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Research paper

Enhancing and sustaining the topical ocular delivery of fluconazole using chitosan solution and poloxamer/chitosan *in situ* forming gel

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ABSTRACT

Fungal keratitis is a serious disease that can lead to loss of vision. Unfortunately, current therapeutic options often result in poor bioavailability of antifungal agents due to protective mechanisms of the eye. The aim of this work was to evaluate the potential of a chitosan solution as well as an *in situ* gel-forming system comprised of poloxamer/chitosan as vehicles for enhanced corneal permeation and sustained release of fluconazole (FLU). For this, *in vitro* release and *ex vivo* corneal permeation experiments were carried out as a function of chitosan concentration from formulation containing the chitosan alone and combined with the thermosensitive polymer, poloxamer. Microdialysis was employed in a rabbit model to evaluate the *in vivo* performance of the formulations. The *in vitro* release studies showed the sustained release of FLU from the poloxamer/chitosan formulation. *Ex vivo* permeation studies across porcine cornea demonstrated that the formulations studied have a permeation-enhancing effect that is independent of chitosan concentration in the range from 0.5 to 1.5% w/w. The chitosan solutions alone showed the greatest *ex vivo* drug permeation; however, the poloxamer/chitosan formulation presented similar *in vivo* performance than the chitosan solution at 1.0%; both formulations showed sustained release and about 3.5-fold greater total amount of FLU permeated when compared to simple aqueous solutions of the drug. In conclusion, it was demonstrated that both the *in situ* gelling formulation evaluated and the chitosan solution are viable alternatives to enhance ocular bioavailability in the treatment of fungal keratitis.

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1. Introduction

Fungal keratitis is a serious disease that can lead to loss of vision if not diagnosed and treated promptly and effectively [1]. Previous ocular surface disease and trauma are the leading causes of fungal infection in the cornea [2], but prolonged chemo- or immunosuppressive therapy [3] and contact lens use [4] have also been reported as predisposing factors.

Unfortunately, current therapeutic options are limited [5]. Oral therapy requires high doses of an antifungal agent to reach therapeutic concentrations at the site of action, which may cause unwanted side effects. Antifungal subconjunctival and intracameral injections are customarily used but are very uncomfortable and may cause complications such as cataracts [6]. In most cases, surgery is recommended [7] even though surgical options, such as therapeutic keratoplasty, have a high incidence of infection recurrence [8]. In extremely severe cases, enucleation or evisceration is needed [9,10]. Topical treatment, which would be the ideal choice, is not efficient. Eye drops often result in poor bioavailability due to normal protective mechanisms of the eye such as rapid precorneal drainage allied with the poor permeability of the cornea to drugs [11,12].

The problem of short residence time of formulations on the eye surface may be overcome by the use of *in situ* forming gels. These systems are applied as solutions or suspensions and undergo gelation after instillation due to physico-chemical changes they undergo in the eye [13]. This allows for an easily reproducible administration of the formulation into the eye as a drop and an *in situ* phase transition to a gel on the surface of the cornea. This may improve the retention time of the formulation and, consequently, of the drug. Furthermore, the administration of solutions is well tolerated by patients, which could contribute to compliance with the regime.

Thermosensitive amphiphilic block copolymers, namely poly (ethylene oxide)–poly (propylene oxide)–poly (ethylene oxide) (PEO–PPO–PEO, poloxamers), have been extensively investigated as *in situ* forming gels [14–18]. They form micelles in solution that can self-organize and form a viscous gel depending on polymer

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concentration and temperature [19]. Though thermosensitive copolymers are widely employed, they suffer from a major drawback of having weak mechanical strength, which leads to rapid erosion [20]. One interesting approach to solve this problem focuses on blends of poloxamers with chitosan [21].

Chitosan is a biodegradable polymer that has demonstrated excellent ocular compatibility [22–24]. It presents positively charged amine groups in its chemical structure that could interact with the negatively charged mucous layer, conferring a mucoadhesive characteristic [25,26]. Due likely to this characteristic, chitosan solutions have been successfully used in prolonging contact time with rabbit's ocular surface [27].

Experimental evidence that poloxamer and chitosan can be used in combination for the preparation of *in situ* forming gels with improved mechanical and mucoadhesive characteristics for prolonged precorneal residence time *in vivo* was showed in our previous work [21]. At determined polymer concentration, a free-flowing solution at an environmental temperature, which could undergo phase transition upon instillation, was obtained. The adequate mechanical and mucoadhesive properties lengthened the residence time, and the problem of rapid precorneal elimination was overcome [21]. However, the potential of the poloxamer/chitosan gel for sustaining drug release and for overcoming cornea impermeability still needed to be evaluated.

Therefore, the aim of the present work was to evaluate the potential of an *in situ* gel-forming delivery system comprised of poloxamer/chitosan as well as a chitosan solution as vehicles for enhanced permeation and sustained release of drugs. To explore the feasibility of such strategies, fluconazole, which is a well-known and well-tolerated antifungal agent used in the treatment of fungal keratitis [28–30], was chosen. *In vitro* release and *ex vivo* permeation experiments were carried out as a function of chitosan concentration, and microdialysis was employed in a rabbit model to evaluate the *in vivo* performance of the formulations.

2. Materials and methods

2.1. Chemicals

Fluconazole (FLU) was purchased from Galena (Campinas, Brazil). Chitosan MMW (190,000–310,000 Da; 75–85% deacetylated – information provided by the manufacturer) was purchased from Sigma Aldrich (Steinheim, Germany). Poloxamer 407 was purchased from Embrafarma (São Paulo, Brazil), and *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid (HEPES) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Ketamine hydrochloride (Dopalen) was purchased from Vetbrands (Jacareí, Brazil) and xylazine (Dopaser) from Hertape Calier (Juatuba, Brazil). For the release experiments, cellulose acetate dialysis membranes were used, 16,000-molecular weight cutoff (Fisher, Pittsburg, USA). All other reagents were analytical or HPLC grade. Deionized water (Milli-Q Millipore Simplicity 185, Bedford, MA, USA) was used to prepare all solutions. The linear probes used in microdialysis consisted of 0.3 × 10.0 mm, Gambro, Cuprophane, 3000-molecular weight cutoff (CMA Microdialysis, Stockholm, Sweden) glued to nylon tubing (standardized length of 50.0 mm).

2.2. Preparation of gels

Chitosan solutions were prepared dissolving the polymer in 0.5% v/w acetic acid solution. The *in situ* forming gels used in this study were prepared as previously described [21]. Briefly, poloxamer was dissolved into cooled FLU solution and, in cases where chitosan was added, it was first dissolved in a solution of acetic acid, 0.5% v/w. The final solution was kept in the refrigerator for at least 24 h to ensure complete dissolution. The final pH of the formulations ranged from 6.0 to 6.5. All formulations were isotonic with osmolalities ranging from 290 to 310 mOsm kg^{-1}. The amount of FLU added to the formulations was quantified by HPLC before the experiments, by appropriate dilution, to guarantee that the theoretical concentration dispersed in the day before was corrected.

2.3. In vitro drug release

FLU release rates from chitosan solutions (0.5, 1.0 or 1.5% w/w) and from *in situ* forming gels comprised of poloxamer (16% w/w) and chitosan (0.5, 1.0 or 1.5% w/w) were measured through a cellulose membrane in a Franz-type diffusion cell [21], with a diffusion area of 0.64 cm^2. Comparisons were made with the poloxamer (16% w/w) gel alone, and with aqueous solution, all of them containing 2 mg/ml FLU. Aqueous solution containing FLU 2 mg/ml was used as a control.

The donor compartment was filled with 1 ml of studied formulation while the receptor compartment contained 35 ml of pH 7.4 HEPES buffer solution. The system was maintained under magnetic stirring (600 rpm) and at 35 °C with an outer bath. Samples (1 ml) were withdrawn from the receiving solution each hour for 6 h and replaced with fresh receiving fluid. The amount of FLU that permeated across the membrane, i.e., the amount of the drug in the receiving solution, was analyzed by HPLC as described in Section 2.5. The diffusion coefficients (D) of FLU from each vehicle were calculated using the following equation [31,32].

$$Q = 2C_o(Dt/\pi)^{1/2} \quad (1)$$

where Q is the cumulative amount of drug released per unit area, C_o is the initial drug concentration in the vehicle, and D is the diffusion coefficient and t is time.

The drug release rate (K) was also determined by the slope of the linear portion of the plots of the FLU cumulative amount versus the square root of time.

2.4. FLU ex vivo corneal permeation

Corneas used in the *ex vivo* experiments were obtained from porcine eyes, which were collected immediately after the animals were slaughtered (Frigorífico Pontal Ltda, Pontal, SP, Brazil). The eyes had not been heat treated in the abattoir in any way. They were then kept at 4 °C while transported to the laboratory and used within 1.5 h of enucleation. Any eye with a collapsed anterior chamber was discarded. Corneoscleral buttons were dissected using standard eye bank techniques, and care was taken to minimize tissue distortion.

Immediately after corneal preparation, the tissue was placed between the donor and the receiving compartments of the vertical modified Franz-type diffusion cell [33]. The receiving compartment was filled with HEPES buffer solution (25 mM, pH 7.4), and the donor compartment was filled with 1.0 ml of the formulations described in Section 2.3 (the *in situ* chitosan/poloxamer forming gels, the chitosan solutions, the poloxamer gel, and the water) containing 2 mg/ml of FLU. The diffusion-cell system was maintained under identical magnetic stirring and outer bath temperature as described for the *in vitro* release studies (see Section 2.3). Corneal integrity and barrier properties when mounted on the described system are assured for 6 h [33]. For each formulation, six corneas were used ($n = 6$).

During the experiment, samples (1 ml) were withdrawn from the receiving solution each hour for 6 h and replaced with fresh receiving fluid. The amount of FLU that permeated across the cornea, i.e., the amount of the drug in the receiving solution, was analyzed by

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