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^{1}H NMR spectroscopic characterization of inclusion complexes of tolfenamic and flufenamic acids with β-cyclodextrin

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HIGHLIGHTS

- We report the complexation process between tolfenamic and flufenamic acid with β-CD.
- Induced chemical shifts variation and ROESY spectra sustain a bimodal complexation.
- The entrance of both aromatic rings occurs through the secondary rim of the β-CD.
- The microscopic association constants order of magnitude was evaluated.

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ABSTRACT

The complexation between the anionic forms of tolfenamic acid and flufenamic acid with β-cyclodextrin was investigated in solution by 1D and 2D proton NMR spectroscopy. The stoichiometry of the complexes was determined by the method of continuous variation using the chemical induced shifts of both the host and guest protons. An analysis of the spectroscopic data revealed that simultaneous inclusion of both rings of tolfenamic and flufenamic acids occur, giving rise each to two isomeric 1:1 complexes. The view of a bimodal binding between these two drugs and β-cyclodextrin was also supported by ROESY experiments. Using a rough approximation, we have estimated the association constants order of magnitude of the 1:1 complexes.

1. Introduction

Tolfenamic acid N-(3-chloro-2-methyl-phenyl) anthranilic acid (TA) and flufenamic acid N-(3-trifluoromethyl-phenyl) anthranilic acid (FF), (see Fig. 1), are nonsteroidal anti-inflammatory drugs (NSAIDs), which along with the other derivatives of anthranilic acid (mefenamic and meclofenamic acid) are widely used as analgesic, anti-inflammatory and antipyretic drugs. These agents inhibit the biosynthesis of prostaglandins (PGs), as a consequence of interfering within the arachidonic acid cascade, as well as the PG receptors in certain tissues [1]. Like many NSAID drugs, TA and FF are very sparingly soluble in water. Therefore aqueous solubility and wettability of these fenamates gives rise to difficulties in pharmaceutical formulations for oral or parenteral delivery, which may lead to variable bioavailability.

To overcome these drawbacks, increasing the aqueous solubility of TA and FF is an important goal. The complexation of TA and FF with naturally occurring α, β and γ-ciclodextrins, or chemically modified ones, may resolve this problem and may also reduce the irritation or local damage of the gastrointestinal mucosa.

Cyclodextrins are naturally occurring cyclic oligosaccharides [2], known for their effect on stability, solubility and bioavailability of various drugs, as well as for the reduction of drugs side-effects [3]. Their unique macrocyclic structure is a result of the stable chair conformation of the constituent D-glucopyranosyl residues and α-1,4 glicosidic bonds linking the residues with each other. The shape of naturally occurring cyclodextrins can be represented as a toroidal hollow truncated cone having many primary and secondary hydroxyl groups crowning the narrower and wider rims of the hollow cone, respectively. Methine protons and glycoxidic oxygen atoms are located inside the hollow cone, colled the cavity. Hence the interior is relatively apolar and hydrophobic, whereas the exterior is relatively polar and hydrophilic. CDs are capable of forming inclusion complexes with many drugs by taking up a whole drug molecule, or some part of it, into the cavity. Such molecular encapsulation will affect many of the physicochemical properties of the drugs, such as their aqueous solubility and rate of dissolution. During the drug–CD complex formation, no covalent bonds are formed and the complex is readily dissociated and the

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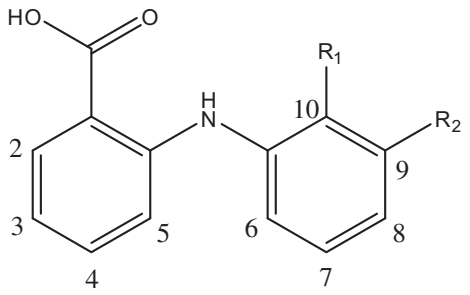

Fig. 1. TA and FF chemical structure and proton notations. TA: R_1 = CH_3, R_2 = Cl; FF: R_1 = H, R_2 = CF_3.

free drug molecules are in rapid equilibrium with the drug molecules bound within the CD cavity. Among α-, β- and γ-CD, β-CD is by far the most widely used compound owing to the optimal size of its internal cavity for the encapsulation of NSAIDs.

Various studies have been carried out on the inclusion of TA and FF by naturally occurring CDs or chemically modified ones in aqueous media and in solid state. Several techniques have been proposed for complex preparation such us freeze-drying [4], kneading [5], or the development of a mucoadhesive film [6], based on chitosan as polymer and glicerol as plasticizer, containing FF as complex with HP-β-CD. During the physicochemical characterization of the inclusion complexes by differential scanning calorimetry and powder X-ray diffractometry, [5] or with spectroscopic and chromatographic methods [7], significant ariations of some experimental parameters were observed and attributed to the complexation process. Thus, the TA was complexed with various CDs (β-CD, HP-β-CD and Me-β-CD) and the stoichiometry of the TA:Me- β-CD complex was determined to be 1:2, using the continuous variation technique, [7]. A validated HPLC method was also applied to obtain the binding constants of the complexes and to evaluate the energetics of the system [8].

^{1}H NMR spectroscopy is one of the most important methods to quantitatively investigate the formation of CD inclusion complexes in solution [9–11]. This paper describes the ^{1}H NMR study of the inclusion of TA and FF with β-CD. Firstly the stoichiometry of the complexes was investigated using the current Job plot method. The type of inclusion process of TA and FF in β-CD was confirmed by 2D ROESY spectroscopy. Using a simplifying approximation, an attempt was made in order to assess the association constants order of magnitude for each 1:1 complex, based on the chemical shift variation of some representative protons belonging to the drug molecules.

2. Materials and methods

2.1. Materials

Tolfenamic acid, flufenamic acid and β-cyclodextrin (water content 8 mol/mol) were purchased from Sigma–Aldrich Chemie GmbH and used without further purification. The β-CD water content was taken into account in the calculation of solute concentration. The deuterium oxide (99.7% D) was obtained from Heavy Water Plant Romag-Prod, Romania.

2.2. Sample preparation

In order to study the complexation process between TA, FF and β-CD, three stock solutions in D_2O, having 10 mM was prepared. The TA and FF stock solutions were prepared containing 5 μl/ml of NaOD 40% for dissolution of the drugs. Based on these equimolar solutions, two series of samples containing β-CD and TA or FF were prepared. This was accomplished by mixing two solutions to constant volume at varying proportions so that a complete range ($0 < r < 1$) of the mole fraction $r = [X]/([G]+[H])$ was sampled. X = H or G and [H] and [G] are the concentrations of the host

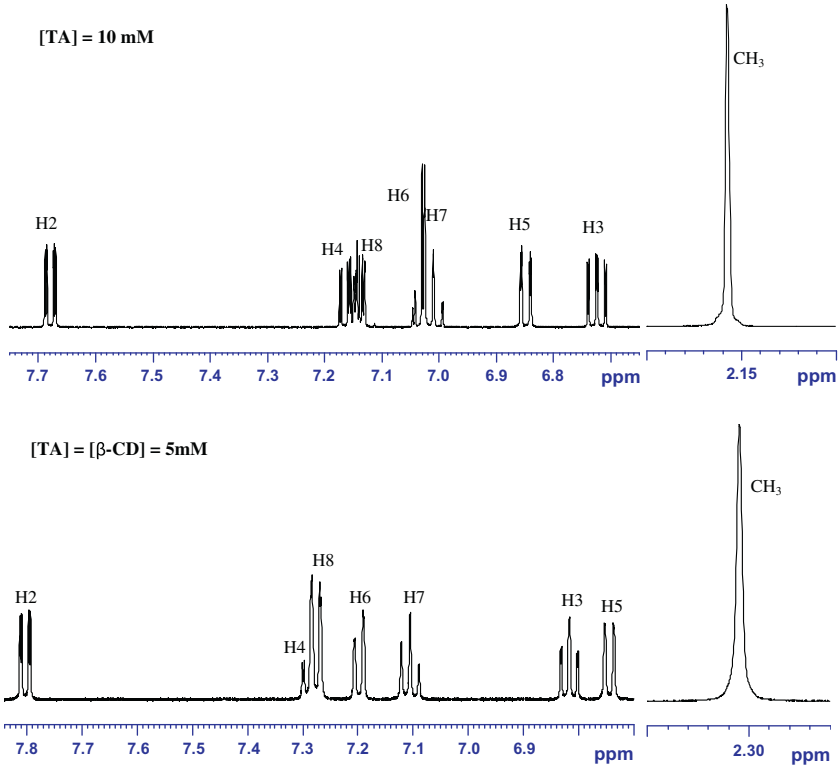

Fig. 2. ^{1}H NMR spectra of TA, in the absence as well in the presence of β-CD.

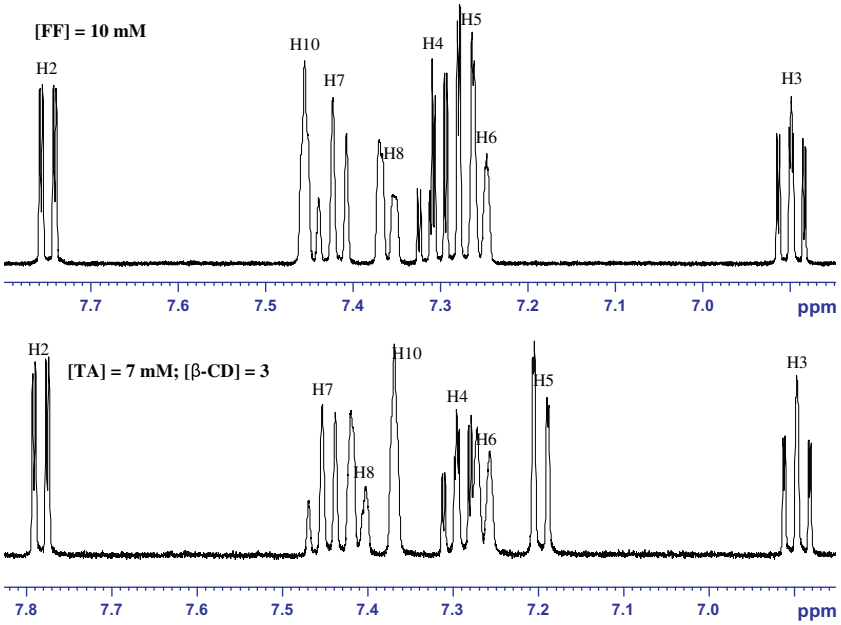

Fig. 3. ^{1}H NMR spectra of FF, in the absence as well in the presence of β-CD.

(β-CD) and guest (TA or FF), respectively. Thus the total concentration $[H]_t + [G]_t = 10$ mM was kept constant for each solution. The two sets of samples were used both for determination of stoichiometry and the evaluation of the association constants.

2.3. Nuclear magnetic resonance measurements

All ^{1}H NMR measurements were carried out on a Bruker AVANCE III spectrometer operating at 500.13 MHz and equipped with a broad band observe probe. The chemical shifts were referenced to TMS. In all experiments the temperature was maintained at 298 K and standard 5 mm NMR tubes were used. For each ^{1}H NMR experiment, 64 transients were collected into 32 K data points over a 3000 Hz spectral window, using a 2 s relaxation delay. The 2D ROESY spectra were acquired in the phase sensitive mode and residual water suppression, using Bruker standard parameters (pulse program roesyphpr). Each spectrum consisted of a matrix of 4 K/4 K data points covering a spectral width of 4000 Hz. The spectra were obtained with a spin-lock mixing time of 200 ms relaxation delay 3 s and 16 scans were recorded.

3. Results and discussion

3.1. Determination of the stoichiometry

NMR is a technique, which provides the most evidence for the inclusion of a guest into the hydrophobic β-CD cavity in solution. Inclusion of TA and FF in β-CD cavity is put in evidence by the change in chemical shifts of some of the guest and host protons, in comparison with the chemical shifts of the same protons in the free components. ^{1}H NMR spectra of pure drugs and drug/β-CD mixture are presented in Figs. 2 and 3.

The ^{1}H chemical shifts variation of some representative β-CD and TA protons as well as β-CD and FF protons as a function of their concentration is presented in Figs. 4 and 5, respectively. It can be seen that in the presence of β-CD, almost all guest proton resonances were affected. Furthermore, there is seemingly no preference of inclusion of a particular aromatic ring, since similar magnitude shifts were observed for the protons belonging to the

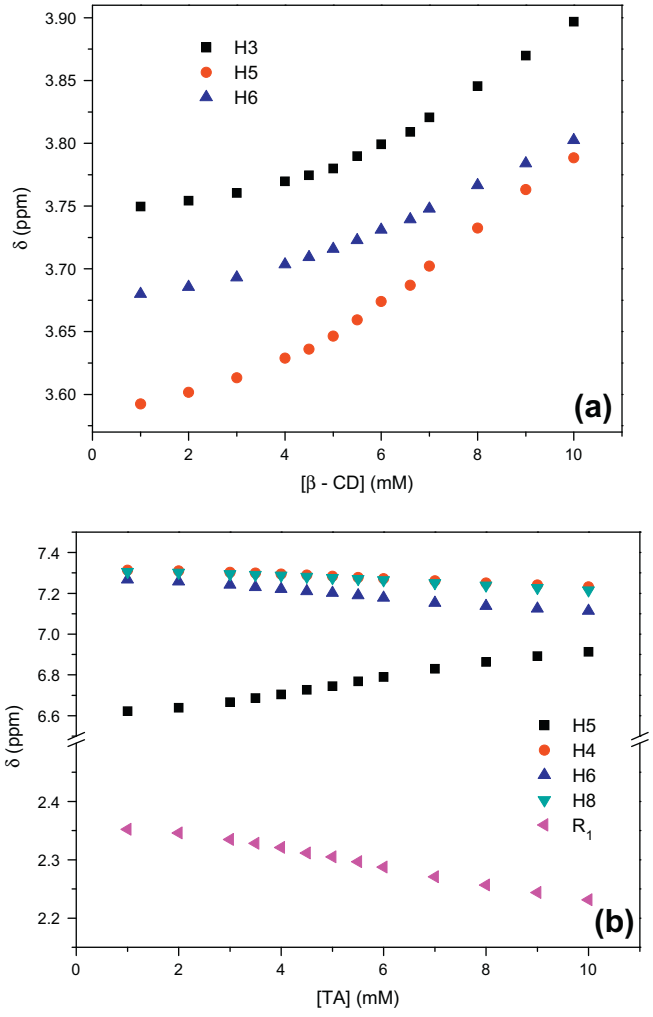

Fig. 4. Chemical shifts variation of some representative: (a) β-CD and (b) TA protons as a function of their concentration, ([β-CD] + [TA] = 10 mM).

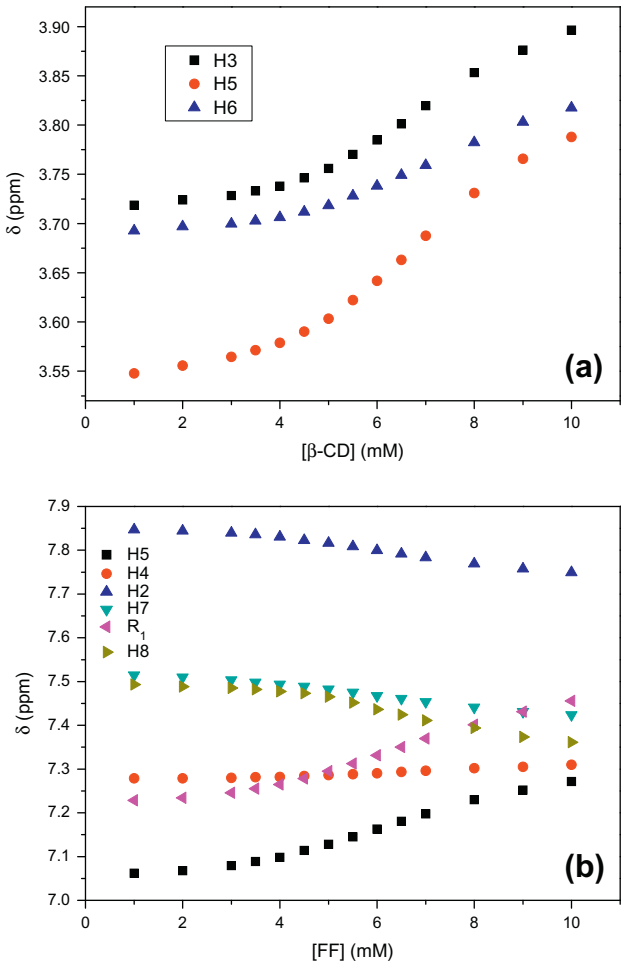

Fig. 5. Chemical shifts variation of some representative: (a) β-CD and (b) FF protons as a function of their concentration, ([β-CD] + [FF] = 10 mM).

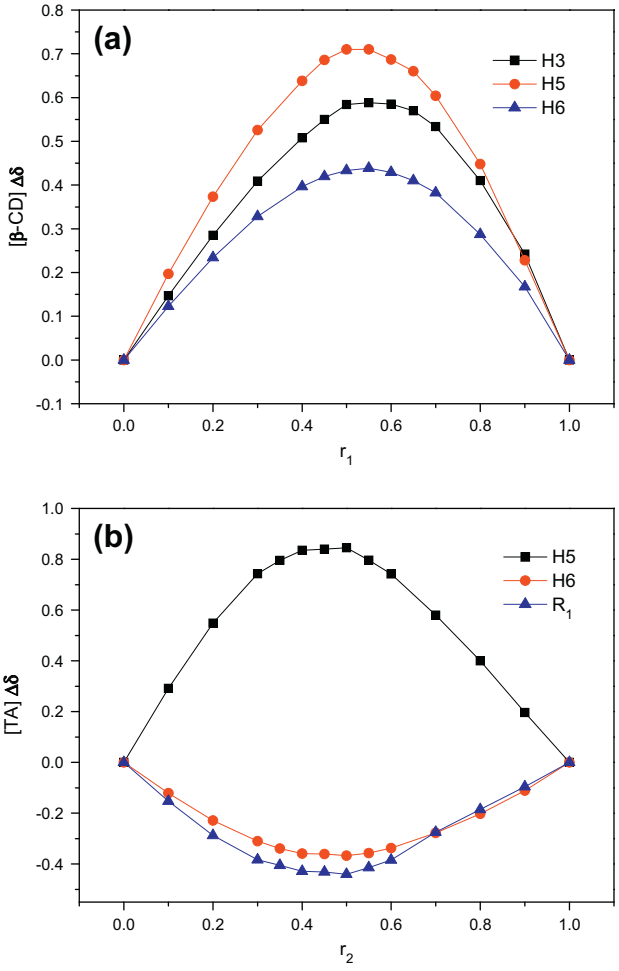

Fig. 6. Job's plots corresponding to the induced chemical shift variation of some: (a) β-CD and (b) TA protons for the TA: β-CD system.

methyl-phenyl or benzoic groups of the TA or trifluorophenyl and benzoic acid moiety in the case of FF.

The aforementioned ^{1}H NMR observations, for the two studied systems, could imply two main possibilities: (a) the presence of two types of 1:1 complexes with the inclusion of both aromatic rings inside the β-CD cavity, which corresponds to a bimodal complexation or, (b) the formation of a 1:2 drug: β-CD complex, where the inclusion phenomena are simultaneously present for the two rings. Considering the chemical structure of TA and FF, the simultaneous inclusion of one guest molecule into two β-CD molecules seems to be sterically unfavorable, which avoids the formation of 1:2 complexes. In order to elucidate which of the two above possibilities is correct the continuous variation method was employed to establish the stoichiometry of the complexes. In our case, the continuous variation method is based on the induced chemical shift variation, $\Delta\delta$, which is directly related to the concentration of the complex. $\Delta\delta$ is defined as the difference in chemical shifts in the absence and in the presence of the other reactant. Thus if a physical quantity, containing $\Delta\delta$, is plotted as a function of the mol fraction of the host or guest, r, (Job's plot), its maximum value will occur at $r_1 = m/(m+n)$ or $r_2 = n/(m+n)$, where m and n are, respectively, the molar ratios of β-CD and drug in the $(\text{drug})_n$:$(\beta\text{-CD})_m$ complex. Under fast exchange conditions, for a signal belonging to β-CD, for example, the calculated quantity $\Delta\delta\cdot[\beta\text{-CD}]$ is proportional to the complex concentration and, can be plotted against r_1, [12]. The continuous variation method was applied for all protons of the different molecules and yielded identical results. For the shake of concision, only several protons (the most markedly affected) have been selected and reported in Figs. 6 and 7. In all cases, Job's plots show a maximum at 0.5, indicating the existence of complexes with a 1:1 stoichiometry.

Thus a binary structure formed by one β-CD molecule surrounding one guest molecule can be considered reliable for both systems. To confirm the hypothesis of bimodal inclusion complexation, ROESY spectra were acquired for TA: β-CD and FF: β-CD complexes. Due to the rapid dynamics of the complexation process, the ROESY effects were only quantitatively used and no conclusions on intermolecular distances were extracted. The ROESY spectrum of TA: β-CD complex is reported in Fig. 8. This spectrum allows us to establish a spatial proximity between the TA protons and the inner protons of β-CD.

The spectrum shows several cross-peaks between H3, H5 and H6 protons of β-CD and protons of both aromatic rings of TA (H4, H5, H6, H7 and H8), demonstrating the inclusion of these groups in the hydrophobic cavity.

In the case of FF: β-CD system, (Fig. 9), no cross-peaks were observed between H2 on the benzoic acid ring and H3, H5 and H6 protons of β-CD. All the other protons belonging to benzoic acid moiety and trifluorophenyl moiety give rise to medium to strong peaks with the inner protons of β-CD, except H3 which has a cross-peak only with the corresponding H6 proton of the β-CD. It is worth mentioning that we found cross-peaks only with the inner β-CD protons, which establishes intra-cavity binding without evidence for outside contributions. These results confirmed the

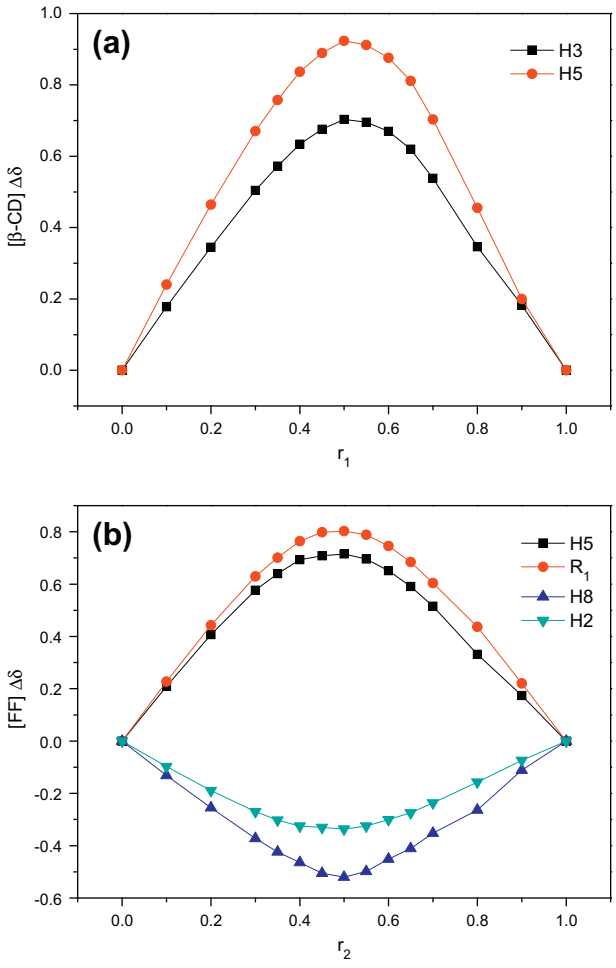

Fig. 7. Job's plots corresponding to the induced chemical shift variation of some: (a) β-CD and (b) FF protons for the FF: β-CD system.

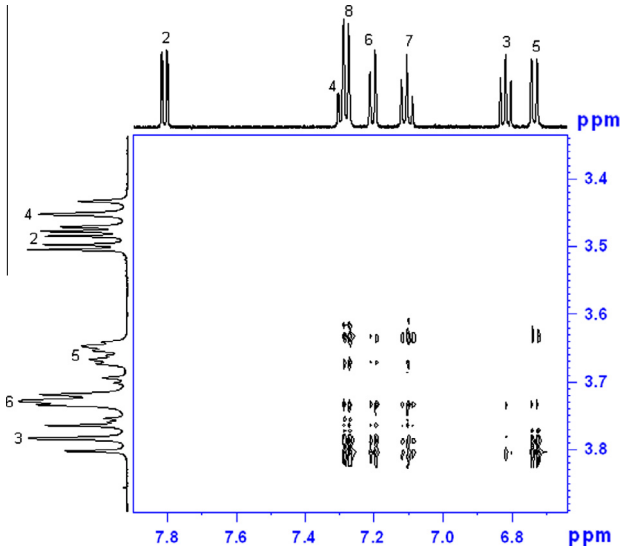

Fig. 8. 2D ROESY spectrum of TA: β-CD system.

hypothesis that two different 1:1 complexes are simultaneously present in solution, each one involving the inclusion of both aromatic ends of TA and FF. A schematic representation of the bimodal inclusion which may exist in solution is presented in Fig. 10.

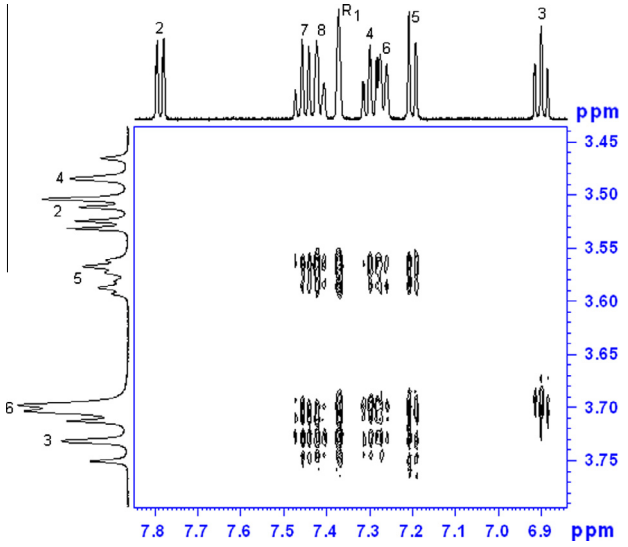

Fig. 9. 2D ROESY spectrum of FF: β-CD system.

Similar results have been reported for other NSAIDs such as piroxicam [13], naproxen [14], diclofenac [15], and mefenamic acid [16] or nicardipine hydrochloride [17].

3.2. Evaluation of the binding constants

As Connors pointed out [18], when a guest molecule possesses two potential binding sites, (*X* and *Y*), a bimodal binding is possible and two isomeric1:1 complexes (*X′Y* and *XY′*) can be formed. A primed site indicates that a CD molecule is bound to that site. The two isomers are characterized by two microscopic binding constants, $K_{X'Y}$ and $K_{XY'}$ and are related to the macroscopic equilibrium constant K_{11}, by $K_{11} = K_{X'Y} + K_{XY'}$. Unfortunately, the determination of microscopic constants is not accessible through NMR, and in some cases only the macroscopic constant can be determined [19]. In order to evaluate the order of magnitude for $K_{X'Y}$ and $K_{XY'}$ we made a rough approximation, assuming that we can treat independently the two isomers by considering two separate 1:1 complexes, and neglecting [*XY′*] in the calculation of $K_{X'Y}$ and [*X′Y*] in the calculation of $K_{XY'}$, respectively. In this case, the association constant, *K*, for a 1:1 complex can be determined according to the following equation [20]:

$$+\Delta\delta^{(i,j)} = \frac{\Delta\delta_c^{(j)}}{2[X]_t^{(i)}}\left\{[G]_t^{(i)} + [H]_t^{(i)} + \frac{1}{K} - \left[\left([G]_t^{(i)} + [H]_t^{(i)} + \frac{1}{K}\right)^2 - 4[H]_t^{(i)}[G]_t^{(i)}\right]^{1/2}\right\} \quad (1)$$

where *i* counts the sample number and *j* the studied proton. If the studied proton belongs to the guest or host molecule, *X* = G or H respectively. $\Delta\delta_c^{(j)}$ represents the chemical shift difference (for a given proton) between the free component and the pure inclusion complex. Eq. (1) involves no approximations and correlates the total concentrations of the guest and host molecules with the observed deference in the chemical shift:

$$\Delta\delta^{(i,j)} = \delta_{free}^{(j)} - \delta_{obs}^{(i,j)} \quad (2)$$

We used a computer program [21] based on an iteration procedure following specific algorithms in order to fit the experimental values of $\Delta\delta^{(i,j)}$ to the appropriate equation. Each iteration step sets up a quadratic function to determine the direction of search and calculates the loss error function *E*:

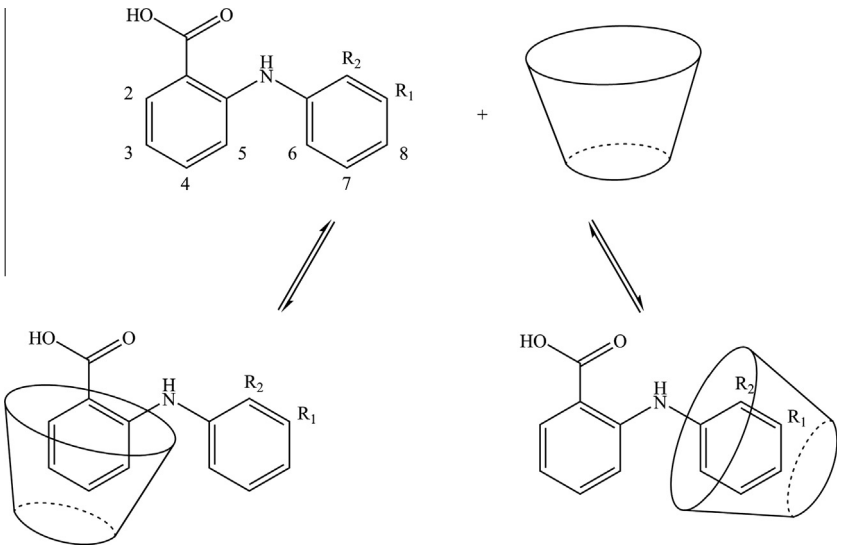

Fig. 10. Proposed model for the bimodal inclusion equilibria of TA and FF with β-CD.

Table 1
Chemical shifts of TA and FF protons in the free and complexed states. $\Delta\delta_c = \delta_{free} - \delta_c$ values were obtained as a result of the fitting procedure.

Proton	TA		FF	
	δ_{free} (ppm)	δ_c (ppm)	δ_{free} (ppm)	δ_c (ppm)
2	7.7572	7.9018	7.7495	7.8659
4	7.2316	7.3889	7.3096	7.2699
5	6.9134	6.3822	7.2713	7.0220
6	7.1142	7.3785	7.2552	7.3558
7	–	–	7.4239	7.5225
8	7.2142	7.3902	7.3610	7.5200
R_1	2.2314	2.4511	7.4757	7.1917

$$E = \sum_{i,j} (\Delta\delta^{(ij)} - \Delta\delta^{(ij)}_{calc})^2 \quad (3)$$

The treatment of the entire set of protons studied produces a single K value for the entire process and a set of calculated $\Delta\delta_c^{(j)}$ values. The program is quite flexible as both the host and the guest can be observed for spectroscopic perturbations, allowing up to 15 protons to be used in the fitting process. For TA: β-CD complex, we applied Eq. (1) first for a set of protons consisting in H2, H4 and H5 of TA and then for H6, H8 and R_1 of TA. This means that we considered first the case when the benzoic acid ring is inserted in the β-CD cavity and then the inclusion of the methyl- phenyl ring. The association constants obtained, using the above described procedure were $K_{X'Y} = 144.1\ M^{-1}$ with $E = 1.53 \times 10^{-3}$ and a correlation factor $r = 0.998$, when the benzoic acid moiety is included in the β-CD cavity and $K_{XY'} = 151.9\ M^{-1}$ with $E = 8.41 \times 10^{-4}$ and $r = 0.994$ for the inclusion of methyl-phenyl moiety. With these values, the macroscopic constant for the TA: β-CD complex has the value $K_{1:1} = 296\ M^{-1}$. In the case of FF: β-CD complex, we applied Eq. (1) for H2, H4 and H5 protons of the benzoic acid moiety and then for H6, H7, H8, and R_1 protons belonging to trifluoromethyl–phenyl ring. The obtained results were $K_{X'Y} = 647.1\ M^{-1}$ with $E = 3.36 \times 10^{-4}$ and a correlation factor $r = 0.999$, when the benzoic acid moiety is included in the β-CD cavity and $K_{XY'} = 802.6\ M^{-1}$ with $E = 1.5 \times 10^{-3}$ and $r = 0.998$ for the inclusion of trifluoromethyl-phenyl moiety. With these values, the macroscopic constant for the FF: β-CD complex has the value $K_{1:1} = 1449.7\ M^{-1}$. Comparing the macroscopic association constants values, we can conclude that FF is more tightly bound to β-CD than TA, in spite of the fact that the structural differences between these two drugs are small. The complete set of chemical shifts in the free state and in the pure complex is reported in Table 1.

Based on the complexation shifts, ROESY cross-peaks and the magnitude of evaluated association constants, we conclude that the inclusion process affects principally the methyl-phenyl and trifluoromethyl-phenyl rings, which enters the β-CD cavity through the secondary hydroxyl rim. In both cases, the interaction of β-CD with the benzoic acid ring seems to be weaker, probably do to the anionic form of this moiety.

4. Conclusions

The complexation between the anionic forms of tolfenamic acid (TA) and flufenamic acid (FF) with β-cyclodextrin has been investigated in solution by 1D and 2D 1H NMR spectroscopy. The constructed Job's plots, based on the induced chemical shifts of the β-CD, TA, and FF protons, confirm the existence of a bimodal binding in both cases. ROESY experiments reveal the correlation between the H3 and H5 protons of the β-CD cavity and both the aromatic ring protons of TA and FF, supporting the bimodal binding process. On the basis of these experimental data we conclude that we have a simultaneous presence of two 1:1 complexes for TA as well as FF, each one involving the inclusion in the β-CD cavity of a different aromatic end. Based on the evaluated microscopic association constants, we believe that the complexation mainly affects the methyl-phenyl or trifluoromethyl-phenyl ring, and to a lesser extent, the benzoic acid moiety.

Acknowledgement

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molstruc.2012.11.021.

References

[1] P.J. Pentikainen, P.J. Neuvonen, C. Backman, Eur. J. Clin. Pharmacol. 19 (1981) 359–365.
[2] H. Dodziuk (Ed.), Cyclodextrins and Their Complexes, Wiley-VCH, Weinheim, 2006.
[3] T. Loftsson, M.E. Brewster, J. Pharm. Sci. 85 (1996) 1017–1025.
[4] P.R. Vavia, N.A. Adhage, Pharm. Dev. Tech. 5 (2000) 571–574.
[5] K. Nakanishi, T. Masukawa, T. Nadai, K. Yoshii, S. Okada, K. Miyama, Biol. Pharm. Bull. 20 (1997) 66–70.
[6] P. Mura, G. Corti, M. Cirri, F. Maestrelli, N. Mennini, M. Bragagni, J. Pharm. Sci. 99 (2010) 3019–3029.
[7] S. Rozou, E. Antoniadou-Vyza, J. Pharm. Biomed. Anal. 18 (1998) 899–905.
[8] S. Rozou, S. Michaleas, E. Antoniadou-Vyza, Pharm. Pharmacol. Comm. 5 (1999) 79–84.
[9] A. Ejchart, W. Kozminski, in: H. Dodziuk (Ed.), Cyclodextrins and Their Complexes, Wiley-VCH, Weinheim, 2006.
[10] K. Hirose, J. Incl. Phenom. Macrocycl. Chem. 39 (2001) 193–209.
[11] L. Fielding, Tetrahedron 56 (2000) 6151–6170.
[12] F. Djedaïne, S.Z. Lin, B. Perly, D. Wouessidjewe, J. Pharm. Sci. 79 (1990) 643–646.
[13] G. Fronza, A. Mele, E. Rendeti, P. Ventura, J. Pharm. Sci. 81 (1992) 1162–1165.
[14] N. Sadlej-Sosnowska, L. Kozerski, E. Bednarek, J. Sitkowski, J. Incl. Phenom. Macrocycl. Chem. 37 (2000) 383–394.
[15] M. Bogdan, M.R. Caira, J. Incl. Phenom. Macrocycl. Chem. 49 (2004) 225–229.
[16] Mihaela Pop, K. Goubitz, Gh. Borodi, M. Bogdan, D.J.A. De Ridder, R. Peschar, H. Schenk, Acta Cryst. B58 (2002) 1036–1043.
[17] Catarina.M. Fernandes, R.A. Carvalho, S.P. da Costa, F.J.B. Veiga, Eur. J. Pharm. Sci. 18 (2003) 285–296.
[18] K.A. Connors, Chem. Rev. 97 (1997) 1325–1357.
[19] A. Jover, J. Phys. Chem. B108 (2004) 18850–18859.
[20] M. Bogdan, M.R. Caira, S.I. Farcas, Supramol. Chem. 14 (2002) 427–435.
[21] C. Floare, F. Balibanu, M. Bogdan, Physica L4a (2005) 451–454 (Special issue).