International Journal of Biological Macromolecules 62 (2013) 543–548

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Effects of benznidazole:cyclodextrin complexes on the drug bioavailability upon oral administration to rats

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ARTICLE INFO

Article history:
Received 23 August 2013
Received in revised form 3 October 2013
Accepted 5 October 2013
Available online xxx

Keywords:
Benznidazole
Chagas' disease
Complexation
Cyclodextrins
Dissolution rate
Bioavailability

ABSTRACT

Benznidazole (BZL) is the drug of choice for the treatment of Chagas' disease, a neglected parasitic infection. It is poorly soluble in water, which may have a direct impact into its bioavailability. Thus, the aim of this study was to evaluate the impact of stoichiometric and non-stoichiometric BZL–cyclodextrins (CDs) complexes on the bioavailability of BZL. The interaction of BZL with the CDs was investigated using differential scanning calorimetry (DSC), scanning electron microscopy (SEM), X-ray diffractometry (XRD), phase solubility and dissolution studies. The oral bioavailability of BZL from these complexes was examined in rats. Both BZL solubility and dissolution increased by CD complexation. The inclusion complexes were found to improve the dissolution rate of BZL by 4.3-fold in comparison with BZL alone. Complexation of BZL with CDs derivatives increased its plasma concentrations when fed to rats, with AUC_{0-5} values increasing up to 3.7-fold and C_{max} increasing 2.5-fold in comparison with BZL alone. It should be note that a remarkable increase in these parameters was observed in the case of the non-stoichiometric complexes. Thus, these CDs complexes could be used to efficiently deliver BZL in patients suffering from Chagas' disease.

1. Introduction

The tropical infectious diseases that are prevalent in the world's least developed nations are known as neglected diseases. Among them, Chagas' disease affects an estimated 10 million people in Latin America. It is a chronic, systemic, parasitic infection caused by the protozoan *Trypanosoma cruzi*. Even though it was discovered more than hundred years ago, Chagas' disease is still the largest parasitic disease burden on the American continent [1]. In the last decade, several programs organized by governments and non-profitable organizations were focused, mainly, on the elimination of the domestic insect vectors. In addition, it was a more rigorous screening of blood donors, as well as, a control and monitoring of chagasic mothers, with parasitological diagnosis and treatment of infected new-bornes [2]. Actually, due to a migration of infected persons from endemic regions to other areas, such as, North America, and Europe, Chagas' disease becomes a global health concern that requires an urgent solution [3]. Despite of it, there are only two approved active compounds, BZL and Nifurtimox (NFX) to treat this infection, to date. In particular, BZL is the drug of choice in the majority of the American countries and Europe and recommended for the acute and recent chronic phases, as well as, for congenital infection [4]. As already described, it is poorly soluble in water [5,6] which may have a direct impact into its bioavailability [7,8]. Thus, the improvement of BZL dissolution rate becomes crucial for achieving improved pharmacokinetic parameters. In this context, complexation with supramolecular hosts, such as cyclodextrins (CDs), is one of the most widely applied strategies to overcome the drawbacks associated with low aqueous solubility [9,10]. Due to the disposition of the primary and secondary hydroxyl groups, the CD cavity is relatively hydrophobic compared to the exterior faces which are hydrophilic. This particular arrangement enables the formation of water-soluble inclusion complexes with a large amount of hydrophobic compounds [11,12]. Due to this complexation property, CDs have been widely used as carrier [13–15] sensor ligands [16], and separation reagents [17]. On the other hand, CDs may improve drug delivery through the membranes by increasing the availability of dissolved drug close to the biological membrane [18,19]. In particular, it should be note that CDs, alone or in combination with other hydrophilic polymers, have been described to increase the solubility/dissolution of BZL through the formation of inclusion complexes [20–22]. Moreover, it was described that BZL–CDs complexes did not modify the drug trypanocidal activity, but diminished the toxicity of the drug on mammals' cells [23]. To our knowledge, however, there is not information so far

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http://dx.doi.org/10.1016/j.ijbiomac.2013.10.007

regarding the impact of BZL–CDs complexes on the bioavailability. Thus, the aim of this study was to evaluate the impact of stoichiometric and non-stoichiometric BZL–CDs complexes on the solubility, dissolution rate and bioavailability of BZL in order to arrive to the most suitable formulation. First, the behavior of complexed BZL in solution was analyzed by means of phase solubility and *in vitro* dissolution studies. BZL–CDs complexes were characterized by differential scanning calorimetry (DSC), X-ray diffractometry (XRD) and scanning electron microscopy (SEM). Then, the evaluation of the pharmacokinetic parameters after the oral administration of BZL alone and the corresponding CDs complexes were described.

2. Materials and methods

2.1. Materials

BZL (lot 260835, 99.45% purity) was a gift from Produtos Roche Químicos e Farmacéuticos S.A. (Jaguare, Sao Paulo, Brazil). β-Cyclodextrin (β-CD), hydroxypropyl-β-cyclodextrin (estimated mol wt ~1396, average degree of substitution 0.9; HP-β-CD), and methyl-β-cyclodextrin (estimated mol wt 1310, average degree of substitution 1.8; Me-β-CD) were purchased from Sigma–Aldrich (Milwaukee, WI). All other chemicals and solvents used in this study were of analytical reagent grade.

2.2. Methods

2.2.1. Preparation of BZL complexes

Binary systems of BZL with β-CD, HP-β-CD, and Me-β-CD (1:1 and 1:2 drug:carrier molar ratio) were prepared using the solvent evaporation method. Briefly, BZL (100 mg) was dissolved in ethanol (10 ml) and CDs in water (10 ml). Both solutions were mixed during 10 min and evaporated under vacuum. The residues were dried at 40 °C and stored in desiccator until their further manipulation. All samples were kept in desiccator until further analysis.

2.2.2. Phase-solubility assay

The phase solubility studies were carried out according to the method already described [21]. Briefly, excess amounts of drug was added to each solution (10 ml) of β-CD, HP-β-CD, and Me-β-CD, in sealed glass containers and shaken at a constant temperature (25 °C) up to equilibrium (3 days). BZL–CDs solutions were prepared at pH 6.8. Aliquots were withdrawn and filtered (pore size 0.45 mm), and the drug concentration was spectrophotometrically determined at 324 nm (LKB Pharmacia Ultrospec II, Cambridge, UK), following the procedure reported by Soares Sobrinho et al. [24]. The presence of CDs did not interfere with the spectrophotometric assay of the drug. Each experiment was performed in triplicate.

2.2.3. Dissolution studies

BZL as raw material, without CDs, was dissolved in ethanol (10 ml) and the resulting solution was evaporated under the same experimental conditions than the corresponding BZL complexes. Dissolution studies of BZL and from the systems prepared with β-CD, HP-β-CD, and Me-β-CD (1:1 and 1:2 drug:carrier molar ratio) were performed in a Hanson Research, SR8 8-Flask Bath equipment (Chatsworth, CA). The following conditions were employed: paddle speed of 50 rpm, 900 ml of HCl 0.1 N as dissolution medium and temperature of 37 °C, according to the methodology described [24]. Powdered samples of each complex, equivalent to 100 mg BZL and BZL alone (100 mg) were spread over the selected dissolution media. At appropriate time intervals (10, 20, 30, 40, 50 60, 90, and 120 min), 5 ml of samples was withdrawn, and filtered (pore size 0.45 mm). Then, each sample was diluted with the dissolution medium, and the samples were assayed spectrophotometrically, as described in "*Phase-solubility assay*". The dissolution profiles were constructed by plotting the cumulative percent drug dissolved against time. In addition, dissolution efficiency (DE) was calculated from the area under the dissolution curve at time t (measured using the trapezoidal rule) and expressed as percentage of the area of the rectangle described by 100% dissolution in the same time [25]. The area under dissolution curve was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software (San Diego, CA). Dissolution experiments were carried out in triplicate.

2.2.4. Differential scanning calorimetry (DSC)

Thermal analysis was performed on a Perkin-Elmer DSC-4 calorimeter (Waltham, MA), using 5-mg samples in crimped aluminum pans. The differential scanning calorimetry instrument was calibrated using indium and zinc as standards. Nitrogen was used as a purge gas and an empty aluminum pan was used as a reference. Each sample was scanned at a rate of 10 °C/min from 25 to 300 °C.

2.2.5. X-ray powder diffraction (XRPD)

X-ray diffraction patterns were recorded on a Philips 1710 X-ray diffractometer (NJ, US) at room temperature, with a monochromatic Cu (Kα) X-ray source operating at 40 kV, 20 mA for all the data acquisitions, at a scan rate of 1° min^{-1}, 2° θ range from 5° to 40°. The STOE powder software package (Darmstad, Hesse, Germany) was used.

2.2.6. Scanning electron microscopy (SEM)

Morphology and surface of BZL, CDs and the binary systems were analyzed by means of scanning electron microscopy using an AMR 1000 Scanning Microscope (Oberkochen, Baden-Württemberg, Germany). Samples were spread on a metal specimen stub and coated with gold prior to the imaging. Pictures were taken at an excitation voltage of 20 kV and a magnification of 550×.

2.2.7. Bioavailability studies

The bioavailability studies were carried out using male Wistar rats 100 days old (250–300 g), housed in standard cages in a room with air, humidity and temperature control and on a 12-h light, 12-h dark cycle and free access to food and water. The formulations analyzed were BZL:β-CD; BZL:Me-β-CD; BZL:HP-β-CD; and BZL dispersed in water was used as control. Rats (n = 3) received *via* buco-gastric tube single dose (10 mg/kg) of each sample containing the same amount of drug. After 0.5, 1, 1.5, 2, 3, 4, and 5 h post administration, blood samples (200–250 μl) from lateral tail vein were withdrawn and stored at −30 °C until analysis. Then, 200 μl of plasma were processed, at 4 °C during 30 min, using 400 μl of a mixture of acetonitrile–dimethylsulfoxide (1:1). After centrifugation at 6000 × g for 15 min, the supernatant was incubated with 25 μl of trichloroacetic acid solution 10% (w/v) for 15 min at −12 °C and centrifuged for 10 min at 10,000 × g [26]. Finally, all samples were concentrated to dryness in a vacuum concentrator and then reconstituted with 200 μl of mobile phase. Baseline plasma samples obtained prior to BZL administration at time 0 served as the blank control. Drug plasma levels were determined by HPLC method with UV detection reading at 324 nm. Chromatography was performed on a Waters chromatographic system equipped with a Waters 600 MS multisolvent delivery system and a Waters 717 Auto sampler. A C18 reversed-phase column (5 μm, 250 mm × 4.6 mm) was used. The corresponding samples (20 μl) were injected and eluted (flow 1.0 ml/min) using a mixture of acetonitrile–water (40–60%, v/v) as mobile phase. The compound was identified by comparison of the corresponding retention time with those of reference compound. The peak concentration (C_{max}) and time to peak concentration (T_{max}) were displayed from the plotted concentration–time curve.

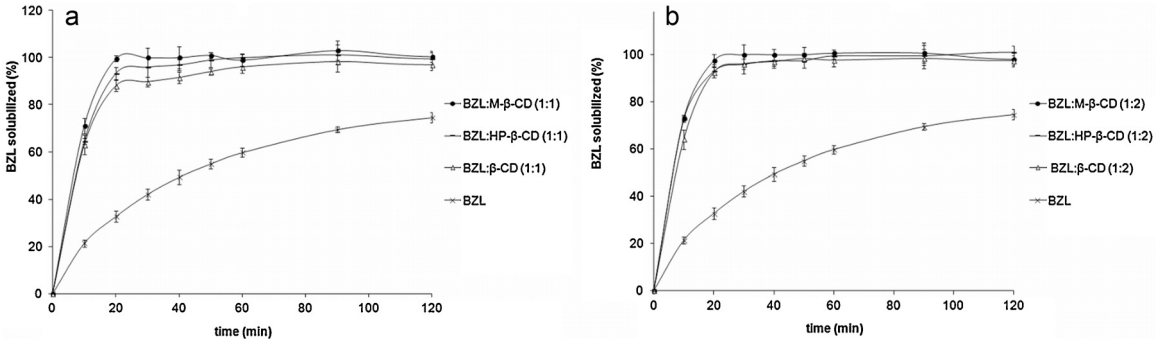

Fig. 1. Dissolution profiles of BZL alone, from BZL:β-CD, BZL:HP-β-CD, and BZL:Me-β-CD complexes at 1:1 (a) and from BZL:β-CD, BZL:HP-β-CD, and BZL:Me-β-CD complexes 1:2 (b) drug:polymer molar ratio.

The area under the concentration–time curve (*AUC*) was calculated by the linear trapezoidal rule.

2.2.8. Statistical analysis

ANOVA tests were used to determine statistical significance of studies, respectively. Differences were considered to be significant for values of $P < 0.05$.

3. Results and discussion

3.1. Phase-solubility assays

The drug solubility increased linearly with CD concentration and the slope was lower than 1. This linear correlation suggested that the complexes could be of the first order with respect to CDs (1:1 stoichiometry). The drug solubility increased from 0.71 mM to about 2.87 mM by increasing CD concentration, which could be due to the formation of water-soluble complexes through intermolecular hydrogen bonding between the host and the drug. Since both the degree and type of chemical substitution on the CDs greatly modify the interaction between the host and the guest molecule, those modifications may have an important role in determining stability constants [21]. In this case, methylation of the carrier enhanced its complexation ability by increasing the hydrophobic property of the CD cavity, in comparison with the other two CDs. Herein, the apparent stability constants (K_s) were higher for Me-β-CD ($72.7 \pm 0.9\,M^{-1}$) and HP-β-CD ($57.5 \pm 0.4\,M^{-1}$), respectively than for β-CD ($8.7 \pm 0.7\,M^{-1}$).

3.2. Dissolution study of BZL from CD complexes

Even though the solubility of BZL increased linearly with an increase in the concentration of each CD, which characterizes the complexation of the drug at 1:1 molar ratio, in this work the complexes were prepared at 1:1 and 1:2 drug:carrier molar ratio to evaluate if an excess of CDs may modify the dissolution profile of BZL. Fig. 1 shows the dissolution profiles of BZL and the corresponding BZL–CD complexes (1:1 and 1:2 molar ratios). It is evident from the data that all the prepared complexes showed better drug release than BZL alone. In particular, the enhanced drug dissolution from the systems prepared with Me-β-CD was higher than from the corresponding ones with the β-CD and HP-β-CD, showing the importance of the proper choice and molar ratio of the carriers. As expected, the amount of BZL dissolved was very low, as a consequence of its very poor aqueous solubility and, after 10 min, only 21% was dissolved in the acidic media. In contrast, BZL:Me-β-CD exhibited 72% and 90% drug dissolution within 10 min, at 1:1 and 1:2 ratio, respectively. These results can be attributed to the combination of factors, such as, the partial or total disappearance of BZL endothermic peak in all the prepared complexes, as observed by DSC analysis (Fig. 2), the detectable loss of BZL crystallinity seen in the different diffractograms (Fig. 3), and/or the novel morphology of the drug particles obtained as a result of the complex formation (Fig. 4). On the other hand, it was found that BZL dissolution rate increased as the ratio of carriers increased. This finding might be related with the formation of both inclusion and non-inclusion complexes [19]. The dissolution efficiency (DE) value at 120 min is shown in Table 1. It is a suitable parameter to compare the '*in vitro*' dissolution of different formulations. As seen, the DE_{120} of all the prepared BZL–CDs complexes were nearly two times higher than those of the plain drug.

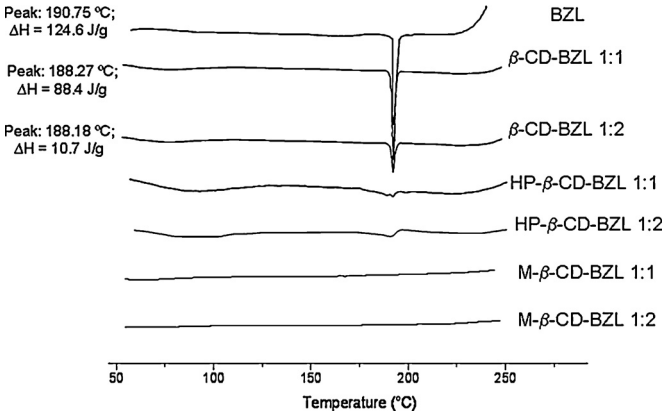

Fig. 2. DSC thermograms of BZL alone, BZL:β-CD, BZL:HP-β-CD, and BZL:Me-β-CD at 1:1 and 1:2 drug:carrier molar ratios.

Table 1
Dissolution efficiency values of BZL and BZL–CDs complexes.

Sample	DE_{120}[b]
BZL[a]	53.31 ± 1.23
BZL-β-CD (1:1)	93.43 ± 1.78
BZL-HP-β-CD (1:1)	88.17 ± 0.93
BZL-Me-β-CD (1:1)	91.87 ± 2.03
BZL-β-CD (1:2)	90.25 ± 1.90
BZL-HP-β-CD (1:2)	92.17 ± 2.45
BZL-Me-β-CD (1:2)	93.43 ± 1.14

[a] Raw BZL was treated in the same manner than the corresponding BZL–CDs complexes.

[b] DE_{120}, dissolution efficiency at $t = 120$ min (calculated from the area under the dissolution curve at $t = 120$ min and expressed as % of the area of the rectangle described by 100% dissolution in the same time). Each value is the average of three determinations.

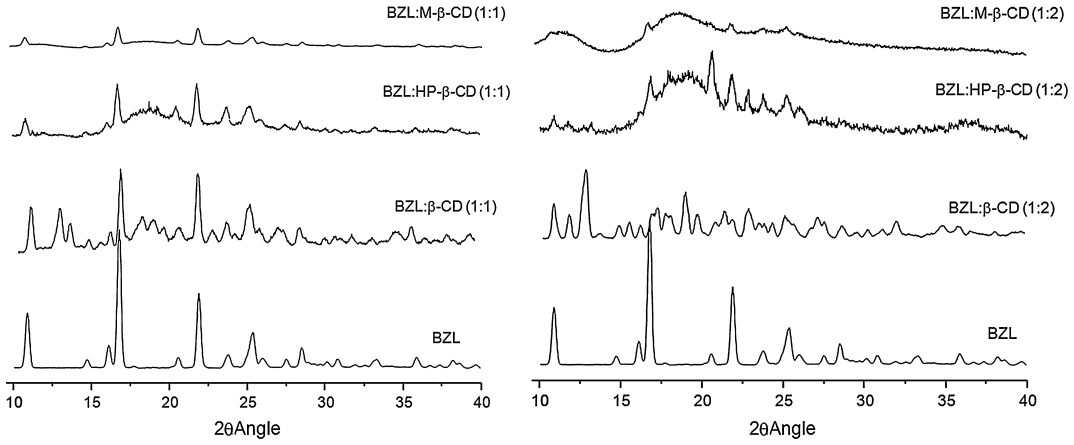

Fig. 3. X-ray diffraction patterns of BZL alone, BZL:β-CD, BZL:HP-β-CD, and BZL:Me-β-CD at 1:1 and 1:2 drug:carrier molar ratios.

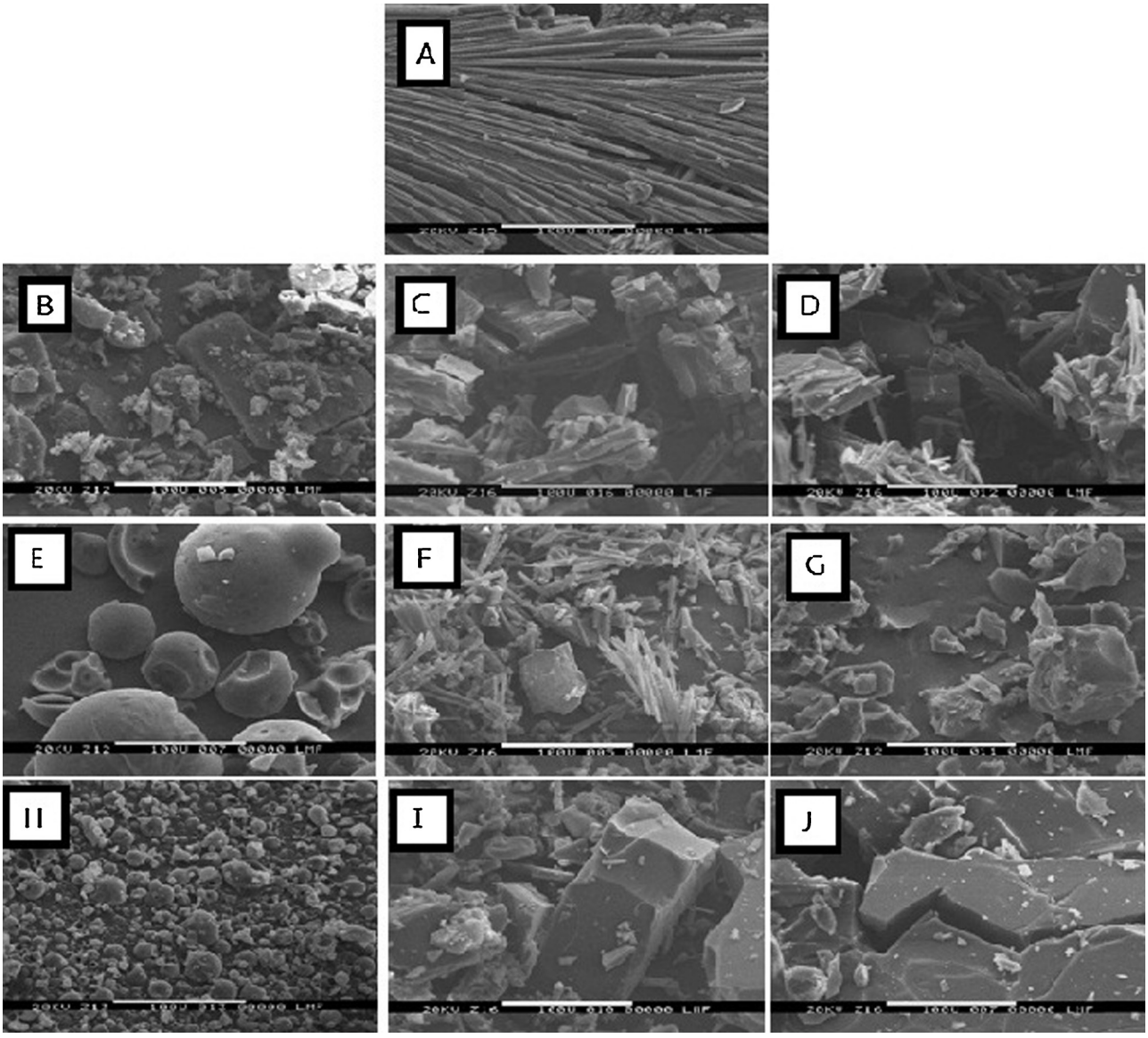

Fig. 4. SEM of (A) BZL, (B) β-CD, (C) HP-β-CD, (D) Me-β-CD, (E) BZL:β-CD 1:1 molar ratio, (F) BZL:HP-β-CD 1:1 molar ratio, (G) BZL:Me-β-CD 1:1 molar ratio, (H) BZL:β-CD 1:2 molar ratio, (I) BZL:HP-β-CD 1:2 molar ratio, (J) BZL:Me-β-CD 1:2 molar ratio.

3.3. Differential scanning calorimetry

DSC profiles of BZL and of the respective binary systems are shown in Fig. 2. BZL alone showed a typical behavior of anhydrous crystalline drug with a melting peak at 191.7 °C [7]. For the inclusion complex with β-CD at 1:1 ratio, both characteristic peaks of BZL (191.7 °C) and β-CD (water loss at around 90 °C) were clearly observed. Marked reduction of area and broadening of drug melting endotherm were observed in the complex with HP-β-CD, indicative of a more evident loss of drug crystallinity. Contrary to previous data, the complete disappearance of the drug endothermal effect was instead observed for the system prepared with Me-β-CD [20]. These modifications confirm a stronger interaction between the drug and the methylated CD in comparison with β-CD and HP-β-CD. On the other hand, the non-stoichiometric complexes were analyzed in order to observe if an increased amount of CDs might further reduce the crystalline state of the drug. The DSC profiles indicated that the thermal behavior of BZL from non-stoichiometric complexes were very similar to those of the stoichiometric complexes, suggesting the absence of novel and/or different thermal interactions by increasing the amount of CDs.

3.4. X-ray diffraction

X-ray diffractometry is a useful method for the detection of cyclodextrin complexation. The diffraction patterns of BZL and CDs complexes are shown in Fig. 3. The XRD of BZL revealed its crystalline character showing the major peaks at 11, 17 and 22.5° 2θ [21]. The diffractogram of the stoichiometric BZL:β-CD complex showed the typical diffraction peaks of the drug. In contrast, a lowered intensity of the peaks at 11, 17° and 22.5° 2θ were observed in the stoichiometric complexes with the HP-β-CD and Me-β-CD, suggesting reciprocal interactions between the components in the solid state, as reported. On the other hand, the non-stoichiometric complexes of BZL with β-CD and HP-β-CD displayed a partial crystalline state, where it was possible to observe the disappearance of some diffraction peaks, particularly at 17° and 22.5° 2θ. Complete drug amorphization was instead observed in the non-stoichiometric complex prepared with Me-β-CD, indicating the strong ability of this carrier to induce drug amorphization. These observations were in accordance with the results of the DSC analysis and confirmed the interactions between the drug and CDs.

3.5. Scanning electron microscopy

Fig. 4 shows SEM micrographs of the pure components and different drug:CDs systems. BZL (A) existed as irregular needle-type crystals while β-CD (B) existed in a crystalline mixture of smooth-surfaced particles with few smaller particles. HP-β-CD and Me-β-CD (E and H) were observed as "shrinked" spheres however, the size of Me-β-CD was notoriously smaller than the HP-β-CD. The systems obtained employing β-CD (C and D) showed similar morphologic characteristics to the raw materials. On the other hand, some changes in the morphology and shape of particles were observed in the complex prepared with HP-β-CD (F and G). In the case of BZL:Me-β-CD complexes (I and J), a poor crystal structure with several cracks and fissures was seen, where pieces of irregular size were present and it was no longer possible to differentiate the two components. These novel arrangements between them would favor the aqueous solubility and dissolution properties of the drug from the corresponding complexes.

3.6. Bioavailability study

It has been reported that CDs may increase or decrease the bioavailability of drugs by different mechanisms [18]. On the other hand, it should be note that an excess of carrier (non-stoichiometric complexes) may decrease the bioavailability of drugs [27]. Thus, the *in vivo* study was carried out to determine the impact of stoichiometric and non-stoichiometric BZL:CDs complexes on the oral bioavailability of the drug, in comparison with BZL alone. As observed in Table 2, T_{max} was similar for the assayed systems indicating that the *in vivo* absorption rate of BZL was nearly the same in those formulations. On the other hand, the complexation with Me-β-CD (1:1 ratio), significantly ($p < 0.05$) increased the AUC_{0-5} of drug from 455.7 ng/ml to 1184.8 ng/ml (2.6-fold), and the C_{max} increased from 665.2 ng/ml to 1536.1 ng/ml (2.3-fold) ($p < 0.05$). Similarly, the complex with HP-β-CD (1:1 ratio) improved significantly ($p < 0.05$) the plasma concentration of BZL, since the AUC_{0-5} increased up to 1051.1 ng/ml (2.3-fold) and a C_{max} of 1480.2 ng/ml (2.2-fold). In contrast, the AUC_{0-5} of BZL complexed with β-CD (1:1 ratio) increased up to 665.5 ng/ml (1.4-fold) ($p < 0.05$). Unexpectedly, the 1:2 drug:Me-β-CD complex, the AUC_{0-5} of BZL was increased up to 1667.9 ng/ml (3.7-fold), while the C_{max} was 1651.3 ng/mL (2.5-fold). The 1:2 drug:HP-β-CD complex increased the AUC_{0-5} and C_{max} values (1180.3 ng/ml and 1467.5 ng/ml, respectively), while the 1:2 drug:β-CD complex also enhanced the values of AUC_{0-5} (984.8 ng/ml) and C_{max} (1463.1 ng/ml). These results were directly correlated with the phase solubility study. As seen, the solubility of BZL was linearly increased in a concentration-dependent manner with the increase in CD concentration, being the Me-β- and HP-β-derivatives more efficient than the β-CD, then, an improved oral bioavailability of BZL was seen by the same complexes. Related with this finding, Loftsson reported that hydrophobic CDs, such as Me-β-CD, may increase the drug flux by changing the membranes properties through extraction or fluidization of some of its components [28]. On the other hand, no correlations were found among the bioavailability and dissolution behavior for all complexes. Considering the dissolution profiles (Fig. 1), it could be postulated that stoichiometric and non-stoichiometric systems (1:1 and 1:2 drug:carrier molar ratio, respectively) would have had almost the same relative bioavailability (Table 2). Since the dissolution studies were not able to predict the relative bioavailability, these findings might be attributed to the change in the dissolution rate of BZL from each complex in the presence of gastrointestinal fluids, and/or some kind of potential interactions between the gastrointestinal membrane and the corresponding complex [29]. In this context, one of the most probable mechanisms hypothesized is related with the accumulation of CDs complexes at level of the biological membranes, so that to favor drug absorption simply by increasing the concentration gradient at the two sides of the membrane [26]. On the other hand, several studies have shown that CDs may interact with biological membranes to form an inclusion complex with the corresponding

Table 2
Comparative pharmacokinetic variables after the oral administration of BZL, and BZL:β-CD, BZL:HP-β-CD, and BZL:M-β-CD at 1:1 and 1:2 drug:carrier molar ratios.[a,b,c]

	C_{max} (ng/ml)	AUC_{0-5} (ng/h/ml)	T_{max} (h)
BZL–water	665.2 ± 23.5	455.7 ± 18.2	2 ± 0.0
BZL:β-CD (1:1)	918.6 ± 41.6	665.5 ± 17.9	2 ± 0.0
BZL:HP-β-CD (1:1)	1480.0 ± 62.1	1051.1 ± 20.1	2 ± 0.0
BZL:M-β-CD (1:1)	1536.0 ± 51.4	1184.8 ± 17.5	2 ± 0.0
BZL:β-CD (1:2)	1463.1 ± 26.7	984.8 ± 24.2	2 ± 0.0
BZL:HP-β-CD (1:2)	1467.5 ± 47.2	1180.3 ± 29.1	2 ± 0.0
BZL:M-β-CD (1:2)	1667.9 ± 31.5	1651.3 ± 23.8	2 ± 0.0

[a] BZL means benznidazole. B-CD, HP-β-CD, and M-β-CD mean β, 2-hydroxypropyl-β, and methyl β cyclodextrins, respectively.
[b] Groups $n = 3$.
[c] Statistical significance of the differences between values was assessed by analysis of variance (ANOVA) followed by Schefe's multiple range tests. P values less than 0.05 were considered significant.

sterol molecules of those membranes, thus reducing their resistance to drug permeation [30]. It might be considered a non-toxic effect, since the interaction between them is only transitory. Then, in the case of non-stoichiometric BZL:CDs complexes, an excess of free CDs may be ready to interact with the hydrophobic molecules (sterol and/or phospholipid derivatives) of the membranes, while in the case of stoichiometric BZL:CDs complexes, the interaction with the membrane phospholipids would exist only by competition between BZL and hydrophobic components of the membranes for the interaction with the CD molecule.

In conclusion, these results demonstrate that both stoichiometric and non-stoichiometric BZL:CDs complexes resulted in a significant increase of the drug solubility and dissolution rate. Moreover, and by the first time, it was seen that the oral bioavailability of BZL from those complexes was significantly enhanced in comparison with the drug alone. Furthermore, the non-stoichiometric BZL:CDs complexes produced a further increased bioavailability of BZL, suggesting that these types of CDs complexes may represent an effective approach to enhance the drug bioavailability for the treatment Chagas' disease.

Acknowledgements

The authors express their gratitude to the National University of Rosario (U.N.R.) (Argentina), FONCYT (Argentina), and the National Council Research (CONICET, Argentina).

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