Elsevier Editorial System(tm) for
International Journal of Pharmaceutics
Manuscript Draft

Manuscript Number: IJP-D-20-01635R2

Title: Inclusion complexes of tricyclic drugs and β-cyclodextrin: inherent chirality and dynamic behaviour

Article Type: VSI: T. Loftsson 70th Birthday

Section/Category: Pharmaceutical Nanotechnology

Keywords: NMR spectroscopy, Tricyclic drugs, Host-guest complex, Chiral recognition, Dynamics

Corresponding Author: Dr. Maria Enrica Di Pietro, Ph.D.

Corresponding Author's Institution: Politecnico di Milano

First Author: Maria Enrica Di Pietro, Ph.D.

Order of Authors: Maria Enrica Di Pietro, Ph.D.; Monica Ferro, Ph.D.; Andrea Mele, Prof.

Abstract: Amitriptyline (AMT) and cyclobenzaprine (CBZ) are tricyclic drugs used as antidepressant and muscle relaxant, respectively. They show inherently chirality, i.e. they are chiral due to the lack of any symmetry element. As they are used as racemic mixture, diastereomeric inclusion complexes are formed via encapsulation in homochiral βCD. In this work we show that a suitable combination of NMR methods easily provides details on the chiral recognition, geometry of complexation, rotational dynamics and spatial proximity of selected atom pairs. In particular, we show that 13C NMR can be used to unambiguously assess chiral recognition, demonstrating a higher performance over 1H NMR. The mole fraction of the bound drug and the association constant can be worked out through diffusion experiments, whereas the combination of non-selective, selective and bi-selective relaxation spectra gave insights into the rotational motion of the complexed drug and the spatial proximity of selected proton pairs. The toolkit here proposed provides a thorough characterization of CD/drug inclusion complexes from a physicochemical point of view. This can constructively complement the conventional pharmacological and pharmacokinetic experiments, and can shed light on the understanding of CD/drug formulations.

Dipartimento di Chimica, Materiali e Ingegneria Chimica "Giulio Natta"

POLITECNICO MILANO 1863

Milan, August 12, 2020

Dear Professor Alvarez-Lorenzo,

I am glad to submit the revised version of our manuscript “Inclusion complexes of tricyclic drugs and β-cyclodextrin: inherent chirality and dynamic behaviour” by Maria Enrica Di Pietro, Monica Ferro and Andrea Mele, for consideration for publication in the *Special Issue of the International Journal of Pharmaceutics in Honor of Professor Thorsteinn Loftsson on the Occasion of His 70th Birthday*.

As required, references are formatted according to IJP style and abstract is one single paragraph.

Thank you for your consideration.

Yours sincerely,

Maria Enrica Di Pietro, PhD

Politecnico di Milano
Dipartimento di Chimica, Materiali e Ingegneria Chimica "Giulio Natta"

Piazza Leonardo da Vinci, 32
20133 Milano
Tel. 02 2399 3200
Fax 02 7063 8173

Via Mancinelli, 7
20131 Milano
Tel. 02 2399.3100/3000
Fax 02 2399.3180/3080

www.chem.polimi.it
Partita Iva: 04376620151
Codice fiscale: 80057930150

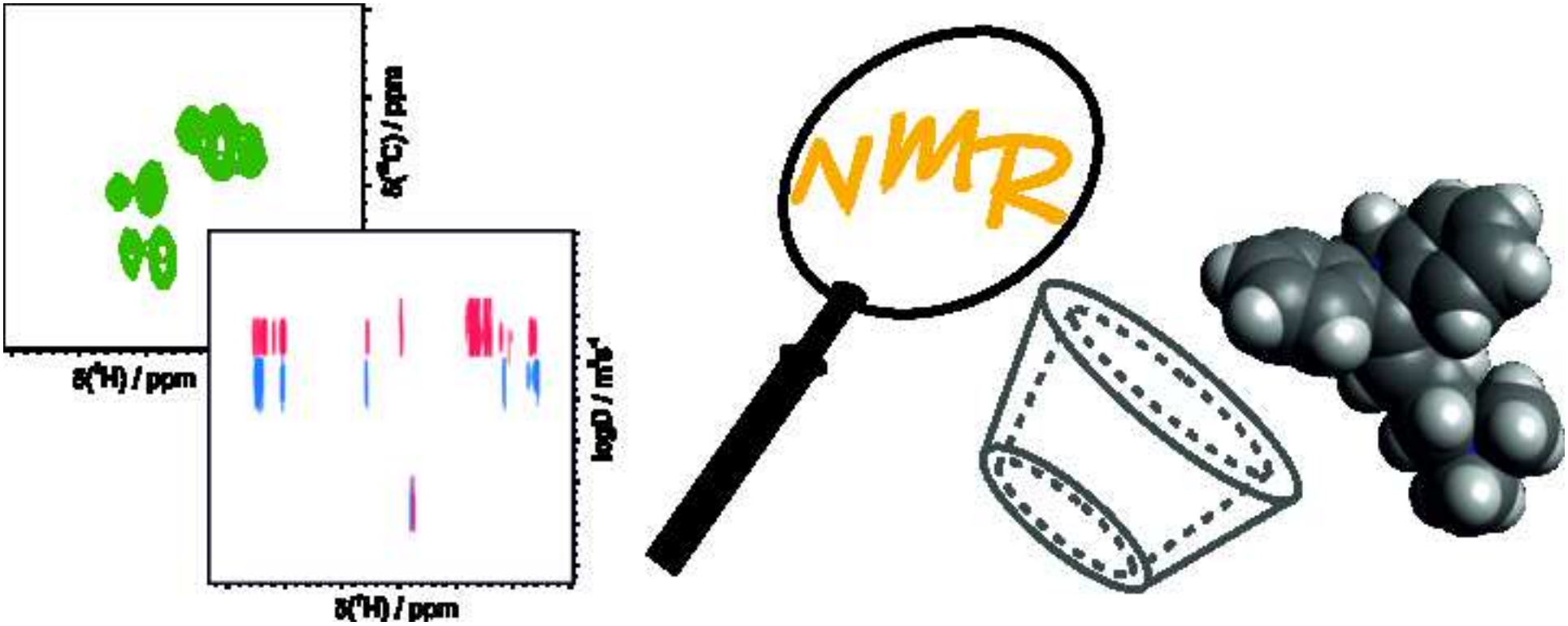
Responses to Reviewers' Comments:

Reviewer #2: It could be accepted

Authors' reply:

We thank Reviewer 2 for her/his appreciation of the work.

*Graphical Abstract (for review)



Inclusion complexes of tricyclic drugs and β-cyclodextrin: inherent chirality and dynamic behaviour

Maria Enrica Di Pietro,*[,a] Monica Ferro,[a] and Andrea Mele*[,a,b]

[a] Department of Chemistry, Materials and Chemical Engineering "G. Natta", Politecnico di Milano, Piazza L. da Vinci 32, 20133 Milano, Italy.

[b] Istituto di Scienze e Tecnologie Chimiche (SCITEC-CNR), Via A. Corti 12, 20133 Milano, Italy.

Abstract

Amitriptyline (AMT) and cyclobenzaprine (CBZ) are tricyclic drugs used as antidepressant and muscle relaxant, respectively. They show inherently chirality, i.e. they are chiral due to the lack of any symmetry element. As they are used as racemic mixture, diastereomeric inclusion complexes are formed via encapsulation in homochiral βCD. In this work we show that a suitable combination of NMR methods easily provides details on the chiral recognition, geometry of complexation, rotational dynamics and spatial proximity of selected atom pairs. In particular, we show that ^{13}C NMR can be used to unambiguously assess chiral recognition, demonstrating a higher performance over ^{1}H NMR. The mole fraction of the bound drug and the association constant can be worked out through diffusion experiments, whereas the combination of non-selective, selective and bi-selective relaxation spectra gave insights into the rotational motion of the complexed drug and the spatial proximity of selected proton pairs. The toolkit here proposed provides a thorough characterization of CD/drug inclusion complexes from a physicochemical point of view. This can constructively complement the conventional pharmacological and pharmacokinetic experiments, and can shed light on the understanding of CD/drug formulations.

Keywords

NMR spectroscopy – Tricyclic drugs – Host-guest complex - Chiral recognition - Dynamics

1. Introduction

Cyclodextrins (CDs) are a family of macrocyclic oligosaccharides that derive from the enzymatic degradation of starch and consist of glucopyranose units in the $^{4}C_{1}$ chair conformation connected with α(1–4) glycosidic

bonds (Crini, 2014; Kurkov and Loftsson, 2013). The most extensively used and studied CDs are α-, β- and γ-CD, which contain six, seven, and eight glucopyranose units, respectively. CDs have a doughnut-shaped structure with all secondary hydroxyl groups O2'-H and O3'-H located on the wider edge, and all primary hydroxyl groups O6'-H on the narrower side, and with a characteristic hydrophobic cavity coated by non-polar C3'-H, C5'-H groups and ether-like O4' atoms (see Fig. 1 for numbering) (Brewster and Loftsson, 1996). Acting as hosts, CDs accommodate various hydrophobic compounds or the hydrophobic moieties present in the molecules (guests) inside the inner cavity to form non-covalently bonded inclusion complexes. The CD's cavity size and the structural conformation and size of the guest are the parameters that mostly affect the formation of the host-guest complex. βCD is the most widely used member of the family due to its cavity size, availability, and low cost, even if its limited aqueous solubility often hinders its successful application as solubilizing agent (Redenti et al., 2000).

Thanks to their vectorization ability and biocompatibility, CDs are of great interest in pharmaceutics, cosmetics, medicine, agriculture, food and textile industry (Bilensoy, 2011; Brewster and Loftsson, 2007; Challa et al., 2005; Crini, 2014; Loftsson and Duchêne, 2007; Rasheed et al., 2008). Indeed, the complexation may significantly improve the physical, chemical, and biological properties of a guest, namely its aqueous solubility, dissolution rate, absorption, bioavailability and stability (Bilensoy, 2011; Brewster and Loftsson, 1996; Diniz et al., 2018; Loftsson and Brewster, 2012; Wouessidjewe et al., 1999). Additionally, the encapsulation in CDs has been advantageously exploited to reduce irritation and toxicity, control the rate of release, and improve palatability and handling. CDs encapsulate also amphiphilic compounds, including surfactants and several drugs (e.g. tricyclic antidepressants or phenothiazine tranquilizers). As a result of the inclusion complex formation, the critical micelle concentration of the guest can be increased. Another strong point of CDs is that they are chiral and can be used to differentiate enantiomeric species through the formation of diastereomeric complexes (Dodziuk et al., 2004; Wenzel and Chisholm, 2011). The enantiorecognition ability made CDs appealing materials for the determination of enantiomeric purity of pharmaceuticals and as supramolecular chiral selectors for chromatographic separations (Wang et al., 2019).

Given the benefits coming from the use of CDs in pharmaceutical chemistry and technology, biochemistry and drug research, intense research activity grew around the study and characterization of CDs/drugs inclusion complexes.

Tricyclic drugs are among the investigated compounds, and here we will focus on two of them, namely amitriptyline (AMT) and cyclobenzaprine (CBZ). AMT and CBZ share a basic butterfly-like structure, with a middle boat-like seven-membered ring C, fused to two adjacent aromatic rings, A and B, as wings, and connected with an N,N-dimethyl-1-propanamino side chain via an exocyclic double bond at C5 (Fig. 1). The

presence of the alkylamine side chain gives AMT and CBZ a surfactant-like behaviour, which may result in the formation of aggregates in aqueous solution.

AMT and CBZ are rather flexible and show inherent chirality (Dalla Cort et al., 2004), due to the bending of the three-ring core and the exocyclic double bond that prevents the free rotation of the side chain with respect to the ring system. As a result, they form diastereomeric inclusion complexes with βCD in aqueous solutions (Castiglione et al., 2017).

AMT belongs to the family of tricyclic antidepressants (TCAs) and is widely used for the treatment of depression (Cano et al., 2007). As all the first-generation TCAs, AMT suffers from several side-effects, such as anti-arrhythmic, anti-cholinergic, cardiovascular and/or hyperthermia side effects (Ali et al., 2012). The disadvantages related to the administration of AMT following a conventional therapeutic protocol (poor water solubility, aggregation and concomitant side effects) can be mitigated if the drug is suitably vectored to the organism with the help of CD as carrier excipient (Ali et al., 2012; Cano et al., 2007; Diniz et al., 2018; Junquera et al., 2001).

CBZ is a muscle relaxant indicated in the relief of muscle spasms. In the presence of βCD, it forms freely soluble and stable inclusion complexes, which allows to reduce the dose as well as the concomitant side effects (Redenti et al., 2000; Szejtli, 1995).

The inclusion complexes of CDs with tricyclic drugs have received attention over the past decades (Ali et al., 2012; Aree, 2020; Cano et al., 2007; Castiglione et al., 2017; Cruz et al., 2008; De Sousa et al., 2008; Georgiou et al., 1999; Junquera et al., 2001; Kundu and Roy, 2017; Nishikawa and Kamimura, 2011; Rajendiran et al., 2014; Redenti et al., 2000; Valsami et al., 1992). However, non-homogeneous results are found in the literature. First, very different binding constant have been estimated. For instance, binding constants of 13.2×10^3 M^{-1}, 368 M^{-1}, or 569 M^{-1} have been reported for 1:1 complexes of doxepin in βCD (Rajendiran et al., 2014; Valsami et al., 1992). For amitriptyline in βCD, association constant values range from 23.9×10^3 M^{-1} to 3.2×10^3 M^{-1} (Junquera et al., 2001; Valsami et al., 1992). Moreover, different geometries for inclusion complexes of tricyclic drugs have been proposed. For example, Junquera *et al.* (2001) found that amitriptyline, imipramine and desipramine insert their side chain in the βCD cavity, while the three cycles reside out of the apolar cavity. Contrarily, the formation of stable inclusion complexes originating from insertion of the aromatic ring of doxepin and dothiepin in βCD's cavity was suggested by Rajendiran *et al.* (2014). Kundu and Roy (2017) reported that nortriptyline and βCD form a 1:1 inclusion complex with two plausible inclusion modes, the side chain and the tricyclic rings. Also, an equilibrium among 1:2, 1:1, and 2:1 βCD/imipramine complexes, with possible inclusion of either the rings or the aliphatic chain, was proposed (De Sousa et al., 2008). Castiglione *et al.* (2017) showed that CBZ and AMT form 1:1 inclusion complexes with βCD, with the A ring embedded in the βCD cavity and the side chain

protruded from the O2'/O3'-side. A similar inclusion scenario in the βCD cavity was proposed by Aree (2020) for nortriptyline and amitriptyline. According to their findings, the two tricyclic drugs insert the A ring into the βCD cavity from the O2'/O3' side vertically and quite deeply to the O6'-side. The A ring moieties are then kept in place by host–guest C5-H···π interactions with the βCD internal protons H5'. Additionally, the inclusion induces conformational changes of βCD, with a narrowing of the O6' end to optimally accommodate the A-ring in the cavity and a simultaneous widening of the O2'/O3' end to make van der Waals contacts with the B-ring and the side chain (Aree, 2020).

It is then clear that the detailed description of tricyclic drugs / βCD inclusion structures still deserves investigation. A deep understanding of the encapsulation process at the molecular level is crucial for a rational design of CD/drug formulations. Next to a structural characterization, dynamic parameters such as association constants and/or rotational mobility are essential to obtain a clear picture of the inclusion complex and the association process (Cano et al., 2007).

Complexes between CDs and guest molecules can be investigated by a variety of methods, including UV-VIS spectroscopy, fluorescence measurements, X-ray crystallography, calorimetry, NMR, electrochemistry, separation techniques, and mass spectrometry. Out of these techniques, only X-ray crystallography and NMR spectroscopy yield information on the inclusion complex on an atomic level. However, X-ray crystallography gives a static view of the complex and depends on the availability of high-quality single crystals. Contrarily, NMR measurements can be run in solution and allow to access dynamic properties together with molecular structural information.

Here we apply NMR spectroscopy to study and characterize 1:1 inclusion complexes of the tricyclic drugs amitriptyline and cyclobenzaprine with βCD. In most NMR studies, complexation-induced chemical shift and nuclear Overhauser enhancement experiments are used to characterize the host-guest inclusion complexes. We aim at demonstrating that very precise qualitative and quantitative information at the molecular level can be accessed by combining several NMR techniques. AMT/βCD and CBZ/βCD have been already studied by Castiglione *et al.* (2017): a 1:1 host–guest stoichiometry was determined using the Job's plot and the analysis of intermolecular NOEs in the rotating frame (ROESY) was applied to describe the inclusion geometry. In the present work, we first show that the enantiorecognition ability of βCD towards the two inherently chiral molecules can be probed by ^{13}C NMR spectroscopy, with significant advantage with respect to ^{1}H NMR. Then, diffusion Ordered SpectroscopY (DOSY) is applied to get the bound fraction and the association constant. Finally, quantitative dynamic information on the inclusion complex in terms of rotational correlation time and host-guest interactions are collected by measurements of non-selective, selective and biselective spin-lattice relaxation times (T_1^{NS}, T_1^{SE}, and T_1^{BS}). We expect that the outcomes will show that the characterization of CD/drug inclusion complexes from a physicochemical point of view

can constructively complement the conventional pharmacological and pharmacokinetic experiments, and can shed light on the understanding of CD/drug formulations.

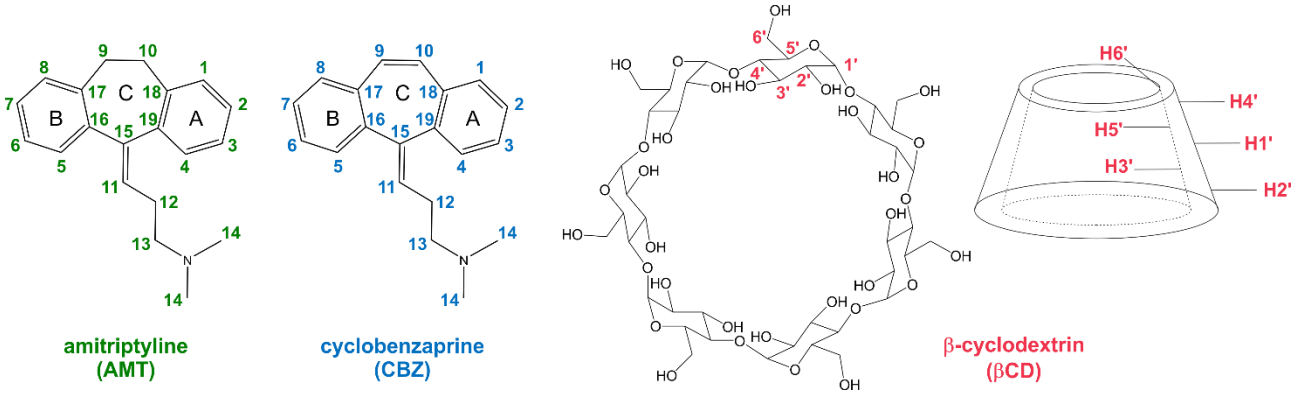


Figure 1. Structure and numbering of guest and host molecules investigated in this work.

2. Experimental

2.1 Materials and sample preparation

β-cyclodextrin (βCD) and D_2O were purchased from Sigma-Aldrich, while amitriptyline hydrochloride (AMT) and cyclobenzaprine hydrochloride (CBZ) from Dipharma. The commercially available HCl salt form was used for both drugs. All chemicals were used without further purification.

Samples AMT/D_2O and CBZ/D_2O were prepared dissolving the drug in D_2O for a concentration equal to 4.0mM. Samples containing βCD (AMT/βCD/D_2O and CBZ/βCD/D_2O) were prepared with a drug's concentration equal to 4.7 and 4.0 mM, respectively, and a final host/guest molar ratio of 1:1. Note that due to the presence of the alkylamine side chain, the drugs possess amphiphilic behaviour, which can result in the formation of aggregates in aqueous solution at or above certain critical concentrations. Critical aggregation concentration (cac) of the free AMT was found to be 33 mM or 43 mM (Ali et al., 2012; Junquera et al., 2001), and is increased by addition of βCD, then we used concentrations well below the cac.

Sample βCD/D_2O was prepared dissolving βCD in D_2O at 9 mg/ml.

2.2 NMR experiments

The samples for NMR analysis were placed in standard 5 mm tubes. All NMR spectra were recorded at 298 K at 11.7 T using a Bruker NEO 500 spectrometer equipped with a 5 mm pulsed-field z-gradient BBFO probe and a variable-temperature unit.

1D ^{13}C-{^{1}H} spectra were recorded using 65536 points and 12288 scans. Raw data were apodized by multiplication with an exponential decay equivalent to 2.0 Hz line broadening.

HSQC spectra were recorded for samples AMT/βCD/D_2O and CBZ/βCD/D_2O, using data matrices of 1024 (t_2) x 1024 (t_1) complex data points. 8 or 16 transients were accumulated per increment, with 50% non-uniform sampling (NUS). Raw data were processed by applying a cosine squared sine window functions in both dimensions.

Self-diffusion coefficients were measured by Diffusion Ordered SpectroscopY (DOSY) experiments by applying sine shaped pulsed magnetic field gradients along the z-direction up to a maximum strength of G = 53.5 G cm^{-1}. All of the experiments were performed using the bipolar pulse longitudinal eddy current delay (BPPLED) pulse sequence, over a spectral width of 9 ppm for samples AMT/D_2O and AMT/βCD/D_2O, 10 ppm for samples CBZ/D_2O and CBZ/βCD/D_2O, and 7 ppm for sample βCD/D_2O. The gradient strength (g) was varied incremented in 32 steps from 2% to 95% of the maximum gradient strength in a linear ramp. The parameters were chosen to obtain 95% signal attenuation for the slowest diffusion species at the last step experiment. A total of 24 or 32 transients per increments was used for samples AMT/D_2O, CBZ/D_2O, AMT/βCD/D_2O and CBZ/βCD/D_2O, and 8 transients per increments for sample βCD/D_2O. Values in the range 2.2-3.2 ms for the gradient duration (δ), and of 0.1 s for the diffusion delay (Δ) were applied. The baselines of all arrayed spectra were corrected prior to processing the data. Data were processed using an exponential filter in F_2 dimension (line broadening of 0.5 Hz) and integrals were used in calculating relaxation times. Diffusion coefficients were computed from experimental raw data by using the Bruker T_1/T_2 relaxation module. Taking into account the experimental S/N ratio, the accuracy of the measurements is estimated at about 5%.

The spin-lattice relaxation rates were measured using data matrices of 16384 (t_2) x 16 (t_1) complex data points with the inversion recovery (IR) (180° - τ - 90° - acqu)$_n$ pulse sequence, having the initial 180° pulse either non-selective, selective or biselective. In all experiments, the relaxation delays were set equal or higher than 5 times the longest T_1. All experiments were carried out over a spectral width of 9 ppm for AMT and 10 ppm for CBZ, for various delay time τ. A total of 16 to 32 transients per increments were collected for each experiment, depending on the S/N ratio. The selective inversion pulse was achieved by replacement of the hard 180° pulse of the inversion-recovery sequence by a Gauss1.1000 shaped π-pulse with a length ranging from 24 ms (corresponding to an excitation width of about 30 Hz) to 9 ms (corresponding to an excitation width of about 80 Hz) and an attenuation power from 32 dB to 40 dB. The biselective pulse was built prior to each experiment using the Bruker WaveMaker tool. The baselines of all arrayed spectra were corrected prior to processing the data. Data were processed using an exponential filter in F_2 dimension and integrals were used in calculating relaxation times. Relaxation times were computed from experimental raw data by using the Bruker T_1/T_2 relaxation module with the standard one-

component fitting function. Taking into account the experimental S/N ratio and the standard deviation in the analysis of T_1 values with both treatments, maximum errors are estimated to be 5%.

3. Results and Discussion

As anticipated in section 1 (Introduction), the stoichiometry and the geometry of the AMT/βCD and CBZ/βCD inclusion complexes have been already investigated using ^{1}H NMR complexation-induced chemical shifts and ROESY spectra, in a joint approach with X-ray diffraction and molecular dynamics simulations.(Castiglione et al., 2017) Here we extend the NMR characterization of the same complexes showing that valuable qualitative and quantitative information can be obtained integrating several NMR techniques.

3.1 Chiral recognition: the advantages of ^{13}C NMR spectroscopy

^{1}H spectra are usually the first NMR tool for the evaluation of the drug inclusion within the βCD cavity, taking into account the variation induced by the complexation on the chemical shifts of both the CD and drug protons. In the case of chiral complexed guests, some additional important structural features can be outlined by the analysis of their ^{1}H NMR spectra. Indeed, CDs and their derivatives are effective chiral NMR solvating agents and can hence discriminate between two enantiomers. This enantioselective recognition ability has been demonstrated towards many molecules of pharmaceutical interest (Dodziuk et al., 2004; Schneider et al., 1998; Wenzel and Chisholm, 2011). Similarly, upon inclusion of the inherently chiral drug CBZ in βCD, the formation of two diastereomeric complexes can be testified by the peculiar modification of its ^{1}H spectrum: the AB quartet corresponding to protons H9 and H10 splits into two AB quartets on passing from the pure drug to the corresponding CBZ/βCD complex (Fig. S1) (Castiglione et al., 2017). Unfortunately, a chemical shift difference for other enantiomeric ^{1}H signals of CBZ and AMT cannot be appreciated in their ^{1}H spectra (Fig. S1).

^{13}C NMR is a helpful alternative method to investigate the inclusion process. Proton decoupled ^{13}C spectra have been used to evaluate the encapsulation through complexation-induced variation of the chemical shifts (Kundu and Roy, 2017), and they are particularly informative to verify chiral recognition (Chankvetadze et al., 1995; Di Pietro et al., 2020). For instance, ^{13}C NMR shifts have been exploited for chiral discrimination of some chiral antidepressant azepine-type drugs, showing larger differences than proton shifts (Chankvetadze et al., 1995). Recently, we have also used ^{13}C-{^{1}H} spectra to demonstrate the retained enantiorecognition ability of βCD in a complex deep eutectic mixture composed of choline

chloride, urea and water (Di Pietro et al., 2020). Proton decoupled ^{13}C spectra acquired on all samples are displayed in Figs. S2-S3. It can be seen that a number of ^{13}C NMR signals of AMT and CBZ are duplicated due to the non-equivalence of complexation-induced chemical shifts of the diastereomeric complexes. Among the aliphatic carbons, the signals corresponding to C12 of CBZ, C9 or C10 of AMT and C13 of both AMT and CBZ, clearly split (Fig. S2). Splittings can be observed also for the majority of signals corresponding to aromatic and quaternary carbons (see for instance C9, C10, C17 and C18 of CBZ in Fig. S3). The main drawback of ^{13}C NMR experiments is that they require long experimental time, much longer than ^{1}H NMR experiments. On the other hand, it is evident that they offer a broader spectral width with respect to ^{1}H NMR, which translates into a better resolution and an easier assignment of signals (Dodziuk et al., 2004; Silva, 2017). Interestingly, ^{1}H-^{13}C HSQC correlation spectra can also be used to verify that ^{13}C NMR signals are duplicated. Fig. 2 and Figs. S4-S5 show correlation peaks corresponding to selected carbons of AMT and CBZ in samples AMT/βCD/D_2O and CBZ/βCD/D_2O. A trivial case is represented by the peaks corresponding to the C9-H9 and C10-H10 correlations in sample CBZ/βCD/D_2O (Fig. 2b, right): a clear differentiation is visible in both ^{1}H and ^{13}C projections, which is confirmed by the cross peaks. The situation is different if one considers the same nuclear pairs 9 and 10 in sample AMT/βCD/D_2O. In this case the signals corresponding to C9 and C10 are duplicated, whereas the difference in the chemical shift of protons H9 and H10 in the two diastereomeric complexes is so tiny that cannot be appreciated in the ^{1}H spectrum. The HSQC experiment (Fig. 2a, left) confirms the assignment of the carbon signals and uncovers the slight difference in the proton dimension. The same holds for the C8-H8 correlation in the AMT/βCD complex (Fig. 2a, right) and for the C13-H13 correlation in the CBZ/βCD complex (Fig. 2b, left). It is then clear that, even though the enantiomeric ^{1}H signals mostly overlap, the wider spectral width on the carbon dimension makes it possible to detect the non-equivalent carbon signals of the two diastereomeric complexes. This is a clear demonstration of the higher performance of ^{13}C over ^{1}H NMR to unambiguously assess chiral recognition.

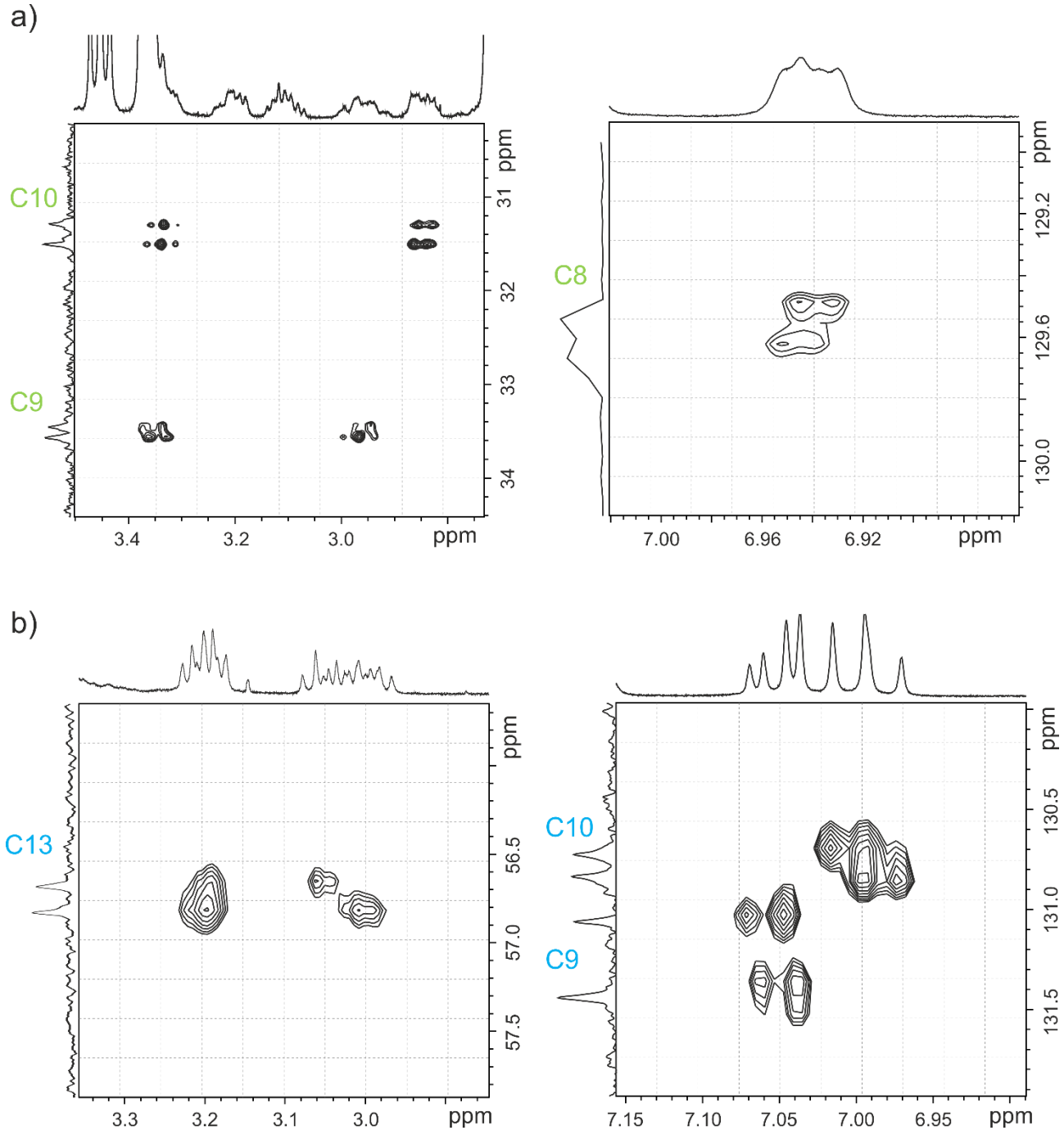


Figure 2. Enlargements of selected signals of 2D ^{1}H-^{13}C HSQC correlation spectra corresponding to (a) C8, C9 and C10 of AMT in sample AMT/βCD/D_2O, and (b) C9, C10 and C13 of CBZ in sample CBZ/βCD/D_2O.

3.2 Association constants: single-point diffusion measurements

^{1}H NMR techniques were used to gather information on the dynamics of the inclusion complexes. It is worth remembering that in ^{1}H spectra signals of the two diastereomeric inclusion complexes are not distinct or, in case of non-equivalence, they overlap. Therefore, in the following relaxation and diffusion analysis, each signal is considered as an average of both diastereomeric complexes.

An advantageous NMR technique used for the determination of association constants in CD inclusion complexes is diffusion NMR (Barhoum et al., 2016; Fielding, 2007; Pagès et al., 2017; Uccello-Barretta et al., 2005). The rationale behind is that the translational diffusion coefficient (D) of a molecule is proportional to its effective size and shape. Broadly speaking, this means that a small molecule diffuses faster than a large one. Analogously, the encapsulation of a small drug into the cavity of a large cyclodextrin leads to a decrease of the guest diffusion coefficient. An easy way to illustrate this concept is via a DOSY (Diffusion Ordered SpectroscopY) experiment, where the chemical shift is plotted in one dimension and the diffusion coefficient in the other one (Morris and Johnson, 1992). Fig. 3 displays DOSY maps acquired for AMT and CBZ as free drugs or complexed with βCD. As a result of the inclusion in the βCD's cavity, the diffusion of both drugs in the samples with βCD (in red) is slower that in the corresponding samples without βCD (in green for AMT and blue for CBZ). More in detail, AMT and CBZ tend to have similar mobilities and diffusivities close to βCD, as also observed for other tricyclic antidepressants (De Sousa et al., 2008).

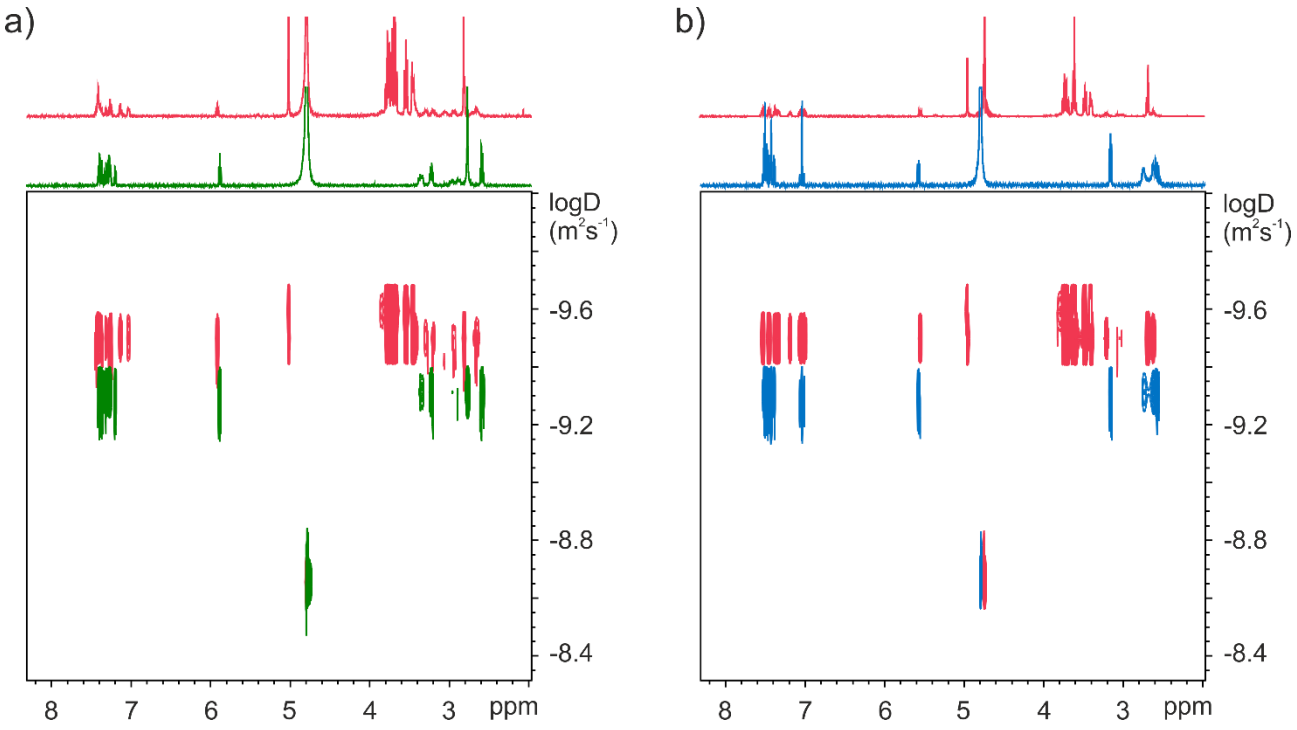


Figure 3. DOSY maps acquired for (a) samples AMT/D_2O (in green) and AMT/βCD/D_2O (in red) and (b) CBZ/D_2O (in blue) and CBZ/βCD/D_2O (in red).

Following a commonly used methodology (see Theoretical toolbox in the SI for more details) (Brand et al., 2005; Cohen et al., 2005; Di Pietro et al., 2019; Lis-Cieplak et al., 2014; Uccello-Barretta et al., 2005), DOSY measurements can be exploited to determine the mole fraction of the bound guest x_G^{bound}. Basically, when

the host and guest are tightly bound in a complex, they diffuse as a single entity and display the same diffusion coefficient. In this case x_G^{bound} is equal to 1. When the association is weak or negligible, x_G^{bound} is equal to 0 and the diffusion coefficients of the host and the guest remain unchanged. For any other case, in the fast exchange limit, the observed diffusion coefficient D_G^{obs} is a weighted average of the diffusion coefficient of the free and bound states (D_G^{free} and D_G^{bound}, respectively) (Brand et al., 2005; Cohen et al., 2005; Uccello-Barretta et al., 2005):

$$D_G^{obs} = (1 - x_G^{bound})D_G^{free} + x_G^{bound}D_G^{bound} \quad (1)$$

As guest molecules are typically much smaller than cyclodextrins, one can assume that D_G^{bound} will be very similar to the diffusion coefficient of the host (D_H). Hence, x_G^{bound} can be determined as follows (Brand et al., 2005; Cohen et al., 2005; Uccello-Barretta et al., 2005):

$$x_G^{bound} = \frac{D_G^{obs} - D_G^{free}}{D_H - D_G^{free}} \quad (2)$$

From x_G^{bound}, it is then possible to determine the association constant K_a (Brand et al., 2005; Cohen et al., 2005; Uccello-Barretta et al., 2005):

$$K_a = \frac{x_G^{bound}}{(1 - x_G^{bound})([H]_0 - x_G^{bound}[G]_0)} \quad (3)$$

where $[H]_0$ and $[G]_0$ are the total concentrations of host and guest, respectively.

Practically speaking, D_G^{obs} and $D_G^{free}$of AMT and CBZ are measured in the equimolar host-guest mixtures (samples AMT/βCD/D_2O and CBZ/βCD/D_2O) and in the absence of the host (samples AMT/D_2O and CBZ/D_2O), respectively. D_H is measured for the cyclodextrin alone (sample βCD/D_2O). In this way, it is possible to determine x_G^{bound} and K_a from single DOSY experiments, without the need of titrations. We have recently reported the diffusion coefficients of AMT and CBZ together with the calculated x_G^{bound} (Di Pietro et al., 2020). Here the K_a are also calculated and listed in Table 1. Taking into account the inaccuracy in the calculations coming from the different approximations and the experimental errors both in sample preparation and in the acquisition and treatment of the experimental data, an error equal to 5% is estimated. A very similar inclusion behaviour can be observed for the two drugs, with most of the drug in the complexed state (mole fraction of the bound drug equal to 0.88 for AMT and 0.84 for CBZ). The values of the association constants obtained for both complexes are relatively high (in the order of 10^3 M^{-1}) and fall within an optimum range from a pharmaceutical point of view. Indeed, binding constants ranging from 200 to 10000 M^{-1} are considered suitable for the encapsulation of drugs by cyclodextrins (Cano et al., 2007). βCD is hence an appropriate vector for the drugs amitriptyline and cyclobenzaprine.

297

298 **Table 1**. Diffusion coefficients, molar fraction of the bound guest x_G^{bound} and association constant K_a
299 determined for AMT and CBZ. Maximum errors are estimated to be 5%

Guest	$\mathbf{D_G^{obs}}$ **[10^{-10}] (m^2s^{-1})**	$\mathbf{D_G^{free}}$ **[10^{-10}] (m^2s^{-1})**	$\mathbf{D_H}$ **[10^{-10}] (m^2s^{-1})**	$\mathbf{x_G^{bound}}$	$\boldsymbol{K_a}$ **[10^3] (M^{-1})**
AMT	2.5 ± 0.1 [a]	4.2 ± 0.2 [a]	2.2 ± 0.1 [a]	0.88 ± 0.04 [a]	8.2 ± 0.4
CBZ	2.7 ± 0.1 [a]	4.3 ± 0.2 [a]	2.3 ± 0.1 [a]	0.84 ± 0.07 [a]	8.5 ± 0.4

300 [a] Data from Di Pietro et al. (2020)

301 It has been argued that the previous methodology may not be fully reliable for medium-sized host
302 molecules, including cyclodextrins (see SI) (Brand et al., 2005; Cameron and Fielding, 2001). Briefly, the
303 method relies on the assumption that, when a small guest molecule binds to a large host molecule, the
304 diffusion coefficient of the host is only insignificantly affected by the complexation. Therefore, the diffusion
305 coefficient of the bound guest D_G^{bound} and the observed diffusion coefficient of the CD host, D_H, are
306 assumed to be equal. This approximation is valid for most studies involving small molecules binding to
307 macromolecules, but may not be true for cyclodextrins and other medium-sized host molecules. To face
308 this criticism we acquired a series of diffusion experiments with solutions having different host:guest mole
309 ratios (Cameron and Fielding, 2001). As discussed in the SI, we found that the apparent diffusion coefficient
310 measured for βCD in all samples was substantially constant within the experimental error over the full
311 range of concentrations. Hence, in the context of the present study the assumption that $D_G^{bound} = D_H$ can
312 be considered valid and the single point approximation appropriate for the evaluation of K_a.

313

314 **3.3 Correlation times and fractional binding: non-selective, selective and biselective spin-lattice**
315 **measurements**

316 The application of ^{1}H relaxation experiments to study aggregation, interactions and/or binding processes
317 with macromolecules, including cyclodextrins, is well known (De Sousa et al., 2008; Fielding, 2007; Li et al.,
318 2007; Reddy et al., 2015; Rossi et al., 2001; Schneider et al., 1998; Tinoco and Figueroa-Villar, 1999; Tošner
319 et al., 2006). Recently, the use of non-selective (R_1^{NS}), selective (R_1^{SE}) and bi-selective (R_1^{BS}) spin-lattice
320 relaxation rates was revised as a tool to derive informative dynamical parameters characterizing the
321 inclusion complexes formed by two drugs, paracetamol and aspirin, with βCD (Kumar et al., 2017). We also
322 demonstrated that non-selective and selective relaxation T_1 experiments can successfully be applied to get
323 information on the rotational motion of encapsulated drugs in complex media such as deep eutectic

systems (Di Pietro et al., 2020).

Following the methodology by Kumar *et al.* (2017), in the present work we measured and analysed R_1^{NS}, R_1^{SE} and R_1^{BS} for the complexes of AMT and CBZ with βCD to access valuable dynamical parameters, namely the molecular rotational correlation times (τ_C) and the cross-relaxation rates (σ_{ij}) of drugs encapsulated in βCD cavities. A detailed theoretical treatment is given in the SI. Here, we directly focus on the dynamical results with the aim of showing how the method allows to get meaningful insights regarding the relaxation mechanism of the drug/βCD complexes and the contribution of other relaxation processes to the dipole−dipole interactions.

Briefly, the molecular rotational correlation time τ_C of the encapsulated drug, which is the average time needed for the molecule to rotate by approximately 1 radian during the isotropic tumbling in the liquid state, can be derived by measuring the R_1^{NS}/R_1^{SE} ratio within the initial rate approximation (Fielding, 2007; Freeman et al., 1974; Hall and Hill, 1976; Tinoco and Figueroa-Villar, 1999):

$$\frac{R_1^{NS}}{R_1^{SE}} = \left[\frac{3\tau_C}{1+\omega_0^2\tau_C^2} + \frac{12\tau_C}{1+4\omega_0^2\tau_C^2}\right] \Big/ \left[\frac{3\tau_C}{1+\omega_0^2\tau_C^2} + \frac{6\tau_C}{1+4\omega_0^2\tau_C^2} + \tau_C\right] \quad (4)$$

Both the R_1^{NS}/R_1^{SE} ratio and τ_C are valuable indicators of the motional regime of the drug. Results obtained for AMT/βCD and CBZ/βCD complexes are summarized in Fig. 4 and Table S1. Note that novel results are integrated with data previously reported (Di Pietro et al., 2020). From the experimental standpoint, to properly measure selective T_1 relaxation times the ^{1}H spectrum has to be sufficiently dispersed so that the inversion pulse can be applied selectively to the chosen resonances (Freeman et al., 1974). Here we selected protons H8, H11, H12 and H14 for AMT and protons H9,10, H11 and H13 for CBZ. As discussed previously (Di Pietro et al., 2020), for protons H8 and H12 of AMT and for protons H9,10 of CBZ the R_1^{NS}/R_1^{SE} ratio in D_2O is in the range 1.5-1.05, indicating a fast to intermediate motion regime for 500 MHz ^{1}H frequency (Kumar et al., 2017; Tinoco and Figueroa-Villar, 1999). In the presence of βCD values close to 1 can be observed, which is symptomatic of intermediate regime. The same holds for the novel data obtained for proton H11 of AMT and CBZ. The slowdown in the dynamics of the drug suggested by the R_1^{NS}/R_1^{SE} ratio can be also quantified in terms of rotational correlation time τ_C, which is inversely proportional to the degree of molecular mobility. Indeed, we found for the free drugs values of τ_C in the range 0.20-27 ns. Upon addition of βCD, the encapsulation in the βCD cavity slows down the rotational mobility of the drugs, so that τ_C values increase to 0.30-0.36 ns. As already reported (Di Pietro et al., 2020), proton H14 of AMT shows a peculiar behaviour, as the R_1^{NS}/R_1^{SE} ratio close to unity both for the free and the encapsulated drug. This was ascribed to the fact that the local rotational motion of the terminal methyl groups is not markedly affected by formation of the inclusion complex (Di Pietro et al., 2020). A somehow intermediate situation is experienced by proton H13 of CBZ, as testified by the small increase of τ_C (from 0.27 to 0.30 ns).

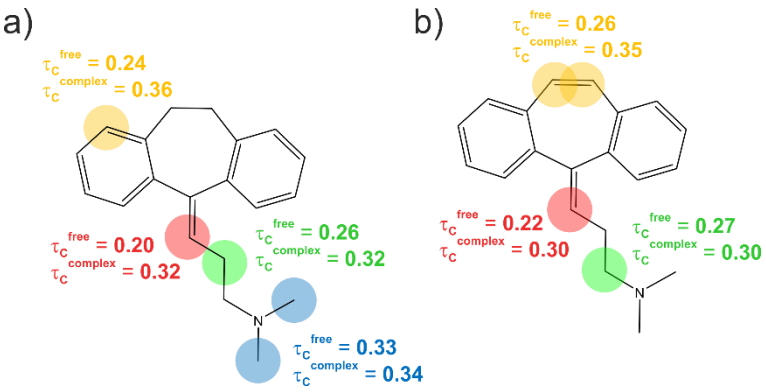


Figure 4. Selected τ_C values (in ns) obtained for (a) amitriptyline (AMT) and its complex with βCD, and (b) cyclobenzaprine (CBZ) and its complex βCD. Maximum errors are estimated to be 5% of the value.

To go further, we run bi-selective inversion recovery experiments in order to extract another valuable dynamical parameter, that is the fractional contribution to relaxation of a proton i via dipolar interaction with a proton j that is simultaneously inverted (see SI) (Hall and Hill, 1976; Kumar et al., 2017):

$$f_{ij}^{i} = \frac{2 \cdot [\mathrm{R}_1^{\mathrm{BS}}(ij) - \mathrm{R}_1^{\mathrm{SE}}(i)]}{\mathrm{R}_1^{\mathrm{SE}}(i)} \quad (5)$$

Using Eq. (5) we quantified the fractional contribution to the cross-relaxation rate of protons H6, H8, H11, H12 and H14 of AMT and protons H9,10 and H11 of CBZ, due to intermolecular dipolar interactions with protons H1' and H5' of βCD in the inclusion complexes. Unfortunately, we could not select the same protons for the two drugs, since H9,10 of AMT and H6, H8, H12 and H14 of CBZ are not isolated enough to be selectively inverted and guarantee an accurate T_1 measurement. Results are reported in Fig. 5 and Table S2. f_{ij}^{i} enclose information on spatial proximity, i.e. the higher the f_{ij}^{i} value, the closer the proton pair ij. In the sample AMT/βCD/D_2O the highest f_{ij}^{i} value with respect to H1' of βCD was calculated for H11, followed by H12 and H14. H6 and H8 showed quite small values, indicating they are located further from H1' of βCD. Contrarily, H6 and H8 show the highest f_{ij}^{i} values with respect to proton H5' of βCD, suggesting a close proximity between the proton pair and hence confirming the insertion of the aromatic ring in the βCD cavity. A similar picture can be derived for sample CBZ/βCD/D_2O, even if only data for protons H9,10 and H11 were accessible. The findings of the bi-selective T_1 measurements alone are not exhaustive to fully describe the geometry of inclusion and undoubtedly assess whether the drug molecule enters the cavity of cyclodextrin from the large opening or the small one. Nevertheless, they are in contrast with the description suggested by Junquera *et al.* (2001) for amitriptyline, with the insertion of the side chain in the βCD cavity and the three cycles out of the apolar cavity. Our experimental findings are instead consistent with the inclusion geometry previously described by Castiglione *et al.* (2017) and Aree (2020), with the A

ring inserted into the βCD cavity from the wider opening, and the seven-membered ring, the alkylammonium chain and the other aromatic ring protruding out of the cavity.

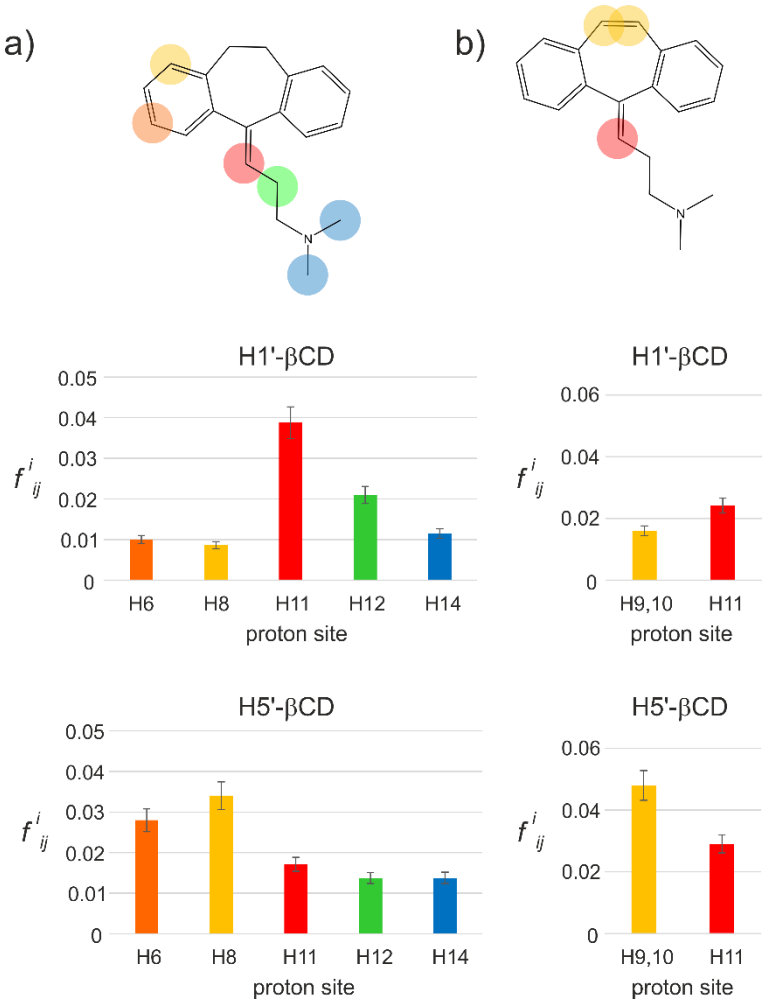


Figure 5. Fractional contribution to cross-relaxation of selected protons of (a) amitriptyline (AMT) and (b) cyclobenzaprine (CBZ), via dipolar interaction with proton H1' (bar diagrams on the top) or H5' (bar diagrams on the bottom) of βCD. Maximum errors are estimated to be 10% of the value.

4. Conclusions

A thorough physicochemical characterization of the interaction and binding between a drug and a potential carrier is highly recommendable for an appropriate controlled release. Here, we exploited multiple NMR techniques to deeply investigate the encapsulation of two tricyclic drugs, amitriptyline (AMT) and cyclobenzaprine (CBZ), in β-cyclodextrin (βCD).

As AMT and CBZ are inherently chiral and are used as racemic mixture, diastereomeric inclusion complexes are formed upon encapsulation in the cavity of the homochiral βCD. Here, ^{13}C NMR was used to unambiguously assess chiral recognition, demonstrating a higher performance over ^{1}H NMR.

The mole fraction of the bound drug and the association constant have been derived through diffusion experiments, whereas the combination of non-selective, selective and bi-selective relaxation spectra gave insights into the rotational motion of the complexed drug and the spatial proximity of selected proton pairs.

Overall, the different NMR methods applied confirm the geometry of inclusion described by Castiglione *et al.* (2017), with an aromatic ring system inserted into the βCD cavity, and the seven-membered ring, the alkylammonium chain and the other aromatic ring protruding out of the cavity. Next to it, in the present work we demonstrated the potential of NMR spectroscopy for a comprehensive characterization of host/guest complexes: each NMR experiment, namely 1D and 2D carbon spectra, PFG NMR and T_1 measurements, provide single pieces of the puzzle, which combined together reinforce themselves and give access to the whole structural and dynamic picture. Such a characterization of CD/drug inclusion complexes from a physicochemical point of view can constructively complement the conventional pharmacological and pharmacokinetic experiments, shedding light on the understanding of CD/drug formulations. An added value is the possibility to extend the methodology not only to other native and substituted cyclodextrins, but also to other supramolecules and macrocyclic compounds, such as calixarenes.

Supporting Information

The following files are available:

1D ^{13}C-{^{1}H} and ^{1}H-^{13}C HSQC spectra, theoretical toolbox and method validation of diffusivity measurements, theoretical toolbox of T_1 measurements, tables of T_1^{NS}, T_1^{SE}, R_1^{NS}/R_1^{SE} ratio, τ_C and f_{ij}^{i} values (PDF)

Corresponding Author

* Dr. Maria Enrica Di Pietro. Phone +39 02 2399 3045. Email mariaenrica.dipietro@polimi.it

* Prof. Andrea Mele. Phone +39 02 2399 3006. Email andrea.mele@polimi.it

Declaration of interest

None

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgment

MEDP thanks Politecnico di Milano for her postdoctoral fellowship in the framework of the "MSCA EF Master Class 2018" funding programme.

References

Ali, M.S., Rub, M.A., Khan, F., Al-Lohedan, H.A., Kabir-Ud-Din, 2012. Interaction of amphiphilic drug amitriptyline hydrochloride with β-cyclodextrin as studied by conductometry, surface tensiometry and viscometry. J. Mol. Liq. 167, 115–118. https://doi.org/10.1016/j.molliq.2012.01.004

Aree, T., 2020. β-Cyclodextrin encapsulation of nortriptyline HCl and amitriptyline HCl: Molecular insights from single-crystal X-ray diffraction and DFT calculation. Int. J. Pharm. 575, 118899–118908. https://doi.org/10.1016/j.ijpharm.2019.118899

Barhoum, S., Palit, S., Yethiraj, A., 2016. Diffusion NMR studies of macromolecular complex formation, crowding and confinement in soft materials. Prog. Nucl. Magn. Reson. Spectrosc. 94–95, 1–10. https://doi.org/10.1016/j.pnmrs.2016.01.004

Bilensoy, E. (Ed.), 2011. Cyclodextrins in Pharmaceutics, Cosmetics, and Biomedicine: Current and Future Industrial Applications. Wiley.

Brand, T., Cabrita, E.J., Berger, S., 2005. Intermolecular interaction as investigated by NOE and diffusion studies. Prog. Nucl. Magn. Reson. Spectrosc. 46, 159–196. https://doi.org/10.1016/j.pnmrs.2005.04.003

Brewster, M.E., Loftsson, T., 2007. Cyclodextrins as pharmaceutical solubilizers. Adv. Drug Deliv. Rev. 59, 645–666. https://doi.org/10.1016/j.addr.2007.05.012

Brewster, M.E., Loftsson, T., 1996. Pharmaceutical applications of cyclodextrins . 1 . Drug solubilization and stabilization. J. Pharm. Sci. 85, 1017–1025.

Cameron, K.S., Fielding, L., 2001. NMR diffusion spectroscopy as a measure of host-guest complex

association constants and as a probe of complex size. J. Org. Chem. 66, 6891–6895. https://doi.org/10.1021/jo010081x

Cano, J., Rodriguez, A., Aicart, E., Junquera, E., 2007. Temperature effect on the complex formation between tricyclic antidepressant drugs (amitriptyline or imipramine) and hydroxypropyl-β-cyclodextrin in water. J. Incl. Phenom. Macrocycl. Chem. 59, 279–285. https://doi.org/10.1007/s10847-007-9328-x

Castiglione, F., Ganazzoli, F., Malpezzi, L., Mele, A., Panzeri, W., Raffaini, G., 2017. Inclusion complexes of β-cyclodextrin with tricyclic drugs: An X-ray diffraction, NMR and molecular dynamics study. Beilstein J. Org. Chem. 13, 714–719. https://doi.org/10.3762/bjoc.13.70

Challa, R., Ahuja, A., Ali, J., Khar, R.K., 2005. Cyclodextrins in drug delivery: An updated review. AAPS PharmSciTech 6, 329–357. https://doi.org/10.1208/pt060243

Chankvetadze, B., Endresz, G., Bergenthal, D., Blaschke, G., 1995. Enantioseparation of mianserine analogues using capillary electrophoresis with neutral and charged cyclodextrin buffer modifiers 13C NMR study of the chiral recognition mechanism. J. Chromatogr. A 717, 245–253. https://doi.org/10.1016/0021-9673(95)00489-4

Cohen, Y., Avram, L., Frish, L., 2005. Diffusion NMR spectroscopy in supramolecular and combinatorial chemistry : An old parameter — New insights. Angew. Chemie - Int. Ed. 44, 520–554. https://doi.org/10.1002/anie.200300637

Crini, G., 2014. Review : A history of cyclodextrins. Chem. Rev. 114, 10940–10975. https://doi.org/10.1021/cr500081p

Cruz, J.R., Becker, B.A., Morris, K.F., Larive, C.K., 2008. NMR characterization of the host-guest inclusion complex between β-cyclodextrin and doxepin. Magn. Reson. Chem. 46, 838–845. https://doi.org/10.1002/mrc.2267

Dalla Cort, A., Mandolini, L., Pasquini, C., Schiaffino, L., Chimica, D., Sapienza, U. La, Roma, B., 2004. ‘“Inherent chirality”’ and curvature. New J. Chem. 28, 1198–1199.

De Sousa, F.B., Leite Denadai, Â.M., Lula, I.S., Nascimento, C.S., Fernandes Neto, N.S.G., Lima, A.C., De Almeida, W.B., Sinisterra, R.D., 2008. Supramolecular self-assembly of cyclodextrin and higher water soluble guest: Thermodynamics and topological studies. J. Am. Chem. Soc. 130, 8426–8436. https://doi.org/10.1021/ja801080v

Di Pietro, M.E., Colombo Dugoni, G., Ferro, M., Mannu, A., Castiglione, F., Costa Gomes, M., Fourmentin, S., Mele, A., 2019. Do cyclodextrins encapsulate volatiles in deep eutectic systems? ACS Sustain. Chem.

483 Eng. 7, 17397–17405. https://doi.org/10.1021/acssuschemeng.9b04526

484 Di Pietro, M.E., Ferro, M., Mele, A., 2020. Drug encapsulation and chiral recognition in deep eutectic
485 solvents/β-cyclodextrin mixtures. J. Mol. Liq. 311, 113279.
486 https://doi.org/10.1016/j.molliq.2020.113279

487 Diniz, T.C., Pinto, T.C.C., Menezes, P. dos P., Silva, J.C., Teles, R.B. de A., Ximenes, R.C.C., Guimarães, A.G.,
488 Serafini, M.R., Araújo, A.A. de S., Quintans Júnior, L.J., Almeida, J.R.G. da S., 2018. Cyclodextrins
489 improving the physicochemical and pharmacological properties of antidepressant drugs: a patent
490 review. Expert Opin. Ther. Pat. 28, 81–92. https://doi.org/10.1080/13543776.2017.1384816

491 Dodziuk, H., Koźmiński, W., Ejchart, A., 2004. NMR studies of chiral recognition by cyclodextrins. Chirality
492 16, 90–105. https://doi.org/10.1002/chir.10304

493 Fielding, L., 2007. NMR methods for the determination of protein-ligand dissociation constants. Prog. Nucl.
494 Magn. Reson. Spectrosc. 51, 219–242. https://doi.org/10.1016/j.pnmrs.2007.04.001

495 Freeman, R., Hill, H.D.W., Tomlinson, B.L., Hall, L.D., 1974. Dipolar contribution to NMR spin-lattice
496 relaxation of protons. J. Chem. Phys. 61, 4466–4473. https://doi.org/10.1063/1.1681764

497 Georgiou, M.E., Georgiou, C.A., Koupparis, M.A., 1999. Rapid automated spectrophotometric competitive
498 complexation studies of drugs with cyclodextrins using the flow injection gradient technique: Tricyclic
499 antidepressant drugs with α-cyclodextrin. Analyst 124, 391–396. https://doi.org/10.1039/a808426b

500 Hall, L.D., Hill, H.D.W., 1976. Spin-lattice relaxation of protons. A general, quantitative evaluation of
501 contributions from the intramolecular dipole-dipole mechanism. J. Am. Chem. Soc. 98, 1269–1270.
502 https://doi.org/10.1016/b978-0-408-01434-2.50020-6

503 Junquera, E., Romero, J.C., Aicart, E., 2001. Behavior of tricyclic antidepressants in aqueous solution: Self-
504 aggregation and association with β-cyclodextrin. Langmuir 17, 1826–1832.
505 https://doi.org/10.1021/la000819q

506 Kumar, D., Krishnan, Y., Paranjothy, M., Pal, S., 2017. Analysis of molecular interaction of drugs within β-
507 cyclodextrin cavity by solution-state NMR relaxation. J. Phys. Chem. B 121, 2864–2872.
508 https://doi.org/10.1021/acs.jpcb.6b11704

509 Kundu, M., Roy, M.N., 2017. Preparation, interaction and spectroscopic characterization of inclusion
510 complex of a cyclic oligosaccharide with an antidepressant drug. J. Incl. Phenom. Macrocycl. Chem. 89,
511 177–187. https://doi.org/10.1007/s10847-017-0745-1

512 Kurkov, S. V., Loftsson, T., 2013. Cyclodextrins. Int. J. Pharm. 453, 167–180.
513 https://doi.org/10.1016/j.ijpharm.2012.06.055

514 Li, Y., Yin, G., Wei, W., Wang, H., Jiang, S., Zhu, D., Du, W., 2007. Interactions of Lycopodium alkaloids with
515 acetylcholinesterase investigated by 1H NMR relaxation rate. Biophys. Chem. 129, 212–217.
516 https://doi.org/10.1016/j.bpc.2007.05.020

517 Lis-Cieplak, A., Sitkowski, J., Kolodziejski, W., 2014. Comparative proton nuclear magnetic resonance studies
518 of amantadine complexes formed in aqueous solutions with three major cyclodextrins. J. Pharm. Sci.
519 103, 274–282. https://doi.org/10.1002/jps.23802

520 Loftsson, T., Brewster, M.E., 2012. Cyclodextrins as functional excipients: Methods to enhance
521 complexation efficiency. J. Pharm. Sci. 101, 3019–3032. https://doi.org/10.1002/jps

522 Loftsson, T., Duchêne, D., 2007. Cyclodextrins and their pharmaceutical applications. Int. J. Pharm. 329, 1–
523 11. https://doi.org/10.1016/j.ijpharm.2006.10.044

524 Morris, K.F., Johnson, C.S.J., 1992. Diffusion-ordered two-dimensional nuclear magnetic resonance
525 spectroscopy. J. Am. Chem. Soc. 114, 3139–3141.

526 Nishikawa, S., Kamimura, E., 2011. Dynamic characteristic of amitriptyline in water by ultrasonic relaxation
527 method and molecular orbital calculation. J. Phys. Chem. A 115, 535–539.
528 https://doi.org/10.1021/jp1107729

529 Pagès, G., Gilard, V., Martino, R., Malet-Martino, M., 2017. Pulsed-field gradient nuclear magnetic
530 resonance measurements (PFG NMR) for diffusion ordered spectroscopy (DOSY) mapping. Analyst
531 142, 3771–3796. https://doi.org/10.1039/c7an01031a

532 Rajendiran, N., Sankaranarayanan, R.K., Saravanan, J., 2014. A study of supramolecular host-guest
533 interaction of dothiepin and doxepin drugs with cyclodextrin macrocycles. J. Mol. Struct. 1067, 252–
534 260. https://doi.org/10.1016/j.molstruc.2014.03.051

535 Rasheed, A., Kumar C.K., A., Sravanthi, V.V.N.S.S., 2008. Cyclodextrins as drug carrier molecule: A review.
536 Sci. Pharm. 76, 567–598. https://doi.org/10.3797/scipharm.0808-05

537 Reddy, R.R., Phani Kumar, B.V.N., Shanmugam, G., Madhan, B., Mandal, A.B., 2015. Molecular level insights
538 on collagen-polyphenols interaction using spin-relaxation and saturation transfer difference NMR. J.
539 Phys. Chem. B 119, 14076–14085. https://doi.org/10.1021/acs.jpcb.5b07911

540 Redenti, E., Szente, L., Szejtli, J., 2000. Drug/cyclodextrin/hydroxy acid multicomponent systems. Properties
541 and pharmaceutical applications. J. Pharm. Sci. 89, 1–8.

542 Rossi, C., Bonechi, C., Martini, S., Ricci, M., Corbini, G., Corti, P., Donati, A., 2001. Ligand-macromolecule
543 complexes: Affinity index determination by selective nuclear relaxation analysis. Magn. Reson. Chem.
544 39, 457–462. https://doi.org/10.1002/mrc.876

545 Schneider, H.-J., Hacket, F., Rüdiger, V., Ikeda, H., 1998. NMR studies of cyclodextrins and cyclodextrin
546 complexes. Chem. Rev. 98, 1755–1786. https://doi.org/10.1021/cr970019t

547 Silva, M.S., 2017. Recent advances in multinuclear NMR spectroscopy for chiral recognition of organic
548 compounds. Molecules 22. https://doi.org/10.3390/molecules22020247

549 Szejtli, J., 1995. Selectivity/structure correlation in cyclodextrin chemistry. Supramol. Chem. 6, 217–223.
550 https://doi.org/10.1080/10610279508032537

551 Tinoco, L.W., Figueroa-Villar, J.D., 1999. Determination of correlation times from selective and non-
552 selective spin-lattice relaxation rates and their use in drug-drug and drug-albumin interaction studies.
553 J. Braz. Chem. Soc. 10, 281–286. https://doi.org/10.1590/S0103-50531999000400005

554 Tošner, Z., Aski, S.N., Kowalewski, J., 2006. Rotational dynamics of adamantanecarboxylic acid in complex
555 with β-cyclodextrin. J. Incl. Phenom. Macrocycl. Chem. 55, 59–70. https://doi.org/10.1007/s10847-
556 005-9019-4

557 Uccello-Barretta, G., Balzano, F., Paolino, D., Ciaccio, R., Guccione, S., 2005. Combined NMR-
558 crystallographic and modelling investigation of the inclusion of molsidomine into α-, β- and γ-
559 cyclodextrins. Bioorganic Med. Chem. 13, 6502–6512. https://doi.org/10.1016/j.bmc.2005.07.009

560 Valsami, G.N., Koupparis, M.A., Macheras, P.E., 1992. Complexation studies of cyclodextrins with tricyclic
561 antidepressants using ion-selective electrodes. Pharm. Res. 9, 94–100.

562 Wang, S., Li, L., Xiao, Y., Wang, Y., 2019. Recent advances in cyclodextrins-based chiral-recognizing
563 platforms. Trends Anal. Chem. 121, 115691.

564 Wenzel, T.J., Chisholm, C.D., 2011. Using NMR spectroscopic methods to determine enantiomeric purity
565 and assign absolute stereochemistry. Prog. Nucl. Magn. Reson. Spectrosc. 59, 1–63.
566 https://doi.org/10.1016/j.pnmrs.2010.07.003

567 Wouessidjewe, D., Ponchel, G., Duchene, D., 1999. Cyclodextrins and carrier systems. J. Control. Release 62,
568 263–268.

569

***Conflict of Interest**

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

***Credit Author Statement**

Maria Enrica Di Pietro: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing - original draft; Writing - review &editing.

Monica Ferro: Conceptualization; Investigation; Writing - review &editing.

Andrea Mele: Conceptualization; Supervision; Writing - review &editing.

Supplementary Material

Click here to download Supplementary Material: IntJPharm_bCD-drugs_Special-Issue_SI_rev2.pdf