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Short communication

Theoretical and experimental study of a praziquantel and β-cyclodextrin inclusion complex using molecular mechanic calculations and ^{1}H-nuclear magnetic resonance

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Abstract

Praziquantel (PZQ) is a broadly effective anthelminthic drug available for human and veterinary use, being the drug of choice for the treatment of all forms of schistosomiasis. Nevertheless, large doses are required in order to achieve adequate concentrations at the target site due to the poor solubility of PZQ and its significant first pass metabolism. To improve it, avoiding efficiency loss, we have designed a controlled-release system, in which PZQ was encapsulated in β-cyclodextrin (β-CD). The inclusion complexes between PZQ/β-CD were studied at two different stoichiometries 1:1 and 1:2, through experimental and theoretical analysis. Molecular modeling calculations were used to foresee the better stoichiometry of the complex formed as well as the possible orientations of PZQ inside the β-CD cavity. The complexes prepared were analyzed through ^{1}H two-dimensional nuclear magnetic resonance (^{1}H 2D-NMR) experiments, which provide (evidences) for the 1:1 complexation of PZQ/β-CD. ^{1}H 2D-NMR also revealed details of PZQ/β-CD molecular interaction, in which the isoquinoline ring of praziquantel is located inside the β-CD cavity. Finally, phase-solubility diagrams revealed a five-fold increase in praziquantel water solubility upon addition of increasing β-CD concentrations up to 16 mM, corresponding to the solubility of β-CD itself. The solubilization profile is consistent with 1:1 stoichiometry of the PZQ/β-CD complex while the solubilization effect will certainly increase the pharmacological activity of praziquantel.

Keywords: Praziquantel; β-Cyclodextrin; Molecular mechanics; Inclusion compounds; Nuclear magnetic resonance

1. Introduction

Schistosomiasis is an infectious disease with enormous public health and socio-economic importance in the developing countries [1]. It was recently estimated that the global number of people infected with *Schistosoma* spp. is around 200 million, with 600 million being at risk of infection and 20 million suffering severe debilitating illness [2]. PZQ is the drug of choice for the treatment of all forms of schistosomiasis, but its aqueous solubility is considerably low, a fact that restricts PZQ delivery only via the oral route [3]. Searching for a controlled-release system that could improve PZQ bioavailability, we have investigated formulations where β-cyclodextrin (β-CD) was the drug-carrier. Cyclodextrins (CDs) are cyclic oligosaccharides composed by α-(1,4)-linked glycosyl units; they are produced from starch or its derivatives by cyclodextrin glycosyl transferase (CGTase, EC 2.4.1.19), a bacterial enzyme [4]. There are three different types of natural CDs, according to the number of glucosyl residues in the molecule: α-, β- and γ-CDs (with 6, 7 or 8 glycosyl units, respectively) [5]. The toroidal shape of the CDs allows it to accommodate simple molecules with apolar groups into its cavity.

The 7 Å internal cavity diameter of β-CDs [6], for instance, can accommodate guest molecules with one benzene ring: chlorhexidine [7], *p*-iodophenolate [8], benzoic acid [9], benzocaine [10,11], in a 1:1 stoichiometry as well as molecules with more than one single ring, such as the local anesthetics bupi-

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vacaine [12] and the anti-inflammatory agent piroxicam [13]. Steric hindrances impede larger molecules to fit in β-CD cavity, favoring more complex structures such as the 1:2 (drug:CD) stoichiometries to be formed [14,15]. Here, we have employed molecular mechanics (MM) calculations to predict the possible orientations of PZQ inside the β-CD cavity at 1:1 or 1:2 stoichiometries. Considering that the entropy remains approximately constant for the same host molecule, enthalpy of formation was used to determine the most stable stoichiometry of complexation [16]. The differences between the enthalpy of formation for the inclusion compounds and the plain molecules were also used to determine details of the association model of complexation. Supporting evidences for the complexation of PZQ with β-CD were also obtained from experimental NMR results [17].

2. Experimental and theoretical

2.1. Molecular modeling

Molecular modeling was used to determine the 3D geometry of PZQ and β-CD molecules, alone and in the complex. HyperChem [18] was used to design the starting point molecules. MM calculations were performed in MacroModel/Maestro 7.3 [19] software using MM2 force field to optimise them in water. To include the solvent effect, we used the generalized Bohr surface area (GB/SA) protocol and Block-Diagonal Newton–Raphson gradient, with a rms less than 0.01 kcal mol^{-1}. The PZQ molecule was positioned into the β-CD cavity at different orientations. MM calculations considered the center of mass the isoquinoline ring system and denote "isoquinoline in" and "isoquinoline out" conformers. To β-CD we used "tail" and "head" to describe the regions of the cavity (Fig. 1). According to these denotations, the isoquinoline system was taken as the reference position for PZQ molecule and "in tail" denotes the conformer that has its ring next to the secondary hydroxyl group of the CD – the large face of the CD cavity – while "in head" refers to the conformer next to the narrower face of the CD cavity. In the MM calculations, we consider the relative thermodynamic relationship as the proportional stability of the complex formation. The stability of the complexes was measured assuming the proportionality $\Delta G \propto \Delta H$ and applying the general thermodynamic relationship:

$$\Delta G = -RT \ln K \quad (1)$$

So that,

$$\Delta H \propto -RT \ln K \quad (2)$$

For the general reaction of inclusion compounds, the formation of 1:1 stoichiometry can be described by

$$S + CD \rightleftharpoons S{:}CD \quad (3)$$

where S is the guest molecule (PZQ), CD the macromolecular system (host) and S:CD the final inclusion compound formed. According to Eq. (2), the stability of the reaction, represented by the equilibrium constant (K), can be taken from the enthalpy of formation for all the species presented in the reaction, as follows:

$$\Delta H = \Delta H_{fS:CD} - (\Delta H_{fS} + \Delta H_{fCD}) \quad (4)$$

The enthalpy of formation (ΔH_f) can be obtained from the MM calculations, so that the final ΔH is proportional to the stability constant of the inclusion compound.

By the same approach, the equilibrium for the 1:2 stoichiometry can be described by

$$S{:}CD + CD \rightleftharpoons S{:}CD_2 \quad (5)$$

where S:CD is the low energy molecular geometry of the 1:1 stoichiometry, CD is the macromolecular system and S:CD$_2$ is the inclusion compound formed in the 1:2 stoichiometry. In this situation, we can calculate the enthalpy of formation from:

$$\Delta H = \Delta H_{fS:CD_2} - (\Delta H_{fS:CD} + \Delta H_{fCD}) \quad (6)$$

In both cases the entropy contribution was considered to be the same [20].

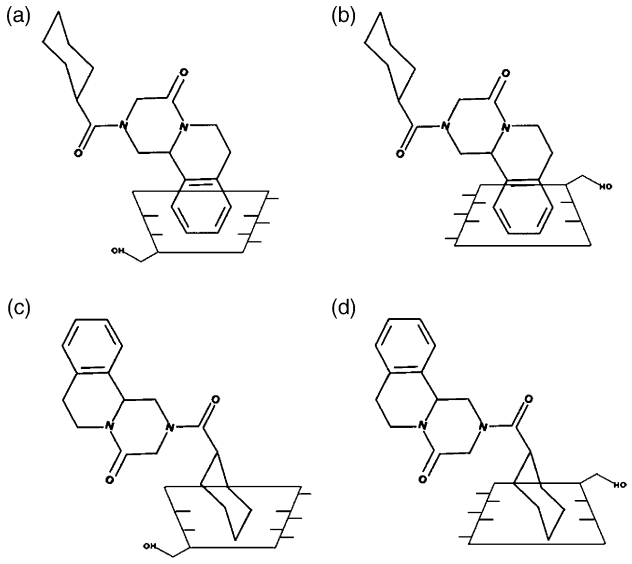

Fig. 1. Schematic representation of the starting orientations of PZQ/β-CD (1:1 stoichiometry) used in the molecular modeling calculations. (a) Isoquinoline in tail, (b) isoquinoline in head, (c) isoquinoline out tail and (d) isoquinoline out head.

2.2. Inclusion complex preparation

PZQ was donated by Merck KGaA (Darmstadt, Germany) and β-CD was purchased from Sigma (St. Louis, USA). Inclusion complexes were obtained by mixing appropriate amounts of PZQ and β-CD, with adaptations in the protocol proposed by de Azevedo et al. [21,22]. Briefly, the protocol consists in solubilizing PZQ and β-CD for the desired molar ratios (1:1 or 1:2). Then, the solutions were mixed until the equilibrium was reached and the samples were freeze dried and stored at −20 °C for further use.

2.3. Nuclear magnetic resonance

NMR experiments were carried out at 298 K on a Varian Inova 500 MHz spectrometer, operating at 11.7 T at the National Laboratory of Synchrotron Radiation (Campinas, SP, Brazil). One-dimensional ^{1}H-NMR spectra of PZQ, β-CD and PZQ/β-CD complexes were recorded at 25 °C. Sample were suspended in 0.6 mL of 99.9% D_2O to a final concentration of 1 mM; the PZQ/β-CD complex was performed in 1:1 stoichiometry. The residual water signal was used as the internal reference, at 4.81 ppm.

Two-dimensional (2D) rotating frame Overhauser effect spectroscopy (ROESY) experiments were performed using a spin-lock field of 3 kHz and 300 ms of time delay. The spectra were collected using 2048 complex data points in the F2 dimension and 324 increments. The spectral width was 10 ppm in both dimensions and eight free induction decays were acquired per increment. The NMR data have been processed using the NMRpipe, NMRview and VNMR 6.1 (Varian Inc.) programs [11].

2.4. Phase solubility test

This methodology was based on the solubility of the guest molecule [23], in which an excess of PZQ (8 mM) was added to aqueous solutions (20 mL) containing increasing concentrations of the CD and shook at 25 ± 0.5 °C at 60 oscillations:min. This test was performed for 7 days, until equilibrium was reached. Daily aliquots were filtered with a Millex-GP, 0.22 μm filter unit (Millipore Carrighwohill, County Cork, Ireland) and total PZQ was analyzed by spectrophotometry (270 nm).

3. Results and discussion

Fig. 1 shows four starting orientations of the most stable PZQ/β-CD complexes (1:1 stoichiometry) used in the calculations. In Table 1 are listed the enthalpy of formation for these four possibilities of 1:1 complexation, calculated according to Eq. (4). The ΔH values indicate that the four complex types are stable in water. The most stable complexes among the four 1:1 complexes, corresponding to the greatest absolute value of heat of formation (ΔH), was found to be the "isoquinoline in" complexes. Although the ΔH values for the "isoquinoline in head" were also favorable, the docking of the "isoquinoline in tail" (Fig. 2) revealed to be the most stable, with a ΔH numeric data of -18.12 kcal mol^{-1}, followed by the "isoquinoline in head" (ΔH, -16.03 kcal mol^{-1}), and by the "isoquinoline out of tail" complex (ΔH, -14.12 kcal mol^{-1}).

Table 1
ΔH for the (1:1) PZQ/β-CD complex in water as calculated by MM2

	ΔH (kcal mol^{-1})
Isoquinoline in tail	−18.12
Isoquinoline in head	−16.03
Isoquinoline out tail	−14.12
Isoquinoline out head	−9.34

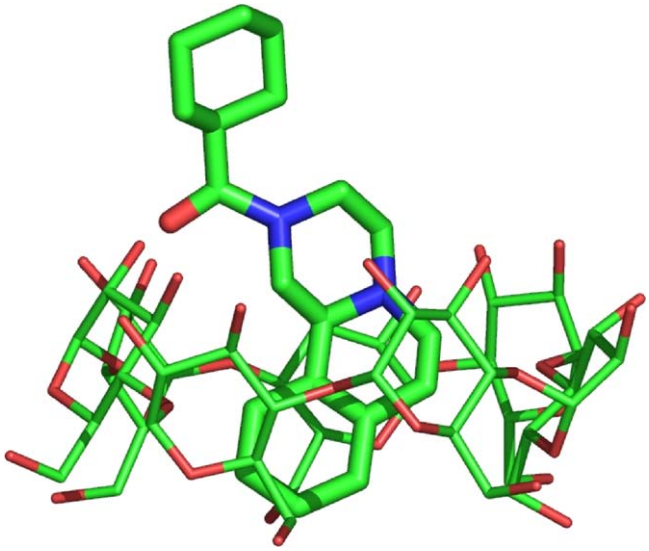

Fig. 2. 1:1 PZQ/β-CD complex by MM2 with GB/SA calculation.

The values found for the 1:2 stoichiometry calculations, carried out with Eq. (6), were close to 0 kcal mol^{-1} (Table 2), indicating that the 1:2 complexes are not stable in water due to the small energy necessary to displace them.

NMR studies allowed us to distinguish between inclusion and other possible external interaction processes. In fact, NMR is the most powerful technique used to determine the inclusion of a guest molecule into the hydrophobic CD cavity, in solution. It is well known that the chemical shifts of the hydrogen atoms located in the interior of the β-CD cavity (H-3 and H-5) become shielded and usually show significant upfield shift in the presence of a guest molecule [8,11], whereas the hydrogen atoms on the outer surface (H-1, H-2, H-4 and H-6) are not affected or experience only a marginal shift upon complexation [5].

Assignment of the hydrogen NMR peaks is in good agreement with the literature, both for β-CD [5] and PZQ [17,24]. We have first tried to evaluate the inclusion of PZQ into the β-CD cavity analyzing changes in the chemical shifts of the hydrogens in the complex, in comparison to free PZQ and β-CD. Although many peaks belonging to PZQ hydrogens shifted after complexation, the $\Delta\delta$ observed were not significant, even for the aromatic protons (<0.05 ppm), even though these last ones presented a higher $\Delta\delta$ than the one observed for the cyclic-aliphatic protons (<0.005 ppm). As for the β-CD molecule, the greatest chemical shift were up-field changes, detected for H-5 and also for H-3 (data not shown) in a similar way as the $\Delta\delta$ reported for chlorhexidine [7].

A definitive proof of the stoichiometry and insersion type of PZQ/β-CD complex was given by two-dimensional ROESY

Table 2
ΔH for the (1:2) PZQ/β-CD complex in water as calculated by MM2

	ΔH (kcal mol^{-1})
Tail:tail	−1.23
Head:tail	−0.31
Head:head	−0.05

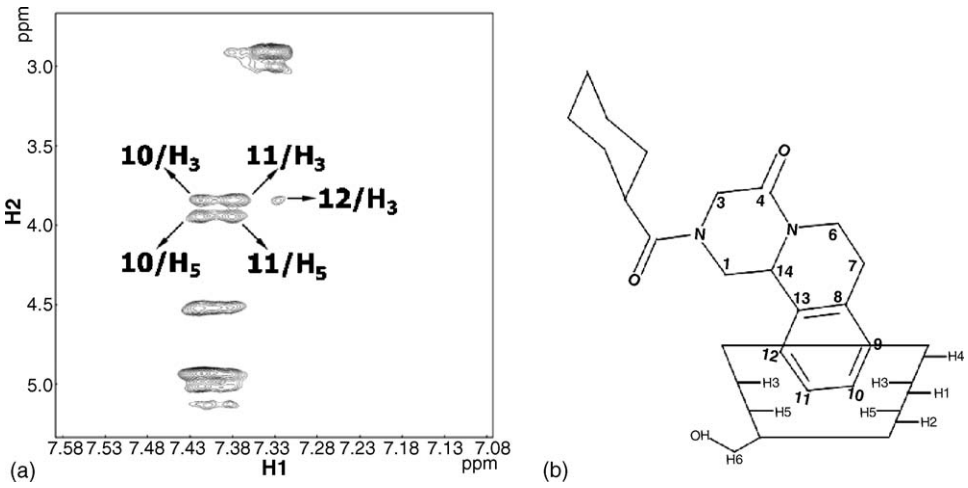

Fig. 3. (a) Expansion of the ROESY spectrum for the PZQ/β-CD complex (1:1) in D_2O at 500 MHz. The cross-peaks represent the intermolecular interaction between aromatic hydrogens of PZQ (10, 11 and 12) and β-CD hydrogens (H-3 and H-5). (b) Molecular numbering and representation of the "isoquinoline in tail" PZQ/β-CD association, according to MM and NMR data.

experiments. As shown in Fig. 3a, Nuclear Overhauser Effects (NOE) were detected between hydrogens 10, 11 and 12, belonging to the isoquinoline ring of PZQ and, H-3 and H-5 hydrogens of the β-CD cavity. ROESY spectra revealed no other intermolecular cross-peaks between PZQ and β-CD hydrogens.

The presence of NOE cross-peaks between PZQ, H-3 and H-5 hydrogens of β-CD suggests a geometry of complexation where the aromatic part of the PZQ isoquinoline ring is deeply inserted in the β-CD cavity, explaining this *through-the-space* intermolecular coupling (Fig. 3). This result is in accordance with those obtained by MM calculations for the 1:1 PZQ/β-CD inclusion complex (Table 1), which pointed out to the "isoquinoline in tail" conformer to be the most probable complexation arrangment.

Fig. 2 depicted the "isoquinoline in tail", preferential insertion of PZQ into the β-CD cavity, according to MM calculations. An analysis of this proposed model for the PZQ/β-CD complex (Fig. 3b) reveals that the 'isoquinoline in tail' insertion plently satisfies the experimental NMR results (Fig. 3).

Finally, phase-solubility studies demonstrate that complexation has increased ca. five-fold the amount of PZQ in water. The increase in solubility occurred as a linear function of β-CD concentration ($r = 0.992$) up to 16 mM. This concentration corresponds to the aqueous solubility of β-CD [5]. The linear relation between PZQ solubility increase and β-CD concentration corresponds to the A_L-type phase-diagram, defined by Higuchi and Connors [23] that are characteristic of 1:1 complexation [25].

In a previous work, Becket and coworkers have examined the complexation of α, β and γ cyclodextrins with PZQ and found that β-CD would form the most stable complex among the natural CD [24]. Those authors also described the phase-diagram of PZQ in β-CD as a B_S type [24]. Nevertheless, taking into account the limiting solubility of β-CD in water (ca. 16 mM [5]), we could assert that the change in profile of the PZQ solubility in excess β-CD concentrations (up to 22 mM) does not characterize the solubilization type proposed by those authors. Instead, as discussed before by Frömming and Szejtli [25], it is a A type phase-diagram, with enhancement in PZQ solubility up to the β-CD solubility.

Herein, we show, using theoretical and experimental approaches, that β-CD forms 1:1 complexes, increasing the amount of PZQ solubilized in water up to its own solubility limit.

4. Conclusion

The molecular modeling data suggests that PZQ/β-CD inclusion complexes have a 1:1 stoichiometry and that the isoquinoline ring system of PZQ is embedded in the cavity of β-CD, with a preferential "isoquinoline in tail" mode of insertion (Fig. 3). MM was useful to predict the geometry of the final complex and to calculate the energy of association between host and guest molecules. Changes in the chemical shifts of internal hydrogens of the β-CD cavity were indicative of the insertion of PZQ into β-CD cavity. ROESY experiments detected molecular proximities (≤5 Å) between hydrogens 10, 11 and 12 of the aromatic ring of PZQ isoquinoline system and hydrogens H-3 and H-5, in the internal cavity of β-CD (Fig 3). Moreover, phase-solubility has shown a five-fold increase in PZQ solubility, in agreement with the proposed 1:1 complexation.

The results reported here show a good agreement between MM calculation and experimental data (NMR data) revealing that, together, these are really powerful tools for the study of the molecular details of CD inclusion complexes. This work demonstrates how theoretical (molecular modeling calculations) and experimental (solubility and NMR experiments) approaches can give complementary and concordant results that reveal useful molecular details of a given drug/β-CD system. The PZQ inclusion complex was designed as a new pharmacological formulation in the treatment of schistosomiasis, since β-CD complexation increases the solubility of PZQ. The results obtained prompted us to perform in vivo experiments (with mice infected with *Schistosoma mansoni*), that are now under course and will allow us to validate the efficiency of this novel formulation.

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