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Host–guest interactions between benznidazole and beta-cyclodextrin in multicomponent complex systems involving hydrophilic polymers and triethanolamine in aqueous solution

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ABSTRACT

Association of hydrophilic compounds with cyclodextrins to increase drug solubility has been extensively studied in aqueous solution. However, the mechanism of interaction among these components remains unclear. In this study, the mechanism of interaction of seven different hydrophilic polymers (HPs) and triethanolamine (TEA) in aqueous solution with beta-cyclodextrin (β-CD) to modify the aqueous solubility of benznidazole (BNZ) was well investigated using solubility diagrams, thermodynamic experiments, molecular modeling and NMR studies. Solubility diagrams in different pH values confirmed linear soluble BNZ-β-CD inclusion complexes, with 1:1 stoichiometry (AL type). A synergistic effect in the association of TEA with BCD did not occur, due to competition between TEA and BNZ β-CD cavity, which led to obtain inclusion complexes with limited solubility (B type). The increment of BNZ solubility occurred only at higher TEA concentrations by cosolvency mechanism, which was evidenced by solubility diagrams, molecular modeling and NMR studies. The association of different hydrophilic polymers with β-CD contributes thermodynamically to stabilize the formed complexes, in which POL 407 and PVA increased considerably the observed $K_{1:1}$ value. An enthalpic contribution of hydrophilic polymers led to enhance the spontaneity of BNZ-β-CD interaction and a slight increasing in entropy change (ΔS) did possible to stabilize the interaction between BNZ and β-CD.

1. Introduction

Chagas disease is an endemic infection caused mainly by the parasite *Trypanosoma cruzi*. According to the World Health Organization (WHO), more than 10 million people are infected, mainly in Latin America. Furthermore, more than 25 million people are at risk of infection. Benznidazole, N-benzyl-2-(2-nitroimidazol-1-yl) acetamide (BNZ — Fig. 1), is the mainly available drug for its treatment [1], which is classified as a poor soluble drug with high permeability through biological barriers. Due to these characteristics, alternatives that lead to an increment in drug solubility can considerably increase its efficacy. Among several techniques that have been studied for enhancing equilibrium solubility of non-polar drugs in aqueous vehicles, cosolvency and complexation with cyclodextrins are well established for this purpose [2,3].

Cyclodextrins (CD) are cyclic oligosaccharides with hydroxyl groups on the outer surface and a cavity in the center. The three most common naturally occurring CD are α-cyclodextrin (α-CD), β-cyclodextrin (β-CD) and γ-cyclodextrin (γ-CD) with six, seven and eight (α-1,4)-linked D-glucopyranose units, respectively. Due to their cone-shaped structure with an outer hydrophilic surface and a cavity with a lipophilic character, these molecules can host hydrophobic or water insoluble compounds by "host-guest" mechanism, with a favorable change in enthalpy by reducing the free energy of the aqueous environment [4–6]. Thus, the cyclodextrin inclusion complexes may alter some physicochemical properties of drugs, such as stability, solubility and consequently bioavailability [4–6].

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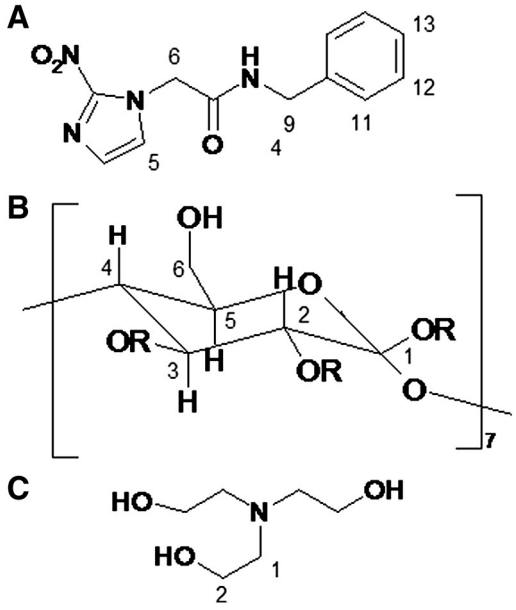

Fig. 1. Schematic representation of chemical structures of (A) benznidazole, (B) β-CD and (C) TEA.

The phase-solubility diagram described by Higuchi and Connors is the most used approach in inclusion complex characterization. This is a well-established method to estimate not only the value of the stability constant but also to give insight into the stoichiometry involved with components in equilibrium. Some types of diagram can be identified in solubility studies, which may be classified into two main categories; A- and B-types. A-type diagram is observed when the apparent solubility of the substrate increases as a function of CD concentration. B-type diagrams are indicative of the formation of complexes with limited water solubility [6–8].

Other strategies have been investigated to enhance both the apparent aqueous solubility and dissolution rate of poor water-soluble drugs. Among them, solid dispersion using hydrophilic polymers (HP) is well established for this purpose [9]. Some studies have also found that the use of hydrophilic compounds such as triethanolamine (TEA) isolated or associated with cyclodextrins has demonstrated interesting results [10,11]. It has also been described in literature papers aiming to increase BNZ solubility by applying several types of CD [12,13] and studies using ternary complexes with CD and hydroxyethylmethylcellulose to increase the BNZ aqueous solubility [14]. However, the effect of TEA and the involved mechanism of its interaction in inclusion complex formation have not been considered. These liquid hydrophilic compounds may interact with insoluble drugs by cosolvency or complexant mechanisms. Some models have been proposed for the prediction of this cosolvency, in which the log-linear model still remains to be the most useful.

Hence, the purpose of this study was to investigate the interaction of BNZ with β-cyclodexdrin (β-CD) in aqueous solution containing hydrophilic compounds, such as TEA and hydrophilic polymers. The polymers studied included polyethyleneglycols with different molecular weights (PEGs 1500, 4000 and 10000); hydroxypropylmethylcellulose (HPMC); Polyvinyl alcohol (PVA); polyvinylpyrrolidone (PVP) and poly (oxyethylene) block copolymer (poloxamer 407). The mechanisms of interaction among these components were followed and clarified using, solubility phase diagrams, thermodynamic experiments, molecular modeling and NMR studies. The analytical results were checked using a validated methodology to access analytical concentration of drug in the samples.

2. Experimental

2.1. Materials

BNZ, N-benzyl-2-(2-nitroimidazol-1-yl) acetamide was obtained from Roche (Brazil); βCD was a gift from Roquette® (Labonathus, Brazil); polyethyleneglycol (PEG) 1500, 4000 and10000, polyvinilpirrolidone PVP-k 30 and triethanolamine were purchased from Synth (Brazil); polaxamer (POL 407) and hydroxypropylmethylcellulose (HPMC) were purchased from Sigma-Aldrich (USA) and polyvinyl alcohol from Vetec (Brazil). All other reagents were analytical grade. The purified water (1.3 μS) was prepared from reverse osmosis purification equipment, model OS50 LX, Gehaka (Brazil).

2.2. Method validation

2.2.1. Instrumental conditions

An UV spectrophotometric method for quantitative analysis of BNZ was validated according to the guidelines established by the International Conference on Harmonisation (ICH) [15] and Brazilian regulatory National Agency of Sanitary Monitoring (ANVISA) [16]. The equipment used consisted of a UV–Vis spectrophotometer Thermo Fisher Scientific, Evolution 60S, USA. All absorbance readings were taken in a 1-cm path-length cuvette at room temperature, at wavelengths between 200 and 400 nm.

2.2.2. Analytical parameters

The standard curve was obtained from different aliquots taken from a BNZ stock solution of 500 μg ml^{-1} prepared in ethanol, which were diluted with water to prepare solutions with different concentrations (2.5 to 40 μg ml^{-1}). A 20 μg ml^{-1} solution was scanned from 200 to 400 nm, to find the best wavelength (324 nm) for BNZ quantifications. Furthermore, the data used to build the standard curve were subjected to analysis of variance (one-way ANOVA) to assure the linearity of method. Specificity of method was determined by comparing the analytical plots of absorbance, of a matrix solution containing different compounds (TEA + β-CD + HP/TEA).

Intra and inter-day precision tests were performed by calculating the relative standard deviation (RSD) of analyses of BNZ solutions at five different concentrations (5, 10, 20, 30 and 35 μg ml^{-1}) (n = 5). Analyses were carried out on the same day (intra-day test) and on five different days (inter-day test), at intervals of at least two days, with the same spectrophotometer equipment. Accuracy was accessed using the standard addition method, in which solutions containing the components of matrix (β-CD + HP/TEA) were mixed with distinct amounts of standard BNZ solution to obtain five different drug concentrations (5, 10, 20, 30 and 35 μg ml^{-1}). Accuracy level was calculated as the mean of five tests at each level (n = 5), from the relationship:

$$\text{Accuracy} = \left[\frac{\text{mean experimental concentration}}{\text{theoretical concentration}}\right] \cdot 100. \quad (1)$$

In the apparent robustness, the influence on precision of several analytical parameters, such as different days was described in previous sections. Additionally, the effect of pH of the analytical solution on the accuracy and precision of the method was investigated. To this end, the pH of the analytical solutions was about pH 6.5, which was adjusted to lower and higher levels (pH 4.5 and 8.5) with 0.05 mol l^{-1} phosphate ($KHPO_4$) buffered solution.

2.3. Solubility studies

2.3.1. Phase solubility studies

The solubility diagrams were obtained according to the method established by Higuchi and Connors [17], in which an excess of BNZ was added to flasks containing different β-CD concentrations (0 to

0.015 mol l^{-1}). All flasks were hermetically closed and placed in a thermostatic bath at 25.0 ± 2.0 °C during 72 h, in which every 12 h, the flasks were shaken in ultrasonic bath for 15 min. Sequentially, the different solutions were filtered through 0.45 μm membranes of cellulose acetate (Sartorius® Biolab Products, Germany). The pH of each solution was measured and BNZ concentration was analytically determined using the UV–Vis spectrophotometric method previously validated. The apparent stability constant (K_c) was estimated using the slope of linear regression from phase solubility diagram, assuming 1:1 mol l^{-1} stoichiometry, according to Eq. (2), in which S_o is the solubility of the pure drug in water. The experimental results were expressed as the mean values of three replicates (n = 3).

$$K_c = \frac{\text{slope}}{S_o \cdot (1 - \text{slope})} \tag{2}$$

2.3.2. Cosolvency or complexant effect involved with TEA

The cosolvency or complexant effect of TEA involved in the BNZ solubility increment was investigated using the log linear model [3,18], in which the apparent solubility of BNZ was determined in different TEA concentrations (0 to 40% w/v). Generally, the enhancement of the cosolvent volume fraction leads to an exponential increment on drug solubility. In this approach, a linear correlation was performed between log of drug solubility in the specific aqueous solution ($\log S_{mix}$) and the volume fraction of used cosolvent (f). The cosolvency power (σ) was the slope of linear fitted plot (Eq. (3)). In this model, a linear increment on drug soluble fraction occurs when the cosolvency mechanism is involved, and thus can be assessed by substituting in the Eq. (3) the log S_{mix} with log (S_{mix}/S_w), in which S_w means the drug solubility in water.

$$\log S_{mix} = \log S_w + \sigma f \tag{3}$$

2.3.3. Effect of pH and TEA on BNZ-β-CD complexation

The effect of TEA was analyzed on BNZ-β-CD complexation using the same TEA concentration (0.67 mol l^{-1}) for different β-CD concentrations (0 to 0.015 mol l^{-1}). Due to the use of TEA as an alkalinizing agent, the effect of pH of the aqueous solutions was also studied. The solubility diagrams at the same pH of solution in the presence of TEA (pH = 10.5, KH_2PO_4 at 0.05 mol l^{-1}) and in acid medium (pH = 3.0, HCl at 0.1 mol l^{-1}) were obtained in the presence of β-CD (0 to 0.015 mol l^{-1}). The solubility studies were conducted as described in the solubility diagrams (n = 3).

2.3.4. Effect of HP association with β-CD in BNZ aqueous solubility

A study of the effect of the association of β-CD with each HP (PEGs 1500, 4000 and 10000; HPMC, PVA, PVP and POL 407) in the apparent aqueous solubility of BNZ was also conducted, in which a fixed HP concentration (0.5% w/v) was combined with different β-CD concentrations (0 to 0.015 mol l^{-1}). The effect of isolated HPs at different concentration (0 to 0.5% w/v) was also studied. The solubility studies were conducted as described in the solubility diagrams (n = 3).

2.3.5. Thermodynamic parameters

Phase solubility diagrams were obtained in three different temperatures (20, 25 and 30 °C) which aim to understand the thermodynamic contribution of hydrophilic polymers in BNZ-β-CD complexation. Thus, standard free energy change (ΔG) was identified in temperature dependence of observed stability constant ($K_{1:1}$) from BNZ-β-CD complexes obtained under different conditions. Standard enthalpy change (ΔH) and standard entropy change (ΔS) were obtained in temperature dependence of $K_{1:1}$ from BNZ-β-CD complexes obtained in water or aqueous solution of polaxamer 407 (POL407). These thermodynamic parameters made it possible to identify an enthalpic or entropic contribution of this copolymer, which was HP that showed the best $K_{1:1}$ increment. The free energy of reaction is derived from the equilibrium constant using the relationship:

$$\Delta G = -RT\ln K_{1:1}. \tag{4}$$

The ΔH values were obtained from a plot of ln $K_{1:1}$ versus 1/T using the following relationship, where the slope will provide the enthalpy data.

$$\log K_{1:1} = \frac{\Delta H}{2.303R} \cdot \frac{1}{T} + \log A \tag{5}$$

Finally, the entropy values (ΔS) for the complexation were calculated using the expression:

$$\Delta G = \Delta H - T\ \Delta S. \tag{6}$$

2.4. Molecular modeling

Possible inclusion complexes between BNZ and β-CD were obtained by molecular docking using Autodock Vina [19]. The complexes were optimized using semi-empirical quantum mechanics level of theory PM6 [20] using MOPAC2009 program. The self-consistent-reaction-field (SCRF) model was employed to simulate the presence of water solvent. The optimized geometries were submitted to frequency calculations to certify that energy minima were obtained. The zero point energy correction to the electronic energy was used to determine the relative conformational energies and to decide the most stable BNZ-β-CD complex.

The interactions of BNZ, β-CD, TEA in solvent were further investigated at atomic level by performing molecular dynamic simulations using explicit TIP3P water models. The molecular topologies were created using topolbuid_1.3 and AM1-BCC [21], atomic charges were computed using antechamber and atom types were defined by the GAFF force field [33]. Covalent bonds were constrained using the LINCS algorithm. The simulations were performed at constant temperature and pressure in a periodic truncated triclinic box. Prior to molecular dynamic simulation the geometries were optimized with energy minimization using a steepest descent algorithm, followed by conjugated gradient minimization. A 100-ps protein position restrained molecular dynamics was performed at 300 K to gently relax the water molecules. Unrestrained molecular dynamics were then performed at 310 K for at least 5 ns to assess the stabilization of the density of the box. A productive simulation was performed for 200-ns simulation in which the temperature and the pressure were maintained at 310 K and 1 bar by coupling with rescaling velocities and using isotropic pressure bath with Parrinello–Rahman barostat.

2.5. Nuclear magnetic resonance (NMR) studies

All experiments were performed on a Bruker Avance II High Resolution Spectrometer, equipped with a Broad Band Inverse probe (BBI) and a Variable Temperature Unit (VTU). All experiments were carried out at 298 K, using 5 mm sample tubes. The NMR data were processed with the Bruker TOPSPIN 2.0 software.

^{1}H NMR spectra were obtained at 400.16 MHz. The chemical shifts (δ) were reported as ppm, and the residual solvent signal (4.80 ppm) was used as the internal reference. Induced changes in the ^{1}H NMR chemical shifts (Δδ) for BNZ originated due to their complexation were calculated according to the following equation: $\Delta\delta = \delta_{complex} - \delta_{free}$; NMR spectra of pure BNZ and their combinations with β-CD and β-CD:TEA were taken in D_2O. The concentration of components was 0.015 mol l^{-1}.

2.6. Statistics

The RSD was calculated as RSD = 100 · (sd/mean). The experimental results of quantitative analysis of drug were initially subjected

to one-way analysis of variance (one-way ANOVA) for multiple comparisons. When F values were significant, data were further analyzed by Tukey test post-hoc test for the comparisons among different sample sets or by Dunnett's test post-hoc test for comparisons with a control sample set. For pairwise comparisons, a Student-*t* test was performed. A $p < 0.05$ was required for significance, i.e. there is difference between samples.

3. Results and discussion

3.1. Validation study

Several analytical methods have been proposed for BNZ, which includes electrochemical analysis, UV–Vis spectrophotometry and HPLC [22,23]. Streck et al. [24] proposed the UV–Vis spectrophotometry for BNZ assay into an emulsion system. The Brazilian Pharmacopoeia establishes the assay of BNZ from tablets by UV–Vis spectrophotometric analysis at 316 nm in acid solution (HCl 0.1 mol l^{-1}) [25]. Development and validation of the analytical methodologies for quantitative analysis of drug from delivery systems or inclusion complexes is a crucial step to assure the experimental results from stability or solubility studies [24,26]. The aim of this part of study was to guarantee the analytical results from solubility studies. In the specificity study, the scanning plots demonstrated that the components of investigated inclusion complexes (β-CD, HP and TEA) did not provoke any interference in the range of maximum wavelength for BNZ (324 nm) (Fig. 2). Moreover, the inclusion complexes of BNZ with β-CD in the presence of hydrophilic polymers (BNZ-β-CD-HP) or in TEA (BNZ-β-CD-TEA) showed a similar UV scanning plot, which demonstrates non relevant changes in the photochemical behavior of drug on the UV region.

The correlation coefficient ($r^2 > 0.999$) identified in the linearity study established the upper (40 μg ml^{-1}) and lower (2.5 μg ml^{-1}) limits for analytical determinations with precision and accuracy. These data were used to build the fitted standard curve for five independent sets of data. In addition, these experimental data were subjected to analysis of variance (one-way ANOVA), which showed significant linear regression and no significant linearity deviation [$F(1,7) = 35515.077$; ($p < 0.05$)]. The experimental results from different analytical parameters are shown in Table 1. All analytical parameters were investigated at each concentration (5, 10, 20, 30 and 35 μg ml^{-1}) of at least three replicate assays.

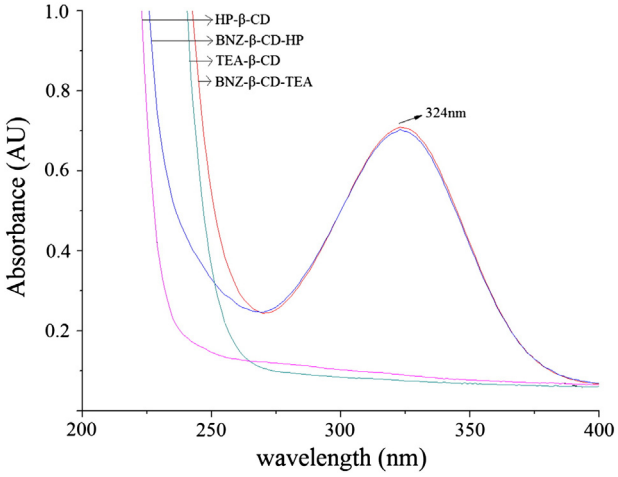

Fig. 2. UV spectrum in aqueous solution for different samples: HP–β-CD [hydrophilic polymer mixture (0.5% w/v) and β-CD (0.015 mol · l^{-1}) without drug]; BNZ–β-CD–HP [inclusion complex of BNZ with β-CD in the presence of hydrophilic polymer mixture (0.5% w/v)]; TEA–β-CD [triethanolamine (10% w/v) and β-CD (0.015 mol · l^{-1}) without drug]; BNZ–β-CD–TEA [inclusion complex of BNZ with β-CD in the presence of triethanolamine (10% w/v)].

The experimental results (Table 1) demonstrated that the UV–Vis spectrophotometric method used can be considered precise for the quantitative analysis of BNZ in the multicomponent complexes. Since, for all tested concentration levels, RSD values calculated were less than 3.5%. In addition, no statistical difference ($p > 0.05$; Student-*t* test) was observed between the experimental data from intra and inter-day studies. Accuracy for all recovery values was satisfactory, ranging from 91.8% to 110.1%, which are inside the established limits (80–120%) for quantitative analysis of drugs and pharmaceuticals. The recovery values were subjected to statistical analysis (one-way ANOVA), which did not reveal statistical differences for different matrix analyzed in the study when compared with BNZ water solution. Robustness consists of the ability to resist being affected by alteration of some analytical parameter, such as pH, ionic strength or temperature [24–26]. Due to the alkalinizing effect of TEA, the effect of a wide range of pH (4.5, 6.5 and 8.5) on the quantitative analysis of BNZ was investigated at five concentrations, by assessing the accuracy and precision at these pH values (Table 1).

Table 1
Experimental results of validation study. Analytical equation ($y = 0{,}031062\ x + 0.055136$); correlation coefficient, $r^2 \geq 0.9999$ for the analysis of BNZ through UV–Vis spectrophotometry.

Analytical parameters	BNZ concentration (μg ml^{-1} ± sd) (RSD%)				
	5 μg ml^{-1}	10 μg ml^{-1}	20 μg ml^{-1}	30 μg ml^{-1}	35 μg ml^{-1}
Precision					
Intra-day	4.9 ± 0.1	9.8 ± 0.2	20.1 ± 0.4	29.4 ± 0.1	34.8 ± 0.3
	(2.6)	(2.3)	(1.8)	(0.5)	(1.0)
Inter-day	5.0 ± 0.1	9.7 ± 0.1	19.0 ± 0.6	29.5 ± 1.0	33.9 ± 0.9
	(2.4)	(1.3)	(2.9)	(3.4)	(2.8)
Student-*t* test (*p*)	0.288	0.482	0.057	0.872	0.176
Accuracy (%)					
β-CD + TEA	109 ± 5	101 ± 2	93 ± 2	95 ± 3	94 ± 2
	(4.8)	(2.1)	(1.8)	(3.4)	(1.8)
β-CD + HP	103 ± 4	99 ± 3	91.8 ± 0.6	93.9 ± 0.4	94 ± 2
	(3.5)	(3.2)	(0.6)	(0.5)	(1.9)
ANOVA	$F(2,12) = 0{,}897$; ($p = 0.433$)				
Apparent robustness					
pH 4.5	5.3 ± 0.2	10.0 ± 0.3	20.0 ± 0.5	29 ± 1	35 ± 1
	(3.2)	(3.0)	(2.8)	(3.7)	(3.7)
pH 6.5	4.9 ± 0.1	9.8 ± 0.2	20.1 ± 0.4	29.4 ± 0.1	34.8 ± 0.3
	(2.6)	(2.3)	(1.8)	(0.5)	(1.0)
pH 8.5	5.4 ± 0.2	10.1 ± 0.2	19.9 ± 0.1	30 ± 1	35 ± 1
	(4.5)	(2.6)	(0.7)	(3.5)	(3.6)
ANOVA	$F(2, 12) = 0.000131$; ($p = 1.00$)				

The analytical method was considered to be robust to inserted variations in pH of the studied solutions (Table 1). No statistical differences were observed among the experimental drug concentrations for different pH values (4.5 and 8.5) compared with values obtained from BNZ solutions in water (pH 6.5). The experimental results shown in Table 1 demonstrate that the method can be considered suitable for the quantitative analysis of BNZ in the studied multicomponent complexes. All tested analytical parameters established that the method may be safely employed in the quantitative analysis of BNZ in solubility studies with the proposed multicomponent complex systems.

3.2. BNZ-β-CD-TEA interaction in solution

The solubility diagrams described by Higuchi and Connors [17] are the most common approach in the evaluation of inclusion complex formation with cyclodextrins. It is well established that from these diagrams it is possible to estimate the stoichiometry involved and calculate the stability constant (K_c) of the formed inclusion complexes. The determination of apparent solubility of BNZ was carried out by adding excess of drug in hermetically closed flasks containing water or aqueous solution with the specific compound at the investigated pH. Fig. 3 shows the solubility diagrams of BNZ in the presence of different β-CD concentrations in water, at two different pH values (pH 3.0 and 10.0) and in 0.67 mol l^{-1} TEA aqueous solution. A linear relationship between the BNZ apparent aqueous solubility and the used β-CD concentration was identified in water and at different pH values, which may be verified by the very high correlation coefficient (r) obtained from the straight line fitted plot of each solubility diagram. In addition, the analysis of variance (*one-way* ANOVA) of the experimental data used to build the fitted solubility diagrams showed significant linear regression and no significant linearity deviation. The r values and statistical analysis for different studies were carried out in water ($r = 0.98763 \pm 0.01128$; [$F = (1,5) = 630.413$; ($p < 0.001$)]), at pH 3.0 ($r = 0.943207 \pm 0.038968$; [$F = (1,5) = 143.316$; ($p < 0.001$)]), and at pH 10.5 ($r = 0.987013 \pm 0.005439$; [$F = (1,5) = 512.154$; ($p < 0.001$)]). The same behavior did not occur in the presence of 0.67 mol l^{-1} TEA aqueous, because concentrations higher than 0.002 mol l^{-1} solubility reached a plateau (0.63973 ± 0.054744 [$F = (1,5) = 9.052$; ($p = 0.030$)]).

The experimental results obtained with the phase solubility studies with β-CD and BNZ in different pH values (Fig. 3) demonstrated the obtaining of inclusion complexes with typical A_L profile and suggested an occurrence of soluble complexes with 1:1 stoichiometry. The apparent stability constant was estimated using Eq. (2). In water, the $K_{1:1}$ value was calculated (51.48 M^{-1}), which confirms the interaction between BNZ and β-CD. The increment in the apparent BNZ aqueous solubility provoked by the maximum studied β-CD concentration was about 1.7 times more than the value observed in water. In aqueous solution, the slightly non-polar CD cavity is occupied by water molecules, which is energetically disadvantaged due to possible polar-non-polar interactions. Therefore, the water molecules can easily be replaced by an appropriate guest molecule with lipophilic character such as BNZ. A cyclodextrin molecule can include one or more drug molecules, but the most frequently observed relationship between CD and guest molecule is 1:1 [27,28], which was confirmed in the inclusion complex of BNZ with β-CD. Although, majority of $K_{1:1}$ values for binary inclusion complexes are ranged from 50 to 2000 mol l^{-1}. Brewster and Loftsson (2007) [29] identified in the literature a medium value about 490 M^{-1} for drug inclusion complexes with β-CD. Experimental results demonstrate a weak interaction of BNZ with β-CD, which is dependent not only of hydrophobic character of the drug substrate, but they are also involved with structural factors of host molecule, such as its tridimensional arrangement of functional groups [30]. It was reported that BNZ interacts with β-CD mainly by imidazolic portion and benzene group inclusion in the CD cavity [31]. However, a weak interaction of BNZ with β-CD was also identified in a recent study [32]. The $K_{1:1}$ value identified in the present study for inclusion complex of BNZ with β-CD confirms the obtaining of an unstable inclusion complex. This experimental result justifies the inevitable use of other compounds such as hydrophilic polymers or TEA and its association, with the aim of having a more significant increment on the apparent aqueous solubility of BNZ.

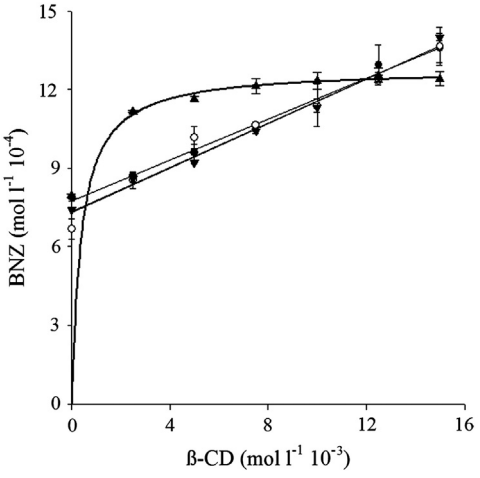

Fig. 3. BNZ phase solubility diagrams with (●) β-CD in water, (○) β-CD at pH (3.0), (▼) β-CD at pH (10.0) and (▲) β-CD + 0.67 mol l^{-1} TEA.

The interaction between BNZ and β-CD was also investigated at different pH values, and it was observed that in acid medium (pH 3.0) the solubility of BNZ was about 2 times higher than its solubility in water, while in alkaline medium (pH 10.5) this value was about 1.9 times. The $K_{1:1}$ stability constants calculated from A_L type solubility diagrams in acid and alkaline medium were 67 ± 3 and 60 ± 5, respectively. The stability constant calculated in acid medium was statistically greater than that observed in water [$F(2,6) = 7.024$; ($p = 0.027$)]. However, considering the aim of cyclodextrin use, these results were similar to those identified in water and the alteration of pH was not sufficient to increase relevantly the effect of β-CD on the aqueous solubility of BNZ. Furthermore, the alkaline pH was studied with the aim of simulating the alkalizing effect of TEA.

The simultaneous effect of TEA associated with β-CD on the increment of apparent aqueous solubility of BNZ was also investigated with the aim to evaluate a possible synergic effect, which is well related in the literature for other drugs when these two solubilizers are simultaneously used [33,34]. However, the association of 0.015 mol l^{-1} of β-CD with 0.67 mol^{-1} TEA in water (Fig. 3), led to an enhancement by a factor of 1.5 times more than apparent solubility of BNZ in water, which was smaller than that observed with aqueous solution with only β-CD. The solubility diagram observed for β-CD inclusion complex with BNZ in the presence of 0.67 mol l^{-1} TEA was B-type, in which a complex with limited solubility is formed. So, it was not possible to calculate the $K_{1:1}$ constant. The mechanism of drug interaction with β-CD in the presence of TEA has not been well established yet. Nonetheless, the experimental results indicate that the TEA led to attenuation of BNZ-β-CD interaction. Another possibility is that TEA and BNZ may be competing with β-CD cavity. TEA is an alkaline chemical reagent widely used in the pharmaceutical and cosmetic industries. Its alkalinizing effect of TEA should be not discharged, since the apparent aqueous solubility of BNZ in 0.015 mol l^{-1} β-CD in the presence of 0.67 mol l^{-1} TEA [$(124 \pm 3)\ 10^{-5}$ mol l^{-1}] was inferior to that identified in alkaline medium at the same pH [$(140 \pm 1)\ 10^{-5}$ mol l^{-1}]. It is interesting to observe that its compound has a molecular mass of about 149 g/mol, it is liquid at ambient temperature and its chemical structure can offer three free hydroxyl groups, which contribute to its characteristic of a cosolvent or complexant agent.

A solubilization curve of BNZ was plotted in function of volume fraction of TEA present in medium (f) with the aim to understand

better this effect of TEA on the solubility of drug and explain its interaction with β-CD in the presence of BNZ (Fig. 4). A linear increment on the apparent solubility of drug was observed [F (1,5) = 336.324 ($p < 0.001$)], in which an enhancement of 4.9-times was achieved for the maximum TEA concentration (40% m/v). The plot of log (S_{mix}/S_w) versus TEA volume shows a linear relationship with a correlation coefficient of $r^2 = 0.9927$ (Fig. 4), which indicates a prevalent cosolvency effect. Drug solubilization by cosolvency occurs when a linear relationship for log (S_{mix}/S_w) versus the solubilizer fraction (f) is observed [3,18]. Generally, the organic cosolvent contributes to disrupt the self-association of water, which increases the solubility of non-polar compounds due to the reduction of the polarity in the aqueous environment. This may lead to a reducing in the complexation efficiency of lipophilic compounds inside the cavity of β-CD, and consequently increase the amount of not included soluble molecules, which explains the low K_c ($1.3 \pm 0.2\ M^{-1}$) observed in the phase solubility studies with the presence of TEA. Due to the characteristics of TEA molecule, a competition between TEA and BNZ in the cavity of β-CD may be also occurring. For example, it is known that CD can be associated with surfactants or amphiphilic drugs acting as modulators in aggregate formation, such as amphiphilic vesicle or other self-assembly aggregates. This interaction is due to a high affinity of the alkyl chains of these molecules with the CD inner portion, which can interact via hydrophobic interactions, Van der Waals forces, among others [35,36]. Thus, TEA can interact with CD through a similar mechanism observed in surfactants, competing with BNZ by the oligosaccharide cavity, and therefore reducing the aqueous drug solubility.

The possibility of competition between TEA and BNZ leads to suggest that interaction of TEA with the BNZ in the presence of β-CD may be dependent on its concentration in the medium. Thus, a solubility study of BNZ in a fixed 0.015 mol l^{-1} β-CD in the presence of different TEA concentrations was performed (Fig. 5) in order to observe if a higher concentration of TEA (0.67 mol l^{-1}) is a limiting factor in inclusion complex formation, and also elucidate the mechanism whereby the TEA works in different concentrations. The presence of TEA at lower concentrations declined BNZ solubility, in a fixed 0.015 mol l^{-1} β-CD. However, from 0.335 mol l^{-1} of TEA, the drug concentration increased without reaching a maximum value of BNZ solubilization in β-CD solution (Fig. 5). This experimental result strongly suggests that a prevalent competition of TEA with BNZ by the cavity of β-CD occurred at lower TEA concentrations, leading to decrease the BNZ solubility due to drug-β-CD association displacement. On the other hand, the rising of TEA concentration in medium led to additional contribution of TEA in the BNZ solubility, probably by cosolvency

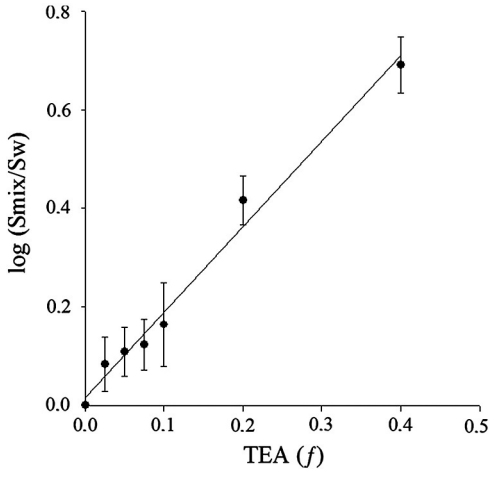

Fig. 4. Solubility curve of BNZ in the presence of TEA in water. S_{mix} is the solubility of the drug in the cosolvent–water mixture and S_w is its apparent aqueous solubility.

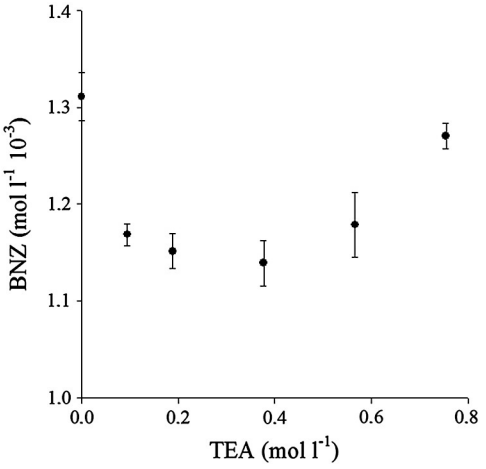

Fig. 5. BNZ phase solubility diagrams in the presence of fixed 0.015 mol l^{-1} β-CD with different TEA concentrations.

mechanism, due to excess of TEA molecules not complexed with β-CD. Furthermore, these observations may be grounded by molecular modeling studies and should be confirmed by NMR studies.

The molecular docking provided several binding modes for inclusion complex of BNZ with β-CD. Every conformation was energy minimized and the relative energies were used to determine the relative stability. It was found that the most suitable binding mode for BNZ inclusion complex can perform three hydrogen bonding with free hydroxyl groups situated in the minor crown of the β-CD (Fig. 6). In the second most stable conformation the imidazolic moiety of the BNZ molecule interacts with the major crown hydroxyl groups with two hydrogen bonds. The energy difference between the two conformations was of 1.10 kcal mol^{-1}. The β-CD cavity could not be completely filled by BNZ and the extra space inside the cavity may have a detrimental effect on the complex stability. This may contribute to the low $K_{1:1}$ value found for inclusion complexes of BNZ with β-CD due to the weak interaction of BNZ with β-CD.

In order to investigate BNZ–β-CD and BNZ–β-CD–TEA interactions in a dynamic fashion, 200-ns molecular dynamic simulations were performed. The simulation with BNZ–β-CD in solvent demonstrated a complex formation similar to the structure predicted using semi-empirical quantum mechanics calculations (Fig. 6). During the simulation BNZ phenyl moiety interacted with the hydrophobic cavity of CD and the imidazole moiety performed alternating hydrogen bond interactions with the hydroxyl groups of the minor crown of β-CD. On the course of the simulation involving BNZ–β-CD–TEA, TEA rapidly inserts into the β-CD hindering the BNZ interaction (Fig. 7). On the contrary of anterior studies using ternary complex approaching involving β-CD and TEA, in which was related that TEA contributes in drug-β-CD complex formation, TEA acts impairing the BNZ–β-CD complex formation in accordance with experimental results, which showed lower solubility of BNZ due to such competitive phenomena in TEA concentrations below 0.335 mol l^{-1} (Fig. 5).

A NMR study was performed with the aim to confirm the experimental findings on both solubility studies and molecular modeling. Evidence of interaction of BNZ with β-CD and β-CD:TEA in aqueous solution was based on the change of the NMR spectra of the mixtures with respect to the spectra for the individual components. Table 2 summarizes chemical shifts (δ) identified for protons of individual components, and the changes induced on them as a result of interactions in the binary and ternary systems.

In the ^1H NMR spectra of the studied systems, appreciable shifts were observed in the BNZ signals, probably due to conformational changes caused by complexation. Since there are no new peaks that could be assigned to the complexes, suggesting that complexation of BNZ with β-CD or β-CD:TEA appears to be a dynamic process with the

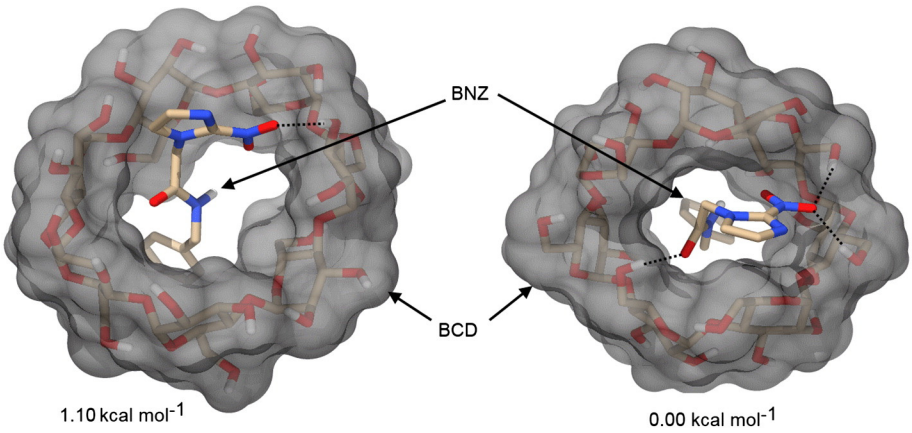

Fig. 6. Two proposed inclusion complexes between BNZ and β-CD optimized by means of the semi-empirical quantum chemistry method PM6 with SCRF water model. The dotted lines are denoting hydrogen bond formation.

BNZ being in a state of fast exchange (relative to the NMR timescale). This was previously reported in the molecular modeling studies (Fig. 7).

^{1}H NMR results for the BNZ:β − CD system revealed that all BNZ protons showed shielding effects, indicating that they are close to a host atom rich in π-electrons. In this case, such effects can be associated with oxygen atoms, which can also reflect conformational changes occurring due to inclusion phenomena. On the other hand, appreciable deshielding effects for β-CD protons were observed, giving evidence of the insertion of an electronegative moiety into the β-CD hydrophobic cavity. In fact, these shifts suggest that the BNZ molecules produce paramagnetic anisotropy effects in the interior of the cavity due to weak interactions (van der Waals forces). The modifications observed for β-CD protons indicated that the CD cavity was deformed as a consequence of interactions between the guest and the host molecules, and gave evidence of the existence of inclusion. In addition, the signals corresponding to the protons located at the outer surface of β-CD were also modified, which might have been due to a conformational rearrangement in the host molecule.

Moreover, the ^{1}H NMR spectrum of the BNZ:β − CD:TEA system presented a considerable complexity. All BNZ protons showed more shielding effects when TEA was added to the binary system with β-CD, demonstrating a strong interaction between BNZ and TEA, which confirms the cosolvency effect of TEA with BNZ as previously reported in solubility studies (Figs. 3 and 5). In addition, the shielding effects for β-CD protons induced by the interaction were observed. This suggests that a hydrophobic interaction prevails between BNZ and β-CD.

3.3. BNZ-β-CD-hydrophilic polymer interaction in solution

The effect of hydrophilic polymer (HP) association with cyclodextrins to increase the solubility of insoluble drugs is well established in the literature [37]. However, the involved mechanism is not yet totally clear. In this study, the influence of isolated HP or combined with β-CD in the BNZ solubility was investigated at a concentration range of 0 to 0.5% w/v. The BNZ solubility curves were obtained for distinct HPs (polyvinyl alcohol, PEG 4000, PEG 1500, PEG 10000, POL 407, PVP-k 30 and HPMC) (Fig. 8).

Generally, hydrophilic polymers may increase dissolution rate of poor soluble drug from solid dispersions by three different mechanisms: (a) drug may be dispersed into polymeric matrix in an amorphous or disorganized crystalline phase; (b) polymer contributes to an increase in the interaction of water with drug molecules or (c) the dissolution of polymer leads to an increase in drug solubility in aqueous environment by the occurrence of soluble complex formation or by cosolvency effect. Thus, to explain the mechanism involved is very important to predict the contribution of the HP in the increment of drug solubility in solid dispersions or from multicomponent complex

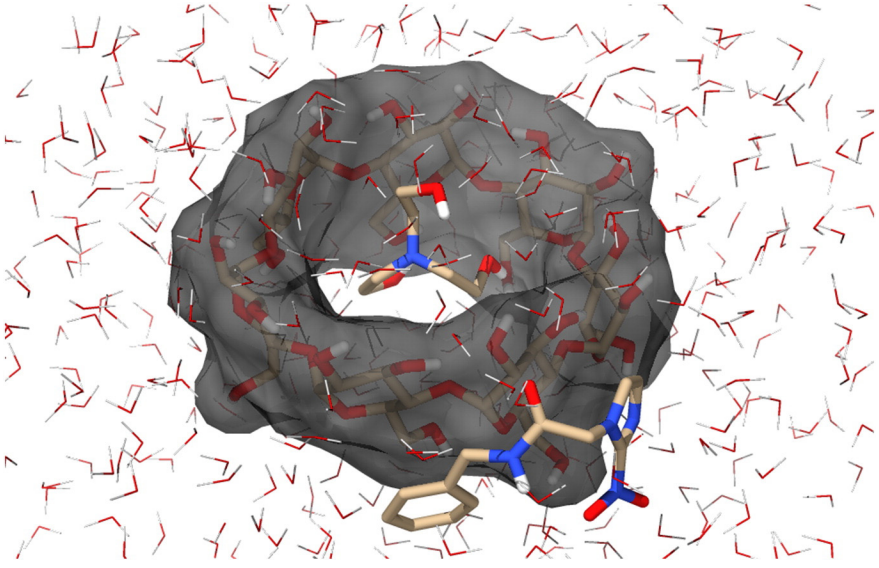

Fig. 7. Molecular dynamics snapshot that shows TEA competitive interaction with BNZ for the β-CD hydrophobic groove.

Table 2
^{1}H NMR chemical shifts (δ) of the individual components and changes (Δδ) in the presence of the binary and ternary systems (see proton numbering in Fig. 1).

Assignment	δ_{free}	$\Delta\delta = \delta_{complex} - \delta_{free}$	
		BNZ:β − CD	BNZ:β − CD:TEA
BNZ			
H4	7.3237	−0.0237	−0.0632
H5	7.5419	−0.0101	−0.0537
H11	7.4914	−0.0312	−0.0663
H12–H13	7.4156	−0.0197	−0.0590
β − CD			
H1	5.0681	0.0354	–
H2	3.6470	0.0369	−0.0595
H3	3.9648	0.0300	−0.0361
H4	3.5824	0.0345	–
H5	3.8541	0.0293	−0.0360
H6	3.8769	0.0318	0.0036
TEA			
H1	2.7054		0.0308
H2	3.6533		–

systems with cyclodextrins. The majority of used polymers did not lead to a relevant change in BNZ aqueous solubility. No difference was identified among the distinct investigated PEGs (1500, 4000 and 10000) (Fig. 8A). This result may have occurred because of low studied polymeric concentrations (0–0.5% w/v). In a previous study, the use of a concentrated aqueous solution of PEG 400 (70% w/v) was able to increase the BNZ solubility from 0.4 mg ml^{-1} to 10 mg ml^{-1} [38]. However, it is impossible to use a high polymeric concentration to obtain solid dispersion or multicomponent complex systems. Moreover, the use of a high concentration of HPs can increase the viscosity of the aqueous solution, which decreases diffusion coefficient of drug and consequently its dissolution rate.

Previous studies reported the increment of aqueous solubility of poorly soluble drugs, such as hydrocortisone in the presence of HPMC and PVP at different concentrations with a dependence on pH [39]. On the other hand, the same did not occur with BNZ. The HPMC and PVA did not change the BNZ solubility (Fig. 8B). On the contrary, PVP-K 30 and POL 407 led to statistically significant enhancement of BNZ apparent aqueous solubility when comparing its solubility in water (ANOVA [$F(7,34) = 7.569$; ($p < 0.001$)] followed by Dunnett's method versus solubility in water ($p < 0.05$). Furthermore, the solubility diagrams of BNZ in different concentrations of each investigated polymer did not show a linear relationship with drug solubility, which makes it impossible to calculate K_c for any polymer. These results suggest an absence of any involved complexation of BNZ with different studied HPs.

The usefulness of natural cyclodextrins has been limited due to its relatively low aqueous solubility, especially β-CD. As previously reported, the use of water-soluble polymers has been extensively studied to enhance the complexation abilities of different types of CDs. Moreover, in a previous section of this study a weak interaction of β-CD with BNZ was established and that the isolated use of β-CD did not lead to relevant increment of BNZ aqueous solubility. Subsequently, to investigate the effect of isolated HPs, the influence of the distinct HPs in a fixed concentration (0.5% w/v) in the inclusion complex formation of BNZ with β-CD was also evaluated. The most relevant analytical parameters, such as $K_{1:1}$, pH of aqueous solution, and solubility increment (ΔSol) achieved by the maximum β-CD concentration were compared with the drug solubility in water. In addition the standard free energy change (ΔG_{free}) was calculated and experimental results are shown in Table 3.

The majority of polymers presented some contribution in the complexation of BNZ with β-CD, which was observed by the identified $K_{1:1}$ value from different systems (Table 3). However, the POL 407 and PVA led to a significant enhancement in the stability of the complexation between drug and CD when compared with β-CD in water [$F(8,18) = 10.856$;($p = 0.001$)]. These isolated polymers in water were not able to increase the BNZ solubility (Fig. 8), but both PVA and POL 407 contribute to improve the interaction of BNZ with β-CD molecules, which may have occurred due to the amphipathic character of these polymers. The mechanism by the $K_{1:1}$ values' increase remains unclear. Despite this the mechanism of polymer–CD interactions is not yet well known in the literature. A thermodynamic approach may be carried out by this interaction of HPs with inclusion complexes of BNZ with β-CD. It is well established that the formation of inclusion complexes of CDs with drugs is favorable with the decrease of the free energy of aqueous environment, which may be proved by the reduction of stability constant (K_c) associated with the temperature enhancement [39].

In the presence of all studied polymers, an increase in free energy change (ΔG_{free}) was calculated and consequently a decrease of the free energy of aqueous environment was observed in comparison with the experiments in the absence of polymers (Table 3). This information showed that the polymers increase the spontaneity drug–CD interaction, even in systems where a significant enhancement of stability constant ($K_{1:1}$) was not observed. Moreover, in the systems obtained with PEGs, a slight relationship was observed between the molecular mass and the observed $K_{1:1}$ and ΔG_{free} values (Table 3). Thus, the polymer addition in the aqueous solution may increase the stability of the interaction of drug with β-CD and this effect may be connected with the solubility character of these macromolecules.

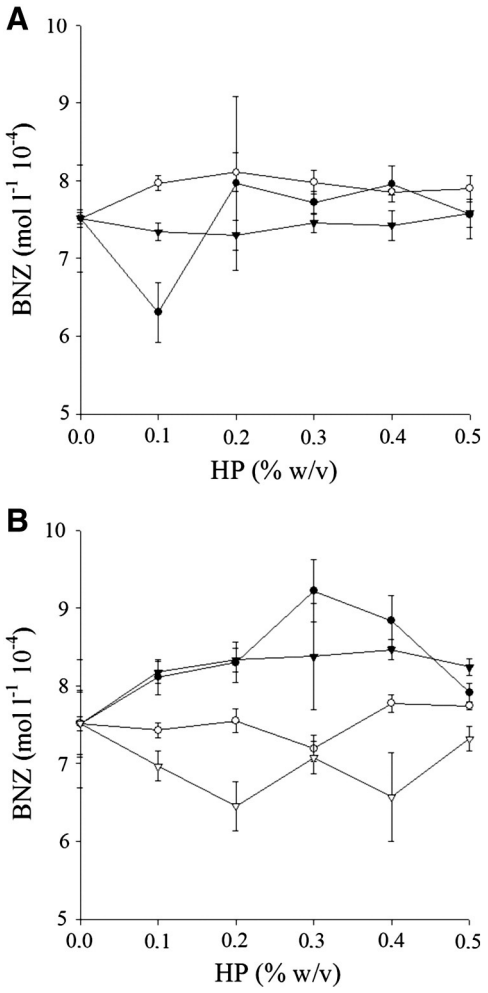

Fig. 8. (A) BNZ solubility curves in the presence of (●) PEG 4000, (○) PEG 10000 and (▼) PEG 1500 and (B) other polymers: (●) POL 407, (○) PVA, (▼) PVP-K 30 and (▼) HPMC.

Table 3
$K_{1:1}$ values, solubility increment (ΔSol), pH of aqueous solution and standard free energy change (ΔG_{free}) for different investigated conditions of inclusion complexes of BNZ with β-CD.

β-CD solubility diagrams	$K_{1:1}$ ($mol \cdot l^{-1}$)	Solubility increment (ΔSol)	pH of aqueous solution	ΔG_{free} (−)
Water	51.5	1.7	6.8	4474.7
pH = 3	66.6*	2.0	3.0	4767.5
pH = 10.5	59.6	1.9	10.0	4641.8
PEG 1500	53.8	1.7	6.8	4525.2
PEG 4000	55.1	1.8	6.5	4551.3
PEG 1000	57.6	1.8	3.2	4602.1
POL 407	74.2*	2.0	6.5	4889.1
PVP	57.6	1.8	4.6	4601.5
PVA	61.3*	1.9	6.9	4672.1
HPMC	56.3	1.7	6.7	4577.2

* Statistically different versus water ($p < 0.05$).

The experimental results (Table 3) permit to observe that the association of these molecules modifies the BNZ–β-CD interaction in solution, possibly due to any change in drug diffusion due to any thermodynamic alteration of solution in the presence of these macromolecules. Thus, some thermodynamic parameters such as entropy change (ΔS), enthalpy change (ΔH) and free energy change (ΔG_{free}) values were calculated at different temperatures (293–303 K) for the POL 407 and compared with values identified in water which aim to better understand whereby these HPs change the formation of inclusion complex BNZ:β-CD (Table 4).

The experimental results (Table 4) demonstrate that the enhancement of temperature led to decrease of the BNZ–β-CD interaction, which was observed by the reduction of stability constants ($K_{1:1}$) and consequent increasing of involved free energy (ΔG_{free}) values. It can also be observed that the complexation of BNZ with β-CD is exothermic judged from the negative enthalpy changes (ΔH) in the presence or absence of polymer. Exothermic judged drug–CD interaction was also observed in literature for different drugs and CDs [39,40].

For β-CD with BNZ, a deep and snug-fitting complex was formed with large negative ΔH and a near-zero ΔS involved in this interaction in solution. It is interesting to observe that negative or slightly positive ΔS values may indicate that the inclusion of guest molecule does not lead to a very strong solvent displacement from CD cavity, and that complex formation is enthalpically driven [41,42]. The presence of POL 407 in solution decreasing ΔH and ΔG_{free} values, which indicates some observations as, a) the polymer presence makes the complexation more spontaneous; b) the dissociation of the complex will increase with the enhancement of the temperature [43]; and c) it may be experiencing a greater hydrophobic interaction between the BNZ and β-CD, with the establishment of a greater number of Van der Waals interactions and hydrogen bonds [44,45]. Moreover, a slight increase was observed in entropy change (ΔS) value in POL 407 presence, which means a slight enhancement in degrees of freedom of translation and rotation of the complex, contributing to stabilize the interaction between the guest and host molecule and lead to reduction of free energy [45]. Thus, the free energy (ΔG_{free}) involved in the formation of inclusion complexes of BNZ with β-CD may be predicted by $\Delta G_{free} = \Delta H_{complex\ formation} - T\Delta S$, in which this inclusion complex formation was exothermically judged, and the addition of a hydrophilic polymer such as POL 407 contributes to spontaneity of drug–CD interaction by decreasing ΔH value.

Table 4
Thermodynamic parameters: ΔG_{free}, ΔH and ΔS in inclusion complex formation in the absence and presence of a hydrophilic polymer.

Systems	T (K)	$K_{1:1}$ ($mol\ L^{-1}$)	ΔG_{free} (J/mol)	ΔH (J/mol)	ΔS (J/mol · K)
Water	293	63.2	−4627.6	−4236.7	0.8
	298	51.5	−4474.7		
	303	46.4	−4429.1		
POL 407	293	80.5	−4899.3	−4629,62	0.9
	298	74.1	−4889.1		
	303	54.9	−4623.2		

4. Conclusion

An increment in the apparent aqueous solubility of BNZ was achieved from inclusion complex with β-CD at different pH values, in which the acid medium was more favorable to inclusion complex formation. Although previous studies reported a synergistic effect in the association of TEA with BCD in increasing of the solubility of drugs, this did not occur with BNZ. The TEA increases the BNZ solubility by a cosolvency mechanism, but its association of β-CD with TEA led to obtaining inclusion complexes with limited solubility (B type). A competition between TEA and BNZ by the cavity of β-CD was evidenced at lower TEA concentrations, which decreased BNZ solubility due to drug–β-CD association displacement. However, the rising of TEA concentration in medium led to additional contribution due its cosolvency. The association of different hydrophilic polymers with β-CD contributes to stabilize the formed complexes probably by a thermodynamically favorable mechanism, in which POL 407 and PVA increased considerably the observed $K_{1:1}$ value. Thermodynamic experiments indicate an enthalpic contribution of hydrophilic polymers, which leads to the enhancement of the spontaneity of BNZ–β-CD interaction. Moreover, POL 407 led to a slight increasing in entropy change (ΔS), which make the stabilization of its interaction between BNZ and β-CD possible.

Acknowledgments

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