European Journal of Pharmaceutics and Biopharmaceutics 75 (2010) 451–454

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Note

In vivo evaluation of albendazole microspheres for the treatment of *Toxocara canis* larva migrans

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ARTICLE INFO

Article history:
Received 3 September 2009
Accepted in revised form 23 March 2010
Available online 27 March 2010

Keywords:
Chitosan microparticles
Albendazole
Toxocariasis
Nematocide activity
Migrating larvae

ABSTRACT

Albendazole is a benzimidazole derivative with proven efficacy against many parasites such as intestinal helminths. Toxocariasis is one of the important parasitic diseases in humans and animals caused by *Toxocara canis*. It is well known that *T. canis* larvae migrate in paratenic hosts, including humans where it may cause visceral larva migrans. Thus, the present research was carried out using *in vivo* experiments with the aim of finding whether novel albendazole microparticles would be active against migrating larvae of the parasite. Albendazole–chitosan microparticles were prepared by ionotropic gelation with sodium lauryl sulphate or by a liquid–liquid phase separation with sodium hydroxide. Mice were infected with *T. canis* and then treated with both albendazole–chitosan microparticles. After treatment (28 days post-infection), it was examined the anthelmintic effect in mice after oral administration of microparticulate preparations. The number of larvae recovered from mice treated with albendazole formulations were compared with placebo. The results showed that albendazole microparticles were easily prepared in high yield using both aqueous solutions of sodium lauryl sulphate or sodium hydroxide. *In vivo* evaluation of larva migration showed that albendazole microparticles exhibited a greater anthelmintic effect in the brain (0 larva/mouse). In addition, it was also found that liver and lung showed a significant decrease in the number of larvae. Therefore, these data suggest that albendazole–chitosan microparticles are effective formulations for the treatment of toxocariasis infection by reducing the number of larvae in liver and lung. Particularly, these polymeric preparations were able to totally prevent migration of larvae to the mice brain.

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1. Introduction

Human and animal diseases caused by helminthes parasites have great impact on public health. Toxocariasis, caused by *Toxocara canis* (*T. canis*), is one of the important parasitic diseases in humans and animals where it may cause visceral larva migrans (VLM) and ocular toxocariasis (OT) [1]. The larvae reach the liver and are distributed to other tissues such as lungs, eyes, heart and brain, in which the living or dead larvae cause eosinophilic inflammation. This parasitic disease is widely disseminated and has been reported in North America, Europe and South America. Up to date, the control of the parasitic infections is based on the use of anthelmintics; however, its treatment is still deficient. In this context, one of the current recommended drugs is albendazole (ABZ), a benzimidazole carbamate with a broad-spectrum activity against human and animal helminth parasites widely used for the treatment of *T. canis* larva migrans [2]. In order to deliver ABZ in an effective amount to the gastrointestinal tract, several approaches such as cosolvents, liposomes and cyclodextrins were successfully applied to the improvement of its bioavailability [3–6]. To our knowledge, however, no article has been published so far regarding the *in vivo* efficacy of ABZ–chitosan (CH) microparticles for the treatment of toxocariasis. Therefore, the aim of this work was to evaluate the effectiveness of ABZ–CH microparticles systems in the treatment of *T. canis* larva migrans. Microparticles were prepared by dripping or spraying the polymeric solution into sodium

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doi:10.1016/j.ejpb.2010.03.017

lauryl sulphate (SLS) or sodium hydroxide (NaOH). The antiparasite efficacy of ABZ formulations in aqueous solution was evaluated in mice infected with *T. canis* larvae, in comparison with placebo and control.

2. Materials and methods

ABZ and CH (medium molecular weight, 75–85% deacetylated) were obtained from Sigma–Aldrich (Aldrich Chemical Co., WI, USA). All other chemicals were of analytical grade.

2.1. Preparation of the microparticles

ABZ (100 mg) was dissolved at room temperature in acetic acid (25 mL) and water (25 mL). CH (3.0%, w/v) was dispersed in the acidic solution. The resulting suspension was stirred to allow the complete CH dissolution in the acidic medium. The microspheres were formed by dropping the bubble-free dispersion of ABZ–CH through a disposable syringe needle (0.30 mm in diameter) onto a gently agitated (magnetic stirrer) of either NaOH (pH 12) or SLS (pH 8) solutions (5.00%, w/v). In addition, ABZ–CH solution was sprayed through a nozzle (0.30 mm in diameter) into a precipitation bath containing either NaOH (pH 12) or NaLS (pH 8) solutions (5.00%, w/v), under magnetic stirring. The pH determinations were carried out in a pH meter Metrohm 744 (Herisau, Switzerland). The polymeric microparticles were filtrated, washed with water, centrifuged twice and finally collected in a drying chamber at 40 °C.

2.2. Determination of ABZ content in microparticles

The encapsulation efficiency (EE) is defined as the percentage of the actual mass of drug encapsulated in the polymeric carrier, relative to the initial amount of loaded drug. For the EE determination, microparticles were dissolved in 0.1 N HCl for 24 h. The amount of loaded drug was determined by spectrophotometric measurements at 291 nm using a LKB-Pharmacia UV spectrophotometer, according to:

$$\text{Encapsulation efficiency } (\%) = 100 \times (W_{ABZ}/W_t) \quad (1)$$

where W_{ABZ} is the actual ABZ content and W_t is theoretical ABZ content in the microparticles.

2.2.1. Dissolution studies

ABZ–CH microparticles and the pure drug (100 mg) were subjected to dissolution assays in an USP Standard Dissolution Apparatus (Hanson Research SR8 Plus, Chatsworth, USA), equipped with a rotational paddle (50 rpm). The dissolution medium (900 mL of 0.1 N HCl) was maintained at 37 °C. Microparticles containing ABZ (100 mg) were introduced into the flasks, and the time counter was set to zero. At 30 min, samples of 5 mL were taken through a filter, and the amount of ABZ released was determined by spectrophotometric measurements at 291 nm. It was found that CH did not interfere with the assay at the working wavelength.

2.3. Scanning electronic microscopy (SEM)

Morphology and surface of the microparticles were analyzed using scanning electron microscopy in a Leitz SEM AMR 1600 T. Samples were previously sputter-coated with a gold layer in order to make them conductive.

2.4. Preparation of T. canis larvae and mice infection

T. canis adult worms were collected from naturally infected dogs. Infective embryonated eggs were cultured according to the method previously described [7]. Mice were anesthetized with diethyl ether and orally infected by a bucco-gastric tube with 300 embryonated eggs. Prior to the inoculation, the viability of eggs was confirmed by microscopic observation of larval movement. For infection confirmation, egg presence was determined by fecal analysis at day 28 post-inoculation. Animals maintained in our breeding facilities were used for the experiments that were conducted following the Institutional Experimental Guidelines for Animal Studies.

2.5. Pharmacological studies

Male BALB/c mice (National University of La Plata, Argentina) weighing 20–25 g were kept under a 12-h light–dark cycle, with free access to food and water. Mice ($n = 5$) received via a bucco-gastric tube a water suspension (0.1 mL) ABZ–CH microparticles, at a dose of 100 mg/kg. As control was used, ABZ suspended in water (ABZ–W), vortexed and immediately administered. The mice were treated daily for 5 days (from 1 to 5 days post-infection (p.i.)). Then, the brain of the mice were taken out and interposed between two slides to count the larvae. The larvae of the liver and lung were collected by artificial digestive juice treatment, as reported previously [4]. Observation of the larva was immediately carried out after the necropsy. Effectiveness of the formulations was compared using an ANOVA test.

3. Results and discussion

3.1. Preparation of microparticles

The selection of the microencapsulation techniques depends largely on the physicochemical properties of the drug and the carrier. It is well known that CH is a natural polysaccharide widely applied to the development of microparticulate systems owing to its good biodegradability, biocompatibility and non-toxicity [8]. CH microspheres are frequently prepared by the solvent evaporation using organic solvents, such as toluene or acetone, and chemical crosslinking reagents, such as glutaraldehyde or formaldehyde [8]. However, residual solvent and/or crosslinking agents in the CH microspheres may cause undesirable effects including irritation to mucosal membranes. To overcome these drawbacks, both non-toxic aqueous solutions of NaOH and SLS were applied to the production of CH microparticles. These microparticles were prepared by ionotropic gelation using a high molecular weight ion, SLS [9]. Also, the microparticles were obtained by a liquid–liquid phase separation with NaOH [10]. In both cases, the polymeric solution was added by dripping or spraying methods into the counter ion aqueous solution. In an inverse experiment, when the aqueous solution of either NaOH or NaLS was added into the acidic CH solution, since the concentration of acetic acid was higher than of the amino groups in CH, the NaOH reacted preferable with acetic acid instead the CH amino groups. Thus, the CH gel did not totally coagulated, and aggregates with concentrated polymer regions were also visible. As a consequence, it was decided to select the first procedure already described in the methodology section for further *in vitro*/*in vivo* studies. The variables involved in this process and their effects on dissolution rate and drug-loaded content of microparticles are presented in Table 1.

3.2. Morphology of the ABZ–CH microparticles

The shape and surface morphology of pure CH, pure ABZ and ABZ–CH microparticles, obtained using NaOH or SLS as counterions, are shown in Fig. 1. The polymer (A) is formed by multi-laminar layers of different forms and sizes. Its surface is smoothly without porosity. ABZ existed as small irregular particles with a

Table 1
ALB–CH microparticles prepared with Na(OH) or SLS.[a]

Preparation method	Polymer amount (%w/v)	Stirring rate (rpm)	$EE_{Na(OH)}$	Q_{30} (%)$_{Na(OH)}$	$Size_{Na(OH)}$	EE_{NaLS}	Q_{30} (%)$_{SLS}$	$Size_{SLS}$
Dripping	3.0	1000	44.45	5.00	200	36.45	4.78	250
Spraying	3.0	1000	68.15	21.70	50	69.13	15.16	100

[a] Q_{30} means percent of ABZ dissolved at 30 min.

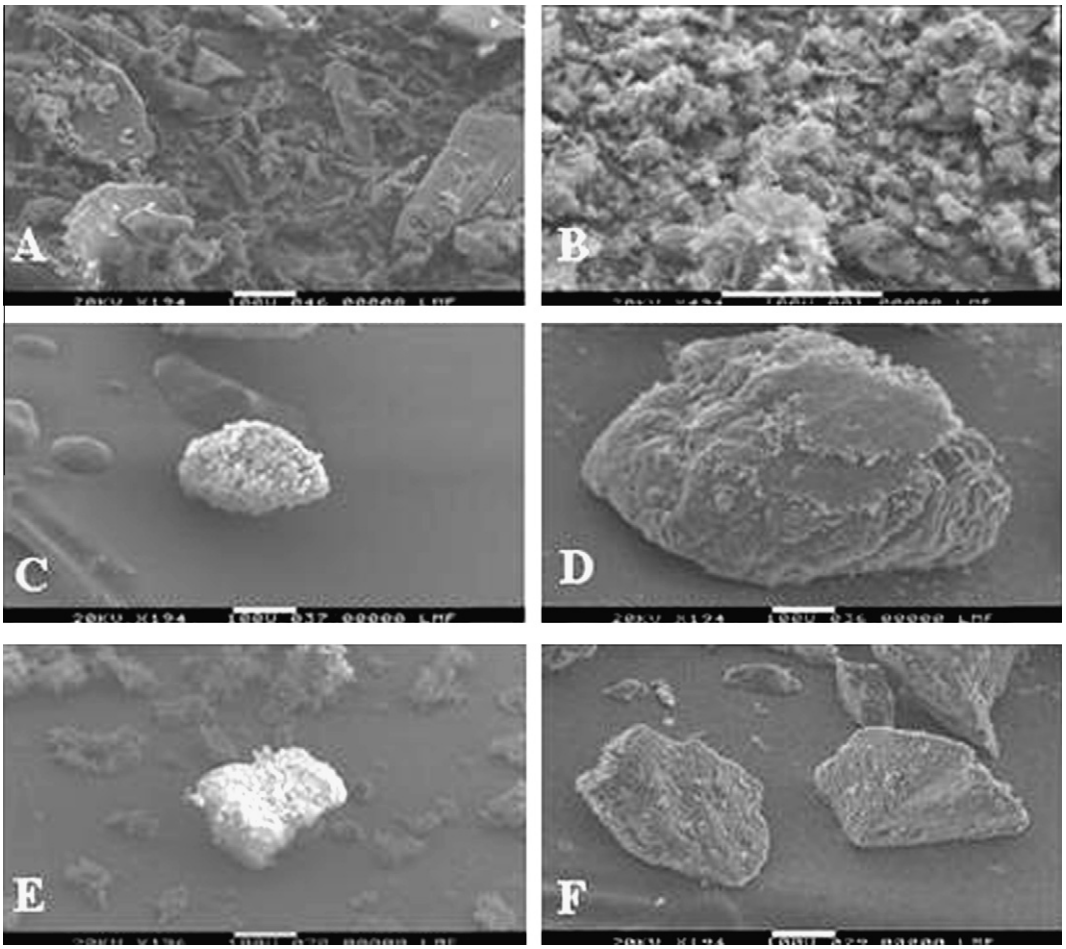

Fig. 1. Scanning electron microscopy (SEM): (A) chitosan, (B) pure ABZ, (C) ABZ–CH with SLS (spraying), (D) ABZ–CH with SLS (dripping), (E) ABZ–CH with NaOH (dripping), (F) ABZ–CH with NaOH (spraying).

smooth surface (B). The applied technique for the preparation of ABZ–CH microparticles affected the particle shape, size and the morphology of these polymeric systems. ABZ–CH microparticles prepared with either SLS (C) or NaOH (F), by the spraying method, showed an acceptable spherical shape with a porous surface. On the other hand, the microparticles obtained by dripping technique, with either SLS (D) or NaOH (E), exhibited a quasi-prismatic shape with a regular and flat surface. The use of a spray device instead of a syringe-needle system led to a significant decrease in particle size range, probably due to a more effective dispersion of the aqueous phase within the NaOH or SLS solution. Also, the increased surface area of the smaller polymeric solution droplets that are accessible for the counter ions might result in smaller CH microparticles.

3.3. In vivo experiments using infected animals

A basic requirement for an excipient that is going to be used in humans or animals is its ability to degrade with no toxicity for biological environments. To better assess the usefulness of the CH microparticles systems on the ABZ dosage forms development, the CH formulations without ABZ (placebo) were orally administered to the animals. It was observed a mice survival rate of 100%, and there were no macroscopically detectable differences between non-treated and CH microparticle–treated animals. Then, the anthelmintic effect in mice after administration of ABZ–CH formulations was carried out. The examination for larvae was done immediately after the necropsy to minimise the possibility of reduced mobility and/or mortality of the larvae. After 28 days of infection, the number of larvae seen in the liver, lung and brain during the administration of ABZ–W was significantly reduced in comparison with the control, as expected. ABZ–CH microparticles were found to decrease the number of larvae in the liver (12 and 10 larva/mouse, respectively), in comparison with ABZ–W (14 larva/mouse) and the control group (36 larva/mouse) ($P < 0.001$). In lung, ABZ–CH formulations (9 and 8 larva/mouse) showed a very good nematocidal activity in comparison with the control group (24 larva/mouse) and ABZ–W (11 larva/mouse) ($P < 0.001$). How-

Table 2
Number of *T. canis* larvae recovered following treatment with ABZ–CH microparticles in BALB/c mice.[a,b]

Localization	ABZ suspension	Control	Groups $n = 5$			
			ABZ–CH–Na(OH)^S	ABZ–CH–Na(OH)^D	ABZ–CH–SLS^S	ABZ–CH–SLS^D
Liver $x \pm SD$	14 ± 3.9	36 ± 1.6	12 ± 5.1	15 ± 3.5	10 ± 2.8	14 ± 2.8
Lungs $x \pm SD$	11 ± 3.7	24 ± 3.1	8 ± 3.4	8 ± 3.7	6 ± 3.7	9 ± 1.8
Brain $x \pm SD$	1 ± 0.1	8 ± 0.8	–	1 ± 0.1	–	1 ± 0.4

[a] ABZ–CH–Na(OH)^S ABZ–CH–NaLS^S means ABZ–CH microparticles obtained by spraying technique using NaOH and SLS, respectively.
[b] ABZ–CH–Na(OH)^D ABZ–CH–NaLS^D means ABZ–CH microparticles obtained by dripping technique using NaOH and SLS, respectively.

ever, the most important results were obtained when the migration of larvae was evaluated in brain. ABZ–W decreased the number of larva (1 larva/mouse) when compared to the control (8 larva/mouse), while ABZ–CH microparticles were able to prevent completely the migration or larvae to the brain (0 larva/mouse) ($P < 0.001$) (Table 2). From these results and having on mind that BALB/c mice have a tendency to accumulate larvae in the brain, it can be postulated that ABZ–CH microparticles would be a novel alternative to deliver an effective drug concentrations to produce the therapeutic effect. Clearly, the anthelmintic property of these formulations resulted in significant retention of larvae in the liver and lung, causing a total reduction in the numbers of larvae migrating to the brain of treated animals compared to untreated (Table 2).

4. Conclusions

ABZ microparticles were successfully prepared using CH as carrier and NaOH or SLS as a counterion. The applied methodology was simple, non-toxic and involved a mixture of aqueous phases at room temperature without using organic solvents and/or cross-linking reagents. The use of the spray device resulted in small and more spherical microparticles, whereas the dripping technique led to significantly larger particles that were more irregular in shape. These CH microparticles were well tolerated by uninfected and infected mice regardless of the dose administered, and all animals survived until the end of the study. Regarding the *in vivo* evaluation of these CH microparticles, a reduction in total larvae in the liver and lung of treated mice in comparison with the treated and placebo controls was observed. Interestingly, ABZ–CH microparticles were able to prevent the arrival of the larvae to the brain, in comparison with the infected mice treated with ABZ–W or placebo controls. These results show that ABZ–CH formulations are significantly more effective than ABZ alone for treatment of chronic infections produced by larva migrans.

Acknowledgements

The authors express their gratitude to the National University of Rosario (U.N.R.) and The National Council Research (CONICET, Argentina). D.L. gives thanks to The National Council Research (CONICET, Argentina) for a fellowship.

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