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Development and *in vitro*/*in vivo* evaluation of a novel benznidazole liquid dosage form using a quality-by-design approach

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Abstract

OBJECTIVES To develop an alcohol-free solution suitable for children of benznidazole, the drug of choice for treatment of Chagas disease.
METHODS In a quality-by-design approach, a systematic optimisation procedure was carried out to estimate the values of the factors leading to the maximum drug concentration. The formulations were analysed in terms of chemical and physical stability and drug content. The final preparation was subjected to an *in vivo* palatability assay. Mice were infected and treated orally in a murine model.
RESULTS The results showed that benznidazole solubility increased up to 18.38 mg/ml in the optimised co-solvent system. The final formulation remained stable at all three temperatures tested, with suitable drug content and no significant variability. Palatability of the preparation was improved by taste masking of BZL. *In vivo* studies showed that both parasitaemia and mortality diminished, particularly at a dose of 40 mg/kg/day.
CONCLUSION Quality by design was a suitable approach to formulate a co-solvent system of benznidazole. The *in vivo* studies confirmed the suitability of the optimised such solutions to diminish both parasitaemia and mortality. Thus, this novel alternative should be taken into account for further clinical evaluation in all age ranges.

keywords Chagas disease, benznidazole, co-solvency, solubility, *in vivo* study

Introduction

Chagas disease or American trypanosomiasis is a neglected parasitic disease caused by *Trypanosoma cruzi (T. cruzi)*. Chagas disease affects around 7 million people causing about 12 000 deaths per year. Around 40 million people in America live in areas of exposure and are at risk of infection [1, 2]. Chagas disease has become a major concern worldwide due to migration of infected populations from Latin America to North America and Europe [3].

T. cruzi parasites are mainly transmitted by triatomids (kissing bugs), but can also be transmitted congenitally, through blood transfusion and/or transplantation of organs from infected donors [4]. Benznidazole (BZL) is one of the two active compounds with significant trypanocidal activity [5]. It is widely recommended in the acute phase of the disease for any infected patients [6, 7]. Even though most infections occur during childhood, including newborn babies, to date BZL is only available in tablets, a highly inappropriate dosage form for children. As reported, the recommended oral paediatric dose of BZL is 5-8 mg/kg/day for 60 days [8]. Thus, commercially available tablets are usually divided or fractioned by hand, which may result in improper dosage increasing the risk of side effects. Recently, a consortium created by LAFEPE and the Drugs for Neglected Diseases initiative (DNDi) produced a 12.5 mg BZL dispersible tablet [9]. Also, an extemporaneous BZL suspension prepared from commercial tablets was recently reported [10]. Although it is a convenient alternative to the available solid dosage forms, there are still some important concerns in terms of drug content, variation of viscosity during storage, crystallisation processes and both short-term and microbial stability [11, 12]. Thus, alternative age-appropriate medicines including oral solutions formulated as syrups

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and/or drops, particularly for children up to 6–8 years of age, are required [13, 14]. However, the low aqueous solubility of BZL (0.23 mg/ml) clearly restricts the production of oral solutions and thus co-solvents and/or surfactants may have to be added [15–19].

Previously, BZL solutions were developed using co-solvency, a technique widely applied to several marketed pharmaceutical products [19]. Such formulations based on polyethylene glycol 400 (PEG 400), with the addition of ethanol and potassium biphthalate buffer, increased BZL solubility up to 10 mg/ml [20]. *In vivo* studies revealed promising results of drug efficacy in solution [21]. However, as recommended the American Academy of Paediatrics (AAP), ethyl alcohol should be avoided in paediatric formulations [22]. Hence, our aim was to develop a set of BZL alcohol-free solutions at neutral pH, following a quality-by-design rational approach.

Materials and methods

A mixture design was constructed using independent components, namely PEG 400 (X1), propylene glycol (PPG, X2) and water (W, X3). Then, a final liquid dosage form was designed using the optimised co-solvent formulation. Finally, the efficacy of the optimised co-solvent formulation was evaluated at three doses (20, 40 and 60 mg/kg body weight) in a murine model of acute Chagas disease.

Materials

BZL (lot 260835, 99.45% purity) was a gift from Produtos Roche Químicos e Farmacêuticos S.A. (Sao Paulo, Brazil). Sodium dihydrogen phosphate (lot 11585, 99.60% purity), sodium hydrogen phosphate (lot 21100, 99.60% purity), methyl paraben sodium salt (lot 23585, 99.55% purity), cherry flavour (lot 45616, 99.40% purity) and sucralose (lot 41349, 99.55% purity) were purchased from Saporiti (Buenos Aires, Argentina). PEG 400, PPG and MTT reagent were purchased from Sigma-Aldrich (GmbH, Germany). Fetal bovine serum (FBS), Hanks' balanced salt solution (HBSS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Rockville, MD). Vero cells were obtained from ABAC (Pergamino, Argentina). All other chemicals and solvents were of analytical grade.

Methods

Mixture design

A mixture-simplex centroid design was applied employing the software design-Expert version 7.0.0 (Stat-Ease Inc, Minneapolis, MN, USA). The factors analysed were as follows: PEG 400, PPG and W concentration (see Figure 1). A systematic optimisation procedure was done to estimate the values of the factors leading to the maximum BZL concentration. The evaluation consisted in analysing the responses in all the conditions quoted in Table 1 (14 experiments, which are combinations of the selected factors in the following ranges: PEG 400, PPG and W 0.00–1.00). An excess of BZL was necessary to secure the maximum solubility of BZL reached in each condition. The analysed response was BZL liquid concentration.

Solubility assays

Solubility studies for BZL were tested with the solvent mixtures given in Table 1. BZL (250 mg) was accurately weighed in each screw-capped vial, and then, 10 ml of solutions containing solvents was added. The flasks were shaken at room temperature (210 rpm) in a Boeco shaker (Hamburg, Germany). After equilibration (48 h), an aliquot was filtered with a 0.2 m membrane filter, diluted and analysed spectrophotometrically at 326 nm in a UV Boeco (Hamburg, Germany).

BZL final dosage form

In agreement with the mixture-simplex centroid design analysis, PEG 400 and PPG were chosen to prepare the oral liquid dosage form. Methyl paraben sodium salt (0.1% w/v) was solubilised in distilled water (2 ml) at 90 °C. Once the preservative agent was dissolved, the solution was cooled down, and cherry flavour (0.1% w/v) and sucralose (0.2% w/v) were added. Then, BZL solubilised in a mixture of PEG 400 (85%) and PPG (15%) was added under magnetic stirring (300 rpm). After

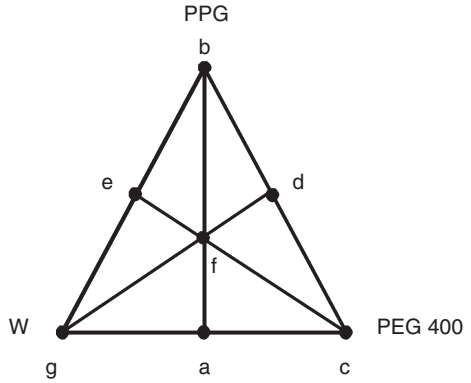

Figure 1 Mixture-simplex centroid design applied employing the software design expert version 7.0.0.

Table 1 Concentration of the different solvents (factors) at each point of the design and BZL solubility (response).

Code	Factors			Response
	PEG 400	PPG	H_2O	BZL (mg/ml)
1	0.67	0.17	0.17	16.600
2	0.00	0.00	1.00	0.149
3	0.00	0.50	0.50	0.723
4	0.17	0.17	0.67	6.490
5	1.00	0.00	0.00	16.000
6	0.00	1.00	0.00	5.770
7	0.50	0.00	0.50	1.372
8	0.33	0.33	0.33	3.518
9	0.00	0.50	0.50	0.937
10	0.17	0.67	0.17	6.490
11	0.00	1.00	0.00	4.465
12	0.00	0.00	1.00	0.148
13	1.00	0.00	0.00	17.820
14	0.50	0.50	0.00	17.300

homogenisation (5 min), a solution of monobasic sodium phosphate and dibasic sodium phosphate was added to adjust the formulation to pH 7. Solutions were filtered and stored in amber-coloured bottles in a dry place at room temperature until further studies. The composition of the BZL alcohol-free formulation is presented in Table 2.

pH measurements

The pH of the BZL solutions was measured using a pH metre (Metrohm, 744-pH metre, Herisa, Switzerland) on days 1, 15, 30, 45, 60, 75 and 90 at 25 °C.

Stability studies

Stability of six BZL formulations was evaluated for 90 days in terms of appearance, pH and drug content. Three of them contained just the co-solvent system (PEG 400 and PPG), and the other three contained all excipients of the final formulation, detailed in Table 2. Drug content as a function of time was assessed following BZL concentrations in each sample determined by UV spectrophotometry at 326 nm. The per cent residual drug for each formulation at different time intervals was calculated considering the initial drug content for each solution to be 100%. The sealed vials of the BZL formulations were visually inspected against black and white backgrounds for changes in colour or turbidity and to detect precipitation under storage at 4 ± 2 °C in a refrigerator, at 25 ± 2 °C in a chamber and at 45 ± 2 °C in an oven. Three samples of each storage condition were withdrawn after 0, 10, 20, 30, 45, 60, 75 and 90 to evaluate its stability.

Table 2 Composition of the BZL final dosage form.

BZL final dosage form (20 ml)	
BZL	360 mg
PEG 400	15.3 ml
PPG	2.7 ml
NaH_2PO_4	48 mg
Na_2HPO_4	132 mg
Methyl parahydroxybenzoate	20 mg
Saccharin sodium	40 mg
Raspberry essence	20 mg
Distilled water	2 ml

Taste masking analysis

A blind taste assay of BZL final formulation was conducted in six healthy human volunteers aged 30-50 years. Sample A (final formulation without BZL), sample B (BZL final formulation), sample C (BZL-PEG 400-PPG optimised solution) and tap water, as a reference, were included in the set of samples. The participants rinsed their mouths with tap water before testing the different samples. Each participant took a sample equivalent to 180 mg of BZL in the mouth for 20 s and then spat out. They were asked to record the bitterness score through a numerical scale between 0 and 4, where 0, 1, 2, 3 and 4 were not bitter, slightly bitter, moderately bitter, bitter and very bitter, respectively. A final score of bitterness was calculated by the average of the value measured for each sample [23]. The protocol of this assay was reviewed and approved by the Bioethics Committee of the National University of Rosario (Argentina) according to the Declaration of Helsinki. Written informed consent was obtained from all participants before enrolment.

Cell viability assays

Vero cells were seeded onto 96-well microtitered plate at a concentration of 1×10^4 cells per well in DMEM supplemented with 2% FBS. After 24 h, different concentrations of each sample were added. After incubation at 37 °C with 5% CO_2 for 48 h, cell growth was evaluated by the MTT assay [24]. Optimised conditions for the assay were those described by Vistica *et al.* (1991) [25]. Each experiment was performed by triplicate.

Animals

BALB/c mice aged 3–4 weeks were obtained from the Department of Parasitology, Institute of Biomedical

Sciences, University of São Paulo (ICB-USP). Mice were given water and food *ad libitum* during the experiments. The protocol was approved by the Ethical Committee for Animal uses in research of the Institute of Biomedical Sciences at University of São Paulo (ICB/USP). All procedures followed Brazilian regulations and were approved under protocol 0107/2013. The recommendations for the use of laboratory animals (World Medical Association, Declaration of Helsinki) were also considered.

Parasites

For this study, 20 mice were used. *T. cruzi* bloodstream trypomastigotes (Y strain) were obtained by infection of BALB/c mice with 1000 blood-derived trypomastigotes/animal intraperitoneally. At day 7 post-infection, blood from infected animals was collected by cardiac punction in the presence of heparin (5000 UI). Trypomastigotes were counted in a Neubauer chamber, and the material was stored in liquid nitrogen [6].

Monitoring of acute infection

Bloodstream forms of *T. cruzi* were assessed under standardised conditions, by direct microscopic observation of 5 ml of heparinised tail venous blood. Data were expressed as number of parasites/50 fields. Mice were also weighed every other day following infection to monitor the systemic repercussion of the acute disease.

Experimental groups

Mice were separated into the four groups with five mice each: A: Untreated mice PPG-PEG 400 solution; B: BZL-treated mice 20 mg/kg/day; C: BZL-treated mice 40 mg/kg/day; and D: BZL-treated mice 60 mg/kg/day. The animals were treated for 10 consecutive days. The effect of the treatments was evaluated by following-up parasitaemia (determined by counting the parasites in a Neubauer chamber) and mortality (determined by counting the animals that survived infection). All experiments were repeated four times with a minimum of five animals/group.

Statistical analysis

GraphPad Prism 4.0 software (GraphPad Software, USA) was used. ANOVA test and Tukey post-test were used for the multiple statistical comparisons of the data obtained from the different groups. A value of $P < 0.05$ was considered statistically significant. Survival data were analysed using the log-rank test.

Software

The software applied to the experimental design and ANOVA test was Design-Expert version 7.0.3 (Stat-Ease Inc., MN, USA).

Results and discussion

Experimental design

The quality-by-design approach for developing pharmaceutical dosage forms is useful in optimising and controlling the production of a final formulation with the required quality. Previously, a pre-formulation study using ethanol, propylene glycol, polyethylene glycol, benzyl alcohol, diethyleneglycolmonoethyl ether (Transcutol) and surfactants (polysorbate 40 and 80, and sodium dioctyl sulfosuccinate) as solubilising agents for BZL was performed in our laboratory. These systems were able to increase the drug solubility from 0.23 up to 10 mg/ml and were not toxic against parasite or mammalian cells *in vitro* when assayed at 1% [20]. Due to concerns related to ethanol as solvent in paediatric formulations, in this work, PPG and PEG 400 were used to formulate BZL alcohol-free solutions. As reported, PPG proved to be suitable as a liquid vehicle when used up to 1000 mg/kg [26] and, even though there are some concerns about the use of PPG in high concentrations, there is a variety of licensed commercial formulations containing this glycol [27]. In this study, a quality-by-design method was applied to develop BZL alcohol-free solutions using PEG 400, PPG and W as selected solvents. The results obtained from the experiments (Table 1) reveal a direct relationship between the PEG 400 concentration and BZL solubility.

Response analysis was performed using response surface methodology (RSM). This model predicted that BZL solubility would increase in the presence of PEG 400 (100%) and PEG 400/PPG (50%/50%). It is due to the fact that BZL is a non-polar compound and it would be more easily dissolved in less polar solvents, such as PPG and PEG 400 than in a highly polar one (water). As discussed, the polarity of the solute/solvent is a key factor in determining solute solubility [28]. However, the hydrophobic character of BZL was clearly observed because the addition of water led to the formation of drug aggregates or precipitates. High values of octanol-water partition coefficients (log *P*) of BZL also indicated a better solubility in less polar solvents than water, as reported [29]. After prediction by software, BZL solutions were prepared with the quantities of the solvents suggested by the model and evaluated in terms of drug

solubility. As seen in Figure 2, the optimum BZL value was 18.38 mg/ml, corresponding to the following values of the influencing factors: PEG 400: 0.85, PPG: 0.15 and W: 0.00. ANOVA test was applied to the experimental data shown in Table 1, using the effect of the dummy variables to obtain an estimate of standard errors in the coefficients.

BZL oral dosage form

BZL, one of the two active compounds with significant trypanocidal activity, is available only as tablets. Despite the large number of newborn and children infected with *T. cruzi*, there are no any liquid dosage forms available to treat paediatric trypanosomiasis. Thus, tablet splitting is a common practice to adjust the administered doses, but it may lead to unequal tablet fragments, inaccurate dose regimen, a decrease in patient compliance and/or modification of biopharmaceutical drug performance [30]. For paediatric treatment, liquid oral medicines are more adequate due to their easier swallowing and dosing scheme. In this study, once the optimised co-solvent system was obtained, a liquid dosage form was prepared by adding the corresponding additives (Table 2). In agreement with the mixture-simplex centroid design analysis, PEG 400 and PPG were chosen, as solubilising agents (vehicle mixture) to prepare the oral liquid formulation. Methyl paraben sodium salt was selected as preservative, and cherry flavour was selected due to its palatability high scores compared with other flavours [31]. Considering that BZL may taste bitter or unpleasant, as other benzimidazole-related compounds, sucralose, a calorie-free artificial sweetener derived from sucrose and nearly 650 times sweeter than sugar, was selected following previous recommendations [32]. The palatability of BZL liquid formulation was also determined in a randomised study by six healthy human volunteers in an intake and spit test [33]. The bitterness level of placebo cherry flavour solution (sample A) was recorded against the BZL final formulation (sample B) and the optimised BZL co-solvent solution (sample C) using a numerical scale [23]. As seen in Figure 3, the placebo formulation and the flavoured BZL final solution displayed scores between 0 and 2, which suggested that the final formulation was slightly bitter. As expected, the non-flavoured BZL-PEG 400-PPG solution displayed scores higher than 2, indicating a considerable perception of bitterness. These results suggest that cherry flavour would be an effective excipient to mask and/or reduce the bitter taste of BZL. The final formulation showed adequate organoleptic characteristics, and neither precipitates nor turbidity was observed. The co-solvent mixture did not alter the solubility of the other excipients detailed in Table 2. Regarding the use of pharmaceutically accepted solvents, the daily intakes of PPG and PEG400 according to children age and body weight by prescribing a BZL dose of 5 mg/kg/day are shown in Table 3.

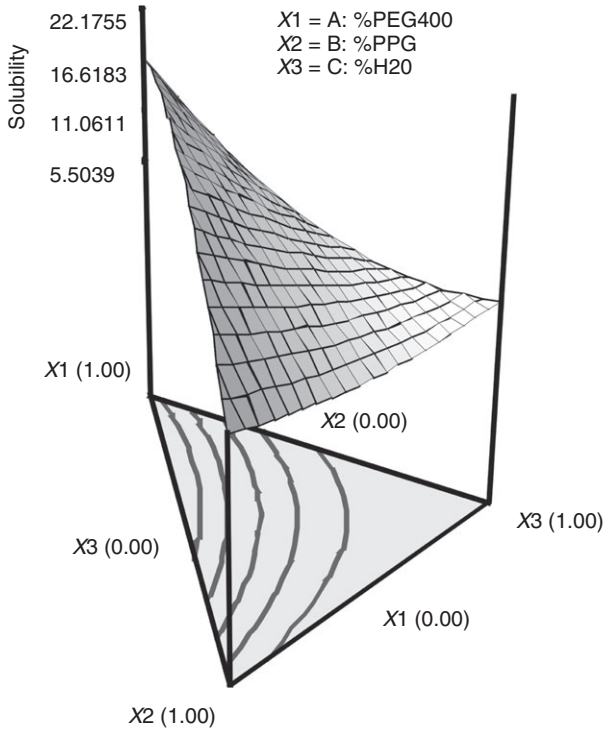

Figure 2 Response surface plot for the BZL solubility.

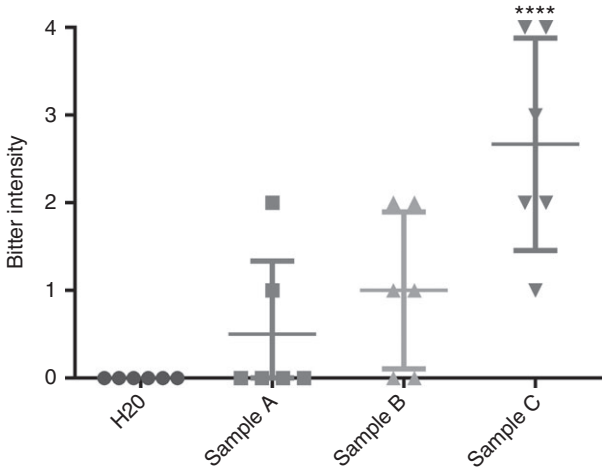

Figure 3 Palatability blind assay on healthy adults volunteers (n = 6).

Table 3 Daily intakes of PPG and PEG 400 according children age and body weight by prescribing a BZL dose of 5 mg/kg/d.

Patient age (Patient weight)	1 year (10 kg)	2 years (12.5 kg)	4 years (16 kg)	5 years (18 kg)	7 years (23 kg)
PPG†	0.375	0.47	0.60	0.67	0.85
PEG 400†	2.12	2.65	3.14	3.81	4.87

†In ml.

Standing and Tuleau published a review related to the oral paediatric cardiovascular medicines licensed and non-licensed in the UK. As described, in both cases, PPG was one of the excipients used to formulate elixirs and sugar-free oral solutions [27]. Moreover, the European Medicines Agency (EMA) recently published a new report concerning the safety of PPG in paediatric formulations. New safety limits were set, expressed in terms of maximum daily doses that are considered to be safe whatever the duration and the route of administration [34]. As reported by Strickley *et al.* [35]., PEG 400 is one of the most common water-soluble organic solvents included in many commercial available medicines, suggesting that it is safe and can be orally administered. Additionally, PEG 300 and PEG 400 are generally considered to be among the safest co-solvents and are widely used in preclinical *in vivo* pharmacokinetic and efficacy studies [36, 37]. In agreement with these data and keeping in mind the concentration of such co-solvents used in this BZL liquid formulation, it would be suitable, in terms of potential toxicity, for children aged 4–5 years and up. However, the exact safety profile of the developed BZL formulation could only be obtained after further clinical studies.

Stability studies

Stability is a key parameter for a quality product. Thus, in this study, the stability of the BZL solutions was analysed under three storage conditions (4 ± 2 °C, 25 ± 2 °C and 45 ± 2 °C) up to 90 days. It was found that the samples containing the co-solvent system (PEG 400 and PPG) and the samples of the final formulation behaved similarly in terms of colour. No turbidity or precipitate formation was detected at storage conditions, while pH values ranged between 6.9 and 7.5 without significant changes for up to 3 months. As shown in Figure 4, the stability data in drug content studies performed for the BZL final formulation at 4 ± 2 °C, 25 ± 2 °C and 45 ± 2 °C, revealed that no considerable differences in drug content were observed at days 0, 15, 30, 45, 60, 75 and 90. Mean stability was above 98% at all three assayed temperatures. It should be mentioned that no crystallisation of BZL was seen in samples stored at 4 ± 2 °C, indicating the suitability of the selected co-solvent system as solubilising mixture, even at low temperature.

In vitro/in vivo evaluation of BZL formulations

First, both biocompatibility and toxicity issues of the optimised BZL formulation were *in vitro* evaluated by means of MTT assay. The mixture of solvents, either alone or mixed with BZL, did not affect cell growth in concentrations up to 1% of the cell culture media, indicating its suitability for further preclinical and clinical studies. As seen in Table 4, the IC_{50} of each solvent and the co-solvent mixture alone were between 4.0 and 5.0%.

Table 4 IC_{50} of the solvents and co-solvents in the Vero cell line. The valued obtained are expressed as percentage in the culture media.

Sample	IC_{50} (% in culture media)
PEG400	5.26 ± 0.4
PPG	4.53 ± 0.3
PEG400/PPG/Water	4.27 ± 0.5

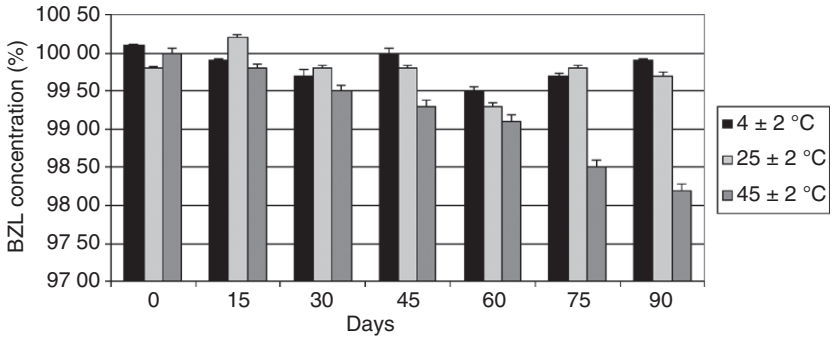

Figure 4 Stability of BZL final dosage formulation stored up to 90 days at 4 ± 2 °C, 25 ± 2 °C and 45 ± 2 °C. (mean values, n = 3).

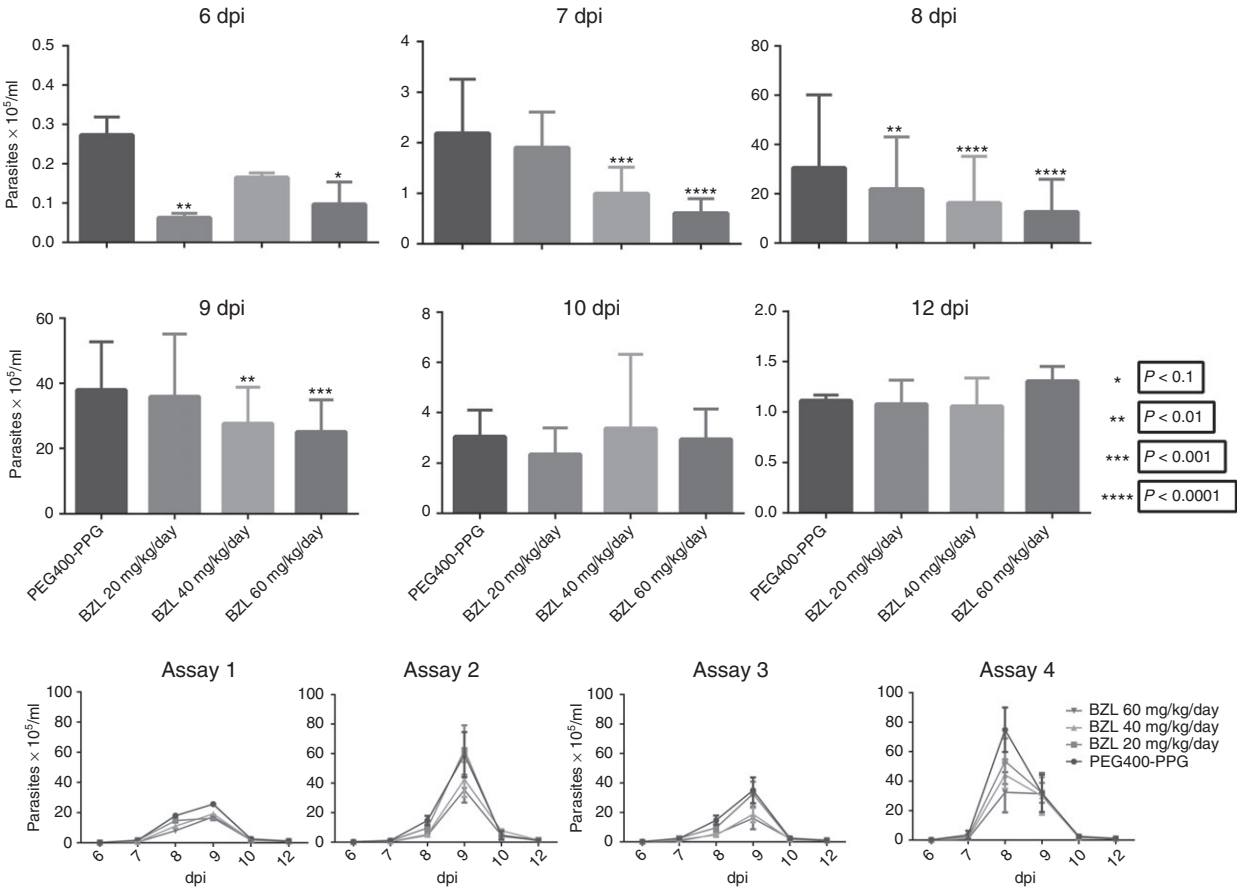

Figure 5 Parasitemia at 6, 7, 8, 9, 10 and 12 days post-infection in different mice groups.

Next, to evaluate the efficacy of the optimised BZL formulation, a dose–response treatment was applied to the experimentally infected mice. Four groups of mice ($n = 5$) were intraperitoneally infected and treated orally once a day, for 10 days, as follows: (i) control of infection (infected mice only treated with the vehicle), (ii) infected mice treated with BZL 20 mg/kg/day, (iii) infected mice treated with BZL 40 mg/kg/day and (iv) infected mice treated with BZL 60 mg/kg/day. Parasitaemia was registered between days 6 and 12, with a peak observed at day 9 [38]. As shown in Figure 5, the three concentrations of BZL significantly diminished parasitaemia before the peak (day 8) when compared to control (PPG-PEG 400), and, to a lesser extent, doses of 40 and 60 mg/kg/day also diminished parasitaemia at day 9. In order to obtain the value corresponding to the total infection yield, the area below the peaks was integrated. All the treatments proved to be effective with respect to control in a dose-dependent way, as reported for other BZL formulations [39].

The ability of BZL formulation to extend the mice survival is a fundamental parameter to evaluate the effectiveness of the treatment. The total numbers of alive and dead mice were recorded over 35 days starting at day 15 post-infection. A significant difference was only observed between the treatment with 40 mg/kg/day and the control group (Figure 6).

These facts reinforce previous observations that a decrease in parasitaemia does not necessarily imply higher survival. Both 20 and 60 mg/kg/day treatments showed a very similar death profile with respect to controls. The lower dose of BZL (20 mg/kg/day) clearly is insufficient to increase the mice survival fraction. For the 60 mg/kg/day dose, the presence of the hermetic dose–response might be postulated as a result of its minor effectiveness in terms of animal survival compared to the 40 mg/kg/day treatment. Hormetic dose–response has been applied to other drugs indicating that a dose–response data for both beneficial and side effects might offer important information including a narrow range of

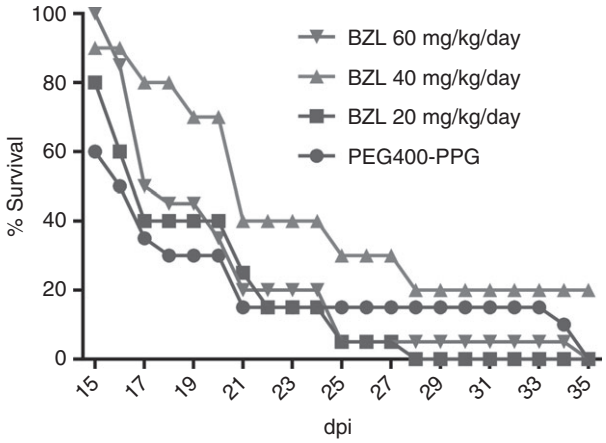

Figure 6 Total number of alive mice recorded over 35 days starting at day 15 post-infection.

doses which would have a direct impact over the benefit-to-risk ratio [40, 41]. At present, there is no information about this phenomenon during the treatment of Chagas disease with BZL. It could be related to the mode of action of BZL through the generation of free radicals and electrophilic metabolites within the parasite which may act as antagonist of BZL. A similar effect was observed for another antiparasite drug, delamanid, for the treatment of visceral leishmaniasis [42]. However, it should be stressed that BZL usually is used in limited concentrations due to its solubility properties. In this sense, the possibility of using unusually high concentrations of BZL could be unmasking its hormetic properties. As a whole, these results show that the formulation was efficient in a narrow range of concentrations in terms of diminishing both parasitaemia and mortality, being 40 mg/kg/day the most efficient treatment.

Conclusions

Quality by design was a suitable approach to formulate a co-solvent system of BZL for the first time. BZL aqueous solubility was increased from 0.23 mg/ml to 18.38 mg/ml (80 fold). The formulation remained stable at 4 ± 2 °C, 25 ± 2 °C and 45 ± 2 °C, with suitable drug content and no significant variability. Moreover, the excipients included in the final formulation effectively covered the bitter taste of BZL. *In vivo* studies confirmed the suitability of the developed BZL formulation to reduce both parasitaemia and mortality, particularly at a dose of 40 mg/kg/day. Taking into account these results, this approach should be seriously considered for further evaluation of its safety and efficacy in all age ranges.

Acknowledgements

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