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Evidence of a Bimodal Binding between Diclofenac-Na and β-Cyclodextrin in Solution

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Abstract

The complexation between diclofenac sodium and β-cyclodextrin was investigated in solution by 1D and 2D proton NMR. The continuous variation method was used to establish the stoichiometry. The obtained results indicate that simultaneous inclusion of both rings occur giving rise to two isomeric 1:1 complexes. The view of a bimodal binding between diclofenac sodium and β-cyclodextrin was also supported by ROESY experiments. The association constants for the two 1:1 complexes were calculated by non-linear least-squares regression analysis, applying an iteration procedure. The geometry of the two 1:1 complexes, according to the obtained NMR data is given.

Introduction

Diclofenac, (DCF), 2-(2,6-dichloroanilino) phenylacetic acid, is one of the most efficient non-steroidal anti-inflammatory drug used in different diseases such as osteoarthritis, rheumatoid arthritis and acute pain in musculoskeletal disorders [1]. However, the very low aqueous solubility of DCF, especially in gastric juice (about 15 μg/ml at pH = 2) causes undesirable effects on the gastric mucosa when it is orally administered. In order to improve the absorption and possibly to increase the gastrointestinal tolerability of DCF, inclusion compounds of DCF and its sodium salt (DCF-Na) with β-cyclodextrin (β-CD) and its derivative, hydroxypropyl-β-cyclodextrin, have been prepared and studied both in solution [2–6] and in the solid state [7–9].

Cyclodextrins are cyclic oligosaccharides with a hydrophilic outer surface and a lipophylic inner cavity. Cyclodextrins can increase water solubility of poorly water soluble drugs and reduce the severe irritation of the stomach mucosa, by taking up the whole molecule or part of it into cavity [10]. A schematic representation of the structure and proton notations of DCF-Na and β-CD is shown in Figure 1.

In a thorough study of an inclusion complex, three main points need to be clarified: stoichiometry, association constant and geometry of the complex. From this point of view, DCF is subjected to a great controversy with respect to its stoichiometry, association constant, K_a, and mode of insertion. Thus, in the solid state the DCF-Na : β-CD complex crystallizes in a unique hexagonal crystal system [7], with the phenylacetate ring preferentially inserted from primary

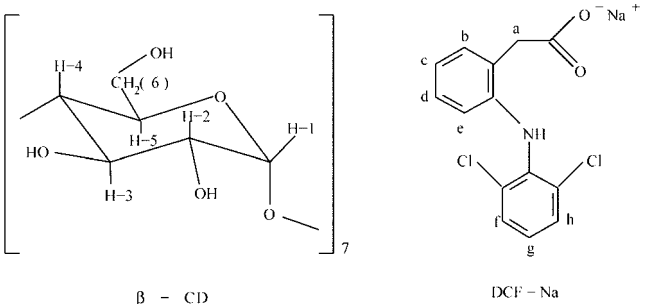

Figure 1. Structure and proton notations of β-CD and DCF-Na.

hydroxyl side and the dichlorophenyl substituent resting on the secondary site of the host. In solution the situation is, however, less clear. Some authors [2, 4, 5] have suggested the inclusion of the dichlorophenyl ring, whereas others [3, 6] are in favor of the phenylacetate moiety. More than that, based on 1D and 2D NMR experiments in solution, some authors [2, 4] concluded that simultaneous inclusion of both rings occurs, even at low DCF-Na/β-CD ratio, giving rise to multiple equilibria involving 1:1 and 1:2 guest–host complexes and other [5] supporting the formation of 1:1 and 2:1 complexes. The same diversity can be found in the reported values of the association constant, K_a, as is presented in Table 1. In principle, this situation can in part be attributed to the fact that the binding process has been examined by many different experimental methods and at different pH values. As Orienti *et al.* [12] pointed out, the DCF : β-CD complex association constant decreases substantially with increasing pH (from approx. 1500 M^{-1} at pH = 2 to approx. 100 M^{-1} at pH = 7). Another cause of inconsistency can be the numerical method used to determine them. It is well

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Table 1. Association constant, K_a, for DCF: β-CD inclusion complex obtained by different methods

Method	pH	K_a (M^{-1})	Reference
CD[a]	7	340	11
UV-VIS	7	510	11
Solubility		150	11
Solubility	1.2	100.6	4
P[b]	5	1259	6
UV-VIS	3.5	1000	6
SF[c]	3.5	1288	6
NMR	$K_{1:1}$ = 170	5	
	$K_{2:1}$ = 22		

[a] Circular dichroism.
[b] Potentiometry.
[c] Spectrofluorimetry.

known [13] that different linearization methods are based on some approximations, which are not always either possible or mathematically correct. Nowadays, the necessity of a careful nonlinear regression analysis of the experimental data is well-established [14, 15], whatever technique may be used, when accurate and meaningful association constants are required.

The aim of this work is to gain further insight into the mode of inclusion and stability of the complex, in order to clarify the most probable conformation of the DCF-Na : β-CD complex in aqueous solution. For this reason we utilized 1D and 2D ^{1}H NMR techniques that are particularly sensitive to small changes in the electronic environment of a proton that occur upon intimate contact or short-range association in the complex.

Experimental

Materials

Diclofenac sodium with 99.4% purity was generously supplied by Terapia SA (Cluj-Napoca, Romania). β-cyclodextrin containing an average of 8 water molecules/molecule, was purchased from Sigma Chemie GmbH (Germany). The β-cyclodextrin was used without further purification and the water content was considered in the calculations of solute concentrations. The D_2O (deuterium content 99.7%) was purchased from Institute for Cryogenics and Isotope Separations (Rm. Valcea, Romania).

Apparatus

The NMR experiments were performed at 300 MHz and 400 MHz with Varian-Gemini 300 and Bruker AMX 400 spectrometers, respectively. The ^{1}H NMR spectra were recorded in D_2O solution at 293 $\pm$ 0.5 K and all chemical shifts were measured relative to external TMS. Typical conditions were as follows: 16 K data points, sweep width 4500 Hz giving a digital resolution of 0.28 Hz/point. The 90° pulse width was 13 μs and the spectra were collected by co-addition of 32 or 64 scans. In some cases, an appropriate Gaussian function was applied before Fourier transformation to enhance the spectral resolution.

The two-dimensional NMR spectra at 400 MHz were obtained without degassing, through standard Bruker software. The conditions for ROESY [16] phase-sensitive spectra via time proportional phase incrementation (TPPI) were: spectral widths of 6.34 ppm in both dimensions with a resolution of 0.62–4.96 Hz/point in f_2 and f_1 respectively; 4 kHz spin-lock field and a mixing time of 300 ms. The experiments were performed using 4096 data points in f_2, $512t_1$ increments with 16 scans per t_1 value and a relaxation period of 3 s. A sine function (SSB = 3) was applied in f_1 and f_2 before Fourier transformation.

Procedure

In order to study the complexation process between DCF-Na and β-CD in solution, two stock solutions in D_2O, both having 10 mM were prepared. Based on these two equimolar solutions, a series of nine samples ($i = 1 - 9$) containing both the DCF-Na and the β-CD molecules were prepared. This was accomplished by mixing the two solutions to constant volume (2 ml) at varying proportions, so that a complete range ($0 < r < 1$) of the ratio $r = [X]/([H] + [G])$ was sampled. $X = G$ or H and $[H]$ and $[G]$ are the total concentrations of the host (β-CD) and guest (DCF-Na), respectively. Thus the total concentration $[H] + [G] = [M] = 10$ mM was kept constant for each solution. The same set of samples was used both for the determination of the stoichiometry and association constant, K_a.

Results and discussion

Determination of the stoichiometry

Several techniques, like DSC, IR and UV-VIS spectroscopy can establish if guest molecules form an inclusion complex with CD, but they cannot provide information about the structural configuration of the complex. In contrast, NMR is a technique, which provides the most evidence for the inclusion of a guest into the hydrophobic CD cavity in solution. Inclusion of DCF-Na in β-CD is shown by the change in the chemical shifts of some of the guest and host protons, in comparison with the chemical shifts of the same protons in the free components. Partial ^{1}H NMR spectra of pure components and diclofenac sodium : β-cyclodextrin mixture in a 1:1 molar ratio are shown in Figures 2 and 3. The absence of new peaks that could be assigned to the complex, suggested that complexation is a dynamic process, the included DCF-Na being in a fast exchange between the free and bound states. Before proceeding with binding constant calculations or with an analysis of the geometry of the DCF-Na : β-CD complex, the stoichiometry of the complex must be calculated. Determination of the stoichiometry of the DCF-Na : β-CD complex by continuous variation method, was based on ^{1}H NMR spectra obtained for DCF-Na and β-CD mixtures in which the initial concentrations of the two species

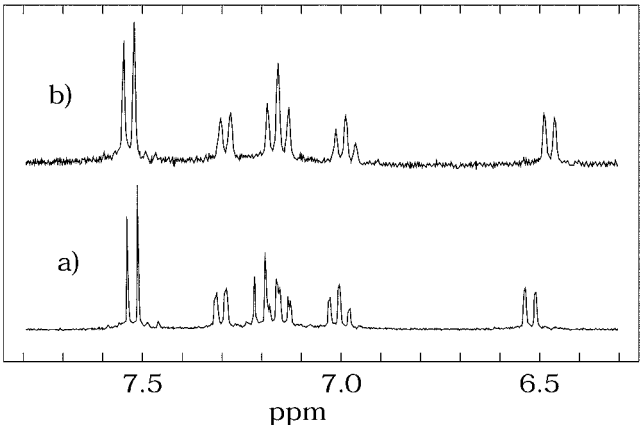

Figure 2. Partial ^{1}H NMR spectra (only the aromatic protons) of DCF-Na (a) 10 mM DCF-Na and (b) 5 mM DCF-Na and 5 mM β-CD.

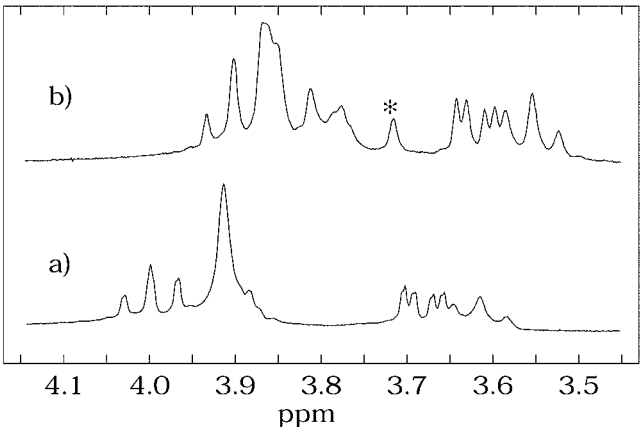

Figure 3. Partial ^{1}H NMR spectra (without H-1 proton) of β-CD. The (*) denotes the Ha signal of DCF-Na (a) 10 mM β-CD and (b) 5 mM β-CD and 5 mM DCF-Na.

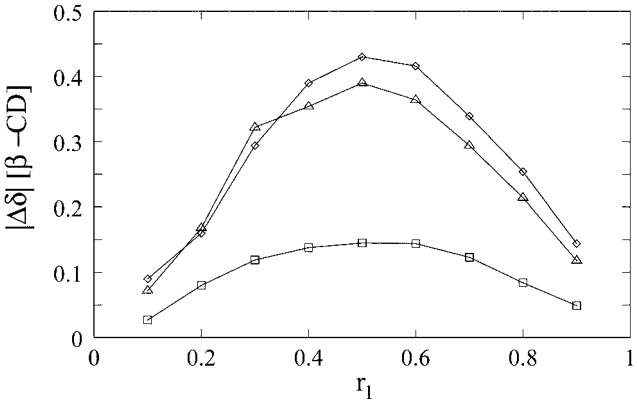

Figure 4. Continuous variation plot (Job plot) for H-3 (△), H-5 (◊) and H-6 (□) protons of β-cyclodextrin.

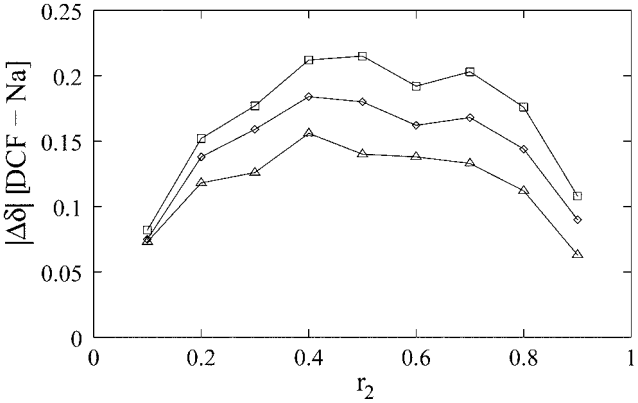

Figure 5. Continuous variation plot (Job plot) for Ha (□), Hd (△) and Hf,h (◊) protons of diclofenac sodium.

were maintained constant and the ratio r varied between 0 and 1 (see Procedure). The continuous variation plots of $|\Delta\delta|$ [β-CD] against $r_1 = m/(m+n)$, where m and n are, respectively, the proportions of β-CD and DCF-Na in the (DCF-Na)$_n$: (β-CD)$_m$ complex, are presented in Figure 4. Similarly, the Job's plots of $|\Delta\delta|$ [DCF-Na] as a function of $r_2 = n/(m+n)$ are presented in Figure 5. The induced shift, $\Delta\delta$, is defined as the difference in chemical shifts in the absence and in the presence of the other reactant for a given ratio r. For the sake of concision, only several protons (the most markedly affected) have been selected in the host and guest molecules.

Thus, for the H3 and H5 protons of β-CD, significant upfield shifts, attributable to the inclusion of an aromatic part were observed. The Job plots show a maximum at $r_1 = 0.5$ and highly symmetrical shapes, indicating that the complex has 1:1 stoichiometry. The DCF-Na protons can be split into two groups, one shifted upfield (He and Hg) and the others downfield. As Veiga *et al.* [17] pointed out, a down field displacement of the guest protons indicates that they are close to an electronegative atom like oxygen, whereas an upfield shift displacement indicates weaker interactions with the hydrogen atoms (shielding effect due to van der Waals forces). Because the protons belonging to both the aromatic rings of DCF-Na show chemical shift differences upon inclusion, suggests that multiple equilibria may exist in solution. Although the shapes of the Job plots for DCF-Na protons are not smoothly and highly symmetrical, the maximum does not deviate significantly from $r_2 = 0.5$, indicating in our opinion, the existence of two isomeric 1:1 complexes. The fact that the Job plots do not present a smooth variation can be attributed in part to chemical shift reading errors, because the DCF-Na protons induced shifts are much more smaller and the absorption signals broader than those of β-CD. We consider that the slight skewing of the curves for some DCF-Na protons to the left of $r_2 = 0.5$ is not enough to sustain [2, 4] the presence of a 1:2 complex or a mixture of 1:1 and 2:1 complexes [5].

To confirm the hypothesis of bimodal inclusion complexation, ROESY experiments were performed at 1:1 DCF-Na/β-CD molar ratio. The obtained results are very similar with those already published by Mucci *et al.* [5] and shows intermolecular cross-peaks between H3 and H5 protons of β-CD and protons belonging to both DCF-Na aromatic rings. Thus, H3 gives ROE cross-peaks with Hf,h, Hd(g) and He. The H5 proton of β-CD gives medium cross-peaks with Hf,h, high cross-peaks with Hb and a low cross-peak with Hd(g). In our case, the contribution for H6 of β-CD which partially overlapped to β-CD H3 proton is difficult to estimate. No cross-peaks were observed between Hc on the

phenylacetate ring, the methylene protons and H3 and H5 protons of β-CD.

To summarize, the Job plots and ROESY results clearly provide evidence for simultaneously presence in solution of two isomeric 1:1 complexes, each one involving inclusion of a different end of the DCF-Na. Similar behaviour were reported for other nonsteroidal anti-inflammatory agents such as piroxicam [18] and naproxen [19].

Evaluation of the binding constants

In order to determine the extent of the intermolecular binding between the two aromatic rings of DCF-Na and β-CD, the association constants have been evaluated. The association constant, K_a, for an 1:1 complex can be determined according to the following equation [20]

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

where i counts the sample number and j the studied proton. If the studied proton belongs to the guest or host molecule, then $X = G$ 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. Equation (1) involves no approximations and correlates the total concentrations of the guest and host molecules with the observed difference in chemical shift $\Delta\delta^{(i,j)}$.

We developed a computer programme 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 sets up a quadratic programme to determine the direction of search and the loss function

$$E = \sum_i \sum_j (\Delta\delta^{(i,j)} - \Delta\delta_{\text{calc}}^{(i,j)})^2 \quad (2)$$

until the search converges. The fitting procedure reaches an end when the difference between two consecutive E values is smaller than 10^{-6}. The treatment of the whole set of protons studied yields one single K_a value for the whole process and a set of calculated $\Delta\delta_c^{(i,j)}$ values. The programme is quite flexible since it allows to observe the chemical shift variation of the host, guest or both molecules as a function of variable guest or host concentrations.

In our particular case, we applied Equation (1) first for a set of protons consisting in H3, H5 and H6 of β-CD and Ha, Hb, Hc, and Hd of DCF-Na and then for H3, H5 and H6 of β-CD and Hf,h of DCF-Na. This means that we considered first the case when the phenylacetate ring is inserted in the β-CD cavity and then the inclusion of the dichlorophenyl ring. At this point is interesting to note that Hc proton is subjected to a relatively high chemical shift variation but gives no cross-peaks with one of β-CD inner protons in the ROESY spectrum. On the contrary, He proton gives a cross-peak with H3 but has a very small chemical shift variation (below 0.02 ppm).

Table 2. Chemical shifts of the protons of DCF-Na and β-CD in the free and complexed states

Proton	δ_{free} (ppm)	δ_c[a] (ppm)	
		1:1	1:1′
Ha	3.698	3.960	
Hb	7.346	7.477	
Hc	7.043	7.150	
Hd	7.228	7.431	
Hf,h	7.598		7.718
H3	4.004	3.720	3.794
H5	3.888	3.557	3.644
H6	3.917	3.803	3.833

[a] $\delta_c = \delta_{\text{free}} - \Delta\delta_c$.
$\Delta\delta_c$ values were obtained as a result of the fitting procedure.

The association constants obtained, using the above described procedure were $K, 1{:}1 = 88.8\ \text{M}^{-1}$ with $E = 1.03 \times 10^{-3}$ and a correlation factor $r = 0.997$, when the phenylacetate is included in the β-CD cavity and $K_{1:1'} = 161\ \text{M}^{-1}$ with $E = 4.76 \times 10 - 4$ and $r = 0.998$ for the inclusion of dichlorophenyl ring. The complete set of chemical shifts in the free state and in pure complex is reported in Table 2. It is also worth mentioning that evaluating K_a only from the observed differences in chemical shifts of H3, H5 and H6 β-CD protons, we obtained $K_a = 250\ \text{M}^{-1}$ indifferently if we used one, two or all three chemical shift variation. This value is almost identical with the overall association constant $Ka = K_{1:1} + K_{1:1'} = 249.8\ \text{M}^{-1}$. In our opinion this result supports the existence of a bimodal binding between DCF-Na and β-CD.

Based on the ^{1}H complexation shifts, ROESY cross-peaks and the magnitude of the association constants, we conclude that the inclusion process affects principally the dichlorophenyl ring, which enters the β-cyclodextrin cavity through the secondary hydroxy rim. The interaction of β-cyclodextrin with the phenylacetate ring seems to be weaker, the complexation process involving the primary hydroxy rim. Preliminary molecular modeling also indicates the possibility of two mutual orientations of diclofenac sodium and β-cyclodextrin. Detailed results of the molecular modeling study will be published elsewhere. At this point we want to underline that our findings concerning the geometry of the complexes are in complete agreement with the model proposed by Mucci *et al.* [5], except the existence of a 2:1 complex. More than that, there is a striking similarity between the association constant calculated by us for the inclusion of dichlorophenyl ring ($K_{1:1'} = 161\ \text{M}^{-1}$) and the value reported by Mucci *et al.* [5] for the 1:1 inclusion complex.

Conclusions

The diclofenac sodium : β-cyclodextrin system in aqueous solution has been studied by 1D and 2D ^{1}H NMR. The induced chemical shifts in the 1D NMR spectra confirm the existence of a bimodal binding between DCF-Na and β-CD and give values for the binding constants obtained with specific protons of both compounds. ROESY experiments reveal the correlation between the protons H3 and H5 of the β-CD cavity and both the aromatic rings of the DCF-Na, supporting the bimodal binding process. The geometry of the two 1:1 complexes according to the NMR data is given, indicating a certain preference of the β-cyclodextrin to the dichlorophenyl ring.

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