Molecular Modelling and ^{1}H-NMR: Ultimate Tools for the Investigation of Tolbutamide : β-Cyclodextrin and Tolbutamide : Hydroxypropyl-β-Cyclodextrin Complexes

Francisco José Baptista VEIGA,*[,a] Catarina Marques FERNANDES,[a] Rui Albuquerque CARVALHO,[b] and Carlos Frederico Gusmao Campos GERALDES[b]

Laboratory of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra,[a] Coimbra (Portugal) and Department of Biochemistry, University of Coimbra,[b] Portugal. Received February 27, 2001; accepted July 2, 2001

A structural study of the inclusion compound of tolbutamide (TBM) with β-cyclodextrin (β-CD) and hydroxypropyl-β-cyclodextrin (HP-β-CD) was attempted by means of ^{1}H-nuclear magnetic resonance (^{1}H-NMR) experiments and computer molecular modelling. To establish the stoichiometry and stability constant of the β-CD:TBM complex, the continuous variation method was used. The presence of true inclusion complexes between TBM and β-CD or HP-β-CD in solution was clearly evidenced by the ^{1}H-NMR technique. Changes in chemical shifts of H-3 and H-5 protons, located inside the CD cavity, associated with variations in the chemical shifts of TBM aromatic protons provided clear evidence of inclusion complexation, suggesting that the phenyl moiety of the drug molecule was included in the hydrophobic cavity of CDs. This view was further supported by the observation of intermolecular NOEs between TBM and β-CD and by the aid of a molecular modelling program, which established the most probable structure of the complex. The molecular graphic computation confirmed that the minimum energy, positioning TBM relative to β-CD, occurs when the aromatic ring of TBM is included within the β-CD cavity by its wider side, leaving the aliphatic chain externally, which is in good agreement with the results of ^{1}H-NMR studies.

Key words tolbutamide; cyclodextrin; ^{1}H-NMR; molecular modelling

Cyclodextrins (CDs) are cyclic oligosaccharides composed of α-1,4-linked D-glucopyranose units. The most common of these ring-shaped molecules are the α-, β-, and γ-CDs formed by six, seven, and eight glucose units, respectively.[1)] CDs are toroidal molecules with a truncated cone structure where the secondary hydroxyl groups are located on the wider side of the ring, while the primary hydroxyl groups are positioned on the opposite, narrower side of the torus. The –CH groups carrying the H-1, H-2, and H-4 protons are located on the exterior of the molecule and the hydroxyl groups are oriented to the cone exterior, which makes the external faces of CDs decidedly hydrophilic. The interior of the torus, which offers an environment of much lower polarity than that present in water, is lined by two rings of –CH groups (H-3 and H-5) and by a ring of glycosidic "ether oxygens" (O-4), with H-6 located near the cavity.[2)] A great variety of "guest" molecules of suitable size and shape may be entirely or partially included in this hydrophobic cavity, resulting in a stable association without formation of covalent bonds. The complexation driving forces have been attributed to hydrophobic interactions, van der Waals-London dispersion forces, and hydrogen bonds.[3,4)] In the pharmaceutical field this complexation phenomenon has been extensively applied to enhance the solubility, dissolution rate, and bioavailability of sparingly soluble drugs in gastrointestinal fluids.[5—10)] Because of the increasing interest in CDs and their inherent usefulness, several studies have been conducted to clarify the mechanism of complexation.

In previous studies, the ability of β-CD and hydroxypropyl-β-cyclodextrin (HP-β-CD) to form inclusion complexes with tolbutamide (TBM), in order to increase its solubility, dissolution rate,[11)] and oral bioavailability[12)] was evaluated. Important information on the solid-phase structure of these complexes may be obtained *via* X-ray analysis when a suitable crystal of the product is available. However, X-ray diffractograms of the lyophilized TBM:CD complexes previously reported[11)] have demonstrated the complete amorphousness of the product obtained, making it impossible to gain more detailed information from them. Although solubility studies indicated the existence of complexation between TBM and β-CD or HP-β-CD in solution,[11)] the exact mechanism of complexation could not be deduced.

The use of computer-aided molecular modelling and ^{1}H-NMR studies has proved to hold promise for such purposes, especially for systems or molecules of biological interest which "act" in aqueous medium. These techniques have been used as important tools for investigating the conformation of the most favored complexes and to obtain a better knowledge of the geometry of the system and the topology of the interactions between guest and CDs.[13—17)] Therefore in the present study we coupled these techniques, which allowed us to compare and to integrate the theoretical findings obtained in a vacuum (molecular modelling) with the experimental data (^{1}H-NMR) to gain insight into the mode of inclusion and to clarify the most probable conformation of the TBM : CD complexes in aqueous medium.

Experimental

Materials TBM (Sigma, St. Louis, MO, U.S.A.), β-CD (Roquette Frères, Lestrem, France), and HP-β-CD with a molar substitution of 0.39 (Janssen, Beerse, Belgium) were used as received. All the other reagents were of analytical grade.

^{1}H-NMR Studies TBM, β-CD, and HP-β-CD solutions (10 mM) were prepared in D_2O following adjustment of pD to 7.5 with 40% NaOD. The drug is insufficiently soluble in water at pD values below 7.5 ($pK_{a\,TBM}=5.4$) to allow ^{1}H-NMR studies. TBM and CD solutions were mixed in a 1 : 1 molar ratio in 5-mm NMR tubes. The ^{1}H-NMR spectra of the pure components and their respective mixtures were obtained at 298 K on a Varian Unity-500 spectrometer using a 5-mm reverse detection NMR probe. The resonance at 4.8 ppm due to residual solvents (H_2O and HDO) was used as

* To whom correspondence should be addressed. e-mail: fveiga@ci.uc.pt

an internal reference. Chemical shifts were calculated according to the formula

$$\Delta\delta = \delta_{(\text{complex})} - \delta_{(\text{free})}$$

The stoichiometry and stability constant (K_s) of the TBM : β-CD inclusion complex were provided using the continuous variation technique (Job's plot).[18] For this system, solutions with other β-CD and TBM molar ratios (3 : 1, 2 : 1, 1 : 2, 1 : 3) were prepared as described for the 1 : 1 mixture. The total molar concentration was kept constant at 10 mM ([β-CD]+[TBM]= 10 mM). The differences in chemical shifts ($\Delta\delta$) of the NMR spectra were measured for a given molar ratio.

The K_s of the TBM : β-CD complex was determined according to the following equation for 1 : 1 complexes[19]:

$$\Delta\delta = \frac{\Delta\delta^*}{2[\beta\text{-CD}]}\left\{[\text{TBM}]+[\beta\text{-CD}]+\frac{1}{K_s} - \left(\left([\text{TBM}]+[\beta\text{-CD}]+\frac{1}{K_s}\right)^2 - 4[\text{TBM}][\beta\text{-CD}]\right)^{1/2}\right\}$$

Experimental data ($\Delta\delta$) and the corresponding concentrations of TBM ([TBM]) and β-CD ([β-CD]) were fitted to this expression using a nonlinear least-squares computer program to obtain the K_s and the chemical shift difference between a pure sample of the complex and the free component ($\Delta\delta$*).

A rotational Overhauser enhancement experiment (ROESY) for detection of intermolecular nuclear Overhauser effects (NOEs) between TBM and β-CD, was acquired for the 1 : 1 molar ratio at 298 K using the same probe. The ROESY spectrum consisted of a 2048 (t_2, complex) by 750 (t_1, real) matrix covering a 4500-Hz sweep width. Gaussian weighting functions were used in both dimensions to improve the signal to noise ratio and zero filling to 4096×4096 was applied before Fourier transformation.

Molecular Modelling The most probable structure of the inclusion compound was predicted using the Cerius$^{2\,TM}$ (Biosym/Molecular Simulations) program and the DREIDING force field.[20] Simulation was carried out for the TBM:β-CD system. After drawing the individual molecules, their geometry was optimized producing thermodynamically possible conformations. Then the guest molecule was inserted into the CD cavity and this system was optimized in vacuum.

Results and Discussion

^{1}H-NMR Studies Several techniques, like differential scanning calorimetry and infrared and ultraviolet spectroscopy, can to either suggest or establish if guest molecules form a complex or not, but they cannot provide a clear answer about the type of complex (inclusion or adsorption) or the structural conformation of the molecules.[21,22] In contrast, NMR studies allow a clear distinction between inclusion and other possible external interaction processes. This technique provides the most evidence for the inclusion of a guest into the hydrophobic CD cavity in solution. If a guest is incorporated into the CD cavity, the hydrogen atoms located in the interior of the cavity (H-3 and H-5) will be considerably shielded by the guest molecule and show a significant upfield shift, whereas the hydrogen atoms on the outer surface (H-1, H-2, H-4, and H-6) will be unaffected or experience only a marginal shift.[1]

In the present study, evidence of TBM : CD complex formation in aqueous solution was based on the modification of the ^{1}H-NMR spectrum of the pure components, following the interaction between TBM and CDs. The ^{1}H-NMR spectra reporting the protons of β-CD alone, TBM alone, and TBM:β-CD mixture (1 : 1) in D_2O solution are presented in Fig. 1. These spectra revealed that under the present conditions only changes in chemical shifts occurred. The smooth variation in chemical shifts and the absence of new peaks that could be assigned to the complex suggested that, on the NMR timescale, the inclusion complexation of TBM with CDs is a fast exchange process. In this dynamic process, TBM is in fast exchange between free and included forms, at a rate that exceeds the reciprocal of the largest observed shift difference (in Hz) for any of the protons of the molecules.[23,24]

The ^{1}H-NMR data of β-CD protons in the presence and absence of equimolar amounts of TBM are listed in Table 1. The induced shift, $\Delta\delta$, is defined as the difference in chemical shifts in the presence and absence of the other reactant. In this convention, a positive sign of $\Delta\delta$ (ppm) shows a downfield displacement and a negative sign shows an upfield one.

Although the multiplicity of β-CD signals remained constant, the protons located inside the β-CD cavity (H-3 and H-5) clearly undergo greater TBM-induced chemical shift changes than those on the exterior of the torus. The upfield shift of the signals due to H-1, H-2, and H-4 and the downfield shift of the signal due to H-6 were minimal and of similar magnitudes, confirming that TBM only interacts with the inside of the cavity (formation of an inclusion complex). The clear upfield shift of the signals of H-3 and H-5 protons have been attributed to magnetic anisotropy effects in the β-CD cavity, due to the inclusion of groups rich in π-electrons.[23] The only large group with π electrons in the TBM molecule is the benzene ring, suggesting that this group is included in the CD macrocycle. The magnetic anisotropy of an aromatic nucleus results in an upfield ^{1}H-chemical shift of protons located above or below the π-electron cloud. The large upfield shift of protons H-3 and H-5, with nearly to central location inside the β-CD cavity, can only be explained by assuming that the aromatic benzene ring of the TBM molecule is located inside the cavity.[13] The fact that H-3 and H-5 protons have a stronger shift than the H-6 protons, which are located on the cavity rim at the narrow end of the CD molecule, indicates that during complex formation the TBM molecule enters the β-CD cavity from the more accessible wider side.

Thus, although of low magnitude, the observed H-3 and H-5 proton shifts are nevertheless indicative of inclusion occurrence, especially as the δ values for the external β-CD protons remained essentially unchanged. Moreover, the effects of β-CD on the ^{1}H-NMR chemical shifts of TBM were also investigated. The chemical shifts for the protons of TBM both in the absence and presence of β-CD are summarized in Table 2 as $\delta_{(\text{free})}$ and $\delta_{(\text{complex})}$, respectively. In the presence of β-CD, the TBM protons were split into two groups, one shifted upfield and the other downfield. A downfield displacement of the drug protons indicates that they are close to an electronegative atom, like oxygen.[25] An upfield shift displacement is probably due to a variation in local polarity when the protons are inside the CD cavity[23,26] and indicates weaker interactions with hydrogen atoms (shielding effect due to van der Waals forces between the drug and carbohydrate chains).[27]

The D and E protons, both belonging to the aromatic ring of TBM, experienced a pronounced shift variation, although with opposite signs. The positive sign of the variation for proton E suggests that it is located near an oxygen atom in the β-CD cavity, while the negative sign for the displacement of proton D indicates a location at some distance from the

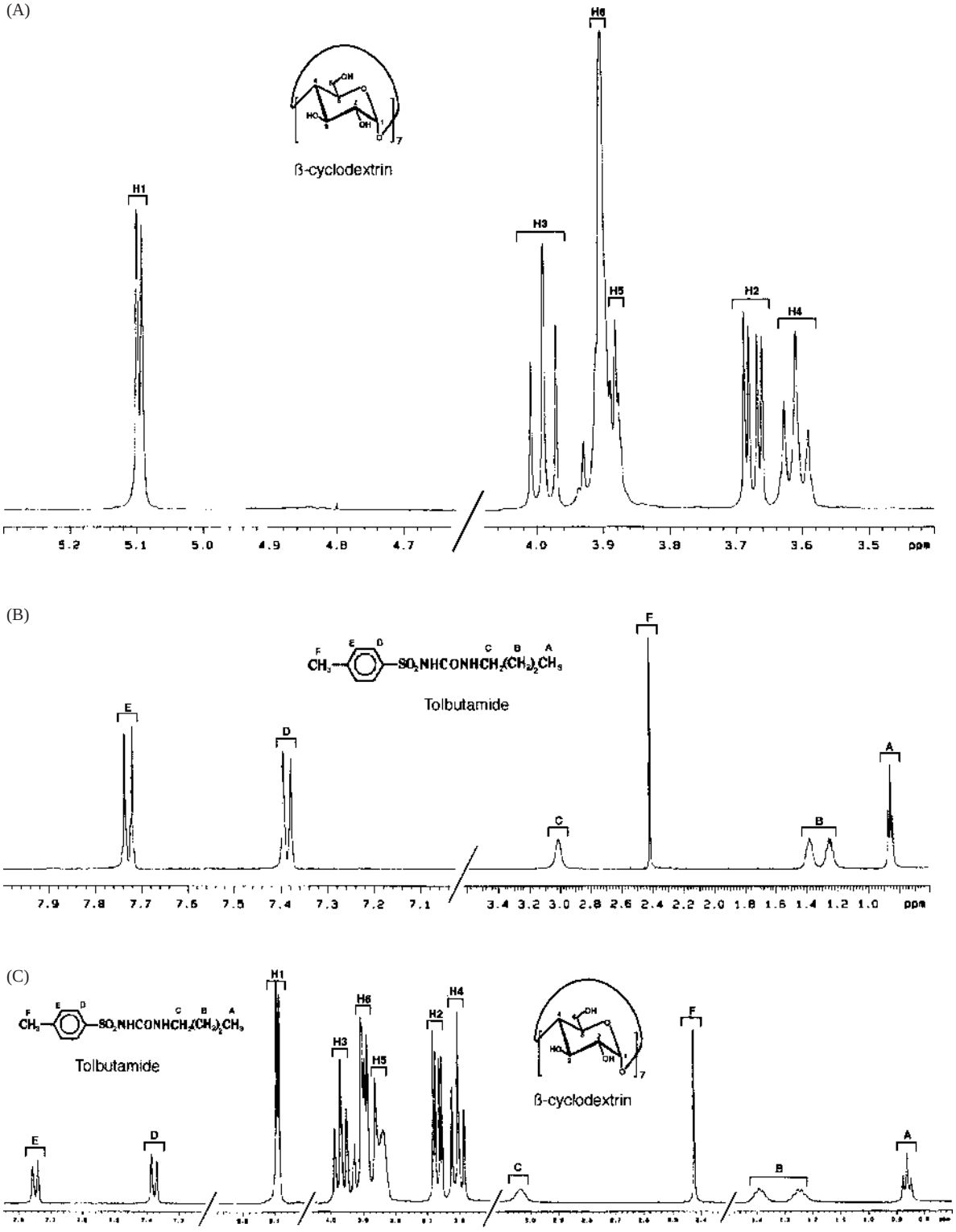

Fig. 1. 500 MHz ^{1}H-NMR Spectra of (A) β-CD, (B) TBM, and (C) TBM : β-CD (1 : 1) Mixture in D_2O at 298 K

oxygen atoms and close to a hydrogen atom. The magnitude of the shift difference for these aromatic protons is dependent on the relative strength of the two types of interaction, with electron-rich or hydrogen atoms of the β-CD cavity.[25] The modification observed for these TBM protons is another demonstration of inclusion occurrence and it also suggests that the phenyl moiety of the drug molecule is included in the cavity of CD.

The downfield displacement of proton C could be attributed to an interaction with an oxygen atom outside the β-CD cavity or to a structure rearrangement of the TBM molecule. TBM structural studies were carried out (PDMODEL, Serena Software) to explain this behavior and demonstrate that proton C is close to the sulfoxide group. Considering this finding and the fact that β-CD has a truncated cone structure, this downfield shift may be due to steric perturbation by the

Table 1. ^{1}H-Chemical Shifts Corresponding to β-CD in the Presence and Absence of TBM

β-CD proton	$\delta_{(free)}$	$\delta_{(complex)}$	$\Delta\delta^{a)}$
H-1	5.099	5.094	−0.005
H-2	3.668	3.661	−0.007
H-3	3.991	3.970	−0.021
H-4	3.609	3.604	−0.005
H-5	3.882	3.839	−0.043
H-6	3.902	3.906	+0.004

a) $\Delta\delta=\delta_{(complex)}-\delta_{(free)}$.

Table 2. ^{1}H-Chemical Shifts Corresponding to TBM in the Presence and Absence of β-CD

TBM proton	$\delta_{(free)}$	$\delta_{(complex)}$	$\Delta\delta^{a)}$
H-A	0.860	0.862	+0.002
H-B	1.259	1.254	−0.005
H-C	3.015	3.036	+0.021
H-D	7.395	7.386	−0.009
H-E	7.736	7.759	+0.023
H-F	2.421	2.424	+0.003

a) $\Delta\delta=\delta_{(complex)}-\delta_{(free)}$.

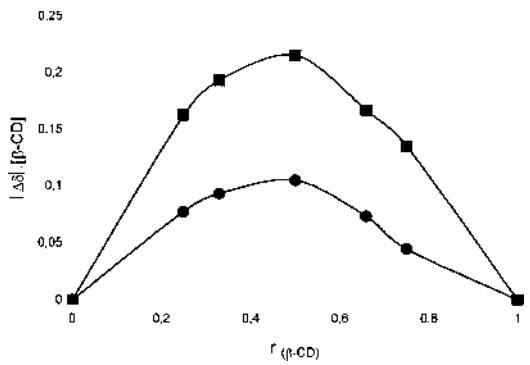

Fig. 2. Job's Plot Corresponding to the Chemical Shift Displacement of H-3 (●) and H-5 (■) Protons of β-CD for the TBM : β-CD Complex

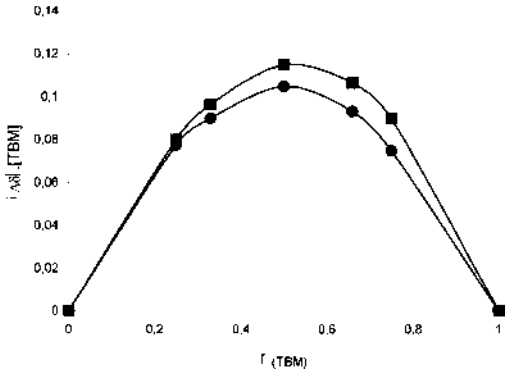

Fig. 3. Job's Plot Corresponding to the Chemical Shift Displacement of H-C (●) and H-E (■) Protons of TBM for the TBM : β-CD Complex

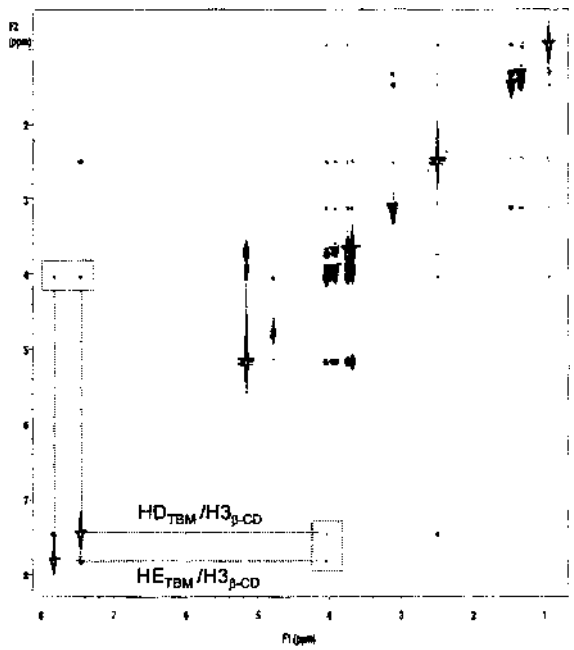

Fig. 4. 500 MHz ROESY Spectrum of a 1 : 1 Mixture of TBM : β-CD Showing the Intermolecular NOEs between the Ring Protons of TBM (D and E) and the H-3 Protons of β-CD

sulfoxide group and/or to a dislocation of charge of the TBM aromatic ring occurring as a result of inclusion.[28—30] The other protons of TBM (A, B and F) presented an insignificant variation in chemical shifts and under the present experimental conditions they could not be interpreted.

The continuous variation plots of $|\Delta\delta|\cdot[\beta\text{-CD}]$ against the mole fraction of β-CD, r_1 ($r=m/[m+n]$), where m and n represent the stoichiometric ratios of β-CD and TBM, respectively) for the most markedly affected protons of β-CD (H-3 and H-5) are presented in Fig. 2. Similiary, the Job's plots of $|\Delta\delta|\cdot$[TBM] *versus* r_2 ($r=n/[m+n]$) for the most affected protons of TBM (H-C and H-E) are presented in Fig. 3. All these plots demonstrated a 1 : 1 stoichiometry for the β-CD:TBM complex, since the maximum was at r_1 or $r_2=0.5$, which is in agreement with the previous phase solubility and ultraviolet spectral studies.[11]

The monitored tritation of the proton H-5 of β-CD was used to estimate the K_s of the TBM : β-CD complex. The K_s value obtained at 298 K in alkaline medium (pD 7.5) was 51.9 M^{-1} (S.D.=5.26%), which was consistent with the value of 50.2 M^{-1} calculated from the phase solubility diagram for the inclusion compound β-CD : TBM (1 : 1) in a pH 7.0 buffer solution.[11]

ROESY experiments were carried out to confirm the results of the ^{1}H-NMR studies. ROESY is a two-dimensional technique based on NOE, in which cross-peaks may be observed between protons if the corresponding internuclear distance is smaller than 3—4 Å.[31] The ROESY spectrum of the 1 : 1 mixture TBM : β-CD (Fig. 4) shows the existence of intermolecular NOEs between the H-3 protons of β-CD and the ring protons (D and E) of TBM. This is in agreement with the ^{1}H chemical shift data, confirming the type of interaction between the two compounds. A lower threshold representation of the ROESY spectrum (not shown) allows the observation of intermolecular NOEs between the ring protons of the drug and the H-2 protons of β-CD. These NOEs confirm that during complex formation the TBM molecule enters the β-CD cavity from the more accessible wider side.

In the ^{1}H-NMR studies of the interaction between TBM and HP-β-CD only the ^{1}H-chemical shift changes of TBM

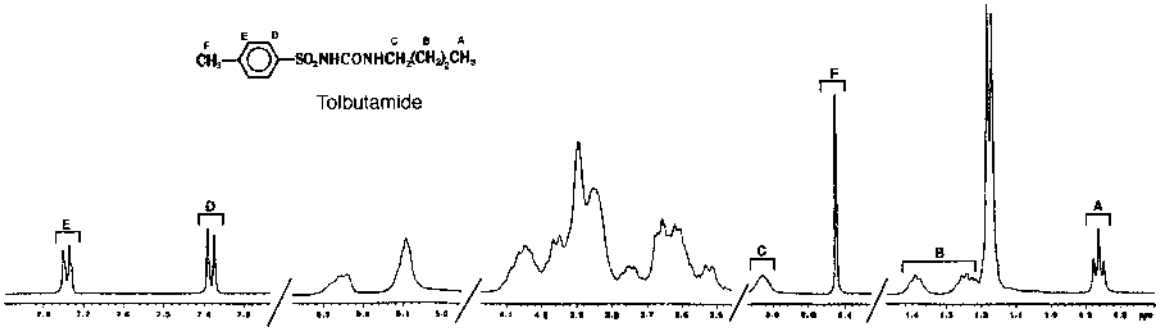

Fig. 5. 500 MHz ^{1}H-NMR Spectrum of TBM : HP-β-CD (1 : 1) Mixture in D_2O at 298 K

Table 3. ^{1}H Chemical Shifts Corresponding to TBM in the Presence and Absence of HP-β-CD

TBM proton	$\delta_{(free)}$	$\delta_{(complex)}$	$\Delta\delta^{a)}$
H-A	0.860	0.862	+0.003
H-B	1.384	1.254	+0.006
H-C	3.015	3.036	+0.015
H-D	7.395	7.386	−0.005
H-E	7.736	7.759	+0.016
H-F	2.421	2.424	+0.005

a) $\Delta\delta = \delta_{(complex)} - \delta_{(free)}$.

protons resonances were analyzed. Unfortunately, the individual HP-β-CD protons, specially H-3 and H-5, could not be assigned to resonance signals because this β-CD derivative consists of a multitude of isomers.[32)]

The ^{1}H-NMR spectra of the TBM : HP-β-CD (1 : 1) mixture in D_2O solution is presented in Fig. 5 and the effect of HP-β-CD on the chemical shifts of TBM protons is shown in Table 3. The chemical shifts of the TBM protons in the presence of HP-β-CD were similar to those obtained upon interaction with β-CD. However, the magnitude of the shifts presented by the TBM : β-CD complex was larger than for TBM : HP-β-CD. This difference was also observed when the stability constants of TBM : β-CD (195.4 M^{-1}) and TBM : HP-β-CD (144.8 M^{-1}) were evaluated in water by previously reported phase solubility studies.[11)] In addition, the low values of these stability constants and the small changes in the chemical shifts observed for both complexes by NMR spectroscopy could be attributed to a weak interaction between the guest molecule and these CDs.

Molecular Modelling Molecular mechanics studies are commonly associated with NMR spectroscopy since they represent a complementary method for rationalizing the experimental information. In the present study, the main goal of the molecular modelling experiments was to obtain a reasonable representation of the structure of the complex and to correlate this finding with results obtained using NMR. Molecular modelling studies were conducted for the TBM : β-CD complexes and it was assumed that the mechanism of inclusion formation is similar for the TBM : HP-β-CD complexes. This is a reasonable assumption since ^{1}H-NMR experiments demonstrated that the same groups of TBM were affected in the presence of both CDs.

The most probable structure of the inclusion complex was determined using the Cerius$^{2\,TM}$ (Biosym/Molecular Simulations) and the DREIDING force field.[20)] The philosophy in DREIDING is to use general force constants and geometry parameters based on simple hybridization considerations rather than individual force constants and geometric parameters that depend on the particular combination of atoms involved in the bond, angle, or torsion terms. With this type of molecular simulation the theoretical findings are strictly related to complexes in vacuum and not in solution. Various researchers have also carried out molecular dynamic simulations of CDs and have shown that these molecules in solution exhibit a greater conformational variability than in their crystal structures. Least-squares comparisons of the crystal and solution structures, however, demonstrated that the differences in their atomic coordinates are only of the order of 1 Å. For most practical purposes, therefore, it seems reasonable to assume that the crystal structures of CDs represent suitable models of their conformations in solution, and that in CD inclusion complexes the variations in conformation will be damped, not only by the solvent, but also by the presence of guest molecules.[33)]

The global minimum energy conformation of the TBM : β-CD complex is illustrated in Fig. 6. The optimum structure was obtained when the aromatic ring of the TBM molecule was included in the CD cavity. Results of molecular modelling indicated that TBM is more likely to enter the larger cavity side of the β-CD molecule, which is in perfect correlation with the ^{1}H-NMR data. This optimized model also revealed the existence of a hydrogen bond between the carbonyl oxygen of TBM and the secondary hydroxyl group on the C-2 atom of a glucose unit of β-CD. In addition, with these molecular mechanics studies it was possible to conclude that the TBM molecule does not act as a rigid system. The TBM molecular geometry is altered upon inclusion in the β-CD cavity: the drug molecule becomes more elongated, due to its various internal degrees of freedom.

Assuming that the complex has the structure described in Fig. 6, it is understandable that the upfield shift of CD proton H-5 is the most prominent, followed by that of proton H-3. This type of complex structure also explains the modification of the chemical shifts of the aromatic protons in the TBM. However, the investigation using molecular modelling, while allowing the construction and three-dimensional manipulation of the molecular complex, is insufficient to indicate the positioning of the whole TBM molecule in relation to β-CD and for describing the real structure of this complex in solution.

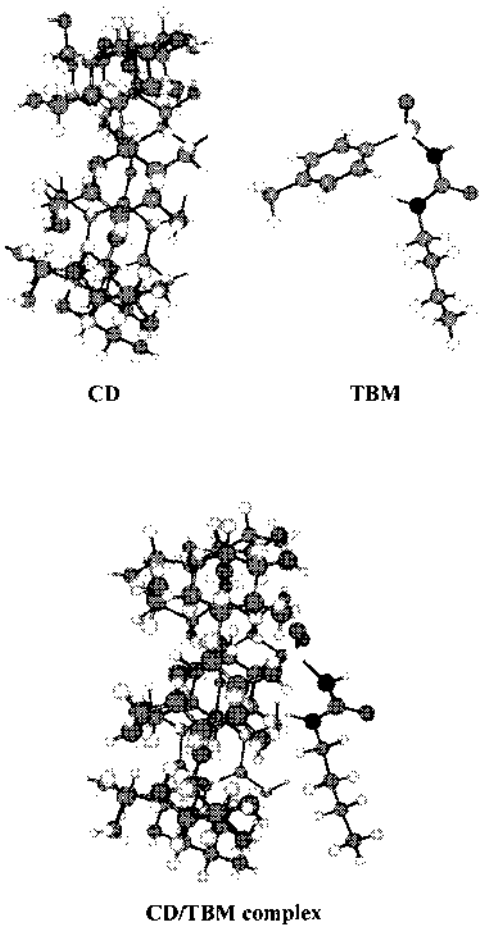

Fig. 6. Proposed Structure of the Inclusion Complex of TBM with β-CD or with HP-β-CD

Conclusions

The insertion of a guest molecule into the CD cavity was clearly demonstrated by changes in NMR proton chemical shift values. The experiments performed with ^{1}H-NMR, ROESY, and molecular modelling suggested that the phenyl moiety of TBM is most probably included inside the hydrophobic β-CD cavity of the solid state as well as in the liquid state. This view is also supported by the change of C-H out-of-plane vibration of the phenyl moiety of TBM upon complexation in the Raman spectrum, as previously described.[11] The association of these two methodologies is becoming an important tool for evidence of drug/CD interaction with a better description of the supramolecular assemblies in solution and also to characterize structurally the inclusion compound formed in a liquid medium.

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