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Preclinical pharmacokinetics of benznidazole-loaded interpolyelectrolyte complex-based delivery systems

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ABSTRACT

Benznidazole (BZ), first-line drug for Chagas treatment, is available as immediate-release tablets. High frequency of administration, long-term therapy, and side effects of BZ conspire against treatment adherence, and negatively impact in therapeutic success. We have developed BZ-loaded interpolyelectrolyte complexes (IPECs) composed of polymethacrylates (EE-EL-BZ) or polysaccharides (Ch-AA-BZ) for controlled BZ release. This work aimed to evaluate their preclinical pharmacokinetics compared to Abarax® (reference treatment) and to correlate them with the *in vitro* BZ release. A randomization schedule with a 3 × 2 cross-over design was used. Each healthy dog received a single oral dose of 100 mg of BZ from EE-EL-BZ, Ch-AA-BZ or Abarax®. BZ quantification was performed in plasma by a validated HPLC-UV method. Moreover, *in silico* simulations using the pharmacokinetic software PK Solutions 2.0™ were calculated for the multiple-dose administration at two dose regimens: 100 mg of BZ administered every 12 and 24 h. Also, the relationship between *in vitro* dissolution and *in vivo* plasma BZ concentration profiles in a single step was model for IVIVC analysis. BZ was rapidly absorbed from all formulations. The C_{max} value for Ch-AA-BZ was 32% higher than reference ($p < 0.05$) and an earlier T_{max} (4.2 h) was observed as compared to EE-EL-BZ (6.0 h). For both IPECs, the T_{max} values were higher ($p < 0.05$) and the areas under the curve were 25% greater than those of Abarax® ($p < 0.01$). Despite these variations in pharmacokinetics parameters, simulations of once or twice daily dosing showed that all formulations reached a steady-state range concentration above of the minimum therapeutic dose while avoiding high BZ concentrations related to increased side effects. A linear level A IVIVC model was established using plasma concentration profiles and dissolved data obtained. Thus, BZ-loaded IPECs prolonged drug release and formulated as capsules showed improved *in vivo* performance, in terms of bioavailability and T_{max} values, which were significantly higher compared to Abarax®. These BZ carrier systems would be useful for oral administration in the treatment of Chagas disease.

1. Introduction

Chagas disease is a potentially life-threatening illness caused by the protozoan parasite *Trypanosoma cruzi* and it constitutes a major public health problem in Latin America due to its prevalence, morbidity and mortality, socioeconomic impact and geographic distribution (Campi-Azevedo et al., 2015; World Health Organization, 2015). Besides, the disease has been spread to non-endemic regions and thousands of new cases are diagnosed each year (Cencig et al., 2012; Rassi et al., 2010).

During the acute phase, the parasites replicate in tissues throughout the body, showing a strong tropism for the myocardium (Coura and Borges-Pereira, 2012; Rassi et al., 2010). Immune responses control the parasite levels, but are insufficient to completely clear the infection and thus most individuals remain infected for life without an efficient treatment (Bustamante et al., 2014; García et al., 2016).

It is a systemic disease with certain therapeutic limitations. In fact, to date, the efficacy and safety of Chagas disease pharmacotherapy is still unsatisfactory, and an effective prophylactic vaccine has yet to be

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developed (García et al., 2016). Specific etiological treatment is based mainly on two drugs developed five decades ago: benznidazole (BZ; Radanil®, Roche, and Abarax®, ELEA) and nifurtimox (NFX; Lampit®, Bayer) (Bellera et al., 2015). BZ and NFX have been shown to result in a parasitological cure in > 95% of congenital cases, 60–80% of adult acute *T. cruzi* infections and 60–70% of early chronic infections (Urbina, 2010). Nevertheless, the recommended treatment with these drugs has poor compliance, especially in adult patients, due to high dose and long-term treatment, frequent undesirable side effects and biochemical damage to mammalian tissues (Davies et al., 2014). Different reports about the effectiveness and differential toxicity of both drugs are controversial. However, it has been proposed that BZ is frequently better tolerated than is NFX (Rassi et al., 2010; Rojo et al., 2014).

BZ is commercially provided as immediate-release tablets and the treatment is administered 2–3 times daily for 60 days (Molina et al., 2017; Soy et al., 2015) in most cases. This therapeutic scheme implies high frequency of administration and long-term treatment (Bustamante et al., 2014; Coura, 2009; Viotti et al., 2009). Moreover, the side effects of BZ conspire against treatment adherence (Davanço et al., 2016) with treatment abandonment ranges from 7.6 to 29.7% (Pinazo et al., 2010), which conditions the efficacy and safety, and in consequence, negatively impact the therapeutic success.

In this sense, the development of novel strategies employing new controlled-release drug systems is an attractive alternative to be considered, in order to improve the current treatment of Chagas (Chatelain and Ioset, 2011). Controlled BZ release would reduce its adverse effects by avoiding high plasmatic BZ concentration (close to 20 mg/L), which are related to a greater risk of toxicity (Soy et al., 2015) and would allow a reduction in its frequency of administration, by maintaining effective concentrations (in the range of 3–6 mg/L) (Soy et al., 2015) for a longer period of time. Moreover, BZ is very slightly soluble in water (García et al., 2015; Kasim et al., 2004) which may have a direct impact on its bioavailability (Sá-Barreto et al., 2013).

Considering the aforementioned, great interest has been focused on the development of novel controlled-release drug systems based on polymers, which involve matrix or coated drug particles based on one or more polymeric carriers to achieve controlled/sustained drug release (Olivera et al., 2017). In this context, several types of oppositely charged polyelectrolytes can interact electrostatically in aqueous media to form soluble or insoluble interpolyelectrolyte complexes (IPECs) (Bani-Jaber et al., 2011; Palena et al., 2015). These complexes have the ability to achieve more sustained drug release than do single polymers, with improved drug delivery behavior in comparison to homologous binary complexes, showing a remarkable robustness towards changes of release media pH, from 1.2 to 6.8 (Palena et al., 2015; Palena et al., 2012).

We hypothesized BZ-loaded IPECs for controlled BZ release could lead to promising results and prospects *in vivo*. In this sense, the goals of the present work were to evaluate comparatively the preclinical pharmacokinetic parameters of BZ-loaded IPECs contained in hard gelatin capsules with respect to Abarax® (BZ 100 mg) tablets (reference treatment), following administration of a single oral dose of BZ in healthy dogs, and to correlate those results with the *in vitro* release of BZ towards simulated gastric fluid (SGF).

Even when the most widely used drug for treating Chagas disease is BZ, there is little information on its pharmacokinetics in dogs (White et al., 1982; Workman et al., 1984). In consequence, this work also usefully contributes to this knowledge.

2. Materials and methods

2.1. Materials: drugs, polymers and reagents

The BZ was extracted and purified from commercially available tablets (Radanil®, Roche, Argentina) (García et al., 2016). Benzocaine (PA grade) internal standard (IS) and metronidazole were obtained from Parafarm® (Buenos Aires, Argentina). Abarax® immediate-release tablets of BZ at 100 mg (Elea, batch number 9878) were used as reference.

Two polymethacrylates were kindly supplied by Etilpharma (Buenos Aires, Argentina): Eudragit® EPO (EE) and Eudragit® L100 (EL) were used to develop the IPEC, and two polysaccharides: alginic acid (AA) from *Macrocystis pyrifera* (PA grade, Sigma Aldrich®) and chitosan (Ch) (PA grade, Sigma Aldrich®) were also used. The proportions of ionizable groups of these polyelectrolytes, determined by potentiometric titration and the equivalents of amino or carboxylic groups, expressed as mmol/g of polyelectrolytes, were 3.15, 4.85, 5.11, and 4.40 for EE, EL, Ch and AA, respectively.

The following reagents: KH_2PO_4 and K_2HPO_4 (PA grade, Anedra®), NaCl (PA grade, Parafarm®), dimethyl sulfoxide (DMSO), absolute ethanol, 1 N NaOH and HCl solutions (Anedra®) were used as purchased without further purification. Acetonitrile (Sintorgan®, HPLC grade) and Milli Q water were used for HPLC mobile phase.

All experiments were carried out with distilled and purified water.

2.2. Preparation of benznidazole-loaded IPEC

The BZ-loaded IPEC was prepared as previously described (García et al., 2018a; García et al., 2018b). Briefly, a two-step process was used: a casting solvent method to obtain the IPEC loaded with BZ, using water or hydroalcoholic solvent as interaction media, followed by a wet granulation to obtain the multiparticulated BZ-loaded IPECs, using water as wetting agent. Briefly, the solids of BZ and the two polyelectrolytes were put in contact in a mortar and the interaction medium (water and water/ethanol 1:1, v/v) was added in small aliquots. The semi-solid paste formed was subjected to kneading for 10 min and left overnight at room temperature. After 24 h, the material was dried at room temperature until constant weight was achieved. Once dry, the solid materials were milled and passed through 210 and 400 μm analytical sieves. After sieving, the powders were subjected to a wet granulation process in order to obtain the multiparticulated BZ-loaded IPECs. For this, each IPEC was placed in a mortar and moistened with (42 ± 3)% v/w of water, with respect to the total amount of solid, and the semi-solid mass was extruded using a 850–1000 μm analytical sieve. Then, the BZ-loaded IPECs were dried to constant weight in an oven at 40 °C.

In order to perform the *in vivo* studies, both multiparticulated BZ-loaded IPECs, at doses of 100 mg of BZ, were formulated in hard gelatin capsules. On the one hand, a system composed of a mix of two BZ-loaded IPEC based on polymethacrylates was evaluated (IPEC EE-EL-BZ), where the 25% of the total dose of BZ was incorporated from an IPEC obtained in water as interaction medium, and the remaining 75% of the total dose of BZ was from an IPEC prepared in hydroalcoholic medium. On the other hand, the IPEC based on polysaccharides obtained in hydroalcoholic mixture as interaction medium was selected (IPEC Ch-AA-BZ).

2.3. Benznidazole release studies

The release of BZ from both IPEC and Abarax® tablets was evaluated in triplicate, in a dissolution apparatus (SOTAX®AT 7 Smart, Switzerland). For both IPECs contained in hard gelatin capsules, the dissolution assay was carried out using *Apparatus 1*, rotating basket, at 100 rpm.

The preparation of hard gelatin capsules was necessary to contain the multiparticulated IPEC systems in a dosage form to be compared with the reference treatment. The capsules were filled with appropriate amount of BZ-loaded IPEC (approximately 200 mg of multiparticles, which represents 100 mg of BZ).

For Abarax® tablets (used as a reference) the assay was performed in *Apparatus 2*, paddles, at 75 rpm. The dissolution vessels were filled with

900 mL of SGF, without pepsin, as dissolution medium, at pH 1.2 ± 0.2 and 37.0 ± 0.5 °C (U.S. Pharmacopoeial Convention, 2015).

At predetermined times (5, 15, 30, 60, 90 and 120 min), samples of 4 mL were withdrawn from the dissolution apparatus media, filtered, conveniently diluted and spectrophotometrically quantified at 324 nm (UV–Vis Evolution 300 spectrophotometer, Thermo Electron Corporation, USA).

All the samples were replaced with preheated fresh dissolution medium. The cumulative percentage of BZ release was calculated and expressed as a function of time. The results were expressed as the % average of three determinations, with their SD.

The release profiles of BZ from the IPEC and Abarax® tablets were compared statistically using the difference factor (f_1) and similarity factor (f_2) (Eqs. (1) and (2), respectively). According to this methodology, an f_1 value above 15 and f_2 value of 0–49 implies a difference between the release profiles (Costa and Lobo, 2001; Food and Drug Administration, 1997).

$$f_1 = \frac{\sum_{t=1}^{n} |R_t - P_t|}{\sum_{t=1}^{n} R} \times 100 \quad (1)$$

$$f_2 = 50 \times \log\left\{\left(1 + \left(\frac{1}{n}\right)\sum_{t=1}^{n} (R_t - P_t)^2\right)^{-0,5} \times 100\right\} \quad (2)$$

where n is the number of sampling time points, Σ is the summation over all time points and R_t and P_t are the cumulative percentages of drug released at each of the n time points of the reference and test product, respectively. The CV was below 15% in all cases. Only one point after 85% of drug release was used for the equation.

2.4. High-performance liquid chromatography validation for benznidazole determination

The high-performance liquid chromatography with UV–visible detection (HPLC-UV) method used in this study was based on the method proposed by Guerrero et al. (2011).

In accordance with the US Food and Drug Administration's "Guidance for Industry, Bioanalytical Method Validation" the following criteria were evaluated: limit of detection (LOD), lower limit of quantitation (LLOQ), linearity, accuracy, precision, recovery, specificity, and short-term and freeze/thaw stability (Department of Health and Human Services Food and Drug Administration (U.S.), 2010).

For quantification validation methodology, drug-free plasma was employed. The observed peak area ratio with reference to the IS was used to assess drug concentrations. The BZ concentration in dog plasma samples was calculated using the linear regression equation of the peak area ratio against the concentration ratio for the calibration curve, according to Eq. (3):

$$\frac{A_{BZ}}{A_{IS}} = a\frac{[BZ]}{[IS]} + b \quad (3)$$

where A_{BZ} is the benznidazole area; A_{IS} is the benzocaine area; [BZ] is the benznidazole theoretical concentration (μg/mL of plasma); [IS] is the benzocaine theoretical concentration (μg/mL of plasma); a is the slope; and b is the ordinate where the BZ concentration equals 0.

For preparation of stock solutions, 2 mg of BZ were weighed and 1 mL of DMSO was added. This solution was diluted four times with Milli Q water to obtain stock solution A ([BZ] = 500 μg/mL of plasma). The IS stock solution was prepared by adding 10 mg of benzocaine to 1 mL of DMSO. This solution was diluted ten times with Milli Q water to obtain stock solution B ([IS] = 1 mg/mL of plasma). Both solutions were prepared freshly just before analysis. Stock solution A was used to prepare both standard and quality control solutions.

In order to construct the calibration curve, several standard solutions were prepared with different concentrations of BZ (concentration range studied 0.4–100.0 μg/mL) by spiking an appropriate volume of stock solution A into drug-free plasma. These dilutions were also spiked with IS (stock solution B) to achieve a final concentration of 300 μg/mL. These standards were mixed, and afterwards the mixture was precipitated with 2 mL of acetonitrile. After that, the samples were centrifuged at 8000 × *g* for 10 min. Finally, the supernatants were filtered and 100 μL of each was injected into the chromatographic system.

The specificity of the method was evaluated by addition of metronidazole to some samples of calibration curve solutions. This drug was selected because it has a chemical structure similar to BZ.

The HPLC chromatographic analyses were performed using a Waters® HPLC system equipped with an isocratic Waters® 1525 pump, an autosampler Waters 717 Plus and a PDA-UV detector (PDA 2296 detector) at 324 nm, with data acquisition and processing being performed using Empower® system software. Chromatographic separations were carried out using a Phenomenex® C18 reverse phase column (250 × 4.6 mm, 5 μm particle size) and a Phenomenex® guard column (C18 4 × 3 mm ID). Analysis was performed with water/acetonitrile (60:40, v/v) as the mobile phase at a flow-rate of 0.9 mL/min in the isocratic mode. For analysis, 100 μL of each sample was injected, and the run time was set at 15 min. Peak areas were used for quantitative analysis.

2.5. Animals and ethics statement

This project was approved by the Commission of Bioethics and Animal Welfare of the Faculty of Agricultural Sciences of Catholic University of Cordoba (Ethical Committee Approval Number CBBA.01.2016UCC).

Six healthy adult mixed-breed dogs (4 males, 2 females), aged 3 to 4 and with body weights of 22 ± 2 kg were included in this study. Dogs were not exposed to any drug treatment for two months prior to the experiment. The animals had *ad libitum* access to water and feed after 6 h of the beginning of these studies at each point.

2.6. Experimental design

The study was carried out with a 3 × 2 cross-over design (3 different treatments and 2 animals assigned per treatment at each experiment). For that purpose, each animal received all treatments after three experiments, with a washout period of 15 days. The treatments were administrated orally by a vet. The animals received a single oral dose of 100 mg of BZ from each IPEC (EE-EL-BZ or Ch-AA-BZ) or Abarax® tablets, according to a randomization schedule (Fig. 1).

2.7. Sampling

Using heparinized syringes, blood samples (3 mL) from the jugular vein were collected at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 24, 36 and 48 h post-administration. The samples were centrifuged at 2000 × *g* (15 min) and the plasma was stored at −20 °C for further processing and BZ quantitation by HPLC within 30 days from the pharmacokinetics experiment.

For HPLC analysis, all dog plasma samples (500 μL) were spiked with an appropriate amount of stock solution B to achieve a final concentration of 300 μg/mL of benzocaine. This mixture was precipitated with 2 mL of acetonitrile and after that, the procedure was similar to that detailed in Section 2.4.

2.8. Pharmacokinetic parameters

The maximum plasma BZ concentration (C_{max}) and the time to reach this concentration (T_{max}) were directly determined from the plasma concentration–time curves. The software PK Solutions 2.0™, which relies on the use of non-compartmental methods of analysis, was used for estimation of pharmacokinetic parameters. The areas under the curve from zero to the last sampling point (AUC_{0-72}) and from zero to

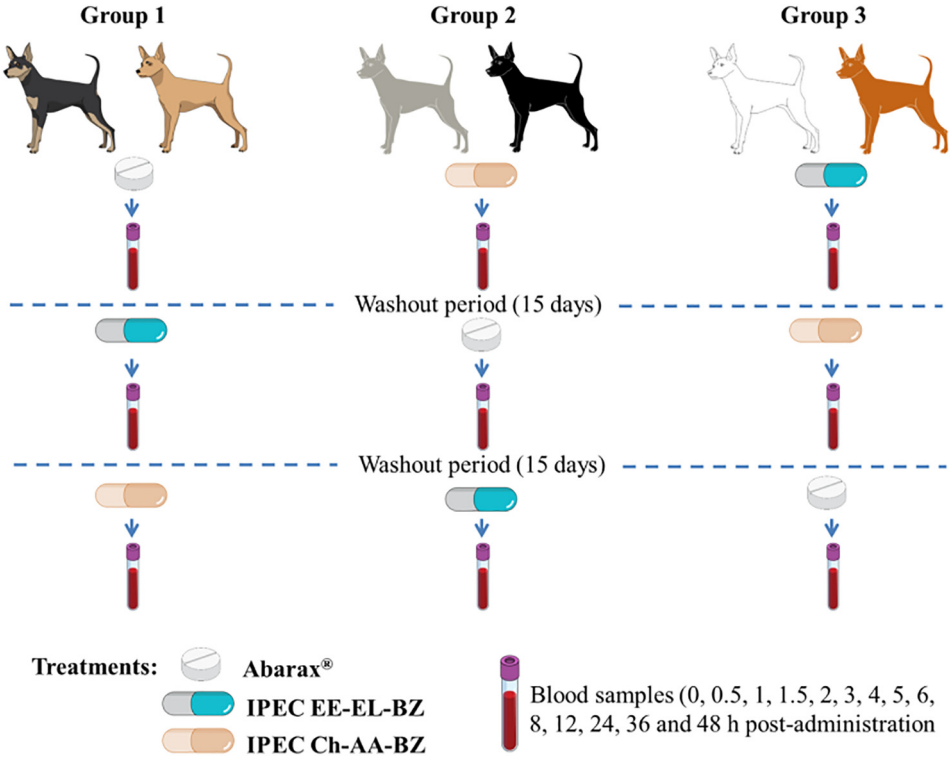

Fig. 1. Scheme of randomization used in the pharmacokinetic experimental design.

infinity ($AUC_{0-\infty}$), and the area under the first moment curve (AUMC) were estimated by the trapezoidal rule. Mean residence time from zero to infinity ($MRT_{0-\infty}$) was calculated as the ratio of AUMC and AUC. Other pharmacokinetic variables as absorption rate constant (k_a); elimination half-life ($t_{1/2}$) were also obtained. In addition, maximum and minimum concentration at steady-state conditions ($C_{max,ss}$ and $C_{min,ss}$, respectively) were predicted by simulation of multiple-dose administration every 12 h and 24 h. $C_{max,ss}$ was calculated from a simplification of the graphing function (which involves the addition of a decay function to the initial concentration at repeated time points for a progressive series of doses. Assumes constant dose intervals given during the post-distribution phase) to a steady state form Eq. (4).

$$C_{max,ss} = C_{1(t)} + \frac{C_z \times e^{-\lambda_z \tau} \times e^{-\lambda_z t}}{1 - e^{-\lambda_z t}} \text{where} \, C_{1(t)} = \sum C_n e^{-\lambda_n t} \quad (4)$$

$C_{1(t)}$, C_z and C_n are the initial concentration at repeated time point, the concentration considered for the decay function and the concentration from y-intercept (extrapolation), respectively, λ_n and λ_z are rate constant and elimination rate constant, respectively, τ is the dosing interval and t is the time.

$C_{min,ss}$ was calculated by the software using the same steady state equation as $C_{max,ss}$, but considering the minimum concentration during a steady state dose interval.

The accumulation factor (R) was determined according to Eq. (5) (Tozer and Rowland, 2006).

$$R = \sum \frac{1}{1 - e^{-\lambda_z \tau}} \quad (5)$$

Fluctuations of plasma concentrations (peak and trough concentrations, P and T respectively) at steady-state were calculated as $C_{max,ss}$ and $C_{min,ss}$ ratio according to Eq. (6) (Toutain and Bousquet-Mélou, 2004).

$$\frac{P}{T} \text{ratio} = \frac{C_{max,ss}}{C_{min,ss}} \quad (6)$$

2.9. In vitro-in vivo correlations (IVIVC)

For IVIVC an approach based on a convolution procedure specified by the US Food and Drug Administration's "*Guidance for Industry, Extended Release Oral Dosage Forms: Development, Evaluation, and Application of In Vitro/In Vivo Correlation* (US Department of Health and Human Services et al., 1997) was used. For that purpose, the relationship between *in vitro* dissolution and *in vivo* plasma BZ concentration profiles in a single step was model. Plasma BZ concentrations *versus* cumulative percentages of BZ released towards SGF for each formulation evaluated were plotted considering up to 2 h of both assays. The regression equations and correlation coefficients (R^2) were determined from linear analysis. To further assess the predictability and the validity of the correlation, observed and predicted pharmacokinetic profiles were determined for each formulation. The percent prediction errors (%PE) for plasma BZ concentrations were calculated according to Eq. (7):

$$\%PE = \frac{[BZ]^{obs}_{plasma} - [BZ]^{pred}_{plasma}}{[BZ]^{obs}_{plasma}} \times 100 \quad (7)$$

where $[BZ]_{plasma}{}^{obs}$ and $[BZ]_{plasma}{}^{pred}$ are BZ plasma concentrations at each time of assay observed *in vivo* or predicted by the IVIVC model, respectively. The IVIVC model was considered valid if the % mean absolute prediction error did not exceed 10% (US Department of Health and Human Services et al., 1997).

2.10. Statistical analysis

Pharmacokinetic parameters were analyzed using the statistical program GraphPad® Prism 6. Normally distributed parameters were analyzed with one-way ANOVA for paired samples test. Shapiro-Wilk and ANOVA tests were used to evaluate normality and homoscedasticity assumptions, respectively. In case that the data did not meet the assumptions of ANOVA, a nonparametric Kruskal-Wallis test was performed. The significance level was 5% ($p < 0.05$) throughout.

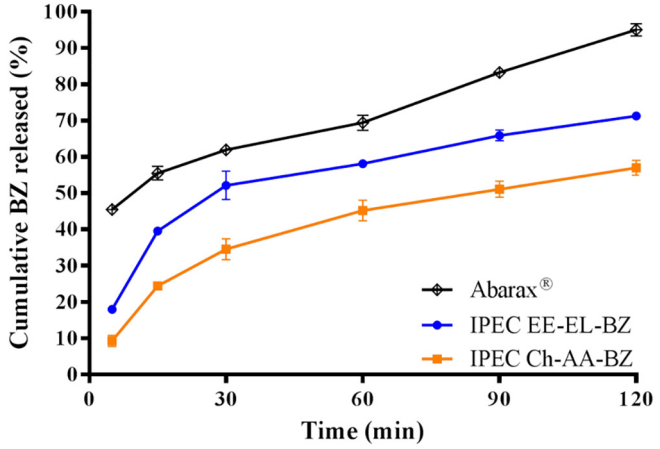

Fig. 2. Release profiles of BZ from both IPEC and Abarax® (used as reference), containing 100 mg of BZ, towards SGF without pepsin.

3. Results

The multiparticles of BZ-loaded IPEC were developed using two methodologies applied consecutively. The first one was the casting solvent method, using water or hydroalcoholic mixture as interaction media, to obtain BZ loaded-IPEC in the solid state. The second was the wet granulation process, using purified water as wetting agent, to obtain the multiparticulated BZ-loaded IPEC with particle sizes of 850–1000 µm. These methods were simple and allowed yields of 83 ± 8% with respect to the initial amount of solids used (García et al., 2018a; García et al., 2018b).

3.1. In vitro drug release studies

The BZ release behavior from the multiparticles was studied to evaluate their performance as an oral drug delivery dosage form. Fig. 2 shows the BZ release profiles from both IPECs based on polymethacrylates and polysaccharides in comparison to Abarax®. For both IPEC systems, a slow and extended release of BZ towards dissolution medium was observed. In assay conditions, Abarax® did not meet the general requirements for immediate-release tablets (U.S. Pharmacopoeial Convention, 2015), and showed only ~60% of BZ dissolved after 30 min.

The analysis of similarity test denoted that all the systems evaluated presented non-similar release profiles of BZ. The comparison between Ch-AA-BZ and Abarax® revealed an f_1 value of 68.1, and an f_2 value of 27.8; and between EE-EL-BZ and Abarax®, values of 26.8 and 39.8, respectively. The f_1 and f_2 values between both IPECs were 37.6 and 42.4, respectively. The cumulative BZ released reached up to 95, 57 and 71% for Abarax®, EE-EL-BZ and Ch-AA-BZ, respectively, after 2 h of release studies.

3.2. Benznidazole bioanalytical method validation

As can be seen in Table 1, all the criteria analyzed to validate the bioanalytical method were successfully fulfilled.

Under the experimental conditions used, BZ and IS were eluted within the retention windows of 6.5–7.5 min and 10.8–11.5 min, respectively. Linearity was found over the concentration range of 0.4–100.0 µg/mL; $R^2 = 0.9992$. The specificity of the method was verified, because metronidazole showed no interfering peaks in the analysis of BZ (metronidazole retention time: 3.2–4 min) (Fig. 3). The inter- and intra-day variations of all values of precision and accuracy were < 9%. Short-term stability after 24 h at room temperature as well as after three cycles of freeze/thaw were verified, with a coefficient of variation < 10% in all cases.

Table 1
Parameters of the bioanalytical method for quantification of BZ in dog plasma.

Parameters	Results
Linearity (n = 8)	
Concentration range [µg/mL]	0.4–100.0
Correlation coefficient [R^2]	0.9992
Equation	$y = 0.0146x + 0.0069$
LOD [µg/mL]	0.1
LLOQ [µg/mL]	0.4
Precision, %CV	
Intra-assay	4 ± 2
Inter-assay	9 ± 3
Accuracy,% E_R	
Intra-assay	3 ± 2
Inter-assay	4 ± 2
Recovery, %R	101 ± 6
Stability, %S	
Short term (24 h at room temperature)	104.75 ± 0.01
Freeze/thaw (3 cycles)	111.000 ± 0.002

LLOQ: lower limit of quantitation, LOD: limit of detection, %CV: coefficient of variation, $\%E_R$: accuracy.
For %CV and $\%E_R$ the values reported correspond to mean ± SD of the values obtained for the lowest, medium and highest BZ plasma concentration evaluated.
For %R and %S the values reported correspond to mean ± SD of the values obtained for the medium and highest BZ plasma concentration evaluated.

Also, the BZ and IS were quantitatively extracted from dog plasma with a recovery of 100%.

3.3. Pharmacokinetic parameters

Fig. 4 shows the mean plasma concentration–time profiles that were obtained from pharmacokinetic studies after a single 100 mg oral dose of BZ. It can be observed that both IPECs exhibited plasmatic concentrations above 3 µg/mL (dotted lines) up to 25 and 27 h for Ch-AA-BZ and EE-EL-BZ, respectively; while Abarax® showed plasmatic concentrations higher than 3 µg/mL only up to 16 h post-administration.

Thirty-six hours post-administration, the elimination profiles in all groups became similar, with plasmatic levels of BZ around 1 and 1.5 µg/mL for Abarax® and both IPECs, respectively (Fig. 4).

As can be seen in Table 2, BZ was absorbed rapidly from all formulations evaluated, with an absorption rate expressed by the k_a values increasing in the order EE-EL-BZ < Ch-AA-BZ~ Abarax®. The C_{max} value for Ch-AA-BZ was 32% higher than the reference treatment. However, the C_{max} value obtained for EE-EL-BZ was similar to that for Abarax®. In addition, an earlier T_{max} was observed with Ch-AA-BZ compared to EE-EL-BZ. For both IPECs, the T_{max} values were significantly higher than for Abarax®. After C_{max}, a faster decrease was observed in BZ plasma levels from Abarax®; while more flattened profiles were observed for both IPECs. The $t_{1/2}$ showed an elimination in the order Ch-AA-BZ~ Abarax® > EE-EL-BZ. However, non-significant differences were observed in the MRT parameter. The AUC_{0-72} and $AUC_{0-\infty}$ of both IPECs were significantly higher, at about 25% greater than those of the reference treatment, and non-significant differences were observed in the calculated AUC parameters between both IPECs (Table 2).

Results from *in silico* simulations are displayed in Table 3. Schematic results of median BZ concentrations at steady-state *versus* time after oral administration every 12 and 24 h are shown in Fig. 5. With a degree of accumulation no > 50% in dose regimen 100 mg/24 h and around 100% in dose regimen 100 mg/12 h, as shown in Table 3, the R values apparent to be similar among the three formulations, which was predictable considering their very close $t_{1/2}$. In addition, a reduction of the amplitude of fluctuations of plasma concentration at steady state was observed in BID schema.

Regarding IVIVC and considering the first 2 h of *in vitro* and *in vivo*

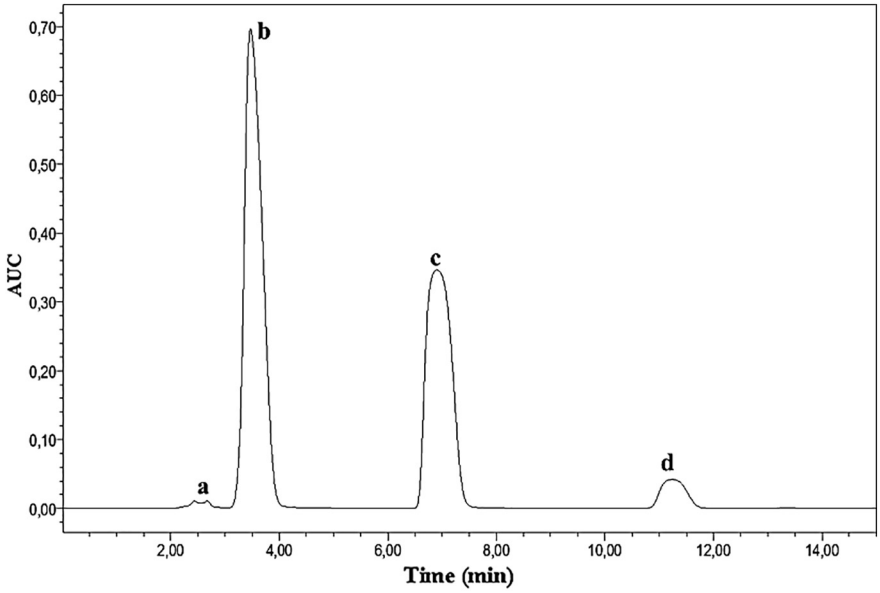

Fig. 3. Representative chromatogram of dog plasma spiked with BZ, IS and metronidazole to evaluate the specificity of the bioanalytical method. The peaks correspond to: a) plasma impurities, b) metronidazole, c) BZ and d) SI.

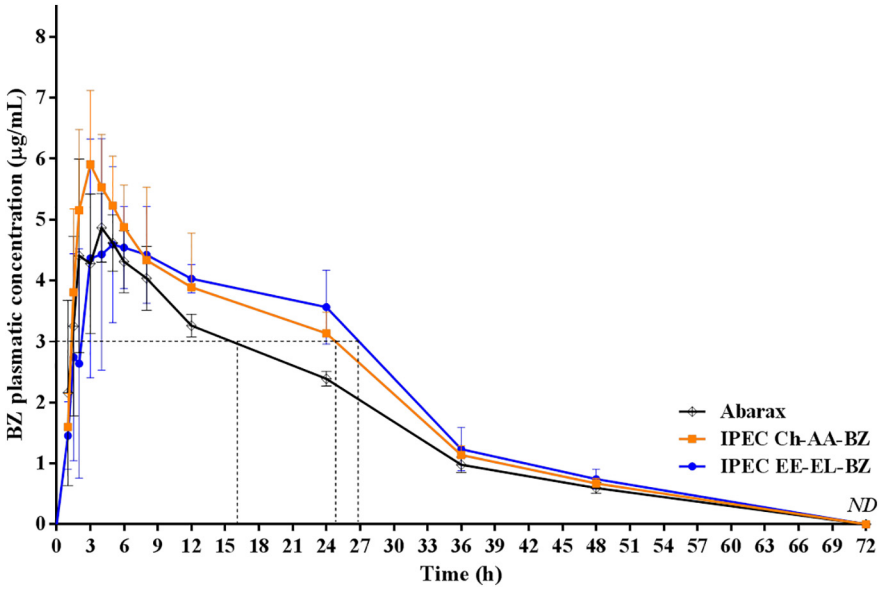

Fig. 4. Systemic exposure of BZ after oral administration of all formulations (at 100 mg dose) to dogs. Dotted lines indicate the minimal effective concentration (trypanocidal concentration) of BZ according to studies performed *in vitro* (Soy et al., 2015).

Table 2
Pharmacokinetic parameters calculated for BZ from IPECs and Abarax® reference tablets using the non-compartmental model by the pharmacokinetic software PK Solutions 2.0™.

Parameter	Abarax®	IPEC Ch-AA-BZ	IPEC EE-EL-BZ
AUC_{0-72} (µg·h/mL)	109 ± 4	137 ± 9**	135 ± 6**
$AUC_{0-\infty}$ (µg·h/mL)	118 ± 5	152 ± 9**	149 ± 9**
C_{max} (µg/mL)	4.9 ± 0.3	6.5 ± 0.5*	5.5 ± 0.9
T_{max} (h)	2.8 ± 0.9	4.2 ± 0.8*	6.0 ± 0.7*
k_a (h^{-1})	0.7 ± 0.3	0.7 ± 0.2	0.3 ± 0.2
$t_{1/2}$ (h)	14 ± 1	15 ± 2	12 ± 2
$^{\#}MRT_{0-\infty}$	21 ± 2	22 ± 2	21 ± 3

Asterisks indicate significant differences with respect to the control group (Abarax®) (*$p < 0.05$; **$p < 0.01$). $^{\#}MRT_{0-\infty}$ was calculated as AUMC and AUC ratio.

Table 3
Predicted parameters under steady-state conditions obtained by *in silico* simulations using the pharmacokinetic software PK Solutions 2.0™.

Predicted steady state parameters	Dose regimens of 100 mg of BZ administered every 12 h and 24 h					
	Abarax®		IPEC Ch-AA-BZ		IPEC EE-EL-BZ	
	12 h	24 h	12 h	24 h	12 h	24 h
$C_{max,ss}$	11.7	7.1	11.8	7.3	14.5	8.2
$C_{min,ss}$	9.4	3.5	8.7	4.0	11.6	4.1
R	1.9	1.3	2.4	1.5	1.8	1.3
$\frac{P}{T}$ ratio	1.2	2.0	1.4	1.8	1.3	2.0

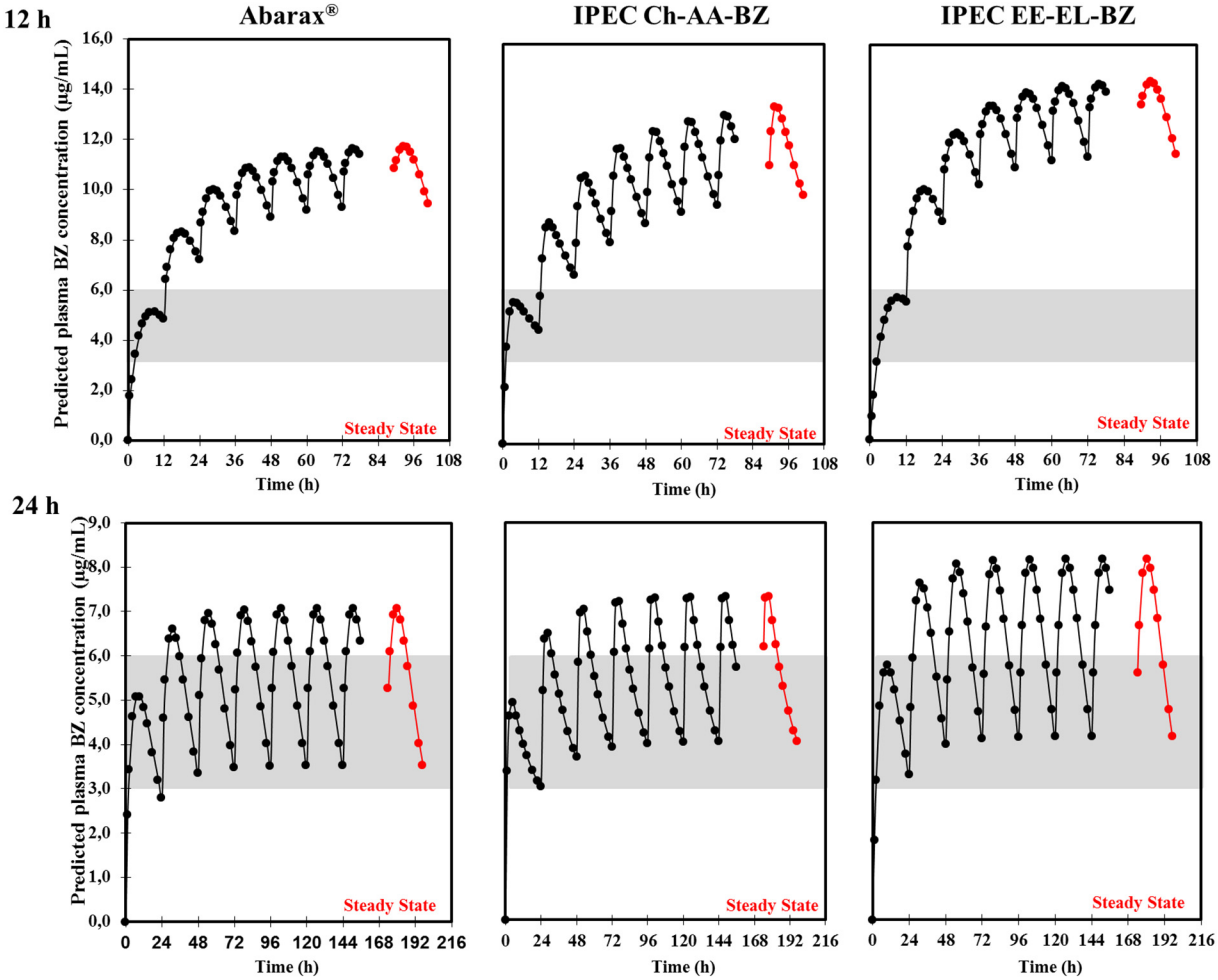

Fig. 5. Schematic *in silico* simulation results of median BZ concentrations *versus* time at steady-state for oral administration of BZ at 100 mg every 12 and 24 h for Abarax®, IPEC EE-EL-BZ and IPEC Ch-AA-BZ. Shaded areas between dashed lines represent the classically accepted optimal therapeutic range between 3 and 6 µg/mL.

results, a satisfactory linear regression was obtained and direct correlations in a single step between the *in vitro* dissolution and *in vivo* plasma concentration profiles for each formulation were observed. The mathematical models of the linear correlations and corresponding factors (R^2) to the least squares regression line were: $y = 0.1226x - 7.0377$, $R^2 = 0.92$, $y = 0.1429x - 7.1279$; $R^2 = 0.89$, and $y = 0.2353x - 8.4102$; $R^2 = 0.97$ for Abarax®, IPEC EE-EL-BZ and IPEC Ch-AA-BZ, respectively. The mean absolute prediction errors for Abarax®, IPEC Ch-AA-BZ and IPEC EE-EL-BZ were 9.86, −7.21 and 6.68% respectively. It is important to note that positive and negative signs of %PE indicate that predicted values are greater than or lesser than observed values.

4. Discussion

The present work provides new knowledge regarding preclinical pharmacokinetics in dogs from BZ-loaded delivery systems based on IPECs, which could contribute to improve Chagas pharmacotherapy.

The treatment of Chagas disease has relied on the use of BZ and NFX since five decades ago (Bellera et al., 2015; Bustamante et al., 2014; Viotti et al., 2009). These drugs are recommended for acute-stage, early-chronic-stage, and reactivated cases (Coura and de Castro, 2002; Urbina, 2010; Urbina and Docampo, 2003; Von et al., 2007). Nevertheless, despite current recommendations, < 1% of *T. cruzi*-infected persons have received any treatment (Ribeiro et al., 2009). Although the treatment with BZ is recommended clinically, the evidence-based medicine has not been fully validated. In addition, evidence on the use of BZ in the chronic phase is still controversial (Rassi et al., 2010).

It has been proposed that BZ is underused because of its side effects, long-term treatment, and unpredictable treatment outcomes (Bustamante et al., 2014; Coura, 2009). Even when a BZ daily treatment has been approved, its duration is highly variable (Viotti et al., 2009). In this sense, new approaches to address this illness should be developed (Bermudez et al., 2016; García et al., 2016) and different pharmaceutical strategies with the overall purpose of finding improved treatments and therapeutic schemes for Chagas disease are currently under investigation (Bustamante et al., 2014; Morilla and Romero, 2015).

Recently, the improvement of Chagas disease treatment has been evaluated experimentally with new formulations of BZ, such as extended-release tablets and solid dispersions, to increase the maintenance of the drug concentration *in vivo* or to increase the anti-*Trypanosoma cruzi* effect, respectively (Davanço et al., 2016; Palmeiro-Roldán et al., 2014).

The multiparticulated BZ-loaded IPECs were obtained by two-step manufacturing process (casting solvent and wet granulation), which allowed yields higher than 80% with respect to the initial amount of polymer and drug solids employed in their preparation. Multiparticulated systems provide several advantages over single-unit dosage forms such as tablets, because of their multiplicity and small sizes, including reduced risk of systemic toxicity, low risk of dose dumping, and more uniform and reliable gastrointestinal transit

(AlHusban et al., 2011; Auriemma et al., 2013; Dey et al., 2008; Pan et al., 2010; Severino et al., 2012). Indeed, the usefulness of multiparticles for controlled drug release has been reported (Adeleke et al., 2014; AlHusban et al., 2011; Auriemma et al., 2013; Severino et al., 2012), where the selected polymer can offer different drug delivery behaviors.

As mentioned, both release and pharmacokinetic studies were conducted with the BZ-loaded IPEC contained in hard gelatin capsules, compared to Abarax® tablets. In this sense, it is important to stress that the multiparticulated BZ-loaded IPEC showed adequate rheological properties, considering that Carr's Index and the Hausner's Ratio were excellent (Vila Jato, 1997), and the angle of repose was good (European Pharmacopoeia, 2017), which indicate that the methodologies employed for obtaining the multiparticles are suitable for the production of monolithic solid dosage forms as capsules (Vila Jato, 1997).

The release behavior of BZ from a multiparticulated IPEC compared to Abarax® tablets was studied to evaluate its performance as an oral drug delivery dosage form. From both IPECs, the release of BZ was controlled towards SGF (Fig. 2) and significant differences were observed in their release profiles according to f_1 and f_2 values. Under the evaluated conditions, only ~60% of BZ was dissolved from Abarax® after 30 min of assay. Nevertheless, a higher amount of cumulative BZ released at 2 h of release studies was observed from Abarax® (95.7%) in comparison to both IPECs (up to 57%). Moreover, significant differences in the BZ release profiles between Abarax® and both IPECs were observed, in which f_1 and f_2 values above 15 and between 0 and 49, respectively, were achieved (Costa and Lobo, 2001; Food and Drug Administration, 1997).

As detailed, the different compositions of the IPECs led to significant differences in the BZ release profiles (Fig. 2). After the dissolution studies, the remaining Ch-AA-BZ system contained in the rotating basket indicated that the multiparticles were swollen, and a viscous gel mass was observed, as previously detailed in other reports of delivery systems based on AA (George and Abraham, 2006; Hodsdon et al., 1995). On the other hand, the remaining solid EE-EL-BZ indicated that although multiparticles maintained their form, significant erosion with minimal swelling or gel layer was produced. These results can be explained by considering the nature of the polymers that compose the IPECs. In the case of the polysaccharides, slower release of BZ was observed compared to the IPEC composed of polymethacrylates.

At low pH, such as the SGF, Ch can easily be dispersed, due to the protonation of the amine groups, which leads to chain repulsion, diffusion of protons and counter ions together with water inside the gel, and dissociation of secondary interactions. This behavior would allow the delivery of the drugs in the stomach (George and Abraham, 2006). In contrast, at gastric pH, the water molecules are physically entrapped inside the AA matrix, which undergoes an almost immediate hydration to produce a hydrocolloidal layer of high viscosity. This makes up a diffusion barrier that decreases the migration of small molecules and is the reason why AA has mainly been applied in drug controlled delivery systems (George and Abraham, 2006; Hodsdon et al., 1995).

In view of these encountered behaviors for pure AA and Ch delivery systems, IPECs composed of these polymers have become interesting platforms for the controlled release of drugs. Upon mixing, the carboxyl residues of AA and the amino groups of Ch interact ionically to form the polyelectrolyte complex. In these IPECs, the solubility of Ch at gastric pH is prevented by the AA network, since AA is insoluble in low-pH conditions (George and Abraham, 2006).

On the other hand, the IPEC composed of polymethacrylates also allowed controlled BZ release, but the percentage released was higher compared to Ch-AA-BZ. Analogously, EE is a cationic copolymer, which is soluble in gastric pHs up to 5 (Patra et al., 2017) and it has been widely used in formulations such as solid dispersions, to improve the dissolution behavior of the payload (Li et al., 2015; Pradhan et al., 2016; Salmani et al., 2015). The EL is an anionic copolymer, soluble at pH above 6 and it has been used for enteric coating, extended release, and bioavailability enhancement (Patra et al., 2017).

Considering the contrasting behavior of these pH-sensitive polymethacrylates, IPECs based on them have been widely studied in the pharmaceutical field due to their demonstrated low toxicity, high biocompatibility, easy availability and low cost (Moustafine, 2011; Patra et al., 2017). Several IPECs, containing different types and proportions of Eudragit® (L100-55, L100, S100, E100, EPO, among others), were prepared and studied for their potential use as drug delivery systems in different oral pharmaceutical dosage forms (Moustafine and Bobyleva, 2006; Moustafine et al., 2011a; Moustafine et al., 2011b; Moustafine et al., 2008). These IPECs allow more sustained drug release than do single polymethacrylates in homologous binary complexes (Bani-Jaberm et al., 2011; Jeganathan and Prakya, 2015; Palena et al., 2015; Palena et al., 2012).

The development of controlled-release systems should be based on a clinical-pharmacological rationale, such as increased compliance, reduced side effects and improved efficacy. Thus, the pharmacokinetic profiles of a these systems should be compared to reference treatments (Steinijans, 1990).

Regarding the bioanalytical method, our results indicated that the method proposed in this work to quantify BZ in dog plasma was in agreement with the specifications of US Food and Drug Administration's "Guidance for Industry, Bioanalytical Method Validation" (Department of Health and Human Services Food and Drug Administration (U.S.), 2010). By following these validation guidelines, the stability of BZ under different conditions required by the method was confirmed (Table 1). Furthermore, the method was sensitive and selective (Fig. 3), precise, accurate, and linear for BZ in the evaluated range concentrations (Table 1).

Analysis of pharmacokinetic data of BZ was performed using a single oral dose of 100 mg (equivalent to approximately 5 mg/kg) administered to healthy dogs, based on its human dose for Chagas treatment by each administration. Currently, Chagas disease patients are treated with a BZ dosage of 5 to 10 mg/kg/day, not exceeding 300 mg/day (Perin et al., 2017). BZ is only available as 50 and 100 mg immediate-release tablets and the treatment is administered 2–3 times daily for 60 days (Molina et al., 2017; Soy et al., 2015).

The dog has been commonly employed as an animal model to evaluate the performance of oral solid dosage forms, assuming that the results obtained may be successfully extrapolated to humans (Chiou et al., 2000). Dogs provide a particularly convenient animal model for evaluating oral dosage forms in terms of their ability to swallow human-scale dosage forms and ease of breeding (Lui et al., 1986). Also, dogs have suitable volemia for sampling. According to our search in the published literature, poor data about pharmacokinetics studies of BZ oral treatment in dogs are reported. The only published results are from Workman et al. (1984) and White et al. (1982), who reported plasma pharmacokinetics data in dogs after an oral dose of 25 mg/kg of BZ, which was given in gelatin capsules (White et al., 1982; Workman et al., 1984). In this sense, our work usefully contributes to the knowledge of preclinical pharmacokinetics of BZ in dogs.

As can be observed in Fig. 4, when BZ was administered from both IPEC systems, maintenance of an effective concentration, above 3 μg/mL (Soy et al., 2015), was achieved for a longer period of time (up to 27 h post-administration) in comparison to the reference treatment (up to 16 h post-administration). This result is in agreement with the *in vitro* release studies.

Table 2 shows that when BZ was administered from both IPECs, an increase in AUC was obtained (25% greater than from Abarax®), which indicates a higher degree of systemic exposure. Interestingly, both IPECs contain components that may well affect drug absorption, by interacting with the absorption site. It is well known that the polymers used for both IPEC delivery systems can interact with the intestinal epithelium mucin, prolonging the residence time and therefore favoring the absorption (Boddupalli et al., 2010; George and Abraham, 2006). Ch is a potential absorption enhancer across mucosal epithelia (George

and Abraham, 2006). The mechanism of Ch was suggested to be a combination of bioadhesion and widening of the tight junctions of the epithelial membrane. The positive charge provided by the amine groups of Ch would allow it to bind to negatively charged zones at the paracellular junctions of the intestinal epithelium. This interaction generates some structural reorganization of tight-junction-associated proteins, and consequently an opening in the paracellular pores, allowing the paracellular transport (Cano-Cebrian et al., 2005; George and Abraham, 2006). Analogously, ionic interactions between positively charged amino groups in EE and negatively charged mucus gel may occur, and several applications have been reported of EE as carrier to improve the bioavailability of drugs due to a permeability enhancing effect (Patra et al., 2017).

In turn, the increase in AUC occurred without modification of MRT and $t_{1/2}$ parameters (non-significant differences being found respect to with Abarax®, Table 2), which indicates that, in this case, the increase in oral bioavailability would not condition the metabolic rate of BZ (Page, 2008). Our results regarding $t_{1/2}$ of BZ are in agreement with a previous work, where a value of 12 h has been reported (Page, 2008). Moreover, according to the literature, after oral administration, BZ is rapidly and completely absorbed (oral bioavailability of 92%), with a peak plasma concentration being reached at 3–4 h. BZ appears to be extensively metabolized, with only 5% of the unchanged drug excreted in the urine (Page, 2008). In fact, when BZ is administered orally, it is metabolized by the hepatic cytochrome P450 (CYP450) enzymes (CYP3A4), generating 2-aminoimidazole by reduction of the nitro group and 2-hydroxyimidazole by hydrolytic replacement of the nitro group, which are excreted in the urine (Perin et al., 2017). This is important, since some drugs are metabolized by the CYP450 enzymes both in humans and in dogs, but some different enzyme functionalities can occur depending on the model employed. Regarding to this, it has been proposed that the toxic effects of BZ against *T. cruzi* may be due to the production of nitro reduction metabolites that bind to cell macromolecules (Maya et al., 1997). However, there was no evidence of metabolites in the chromatograms, since only a single peak was observed at 324 nm. Similar results were previously reported (Workman et al., 1984).

From both IPECs, significantly higher T_{max} values than that of Abarax® were observed (Table 2). These results may be directly related to BZ release profiles. Both IPECs promoted prolonged drug release, causing greater systemic exposure and drug absorption time, and higher maintenance of the effective plasma drug concentration compared to Abarax®.

Contrary to expectation from *in vitro* release studies, an earlier T_{max} was observed for Ch-AA-BZ compared to EE-EL-BZ. Also, for Ch-AA-BZ a higher C_{max} value (Table 2) was observed compared to reference treatment and EE-EL-BZ. These results can be explained by taking into consideration the nature of the polymers that compose both IPECs.

Even though the four polymers employed are biocompatible, only the polysaccharides are biodegradable (Guarino et al., 2015), while the polymethacrylates cannot experience *in vivo* degradability (Patra et al., 2017). It has been reported that Ch and AA can experience acidic hydrolysis as the primary mechanisms involved in their degradation. But most importantly, enzyme degradation may play a key role in the *in vivo* behavior of these natural polymers. Living organisms have lysozymes, for example in saliva, which are able to hydrolyze Ch (Guarino et al., 2015). Also, it has been reported that Ch interacts with pepsin, an acidic protein and digestive enzyme produced in the stomach forming a soluble protein–polyelectrolyte complex in acidic microenvironments (Boeris et al., 2011). These *in vivo* processes would explain the contrasting *in vitro* and *in vivo* results obtained for Ch-AA-BZ (Figs. 2 and 4, Table 2).

The T_{max} values obtained are in agreement with White et al. (1982) and Workman et al. (1984) previous reports, where peak plasmatic concentrations were reached between 1 and 5 h after oral administration in dogs. However, we obtained lower C_{max} values (up to 6.5 μg/mL *versus* 50 μg/mL) and 6–8 folds lower AUC values compared with their results. These differences can be explained by the lower oral dose administered (100 mg *versus* 500 mg) (White et al., 1982; Workman et al., 1984).

According to literature data, high plasmatic BZ concentrations equal to or > 20 μg/mL increase the risk of adverse effects, mainly dermal manifestations (Soy et al., 2015). In this context, our results indicated that although an increase in C_{max} from Ch-AA-BZ was observed, it did not exceed 20 μg/mL.

The terminal half-life can be used to predict drug accumulation (Toutain and Bousquet-Mélou, 2004). From the experimental data of plasma BZ concentration, and using *in silico* simulations, both therapeutic regimens will attain the classically accepted trypanocidal concentration (Fig. 5). Moreover, it could be observed that the simulations for all the formulations avoid plasma concentrations of BZ higher than 20 μg/mL, which are related with increased risks of side effects or toxicity (Soy et al., 2015). It should be noted that although there were no apparent differences between the steady-state calculated parameters R, this theoretical prediction is based on pharmacokinetic values in a model in dogs. Therefore, more studies in humans would be required to confirm whether the administration of one or the other formulation would give different results.

Even though there was no direct relationship between the C_{max} and T_{max} values and the *in vitro* release profiles as stated above, mainly for Ch-AA-BZ, a level A IVIVC was established between the *in vitro* and the *in vivo* results, considering only the first 2 h of both assays. The results obtained indicate that the IVIVC was good enough for predicting the plasma BZ concentration profiles for each formulation, with prediction errors lower than 10%, which are acceptable according to the US Food and Drug Administration's Guidance for Industry (US Department of Health and Human Services et al., 1997). In addition, as far as the author's knowledge, not reports concerning IVIVC for formulations containing BZ have been informed; thus, this work provides the first attempt to establish correlations between *in vitro* and *in vivo* data for this drug. However, it should be noted that only the results up to 2 h were used for the IVIVC. Considering that the C_{max} and T_{max} values obtained were achieved at later times of assay (after 2 h), it could be explained why the *in vitro* release studies do not show direct relationship with these pharmacokinetic parameters. Thus, even when the obtained data may be useful for guiding new product development and ensuring the quality of the BZ formulations, more data of dissolution studies at different pHs as well as for longer periods of assays would be necessary to fully study the IVIVC.

Bearing in mind that Chagas disease is a life-threatening illness caused by the parasite *Trypanosoma cruzi*, it would be expected some results regarding the efficacy of the developed IPECs to reduce or override the parasitemia levels. However, that is not possible due to the animal model used in this study is a healthy dog, which was selected to study the performance of our oral solid dosage forms compared to the reference treatment. The advantages of using this model were already explained. Nevertheless, it is important to stress that we studied the *in vivo* efficacy and safety of IPEC EE-EL-BZ in a murine model of Chagas disease. According to our previous report, the results clearly suggest that the BZ-loaded IPEC is efficient to override the parasitemia, but also seems to generate lower liver damage in comparison to the free drug (García et al., 2018b).

In summary, these results allowed comparative evaluation of the *in vitro* release of BZ from both IPEC and the reference (Abarax®) towards an SGF, and determination of preclinical pharmacokinetic parameters of BZ from both IPECs from dog plasma samples. It was demonstrated that the BZ-loaded IPEC prolonged drug release and the formulated capsules containing the IPEC showed an adequate *in vivo* performance, considering that the bioavailability of BZ and T_{max} values were significantly higher after administration of both IPECs compared to Abarax®. Thus, the developed BZ carrier systems would be useful for oral administration in the treatment of Chagas disease.

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