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International Journal of Pharmaceutics 307 (2006) 239–243
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Development of parenteral formulations and evaluation of the biological activity of the trypanocide drug benznidazole

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Received 27 May 2005; received in revised form 5 October 2005; accepted 6 October 2005
Available online 15 November 2005

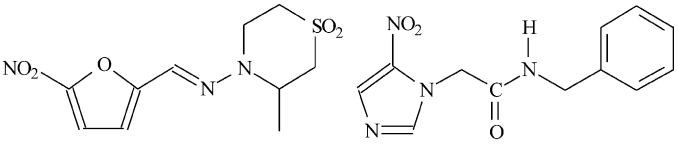
Abstract

Chagas disease, caused by *Trypanosoma cruzi*, is a major public health problem in Latin America. According to the World Health Organization, around 20 million people are infected and another 40 million are at risk of acquiring the disease. One of the drugs most frequently used for the treatment of Chagas disease is benznidazole (BZL). It is practically insoluble in water (0.4 mg/ml), which precludes the preparation of liquid dosage forms, in particular, parenteral formulations. Thus, the aim of this work was to investigate the solubilization of BZL at two pH values using various cosolvents such as ethyl alcohol, propylene glycol, polyethylene glycol 400, benzyl alcohol, diethylene glycol monoethyl ether (Transcutol) and surfactants such as polysorbates (Tween) 40 and 80, and sodium dioctyl sulfosuccinate (AOT). Solvent systems based on PEG 400, with the addition ethyl alcohol and/or potassium biphthalate buffer solution, increased the BZL solubility up to 10 mg/ml. These alcoholic vehicles showed no toxicity against parasite when assayed at 1%. Physical and chemical stability studies showed that the formulations were stable for at least 1.5 years. In agreement with the biological activity results, the selected formulations are suitable for further clinical studies. Moreover, increasing the aqueous solubility of BZL reduced the problems in vitro testing techniques and bioassays leading to more reliable results and/or reproducibility.

Keywords: Chagas disease; Benznidazole; Water solubility; Cosolvent systems; Parenteral formulation

1. Introduction

Chagas' disease, a protozoan infection caused by the kitenoplastid *Trypanosoma cruzi*, constitutes a major public health problem for developing nations. According to the World Health Organization, an estimated 20 million people are infected with this parasite and another 40 million are at risk of acquiring the disease (WHO, 2002). The morbidity and mortality associated with Chagas' disease are more than one order of magnitude higher than those caused by malaria, schistosomiasis or leishmaniasis in Latin America (Morel, 2000). After the initial acute phase, a chronic condition develops, causing irreversible damage to heart, oesophagus and colon, with severe disorders of nerve conduction in these organs; patients usually die from heart conditions (Urbina and Docampo, 2003). Currently, there is no effective treatment for chronic cases, no vaccine, and no preventive treatment (Cuellar et al., 2003). In the acute, recent or congenital disease, the most important drugs available for treatment are: nifurtimox (NX) (3-methyl-4-[(5-nitrofurfurylidene) amino] thiomorpholine-1, 1-dioxide), a nitrofuran derivative (**1a**), and benznidazole (BZL) (*N*-benzyl-2-nitroimidazolylacetamide), a nitroimidazole derivative (**1b**) (Barbeira et al., 1999).



1a **1b**

The only trypanocidal chemotherapies available for Chagas' disease are solid dosage forms, but they have the disadvantages associated with oral absorption of poorly soluble drugs.

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doi:10.1016/j.ijpharm.2005.10.004

Moreover, both drugs have significant side effects, including anorexia, vomiting, peripheral polyneuropathy, depression of bone marrow, and allergic dermopathy. As a consequence of these adverse reactions, frequently oral treatments have to be discontinued (Murta et al., 1998). If the pharmacokinetics of these drugs improves, the therapeutic dose is expected to decrease, with the consequent diminution of side effects. The reasons for the pronounced difference in the antiparasitic efficacy of nitro heterocyclic compounds in acute and chronic stages of the disease are not yet understood, but they could be related to unfavourable pharmacokinetic properties of the drugs in the chronic stages (Molina et al., 2001). Recently, the development of a BZL liposomal formulation has been reported. The multilamelar liposomes were prepared by mixing a solution of lipids in Cl_3CH:CH_3OH and BZL dissolved in DMSO or in a mixture of Cl_3CH:CH_3OH:H_2O (Morilla et al., 2002). Despite the frequent use of these solvents for liposomal formulations, there is some concern regarding toxicity in their residual amount after evaporation, in particular, chloroform that is possibly carcinogenic to humans (Ran and Yalkowsky, 2003). In addition, the BZL delivery was not greatly increased after incorporation in these liposomes (Morilla et al., 2004). Thus, an urgent need exists for the design and development of safe and effective delivery systems of BZL, aimed at reducing the administered dose while improving the absorption and bioavailability. Therefore, the purpose of this study was to develop an improved parenteral dosage form of BZL. To overcome the poor aqueous solubility of this antichagasic agent, several cosolvents such as ethanol, propylene glycol, polyethylene glycol, benzyl alcohol, diethylene glycol monoethyl ether (Transcutol), and surfactants (polysorbate 40 and 80, and sodium dioctyl sulfosuccinate) were assayed. Three selected liquid formulations were developed and studied for physical and chemical stability. These dosage forms were evaluated against infections caused by three different strains of *T. cruzi*.

2. Materials and methods

2.1. Materials

Polyethylene glycol 400 was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA), potassium biphthalate [$KHC_6H_4(COO)_2$], propylene glycol, ethyl and benzyl alcohols were obtained from Merck (Schuchadt, Germany), diethylene glycol monoethyl ether (Transcutol), was purchased from Gatefosse (Westwood, NJ, USA), polysorbate (Tween) 40 and 80, and sodium dioctyl sulfosuccinate (AOT) were purchased from Aldrich Chemical Co. Dimethyl sulfoxide (DMSO) was obtained from Anedra (Buenos Aires, Argentina) and benznidazole was a gift from Roche (Brazil). RPMI medium was purchased from Invitrogen (CA, USA) and AlamarBlue^{TM} was from BioSource International (CA, USA). The 0.45 μm syringe filters were from Millipore (Millipore Inc., MA, USA). All reagents were analytical grade.

2.2. Solubility studies

An excess amount of BZL was added to 30 ml glass vials containing the different cosolvents (ethyl and benzyl alcohol, propylene glycol, polyethylene glycol 400, and Transcutol) and surfactants (Tween 40 and 80, and AOT). They were assayed for their ability to enhance the solubility of BZL in aqueous phase. These solutions were magnetically stirred for 6 h at $40 \pm 2\,^{\circ}C$ for complete solubilization and equilibrated for additional 12 h. The supernatant of each vial was filtered, and analysed spectrophotometrically at 326 nm. The solubility of BZL was determined in duplicate. Details of cosolvent systems proposed to study the BZL solubility are presented in Table 1.

2.3. Formulation of the parenteral dosage forms

Three vehicles were selected to prepare the parenteral dosage forms, focusing on the best solubility parameters, and low solvent toxicity. Details of compositions proposed to design the BZL parenteral formulations are presented in Table 2.

Tonicity was not adjusted, and the final concentration of the parenteral dosage forms was 10 mg/ml theoretically. These solutions were filtered through 0.45 μm membrane filter and analysed spectrophotometrically at 326 nm for drug content after appropriate dilutions (Constantinides et al., 2004).

2.3.1. Treatment of packing material

All the vials of 5-ml capacity and the rubbers stoppers were first washed several times with distilled water and then dried by dry heat in an oven at $45 \pm 2\,^{\circ}C$ in inverted position.

Table 1
Cosolvent systems

System no.	Propylene glycol (ml)	Benzyl alcohol (ml)	Transcutol (ml)	Distilled water (ml)	Tween 40 (g)	Tween 80 (g)	AOT (g)
1	0.50			0.50			
2	0.75			0.25			
3		0.10	0.90	9.00			
4		1.60	8.40				
5		1.40	2.80	5.80			
6				9.90	0.10		
7				9.90		0.10	
8				9.90			0.10
9		0.10	0.90	8.90			0.10

Table 2
Solubility of benznidazole in selected cosolvent systems

System no.	PEG 400 (ml)	Ethyl alcohol (ml)	Buffer potassium biphthalate (ml)	Solubility (mg/ml)	pH
10	7.0	3.0		8.42	7.8
11	7.0	1.0	2.0	9.90	2.5
12	7.0		3.0	8.04	2.5

2.4. Sterilization

The solutions were sterilized by steam sterilization for 20 min at 110 °C, 0.5 atm in a model chamberlain autoclave.

2.5. Physical stability studies

The sealed vials of the formulations were visually inspected against black and white backgrounds to see the changes occurring, like colour, turbidity, precipitation on storage at 4 ± 2 °C in a refrigerator, and 45 ± 2 °C in an oven, during 30 days. The vials were kept alternately at 45 ± 2 and 4 ± 2 °C for 24 h and shaken every day for 10 min on a mechanical shaker (Agrawal et al., 2004).

2.6. Chemical stability studies

Chemical stability was studied at room temperature conditions. The parenteral formulations were analysed spectrophotometrically initially and at intervals of 10 days during 1.5 years to calculate the drug content. The percent residual drug for each formulation at different time intervals was calculated considering the initial drug content for each formulation to be 100%.

2.7. Trypanocide assays

Epimastigotes from Tulahuen 2, CL Brener and Y strains were cultured in LIT medium supplemented with 5% fetal bovine serum (FBS) at 37 ± 0.5 °C in 12 or 96 wells culture flasks (Camargo, 1964). Cultures ($3–4 \times 10^6$ parasite/ml) were incubated with increasing amounts of BZL (1–50 μg/ml) solubilized in the different formulations and DMSO. In all the experiments, the concentration of cosolvent systems and DMSO were fixed to 1%. Parasite growth was monitored by 600 nm absorbance and by countage in a Neubauer chamber. Inhibition was calculated as the ratio between parasite growth in presence or absence of BZL, after 72 h of culture. Inhibitory concentration 50 (IC_{50}) was obtained by plotting percentage of inhibition against drug concentration. Each experiment was done in triplicate.

2.8. Mammalian cells toxicity assays

Monocyte-derived RAW 264.7 cell line was maintained in RPMI culture medium supplemented with 10% FBS in a 5% CO_2 atmosphere, at 37 ± 0.5 °C. Toxicity assays were performed by incubation cells (10^5 cells/cm^2) with increasing concentrations of different formulations and DMSO, in 96 well culture flasks. After 48 h, the culture medium was gently removed, 200 μl of a solution of 0.2% Trypan Blue was added and cells were counted in an inverted microscope during the following 30 min. Percentage of dead cells was estimated after counting 400 cells in at least three different microscope fields. Each experiment was done in triplicate. AlamarBlue™ measure of cell metabolic activity was performed as indicated by the manufacturer.

3. Results and discussion

3.1. Solubility studies

In agreement with the Biopharmaceutics Classification System (BCS), and the World Health Organization (WHO) Essential Drug List, BZL is a very slightly soluble (vss) drug with an aqueous solubility of 0.4 mg/ml (Kasim et al., 2004). The choice of organic solvents used in pharmaceutical parenteral formulations has already been described, when aqueous solutions cannot be considered owing to drug solubility limitations. At the same time, low aqueous solubility also affects quantities of solvents necessary for dissolution. Several solvent systems were tested to achieve complete dissolution of the drug, as summarized in Tables 1 and 2. Propylene glycol and benzyl alcohol are among the most frequently used cosolvents for parenteral formulations (Yalkowsky and Roseman, 1981), and Transcutol is a powerful solubilizing agent used in several dosage forms since it has an excellent miscibility with polar and non-polar solvents and optimal solubilizing properties for a number of drugs (Torrado et al., 1997; Kim and Park, 2004). Moreover, poorly water-soluble drugs can be solubilized by incorporation into micelles formed by surfactants such as polysorbates and AOT (Ni et al., 2002). Thus, the solvents and surfactants were combined in several ratios in order to improve the BZL solubility. As already described, the aqueous solubility of BZL is 0.4 mg/ml, but when the drug was assayed in different pharmaceutically acceptable cosolvents systems the BZL solubility increased up to 1 mg/ml (Table 1).

Despite these promising results, the solubility of the drug was not sufficient to allow a further development of a parenteral formulation. Therefore, we decided to explore the most common solvents for injections. It is well known that polyethylene glycol of different molecular weights is one of the most useful solubility enhancers, and PEG 200 to PEG 600 are the solvents of choice in liquid formulations (Spiegel and Noseworthy, 1963). PEG 400 is an excipient of choice due to its good solubilization properties and overall acceptability in terms of side-effect profile. Recently,

its uselfulness has been reinforced with the developing of quantitative structure–property relationship (QSPR)-based in silico models to predict the solubility of drug-like organic compounds (Rytting et al., 2004). In addition, it is known that the capacity of solubilization of PEG 400 as a cosolvent is in agreement with the reported dielectric constant or solubility parameters (Etman and Nada, 1999). Thus, we prepared several systems based on PEG 400, and the solubility of BZL was ensured by careful adjustment of the PEG 400 concentration. Based on solubility characteristics and previous results (Stella et al., 1995), three formulations containing PEG 400, ethyl alcohol and/or potassium biphthalate were developed (Table 2). When the cosolvents were mixed in a ratio 7:3, under physiological pH conditions the BZL solubility increased up to 8.42 mg/ml (system **10**). Surprisingly, BZL solubility was higher (9.90 mg/ml) at pH 2.5 when PEG 400, ethyl alcohol, and potassium biphthalate were mixed in a 7:1:2 ratio (system **11**). The last selected formulation containing PEG 400 and potassium biphthalate in a 7:3 ratio (system **12**) showed a similar solubility (8.04 mg/ml) at pH 2.5. Thus, the BZL solubility was found to increase up to 20 times in a 70% PEG 400 solution. By microscopic evaluation it was observed that the systems **10**–**12**, gave clear solutions at pH 2.5 and pH 7.8, without observing any precipitation phenomenon.

3.2. Stability

The physical stability study showed that the formulations **10**–**12** remained unchanged with respect to colour stability, and no turbidity or precipitate formation was detected at the storage conditions. The chemical stability assays at room temperature showed that those formulations continued to be stable after 1.5 years since the BZL concentration decreased less than 2%. A similar result was obtained for DMSO solutions (Table 3).

3.3. Biological activity

Trypanocide activity was measured for all the BZL soluble formulations obtained, on epimastigotes from three different *T. cruzi* strains. The cosolvent systems containing Transcutol killed parasite in a very short time, even in absence of the drug. Thus, Transcutol should not be a suitable vehicle for parenteral formulations of BZL at those concentrations. Moreover, the cosolvent systems based on Transcutol were also toxic against mammal's cultured cells. Results obtained with the vehicles **10**–**12** are shown in Table 4. As can be seen, IC_{50} for all BZL soluble formulations was similar to that obtained for DMSO-solubilized BZL.

Table 3
Chemical stability of benznidazole (1.5 year at 25 ±2 °C)

System no.	PEG 400 (ml)	Ethyl alcohol (ml)	Buffer potassium biphthalate (ml)	BZL (%)
10	7.0	3.0		99.68
11	7.0	1.0	2.0	98.10
12	7.0		3.0	98.45
DMSO				99.71

Table 4
Trypanocide activity of benznidazole

System no.	*Trypanosoma cruzi* strains		
	Tul 2	CL Brener	Y
10	8.30 ± 0.90	13.50 ± 1.20	14.20 ± 0.55
11	6.70 ± 0.55	11.40 ± 1.40	13.20 ± 0.90
12	6.00 ± 0.30	10.70 ± 0.95	15.00 ± 0.45
DMSO	8.00 ± 1.20	7.00 ± 1.55	7.80 ± 1.35

Results are expressed as IC_{50} (μM).

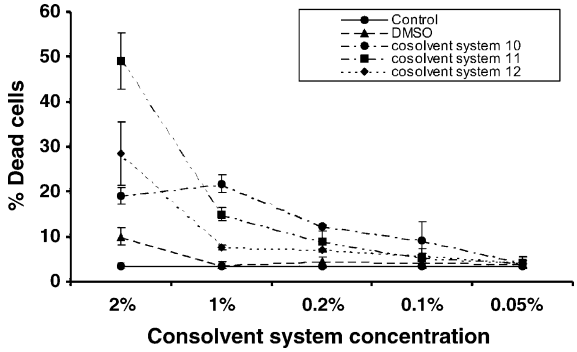

Fig. 1. Cosolvent systems toxicity measured by Trypan Blue dye exclusion method.

DMSO is one of the major solvents used for biological tests due to its physical properties such as high solubilizing capacity, water-miscibility, and low viscosity, and the recommended concentration for biological assays is 0.1%. To better assess the usefulness of our cosolvent systems on the BZL dosage form development, we assayed their toxicity in absence of drug on a monocyte-derived cultured cell line. First, toxicity was determined by a dye-exclusion method using Trypan Blue. As showed in Fig. 1, the systems **10**–**12**, as well as DMSO, showed high toxicity when assayed at 2%. DMSO toxicity decreased to control values at 1%, while cosolvent systems **11** and **12** reached control toxicity level when used at 0.1%. In this assay, cosolvent system **10** showed the lowest toxicity at 2%, but at 0.1% was more toxic for mammal cells than systems **11** and **12**.

Finally, the metabolic activity of cells was estimated by the AlamarBlue™ method (Fig. 2). As it can be seen, the redox

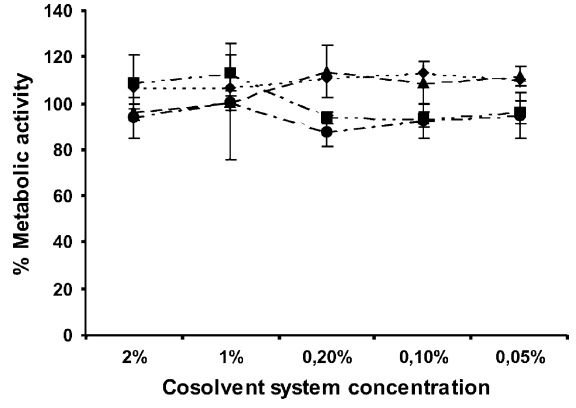

Fig. 2. Influence of cosolvent systems on cell metabolic activity determined by AlamarBlue™ assay. See references Fig. 1.

activity of cells was similar when incubated with DMSO or cosolvent systems **10–12** at concentrations between 2 and 0.2%, the range in which the cosolvent systems showed more toxicity than DMSO when measured by dye-exclusion. All these assays were carried out in absence of BZL. These results suggest that the toxicity observed for our formulations was due to reasons over than that a metabolic perturbation of cells. Taking into account the high concentration of organic solvents used in these assays, toxicity could be attributed to disruption of membrane integrity.

4. Conclusions

The choice of non-aqueous solvents for parenteral formulations of BZL was related with several factors such as solubilizing properties, tolerance, and biological activity. Due to its very poor aqueous solubility (0.4 mg/ml), the formulations were prepared in a non-aqueous vehicle consisting of 70% PEG 400, with the addition of 10–30% ethyl alcohol and/or 20–30% of potassium biphthalate buffer solution (pH 2.5). It was revealed that these PEG 400 based systems were able to increase the BZL solubility up to 10 mg/ml. These alcoholic vehicles were not toxic against parasite when assayed at 1%. Physical and chemical stability studies showed that the dosage forms could be kept at room temperature for long-term storage. In the particular case of cosolvent systems using Transcutol, it was revealed to be toxic per se against parasite, without the inclusion of the drug. Trypanocide activity exhibited by BZL formulations in PEG 400 was similar compared with the activity obtained from BZL in DMSO. Regarding all the systems proposed, it is worth noting that BZL in PEG 400:potassium biphthalate buffer (7:3) showed the highest activity as a trypanocide, when assayed over two strains of *T. cruzi*, Tul 2 and CL Brener. These solvent systems also exhibited low toxicity against a mammal cell line thus increasing interest in them for testing on an animal infection model. Furthermore, these simple, effective and novel formulations may also provide a better pharmacokinetic profile, since the drug is completely dissolved which would improve the absorption.

Acknowledgements

The authors express their gratitude to the National University of Rosario (U.N.R.), the National Council Research (CONICET, Argentina) and Fundación Antorchas for financial support. M.C.L. thanks the Ministry of Health for a Carrillo-Oñativia fellowship. C.J.S. is grateful to Fundación Bunge y Born (Argentina) for a research grant.

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