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Elucidating the guest-host interactions and complex formation of praziquantel and cyclodextrin derivatives by ^{13}C and ^{15}N solid-state NMR spectroscopy

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ABSTRACT

Praziquantel is the drug of choice to treat several parasitic infections including the neglected tropical disease schistosomiasis. Due to its low aqueous solubility, cyclodextrins have been tested as potential host candidates to prepare praziquantel inclusion complexes with improved solubility. For the first time, the interactions of praziquantel with β-cyclodextrin and β-cyclodextrin derivatives (methyl-β-cyclodextrin and hydroxypropyl-β-cyclodextrin) were investigated using high resolution solid-state NMR spectroscopy. The results of this work confirmed that solid-state NMR experiments provided structural characterization, demonstrating the formation of inclusion complexes most probably with PZQ adopting an *anti* conformation, also the most likely in amorphous raw PZQ. Further information on the interaction of praziquantel with methyl-β-cyclodextrin was obtained from proton rotating-frame relaxation time measurements, sensitive to kilohertz-regime motions but modulated by spin-diffusion. Evidences were presented in all cases for praziquantel complexation through the aromatic ring. In addition, 1:2 drug:carrier molar ratio appears to be the most probable and therefore suitable stoichiometry to improve pharmaceutical formulations of this antischistosomal drug.

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1. Introduction

Praziquantel (PZQ, Scheme 1), (*RS*)-2-(Cyclohexylcarbonyl)-1,2,3,6,7,11b-hexahydro-4*H*-pyrazino[2,1-*a*]isoquinolin-4-one, is the basis of the treatment of all forms of the neglected tropical disease schistosomiasis (Dömling and Khoury, 2010) which is the second most prevalent parasitic disease after malaria and has been estimated to affect around 207 million people (Jenkins-Holick and Kaul, 2013). The drug is currently manufactured and administered as a racemate but only *S*-PZQ has pharmacologic effect. The *R* isomer apparently contributes only to the bitter taste of the drug (Meyer et al., 2009). The preparation of PZQ as a single enantiomer was already described (Woelfle et al., 2011) and it was reported on a preparative scale using high-performance liquid chromatography (HPLC) equipped with a chiral column functionalized with β-cyclodextrin (Liu et al., 2004 and references there in). It was found that crystalline enantiomers and the racemic commercialized drug exhibit different thermal properties (el-Arini et al., 1998). While similar melting points and enthalpies of fusion were obtained for both enantiomers (110.6 °C and 18.48 kJ/mol for (*S*)-PZQ), significantly higher values were found for racemic PZQ (136.2 °C and 25.73 kJ/mol) (Liu et al., 2006) and this fact indicated that the drug is a racemic compound, not a racemic conglomerate, as also confirmed by X-ray powder diffraction (XRPD) and infrared (IR) spectra (el-Arini et al., 1998). It is well known that most of the chiral systems (90–95%) form racemic compounds and that, unlike conglomerate forming chiral substances, crystallization does not provide the pure enantiomers (Lorenz et al., 2006). Therefore, PZQ molecules have an affinity for the opposite enantiomer greater than for the same enantiomer, and the two enantiomers are present in the 1:1 ratio in the crystal unit cell.

Abbreviations: PZQ, praziquantel; CD, cyclodextrin; β -CD, β -cyclodextrin; Me-β-CD, methyl-β-cyclodextrin; HP-β-CD, hydroxypropyl-β-cyclodextrin; XRPD, X-ray powder diffraction; CP/MA, Scross polarization/magic-angle spinning; TOSS, total suppression of spinning sidebands; SELTICS, sideband elimination by temporary interrup tion of chemical shift; FWHM, full width at half maximum.

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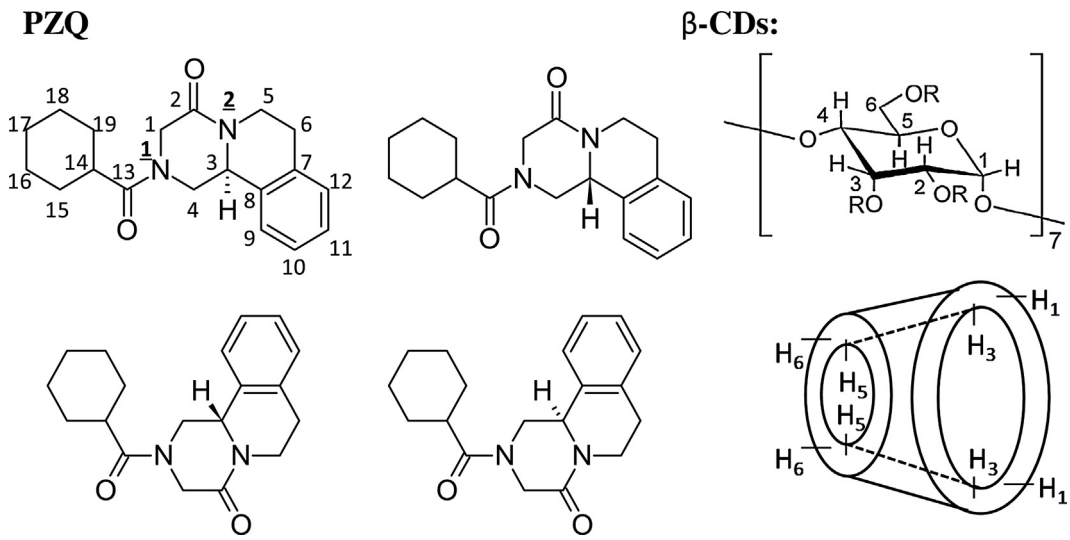

Scheme 1. PZQ: chemical structure of praziquantel enantiomers (*S*-PZQ and *R*-PZQ, on the left- and right-side, respectively) and rotational conformers *anti* (top) and *syn* (bottom). β-CDs: chemical structures of the β-CDs used in this study where R = H (β-CD), R = H or CH_3 (Me-β-CD) and R = H or $CH_2-CH(OH)-CH_3$ (HP-β-CD).

PZQ, the drug of choice for schistosomiasis treatment, is classified as Class II in the Biopharmaceutics Classification System (Amidon et al., 1995), as its low solubility hinders its performance in biological systems (Rodrigues et al., 2011). Large doses are required in order to achieve adequate concentrations at the target site due to the poor aqueous solubility of PZQ (0.04 g/100 ml) (el-Arini et al., 1998; De La Torre and Torrado, 1999) and its significant first pass metabolism. In order to increase drug absorption and effectiveness, formulations that improve the apparent aqueous solubility of PZQ without reducing its lipophilicity are required. Common formulations include drug:cyclodextrin complexation (Loftsson et al., 2005), which is also the approach followed in the present work.

Cyclodextrins (CDs, Scheme 1) are cyclic oligosaccharides with the (α-1,4-)-linked α-D-glucopyranose units rearranged in a toroidal shape. While the outer surface is hydrophilic, the lipophilicity of the inner cavity is comparable to that of an aqueous ethanolic solution (Frömming and Szejtli, 1994). CD complexes have been successful tested as useful tools to increase the solubility and bioavailability of lipophilic drugs in aqueous environments. The drug molecule or just a lipophilic moiety is encapsulated into the toroid cavity. The complexation does not involve the formation or cleavage of covalent bonds but rather release of water molecules from the cavity, loss of conformational strain, the establishment of hydrogen bonds and several other interactions such as electrostatic, van der Waals', hydrophobic, and charge-transfer interactions (Loftsson and Brewster, 1996; Liu and Guo, 2002). In this regard, it was reported that the dissolution of complexes of PZQ with α-, β- and γ-CD was 2.6-, 5- and 8-fold greater than that of the pure drug, respectively (Becket et al., 1999). However, only the β-complex has a stability constant adequate for pharmaceutical use, suggesting that the best candidate for further formulation development would be a water-soluble β-CD derivative (Becket et al., 1999). But a CD with higher aqueous solubility than β-CD (limited to 16 mM due to strong binding of CD molecules in the crystal state) (Loftsson and Brewster, 1996; Jesus et al., 2006) would be certainly a more suitable candidate for the preparation of PZQ inclusion complexes. With random substitution of the hydroxy groups, which includes hydrophobic moieties, occurs a remarkable solubility improvement because amorphous mixtures of isomeric derivatives are then obtained. This is the case of some β-CD derivatives like hydroxypropyl-β-cyclodextrin (HP-β-CD) and the randomly methylated β-cyclodextrin (Me-β-CD) (Loftsson et al., 2005). In solution, the solvent effect must be taken into account and drug molecules in the complex are in rapid equilibrium with free molecules (Wenz, 1994). Therefore, using a non-destructive technique like solid-state NMR (ssNMR), which has already became a helpful technique to the pharmaceutical industry (Berendt et al., 2006), further structural elucidation on the inclusion complexes is expected to be provided. Recently we reported ssNMR studies on albendazole (Ferreira et al., 2015) and benznidazole (Priotti et al., 2015) interactions with several CD derivatives but, to the best of our knowledge, no ssNMR study was ever to date reported on PZQ:CD complexes. Additional evidence on solid PZQ:β-CD complex structure is needed because the presence of solvents may induce a PZQ conformational change as previously reported for PZQ enantiomers in the form of hemihydrates when compared with *RS*-PZQ (Liu et al., 2006; Espinosa-Lara et al., 2013). The objective of this study is to use ssNMR to obtain evidence for the presence of inclusion complexes without the solvent effect, therefore not submitting the samples to any previous treatment.

2. Materials and methods

2.1. Materials

RS-PZQ (lot 090,608, 99.4% purity) was purchased from Romikin (Buenos Aires, Argentina). β-CD (>99% purity), HP-β-CD (estimated mol. wt. ≈ 1396, average degree of substitution 0.9; purity >98%), and Me-β-CD (estimated mol. wt. ≈ 1310, average degree of substitution 1.8; purity >98%) were purchased from Sigma–Aldrich (Milwaukee, WI, USA). All other chemicals and solvents used in this study were of analytical reagent grade.

2.2. Preparation of the PZQ-CDs binary systems and physical mixtures

Binary PZQ, β-CD, HP-β-CD and Me-β-CD (1:1 and 1:2 drug: carrier molar ratio) systems were prepared by means of the solvent evaporation technique, as previously described for benznidazole samples (Leonardi et al., 2013). Briefly, an accurately amount of PZQ (500 mg) was dissolved in 20 ml of ethanol and the corresponding amount of CD was dissolved in 20 ml of water, to obtain the drug:CD ratios of 1:1 and 1:2, respectively. The solvents were removed under vacuum at 40 °C in a rotatory evaporator (150 rpm). The solid residue was dried under vacuum (3 h). Then,

the dry mixture was ground in a mortar and then passed through a 40-mesh metal screen. The resulting powders were kept in a desiccator until further use. For comparison, PZQ:CD physical mixtures (PM) (1:1 and 1:2 drug:carrier molar ratio) were prepared by homogeneous blending in a mortar (15 min). Each resulting mixture was dried under vacuum (2 h) and stored overnight in a desiccator. Then, the solid was ground in a mortar and then passed through a 40-mesh metal screen. The resultant powders were kept under vacuum until further studies. Amorphization of PZQ was performed by heating the drug up to 150 °C and subsequent quench cooling with liquid nitrogen, as described elsewhere (Costa et al., to be published).

2.3. X-ray powder diffraction

XRPD analysis was carried out in a Bruker D8 Advance diffractometer (Bragg Brentano geometry), with a Cu anode source (λ = 1.54060 Å) and a secondary monochromator, operating at 40 kV and 30 mA, and data were acquired at room temperature in the range 2θ = 3–40°.

2.4. ^{13}C Solid-state NMR spectroscopy

PZQ and PZQ:CDs (~200 mg), as powders, were packed into 7 mm o.d. cylindrical zirconia rotors (4 mm rotor was also used just for PZQ observation). ^{13}C cross polarization/magic angle spinning (CP/MAS) spectra were obtained at 75.49 MHz on a Tecmag Redstone/Bruker 300 WB spectrometer at a rate of 3.2–4.1 kHz with 90° RF pulses of about 4 μs, contact time of 3 ms and a relaxation delay of 20 s and 10 s for PZQ and PZQ:CDs samples, respectively. The significant contributions of the ^{13}C spinning sidebands, particularly in the aromatic and carbonyl regions, were eliminated by running the spectra with the SELTICS sequence (Sideband Elimination by Temporary Interruption of Chemical Shift) (Hong and Harbison, 1993), or the TOSS sequence (Total Suppression of Spinning Sidebands) (Dixon et al., 1982). CP/MAS spectra with suppression of ^{13}C non-quaternary signals was achieved by interrupting proton decoupling during 40 μs before the acquisition period. ^{15}N CP/MAS was observed at 30.415 MHz, using the conventional CP RF pulse sequence, a recycle delay of 20 s, 3 ms contact time, 90° RF pulse of 6 μs and a MAS rate of 3.3 kHz; concerning the number of transients, were acquired about 4800 for PZQ, 8600 for β-CD, 12,000 for Me-β-CD and 12,900 for HP-β-CD. ^{13}C and ^{15}N chemical shifts were referenced with respect to external glycine (^{13}CO observed at 176.03 ppm and $^{15}NH_2$ observed at 32.4 ppm in the liquid NH_3 scale). In order to deconvolute ^{13}C CP/MAS signals were used Gaussian functions as input to obtain the fitting curves by the iterative method of nonlinear least squares, available in Origin (Microcal Software, Inc., USA). A similar procedure was followed to simulate ^{15}N CP/MAS spectra.

The proton relaxation time in the rotating frame ($^{H}T_{1\rho}$) was measured by recording the carbon signal as a function of the ^{1}H spin-locking time in the range 10 μs–14 ms (17 values), before the CP period in ^{13}C CP/MAS experiments (for crystalline PZQ were selected 19 values and a maximum spin-locking period of 18 ms). A frequency field of 55.6 kHz was used for the spin-lock field B_1. The recycle delay was 10 s (or 20 s for crystalline PZQ), the number of transients was between 42 and 168. A contact time of only 400 μs was selected to minimize ^{1}H spin-diffusion during this period. $^{H}T_{1\rho}$ data were obtained using the exponential function that characterizes the rate of decay of the spin-locked carbon magnetization, $M(t) = M_0 \exp(-t/T_{1\rho})$, where $M(t)$ is the magnetization at time t and M_0 is the magnetization recorded without ^{1}H spin-locking time previous to the CP period, to fit the experimental data (the intensity of the peaks of interest as a function of the spin-locking

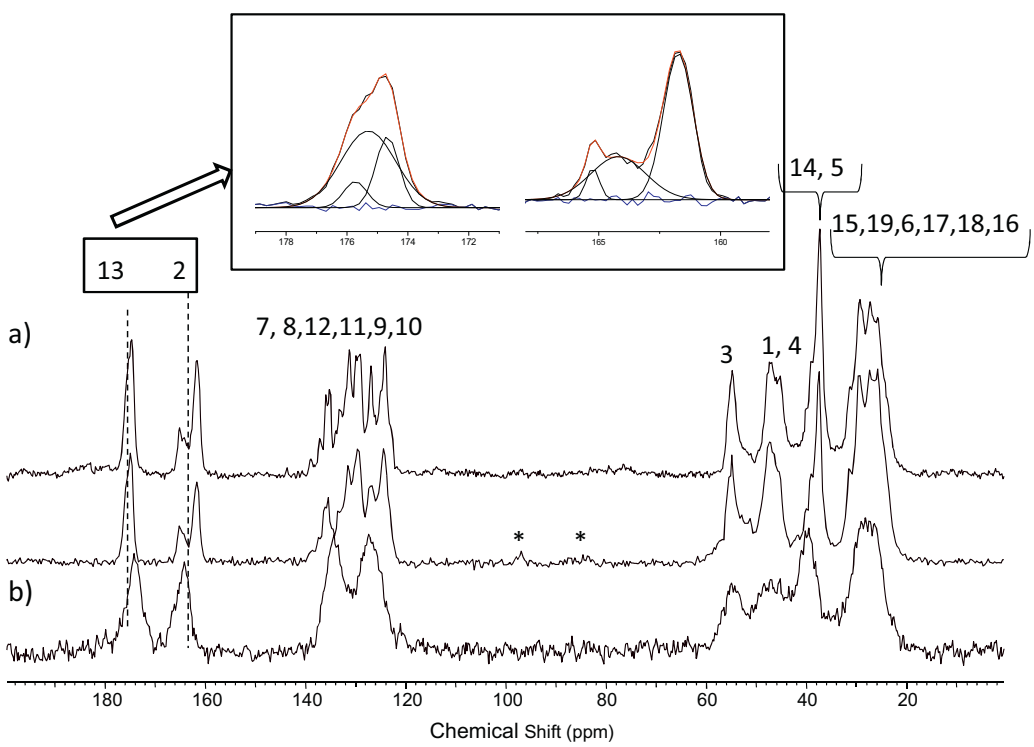

Fig. 1. ^{13}C CP/MAS spectra obtained from PZQ as: (a) crystalline powder with 4.1 kHz MAS rate, using 7 mm o.d. rotor, with the TOSS sequence (top) and 5.9 kHz MAS rate, using 4 mm o.d. rotor (middle); (b) amorphous sample, under 3.2 kHz MAS rate with the TOSS sequence. Dashed lines are just guides to the eye. The inset presents the deconvolution of C2 and C13 resonances using three Gaussian functions for each type of carbon (dashed lines, see Section 2.4 for details); dot lines (red and blue lines on the web) show the fitting curve and the line fitting residues, respectively, and Table 1 presents the fitting parameters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

time) by the least-squares method. For this purpose was used the software delivered by Tecmag Inc. (Houston, USA). The relaxation measurements were always performed at 293 K.

3. Results and discussion

3.1. XRPD analysis of PZQ

Fig. S1 (see Suplementary material) shows the diffractogram obtained from PZQ. The most intense peaks are observed at 15.347°, 16.385° and 20.151° (2θ). Comparison of the XRPD patterns with previous published data (Espinosa-Lara et al., 2013) enables identifying PZQ as (*RS*)-PZQ.

3.2. ^{13}C CP/MAS NMR spectra of PZQ

The ssNMR study of crystalline and amorphous PZQ was performed as an essential part for the understanding of the NMR data obtained from mixtures of PZQ and CDs. Fig. 1a shows typical ^{13}C CP/MAS spectra obtained from crystalline PZQ which were run either with or without suppression of the spinning sidebands. The spectra display more than 19 resonances, consistent with more than one molecule in the asymmetric unit. Table 1 presents the chemical shifts (δ, ppm) of the carbon resonances (also assigned in Fig. 1), and δ values predicted using the software provided by the authors of the algorithm to simulate ^{13}C spectra (Castillo et al., 2011), which are present for comparison.

RS-PZQ crystallizes with four molecules in the asymmetric unit, two of which are disorder, and this fact did not hamper establishing that only *syn*-conformation is present (Espinosa-Lara et al., 2013). X-ray data show that the two PZQ amide groups are just able to participate in hydrogen bonding interactions through the oxygen atoms because the free electron pairs of the nitrogen atoms are involved in π-electron delocalization. Also, there are three types of C—H acidic hydrogen atoms for the generation of attractive secondary C—H···O contacts situated in (i) the

Table 1
^{13}C and ^{15}N CP/MAS chemical shifts (δ/ppm) obtained from PZQ and from PZQ:β-CD, PZQ:Me-β-CD and PZQ:HP-β-CD complexes, with 1:1 (drug: carrier) molar ratio.

^{13}C or ^{15}N					
C or ***N*** number	PZQ (**amorphous**)	PZQ (predicted)[f] (*syn or anti*)	PZQ:β-CD	PZQ:Me-β-CD	PZQ:HP-β-CD
1	47.30	48.90	–	–	–
1	(–)		*127.9*[c],*123.11*	*123.11*	*126.32*[e]
	126.32[c],*123.91*[c],*122.30*		162.31, 165.3[e]	162.5, 165.3	162.3, 165.8
			100.63,*107.85*	*103.04*[e]	*107.05*
2	161.73, 165.24, 164.20[a]	164.50			
2	**(164.3)** *106.64*[c], *107.45*,*110.26*[d]				
3	54.92, 55.80[c]	55.20	54.92	–	55.1
	(55)[e]				
4	45.56	45.40	45.99	45.9	45.8
	(–)				
5	40.30[d],39.24, 37.50	39.00	39.45, 37.93	38.6	–
	(39.9)[e]				
6	27.48	28.80	27.04, 27.69	–	26.4
	(–)				
7	135.30, 135.96	135.00	135.08	135.7	133.1[e]
	(134.2)[e]				
8	131.38	132.80	132.03	–	133.1[e]
	(134.2)[e]				
9	127.02	127.5	127.24	127.2	127.5
	(127.0)[e]				
10	124.19	126.08	124.62	–	–
	(127.0)[e]				
11	129.20	128.48	129.63	129.4	129.4
	(127.0)[e]				
12	130.07	128.96	130.07	134.4	133.1[e]
	(134.2)[e]				
13	174.67, 175.30, 175.73[b]	174.50	174.94, 176.25	174.3[e]	175.1[e]
	(174.1)				
14	40.30[d],39.24, 37.50	40.80	40.54	39.9, 40.5	37.7, 40.3
	(39.9)[e]				
15	31.62	29.20	29.90	–	–
	(–)				
16	24.43[e]	25.33	–	–	–
	(–)				
17	26.82	26.32	26.17	–	26.4
	(–)				
18	25.74	25.33	–	–	–
	(–)				
19	29.43	29.20	29.00	29.7	29.0
	(–)				

^{13}C δ values for the PZQ amorphous form are in bold and between parentheses. ^{15}N parameters are in italic and underlined.
[a] From deconvolution of C2 signal using Gaussian functions with the following parameters (from low to high chemical shift): FWHM 1.22, 2.25 and 0.54 (ppm) and integrals (%) 61, 33 and 6.
[b] From deconvolution of C13 signal using Gaussian functions with the following parameters (from low to high chemical shift): FWHM 0.77, 1.80 and 0.77 (ppm) and integrals (%) 26, 65 and 9.
[c] Shoulder.
[d] Low intensity.
[e] Broad.
[f] Castillo et al., 2011.

tetrahydroisoquinoline segment, (ii) the piperazinone ring, and (iii) the cyclohexylcarbonyl moiety. Three homodimeric motifs were recognized in *RS*-PZQ structure consisting of molecules, designed here as PZQ1 and PZQ2, which are interacting as following: (a) C2O and H6 in PZQ1 are hydrogen bonded to H4 and C2O in PZQ2, respectively, (b) C13O in PZQ1 is hydrogen bonded to the H16, H14, H4 and H9 in PZQ2 and (c) CH5 in PZQ1 forms a CH···π complex with the aromatic ring in PZQ2. In addition, single-bridged CH···O and CH···π complete the characterization of the resulting 2D hydrogen bonded network (Espinosa-Lara et al., 2013). On the whole, in PZQ crystal structure were identified three types of dimers, but only one presents two different hydrogen bonds which involve C2O and acidic hydrogen atoms (Espinosa-Lara et al., 2013).

In the crystalline PZQ spectrum, several signals assigned to C2 were identified that differ by more than 2 ppm. At least two hypotheses can explain such observation:

a.) C2 signals are assigned to one molecule. C2 resonance appears as an asymmetric doublet due to the interaction with the directly bonded quadrupolar nucleus ^{14}N in the isoquinoline ring, which produces a residual dipolar splitting of about 240 Hz; the downfield signal is the less intense one. Unique to ssNMR is the possibility of observing a broadening and/or splitting of the resonance signals of carbons close to quadrupolar nuclei with electric field gradient (EFG) different from zero. EFG is provided by the electrons surrounding the nitrogen nucleus and depends on its chemical environment. However, this effect is usually observed at static magnetic fields lower than 7 T, which was the field used in the present study (Naito et al., 1981).

b.) C2 signals are assigned to more than one molecule. Gaussian functions were used to deconvolute the C2 signals (Fig. 1) and the corresponding parameters are shown in Table 1. The presence of C2 signals at higher resonance frequency (165.24 and 164.20 ppm) reflect the presence of about 39% PZQ molecules (corresponding to the integral of both signals) with C2O involved in hydrogen bonding; other molecules (about 61%, determined from the integral) have a C2 chemical shift of 161.73 ppm. It is worth mentioning that the peak at 164.20 ppm has the highest full width at half maximum (FWHM, 2.25 ppm) thus reflecting the lack of short range order in some PZQ molecules (33%), present in amorphous form.

Several evidences support the hypothesis b that is the assignment of C2 signals to more than one PZQ molecule. First of all, deconvolution data obtained for C13 signal is consistent with the presence of at least three molecules in the asymmetric unit and the integrals (%) and the FWHM (ppm) of the Gaussian functions correspond well to the species identified in C2 signal analysis: integrals (%) 26, 65 and 9, and FWHM (ppm) 0.77, 1.80 and 0.77 (from low to high chemical shift). Moreover, the spectra recorded from the PZQ:CDs binary systems (see next section) display an intensity variation of C2 components, which is consistent with hypothesis b.

The chemical shift ranges obtained for C2O and C13O multiple signals (161.73 to 165.24 ppm and 174.67 to 175.73 ppm for C2O and C13O, respectively) indicate the presence of different types of hydrogen bonding in PZQ structure (higher chemical shifts correspond to stronger hydrogen bonds, involving shorter CO···H distances).

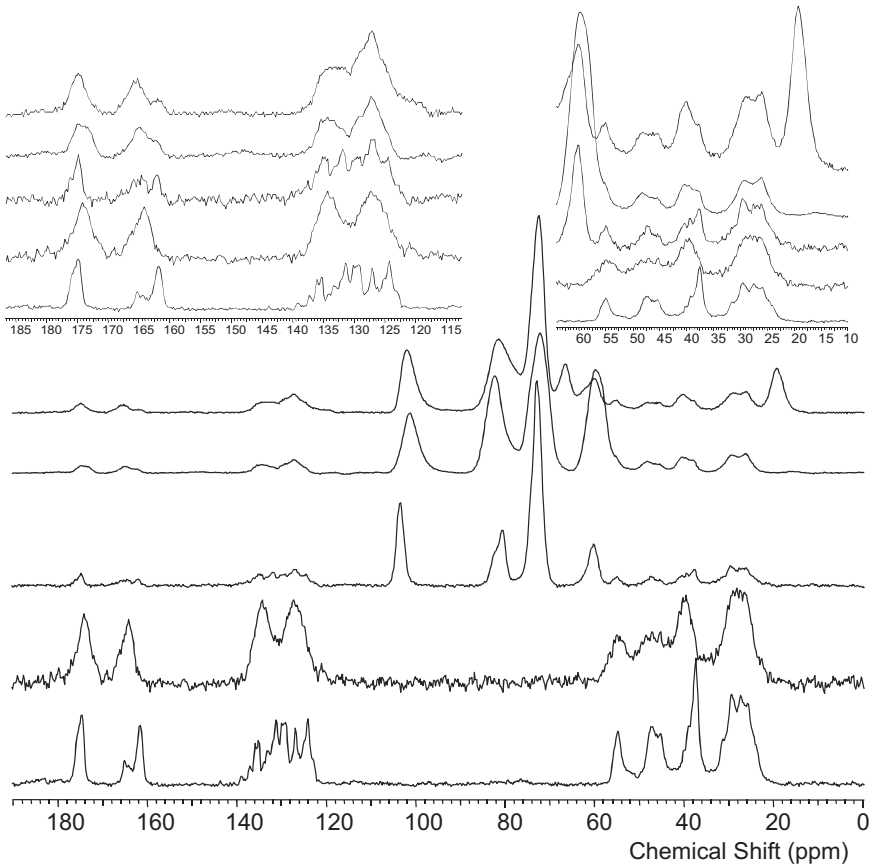

Fig. 2. ^{13}C CP/MAS spectra obtained obtained from crystalline PZQ, amorphous PZQ and from PZQ and β-CD, PZQ and Me-β-CD and PZQ and HP-β-CD (1:1 molar ratio, from bottom to top) systems. All PZQ signals are also shown with an expanded vertical scale.

Such observation could be due to the presence of rotational conformers with *syn* and *anti* orientation (see Scheme 1), which was only reported on X-ray structure of PZQ hemihydrates not on (*RS*)-PZQ (Espinosa-Lara et al., 2013). PZQ enantiomers in the form of hemihydrates, (*R*)-PZQ·0.5H_2O and (*S*)-PZQ·0.5H_2O adopt the *anti*-conformation (Meyer et al., 2009; Liu et al., 2006) while in (*RS*)-PZQ all four asymmetric unit PZQ molecules present *syn*-conformation, two of which being disordered, as already pointed out (Espinosa-Lara et al., 2013). However, according to XRPD data (see Section 3.1) there are no PZQ hemihydrates in the sample under study.

At this stage, it is not possible to assign C2 and C13 signals to the presence of different PZQ conformers. However, this study provides evidence for at least two carbonyls C2O involved in hydrogen bonding because were identified two signals shifted to lower magnetic field. Such C2O interactions are only present in one of the three homodimeric motifs reported on X-ray structure (Espinosa-Lara et al., 2013): C2O and H6 in PZQ1 are hydrogen bonded to H4 and C2O in PZQ2, respectively.

Several differences may be pointed out when comparing crystalline and amorphous PZQ spectra, which are both shown in Fig. 1. Two broad signals are assigned to the carbons in the aromatic ring of amorphous PZQ, with maximum intensities at 134.2 ppm (carbons 7,8,12) and 127.0 ppm (carbons 9,10,11); other broad resonances are observed at about 28, 40, 47 and 55 ppm, while both C13O and C2O carbonyl signals, at 174.1 and 164.3 ppm, are less broad but appear shifted to higher and lower magnetic field, respectively, when compared to crystalline PZQ signals (Fig. 1). As far as amorphous PZQ is concerned, C2O and C13O chemical shifts are consistent with different hydrogen schemes being dominant in this form, which favours the involvement of C2O but not C13O. Moreover, the fact that resonances from carbon next to the carbonyl groups (1,14) and nitrogen atoms (particularly 4,5) are the most affected by amorphization points to the presence of a different conformer (*anti*) being the dominant species.

3.3. ^{13}C CP/MAS NMR spectra of PZQ and PZQ-CDs binary systems

Figs. 2 and 3 show the spectra obtained from mixtures of PZQ and β-CD with 1:1 and 1:2 drug:carrier molar ratio, respectively (crystalline PZQ and β-CD spectra are also presented for comparison). The four major signals were obtained from β-CD and are broad. They are assigned to the different sugar carbons in glucose subunits of β-CD and the chemical shifts (from the 1:1 mixture) are as follows: C6 at 60.15 ppm; C2,3,5 at 72.99 ppm; C4 at 80.62 ppm and anomeric C1 at 103.49 ppm. The spectrum recorded from pure β-CD presented better resolution as reported recently elsewhere (Ferreira et al., 2015; Priotti et al., 2015). Table 1 shows the chemical shifts obtained for PZQ resonances in PZQ: β-CD (1:1) complex. Comparatively to the spectrum of crystalline PZQ, the spectrum from 1:1 sample still presents PZQ resolved resonances (Fig. 2); however, a strong intensity decrease is noticed for both the C2 signal at high magnetic field and C14,5 superimposed signals. Clearly, such result enabling the recognition of several PZQ molecules in different chemical environments, including uncomplexed PZQ, is not compatible with the presence of a unique complex with 1:1 stoichiometry. The C2 signal at high magnetic field and the C14,5 superimposed signals identified in the spectrum of pure PZQ are absent in the 1:2 spectrum (Fig. 3) which also do not display any well resolved PZQ resonance. Therefore, in this system, PZQ molecules are all in the amorphous form and free PZQ is no more detected. This result

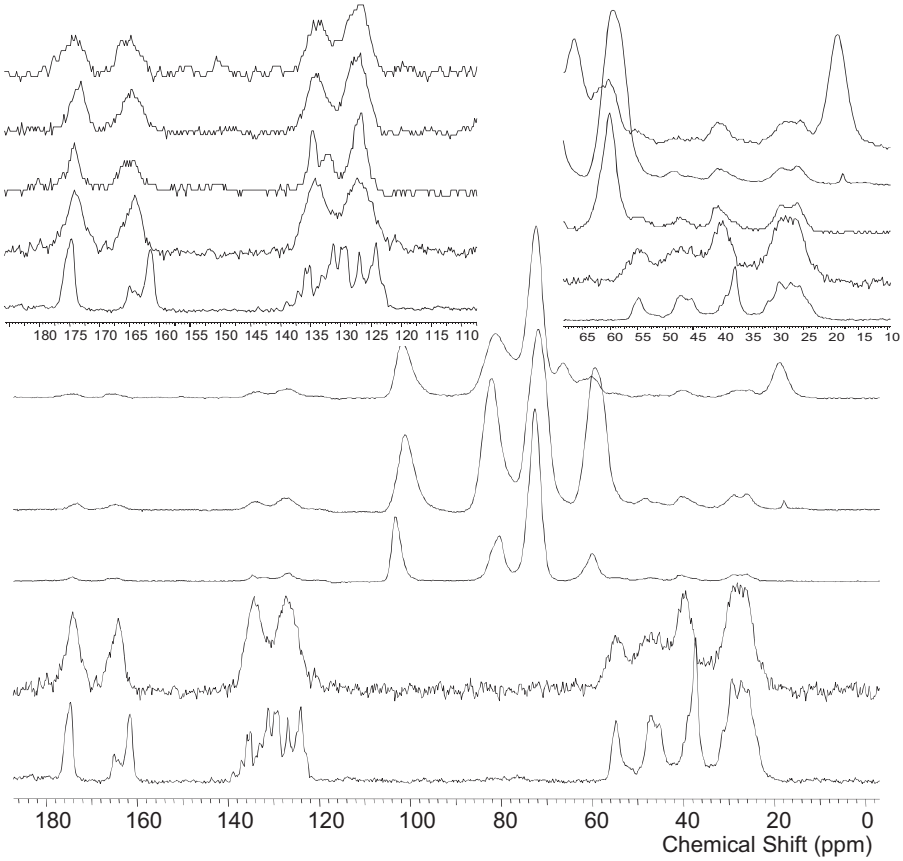

Fig. 3. ^{13}C CP/MAS spectra obtained from crystalline PZQ, amorphous PZQ and from of PZQ and β-CD, PZQ and Me-β-CD and PZQ and HP-β-CD systems (1:2 molar ratio, from bottom to top). The spectral regions showing PZQ resonances are represented with an expanded vertical scale.

is consistent with PZQ and β-CD complexation, most probably involving 1:2 guest:host molar ratio, but it is not possible to infer about a PZQ conformation change having occurred during complexation (more detailed discussion is provided for PZQ:Me-β-CD system). It was already reported a discrepancy on the complex molar ratio obtained from computer modelling and ^{1}H solution NMR data (1:1) with the value from elemental analysis of the solid material (1:1.7) (Becket et al., 1999); such result suggested the presence of uncomplexed CD or formation of complexes of mixed stoichiometry. However, other authors reported that the solubility in water of both PZQ isomers and the racemic PZQ in the presence of β-CD was consistent with 1:1 complex formation (Becket et al., 1999). Further evidences were provided for the presence of 1:1 PZQ:β-CD complex from ^{1}H liquid-state NMR experimental and theoretical analysis data, when studying inclusion complexes at two different stoichiometries (1:1 and 1:2) (Jesus et al., 2006). This ssNMR study clearly reveals that, in the absence of a solvent effect, the complex molar ratio is most probably 1:2. In solution, ^{1}H 2D-NMR revealed that the isoquinoline ring of PZQ is located inside the β-CD cavity and phase-solubility diagrams showed a five-fold increase in PZQ water solubility upon addition of increasing β-CD concentrations up to the limit of the solubility of β-CD itself (Jesus et al., 2006).

Figs. 2 and 3 display the spectra obtained from PZQ:Me-β-CD system at 1:1 and 1:2 drug:carrier molar ratio, respectively. Like in PZQ:β-CD spectra, the signals obtained from Me-β-CD are broad. They are assigned as follows: C6 at 59.71 ppm; C2,3,5 at 72.13 ppm; C4 at 82.37 ppm; anomeric C1 at 101.32 ppm and the group substituent CH_3 at 59.7 ppm. Table 1 presents the chemical shifts obtained for PZQ resonances in PZQ:Me-β-CD (1:1) complex. Clearly, PZQ signals are strongly broadened when the drug is in the presence of CD, in particular those from carbonyl and aromatic groups. The fact that the line widths of PZQ are considerably higher than in the spectrum of the pure drug suggests that PZQ molecules are in a less ordered environment, therefore in an amorphous form. Comparatively to pure PZQ, the C2 signal at higher magnetic field is now much less intense (in 1:1) or missing (in 1:2), and a strong intensity decrease is observed for C14,5 superimposed signals. These evidences point to the presence of a complex PZQ: Me-β-CD most probably with 1:2 molar ratio. A detailed spectral analysis supports this conclusion. Fig. S3 (see the Supplementary data file) shows ^{13}C CP/MAS sub-spectra obtained from PZQ:Me-β-CD binary systems with the molar ratios 1:1 and 1:2. Gaussian functions were used to obtain the curves to fit PZQ signals (carbonyl and aromatic ring resonances) and the signal of C1 in Me-β-CD (anomeric carbon). Fig.S3 also shows that three or single Gaussian functions were used to fit each carbonyl signal from 1:1 or 1:2 systems, respectively. Table S1A presents the NMR parameters with the integrals of these functions expressed as a percentage of the total area of each peak. Clearly, only one type of PZQ molecule is present in 1:2 system because only one Gaussian function was used to fit the signal from each carbon species. The integrals obtained from C1 (Me-β-CD) and from C9,10,11,12 (PZQ aromatic ring) (Fig. S3 and Table S1B) were used to obtain the stoichiometry of the complex based on the fact that carbons with the same multiplicity cross-polarize at similar rates: [PZQ]/[Me-β-CD] = {(integral of C9,10,11,12)/4}/{(integral of C1)/14} = 0.08(4)/0.18(4) = 1/2.19. This result confirms the presence of a complex PZQ:Me-β-CD with the stoichiometry 1:2. The existence of signals with different broadening could reveal molecular motion diversity. On the other hand, because at least three types of PZQ molecules are present in the system 1:1, is not reasonable to perform calculations based on signal deconvolution of the aromatic methine carbons (only four Gaussian functions are plotted in Fig.S3; at least six functions would be needed to obtain the fitting curve but the low spectral resolution does not allow a unique solution to be obtained). However, the identification of free PZQ signals (C2 and C13 at 165.14 and 175.07 ppm, respectively, from Table S1B) excludes the presence of a unique 1:1 complex in this system.

It was already suggested that the association of PZQ with Me-β-CD could be a good alternative for the treatment of schistosomiasis (Rodrigues et al., 2011); phase solubility diagrams showed that there was a 5.5-fold increase in PZQ solubility and no *in vitro* cytotoxicity was observed for the PZQ:Me-β-CD inclusion compound. Physico-chemical characterization of the complex was performed (Rodrigues et al., 2011); in particular, SEM showed a change of the morphological characteristics of PZQ:Me-β-CD crystals and IR data presented changes after interaction with Me-β-CD which included effects on the C–H of the aromatic ring. However, it was reported that complexation occurred in a 1:1 molar ratio, as revealed by the lack of a PZQ transition temperature in DSC thermograms after PZQ inclusion into the Me-β-CD cavity (Rodrigues et al., 2011). It must be pointed out here that the low enthalpy of raw PZQ (ΔH = 86.5 J/g) may prevent the observation of a clear endothermic peak (De La Torre and Torrado, 1999). Also, the presence of a stable amorphous form is not necessary an indication of the presence of a stable complex. Although being metastable, the amorphous state may be stabilized, for example, when dynamic heterogeneity does not favour crystallization (Nunes et al., 2014).

Figs. 2 and 3 present the spectra recorded from PZQ-HP-β-CD systems at 1:1 and 1:2 drug:carrier molar ratio, respectively. The resonances from HP-β-CD are also broad, as already pointed out for the other CDs. The assignment is as follows: C6 at 60.15 ppm; C2,3,5 at 72.56 ppm; C4 at 81.49 ppm; anomeric C1 at 101.97 ppm and the group substituent $CH_2CH(OH)CH_3$ at 61.86 (shoulder), 66.46 and 19.41 ppm. As for PZQ:Me-β-CD spectra (Fig. 3), the influence of CD is evidenced by the PZQ signal broadening, more evident in the cases of carbonyl and aromatic groups. Hence, PZQ is also in an amorphous form. Moreover, the C2 signal observed at higher magnetic field for raw PZQ is now much less intense (1:1) or absent (1:2) and a strong intensity decrease is observed for C14,5 superimposed signals. These evidences point to the presence of a complex PZQ: HP-β-CD most probably with 1:2 molar ratio. An inclusion complex of PZQ with HP-β-CD was prepared and characterized using several techniques, and it was reported a 2.4 fold increase on PZQ aqueous solubility (Chaves et al., 2010). In vitro cytotoxicity tests, using fibroblast cells, evidenced no toxicity for these cells at the concentrations tested. These results demonstrated that there is a potential use of PZQ in formulations with HP-β-CD. In order to facilitate comparing ^{13}C MAS spectra run with CP and without CP mode (single pulse, SP) Fig. S2 shows ^{13}C spectra obtained from PZQ:HP-β-CD (1:1) with the TOSS sequence and SP/MAS or CP/MAS. Clearly, the resonances of quaternary carbons are almost absent in the SP/MAS spectrum.

3.4. ^{15}N CP/MAS NMR spectra of PZQ and PZQ-CDs binary systems

Like ^{13}C, ^{15}N has spin nuclear $^{1}/_{2}$ and consequently narrow lines are expected to be observed from ordered environments. ^{15}N spectra usually require longer acquisition times than ^{13}C experiments particularly because of its natural abundance (0.37% to be compared with 1.1 % for ^{13}C). However, it is worth mentioning that, the presence of only two nitrogen atoms in PZQ, makes ^{15}N a very convenient probe to complement ^{13}C data. Fig. 4 shows representative ^{15}N CP/MAS spectra run from crystalline PZQ and from PZQ:β-CD, PZQ:Me-β-CD and PZQ:HP-β-CD samples with 1:1 drug:carrier molar ratio. Table 1 shows the ^{15}N corresponding chemical shifts which were obtained by fitting Gaussian functions to the experimental spectra (see Fig. S4 in the Supplementary Data file). PZQ spectrum displays two well resolved resonances at

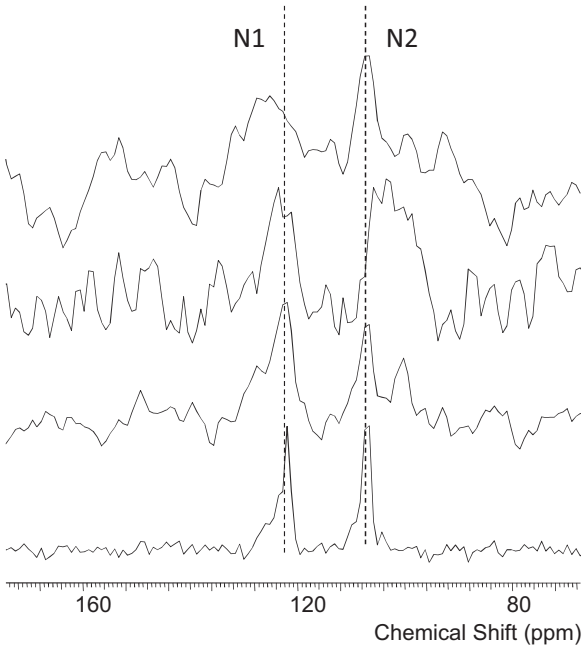

Fig. 4. ^{15}N CP/MAS spectra (from bottom to top) obtained from PZQ and from PZQ:β-CD, PZQ:Me-β-CD, PZQ:HP-β-CD binary systems (1:1 molar ratio).

122.30 ppm and 107.45 ppm assigned to N1 and N2, respectively (the narrowest signal in Fig. 4 is from N1). Other much less intense signals were assigned to N1 and N2 in other electronic environments (126.32 and 123.91 ppm for N1, 106.64 and 110.26 ppm for N2). The N1 and N2 multiple signals, with different intensities and line broadening, correlate well with ^{13}C resonances identified from C2O and C13O.

Concerning the PZQ:β-CD system, the major signals are from uncomplexed PZQ but other resonances are present (at 127.92 ppm and 100.63 ppm), which are assigned to the complex species already identified in ^{13}C spectra. Clearly, a progressive N1 signal broadening is observed when comparing spectra of PZQ:β-CD, PZQ:Me-β-CD and PZQ:HP-β-CD. ^{15}N spectrum from PZQ:Me-β-CD present broad signals both from N1 and N2. In the PZQ:HP-β-CD spectrum is observed a N2 signal with chemical shift similar to raw PZQ but the signal to noise ratio is too low and hampers any further clear conclusion.

Overall, based on the line widths and chemical shifts, ^{15}N spectra are consistent with the presence of amorphous PZQ (particularly PZQ:Me-β-CD) but raw PZQ seems to be present in the three systems with 1:1 (drug:carrier) molar ratio.

3.5. ^{13}C CP/MAS NMR spectra of PZQ and PZQ:CDs physical mixtures

^{13}C CP/MAS spectra were also recorded from PM prepared with the same molar ratios as the complexes. ^{13}C CP/MAS spectra obtained from PZQ and from PM of PZQ:β-CD, PZQ:Me-β-CD, PZQ:HP-β-CD (1:1 and 1:2 molar ratio) are provided in the Supplementary data file (Figs. S5 and S6). These data were recorded with parameters similar to those used to run the spectra from the corresponding complexes (see Figs. 2 and 3) except for the recycle delay which was 10 s instead of 5 s. That value was selected in order to improve the spectral signal to noise ratio, under the reasonable assumption that PMs are more rigid systems than complexes, therefore with longer ^{1}H spin-lattice relaxation time (this parameter determines the equilibrium magnetization recovery of the spin system under CP mode). The comparison of Figs. S5 and S6 with the complex spectra enables concluding that no complexes are present in PMs because PZQ and CD signals are just superimposed signals of the resonances observed from the pure compounds. Similar conclusions were reported on the ssNMR studies of PMs of albendazole or benznidazole with β-CD or β-CD derivatives (Ferreira et al., 2015; Priotti et al., 2015).

3.6. Relaxation study

In addition to structural data, ssNMR enables obtaining information on molecular dynamics. Motions in several frequency ranges may be probed by measuring relaxation times. Such parameters reflect mobility in the solid state and, although being mainly influenced by structural rigidity, they are strongly dependent on the presence of highly mobile groups (methyl groups, for example) and residual solvents. Mobility in the kilohertz frequency range was probed by measuring proton rotating-frame relaxation time ($^{H}T_{1\rho}$). However, a very efficient adiabatic process involving hydrogen nuclei that are nearby in space may prevent obtaining correct information about local motions. This process, which is commonly known as spin-diffusion, involves magnetization transport and causes those nuclei to behave as a single spin reservoir characterized by a single, averaged, $^{H}T_{1\rho}$. It is induced by the dipolar interaction of nuclear spins and the spin-diffusion rate scales with $1/d^6$, where d is the internuclear distance (Koenig, 1999). Although providing a mechanism that may mask local motions, spin-diffusion can add support for the presence of an inclusion complex. Because it is restricted to the nucleus nearby, it is reasonable to expect that $^{H}T_{1\rho}$ may probe the interaction between CD and encapsulated PZQ. While the pure drug and carrier can be characterized by different $^{H}T_{1\rho}$ data, similar $^{H}T_{1\rho}$ values are expected for the guest and the host in an inclusion complex. The 1:1 molar ratio system was chosen for this relaxation study based on the ^{13}C and ^{15}N results.

Table 2
Proton spin-lattice relaxation time in the rotating frame ($^{H}T_{1\rho}$, ms) obtained for both crystalline and amorphous PZQ, Me-β-CD and PZQ:Me-β-CD (1:1) complex. The errors obtained for the fittings are also shown.

Carbon number/δ (ppm)											
Me-β-CD				PZQ							
Pure	1/101.8	2,3,5/71.9	4/83.0	6 & CH_3/59.4	17/26.82	10/124.19	3/54.92	2/161.88	13/174.73	14,5/39.2, 37.5	4,1/45.6,47.33
	8.53 ± 0.22	8.52 ± 0.13	8.29 ± 0.13	7.72 ± 0.17	Crystalline powder						
					291 ± 21	138±13	146 ± 13	40 ± 3	61 ± 4	215 ± 26, 228 ± 22	270 ± 48
	9.8 ± 1.0[b]	11.6 ± 0.8[b]	10.8 ± 1.5[b]	9.8±1.3[b]	Amorphous						
					6.58 ± 0.18[a]	5.81 ± 0.38	6.53 ± 0.40	6.9 ± 1.1	5.4 ± 0.4	6.31 ± 0.20	6.16 ± 0.24
Complex	7.67 ± 0.05	7.70 ± 0.05	7.77 ± 0.06	7.33 ± 0.08	11.90 ± 0.25	7.98 ± 0.29	–	–	–	10.45 ± 0.35, 12.87 ± 0.49	14.61 ± 0.91

[a] Corresponds to relaxation data obtained from carbons 6,15,16,17,18,19.
[b] Priotti et al., 2015.

Hence, the experiments were performed on crystalline PZQ, amorphous PZQ, pure Me-β-CD and PZQ:Me-β-CD.

$^{H}T_{1\rho}$ relaxation times were obtained through ^{13}C observation under CP/MAS mode by varying the ^{1}H spin-lock duration following a ^{1}H 90° pulse. Thus, information was obtained on the relaxation performance of the protons at short distance of the carbons. Relaxation data obtained from crystalline PZQ (Table 2) must be considered as preliminary results because the longest spin-lock duration was just 18 ms (due to equipment constraints), which is much shorter than $^{H}T_{1\rho}$ values and, consequently, large errors are assigned to the fittings. Even so, those data indicate the presence of distinct proton spin reservoirs that are separated from one another in space because several, distinct, $^{H}T_{1\rho}$ values were measured. This finding shows that low-frequency, cooperative molecular mobility, is almost absent in PZQ. Considering that spin-diffusion scales with the distance, it is possible to consider at least three spin proton reservoirs: cyclohexylcarbonyl (C14 at about 39 ppm and C15,16,17,18,19 in the chemical shift range 25–32 ppm), the two heterocyclic rings (C1 at 47.30, C3 at about 55 ppm, C4 at 45.56 ppm, C5 and C6 at about 39 and 27 ppm, respectively) and the aromatic ring (C9,10,11,12 in the chemical shift range 124–130 ppm). Table 2 shows that protons in the cyclohexylcarbonyl moiety relax much slower ($^{H}T_{1\rho}$ exceeds 215 ms) than those in the heterocyclic and aromatic rings ($^{H}T_{1\rho}$ is about 140 ms). Both carbonyl groups present shorter $^{H}T_{1\rho}$ (less than 70 ms).

Table 2 indicates that similar $^{H}T_{1\rho}$ values were obtained for amorphous PZQ and pure Me-β-CD: about 6 ms and 8 ms, respectively. However, different relaxation data were measured for amorphous PZQ and Me-β-CD as pure compounds and in the PZQ:Me-β-CD system. In this system, $^{H}T_{1\rho}$ of Me-β-CD presents a small decrease (less than 1 ms) comparatively to the data obtained for the raw CD; in both cases, a single, averaged, value was obtained which is consistent with a single spin reservoir being present. However, more than one spin reservoir was detected for PZQ in the PZQ:Me-β-CD system because were measured $^{H}T_{1\rho}$ values that differ by more than 7 ms; namely, there is a difference of almost twofold between the values obtained from C4 in the piperazinone ring (14.61 ± 0.91 ms) and C9,10,11,12 in the aromatic ring (7.98 ± 0.29 ms). Hence, only $^{H}T_{1\rho}$ data obtained for the hydrogens in PZQ aromatic ring are similar to the values measured for the host (between 7.3 and 7.8 ms). This result is consistent with the formation of an inclusion complex PZQ:Me-β-CD, stabilized through the encapsulation of the aromatic ring in the cavity of the Me-β-CD, that is, by the interaction of PZQ aromatic protons with CD. Hindered mobility of other groups in the molecule, although not favouring spin-diffusion, explains the corresponding relaxation time increase.

The magnetization of the PZQ resonances in the complexes all display single-exponential decay (data not shown), in agreement with a major population of complexed PZQ molecules.

4. Conclusions

Solid-state NMR studies on PZQ:β-CD or PZQ:β-CD derivatives provided evidence for the presence of complexes most probably with 1:2 guest:host molar ratio. Relaxation studies afford additional support for the presence of an inclusion complex PZQ:Me-β-CD stabilized by the interaction of PZQ aromatic protons with CD. Additional, ssNMR data indicated that PZQ most probably adopts an *anti* conformation both as raw amorphous drug and in β-CD complexes. Free PZQ was identified in PZQ:β-CD samples with 1:1 molar ratio and this observation contradicts conclusions previously reached by other authors. Physico-chemical characterization of CD inclusion complexes has been often limited to 1:1 complexes. However, in some cases, aqueous solubility could be enhanced by the formation of 1:2 complexes but, in spite of such improvement, is missing a systematic study on the driving mechanism that lead to the formation of such complexes. Considering that β-CD is less soluble in water than the β-CD derivatives used in this study and that stable inclusion complexes are formed between PZQ and those β-CD derivatives, improved pharmaceutical formulations of this anti-schistosomal drug may be produced based on the results reported here.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpharm.2015.11.026.

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