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# The self-association of the drug acemetacin and its interactions and stabilization with β-cyclodextrin in aqueous solution as inferred from NMR spectroscopy and HPLC studies

Dimitris Zouvelekis,[a] Konstantina Yannakopoulou,[a,*] Irene M. Mavridis,[a] Ekaterini Antoniadou-Vyza[b]

[a]*Institute of Physical Chemistry, NCSR "Demokritos" Aghia Paraskevi, Gr-15310, Athens, Greece*
[b]*Department of Pharmacy, University of Athens, Zografou, Gr-15771, Athens, Greece*

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**Abstract**

Strongly concentration dependent, $^1$H NMR chemical shifts of the non-steroidal anti-inflammatory drug acemetacin sodium salt (sodium {[1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-yl]acetoxy}acetate), were observed in aqueous solution. Self-titration and nOe experiments, point to a self-association model where stacking takes place via the indole portion of the drug. In addition, conformational isomerism (atropisomerism) of the anti to syn form was confirmed. Further increase of the concentration eventually led to stable chemical shifts and nearly simultaneous appearance of microcrystals. In the presence of βCD, 1:1 inclusion complexation occurred through the *p*-chlorobenzoyl part of the drug, whereas with excess βCD the indole part seemed to participate to a minor degree. The anti isomer is suggested to be involved in the inclusion process. In addition, aggregation of acemetacin was also evident, as competing with the conformational and inclusion equilibria. The present case demonstrates that many competitive processes are simultaneously active in a seemingly simple system. The measurements were strongly dependent upon the pH and use of buffered solutions was mandatory. Finally, for the quantitative analysis of acemetacin in the presence of βCD, a special HPLC method was developed. The stability of the drug, studied by the identification of the degradation products and the pseudo-first order rate of hydrolysis, was found to be unaffected by the presence of βCD. 

*Keywords:* β-Cyclodextrin; Self-association; Acemetacin; NMR; HPLC; Multiple equilibria

## 1. Introduction

Acemetacin ({[1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-yl]acetoxy}acetic acid (**1**)) is a non-steroidal anti-inflammatory drug (NSAID), whose biological action is a consequence of its in situ hydrolysis to indomethacin ({[1-(4-chlorobenzoyl)-5-methoxy-2-methylindol-3-yl]acetic acid (**2**)), also a known NSAID, that is, **1** is a pro-drug of **2**[1] (Scheme 1). Cyclodextrins (Scheme 2) are a family of cyclic oligosaccharides able to include organic molecules in their cavity, thus frequently resulting in greater stabilization, increased aqueous solubility, controlled release and other desirable properties of the included compounds.[2] No reference is found in the literature regarding the interaction/inclusion of acemetacin with cyclodextrins, whereas numerous studies regarding preparation, stabilization and potential pharmaceutical applications of the corresponding inclusion complex with indomethacin have appeared,[3] only a few[4] dealing with the structural characterization of the complex in solution. We have been interested in the complexation of acemetacin with βCD, given the practical importance of formulating NSAIDs with cyclodextrins,[5] as reflected by the growing number of patent applications[5c] on this subject. We have focused on the structure and degradation pathways of the βCD/acemetacin complex, since

* Corresponding author. Tel.: +3010-6503796; fax: +3010-6511766
*E-mail address:* dyanna@chem.demokritos.gr (K. Yannakopoulou).

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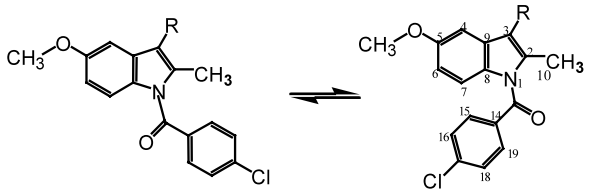
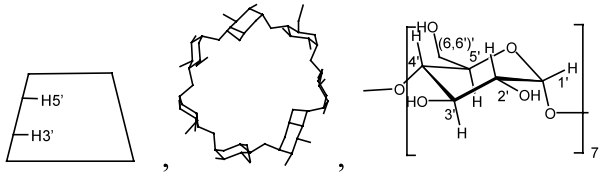



**1** R: -$CH_2COOCH_2COOH$ (acemetacin)

**2** R : -$CH_2COOH$ (indomethacin),

Scheme 1. Numbering scheme and conformational isomerisation of acemethacin (**1**) and indomethacin (**2**).




Scheme 2. Side (left) and top (middle) view of βCD and of the repeating unit (right) with numbering.

these are the crucial parameters towards sustained release and optimized bioavailability formulations of the drug. The present article presents a complete study on the stoichiometry, the association constant, the effect of pH, and the structural characterization of the intermolecular interactions of the sodium salt of **1** with βCD in aqueous solution, using NMR spectroscopy. The chemical stability of the complex in solution, on the other hand, is evaluated using HPLC and the rates of hydrolysis at several temperatures are measured. For this reason, a specific validated and stability indicating HPLC method for the determination of **1** in the presence of βCD has been developed. In the course of the NMR experiments, the enormous dependence of the chemical shifts of **1** on its concentration, and the in situ nucleation, leading to formation of crystals, was recognized.

The present results can be regarded as clarifying and complementing previous studies on the related complex of indomethacin (**2**) with βCD. In the most recent report,[4a] the subject of structure of the possible complexes was comprehensively treated. However in general,[4] experiments regarding stoichiometry have been partly performed, the effect of pH has not been addressed, neither has the self-association of the drug itself been investigated, a factor known[6] to alter the mole-ratio and continuous variation curves significantly, as we also demonstrate in the current case.

## 2. Results and discussion

*NMR studies.*—During preliminary $^1H$ NMR experiments with acemetacin sodium salt—the free acid was very insoluble in water—it became evident that there existed a strong dependence of the drug's chemical shifts on its concentration. Therefore, we had to examine this process by separate experiments.

*Concentration dependence of chemical shifts of acemetacin in $D_2O$.* Self-titration of the sodium salt of **1** from 0.36 to 9.75 mM revealed a pronounced shielding for all protons of the molecule. In order to ensure that acemetacin sodium would remain ionized throughout the experiment, we used a solution buffered at pH 8.2. The shape of the titration curves in Fig. 1 shows that the chemical shifts of all protons decrease uniformly upon increasing the concentration of **1**. The aromatic proton H-7 is the most shielded and the aliphatic methylene protons H-12 are the least shielded. The curves can be grouped into two categories, one of the most affected protons (H-7, H-6, H-16, H-18 and H-15, H-19) and one of the least affected protons (H-4 and aliphatic Me, MeO, H-11, and H-12). The experimental curves do not have the typical hyperbolic shape of binding, as systems with π–π aromatic stacking[7] but

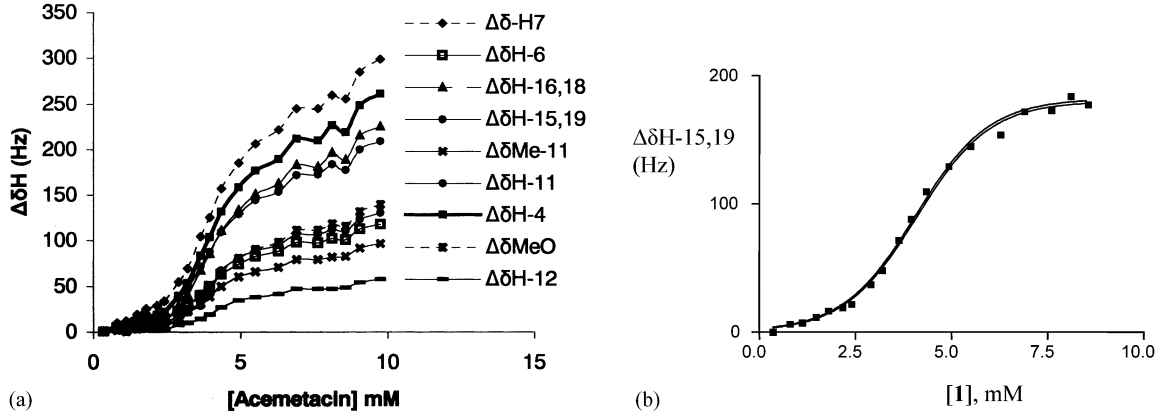



Fig. 1. Chemical shift displacements ($\Delta\delta H = \delta_{initial} - \delta_{final}$) of acemetacin sodium salt during self-titration at pH 8.2, 300 K (500 MHz) (left) and fitting to a sigmoidal-type equation (right), see text.

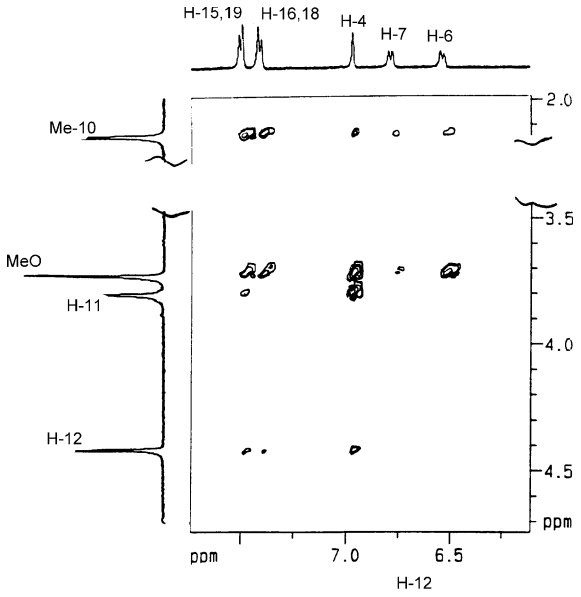



Fig. 2. Partial ROESY spectrum of acemethacin sodium salt (1.8 mM, $D_2O$, pH 8.2, 300 K, 500 MHz).

rather a sigmoidal shape, with a slowly growing part up to about 2 mM ( ~ sixfold concentration increase) followed by a fast rise and a turning point at about 6 mM. The plateau following that point has a non-zero slope. Increasing difficulty was encountered during collection of the last data points due to the appearance of tiny crystals in the NMR tube upon standing, which had to be dissolved by vigorous shaking before acquisition of the next spectrum. We, therefore, suggest that the molecule participates in a self-aggregation process. Upon increasing the concentration, a cooperative event[8,9] sets on, which results not only in dimers but in higher aggregates and finally in crystals. In other words, during the self-titration we are actually witnessing the nucleation and crystallization process. A similar shape of the titration curves has also been reported for the self-aggregation of chlorpromazine[10] and some naphthalene bis(dicarboximide)s[11] both in aqueous solution, probed by NMR spectroscopy. Quantitative treatment using the entire range of the concentration dependence data (see Eq (2) in the Experimental), with the assumption that dimerization prevails and higher aggregates are negligible, did not result to any reasonable fit. This is expected since the crystals formed during the final points of the self-titration cannot be dimers. More reasonable fitting, however, was obtained using the first seven data points giving the dimerization constant, $K = 210 \pm 22$, with $R^2 = 0.986$. On the other hand, fitting of all but the last two data points to the sigmoidal Eq (3) provided excellent fit ($R^2 = 0.996$). The data were also plotted according to the Hill equation[8] and afforded a linear graph ($R^2 = 0.961$) with slope, i.e., the "Hill coefficient" $h = 2.5$, indicating positive cooperativity during the process.[8]

The very strong shielding effect on H-7, H-6 and H-16, H-18, H-15, H-19 (the top group of curves) indicates changes involving the aromatic rings (indole and *p*-chlorobenzoyl). This could be attributed to the continuous shifting of a conformational equilibrium (Scheme 1), such as that reported for indomethacin,[4a] towards the syn conformer[1] (the indole ring becoming nearly perpendicular to *p*-chlorobenzoyl ring to maximize $=C–H\cdots\pi$ interactions, thus resulting in mutual shielding), although this motion is not expected to affect all protons in the same way. After a critical concentration, the syn conformer aggregates fast in a fashion reminiscent of crystal packing, and produces the first crystals. The ongoing conformational equilibrium in solution was detected through intermolecular interactions, as shown next.

The structure of the aggregated species in the solution was investigated by 2D ROESY spectra acquired under conditions identical to the corresponding spectra of **1** with βCD (to be shown later) and at a concentration corresponding to the beginning of the plateau in Fig. 1. The spectra obtained mapped various interactions among the protons (Fig. 2). First of all interactions between the *p*-chlorobenzoyl protons (H-15, H-19, but also H-16, H-18) with both the methyl group Me-10 (Fig. 2) and H-7 (not included) demonstrate the (anticipated) coexistence of the fast exchanging syn and anti conformers in solution. Further, the methylene protons of C-12 correlate with H-4, weakly with H-15, H-19 and H-16, H-18, but also with the methyl group at C-10 (not included in Fig. 2) indicating a motion of the side chain in space. What cannot really be attributed to the intramolecular proximity of the protons but to the formation of aggregates, is the observed correlation of the methoxy group with both sets of *p*-chlorobenzoyl ring protons. In addition, correlation was observed between the protons of Me-10 with diametrically opposite aromatic protons (H-7, H-6, H-4) of the indole ring. It seems, therefore, that the aggregation involves a head-to-tail stacking of two indole rings belonging to adjacent molecules. Towards this model, also point the detectable correlations of MeO with Me-10 and with H-12 (not in Fig. 2). The above are in agreement with what was observed in the crystal structure of indomethacin,[12] in which the indole plane was found almost perpendicular to the *p*-chlorobenzoyl ring of the same molecule, and overlapping with the acetic acid side-chain of an inverted indole ring of another molecule, all in the syn configuration (Scheme 1), this isomer being both the more stable[4a,12] and the bioactive one.[13] Unfortunately, we were unable to obtain crystals

[1] The two distinct conformers should be strictly referred to as atropisomers, since the amide linkage is not coplanar with the indole ring,[12] however we maintain the above symbolism to conform with previous articles.[4a,12]

of good quality for X-ray analysis to verify the structure of the proposed "pre-crystalline" state.

The strong concentration dependence of the chemical shifts of a substance has been reported for several types of small heterocyclic molecules such as quinolines, indoles, benzofuranes, isoquinolines and acridine in various organic solvents,[14] and of conjugated phenylacetylene macrocycles[7] in chloroform. In aqueous solutions, there are also examples of self-aggregation processes evaluated through NMR chemical shift measurements, such as the pre-micellar organization of chlorpromazine (a phenothiazine derivative),[10] the self-stacking of naphthalene diimides,[11] and the color stabilizing self-aggregation of the flavylium cation.[15] The present report of the similar behavior of a commonly used drug enforces the notion that self-association of small organic molecules in aqueous solutions at sub-millimolar concentrations is a widely-spread phenomenon that should be taken into account when trying to assess intermolecular interactions, evaluate binding constants in host–guest systems or, in general, use chemical shifts as a measure of molecular recognition phenomena. On the other hand, the formation of aggregates of acemetacin, and very likely many other NSAIDS (e.g., indomethacin) in aqueous solution, could also affect their physiological behavior, especially in cases where the latter is concentration dependent.[16] The above observations prompted us to further study the complexation of **1** with βCD, and allowed to explain the unusual curves obtained during Job plots.

*Molar ratio titrations*. In order to examine the inclusion of the acemetacin molecule inside the βCD host, titration of a βCD solution with solid acemetacin sodium salt was carried out in both buffered ([βCD] = 3.1 mM, pH 8.2) and unbuffered ([βCD] = 3.0 mM) solutions and the chemical shift displacement of the βCD cavity protons H-5′ and H-3′ (Scheme 2) was monitored. In the buffered solution, only the anionic form of **1** is present during the process, given that the p$K_a$ of acemetacin is 3.91 at 20 °C.[17] The reverse experiment (titration of an aqueous solution of **1** sodium salt) with solid βCD and monitoring of the chemical shift alterations of **1**) would be unreliable given the serious aggregation of acemetacin and the resulting dependence of its chemical shifts on the concentration of the (unknown) free **1**. In addition, complexation might take place preferentially through one of the conformers of the drug, and this would also contribute to alterations of the chemical shifts of **1**. Fig. 3 shows the binding curves and illustrates the behavior of the cavity protons H-5′ and H-3′ in the two solutions. Thus, H-5′ is shielded more strongly than H-3′ upon increasing the concentration of the guest drug. The corresponding smooth curves (Fig. 3), show binding that reflects a stoichiometry close to 1:1. The peaks of the drug became broadened, from the stoichiometry 1:1 and over, probably due to the formation of aggregates of **1**. Indeed, crystallization of **1** out of the solution occurred after addition of about 1.5 equivalents of the drug. The curves corresponding to the unbuffered titrations (pH 6.0–6.8) show a step at the mole ratio 1:1, followed by a plateau above 2:1 and the overall displacements are greater. Apparently the pH variation, and thus the presence of a non-negligible amount of the free acid, is sufficient to cause anomalies in the appearance of the curve (by, e.g., altering the self-association process), and therefore we regard the unbuffered curves unreliable. Fitting of the experimental curve obtained at pH 8.2 with the equation describing the 1:1 model[18] (this stoichiometry also suggested by the Job plots, shown below) gives a binding constant $K_{11} = 1100\ (\pm 31)\ M^{-1}$ and intrinsic $\Delta\delta H$-3′ 0.2012 ($\pm$0.0013) ppm with $R^2 = 0.9991$ at 300 K. This $K_{11}$ value is more than four times higher that the roughly calculated dimerization constant reported above, meaning that complexation with βCD prevails, until the concentration of **1** increases (past the 1:1 point), and then the aggregation process manifests itself again, in agreement with the spectral observations.

An ESI-mass spectrum of the unbuffered solution showed a weak molecular ion corresponding to ([βCD·acemetacinNa] + Na)$^+$ at $m/e$ 1596.7. A fairly strong peak (13%) corresponding to ([acemetacin·acemetacin] + Na)$^+$ at $m/e$ 853 indicated the strong self-association of the guest.

*Job plots*. Continuous variation (Job) plots[9] were calculated for buffered and unbuffered drug/βCD mixtures are shown in Fig. 4. The curves that are produced

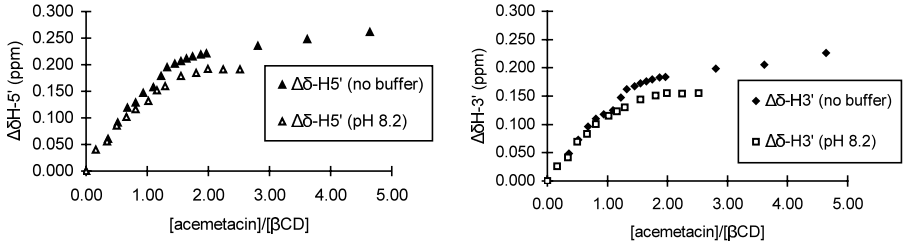



Fig. 3. Titration $^1$H NMR data of βCD (3.1 mM) aqueous solutions with solid acemetacin sodium salt, where $\Delta\delta H' = \delta_{free} - \delta_{complex}$ (βCD protons). For unbuffered $D_2O$ solutions, pH 6.0–6.8; for buffered $D_2O$ solutions, pH 8.2.

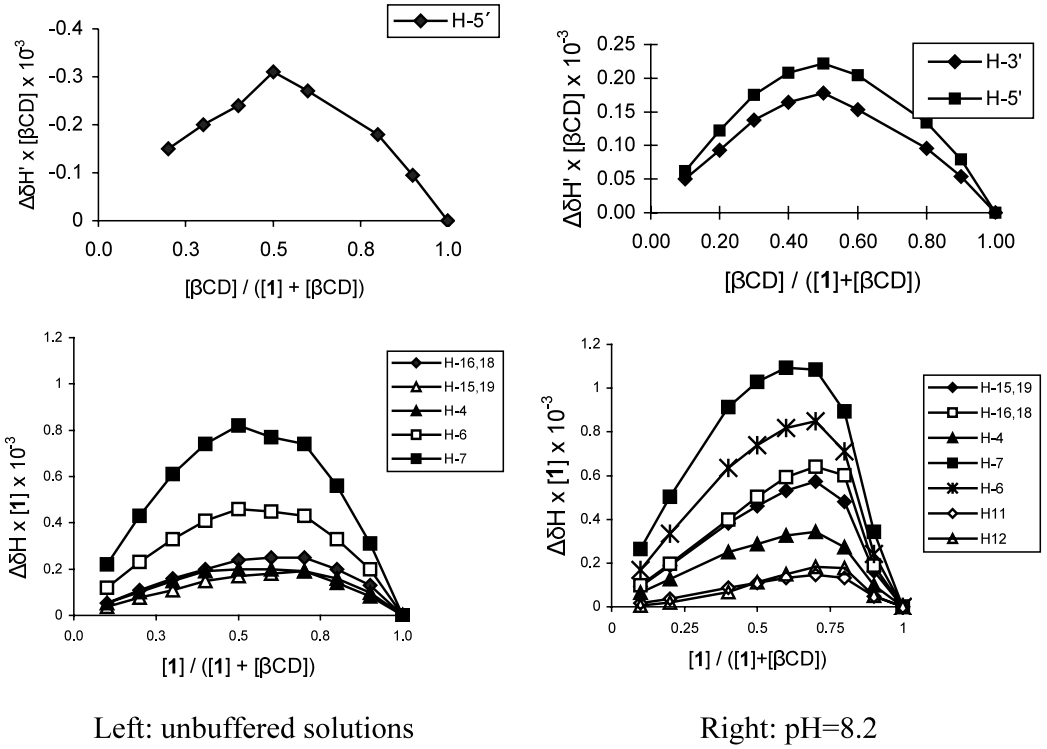



Fig. 4. Job plots of unbuffered (left) and buffered (pH 8.2, right) $D_2O$ solutions of βCD/acemetacin sodium salt.

by monitoring the βCD cavity protons, in either buffered or unbuffered solutions provide 1:1 stoichiometry. The protons of acemetacin, however, behave inhomogeneously in both cases. These anomalies in the chemical shifts of **1** arise from a combination of changes due to de-aggregation, inclusion and possibly conformational syn to anti isomerism, therefore no conclusions can be drawn from the curves of **1**. However, one observes once more that without buffers the results can be erroneous, by comparing for example, the behavior of H-6 and H-7 in the buffered and unbuffered solutions.

*Intermolecular nOe data*. The results presented so far demonstrate that inclusion does take place in solution, that the complex formed is most probably 1:1, and that it is not clear which part (indolyl- or *p*-chlorobenzoyl) is inserted in the cavity and how. This information was derived from 2D ROESY spectra of the complex. The intermolecular interactions were mapped with this experiment at different βCD/acemetacin mole ratios. At mole ratio 3:1 (excess βCD, Fig. 5), we observe very strong interactions of both sets of the *p*-chlorobenzoyl protons with H-3′ and H-5′ of βCD, indicating insertion of this part into the cavity. On closer inspection, we see that this is strictly true for H-15, H-19 whereas H-16, H-18 show a large cross peak that culminates at (H-6, H-6′)′ of the primary side of βCD, suggesting deep insertion of the *p*-chlorobenzoyl part from the secondary side of the host. The indolyl protons, show primarily intramolecular nOes, and weak dipolar correlation with H-5′ and probably with H-3′ of the host. These weak interactions suggest that there could be a second 1:1 complex present in the solution involving inclusion of the indolyl part, since there is an excess of βCD available. It is worth noting that there is only one intramolecular nOe peak present between Me-10 and H-15, H-19 whereas in Fig. 2 (acemetacin alone) all

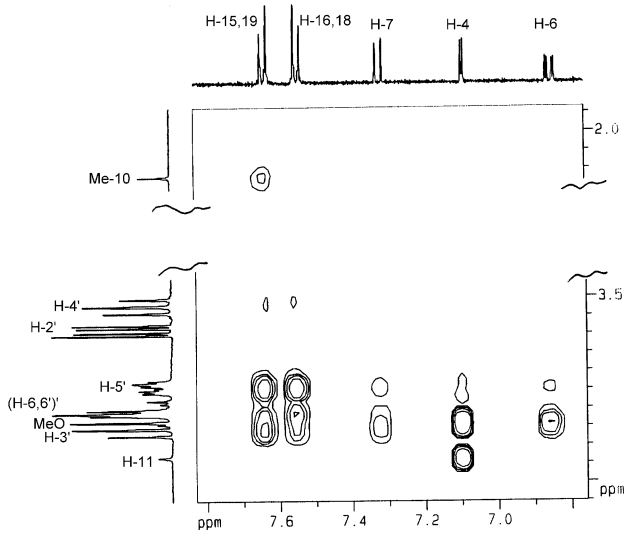



Fig. 5. Partial ROESY spectrum of βCD (6.6 mM) with acemetacin sodium salt (2.2 mM), $D_2O$, pH 8.2, 300 K, 500 MHz.

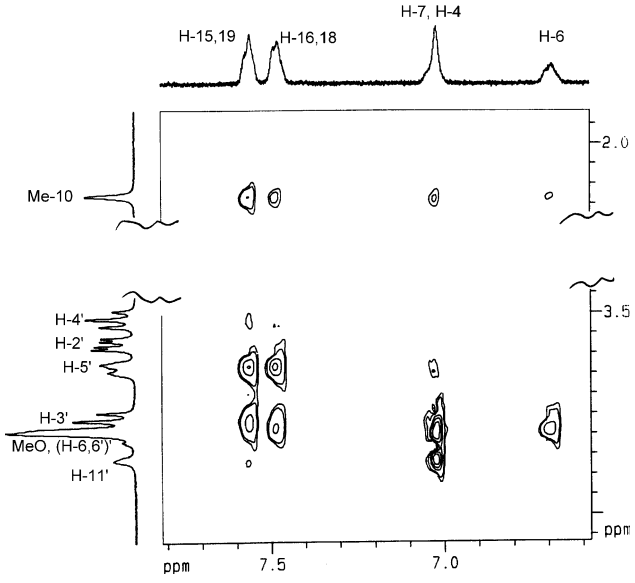
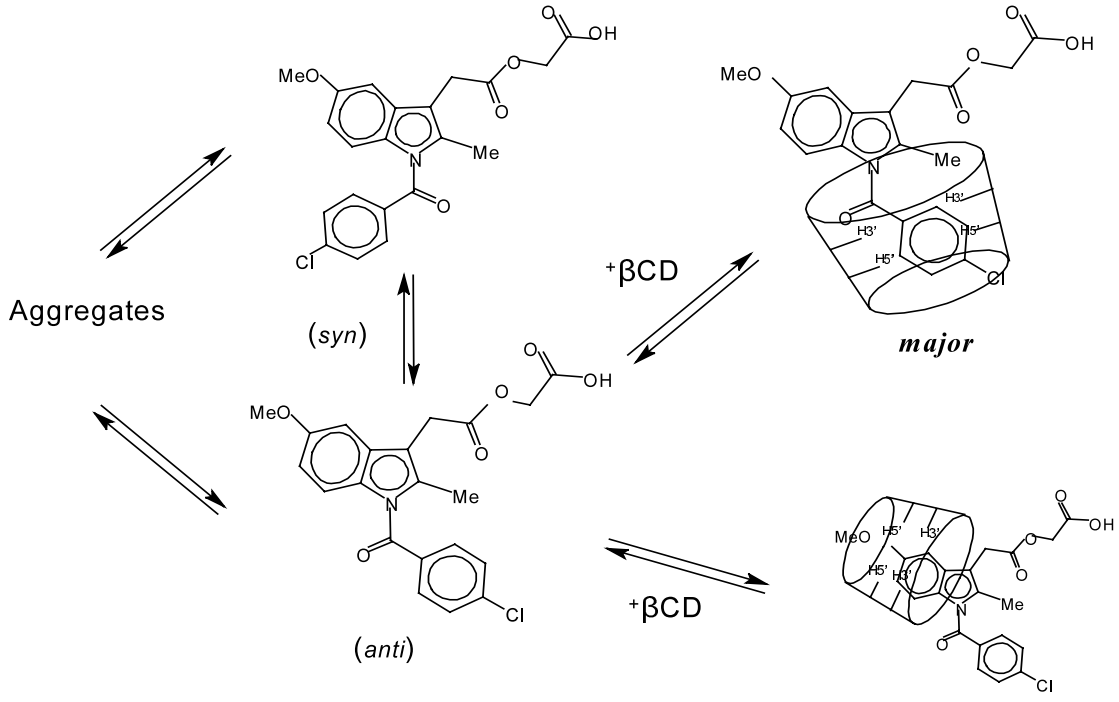



Fig. 6. Partial ROESY spectrum of βCD (3.0 mM) with acemetacin sodium salt (6.0 mM) $D_2O$, pH 8.2, 300 K, 500 MHz.

aromatic protons show interactions with Me-10 and in spite the similar concentration of **1** in the two spectra. This in turn indicates that complexation with βCD breaks up the aggregates of the drug and that there is appreciable presence of the anti-type which is, for steric reasons, expected to complex preferentially with βCD. As the mole ratio of the two complexing partners approaches the value 1:1, the dipolar correlation map (not shown) and the appearance of the drug's spectrum change. The resonance of H-7 moves close to that of H-4, the complexation of the *p*-chlorobenzoyl ring with βCD is strong but the respective cross peaks of the indolyl protons are diminished, and only H-7 shows interactions with H-3′ and H-5′. This could be due to either minimization of the second complex mentioned above or possibly interaction of the H-7 with the *p*-chlorobenzoyl ring-filled βCD cavity due to this proton's proximity with the inserted part. The second scenario does not justify the (weak) interaction H-7/H-5′. At even higher concentrations of **1** and at mole ratio 1:3, there is broadening of the aromatic peaks and the resonances of H-4 and H-7 come together (Fig. 6). The intramolecular correlations of Me-10 with the aromatic protons appear now again as in Fig. 2. The aggregation of the drug is apparently effective again and competes with the inclusion process resulting into a broadening of the peaks of **1**, not observed in Fig. 2. The βCD complex is prominent as the strong cross-peaks *p*-chlorobenzoyl/H-3′, H-5′ show, whereas H-7 interacts weakly with βCD (less than shown in Fig. 5).

The intermolecular interactions, therefore, have shown that in the solution there exist at least four different equilibria, i.e., conformer exchange, aggregation of **1**, and two inclusion processes of which the predominant is βCD including the *p*-chlorobenzoyl group (Scheme 3). The percentage of contribution the second complex (indolyl group included) is small, it depends on the mole ratio of the components and can be considered negligible at or below 1:1 ratio [βCD]/[**1**].




Scheme 3. Equilibria in the aqueous solution of βCD/acemetacin.

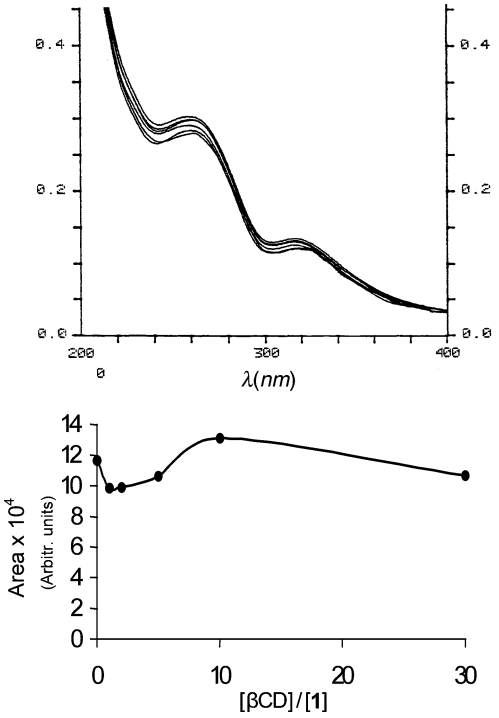



Fig. 7. Non-proportional variation in the UV absorption (top), and in the HPLC peak area determined using the UV-detector (bottom) of acemetacin (**1**) in the presence of increasing amounts of βCD.

Table 1
First order rate constant ($k$, $s^{-1}$) for the hydrolysis of acemetacin alone and in the presence of βCD at various temperatures

| Temperature (K) | 333 | 323 | 308 |
|---|---|---|---|
| $k_{\text{acemetacin}}$ ($\times 10^{-5}$) | 3.33 | 1.33 | 0.20 |
| $k_{\beta\text{CD/acemetacin}}$ ($\times 10^{-5}$) | 3.50 | 1.33 | 0.21 |

*Stability studies.*—The complex βCD/acemetacin was studied, regarding its stability, at different temperatures and under mildly alkaline conditions (pH 8), very close to that used in the structural studies above. The concentration of the drug as well as its products of hydrolysis were determined using HPLC. For these quantitative measurements, a special stability indicating and validated chromatographic method was developed. The UV spectrum of any guest is affected by complexation with cyclodextrins,[19] therefore the accuracy of the chromatographic determination (using a UV detector for the HPLC) can be seriously hampered (Fig. 7). For this reason, conditions suitable for complete de-complexation of **1** from βCD were explored: variations in the composition of the mobile phase regarding the organic modifiers, pH effects, types of reverse phase columns (C-18, phenyl-, amino-) and effect of temperature in the range of 30–38 °C. The method finally developed, involved the use of a C-18 Spherisorb reverse phase column with 50:5:45 methanol–isopropanol–phosphate buffer (pH 4.0) as the mobile phase, a flow rate of 1.35 mL/min at 35 °C and the UV detector set at 238 nm. These conditions gave satisfactory results regarding the accuracy of determination of **1** and also the retention times of the potential degradation products of **1**,[20] indomethacin and *p*-chlorobenzoic acid, in the presence of βCD. The method developed was checked for RSD (1.10%), precision (98.5%) and repeatability for the entire range of its linear region (0.45–4.5 mg/L), all of which were satisfactory. In addition, the method was characterized by a very good sensitivity, and a low detection limit (0.18 mg/L) and quantitation limit (0.54 mg/L).

The stability of a 0.0106 mM solution of acemetacin (**1**) in the presence of βCD was studied under pseudo-first order reaction conditions (large excess of βCD) at pH 8.0, and the first-order reaction constant for the degradation of **1** was measured at different temperatures (Table 1) and compared with the results obtained without βCD. The table shows that there exists a satisfactory linearity among the calculated values of $k$ in both cases ($R^2 = 0.9965$ for **1** and 0.9981 for βCD/**1**) and that the presence of βCD does not alter the rate of hydrolysis of acemetacin. That is, despite the documented inclusion that takes place at this pH ($K_{11} = 1100\ M^{-1}$) and with excess βCD (which ensures high concentration of complex), there is neither stabilization offered by the host, nor acceleration of its degradation. The hydrolysis products determined chromatographically (Fig. 8) were identified using authentic samples, by NMR spectroscopy and by comparing the results with the hydrolysis profile of pure indomethacin. The products were 5-methoxy-2-methylindol-3-yl acetic acid (retention time 0.56 min), 5-methoxy-2-methylindol-3-yl acetic acid carboxymethyl ester (retention time 0.90 min), *p*-chlorobenzoic acid (retention time 1.15 min), and indomethacin (**2**) (retention time 4.35 min). The above show that hydrolysis affects two functions with apparently different rates: (i) of the ester bond and (ii) of the pseudo-amide bond, as previously observed for some other esters of indomethacin.[21]

## 3. Conclusions

Acemetacin sodium salt forms aggregates at mildly alkaline pH, resulting in serious alteration of its chemical shifts. Self-titration curves and nOe data indicate that a head-to-tail stacking of the molecules through their indole parts leads to nucleation and eventually to crystallization of the drug. In aqueous solution and at the same pH, βCD forms a 1:1 complex with acemetacin, characterized by an association constant of $1100\ M^{-1}$. Inclusion in the βCD cavity takes place via the *p*-chlorobenzoyl moiety, as in the case of the βCD/

indomethacin complex. Under conditions involving excess βCD, the indole part also forms a complex, which exists in small concentration. Based on dipolar interaction data, we suggest that the anti conformer is the one that participates in the formation of inclusion complexes, whereas the syn conformer seems to be the dominant conformer in solution, in the absence of βCD. Buffering of the solution for any concentration dependence study is mandatory for accurate results. For the quantitative determination of **1** in the presence of βCD, the special chromatographic method developed was used to measure the pseudo-first order rate of degradation of the drug. In spite of the formation of a fairly strong complex, there is no effect of the βCD host on the rate of hydrolysis of acemetacin.

## 4. Experimental

*General.*—Acemetacin (Sigma) was converted to its sodium salt by treatment with 1 equiv of NaOH in water. The product was dried under diminished pressure and then dissolved in buffered ($K_2HPO_4$–$NaH_2PO_4$) $D_2O$ (pH 8.2). The HPLC was carried out using HPLC grade methanol and isopropanol (E. Merck). Deionized water was purified by a MilliQ (Millipore, USA) unit. The NMR spectra were acquired on Bruker instruments AC 250 and DRX 500 MHz. The ESI mass spectrum was run on an AQA Navigator Finnigan-Thermoquest instrument. HPLC quantitations were carried out on a Waters LC instrument consisting of a model 590 pump, a Waters 996 photodiode array UV detector, and a Reodyne 717S injector fitted with a 20 L loop. The degassed mobile phase of 50:5:45 MeOH–isopropanol–phosphate buffer was vacuum filtered through a 0.45 m pore-size nylon membrane filter (Millipore, USA). All runs were performed at constant temperature 35 °C using a Jones column chromatography oven (UK) and a Spherisorb S3 ODS2, 4.6 mm × 10 cm column. The flow rate was maintained at 1.35 mL/min and the pressure was 400 psi.

*NMR studies.*—All samples were allowed to equilibrate at 300 K for at least 15 min prior to acquisition. The residual HDO peak of the solvent was used as the chemical shift reference for the spectra. For the self-titration studies, the initial concentration of **1** was 0.36 mM and the final 9.75 mM. For the mole ratio studies, the concentration of βCD was held constant at 3.1 mM and acemetacin was added in portions up to 14 mM. Fitting of the mole ratio data points to the theoretical curve derived for 1:1 complex under fast exchange conditions[17] was carried out using the routine provided by the program GRAPHPAD PRISM. For the continuous variation plots, a solution 4.0 mM in **1** and another 4.0 mM in βCD were mixed at different proportions and the chemical shift displacements were recorded. The construction of the continuous variation and mole ratio plots required short 2D COSY experiments (gradient version) for most of the data points (determination of chemical shift displacements for overlapping resonances). 2D ROESY spectra were acquired with 1–2 K size in $F^2$ with 256 experiments, $d_1 = 0.8$–$1$ s and mixing time 350 ms at transmitter attenuation 30 dB. Processing was carried out using zero-filling in $F1$ and the sine-bell squared window function in both dimensions.

$^1$H NMR data analysis of self-titration of acemetacin was carried out using the equation:[7]

$$(c/2[m2]) + (2[m2]/c) - 2 = 1/2cK \quad (1)$$

where $c$ = total concentration of acemetacin sodium salt, $[m2]$ = the concentration of the dimer, and $K$ = the dimerization constant. The observed chemical shift, $\Delta\delta_{obs} = f(m2)\Delta\delta^{\circ}_{m2}$, where $f(m2)$ = mole fraction of dimer, can be expressed as $\Delta\delta_{obs} = 2[m2]\Delta\delta^{\circ}_{m2}/c$, therefore Eq. (1) becomes

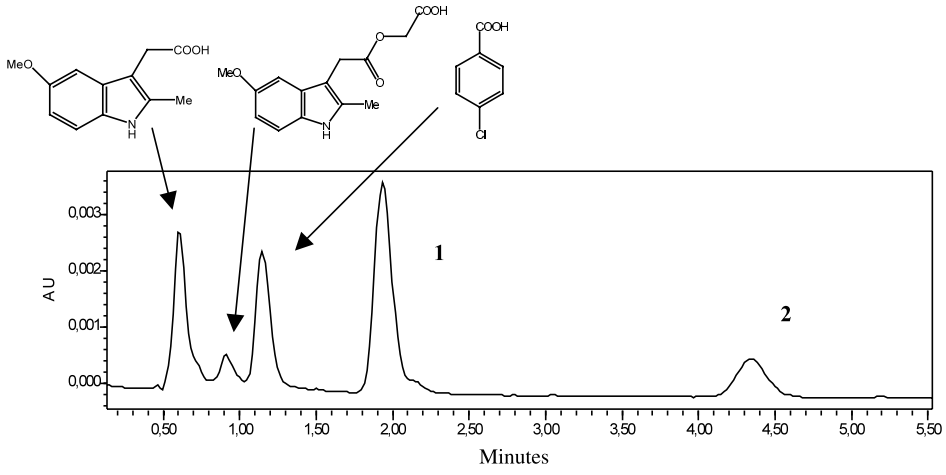



Fig. 8. Chromatogram of **1** and its products of hydrolysis at pH 8, 333 K; detector wavelength 254 nm.

$$c = \frac{2}{2cK} + \frac{\Delta\delta_{obs}\Delta\delta^{\circ}_{m2}}{(\Delta\delta^{\circ}_{m2} - \Delta\delta_{obs})^2} \quad (2)$$

which utilizes the experimental variables $c$ and $\Delta\delta_{obs}$, and provides after fitting the values $K$ and $\Delta\delta^{\circ}_{m2}$ which the limiting chemical shift of the dimer.[7]

The equation

$$\Delta\delta_{obs} = \Delta\delta_{obs,initial} + \frac{(\Delta\delta_{obs,final} - \Delta\delta_{obs,initial})}{1 + \exp[(c' - c)/slope]} \quad (3)$$

where $c'$ = the concentration half-way in the titration, was utilized for the fitting of the self-titration data, and is a suitably corrected built-in equation for sigmoidal response in the GRAPHPAD PRISM software.

*Stability studies.*—Reference solutions of acemetacin, indomethacin, and *p*-chlorobenzoic acid were prepared for quantification of hydrolysis at pH 8. The kinetic studies were performed at 35, 50 and 60 °C using a thermostated oven. The samples were analyzed after careful neutralization using 1 N HCl and vortexed for 10 s.

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