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Enhancement and inhibition effects on the corneal permeability of timolol maleate: Polymers, cyclodextrins and chelating agents

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ABSTRACT

This study investigates how both bioadhesive polymers (chitosan, hyaluronic acid and alginate) and permeability enhancers (ethylene glycol- bis(2-aminoethylether)- N, N, N', N'- tetraacetic acid (EGTA) and hydroxypropyl-ß-cyclodextrin) influence the permeability of the anti-glaucoma drug timolol maleate through *ex vivo* bovine corneas. Our results showed that only the permeability enhancers alone were able to increase drug permeability, whereas the polymers significantly reduced drug permeation, and however, they increased the pre-corneal residence of timolol. Ternary systems (polymer-enhancer-drug) showed a reduced drug permeability compared to the polymers alone. Fluorescence microscopy analysis of the epithelium surface confirmed there was no evidence of epithelial disruption caused by these formulations, suggesting that polymer-enhancer interactions reduce drug solubilization and counteract the disruptive effect of the permeability enhancers on the surface of the cornea. Further mucoadhesive tests, revealed a stable interaction of chitosan and hyaluronic acid with the epithelium, while alginate showed poor mucoadhesive properties. The differences in mucoadhesion correlated with the permeability of timolol maleate observed, *i.e.* formulations containing mucoadhesive polymers showed lower drug permeabilities.

The results of the present study indicate polymers acting as an additional barrier towards drug permeability which is even more evident in the presence of permeability enhancers like EGTA and hydroxypropyl-ß-cyclodextrin. Then, this study highlights the need to adequately select additives intended for ocular applications since interactions between them can have opposite results to what expected in terms of drug permeability.

1. Introduction

The topical application of drugs is the most popular and well-accepted route of administration for the treatment of various eye conditions (Ludwig, 2005). However, the bioavailability of ophthalmic drugs is very poor due to the effective protective mechanisms of the eye (Lee and Robinson, 1986), including blinking, lachrymation, and drainage (Ludwig, 2005). Therefore, frequent instillations of eye drops or high drug concentrations are needed to achieve therapeutic levels in the tissues (Andrés-Guerrero et al., 2011), which might induce toxic side effects and cellular damage at the ocular surface (Baudouin, 1996). In addition, the treatment of certain ocular diseases such as glaucoma follows the administration of combinations of two or more drugs (Bell et al., 2010), and therapies must be continued throughout the lifetime of the patient (Andrés-Guerrero, 2011), leading to a lack of patient's compliance. Glaucoma is the leading cause of irreversible blindness throughout the world and the lowering of intraocular pressure (IOP) at present is the only therapeutic approach proven to be successful (Lorenz and Pfeiffer, 2014). For many years now, ß-adrenergic receptor blocking agents (ß-blockers) have been the first choice for the treatment of ocular hypertension and primary open-angle glaucoma (Lorenz and Pfeiffer, 2014). Timolol maleate is a nonselective ß-blocker (Brooks and Gillies, 1992) used alone or more frequently, in combination with other medicaments (García-López et al., 2014).

Although in general, timolol is well tolerated by patients (Brooks and Gillies, 1992), approximately 80% of topically

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administered eye drops is reported to drain through the nasolacrimal duct and is systemically absorbed (Shell, 1982). Therefore it is necessary to deepen research into new mechanisms focused on increasing the bioavailability of timolol at the ocular surface. In this regard, the use of bioadhesive polymers has been proposed as components of antiglaucoma formulations to reduce ocular toxicity, improve drug efficacy, and protect the ocular surface in long-term therapies (Andrés-Guerrero, 2011). Both the ability to increase the formulation viscosity (Saettone et al., 1982) and the bioadhesive properties (Kaur and Smitha, 2002) of polymers were reported to reduce the drainage after instillation and therefore, increase the therapeutic efficacy of the ophthalmic drugs. The most common biopolymers used in the formulation of ocular solutions include natural, synthetic and semi-synthetic high molecular weight molecules (Kaur and Smitha, 2002), which are capable of forming strong noncovalent bonds with the mucin coating biological membranes (Almeida et al., 2014). Some examples of mucoadhesive polymers for ocular application are derivatives of cellulose (methylcellulose, carboxymethylcellulose, hydroxypropylcellulose, and hydroxyethylcellulose), polyvinyl alcohol (PVA), polyacrylic acid (PAA), chitosan, and hyaluronic acid. Nevertheless the biodegradability, biocompatibility and non-toxicity of the natural biopolymers, mainly glycosaminoglycans, make them excellent candidates for the development of drug delivery devices.

Besides extending the residence time of the drug it is necessary to promote the permeability through the cornea using penetration enhancers or absorption promoters (Kaur and Smitha, 2002), in order to improve drug bioavailability. These compounds include some preservatives such as benzalkonium chloride and cetylpyridinium chloride that were reported to enhance penetration of some active compounds due to the disruption of the hydrophobic barrier of the corneal epithelium (Andrés-Guerrero, 2011). Surfactants, calcium chelators and cyclodextrins are among other penetration enhancers commonly used in ocular formulations. Surfactants are incorporated into the lipid bilayer of the epithelium, resulting in the formation of mixed micelles that cause the removal of phospholipids and hence lead to membrane solubilization (Kaur and Smitha, 2002). Surfactants can also increase the paracellular transport of drugs by affecting the tight junctions between epithelial cells (Deli, 2009). In the same way, calcium chelators disrupt the corneal epithelium by extracting Ca^{2+} ions (Kaur and Smitha, 2002) which are responsible for the maintenance of the effectiveness of the epithelium barrier. The polyaminocarboxylic acids ethylenediaminetetraacetic acid (EDTA) and its analogue ethylene glycol- bis(2-aminoethylether)-N, N, N', N'- tetraacetic acid (EGTA) have ion sequestering properties. Both calcium chelators were reported to reduce the electrical resistance of corneal membranes, confirming their ability to modify the barrier function, and to increase the corneal permeability of riboflavin *in vitro* (Morrison and Khutoryanskiy, 2014). Finally, cyclodextrins are oligosaccharides with a lipophilic central cavity and hydrophilic outer surface which are used as excipients in ocular formulations because of their ability to increase the water solubility of hydrophobic drugs (Loftsson and Stefánsson, 2002), such as riboflavin (Morrison et al., 2013). These authors proposed cyclodextrins are responsible for the extraction of cholesterol and other lipids from ocular cellular membrane being the reason for the observed increase in riboflavin permeability (Morrison et al., 2013).

In the present study, we investigated the effect of different formulations containing biopolymers (hyaluronic acid, chitosan and alginate) and permeability enhancers (calcium chelators and cyclodextrins) on timolol maleate permeability through bovine cornea. We also analysed whether these formulations modified the corneal integrity and how their mucoadhesive properties affected the permeability of timolol. For a better comparison of results between treatments, we developed mathematical models to accurately quantify the apparent permeability of the drug and retention of the polymers on the corneal surface.

2. Materials and methods

2.1. Materials

Timolol maleate was kindly supplied by Fine Chemicals Ltd (Dorset, United Kingdom). Triethylamine hydrochloride was purchased from Fluka. Chitosan medium molecular weight (190,000–310,000 Da, 75–85% deacetylation), sodium alginate medium viscosity (12,000–40,000 Da), fluorescein isothiocyanate–dextran (FITC-dextran) 70,000 Da, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and phosphoric acid were purchased from Sigma-Aldrich (Gillingham, United Kingdom). Hydroxypropyl-β-cyclodextrin (HP-ß-CD), and ethylene glycol-bis(2-aminoethylether)- N,N,N',N' −tetraacetic acid (EGTA) were obtained from TCI Ltd (Oxford, United Kingdom). Hyaluronic acid was obtained by fermentation of *Streptococcus zoeepidemicus* ATCC 35246 (Amado et al., 2016), followed by acid hydrolysis using H_3PO_4 to a final MW of 24,000 Da. Sodium chloride, potassium chloride, sodium phosphate, potassium dihydrogen phosphate, sodium hydroxide, Minisart syringe filters (0.2 μm), optimal cutting temperature compound (OCT) and methanol were obtained from Fisher Scientific (Hemel Hempstead, United Kingdom). Vectashield mounting medium with 4′,6-diamidino-2-phenyl- indole (DAPI) was obtained from Vector Laboratories Ltd. (Peterborough, United Kingdom).

2.2. HPLC analysis

HPLC analysis was conducted using a PerkinElmer series 200 HPLC system comprising of 785 A UV–vis detector, series 200 quaternary pump and series 200 autosampler (PerkinElmer Inc., UK), Ascentis C_{18} column, 150 mm × 4.6 mm, 5 μm (part number: 581324-U) and data acquisition software (Peaksimple, version 4.09, SRI Inc., USA). Analysis of timolol maleate was achieved with a run time of 5 min using the method adapted from El-Kamel (2002). The mobile phase consisted of a mixture of methanol and triethylamine hydrochloride (45:55) under isocratic conditions, a flow rate was used at 1 mL min^{-1} at 30 °C and detected with a UV detector (295 nm). The retention time of timolol maleate was 2.85 min and the detection limit was 0.1 μM. Quantification of timolol maleate concentration in the samples was achieved by linear interpolation in a calibration curve of timolol maleate standards at concentrations ranging from 0.28 to 2.8 μg mL^{-1}.

2.3. Preparation of animal tissues

Bovine eyes were provided by PC Turners abattoirs (Farnborough, United Kingdom) and stored on ice during transport. The eyes were carefully handled and used whole or cornea dissected depending on the experiment. The corneas were dissected using a sharp blade with 2−3 mm of sclera attached, rinsed with PBS, and wrapped in a cling film to prevent dehydration. Fresh tissues were stored at 4 °C in a refrigerator and used within 48 h prior to experiments, preserved according to previous ocular drug permeability tests (Morrison et al., 2013; Morrison and Khutoryanskiy, 2014).

Corneal sections from experiments were prepared by setting the cornea segment in OCT, quick freezing on dry ice, and subsequent microtome sectioning. Specimens were prepared for microscopy using a microtome (Bright, model 5040) within a cryostat (Bright, model OTF). Sections were cut at 7 μm, placed in