12

Review: Cyclodextrin Inclusion Complexes Probed by NMR Techniques

Francisco B. T. Pessine, Adriana Calderini and Guilherme L. Alexandrino
Department of Physical Chemistry, Chemistry Institute, State University of Campinas
Brazil

1. Introduction

Cyclodextrins (CDs) are cyclic oligomers of glucopyranose units that play an important role as a host in inclusion complexes, where non-covalent interactions are involved. They have been extensively studied in supramolecular chemistry. Because of its biocompatibility, relatively non-toxicity and relatively low price, CDs have been widely employed for encapsulation of several substances, being used in food, cosmetic and pharmaceutical industries. Nuclear Magnetic Resonance spectroscopy (NMR) is one of the most useful techniques to study interactions of cyclodextrins with guest compounds. It is relatively easy to apply, the experiments are fast and it is the only technique that provides information on the right orientation of the guest molecule inside the cavity and also on other important parameters related to the physico-chemical characteristics of the inclusion complexes. In this review, it will be discussed the study of inclusion complexes between drugs and cyclodextrins by different NMR techniques. Initially, a brief introduction of the properties of cyclodextrins, its importance as innovative drug carrier systems and its applicability is reviewed. Then different NMR techniques used for characterization of inclusion complexes are detailed, with examples studied in our group, which involves since simple measures of ^{1}H-NMR spectrum to more sophisticated experiments, e.g. Diffusion Ordered SpectroscopY (DOSY), NOE methods (ROESY), T1 measure and solid NMR by ^{13}C Cross-Polarization Magic Angle Spinning (CPMAS).

2. Properties of cyclodextrins

Cyclodextrins (CDs) as complexation agents and its study in supramolecular chemistry have been used in many areas (Steed & Atwood, 2002; Chen & Jiang, 2011). Cyclodextrins are cyclic oligosaccharides classified according to their number of glucopyranose units. The natural and most employed cyclodextrins are crystalline, homogeneous, non-hygroscopic substances and includes α-cyclodextrin (αCD, cyclohexaamylose, 6 units of glucopyranose), β-cyclodextrin (βCD, cycloheptaamilose, 7 units of glucopyranose) and γ-cyclodextrin (γCD, cyclooctaamylose, 8 units of glucopyranose), whose chemical structures are shown in Figure 1 (Szejtli, 1998). They are biocompatible, non-toxic in a wide range of concentration, relatively inexpensive and produced naturally by enzyme degradation of starch (Yorozu et al., 1982).

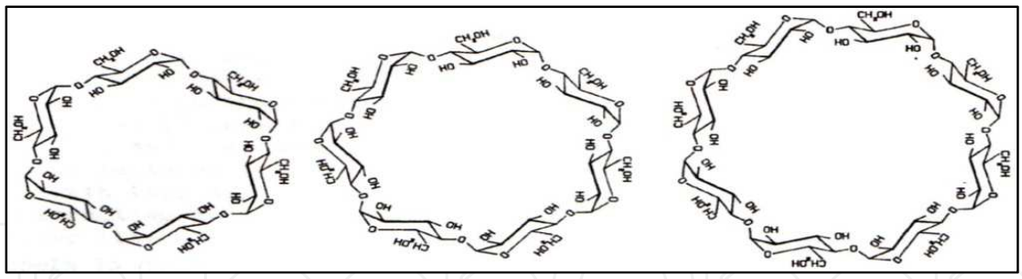


Fig. 1. Chemical structures of (a) αCD; (b) βCD; (c) γCD

These cyclodextrins have torus-like macro ring shape, are relatively low soluble in water and have a hydrophobic cavity. The main factors acting as driving force to form complexes and also responsible for the stability of these complexes are hydrophobic forces, the sizes of molecules/cavity and the guest properties (Griffiths & Bender, 1973).

The glucopyranosides units are in C1 conformation, where the OH groups are linked to the carbon atoms C2 and C3 around the bigger edge and the more reactive OH group (linked to C6) is in the smaller edge. The cavity is delined by the hydrogens atoms and by the glucosidal bridge. The non-ligant electron pairs of the oxygen atoms are inner of cavity, leading to a high electron density and resulting in an environment similar to that of Lewis bases (Szejtli, 1988).

Casu et al. (Casu et al., 1966, 1968), had applying Nuclear Magnetic Resonance and Rotatory Optical Dispersion techniques, to DMSO solution containing CDs, where there is a strong competition with the solvent molecules by the intramolecular hydrogen bridge, showing that the glucose residues kept their conformation and the hydrogen bridge bonds in both solid state and in solution, where this competition is much lower, although, in solution, there is much more conformational mobility than in the solid state.

Bergeron (1977) and Saenger (1980) studied the formation of hydrogen bridges between the OH groups of the C2 and C3 atoms and observed that the conformational mobility of the macro cyclic ring is restricted, contributing to its toughness, both in solid and solution. This array keeps the linkages directed to the core of the cavity, leading to a hydrophobic environment, although the outer surface is hydrophilic, as shown in Figure 2. Some CDs structural and physical-chemical parameters are in Table 1.

Properties	**αCD**	**βCD**	**γCD**
Number of glucopyranose units	6	7	8
Molar mass / (g/mol)	972	1135	1297
Solubility / (g/100 mL)	14.5	1.85	23.2
Inner cavity diameter / (Å)	4.7-5.2	6.0-6.4	7.5-8.3
Outer cavity diameter / (Å)	14.6	15.4	17.5
Cavity height / (Å)	6.7	7.0	7.0
Specific rotation $(\alpha)_D^{25*}$	150.5 ± 0.5	162.5 ± 0.5	177.4 ± 0.5
Volume of the cavity / ($Å^3$)	174	262	427
ΔH^0 (aq) / (kcal/mol)	7.67	8.31	7.73
ΔS^0 (aq) / (cal/(mol K))	13.8	11.7	14.7

Table 1. α, β and γCD water solubility (25°C) and structural parameters (Szejtli, 1988, 1998; Connors, 1997)

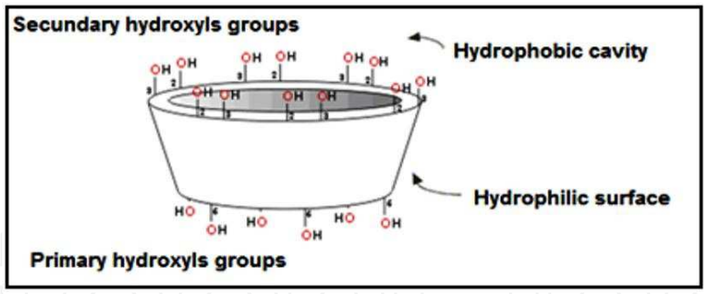


Fig. 2. CDs structure in torus-like macro ring shape, with the hydroxyls groups (Uekama et al., 1998)

The cavity size increases with the number of glucose units, although the height is constant (6.7-7.0 Å). The solubility do not follow this rule: βCD is considerably less soluble in water than αCD and γCD and this low solubility is due to the hydrogen bridges between OH groups of C2 and C3, leading to a rigid structure. In the αCD molecule, one glucose unit in distorted and only 4 of 6 possible hydrogen bridges are formed. In γCD, its glucose units are not in the same plane and its structure is more flexible; therefore, this CD have higher solubility in aqueous solutions (Uekama et al., 1998).

Although the solubility of βCD is smaller, the size of its cavity is more appropriate to encapsulate a great variety of molecules with biological and pharmacological properties. Chemical substitution of the OH groups in C2, C3 and C3 have been done to increase its solubility, as for example, the hydroxipropyl-βCD.

Some factors should be considered to choose the type of the CD for a given study: physical-chemical properties of the guest, size of the CD cavity, solubility, the preparation method and possibility of co-encapsulation. Basically, the inclusion complexes involves interactions of the molecules of both the host and the guest, and it is a combination of different non-covalent interactions as ionic, dipolar, electronic, van der Waals and the hydrophobic effect, besides the size and the shape of the molecules (Chen & Jiang, 2011).

The stability of the inclusion complex is due to, primarily, hydrophobic forces. The association constant is, usually, in the order of 10^{-3} M, typical of weak interactions (Griffiths & Bender, 1973). Hydrophobic molecules, or only the hydrophobic part of a polar molecule, are incorporated in the CD cavity promoting the shift of the water molecules from inside the cavity, which is favored by repulsions between the apolar guest and the water polar molecule. This process leads to the partial or total encapsulation of the guest molecule, increasing aqueous solubility of the sample. However, when the inclusion complex is diluted in a larger volume, the phenomenon is reverted and the species is free in solution.

The main advantages of using CDs in drug delivery systems includes: the increase the biodisponibility, solubility enhancer, improve the stability of the drug, increase the therapeutic index, the efficacy/pharmacokinetics properties, and decrease the drug toxicity (Uekama et al., 1998).

The applicability of CDs includes: analytical chemistry as chromatographic separations (Li & Purdy, 2002), drug delivery systems (Arun et al., 2008), as masking agent in food

(Tamamoto et al., 2010), cosmetics (Schmann & Schollmeyer, 2002), in hydrogels (Hoarea &. Kohaneb, 2008) and in contact lenses (Santos et al., 2009).

3. Nuclear Magnetic Resonance spectroscopy

3.1 ^{1}H-Nuclear Magnetic Resonance spectroscopy

Nuclear Magnetic Resonance spectroscopy has been extensively employed in Chemistry and can be considered as one of the most complete spectroscopic techniques, due to its wide field of applications from structural elucidation of structures to investigations on intra/inter-molecular. Applications of NMR on CDs chemistry is so important that no other spectroscopic technique can provide the same wealth of chemical information on the supramolecular systems. Other spectroscopic techniques, like molecular UV-Vis absorption, fluorescence emission, circular dichroism, etc., are also suitable for thermodynamic study of the host-guest intermolecular interactions, but they give only indirect information on the molecular structure of the inclusion complexes.

The simplest experiment of NMR as an indicative of complexation is the observation of the difference in the proton chemical shifts between the free guest and host species and the suggested complex. There has been a long time since Demarco & Thakkar (Demarco & Thakkar, 1971) started studies on CDs complexes by observing the chemical shifts changes of the protons H3 and H5 inside the cavity of αCD when in presence of aromatic molecules due to the anisotropic effect of the aromatic ring. When there is a host-guest interaction, it leads to a change in the δ of the hydrogens due the complexation. This is a first evidence of the guest inclusion in the CD cavity (Schneider et al., 1998). The CDs protons are named according Figure 3.

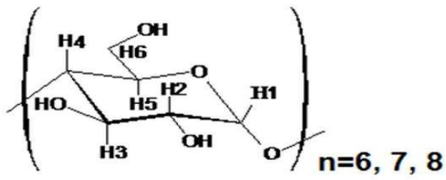


Fig. 3. NMR protons for α; β; γCD (n = 6; 7; 8, respectively)

The ^{1}H-NMR spectra of the three natural cyclodextrins are shown in Figure 4.

One can see that the differences among the spectra of the three CDs, due to the proton shielding, are smaller than 0.1 ppm. The characteristics of these spectra are discussed elsewhere (Schneider et al., 1998).

The stability of the inclusion complex and the orientation of the drug molecule can be inferred by this experiment. Greatbanks & Pickford (Greatbanks & Pickford, 1987) concluded that when $\Delta\delta$ H3 > $\Delta\delta$ H5, occurs partial inclusion of the guest inside the cavity and when $\Delta\delta$ H3 $\leq$ $\Delta\delta$ H5, a total inclusion takes place.

As an example, the stability of the inclusion complex between Minoxidil (MNX), a vasodilator drug also used for treatment of alopecia areata (Gorecki, 1988), and the natural CDs can be suggested by analyzing the differences between the protons chemical shifts of both species. Figure 5 shows the MNX structure and the protons assignment.

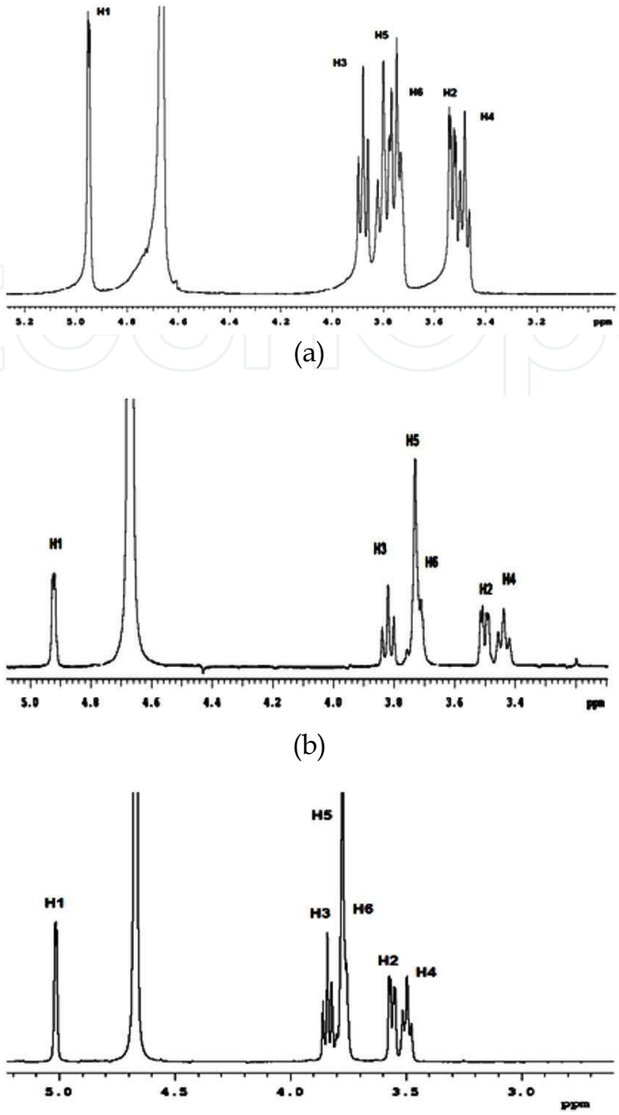


Fig. 4. ^{1}H-NMR spectra of the natural cyclodextrins (298 K; 500 MHz; D_2O; δ_{HOD} 4.67 ppm): (a) αCD; (b) βCD; (c) γCD

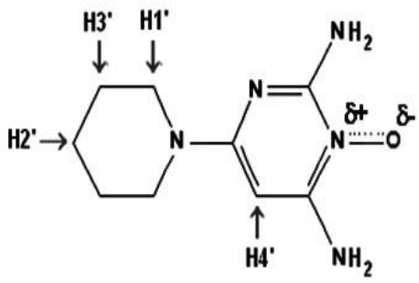


Fig. 5. Structure of MNX and its protons

Tables 2 and 3 summarized the data for this complex.

H	αCD	MNX: αCD		βCD	MNX: βCD		γCD	MNX: γCD	
	$\delta_{\alpha CD}$	$\delta_{\alpha CD:MNX}$	$\Delta\delta_{\alpha CD:MNX}$	$\delta_{\beta CD}$	$\delta_{\beta CD:MNX}$	$\Delta\delta_{\beta CD:MNX}$	$\delta_{\gamma CD}$	$\delta_{\gamma CD:MNX}$	$\Delta\delta_{\gamma CD:MNX}$
1	4.960	4.955	-0.005	4.960	4.965	0.005	5.015	5.015	---
2	3.530	3.540	0.010	3.545	3.550	0.005	3.560	3.555	-0.005
3	3.910	3.890	-0.020	3.830	3.820	-0.010	3.860	3.850	-0.010
4	3.490	3.480	-0.010	3.490	3.480	-0.010	3.495	3.490	-0.005
5	3.765	3.750	-0.015	3.720	3.710	-0.010	3.800	3.800	---

Table 2. CDs chemical shifts (δ) and their difference when in presence of MNX ($\Delta\delta = \delta_{complexed}-\delta_{free}$)

H	MNX	MNX:αCD		MNX:βCD		MNX:γCD	
	δ_{MNX}	$\delta_{\alpha CD:MNX}$	$\Delta\delta_{\alpha CD:MNX}$	$\delta_{\beta CD:MNX}$	$\Delta\delta_{\beta CD:MNX}$	$\delta_{\gamma CD:MNX}$	$\Delta\delta_{\gamma CD:MNX}$
1′	3.340	3.360	0.020	3.420	0.080	3.340	---
2′	1.535	1.550	0.015	1.570	0.035	1.535	---
3′	1.455	1.490	0.035	1.520	0.065	1.455	---

Table 3. MNX chemical shifts (δ) and their difference when in presence of CDs ($\Delta\delta=\delta_{complexed}-\delta_{free}$)

One can see that $\Delta\delta_{\beta CD:MNX} > \Delta\delta_{\alpha CD:MNX} > \Delta\delta_{\gamma CD:MNX}$. Therefore, the complexation between βCD and MNX is stronger than in α and γCD. Considering the values of Δδ for H3 and H5, there is a partial inclusion in α and γCD. For the complex βCD:MNX, the inclusion is total. These results were confirmed by other spectroscopic techniques.

Other drug that we had performed NMR studies with CDs is the anti-neoplasic 5-fluorouracil. No differences in the chemical shifts were observed. This lack of interaction was supposed to be due to the relatively high polarity and solubility of the drug in aqueous media (12.2 mg/mL) (Bayomi & Al-Badr, 1990).

Despite the chemical shift changes of ^1H from CD's inner cavity the characterization of inclusion complexes with NMR could be done. This same experimental evidence also could be observed for ^1H from the guest molecule inserted into the CD cavity. Moreover, this chemical shift analysis could be extended for other types of experiments, and the characterization of different properties around the complex supramolecular organization can be obtained easily.

One of the most employed NMR experiment based on chemical shift changes is NMR titration. In this experiment, NMR spectra are obtained for solutions containing a fixed concentration of guest [G_o], but the initial concentration of the CD [CD_O] is variable. Due to the chemical equilibrium of G:CD complex formation (equation 3.1), the increase on CD concentration in the solution will result in more population of the G:CD inclusion complex, consequently, it is expected more chemical shift changes in interacting host and guest nucleus as may be the higher CD concentration in the solution. Besides the CD concentration, the magnitude of equilibrium constant associated to the complex formation (K) will be directly related to the chemical shift changes, because it represents the thermodynamic tendency through the formation of G:CD complex, equation 3.2.

$$[G] + [CD] = [G:CD] \quad (3.1)$$

$$K = \{[G:CD]/([G]\,[CD]\} \quad (3.2)$$

[G] and [CD] are the concentration of free guest and CD in the equilibrium, respectively, which is defined, for a 1:1 complex, according to their respective mass balance (equations 3.3 and 3.4).

$$[G] + [G:CD] = G_O \quad (3.3)$$

$$[CD] + [G:CD] = CD_O \quad (3.4)$$

As the chemical shift changes in the experiment is defined as the molar weighted average of free and complexed G or CD molecules (equation 3.5), the correlation among the equations 3.2-3.5 can provide a non-linear math relationship between K and δobs, which leads to a thermodynamic application of NMR to cyclodextrin inclusion complexes (Fielding, 2000).

$$\delta exp = X_G\delta_G + X_{G:CD}\delta_{G:CD} \quad (3.5)$$

Examples of the linear relation between K and δ_{obs} are those reported by Mathur et al. (Mathur et al., 1963), Hannah & Ashbaugh (Hannah & Ashbaugh, 1964) and Foster & Fyfe (Foster & Fyfe, 1965a,b). Using the Benesi-Hildebrand and Scatchard linear models they investigated the thermodynamic properties of guest:CD inclusion complex using UV-Vis spectroscopy (equations 3.6 and 3.7).

$$1/\Delta\delta = 1/(K.\Delta\delta max.[CD_O]) + 1/\Delta\delta max \quad (3.6)$$

$$\Delta\delta/[CD_O] = -K.\Delta\delta + K.\Delta\delta max \quad (3.7)$$

Δδ = (δG – δexp), is the chemical shift change of guest proton as the concentration of the CD increase, and Δδmax = (δG – δG:CD), is the maximum possible chemical shift changes supposing all guest molecules are complexed with the CD. Equation 3.6 is known as the double reciprocal plot, and its deduction require an approximation that $[CD_O]$ must be at least 10 times higher than $[G_O]$. Besides, weak inclusion complexes implies significant uncertainly on Δδ, therefore, K estimation become prejudiced. Equation 3.7 is known as x-reciprocal plot, and is less often found in articles of NMR in CD complexes (Fielding, 2000).

Another application of Δδ is the determination of most stable molar stoichiometry of guest:CD in solution, which can be done through the Job plot experiment, that was initially developed for optical spectroscopy and adapted to NMR. Δδ is calculated in solution with different guest molar fraction r, and the stoichiometry relation is obtained for the r value that show the higher Δδ, which corresponds to the maximum population of inclusion complex in solution. If the complex most stable stoichometry is 1:1, Δδ will be maximum in the solution of r 0.5, while if guest:CD complex is 1:2, the higher Δδ will occur for r 0.33. The principle of this method is based on the mass balance of each specie guest, CD and guest:CD complex in equilibrium, that depend on the r value according to equations 3.8 and 3.9 (Loukas, 1997).

$$[G] = rM - [G:CD] \quad (3.8)$$

$$[CD] = M(1 - r) - n\,[G:CD] \quad (3.9)$$

where r is the guest molar fraction, M = [G] + [CD] and n is the number of CD molecules that encapsulate the guest. The maximum [G:CD] can be determined as a function of r calculating d[G:CD]/dr = 0 that gives $r = (n+1)^{-1}$. Therefore, for n = 1 (1:1 guest:CD complex) [G:CD] will be higher in the solution which r must be 0.5.

As the thermodynamic models for K prediction assumes a fixed stoichometry for the guest:CD complex, equations assuming different stoichometries can also be adjusted, which could lead to uncorrected predictions of guest:CD molar relation. Then, this experiment become very important for study of CDs inclusion complexes, as NMR allow any kind of guest to be investigated, because it does not depend on guest optical spectroscopy activity.

3.2 T_1 measurement

The T_1 measurement is directly related to the relaxation phenomenon, then it will be given a brief review about it. In NMR experiments, the irradiation of the resonance frequency disturbs the thermal equilibrium of the spin system, changing the population ratio and causing appearance of transverse field magnetic components (M_x and M_y). The relaxation occurs when the perturbation ceases until it reaches the equilibrium. It can be divided in two different processes:

1. The relaxation in the applied field direction is characterized by the *spin-lattice* or longitudinal relaxation time T_1 and will be discussed further,
2. The relaxation perpendicular to the field direction, which is characterized by the *spin-spin* or transverse relaxation time.

The time needed for the relaxation of nuclear system is very small which may be seconds, minutes or hours. For protons under high-resolution, T_1 is in the order of a second (Friebolin, 1993; Günther, 1994).

As discussed in the previous paragraph, immediately after exposing the spins to B_0 (the external magnetic field), they are in a non-equilibrium state because all spin states are equally populated and M_0 (initially M_z) = 0, i.e., the magnetization vector M_0 is rotated by $90°_{x'}$ pulse into the axis direction y', or by a $180°_{x'}$ pulse into the negative z direction, leading to $M_z = 0$. After a $180°_x$ pulse, its new value is $M_0 = - M_z$, as in Figure 6 (Friebolin, 1993).

The population ratio changes because the $90°_{x'}$ pulse equalizes the population of the two energy levels, whereas the $180°_{x'}$ pulse inverts the population ratio. Therefore, after the perturbation, the equilibrium condition is reached when $M_z = M_0$ and the rate at which it occurs is determined by the spin-lattice relaxation time T_1. This process was described by the Bloch's differential equation (Friebolin, 1993).

$$\frac{dM_z}{dt} = -\frac{M_z - M_0}{T_1} \tag{3.10}$$

where T_1^{-1} is the rate constant of the relaxation, which is a first-order process.

T_1 can be understood by a change in the energy in the spin system, as the energy absorbed form the pulse must be given up again, transferring to the lattice (or the surroundings),

whose thermal energy increases. Intra and intermolecular interactions like dipole-dipole, spin rotation, anisotropy, etc., can contribute to the spin-lattice relaxation. This is recognized as a significant phenomenon related to the dynamic properties of molecules. The most used method to determine T_1 is the inversion recovery method (Günther, 1994), as seen in Figure 7.

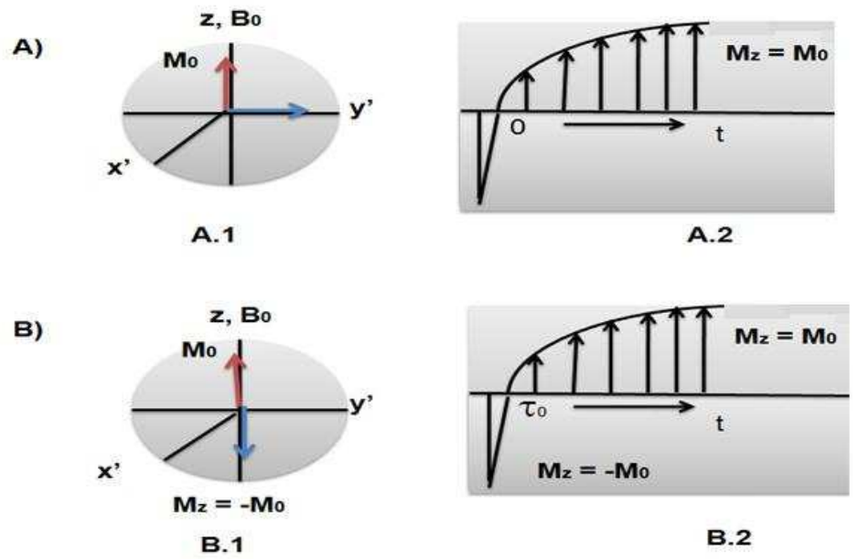


Fig. 6. The longitudinal component M_z in the rotating coordinate system x′; y′; z: A) after a $90°_{x'}$ pulse; B) after a $180°_{x'}$ pulse

Consider the macroscopic magnetization M_0 in the rotating coordinate system.

1. A 180° pulse at the beginning of the experiment brings the vector M_0 to the negative z direction,
2. The value of M_0 decreases due to the spin-lattice relaxation at time τ_1,
3. M_0 passes through zero at time τ_0,
4. M_0 begins to increase in the positive z-direction at time τ_2 reaching its final value.

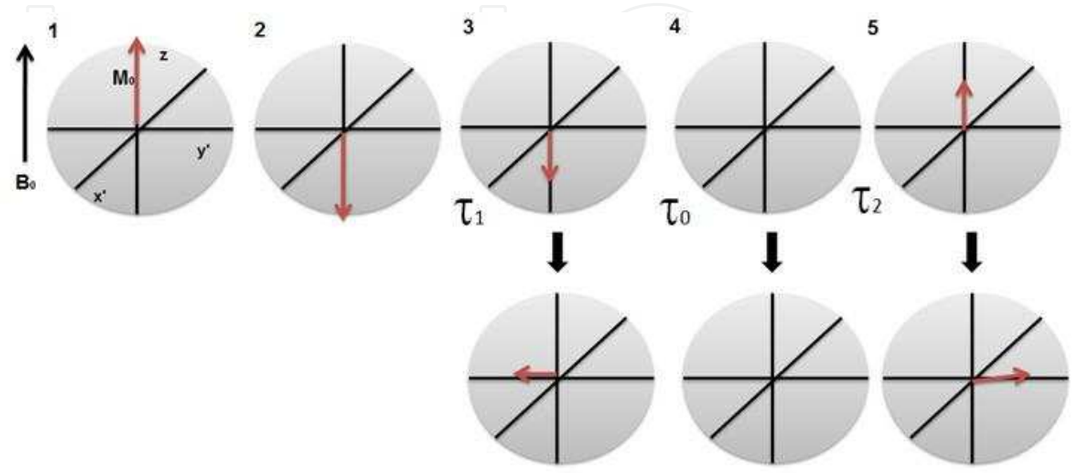


Fig. 7. The inversion-recovery experiment for T_1 measurement

The magnetization can be detected by two signals, applying 90° pulses at time τ_1 and τ_2 which align M_0 along the negative or positive y-direction, respectively, differing in phase by 180°. At time τ_0, there is no magnetization and no signal can be detected. Equation 3.11 is used to determine T_1 (Günther, 1994).

$$\tau_0 = T_1 \ln 2 = 0.693\, T_1 \tag{3.11}$$

In an alternative way, T_1 can be determined more accurately from a semi logarithmic plot of the intensity changes M_0 and M_z against τ, since it can be derived from equation 3.10 by integration (equation 3.12).

$$\ln(M_0 - M_Z) = \ln 2M_0 - \frac{\tau}{T_1} \tag{3.12}$$

Therefore, longitudinal relaxation times (T1) give information about the nucleus mobility in solution and the interaction between host and guest molecules by a qualitative analysis of the decreasing of T_1 values in the complexes (Lambert & Mazzola, 2004). Grillo et al. (Grillo et al., 2007) showed that T_1 lowering of the hydroxymethylnitrofurazone and dimethyl-βcyclodextrin system is a strong evidence of interactions between both molecules.

One of the inclusion compounds studied by this technique in our group was Dapsone:βCD, where the T_1 values for all hydrogens were obtained for each molecule and for the complex (data to be published). The values for each sample are summarized in Tables 4 and 5. The structure of Dapsone (DPS) is in Figure 8.

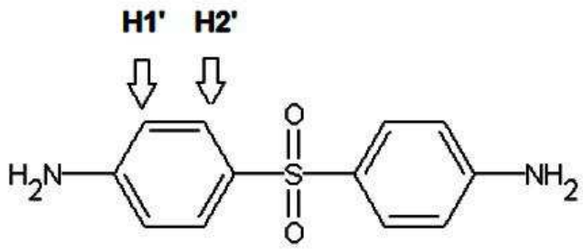


Fig. 8. Structure of Dapsone and the protons probed in NMR experiments

Proton	$T1_{DPS}$ (s)	$T1_{DPS:\beta CD}$ (s)
H1′	3.95 ± 0.54	1.02 ± 0.16
H2′	6.76 ± 3.07	1.73 ± 0.22

Table 4. Longitudinal relaxation times (T1, s) of DPS hydrogens in presence of βCD

Proton	$T1_{\beta CD}$ (s)	$T1_{DPS:\beta CD}$ (s)
H1	1.2 ± 0.1	0.73 ± 0.07
H3	1.56 ± 0.13	1.48 ± 0.07
H5	0.63 ± 0.03	0.39 ± 0.03
H4	0.91 ± 0.06	0.81 ± 0.04

Table 5. Longitudinal relaxation times (T1, s) of βCD hydrogens in presence of DPS

One observed that all T_1 values decrease for the inclusion compound. Note that the T_1 values obtained for the DPS hydrogens became at the same order as the T_1 values of βCD, indicating a strong host:guest interaction.

3.3 DOSY experiment (Diffusion Ordered SpectroscopY)

The molecular diffusion in solution is a phenomenon related to the molecular dynamics in biological and chemical systems, which can be probed by NMR. The diffusion coefficients D provide important information about the molecular organization and the phase structures. Typical D values in liquids (298-303 K) are from 10^{-12} m^2s^{-1} (polymers with high molecular weight) to 10^{-9} m^2s^{-1} (small molecules) (Stilbs, 1987).

Even though the molecular diffusion is not straightway related with interactions and spins energies, the relation of this property with the longitudinal (T_1) and transversal (T_2) times are better understood. Brownian motion perturbs the nuclei magnetic field, changes T_1 and T_2 (Price, 1997).

These studies started with the discovery of the spin echoes by Hahn in 1950. In this experiment, a pulse sequence $[90°\text{-}\tau\text{-}180°\text{-}\tau\text{-}(echo)\text{-}]_n$ is applied and it will result in a perfect refocus of the nuclei magnetization (spin echoes) only if there is any changes in the magnetic field gradient G applied during the time 2τ. However, the molecular diffusion does not allow G to keep totally homogeneous, decreasing the echoes amplitude in 2τ. The relation between the spin echoes and the diffusion coefficient D is given by equation 3.13 (Souza & Laverde, 2002).

$$\frac{A(2\tau)}{A(0)} = \exp\left[-\left(\frac{2\tau}{T2}\right) - \frac{2}{3}\gamma^2 G^2 D\tau^3\right] \tag{3.13}$$

The disadvantage in the Hahn equation is the difficulty in separate the contributions due to the transversal relaxation (T_2) and the molecular diffusion (D). This was solved by Carr and Purcell in 1954, where the pulse sequence $90°\text{-}\tau\text{-}[180°\text{-}\tau\text{-}(eco)\text{-}\tau\text{-}180°\text{-}\tau\text{-}(eco)\text{-}]_n$ can separate the D effect from the T_2 effect (equation 3.14).

$$\frac{A(t)}{A(0)} = \exp\left[-\left(\frac{t}{T2}\right)\right] \exp\left[-\frac{1}{3}(\gamma G\tau)^2 D\tau\right] \tag{3.14}$$

Even though the application of this sequence and the improvement on the echo amplitude with the Stimulated Spin Echo (STE), also discussed by Hahn in 1950, the application of static magnetic field gradients (G) resulted in the necessity to use high values of G to measure small values of D, which led to a increase in the echo and, consequently, a decrease in the noise/signal ratio, causing a difficulty in the detection (Souza & Laverde, 2002).

These difficulties were overcome with application of Pulsed Magnetic Field Gradients (PFG), with spin echoes (PFGSE) (Johnson, 1999) and stimulated spin echoes (PFGSTE) (Stilbs, 1987; Woessner, 1961; Tanner, 1970). Other advantages due to PFG are (Parella, 1998):

1. Reduction of the steps in the cycles phases to suppression of undesired artifacts,
2. Decrease of the signal acquisition time,
3. Noise reduction of the 2D experiment,

4. Improvement of the spectral processing,
5. Efficient suppression of undesired signals, like solvent and heteronuclear coupling.

The advantages of PFG application are (Souza & Laverde, 2002):

a. Separation of the echo attenuation caused by the D effect due to T_2, doing the experiment to a fix τ interval between the RF pulses and changing the area of the gradient pulse,
b. The spin echo is detected in a homogeneous magnetic field condition.

In 1992, Morris and Johnson (Morris & Johnson, 1992) developed an analytical technique based on PFGSE that made possible the distinction among the components of a mixture by the molecular diffusion of each chemical shift. This technique was named DOSY (Diffused Ordered Spectroscopy). In this experiment, D is obtained from the decay of the signal intensity I, which is a function of the area of the magnetic field gradient q (q = ɣgδ). It can be correlated with q and D by equation 3.15.

$$I(q, v) = \sum \left[An(v).exp\left[-Dn\left(\Delta - \frac{\delta}{3} \right) q^2 \right] \right] \quad (3.15)$$

where An(v) is the NMR signal intensity when q=0; Dn is the diffusion coefficient of the component n and Δ is the monitoring time. The DOSY spectrum has two dimensions: the chemical shift in the x axis and Dn in the y axis. It is obtained applying a Laplace inverse transformation after the signal processing by Fourier transformation.

The DOSY experiment allows a global analysis of the sample dynamics, involving since small molecules to aggregates and supramolecular structures. Besides, this technique presents other advantages as impurity detection with no interference, evaluation of the species in equilibrium and selection of the desired specie to analysis.

The small sensibilities of NMR technique, superposition of the signals and similar D values interfere with the acquisition of a good DOSY spectrum. Also, this experiment requires a good stability of the signal, which can be impaired by phenomena as induced vortex currents in the sample, coupling between the gradient coil and the principal magnetic field and perturbation in the system of lock frequency and field. So, to try to minimize such effects, various pulse sequences were developed, each with advantages and disadvantages according to the characteristics of the sample components. The main examples of DOSY pulse sequences are: BPPSTE (Bipolar Pulser Pair STimulated Echo), BPPLED (Bipolar Pulse Pair Longitudinal Eddy currents Decay), GCSTE (Gradient Compensated STimulated Echo) e GCSTESL (Gradient Compensated Stimulated Echo Spin Lock) (Souza & Laverde, 2002).

The implementation of simple PFGSE experiments on cyclodextrins complexes was initiated with Stilbs in 1983 (Stilbs, 1987), and the group of Lin first studied this interaction by DOSY in 1995 (Lin et al., 1995). The formation of inclusion complex in a solution containing a drug and CD can be observed by reduction of its diffusion coefficient. The larger the difference between D in solution with CD compared to D obtained from solution without CD, the higher the fraction of drug inclusion complex.

The diffusion coefficient (D) depends on the size of the molecule and it can be calculated by equation 3.16.

$$D = \frac{\kappa T}{6\,\pi\eta\, r} \tag{3.16}$$

where κ is the Boltzmann constant, T is the absolute temperature, η is the dynamic viscosity, and r is the radius of the molecule. The population of the guest involved in this complexation process can be calculated from the diffusion coefficients observed for the species in the free and complexed forms, using the equations 3.17-3.19 (Lin et al., 1985; Rymdén et al., 1983).

$$\mathrm{D_{observed}} = \mathrm{D_{free}}\ \mathrm{p_{free}} + \mathrm{D_{complexed}}\ \mathrm{p_{complexed}} \tag{3.17}$$

where:

$$\mathrm{p_{free}} + \mathrm{p_{complexed}} = 1 \tag{3.18}$$

and:

$$\mathrm{p_{free}} = 1 - \mathrm{p_{complexed}} \tag{3.19}$$

$D_{observed}$ is the diffusion coefficient of the active in the presence of CD; D_{free} is the diffusion coefficient of the active in the absence of CD; $p_{complexed}$ is the population fraction of completely complexed active, and $D_{complexed}$ is the diffusion coefficient of the completely complexed.

Substituting the equation 3.19 in the equation 3.17, and considering that the diffusion of the active totally complexed is the same as the CD totally complexed and that the diffusion observed to the CD partially complexed is very close to the same free diffusion:

$$D_{CD\ complexed} \approx D_{CD\ observed\ free} \approx D_{CD\ free} \tag{3.20}$$

Substituting the equation 3.8 in the equation 3.5, $p_{complexed}$ can be obtained as:

$$p_{complexed} = \frac{\mathrm{D_{free}} - \mathrm{D_{complexed}}}{\mathrm{D_{free}} - \mathrm{D_{CDobserved}}} \tag{3.21}$$

Knowing $p_{complexed}$ and the molar concentration of each species in solution, it is possible to obtain an estimative of the complex association constant (Ka), if some precautions are taken account as: association constants in the order of 10-10^4 L/mol, the NMR observation and the solution concentration (Fielding, 2000). In our studies, one could observe that if the complex is sparkling soluble, more difficult is to acquire the DOSY data because the diffusion coefficients will have big errors.

The determination of association constants by DOSY provides an additional NMR method and an alternative of the chemical shift titration method (Simova & Berger, 2005). The K_a for a complex between the n mol of drug D and m mol of CD is (Rymdén et al., 1983).

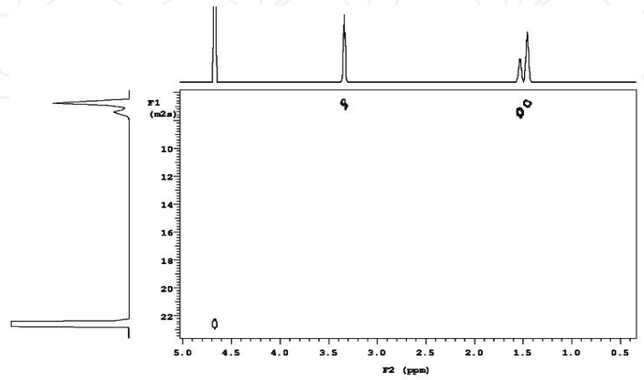
$$n\,D + m\,CD \leftrightarrow Complex\ [DnCDm] \tag{3.22}$$

$$Ka = \frac{[\,Complex]}{[D]^n\,[CD]^m} = \frac{[Complex]}{([D]_0 - n[Complex])^n([CD]_0 - m[Complex])^m} \tag{3.23}$$

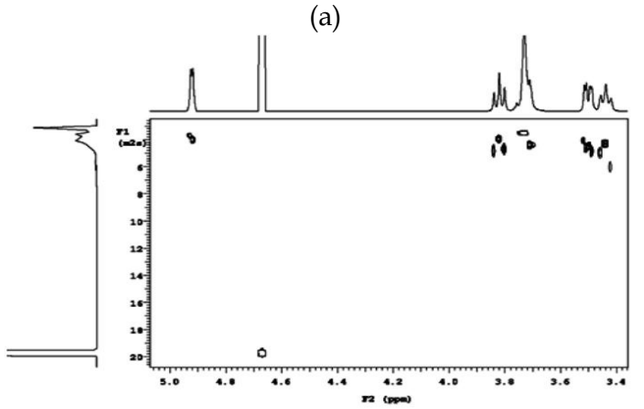
The equation 3.23 can be related with equation 3.21, then:

$$K_a = \frac{\boldsymbol{p_{complexed}}}{(\mathbf{1} - \boldsymbol{p_{complexed}})([\boldsymbol{CD}]_0 - \boldsymbol{p_{complexed}}\,[\boldsymbol{D}]_0)} \tag{3.24}$$

Fraceto et al. (Fraceto et al., 2007) applied DOSY to study the interaction between charged tetracaine in βCD and p-sulphonic acid calix[6]arene, obtaining Ka 1,358 M^{-1} and 3,889 M^{-1},



(a)



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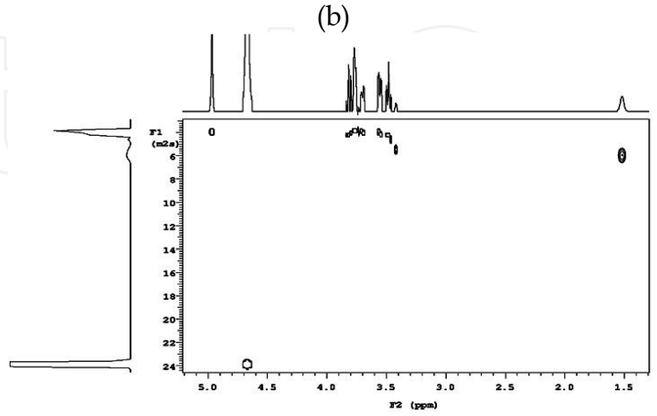

(c)

Fig. 9. ^{1}H-NMR DOSY spectra. (a) MNX; (b) βCD; (c) NMX-βCD 1:1, respectively (500 MHz; D_2O; δ_{HOD} 4.67 ppm)

respectively, indicating a good stability of the complexes. Jullian et al. (Jullian et al., 2007) studied the interaction between (+) cathecin and natural and modified βCDs observing a stronger interaction with natural βCD than with hydroxipropil-βCD or dimethyl-βCD, with association constants of 21,800 M^{-1}, 13,580 M^{-1} and 3,500 M^{-1}, respectively.

Besides the applicability of this technique to measure diffusion coefficients and association constants, DOSY experiments with cyclodextrins can be used with other techniques to predict enantiomeric discrimination (Laverde et al., 2002), to predict drug diffusion with polymeric CD (Bakkour et al., 2006), to conclude by the formation of conjugates with CDs, as for example the conjugate between CD and folic acid, studied by Clementi et al. (Clementi et al., 2010), etc. In our group, the complexes of Minoxidil (MNX) and βCD was investigated by DOSY using the pulse sequence GCSTESL (*DOSY Gradient Compensated* was *Stimulated Echo with Spin Lock)*, 25 different amplitudes of pulse gradients in each experiment with the parameters pw = 6.1s; at = 3.3 s; d1 = 3.0 s; nt = 32; lb = 0.2 Hz. The spectra of MNX, βCD and MNX:βCD are shown in the Figure 9 and the data in Table 6.

Complex	D_{MNX} (/$10^{-10}m^2s^{-1}$)	D_{CD} (/$10^{-10}m^2s^{-1}$)	D_{OH} (/$10^{-10}m^2s^{-1}$)	$P_{complexed}$ (%)
MNX free	6.363 ± 0.019	---	22.538 ± 0.087	---
αCD	---	3.449 ± 0.021	23.366 ± 0.017	19.8
α 1:1	5.811 ± 0.014	3.581 ± 0.006	22.127 ± 0.111	
βCD	---	3.254 ± 0.024	21.943 ± 0.079	91.4
L β 1:1	3.565 ± 0.058	3.302 ± 0.021	23.935 ± 0.118	
γCD	---	3.154 ± 0.012	22.082 ± 0.105	17.5
L γ 1:1	5.778 ± 0.050	3.014 ± 0.033	23.172 ± 0.119	

Table 6. MNX diffusion coefficient (D_{MNX}) free and in the presence of CDs, diffusion coefficient of α, β and γCDs and diffusion coefficients of water (D_{OH}). Values of percentages of complexed MNX with CDs

It turns out that D of HOD, free CD and MNX are quite different. Considering the size of the species in solution, the values are consistent, because the smaller hydrodynamic radius, the greater is tis coefficient. As expected, βCD complexes are those with smaller diffusion coefficient and hence the largest population of complexed species, as the more complex the MNX is with the CDs, the lower its diffusion. Finally, it was also observed that these results have small errors.

DOSY was also employed to study the interaction between 5FU, a water soluble drug and the natural cyclodextrins. The diffusion coefficients data are in Table 7. It is evident that there are no interactions between the CDs and 5FU, as also observed by using other techniques.

Sample	$D_{5\text{-}FU}$ (/$10^{-10}m^2s^{-1}$)	D_{CD} (/$10^{-10}m^2s^{-1}$)	D_{OH} (/$10^{-10}m^2s^{-1}$)	$P_{complexed}$ (%)
5FU	9.216 ± 0.028	---	22.538 ± 0.087	---
αCD	---	3.449 ± 0.021	23.366 ± 0.017	0
α 1:1	9.347 ± 0.179	3.353 ± 0.146	22.871 ± 0.086	
βCD	---	3.254 ± 0.024	21.943 ± 0.079	0
L β 1:1	9.338 ± 0.295	3.356 ± 0.086	22.879 ± 0.087	

Table 7. Free 5FU diffusion coefficient (D_{5FU}) and in the presence of CDs, diffusion coefficient of α and βCDs and diffusion coefficients of water (D_{OH}). Values of percentages of complexed 5FU with CDs

Studies were done on the complex involving the anti-helmintic drug thiabendazole (TBZ) and βCD. TBZ is a poor water soluble drug derived from benzimidazole with wide pharmacological, fungicide and bactericide applicability (Tang et al., 2005). It is believed that the enhancement of its water solubility can be achieved through formation of inclusion complexes with βCD. The data are in Table 8.

Sample	D_{TBZ} (/$10^{-10}m^2s^{-1}$)	D_{CD} (/$10^{-10}m^2s^{-1}$)	D_{OH} (/$10^{-10}m^2s^{-1}$)	$P_{complexed}$ (%)
TBZ	4.614 ± 0.709	---	16.841 ± 0.271	---
βCD	---	2.513 ± 0.038	15.568 ± 0.230	18.4
L β 1:1	4.227 ± 0.636	2.243 ± 0.019	16.821 ± 0.400	

Table 8. Free TBZ diffusion coefficient (D_{TBZ}) free and in the presence of CDs, diffusion coefficient of βCD and diffusion coefficients of water (D_{OH}). Values of percentages of complexed TBZ with CDs

The values of D for free TBZ and for the drug in the 1:1 complex was not statistically different but the lower mean value for the complex indicates encapsulation of the molecules.

3.4 The NOE experiments for structural characterization of CDs inclusion complexes: NOESY and ROESY

When two nucleus, HI and HS, are closely situated (≈ 4 A), which can be in the same molecule or due to intermolecular forces, the local field existing in both nucleus will disturb each one, causing an dipole-dipole coupling that will have a null J-coupling (JIS = 0). However it will change the spin-lattice relaxation time (T1) in the inter-nucleus environment. The dipole-dipole coupling will cause splitting of the spin energy levels of both, HI and HS. When it occurs involving the nucleus of the same specie (as hydrogen nuclei), four new energy levels (αα, αβ, βα, ββ) , as in Figure 10 (Keeler, 2002).

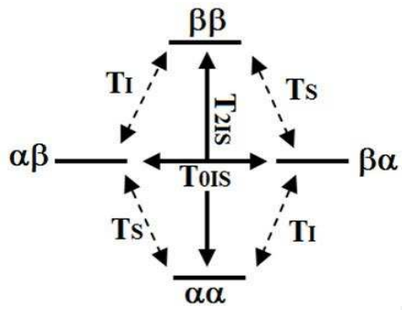

Fig. 10. Two homonuclear spin system energy levels diagram. T is respective transition occurrence probabilities. Spin states are shown for HI and HS nuclei, respectively

NOE can be defined as the change of the HI resonance intensity when HS resonance is perturbed. This can be probed applying a selective pulse into this nucleus (RF pulse applied has the same frequency as the HI Larmour frequency). Due to the dipolar coupling, this change is related to the population transition between the energy levels. While these transitions cannot be induced by another RF pulse, they can occur in the dipolar relaxation process. Initially, only αα and ββ spin energy levels are populated; however, when applying a selective pulse, all the levels become equally populated, but no change on resonance

intensity in both HI and HS is observed. When the selective pulse in HS is ended, the re-equilibrium will occurs through dipolar relaxation, according to the transitions probability T0IS and T2IS or TI/TS, (the last ones are isolated from each nucleus and, therefore, will not cause NOE), during a specific time (*mixing time*). The relaxation through the transition probability T2IS will increase the HI resonance intensity (positive NOE), and the one that occurs through T0IS will result in decrease of HI resonance (negative NOE) (Neuhaus & Williamson, 1989).

NOE measurements can be done in both steady and dynamic states. In the steady-state, HS is irradiated with a weak and continuum RF field that does not affect HI spin, until its resonance become saturated. Then, the NOE enhancement over HI is measured as the difference of its resonance intensities under HS saturation and when this condition is not applied (system in equilibrium). In the dynamic NOE measurement (NOESY, Nuclear Overhauser Enhancement SpectroscopY) HS resonance will not be saturated during the mixing time, and the NOE enhancement will depend to both nuclei magnetization amplitudes after the evolution period t1 (Figure 2). The main difference in the NOESY experiment is that the NOE enhancement intensity will be describe as three different peaks in a 2D-spectrum which can discriminate HI-HS cross-peak correlation from peaks related to other changes on HS resonance. The pulse sequences for each experiment are those reported by Keeler (Keeler, 2002).

ROESY (Rotational Overhauser Enhancement SpectroscopY) experiment was firstly developed by Bothner-By et al. (Bothner-By et al., 1984), as an alternative methodology to study NOE, and it can be done in one or two dimensions. The 1D experiment was named CAMELSPIN (Cross-relaxation Apropriate for Mini-molecules EmuLated by SPIN-locking).

The main advantage of the ROESY experiment over the traditional NOESY is the use of the *spin-lock condition*, that is done applying of a strong, constant and coherent pulse at HS Larmour frequency throughout the *mixing time.* This pulse will saturate the HS resonance (magnetization vectors projections stays precessing in the XY plane), and then, the rOe (rotational Overhauser effect) will not only be enhanced due the longitudinal magnetization components interactions, but also due to the interactions of transversal ones. The main consequence will be a positive rOe condition over all molecules. The NOE signal will be enhanced even for molecules whose w.tc (w is the Larmour frequency and t_c is the rotational correlation time) product is small, in contrast to NOESY, wherein small t_c results on negative nOe (Neuhaus & Williamson, 1989). As this small $w.t_c$ condition exist on NOE measurements of CD inclusion complexes, the ROESY experiment become suitable for structural study of these systems (Schneider et al., 1998).

Both NOESY and ROESY experiments have been widely applied for structural elucidation of guest:CD inclusion complexes, which are done through the internuclear NOE enhancement measures between the guest nuclei and the CD inner cavity nuclei H3, H5 and H6. Besides, NOE cross-peaks can be correlated to their respective internuclear distances (Evans, 1995; Pinto et al., 2005). Therefore more detailed information about the supramolecular organization of these systems can also be obtained beyond just qualitative structural analysis as with the changes in chemical shift.

NOE-based experiments are generally done in two-dimensions, as all cross-peak correlations can be seen in the spectrum, becoming easier the interpretation of the data. In

the 1-D version, the experiment must be done separately by applying the selective pulses for each nucleus, obtaining the corresponding spectrum, which turn this experiment usually more time requesting. However 1D experiment has more sensibility, which can be necessary if detection of weak NOE interactions or complex limited solubility is involved.

The literature reports several NOESY and ROESY experiments, and some examples will be commented. An extent characterization of CDs inclusion complexes with different terpenes employing NMR based on ^{1}H and ^{13}C chemical shifts analyses ($\Delta\delta$) and 2D-ROESY was reported by Bergonzi et al. (Bergonzi et al., 2007).

Interactions involving the steroids prednisolone, ethinyloestradiol and estriol with βCD were studied by the NOE-based and chemical shift analysis NMR experiments (Bednarek et al., 2002). The authors could distinguish steroids affinities with βCD through their different penetration into the CD cavity.

Inclusion complex between the drug Tenoxican and βCD was also characterized by ROESY-2D experiment (Voulgari et al., 2007) and drug molecular dimerization was studied by NOESY, showing that further description of these systems can be obtained with different NMR different experiment.

Another very interesting application of NOE-based experiments on CDs inclusion complexes provides structure elucidation between enantiomers and CDs. With the 1D-ROESY experiment, interactions between Aminoglutethimide (Elbashir et al., 2009) and propranolol (Servais et al., 2010) enantiomers with CDs could have been separately characterized. This kind of study was also done with ROESY-2D experiment for vinca alkaloids enantiomers (Sohajda et al., 2010). In a recent work, de Paula et al. explored 1D-ROESY for studying interactions involved on ternary systems of Prilocaine-cyclodextrin-liposome (Cabeça et al., 2011) and propracaine-βCD-liposome (Cabeça et al., 2008), obtaining information on the topology of the inclusion complex inside the liposome membrane.

3.5 Solid state NMR

^{13}C-Cross Polarization Magic Angle Spinning (CPMAS) NMR is other technique used to study interactions between drugs and cyclodextrins (Schneider et al., 1998). Also CPMAS measurements provides a powerful non-invasive approach to the molecular analysis of starch-related structures, its cyclodextrin characterization provides information on the molecular organization at shorter distance scales (Gidley & Bociek, 1988**).** However, this study is more complex and fails when some drug characteristics are not obey, as it will be shown.

The spectra of solids in normal conditions are broad and unresolved, providing restricted information. This phenomenon happens because not only the indirect spin-spin interaction between nuclei through bonds takes place, but also the nuclear magnets can couple through the direct interaction of their nuclear dipoles, in order of 10^2–10^4 Hz. This effect can be eliminated by applying a strong magnetic field perpendicular to the magnetic field B_0 (B_2). Other factor is the chemical shielding anisotropy, which are in the order of 103–104 Hz, due to the shielding of a specific nucleus in a time to record because the nuclei have to be allowed to relax for several minutes between pulses. This factor was solved by a process

called Cross Polarization (CP). It takes advantage of the properties of the protons coupled to the carbons, as the double irradiation process (B_0 and B_2) is used to transfer some of the proton's faster relaxation and higher magnetization to the carbon atoms (Lambert & Mazzola, 2004; Saito et al., 2006).

When the protons move onto the x-axis by a 90° pulse, a continuous y field is applied, which intensity is controlled in the equipment, to keep the magnetization precessing in that axis (*spin locking).* As soon as the ^{13}C channel is turned on, the Hartmann-Hann condition is set, i.e. ^{13}C frequency become equal to the ^{1}H frequency. In this situation all nuclei precess at the same frequency and magnetization, turning the ^{13}C higher than in normal pulse experiments, enhancing the carbon resonances and the relaxation. Finally, at the maximum intensity, the magnetic field of the ^{13}C channel is turned off and the carbon magnetization is acquired (Lambert & Mazzola, 2004).

However, broad line widths and spectra of compounds with many non-equivalent nuclei are difficult to analyze due to the strong overlap even if the contributions of dipolar ^{1}H, ^{13}C coupling are practically eliminated. So, a technique is used to observe high resolution spectra, where the sample cell is rotated around the magic angle (MAS = Magic Angle Spinning). In this experiment, the rotor is rotated with a high spinning rate around an axis which makes the magic angle of $\theta = 54.7$ ° with the axis of the external field B_o in order to vanish the chemical shift anisotropy. So, combining the MAS technique with the CP technique is possible to narrow the resonance lines and obtain the CPMAS spectra (Günter, 1994).

The confirmation of the inclusion complex between molecules and CDs is doing analyzing the differences in the chemical shifts and modification of the peaks between the free and complexed guest molecules. The appearance of multiple resonances for atoms C2, C3, C5, C4, and C6 of the glucopyranose units indicates the coexistence of different structural arrangements (Lima, 2001). Usually, the multiple resonances of the carbons of the glucose monomers tends to converge to a single peak in the inclusion compound, suggesting that the glucose units adopt a more symmetrical conformation in the complex (Lai et al., 2003). However, one has to take care in this analysis since this phenomenon can also be due to the freeze-drying process, which leads to amorphization of the sample, not indicating the host-guest interaction (Figure 11).

The imazalil :βCD complex was prepared using supercritical carbon dioxide and was characterized by CPMAS by Lai et al. The authors realized not only the conformation changing between the spectra of inclusion compound and physical mixture, but also changes in chemical shift, a marked broadening of all signals, and that several resonances of imazalil split up into multiple signals, indicative of a pronounced structural rearrangement of the imidazole and aryl rings (Lai et al., 2003) inside the CD cavity.

This technique is applied to confirm the inclusion between the CDs and polymers, as poly(ε-caprolactone) (Harada et al., 2007), comblike poly(ethylene oxide) grafted polymers (He et al., 2005) and poly(ε-lysine) (Huh et al., 2001). In these studies, they usually compared the spectrum of the complex with the physical mixture, showing that the CD molecule retain a less symmetrical cyclic conformation in the crystalline uncomplexed state, characterized by resolved C1 and C4 resonances of the glucose units, comparing with the CD in the complexed state, which has a symmetrical cyclic conformation.

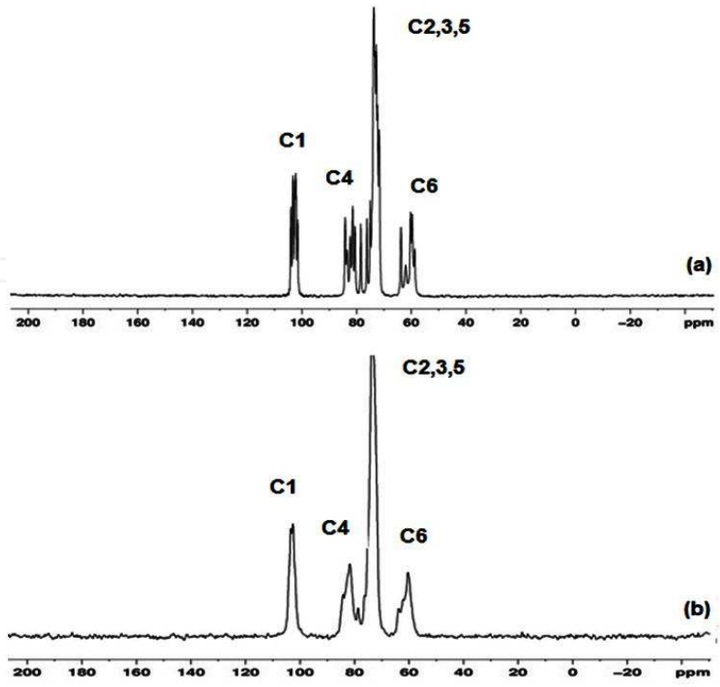

Fig. 11. ^{13}C– CPMAS NMR spectra. (a) natural; (b) freeze-dried βCD (10 kHz, 298 K)

One of the inclusion complex analyzed in our laboratory was Dapsona (DPS) and HP-hydroxypropyl-βCD. DPS (4,4'diaminodiphenylsulfone) is a very effective drug to treat leprosy and inflammatory conditions in *Pneumocystis carinii* pneumonia, toxoplasmosis and tuberculosis. However, the oral administration of this drug leads to serious side effects and treatment failures. It is believed that the complex DPS:HP-βCD would increase the wettability and the solubility of this drug for a supported and gradual release, maximizing its biodisponibility over time (Wozel et al., 1997; Chougule et al., 2008). The spectra of DPS, HP-βCD, the physical mixture and the inclusion compound are in the Figures 12-15.

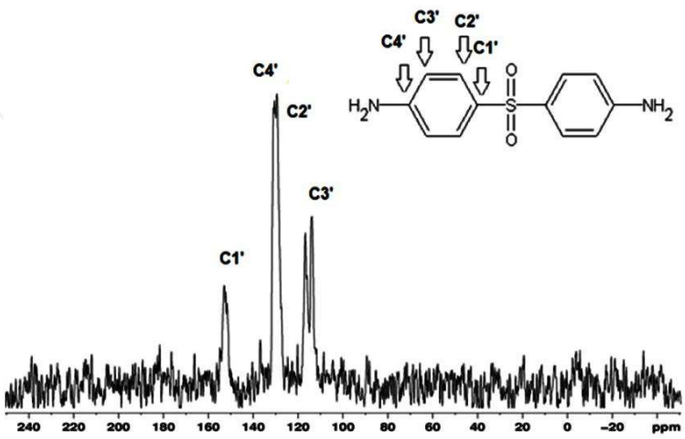

Fig. 12. ^{13}C– CPMAS NMR spectra of DPS (10 kHz, 298 K)

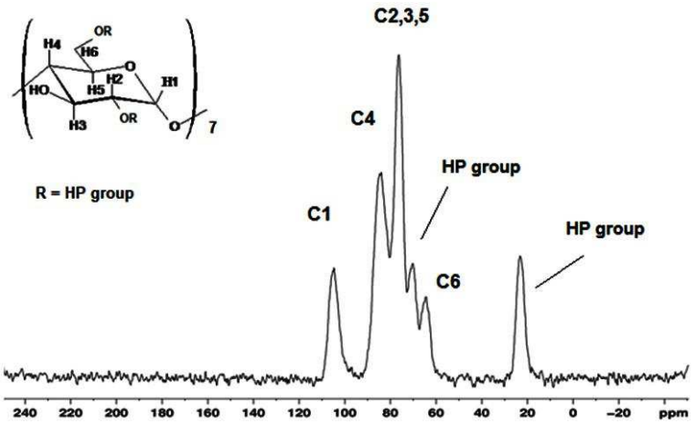

Fig. 13. ^{13}C– CPMAS NMR spectra of HP-βCD (10 kHz, 298 K)

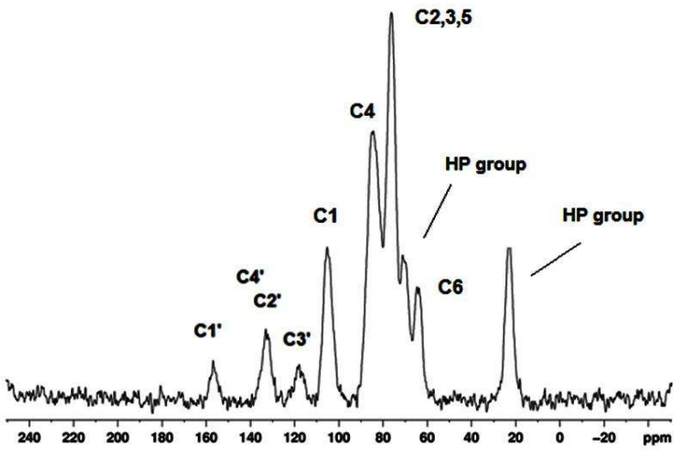

Fig. 14. ^{13}C– CPMAS NMR spectra of DPS :HP-βCD: (10 kHz, 298 K)

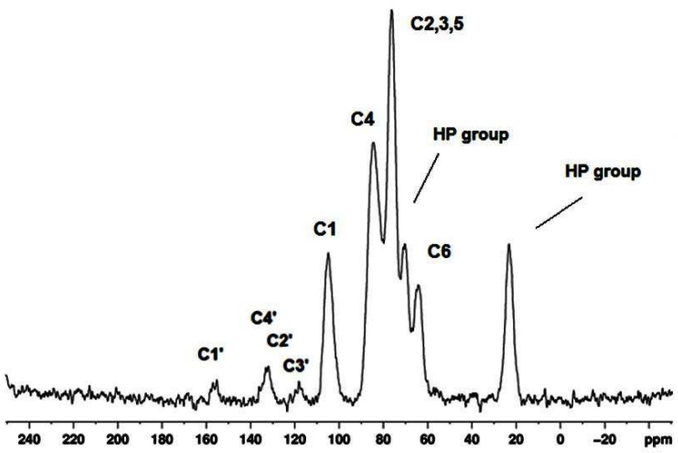

Fig. 15. ^{13}C– CPMAS NMR spectra of DPS and HP-βCD physical mixture (10 kHz, 298 K)

The ^{13}C chemical shifts for βCD, DPS, physical mixtures and complex are in the Tables 9-12.

C	$\delta_{HP\text{-}\beta CD}$	$\delta_{DPS:HP\text{-}\beta CD/PM}$	$\Delta(\delta_{DPS:HP\text{-}\beta CD} - \delta_{HP\text{-}\beta CD})$
C1	104.7	104.9	0.2
C2,3,5	76.3	76.3	0
C4	84	84.7	0.7
C6	64.3	64.3	0
HP-group	70.1; 23.2	70.3; 23.2	0.2; 0

Table 9. ^{13}C-CPMAS NMR chemical shifts of βCD and their change in the presence of DPS in the physical mixture ($\Delta\delta = \delta_{PM} - \delta_{free}$)

C	$\delta_{HP\text{-}\beta CD}$	$\delta_{DPS:HP\text{-}\beta CD}$	$\Delta(\delta_{DPS:\beta CD} - \delta_{HP\text{-}\beta CD})$
C1	104.7	105.3	0.6
C2,3,5	76.3	76.2	0.1
C4	84	84.5	0.5
C6	64.3	64.2	0.3
HP-group	70.1; 23.2	71.2; 22.9	1.1; 0.3

Table 10. ^{13}C-CPMAS NMR chemical shifts of HP-βCD and their change in the in the complex ($\Delta\delta = \delta_{complexed} - \delta_{free}$)

H	δ_{DPS}	$\delta_{DPS\ HP\text{-}\beta CD/PM}$	$\Delta(\delta_{DPS:HP\text{-}\beta CD/PM} - \delta_{HP\text{-}\beta CD})$
C1′	152.8	153.9	1.1
C2,4′	130	131.9	1.9
C3′	115.5	116	0.5

Table 11. ^{13}C-CPMAS NMR chemical shifts of DPS and their change in the presence of βCD in the physical mixture ($\Delta\delta = \delta_{PM} - \delta_{free}$)

H	δ_{DPS}	$\delta_{DPS:HP\text{-}\beta CD}$	$\Delta(\delta_{DPS:\beta CD} - \delta_{HP\text{-}\beta CD})$
C1′	152.8	156.9	4.1
C2,4′	130	133.1	3.1
C3′	115.5	118	2.5

Table 12. ^{13}C-CPMAS NMR chemical shifts of DPS and their variation in the presence of HP-βCD in the complex ($\Delta\delta = \delta_{complexed} - \delta_{free}$)

When DPS is complexed with HP-βCD Δδ is higher than in the physical mixture. Also, there is a peak broadening in the inclusion complex spectrum. As discussed before, both facts suggest that DPS is encapsulated into the cyclodextrin cavity and interacting with the hydroxypropyl group. Moreover, although it is observed a Δδ, one can clearly note that the spectrum of the physical mixture is a combination of the spectrum of HP-βCD and of DPS.

4. Experimental

4.1 Materials and methods

Dapsone was supplied by Ecofarma Farmácia Ltda.; Minoxidil by Galderma Brasil S.A.; Thiabendazole by EMS; α, β and γCD were supplied by Amaizo (American Maize-Products

Co.); hydroxypropyl-βCD and βCD were gifts from ISP Technologies, Inc.; ethyl alcohol 99.5% P.A. was purchased from LabSynth Ltda. Products for Laboratories; 99.9% deuterium oxide was purchased from Cambridge Isotope Laboratories, Inc.; Freeze-dryer FTS Systems; Bruker Avance II 300 MHz and Varian 500 MHz NMR spectrometers; rotary evaporator RE111, water bath 461 and vacuum pump Büchi Labortechnik AG.

4.2 Preparation of inclusion complex

The inclusion complexes were prepared by one of the two method, co-precipitation or freeze-drying, in an equimolar stoichoimetry.

4.3 Preparation of physical mixtures (PM)

Physical mixtures were prepared using the molar ratio of CD and drug by simply mixing the two compounds for 2 min.

4.4 NMR spectroscopy experiments

All experiments with liquid samples were run on a Varian INOVA-500 spectrometer (BO = 11 T), operating at 500 MHz for ^{1}H. The temperature was kept at 297.6 ± 0.1 K in all experiments. The chemical shifts were referenced against the HOD resonance (δ 4.67 ppm). The samples were prepared by dissolving of 2-4 mg of the FD complex in ≈ 0.6 mL of D_2O. The signal of the solvent was used to stop the magnetic field and the radio frequency. The data were acquired using standards Varian software in the following conditions and processed using the program VNMR of the equipment. To obtain the ^{1}H NMR spectra the conditions were: pw 6.1 s; at 3.3 s; d1 3.0 s; nt 32 scans; lb 0.2 Hz.

DOSY: The pulse sequence for DOSY was GCSTESL (DOSY Gradient Compensated Stimulated Echo with Spin Lock). In all analyses 25 different pulsed gradient amplitudes were: d1 6.1 s; at 3.3 s; nt 32 scans; lb 0.2 Hz.

T1 measurement: For ^{1}H-NMR, a 90° pulse was typically of 15 μs, and the recycling time was set to 15 s. Longitudinal relaxation times were obtained by the conventional inversion-recovery method.

ROESY: The ROESY experiment was carried out using the parameters: at 1.0 s; d1 3.0 s; nt 1024 scans; lb 1.0 Hz. The data was obtained applying a sequence of pulses 180^o sel. - 90^o sel. – spin lock-FID, mixing time of 500 ms. FIDs were acquired through the sequence of pulses 90^o sel. - spin lock - FID. A modulator generated the selective pulses and automatically attenuated the power and duration of the pulse.

^{13}C-CPMAS: All spectra of ^{13}C-CPMAS of lyophilized samples, physical mixtures, drugs and CDs were run on a Bruker 300 MHz at 298 K and 10 kHz.

5. Conclusion

NMR is one of the most powerful techniques to investigate interactions between guest and cyclodextrins molecules as it gives extremely useful information on physico-chemical parameters, orientation of the guest molecule inside the cavity and the complex stability.

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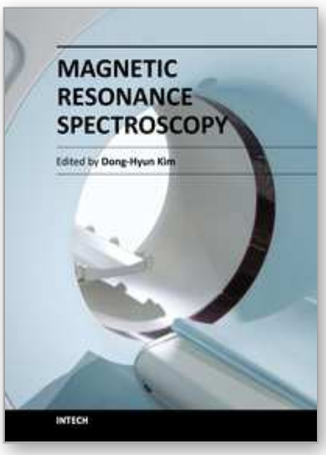

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Magnetic Resonance Spectroscopy (MRS) is a unique tool to probe the biochemistry in vivo providing metabolic information non-invasively. Applications using MRS has been found over a broad spectrum in investigating the underlying structures of compounds as well as in determining disease states. In this book, topics of MRS both relevant to the clinic and also those that are beyond the clinical arena are covered. The book consists of two sections. The first section is entitled 'MRS inside the clinic' and is focused on clinical applications of MRS while the second section is entitled 'MRS beyond the clinic' and discusses applications of MRS in other academic fields. Our hope is that through this book, readers can understand the broad applications that NMR and MRS can offer and also that there are enough references to guide the readers for further study in this important topic.

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Phone: +385 (51) 770 447
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InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
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