 \text{ M}^{-1}\text{cm}^{-1}$), at the limit of a weak $\pi\text{-}\pi^*$ and a forbidden $n\text{-}\pi^*$ transition. According to literature data, this band was considered a benzenic band bathochromically shifted due to the amide substitution (Gratzer, 1967).

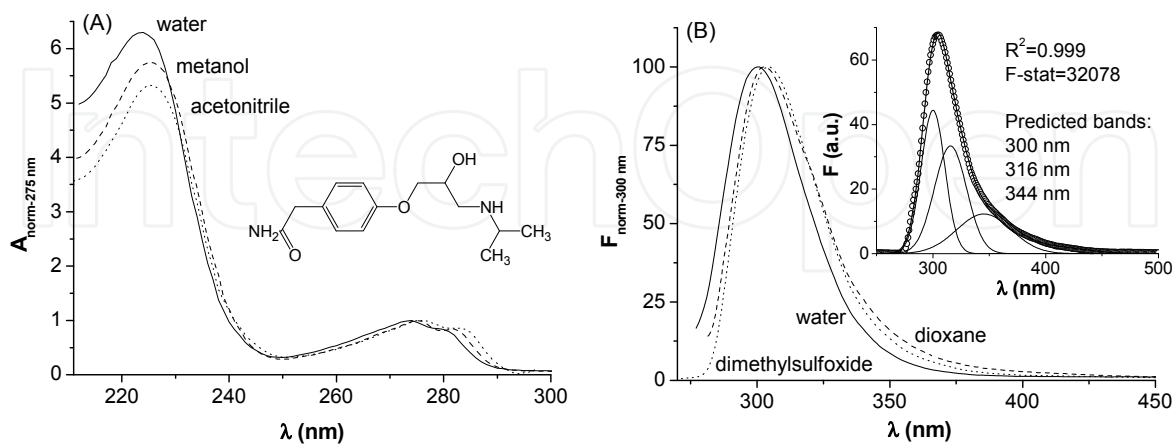


Fig. 8. Absorption (A) and fluorescence (B) spectra of atenolol in different solvents. Inset: Deconvolution of the fluorescence spectrum of atenolol in dimethylformamide. $\lambda_{ex} = 275 \text{ nm}$.

The fluorescence spectrum of atenolol was recorded in nonpolar, polar aprotic and polar protic solvents considering several excitation wavelengths, in the range of the two aforementioned bands. An intense emission band, hereafter labelled B1, slightly influenced by the solvents, was obtained in the range 299–306 nm (Fig. 8B), characterized by a fluorescence quantum yield of 0.11 in water and 0.20 in acetonitrile. A careful examination of the band shape revealed an asymmetry at the longer wavelength range, better evidenced by a deconvolution process, as it can be seen from the inset of Fig. 8B. Besides the main band (300 nm), two other bands were found, one at 316 nm, very close to the first band, and another at 344 nm. This new band, located at 344 nm, much lower in intensity than the former one, will be further called B2. Performing a systematic scanning of the role of different experimental factors (concentration, excitation wavelength, etc.) on the shape of the fluorescence bands, we have found that B2 strongly increases in intensity by dilution, presents in fact two maxima (Fig. 9A) and that it is very sensitive to the excitation wavelength, being much enhanced for $\lambda_{ex} = 250 \text{ nm}$. The ratio of the two bands composing B2 remains constant upon dilution, suggesting a vibrational structure of the band. By comparison with the fluorescence spectrum of a related compound, 4-phenyl-1-N,N-dimethylaminobutane (Xie et al., 2004), B1 was assigned to the emission of the excited aromatic system and B2 to a species in which the excitation is localized on the amino chromophore.

For obtaining supplementary data on the emission process, the fluorescence spectra were recorded at several temperatures in the range 25–75°C using different excitation wavelengths. By cooling back to 25°C, we have observed a reversible decrease of the intensity of the B1 band, which allowed for the estimation of the activation energy of the nonradiative processes. In the limit of experimental errors, the obtained values showed that the process is not influenced by the excitation wavelength ($E_a \sim 1.2 \text{ kcal/mol}$). Analyzing the behaviour of B2 in the same temperature range, a different result was obtained, *i.e.* a larger value of the activation energy of the process, 5.38 kcal/mol, for $\lambda_{ex} = 250 \text{ nm}$.

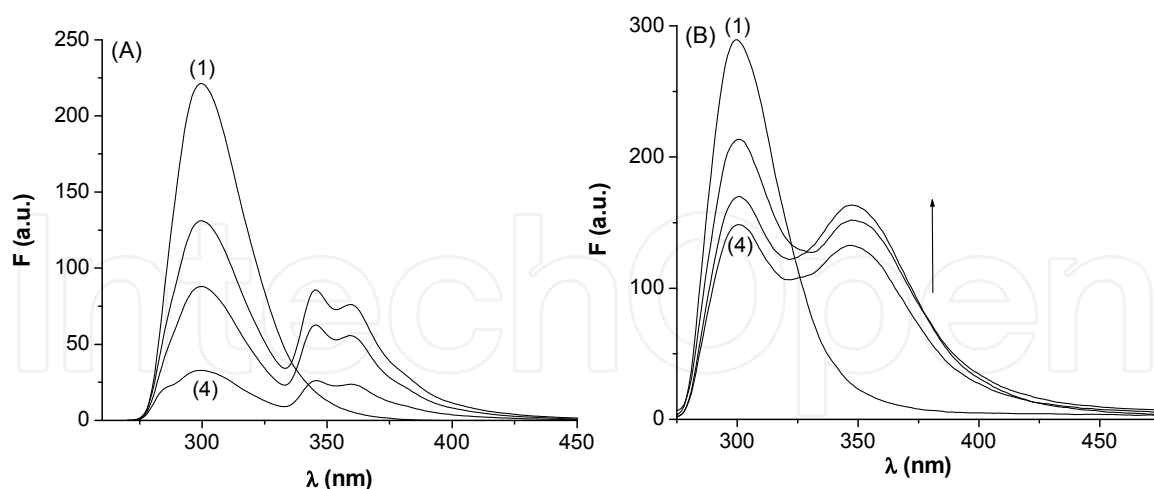


Fig. 9. Evolution of the B1 and B2 fluorescence bands of atenolol in methanol (A) upon dilution with methanol and (B) upon irradiation at 254 nm (three steps of 25 minutes each).

All these data show that atenolol exists in solution as several conformers differently influenced by the solvent, the excitation energy and/or the temperature. Furthermore, considering the pKa value of 9.6 of atenolol (Kasim et al., 2004), we can not rule out the presence of different amounts of protonated/nonprotonated species, especially in water and protic solvents.

In order to obtain more information on the species present in solution, the fluorescence spectrum of atenolol was recorded after irradiation with a mercury lamp at 254 nm. The results are displayed in Fig. 9B and show unambiguously that the irradiation favours the accumulation of the species emitting at 350 nm.

In conclusion, the experimental data on the emission process of atenolol showed the presence of two emitting species, influenced by the concentration, excitation wavelength, temperature and irradiation of the system. All these data must be considered in the further discussion of the atenolol-CD inclusion complexes, especially when the fluorescence spectroscopy is used as the method of investigation.

3.4.2 Characterization of the atenolol-cyclodextrin inclusion complexes

Several spectral methods (FTIR, DSC, SEM, etc.) have been employed for the characterization of the CD inclusion complexes of atenolol in solid state (Borodi et al., 2008; Ficarra et al., 2000a, 2000b). In the following, we will present our results on the interaction of atenolol with α -, β - and γ -CD in solution, studied by means of fluorescence, circular dichroism and absorption spectroscopies. Recording the fluorescence spectra of atenolol in the presence of CDs, we observed slight changes in the intensity of B1 and the strong increase of a band in the same spectral region and with the same shape as the B2 band of uncomplexed atenolol (Fig. 10). This increase indicates that the inclusion process favours the same process as previously discussed for uncomplexed atenolol, *i.e.* the emission correlated to the amino group evidenced in several experimental conditions. This could be due to the inclusion of the isopropylamine fragment in the CD cavity. The fluorescence spectra are strongly dependent on the excitation wavelength (Fig. 11).

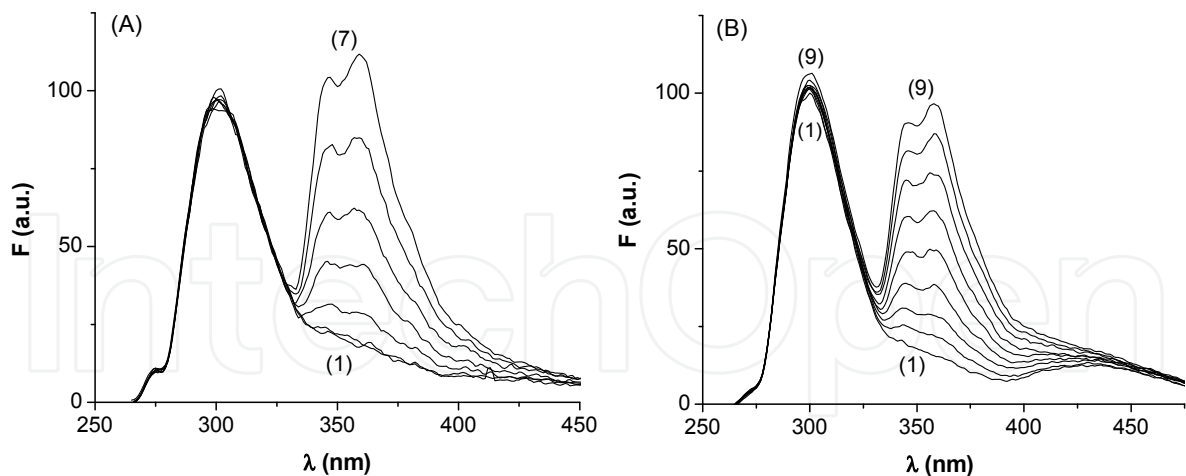


Fig. 10. The fluorescence spectrum of atenolol (2×10^{-5} M in methanol:water 1:9 v:v) in absence (1) and presence (2-9) of increasing amounts of CD: (A) β (up to 5.5×10^{-4} M) and (B) γ (up to 7.3×10^{-4} M). $\lambda_{ex} = 250$ nm.

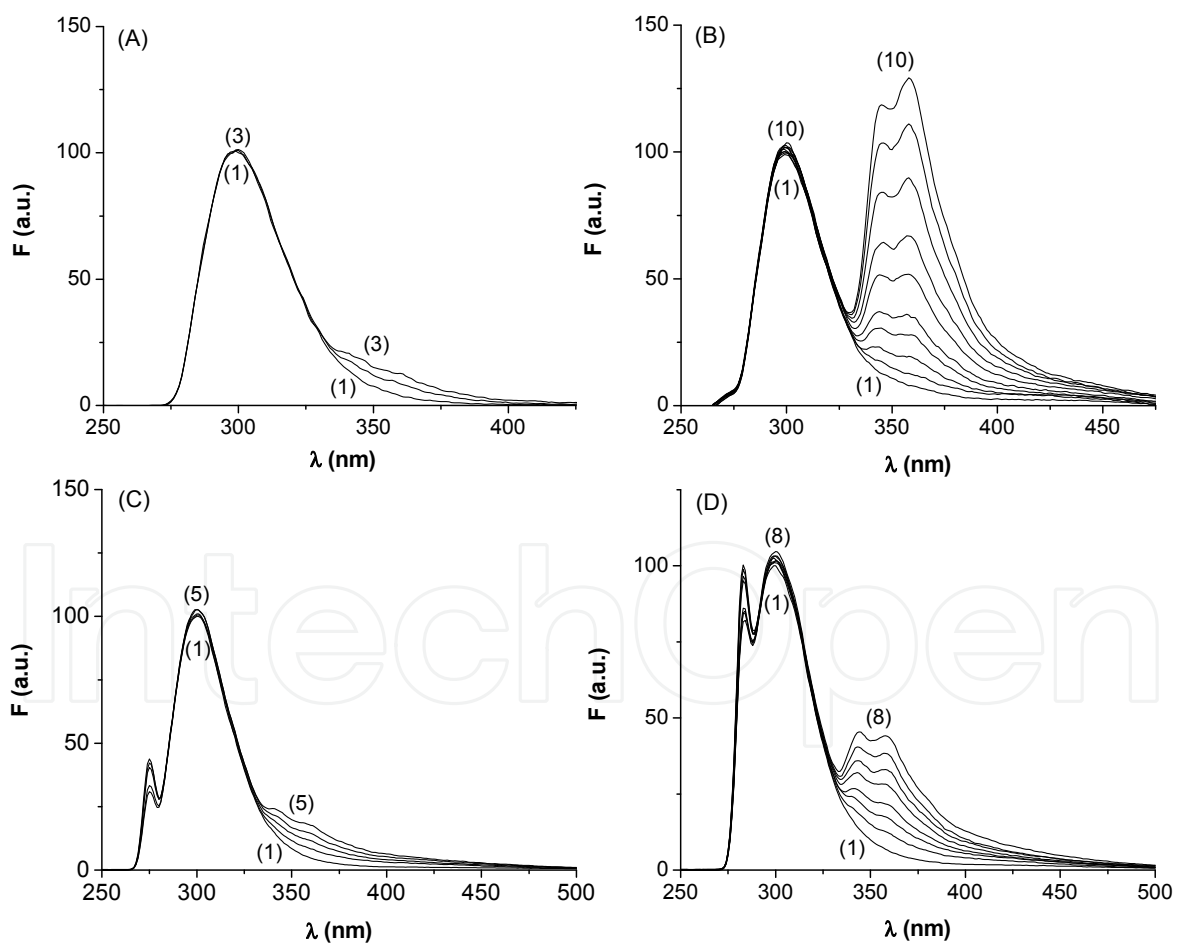


Fig. 11. The fluorescence spectrum of atenolol (2×10^{-5} M in methanol:water 1:9 v:v) in absence (1) and presence (2-10) of increasing amounts of α -CD (up to 7.4×10^{-4} M), at various λ_{ex} (nm): (A) 225, (B) 250, (C) 275 and (D) 282.

The 1:2 stoichiometry of the inclusion complexes in methanol:water has been determined by fitting the experimental data with eq. 6. In the case of the atenolol- α -CD complex, the readings have been made on both B1 and B2, at $\lambda_{ex} = 282$ nm (Fig. 12). In an attempt to obtain a more consistent value of the association constant, for B2, the emission intensities were read at three wavelengths within the band (345, 358 and 380 nm). The obtained data for all CDs are summarized in Table 3. For $\lambda_{ex} = 225$ and 275 nm, the fits resulted in high standard errors, while for $\lambda_{ex} = 250$ nm, due to high data scattering, no reliable results could be obtained. Considering that this band is also evidenced in absence of CD and most prominently at $\lambda_{ex} = 250$ nm, it must be stressed that the great deviations may be due to the superposition of several effects.

The influence of the temperature on the equilibria present in solution is the same, irrespective of the CD type and λ_{ex} (Fig. 13), and it is similar to that discussed for uncomplexed atenolol in different solvents. Upon raising the temperature, we observed an increase in the intensity of B1 and a decrease of B2. Similar data were obtained for all CDs. The trend of B1 is an usually encountered for fluorescent molecules one, and can be explained by an increased magnitude of the nonradiative deactivation processes. Differently, the behaviour of B2 could be rationalized in terms of a conformational change leading to an atenolol molecule in a highly fluorescent conformation. This process could be favoured by the decreased viscosity of the medium, leading to an increased mobility of the molecular fragments. Interesting information can be obtained from the behaviour of B1 and B2 upon cooling. While B1 temperature variation is reversible, the intensity of B2 doesn't return to its original value upon decreasing the temperature. This irreversibility could indicate that the amino group is included in the CD cavity and only successively fixed in a fluorescent conformation.

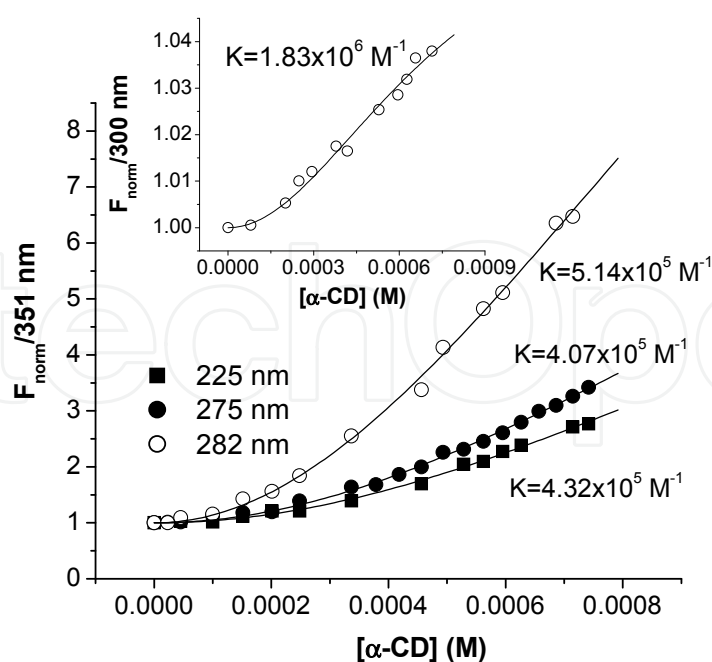


Fig. 12. Plots for the determination of the stoichiometry and association constant of the atenolol- α -CD complex in methanol:water 1:9 v:v using fluorescence data read on B2 at three excitation wavelengths (225, 275 and 282 nm) and B1 (inset) at 282 nm.

| Band | $K_{12} \times 10^{-6} (M^{-1})$ | F_{12}/F_0 | R^2 ; F-stat |
|--------------------|----------------------------------|--------------|----------------|
| <i>α</i>-CD | | | |
| B1, 300 nm | 1.83±0.43 | 1.08 | 0.987; 739 |
| B2, 345 nm | 0.43±0.10 | 15.22 | 0.997; 5291 |
| 358 nm | 0.51±0.09 | 29.13 | 0.998; 7786 |
| 380 nm | 0.81±0.11 | 24.71 | 0.997; 6133 |
| <i>β</i>-CD | | | |
| B2, 345 nm | 3.67±0.77 | 3.47 | 0.991; 1056 |
| 359 nm | 4.91±0.90 | 5.67 | 0.988; 887 |
| 380 nm | 5.54±0.75 | 6.48 | 0.992; 1312 |
| <i>γ</i>-CD | | | |
| B2, 345 nm | 0.94±0.19 | 5.63 | 0.992; 2168 |
| 359 nm | 1.02±0.19 | 10.44 | 0.992; 2252 |
| 380 nm | 1,88±0,30 | 9,19 | 0,988; 1484 |

Table 3. Parameters of the 1:2 atenolol-CD inclusion complexes in methanol:water 1:9 v:v. $\lambda_{ex} = 282$ nm.

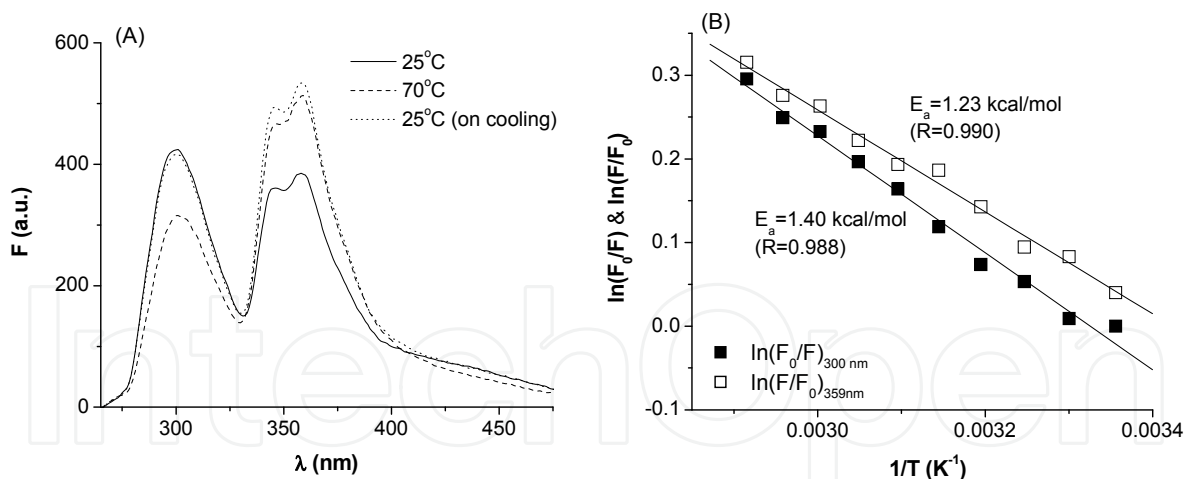


Fig. 13. The temperature dependence of the fluorescence intensity of the atenolol- γ -CD system in methanol:water 1:9 v:v (A) and the activation energies of the respective processes (B). $\lambda_{ex} = 250$ nm.

Differing from the spectra in methanol:water, using dimethylformamide as solvent we observed an enhanced decrease of the B1 band intensity, together with a significant increase of B2 (Fig. 14). Despite the significant intensity change of B1, the readings on this band could not be used for the stoichiometry determination. On band B2, the fits, according to eq. 6, indicated the presence of 1:2 complexes (inset of Fig. 14).

To obtain more data on the atenolol complexation process, several other methods were used, including circular dichroism and absorption spectroscopies, together with molecular modeling. Therefore, the stoichiometry of the inclusion complex of atenolol with β -CD has been determined *via* circular dichroism spectroscopy. While in absence of CD no signal is recorded for atenolol, upon CD addition a negative dichroic signal appears at 275 nm (Fig. 15A). The corresponding calculated transition moment is located in the plane of the aromatic ring, perpendicular to the long molecular axis (*vide infra*). The appearance of an induced circular dichroism signal of atenolol confirms its inclusion into the asymmetric CD cavity and indicates that the aromatic ring is perturbed by CD incorporation. Moreover, its negative sign indicates the perpendicular orientation of the transition moment with respect to the symmetry axis of β -CD.

The data for the system atenolol- β -CD were fitted with eq. 3, revealing the formation of complexes of 1:1 stoichiometry (Fig. 15B). One can observe the greater scattering of the experimental points, characteristic to the measurements by circular dichroism. The data were also analyzed using the Scott model (eq. 2, inset of Fig. 15B), which yielded a K_{11} value in good accordance with the results of the nonlinear model, although the fit was of somewhat lower quality. The value of the association constant also correlates to that obtained by us (Fig. 16) and by Borodi *et al.* (Borodi *et al.*, 2008) ($110 \pm 22 \text{ M}^{-1}$, Scott's model) from UV-vis absorption data.

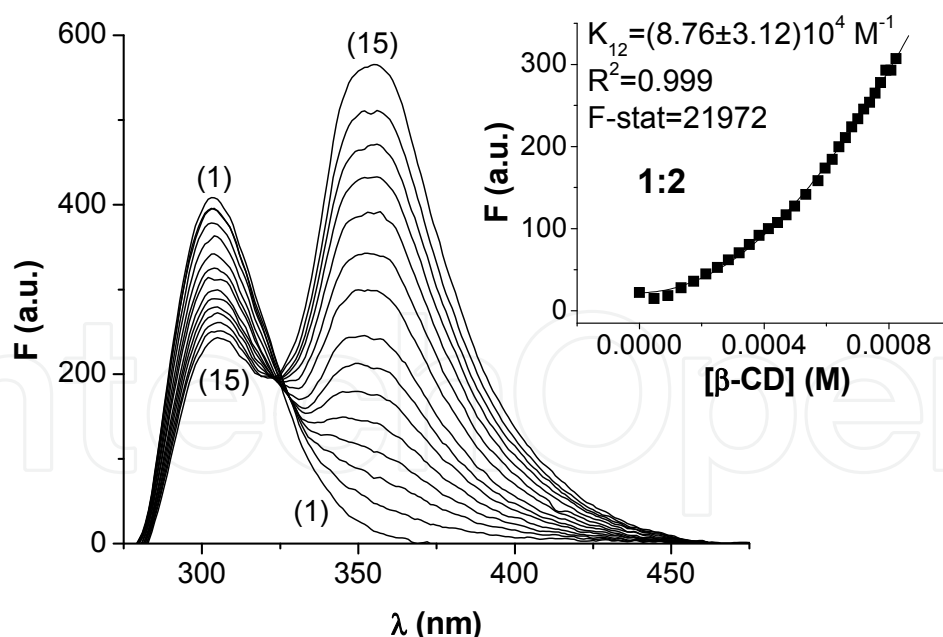


Fig. 14. The fluorescence spectrum of atenolol ($2 \times 10^{-5} \text{ M}$ in dimethylformamide) in the absence (1) and presence (2–30) of increasing amounts of β -CD (up to $8 \times 10^{-4} \text{ M}$). $\lambda_{ex} = 275 \text{ nm}$. The DMF spectrum has been subtracted. Inset: Plot of the fluorescence intensity of B2 *vs.* the β -CD concentration.

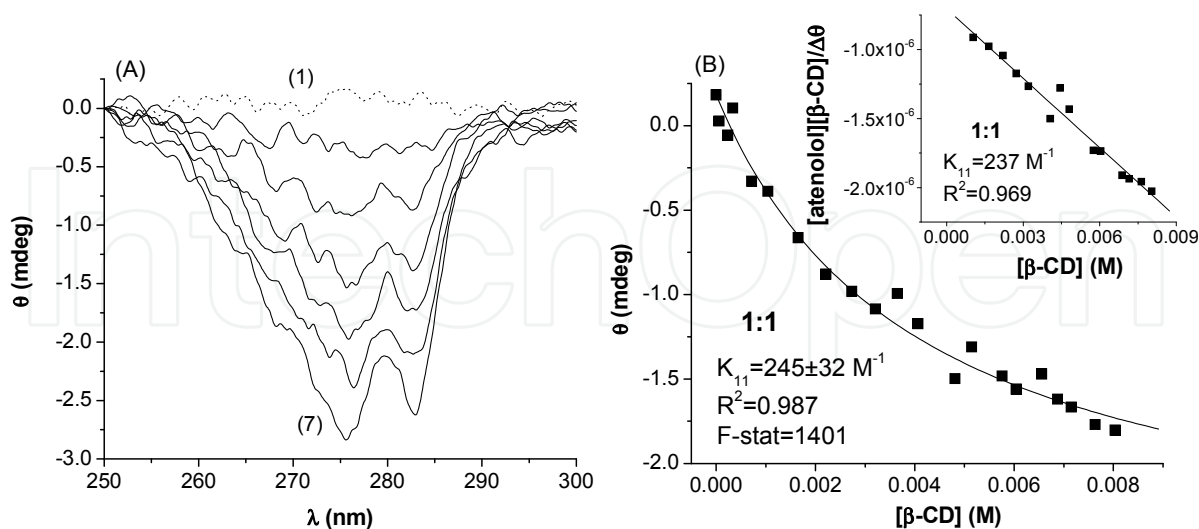


Fig. 15. (A) The circular dichroism spectra of atenolol (5×10^{-4} M in pH 7.4 phosphate buffer) in absence (1) and presence (2–7) of increasing concentrations of β -CD (up to 2×10^{-2} M). (B) Determination of the stoichiometry and association constant of the atenolol- β -CD complex.

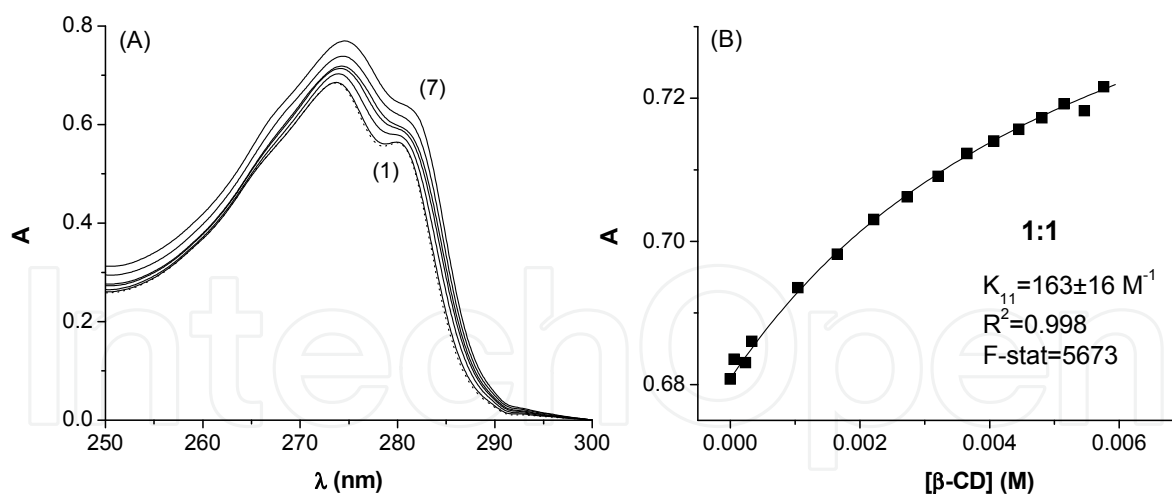


Fig. 16. (A) The absorption spectra of atenolol (5×10^{-4} M in pH 7.4 phosphate buffer) in absence (1) and presence (2–7) of increasing concentrations of β -CD (up to 2×10^{-2} M). (B) Determination of the stoichiometry and association constant of the atenolol- β -CD complex.

As stated above, molecular mechanics calculations were performed using the MM+ force field in order to gain theoretical support for two experimental results:

a. the negative signal of the dichroic band of atenolol in the 1:1 complex.

This is rationalized in terms of the equatorial orientation of the corresponding transition moment of atenolol calculated at the TDDFT level (represented in grey in Fig. 17A) with respect to the β -CD symmetry axis;

b. the increased and temperature irreversible emission correlated to the amino group observed upon the formation of the 1:2 complex.

This behaviour can be explained on the basis of the inclusion of the isopropylamino moiety of atenolol in one of the two CD cavities of the 1:2 complex (Fig. 17B). The molecule is “fixed” by the cavity in a conformation where the excited state is predominantly localized on the amino group.

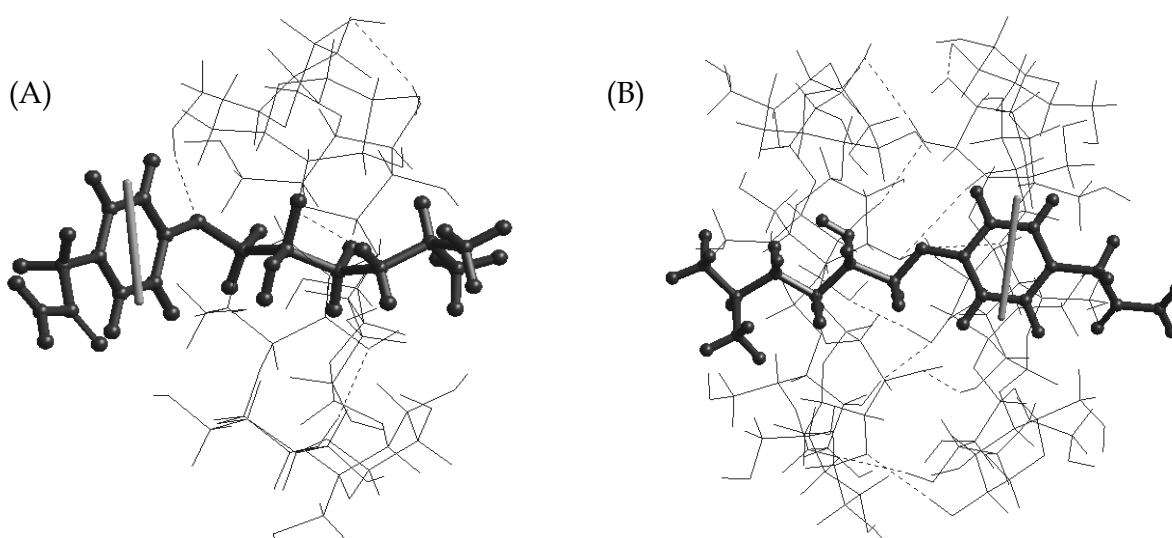


Fig. 17. Proposed structures for the (A) 1:1 and (B) 1:2 atenolol- β -CD inclusion complexes, *via* molecular modeling. The dotted lines represent hydrogen bonds. The transition moment of atenolol is plotted in grey.

4. Conclusions

The estimation of the stoichiometry of a CD inclusion complex represents the first step in the characterization of the complex, leading to the determination of reliable association constants and offering a hint of the supramolecular structure. Unfortunately, the unequivocal estimation of the stoichiometry is not always straightforward and in some cases a single experiment is not sufficient.

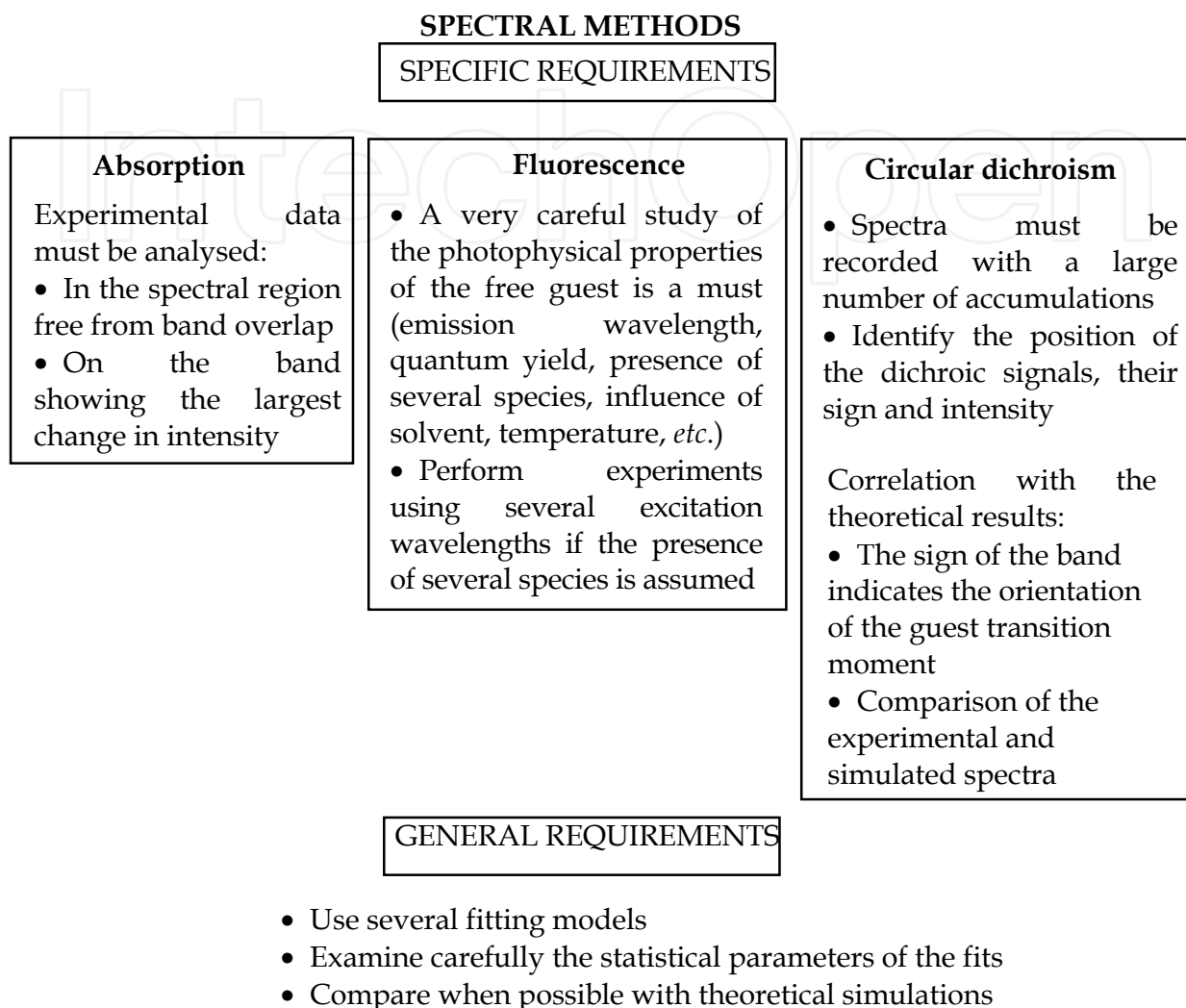
In the four discussed examples we have described some cases we have encountered, in order of increasing complexity. In the first case we have had no problems in determining the stoichiometry, the 1:1 inclusion complex formed by the phenoxathiin derivative being clearly defined, with all the other models leading to absurd results. For the two following guests, DEAC and indapamide, the uncertainties arose in the determination of the stoichiometry were solved in two ways, starting with the careful examination of the results obtained by fitting the data to different models.

For DEAC, the slightly better statistical parameters, R^2 and Fisher statistic coefficient, for the 1:1+1:2 stoichiometry, as compared to 1:1, was a first indication of the real stoichiometry. The presence of a mixture of complexes became clear by examining the residuals of the two corresponding fits. Thus, we have rejected the hypothesis of the 1:2 stoichiometry, for which the residuals were grouped in positive and negative values for different regions of the plot, and accepted the simultaneous presence of a mixture of two complexes, 1:1+1:2, for which the residuals were lower and randomly distributed, attesting the inherent experimental errors and not an inadequate model.

When the values of the statistical parameters are not sufficient to determine the adequate model, a theoretical simulation of the property *vs.* concentration curve for the assumed stoichiometry is necessary. In the case of the indapamide-CD systems, we succeeded to establish the presence of a 1:2 mixture of complexes by coupling an improvement of the experimental conditions, *i.e.* extending the concentration of the host up to the limit of solubility, with theoretical curves built assuming different stoichiometries.

In the last case, the atenolol-CD inclusion complexes represented more difficult systems and some uncertainties still remained. The problems are due to the high complexity of the fluorescence spectrum of the free drug, very sensitive to the experimental conditions such as the solvent, excitation wavelength, temperature, *etc.* We have observed that the emission band that is enhanced in intensity in the presence of CDs, and therefore assigned to the inclusion complex formation, is also present for some experimental conditions and to different extents in the spectrum of the free guest. Therefore, it is difficult to state that the fluorescence intensity measured in the presence of CDs is uniquely due to the inclusion complex and not to another species pre-existent in the system. The presence of several conformational equilibria and/or of inter-/intra-molecular interactions in the free atenolol solution can be influenced by the CD addition. The second problem encountered was the very slight variation in the main emission band intensity, which prevented its use for quantitative estimations. With all these reservations, we considered that the stoichiometry of the inclusion complex, as revealed by the fluorescence measurements, is 1:2. In the circular dichroism spectra, working in other concentration domain and measuring a ground state property, we evidenced the presence of a 1:1 complex but the association constants are different as compared to those obtained by fluorescence. Getting different values for the association constants, by the use of several experimental methods, is a widely discussed topic in literature (Valeur et al., 2007), various explanations being given, the different range of concentrations used being the most frequent (Radi & Eissa, 2011). Another explanation invoked especially for the cases in which association parameters measured using fluorescence, on one side, and absorption and circular dichroism spectroscopies, on the other, are compared consists in the different features of the involved guest state, the excited state for the first method and the ground state for the other two. Last but not least, the isothermal calorimetric titration is a method also suitable for completing the information on the inclusion process, the method allowing for the determination, in a single experiment, of the stoichiometry, association constant and enthalpy change during the process. The experimental data can be fitted to several models, including one or more independent classes of binding sites or sequential binding [Xing et al., 2009].

As a general conclusion, to establish the real stoichiometry of G:H systems, the use of multiple methods is recommended together with considering several required conditions, summarized in the scheme below.



5. Acknowledgement

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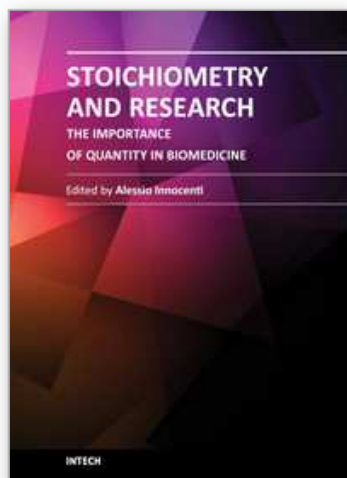
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Stoichiometry and Research - The Importance of Quantity in Biomedicine

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The aim of this book is to provide an overview of the importance of stoichiometry in the biomedical field. It proposes a collection of selected research articles and reviews which provide up-to-date information related to stoichiometry at various levels. The first section deals with host-guest chemistry, focusing on selected calixarenes, cyclodextrins and crown ethers derivatives. In the second and third sections the book presents some issues concerning stoichiometry of metal complexes and lipids and polymers architecture. The fourth section aims to clarify the role of stoichiometry in the determination of protein interactions, while in the fifth section some selected experimental techniques applied to specific systems are introduced. The last section of the book is an attempt at showing some interesting connections between biomedicine and the environment, introducing the concept of biological stoichiometry. On this basis, the present volume would definitely be an ideal source of scientific information to researchers and scientists involved in biomedicine, biochemistry and other areas involving stoichiometry evaluation.

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