Ph.D. Thesis

Application of cyclodextrins and mucoadhesive preservative system in ophthalmic formulations

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List of original publications

I. **Bíró, T.,** Horvát, G., Budai-Szűcs, M., Csányi, E., Urbán, E., Facskó, A., Szabó-Révész, P., Csóka, I., Aigner, Z., Development of prednisolone-containing eye drop formulations by cyclodextrin complexation and antimicrobial, mucoadhesive biopolymer. *Drug Design, Development and Therapy* (2018) 12, 2529–2537.
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II. **Bíró T.**, Aigner Z., Current Approaches to Use Cyclodextrins and Mucoadhesive Polymers in Ocular Drug Delivery – A Mini-Review. *Scientia Pharmaceutica* (2019) 87, 15.
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Presentations related to the thesis

1. Tivadar Bíró, Zoltán Aigner: Megnövelt biohasznosulású szemészeti készítmény formulálása és vizsgálata.
   Local Scientific Student's Association, p. 170, Szeged, Hungary, 2016.

2. Tivadar Bíró, Zoltán Aigner: Megnövelt biohasznosulású szemészeti készítmény formulálása és vizsgálata.
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3. Tivadar Bíró, Zoltán Aigner: Megnövelt biohasznosulású szemészeti készítmény formulálása és vizsgálata.
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4. Tivadar Bíró, Zoltán Aigner: Megnövelt biohasznosulású gyulladáscsökkentő szemcsepp formulálása.
   Hungarian Chemical Society, Crystallization and Drug Formulation Department 10th Conference, p. 29, Balatonszemes, Hungary, 2017.

5. Tivadar Bíró, Gabriella Horvát, Mária Budai-Szűcs, Erzsébet Csányi, Edit Urbán, Andrea Facskó, Piroska Révész- Szabó, Ildikó Csóka, Zoltán Aigner:
   Characterization of prednisolone containing eye drop formulations by mucoadhesive–preservative system and cyclodextrin inclusion complex formation.
   7th BBBB International Conference on Pharmaceutical, Sciences, Acta Pharmaceutica Hungarica 87 (3-4) p. 178, Balatonfüred, Hungary, 2017.

6. Tivadar Bíró, Gabriella Horvát, Alexandra Bocsik, Ilona Gróf, Edit Urbán, Mária A. Deli, Erzsébet Csányi, Piroska Szabó-Révész Ildikó Csóka, Zoltán Aigner: Formulation of steroid containing eye drops with cyclodextrin derivatives and mucoadhesive preservative system

12th Central European Symposium on Pharmaceutical Technology and Regulatory Affairs, Acta Pharmaceutica Hungarica 88 (3) p. 139-140, Szeged, Hungary, 2018.

7. Tivadar Bíró, Zoltán Aigner: Ciklodextrinek és mukoadhezív, antimikróbás biopolimer alkalmazása megnövelt hatékonyságú szemészeti készítmények formulálása céljából
   XIII. Ottó Clauder Memorial Competition, p. 18, Budapest, Hungary, 2018.

8. Tivadar Bíró, Zoltán Aigner: Novel ophthalmic formulations to increase the efficacy and stability
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9. Tivadar Bíró, Zoltán Aigner: *In vitro* and *ex vivo* investigation of steroid containing ocular drug delivery systems
   II. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, p. 6, Szeged, Hungary, 2020.

10. Tivadar Bíró, Alexandra Bocsik, Ilona Gróf, Bisera Jurišić Dukovski, Jasmina Lovrić, Mária A. Deli, Zoltán Aigner: Innovatív szemcseppek toxicitásának és hatóanyag-permeabilitásának vizsgálata *in vitro* és *ex vivo* modellek alkalmazásával
    Congressus Pharmaceuticus Hungaricus XVI; Acta Pharmaceutica Hungarica 90. p. 86, Debrecen, Hungary, 2020.

Abbreviations

API – active pharmaceutical ingredient

BAB – blood aqueous barrier

BK – benzalkonium chloride

BRB – blood retinal barrier

CD – cyclodextrin

CFU – colony-forming units

DMSO – dimethyl sulfoxide

DSC – Differential Scanning Calorimetry

EBA – Evans blue labelled albumin

EGF – epidermal growth factor

EP – European Pharmacopoeia

FTIR – Fourier-transformed Infrared Spectroscopy

HA – hyaluronic acid

HCE-T – human corneal epithelial cell line

HPBCD – hydroxypropyl-β-cyclodextrin

HPGCD – hydroxypropyl-γ-cyclodextrin

HPLC – high-performance liquid chromatography

MWCO – molecular weight cut-off

n.d. – not detectable

n.i. – no increase

PBS – phosphate buffer

PR – prednisolone

SBEBCD – sulfobuthylaether-β-cyclodextrin

SF – fluorescein

TEER – transepithelial electrical resistance

TX-100 – Triton X-100

XRPD – X-Ray Powder Diffraction

ZnGlu – zinc-gluconate

ZnHA – zinc-hyaluronate

ZO-1 – zonula occludens protein-1

1. Introduction

Ocular drug delivery provides a challenging opportunity to develop optimal formulations with proper therapeutic effect and acceptable patient compliance, because it is restricted by many factors, like complex anatomical structure, defensive reflex mechanisms, rapid drainage and applicability issues. The main goal is to meet the requirements of patient-based therapy and technological formulation aspects.

Eyes are one of the most important organs of the human body. In the case of any dysfunction of vision, serious drawback can be appeared in daily activities. Patient compliance is a key factor, thus finding the optimal administration route which is self-applicable by the patients, and optimal formulation with accomplished therapeutic effect and zero irritation is the mission what researchers are trying to complete. The special environment of the eye makes the formulation optimization difficult. Several methods are developed for enhanced ocular drug delivery. Mainly topical eye drop solutions are in the focus of research laboratories. Besides the advantages of these formulations (self-applicable, non-invasive, convenient, economical), many difficulties are known, which need to be overcome: short retention time, low drug absorption, low bioavailability and problematic microbiological stability in multi-dose products.

To increase the efficiency of the ocular delivery of the drug, the enhancement of water solubility and the contact time of the drug on the surface of the cornea are necessary. Addition of solubility enhancer cyclodextrin (CD) derivatives and mucoadhesive polymers, the permeability of active ingredients is improved, and the retention time is increased in the ocular surface. Therefore, preferable efficacy and bioavailability can be achieved. Antimicrobial stability of topical ophthalmic formulations is especially important. According to previous studies, the mostly used preservative, benzalkonium-chloride (BK) is irritant and toxic on corneal epithelial cells, therefore novel non-toxic, antimicrobial agents are required.

2. Aims

Formulation optimization is a major challenge in the field of ocular drug delivery. The aim of this work was the development of innovative eye drop formulation containing prednisolone (PR) in water-soluble CD complex with acceptable physiological rheological and mucoadhesive parameters, adequate microbiological stability, optimal toxicity and drug permeability using CD inclusion complex and preservative, mucoadhesive biopolymer.

The main steps of the project were as follows:

I. Formation and optimization of CD inclusion complex with the chosen active pharmaceutical ingredient (API), PR tested by phase solubility test and membrane diffusion study;

II. Setting the physiological parameters using pH, surface tension and osmolality measurements;

III. Investigation of the viscosity and mucoadhesive properties;

IV. Testing the microbiological stability according to the standards of European Pharmacopoeia (EP);

V. *In vitro* cytotoxicity studies on human corneal epithelial cell line (HCE-T);

VI. Permeability tests on *in vitro* HCE-T and *ex vivo* porcine cornea models.

3. Literature survey

3.1 Anatomical and physiological perspectives of ocular drug delivery

The complex anatomy of eye limits the therapy of different diseases, especially when deeper drug permeation is needed. Eye has two main parts; the anterior segment is from the cornea, aqueous humour, iris, lens, and the posterior segment from the lens to the deeper tissues (vitreous humour, retina, sclera, optic nerve). The cornea consists of five layers: the lipophilic epithelium with tight junctions; Descemet's membrane; the hydrophilic stroma which is the thickest part of the cornea; Bowman's layer and the lipophilic endothelium [1–4]. Considering the optimal drug penetration through the cornea, the balance in the hydrophilicity and lipophilicity of the drug and the delivery system is necessary. Due to the complex anatomical structure, the formed physiological barriers protect the eye from the surrounding exposures. The first barrier built by the tear film, which include a lipid layer, mucin and water. It protects the cornea and conjunctiva. The composition of corneal barrier is mentioned before. It mainly restricts the drug permeation to the anterior tissues. The conjunctival barrier consists of epithelial layers and connective tissue with blood and lymphatic vessels. The Blood Aqueous Barrier (BAB) contains tight junctions of the capillary endothelium of the iris, and ciliary epithelium. It is mildly permeable for low-weight molecules. The drug permeation is restricted from the systemic circulation to the posterior segment of eye by the Blood Retinal Barrier (BRB) due to the tight junctions of retinal pigment epithelium and endothelial membrane of retinal blood vessels [5–7]. After any stimulus, reflex mechanisms, like lachrymal secretion and eye blinking are induced, thus the irritant agents are eliminated in minutes from the eye surface. If the drug is passed through the cornea, the opposite flow of aqueous humour also limits the penetration to the posterior direction [8–10]. Therefore, these mechanisms also limit the therapy by blocking the drug permeation into the targeted tissues.

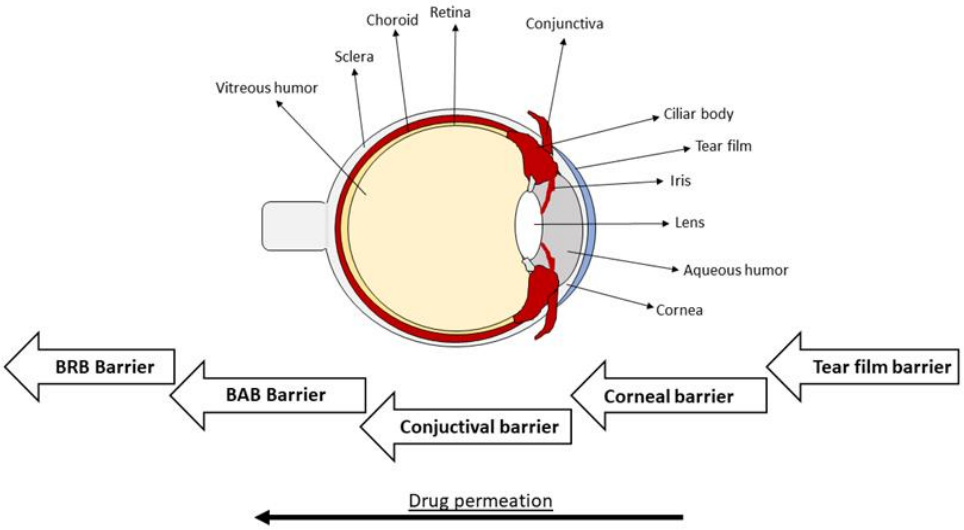

Figure 1. Structure of eye and physiological barriers (BRB: Blood Retinal Barrier, BAB: Blood Aqueous Barrier) [11]

3.2 Conventional routes of administration

Considering the above-mentioned defensive blockade, to ensure the required therapy is excessively difficult in ophthalmology. In the clinical practice, there are invasive and non-invasive methods for administration of the formulation to reach the targeted site. Non-invasive routes, also known as topical formulations, are mainly for reaching the anterior segment. The indications at this site are cataract, glaucoma, dry eye, inflammatory diseases, trauma or surgery induced diseases, injury, tumour.

Topical administration is the easiest, most commonly applied, non-invasive method which is self-applicable for the patient. Mostly eye-drops, semisolid formulations, inserts, contact lens containing API are used [1,12,13]. The requirements are exact for these products: sterile, isotonic, microbiologically stable formulations must be prepared with acceptable pH and viscosity. If any of the parameters differ from the optimal range, the defensive mechanisms are induced in the eye, therefore the expected efficacy would be much worse. After application, the tear film barrier is the first blockade. For optimal drug permeation, sufficient concentration of drug must present at the cornea. The second obstacle is the corneal barrier, where firstly the drug meets the corneal epithelial multilayer. Because of the tight junction proteins between the epithelial cells, penetration of hydrophilic molecules is restricted, lipophilic drugs can

permeate transcellular by passive diffusion. The second part of the cornea is the stroma, which is a hydrophilic environment, therefore penetration of lipophilic drugs is restricted here. The lipophilic endothelial monolayer is more transparent for macromolecules, than the epithelium. The non-corneal pathways also known as conjunctival-scleral route, where the permeation mostly depends on the molecular weight. Through the corneal and non-corneal pathway, the anterior tissues are partly reachable for the active ingredients. From the precorneal area (tear film) the applied formulation is eliminated through the tear turnover and nasolacrimal drainage to the systemic circulation. The corneal and non-corneal pathways for topically applied drug absorption are complex, and consist of lipophilic and hydrophilic layers. For optimal drug penetration the required chemical characteristics of the drug delivery system must be strictly designed. Optimally a lipophilic-hydrophilic balance is needed in the system to induce the required therapeutic effect without minimal precorneal drug elimination [14–17].

The invasive administration methods like intravitreal, subconjunctival injections and inserts have the advantages but also limitations. By these methods, the target tissues are directly reachable, although these invasive administrations are limited because of the required expertise, proper dosage and possible side-effects, like toxic reactions of the cornea. The subconjunctival application is less invasive, although the elimination is decently fast through the conjunctival blood and lymphatic vessels.

Oral and intravenous administration are rather unfavored because of the presence of blood aqueous barrier and first pass metabolism. To overcome the barrier, high concentration of drug needs to be used, which is difficult because of the possible side effects and poor solubility of most active ingredients [18,19].

Table 1. Route of administrations to the eye with advantages and limitations [11]

Route of administration	Advantages	Limitations
Topical	Patient-compliance, self-applicable, non-invasive, simple, no first-pass effect	Frequent administration needed, low bioavailability, short contact time on the eye surface, tear dilution
Subconjunctival	Barely invasive, high efficacy, no first pass effect	Fast clearance, expertise needed, not self-applicable
Intravitreal	High bioavailability, avoiding cornea, no first-pass effect	Critical dosing, very invasive method, expertise needed, not patient compliant, toxic side effects
Intravenous	Avoiding cornea, less frequent application	Invasive, expertise needed, not targeted exposure, large dose needed
Oral	Patent compliant, non-invasive	First pass effect, low ocular efficacy, not targeted exposure, large dose needed

In ophthalmic surgery, glucocorticoid derivatives like PR, dexamethasone and fluorometholone are widely used for postoperative inflammation prophylaxis. Due to their low aqueous solubility, they are present on the market primarily in suspension formulations. When there is a risk of severe inflammation, especially after cornea transplantation, anti-inflammatory steroid therapy is needed by giving a subconjunctival or subretinal injection [20]. Considering the attributes of physiological obstacles and administration routes an innovative solution is required, which is acceptable from the aspects of patient compliance and efficient therapy. A topically self-administrable formulation would be optimal with enhanced drug permeability into the anterior/posterior tissues and increased residence time on the surface of the eye.

3.3 Prednisolone

PR is a synthetic glucocorticoid derivative. Glucocorticoids affect cells through intracellular glucocorticoid receptors. These receptors are found in the cytoplasm complexed with heat shock proteins. The glucocorticoid activates the receptor, detaches it from the heat shock protein, and forms a steroid-receptor complex. These complexes enter the nucleus by forming dimers, where they induce or inhibit gene

transcription, thereby affecting cellular metabolism. The anti-inflammatory and immunosuppressive effect is mediated by inhibiting the transcription of the gene encoding the cyclooxygenase-2 enzyme, the genes of interleukins and cell adhesion molecules, and the NO synthase gene, and by enhancing annexin-1 protein formation. Annexin-1 inhibits the enzyme phospholipase A2 in the arachidonic acid cascade, therefore the synthesis of inflammatory mediators is reduced. The early (redness, oedema, pain) and late (wound healing, fibroblast activity, increased cell proliferation) stages of the inflammatory process are inhibited. PR can be used topically in ophthalmic diseases, anterior and posterior uveitis, in allergic processes in combination with an antiallergic agent, and in post-infection conditions they can help clear corneal opacities. Treatment of bacterial and viral infections should be cautious, as they may mask the symptoms of the infection. Prolonged use may increase intraocular pressure, so frequent monitoring is required [21–24].

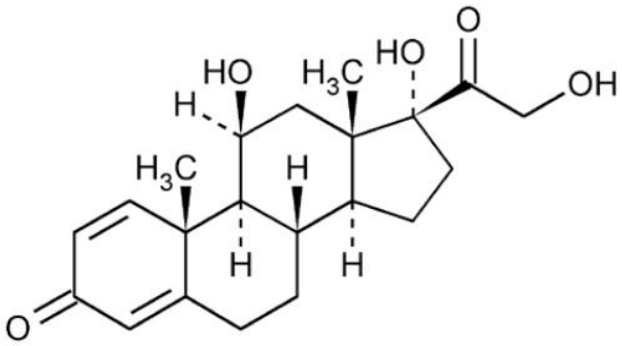

Figure 2. Chemical structure of PR

To reach the optimal penetration of PR, it needs to be dissolved in lachrymal fluid and pass the tear film barrier. If the concentration of API going to be optimal near the corneal epithelium, steady amount is ensured for optimal permeation. A major challenge is that, the applied APIs in ophthalmology are mostly lipophilic molecules, with low water solubility. The eye drops in the market contain 1% or 0.12% PR-acetate in suspension formulations. Although the water-solubility of acetate form is better, it may cause irritation after administration. Application of solubility enhancer additives, like CDs could be the first step for optimization of eye drop formulations [20,25–29].

3.4 Cyclodextrins

CDs are cyclic oligosaccharides with α-(1,4) linked α-D-glucopyranose units. In the nature three types are formed by bacterial digestion of starch: α-CD with 6, β-CD with 7 and γ-CD with 8 glucopyranose units (figure 3.).

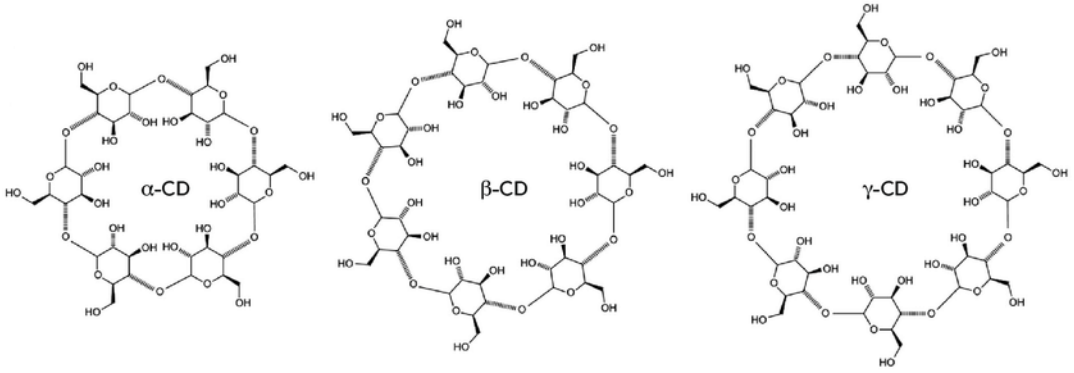

Figure 3. Chemical structure of natural CD derivatives

The external surface of these molecules is hydrophilic due to the orientation of hydroxyl-groups, which form hydrogen bonds with the surrounding water molecules. Inside the cavity of CDs, the environment is hydrophobic, therefore inclusion complex can be formed with lipophilic agents by hydrogen bonds, van der Waals and charge-transfer interactions [30–32]. In aqueous solution dynamic equilibrium is created between the free CD and drug molecules and the complex. After application on the eye surface, only the free lipophilic molecule can permeate through the cornea, the hydrophilic CD remains and going to be eliminated through the nasolacrimal pathway (Figure 4.).

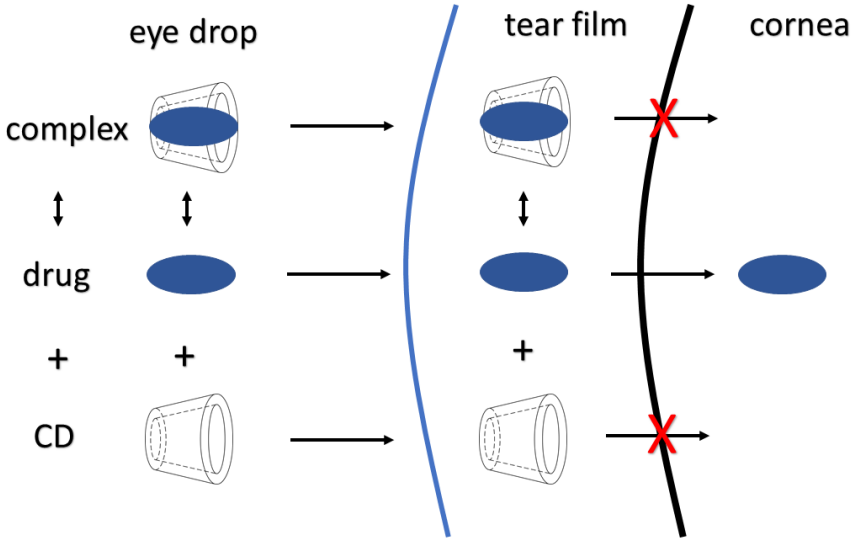

Figure 4. Schematic figure about the cyclodextrin drug permeability enhancer attributes

With formation of inclusion complexes, the API are dissolved in the tear and concentrated near the cornea epithelium. Low or unnecessarily high amount of CD restricts the permeation of drug, thus the CD concentration need to be optimized in the formulation [33–39]. To investigate the formation of inclusion complex in solution, phase-solubility test is well known, which is described previously by Higuchi and Connors. Apparent stability constant of complex (K_S) is calculable from the slope of phase solubility diagram using Higuchi-Connors Equation. By the stability constant, the intensity of binding forces between the API and CD molecules can be established [40–42]. In solid state, the formation of drug-CD inclusion complex can be investigated by Differential Scanning Calorimetry (DSC), Fourier-transformed Infrared Spectroscopy (FTIR), X-Ray Powder Diffraction (XRPD) and C13-NMR methods. Considering the changes in physicochemical attributes, crystallization and the IR spectrum the formation of complex can be assumed [43–46]. CD derivatives have been developed with more favorable attributes like increased solubility, stability and less toxicity. In ophthalmic formulations the hydroxypropyl-β-cyclodextrin (HPBCD), hydroxypropyl-γ-cyclodextrin (HPGCD) and sulfobuthylaether-β-cyclodextrin (SBEBCD) are the most commonly applied derivatives, which are also official in EP. Studies on rabbit corneal epithelial cell-line showed non-toxic attributes after application of these type of CDs [47–50].

Table 2. Recent approaches to use CDs in ophthalmic formulations [11].

API	CD derivative	Formulation	Reference
Flurbiprofen	HPBCD	eye drop	*Shinde et al.* 2019 [51]
Nepafenac	HPBCD HPGCD	eye drop	*Lorenzo-Veiga et al.* 2019 [52]
Amlodipine	HPBCD SBEBCD	eye drop	*Nanda et al.* 2018 [53]
Dexamethasone acetate	HPBCD HPGCD	eye drop	*Mazet et al.* 2018 [54]
Cyclosporine	HPBCD	insert	*Grimaudo et al.* 2018 [55]

3.5 Mucoadhesion

Application of polymers for prolonged ocular drug delivery is a common strategy. Increasing the viscosity until not necessarily high level, the residence time of the eye drop on the surface of the eye is prolonged without any side-effect as visual disorder and irritation. Mucoadhesive polymers are especially useful, because of the possible adhesion due to the interaction of polymer chains and mucin layer of the tear film. It is defined as bioadhesion if the polymer chains are attached to biological surface [56–59]. Several theories are known as the mechanisms of mucoadhesion. The wetting theory describes the effect of drop spread ability and wettability on the eye surface. According to the electrostatic theory, electron transfer is the mechanism of mucoadhesion. Adsorption theory is about primary and secondary chemical bonds between the polymer and mucus. In the case of high molecular weight polymers, the diffusion of polymer chains and glycoproteins of mucus can interpenetrate into each other creating an intermolecular net and mucoadhesion. This mechanism is also known as mechanical theory [60–63].

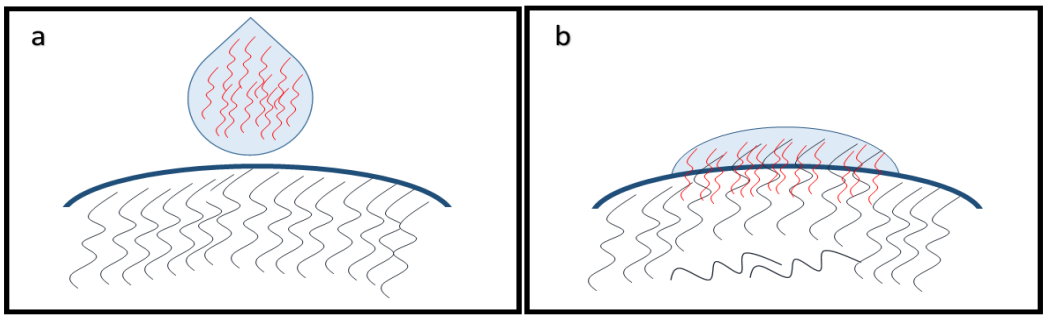

Figure 5. Mechanical theory of mucoadhesion. Before application of eye drop (a) and after interpenetration of polymer chains (b). After application, the polymer chains penetrate during the spreading of eye drop on the ocular surface.

The most commonly used mucoadhesive polymers are carbomers, alginates, methylcellulose, hydroxypropylmethyl cellulose, hydroxyethylcellulose, chitosan, thiolated polymers and hyaluronic acid (HA). Biopolymers like HA are favourable in ophthalmic formulations, because of the biocompatible, non-toxic and biodegradable attributes [55,63–67].

3.6 Preservation of multi-dose ophthalmic formulations

BK is a cationic surfactant additive, which is widely used as a microbiological preservative agent in eye drop formulations. BK may destroy the cell membrane of microorganisms, which results in an antimicrobial effect. Toxicity for corneal and conjunctival epithelial cells and incompatibility with contact lenses were reported earlier. BK causes DNA single- and double-strand breaks in corneal epithelial cells, so the barrier of the eye surface may be damaged. Allergic reaction, eye irritation and increased tear secretion may be caused by application [68–71]. It is also known that resistance of *Pseudomonas aeruginosa* against BK appears due to decreasing the permeation through the cell wall [72]. The antimicrobial properties of Zn^{2+} ion-containing compounds are favorable in pharmaceutical formulations. Marketed products, like Ophylosa® (Gedeon Richter Plc, Budapest, Hungary) contain zinc-hyaluronate (ZnHA) and zinc-gluconate (ZnGlu) for replacing BK. The antimicrobial effect depends on the reactive oxygen species generating mechanism, the cell wall destabilizing effect of cytotoxic, dissolved Zn^{2+} ion in a water-based environment [73–75]. Zinc-containing polymers like ZnHA could be acceptable, combined with a zinc salt of gluconic acid, ZnGlu, to reach the suitable antimicrobial stability. Further investigation is needed to confirm the capability of these compounds as replacements

of the unfavorable BK. Hyaluronic acid has a polyanionic structure and therefore forms intra- and intermolecular bonds with zinc and sodium ions. The zinc cation of ZnHA is surrounded by four oxygen, two oxygen from the carboxyl groups of glucuronic acids, and two from the ring of glucuronic acid molecules [76–78]. ZnHA could be a useful antimicrobial and mucoadhesive additive in ophthalmic formulations.

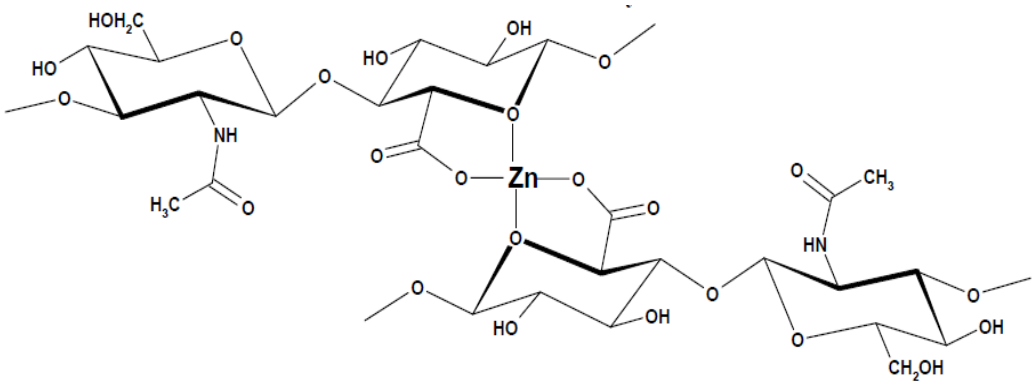

Figure 6. Structure of zinc-hyaluronate

4. Materials

PR was purchased from Henan Lihua Pharmaceutical Company (Henan, China). HPBCD was obtained from Wacker-Chemie GmbH (Munich, Germany), HPGCD was kindly donated by Cyclolab Ltd. (Budapest, Hungary), ZnHA and ZnGlu from Gedeon Richter Plc. (Budapest, Hungary). BK, NaCl, boric acid, borax (for borate buffer) and dimethyl sulfoxide (DMSO) were obtained from Molar Chemical Ltd. (Halásztelek, Hungary). Mucin (porcine gastric mucin type II) was purchased from Sigma Aldrich (Saint Louis, Missouri, USA). Lachrymal fluid of pH = 7.4 was prepared by dissolving 2.2 g/L $NaHCO_3$, 6.26 g/L NaCl, 1.79 g/L KCl, 96.4 mg/L $MgCl_2.6H_2O$ and 73.5 mg/L $CaCl_2 \cdot H_2O$ in distilled water, the pH being adjusted with 1 M HCl [79]. Dimethyl sulfoxide (DMSO), KCl, $NaHCO_3$, D-glucose monohydrate from Kemika (Zagreb, Croatia), $CaCl_2.2H_2O$ and $MgCl_2.6H_2O$ from Sigma-Aldrich, Chemie GmbH (Steinheim, Germany). Ringer-buffer was used as medium during the experiments. The pH of Ringer buffer was set to 7.4.

5. Methods

5.1 Characterization of PR-cyclodextrin inclusion complex

5.1.1 Phase solubility test

The phase solubility of PR was measured by adding it in excess amount to HPBCD- and HPGCD-containing solutions (purified water was used as solvent) with different concentrations (0-150 mM) and allowing it to be intermixed for 48 hours. Thereafter the solutions were filtered with a 0.45 μm membrane filter (Millex-HV Syringe Driven Filter Unit, 0.45 μm, EMD Millipore, Billerica, MA, USA) and analysed with UV spectrophotometry (wavelength: 248 nm, Unicam UV/Vis Spectrometer, ATI Unicam, Cambridge, UK).

The type of diagrams and the ratio of complexes were determined and the apparent stability constants of complexes (K_s) were calculated by Equation (1) [80]:

$$K_s = \text{slope}/\{\text{intercept}(1-\text{slope})\} \quad (1)$$

5.2 Preparation of products

0.1% PR was used in the formulations as API. Considering the probable improvement of the bioavailability this amount is suitable and proper therapeutic effect can be expected. Defined amounts of HPGCD or HPBCD were dissolved in borate buffer (prepared by water for injection filtered on 0.22 μm membrane filter). After addition of PR, products were sonicated for 10 minutes, until total dissolution of API. Then 0.5% ZnHA and 0.5% ZnGlu was added to the system. According to *Horvát et al.*, this amount of ZnHA could not create a viscous, gel-formulation, therefore the unfavorable attributes of high viscosity are not expected [60]. Osmolality was set with NaCl to about 300 mOsm/kg, the pH was about 6.20 in every formulation. Every eye drop was prepared in aseptic environment. The containers were stored in fridge for at least 24 hours for completely wetting of polymer. Final composition is shown in Table 3.

Table 3. Composition of eye drop formulation

Materials	Concentration
prednisolone	0.1%
hydroxypropyl-β-cyclodextrin	5 mM
or	or
hydroxypropyl-γ-cyclodextrin	4 mM
zinc-hyaluronate	0.5%
zinc-gluconate	0.5%
borate buffer	quantum satis
sodium-chloride	quantum satis
water for injection	quantum satis

5.3 Study of diffusion through dialysis membrane

The penetration of API depends on the concentration of CD. Overly high or low amounts of CD can cause a decreased absorption of API, therefore its determination is important. With the investigation of the diffusion of PR through dialysis membrane, we can adjust the optimal CD quantity as a function of drug penetration.

The amount of CD for the optimal penetration of API was determined by drug diffusion monitoring. Zellutrans/Roth cellulose dialysis membrane tube (10 mm wide, 6.4 mm diameter, MWCO: 12000-14000 D) was used for the experiment. The membrane pouches were closed with Spectra/Por Closures. The sample (2.00 mL) was injected into the pouches and put into 25 mL of borate buffer containing aqueous acceptor phase (pH=7.4) tempered at 35 °C. At various time intervals (15, 30, 60, 120, 180 and 240 min) 1.00 mL of sample was removed from the acceptor phase and refilled with the buffered solution. The length of the measurement is reasonable, due to the possible increased retention on the surface of the eye. Four samples were measured parallel at the same time. The PR content was analysed with UV spectrophotometry [81].

5.4 Viscosity

A Physica MCR 101 rheometer with cone-plate measuring device (Anton Paar, Graz, Austria, CP25-1, cone angle 0.997°, 25 mm diameter) was used for the measurement. The formulations were investigated at 25 °C; the shear rate was increased from 0.1 to 100 1/s, the means of the data at 100 1/s shear rate were calculated at the evaluation. The viscosity values were illustrated as a function of the concentration of CD derivatives.

5.5 Surface tension

The surface tension of the samples was measured with OCA 20 contact angle system (Dataphysics Instruments GmbH, Filderstadt, Germany) by analysing the shape of pendant drop. The values of surface tension were determined with SCA 20/22 software module using the Young-Laplace equation.

5.6 Efficacy of antimicrobial preservation

The applicability of ZnHA–ZnGlu as ophthalmic preservative system was investigated versus BK, because the other components in the formulation could affect its antimicrobial effect. The antimicrobial effectiveness of the ophthalmic samples was determined according to the standards of the EP. ZnHA–ZnGlu and BK as preservatives were tested on control strains, *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 9027) and *Candida albicans* (ATCC 10231). Inoculum suspensions of the microorganisms were prepared by using a sterile suspending fluid containing 9 g/L NaCl. The number of colony-forming units (CFU) was determined with plate count. The microbial count was about 10^8 CFU per millilitre. Preserved samples were inoculated with the suspensions of bacteria and fungus by adding 10^6 CFU per millilitre. The volume of the inoculated suspensions of microorganisms did not exceed 1% of the volume of the product. According to the standard method, three parallel samples were removed at zero hours and at appropriate intervals (6 hours, 24 hours, 7 days, 14 days, 28 days), and plated to Sabouraud-dextrose fluid agar (fungus) or tryptic soy fluid agar (bacteria). Bacteria-containing samples were incubated at 30°C–35°C for 24 hours and fungus-containing samples at 20°C–25°C for 48 hours. The reduction of these values was converted to $\log_{10}$ and compared with requirements A and B of the EP (EP-A, EP-B). The requirement of preservative is determined by the EP as the logarithmic reduction of CFU. The effectiveness needed against bacteria and fungus is managed separately. The decrease of CFU needs to be in accordance with the EP-A criteria. In cases when adverse drug reaction can appear with the EP-A criteria, the EP-B criteria are acceptable [82]. The aim was to determine whether the preservative effect of ZnHA meets the requirements of EP in the presence of CD derivatives.

5.7 Mucoadhesion

The mucoadhesion of CD-containing eye drops was determined by the tensile test method, based on the measurement of the forces of detachment and the total work of adhesion needed to separate the surfaces, resulting from the area under the force–distance curve [83,84]. Samples contained two types of CDs (HPBCD and HPGCD) prepared with and without ZnHA–ZnGlu. The purpose was to determine the effect of ZnHA on mucoadhesion and to establish if the presence of the type of CD has an effect in mucoadhesion. The measurement was performed with a TA.XT Plus Texture analyser (ENCO, Spinea, Italy) instrument equipped with a 1 kg load cell and a cylinder probe with a diameter of 1 cm. The sample (20 μL) was attached to the cylinder probe and placed in contact with a filter paper disc wetted with 50 μL of an 8% w/w mucin dispersion or simulated lachrymal fluid (blank, pH=7.4). The mucin dispersion was made with simulated lachrymal fluid [60,85]. 2,500 mN preload was used for 3 minutes. The cylinder probe was moved upward to separate the sample from the substrate at a prefixed speed of 2.5 mm/min.

5.8 Preparation of human corneal epithelial cell line (HCE-T) model

Human corneal epithelial cells (HCE-T; RCB 2280; RIKEN BRC, Tsukuba, Japan) were immortalized by transfection with a recombinant SV40-adenovirus vector, established and characterized by *Araki-Sasaki et al.* [86]. The cells were grown in Dulbecco's Modified Eagle's Medium/F-12 (Gibco, Life Technologies, Carlsbad, California, USA) supplemented with 10% fetal bovine serum (Gibco, Life Technologies, Carlsbad, California, USA), 0.5% DMSO, 5 μg/mL recombinant human insulin and 10 ng/mL recombinant human epidermal growth factor (EGF) in a humidified incubator with 5% CO_2 at 37 °C. All plastic surfaces were coated with 0.05% rat tail collagen in sterile distilled water before cell seeding in culture dishes. The culture medium was changed every second day. The air-liquid interface is crucial for the development of a tight multilayer epithelium in HCE-T cells [87]. HCE-T cells were cultured first in liquid-liquid condition for 5-8 days. To create the air-liquid condition the medium from the upper compartment was removed and only 1 mL of medium was added to the lower compartment to keep the liquid level at the appropriate

height for the next 5-8 days. To measure transepithelial electrical resistance (TEER), cells were fed with 500 µL medium in the upper compartment every second day.

5.9 Treatment of cultured cells

The final concentration of the PR in the formulations for cell culture experiments was 100 µg/mL. The formulations were diluted in Ringer buffer (pH=7.4) (150 mM NaCl, 2.2 mM $CaCl_2$, 0.2 mM $MgCl_2$, 5.2 mM KCl, 5 mM glucose, 6 mM $NaHCO_3$). We tested the following samples (Table 4.):

Table 4. Composition of investigated samples by toxicity and permeability studies. Defined amounts were completely dissolved or suspended in Ringer buffer.

For cell viability measurements		For permeability assay	
I.	PR/HPBCD/ZnHA/ZnGlu	F1	PR
II.	PR/HPGCD/ZnHA/ZnGlu	F2	PR/HPBCD
III.	PR/HPBCD/BK	F3	PR/HPGCD
IV.	PR/HPGCD/BK	F4	PR/HPBCD/ZnHA/ZnGlu
V.	ZnHA/ZnGlu	F5	PR/HPGCD/ZnHA/ZnGlu
VI.	BK		

5.10 Cell viability measurement by impedance

Impedance was measured at 10 kHz by an RTCA SP instrument (ACEA Biosciences, San Diego, CA, USA). This method is label-free, non-invasive and monitors cell adherence, growth and viability real time. We have successfully tested the cellular effects of pharmaceutical excipients and peptides by impedance kinetics in our previous studies [88–90]. For background measurements 50 µL of cell culture medium was added to the wells, then cells were seeded at a density of 5×10^3 cells/well to 96-well plate with gold electrodes (E-plate 96, ACEA Biosciences) coated with collagen. Cells were cultured for 4-5 days in CO_2 incubator at 37 °C and monitored every 10 minutes until the end of experiments. Cells were treated at the beginning of the plateau phase of growth. The treatment solutions were dissolved in Ringer buffer. Triton X-100 (TX-100) detergent (1 mg/mL) was used as a reference compound to induce cell toxicity. Cell index was defined as Rn-Rb at each time point of measurement, where Rn is the cell-electrode impedance of the well when it contains cells and Rb is the background impedance of the well with the medium alone.

5.11 Immunohistochemistry

To evaluate morphological changes in HCE-T cells caused by the different formulations, cell viability assay was followed by immunostaining for junctional proteins zonula occludens protein-1 (ZO-1), occludin, β-catenin and E-cadherin. Cells were grown on glass coverslips (Menzel-Glaser, Braunschweig, Germany) at a density of 4×10^4 cells/coverslips and treated with different formulations containing PR for 30 minutes. After the treatment coverslips were washed with phosphate buffer (PBS) and the cells were fixed with 3% paraformaldehyde solution for 15 minutes at room temperature. The cells were permeabilized by 0.2% TX-100 solution for 10 minutes and the nonspecific binding sites were blocked with 3% bovine serum albumin in PBS. Primary antibodies rabbit anti-ZO-1 (AB_138452, 1:400; Life Technologies, Carlsbad, CA, USA), rabbit anti-β-catenin (AB_476831, 1:400), rabbit anti-occludin (AB_2533977, 1:100; Life Technologies, Carlsbad, CA, USA) and mouse anti-E-cadherin (AB_397580, 1:400; Life Technologies, Carlsbad, CA, USA) were applied as overnight treatment. Incubation with secondary antibodies Alexa Fluor-488-labeled anti-mouse (AB_2534088, 1:400; Life Technologies, Invitrogen, USA) and anti-rabbit IgG Cy3 conjugated (AB_258792, 1:400) lasted for 1 hour. Hoechst dye 33342 was used to stain cell nuclei. After mounting the samples (Fluoromount-G; Southern Biotech, Birmingham, USA) staining was visualized by a Visitron spinning disk confocal system (Visitron Systems GmbH, Germany).

5.12 Permeability study on HCE-T cell culture model

TEER reflects the tightness of the intercellular junctions closing the paracellular cleft, therefore the overall tightness of cell layers of biological barriers. TEER was measured to check the barrier integrity by an EVOM volt-ohmmeter (World Precision Instruments, Sarasota, FL, USA) combined with STX-2 electrodes, and was expressed relative to the surface area of the monolayers as $\Omega \times cm^2$. TEER of cell-free inserts was subtracted from the measured data.

HCE-T cells were seeded at a density of 10^5 cells onto Transwell inserts (polycarbonate membrane, 0.4 μm pore size, 1.12 cm^2 surface area; 3401, Corning Life Sciences, Tewksbury, Massachusetts, USA) and cultured for 5-8 days at liquid-liquid and for 5-8 days at air-liquid interface. The culture medium was changed and TEER was checked every second day.

For the permeability experiments the inserts were transferred to 12-well plates containing 1.5 mL Ringer buffer in the acceptor (lower/basal) compartments. In the donor (upper/apical) compartments 0.5 mL buffer was pipetted containing different formulations (F1-F5) of PR for 30 minutes. To avoid unstirred water layer effect, the plates were kept on a horizontal shaker (120 rpm) during the assay. Samples from both compartments were collected and the PR concentration was detected by high-performance liquid chromatography (HPLC).

To determine the tightness of the cornea epithelial culture model two marker molecules were tested [90]. In the donor compartments 0.5 mL buffer containing fluorescein (10 µg/mL; Mw: 376 D) and Evans blue labeled albumin (167.5 µg/mL Evans blue dye and 10 mg/mL bovine serum albumin; Mw: 67.5 kDa) was added. The inserts were kept in the multiwell plates on a horizontal shaker (120 rpm) for 30 minutes, then the concentrations of the marker molecules in the samples from the compartments were determined by a fluorescence multiwell plate reader (Fluostar Optima, BMG Labtechnologies, Germany; fluorescein: excitation wavelength: 485 nm, emission wavelength: 520 nm; Evans-blue labeled albumin: excitation wavelength: 584 nm, emission wavelength: 680 nm).

The apparent permeability coefficients (P_{app}) were calculated as described previously [90]. Briefly, cleared volume was calculated from the concentration difference of the tracer in the acceptor compartment ($\Delta[C]_A$) after 30 minutes and donor compartments at 0 hour ($[C]_D$), the volume of the acceptor compartment (V_A; 1.5 mL) and the surface area available for permeability (A; 1.1 cm^2) using Equation 2 (2.):

$$P_{app}\ (cm/s) = \frac{\Delta[C]_A \times V_A}{A \times [C]_D \times \Delta t} \qquad (2)$$

5.13 *Ex vivo* permeability assay

The *ex vivo* permeability model was published previously by *Juretić et al.* [91,92]. Fresh porcine eyes were collected from Large White Pigs (weight 90-115 kg, male and female, 6-7 months) from local slaughterhouse. Enucleated eyeballs were washed by isotonic saline solution (NaCl, 0.9%; B. Braun, Melsungen, Germany) and stored in Ringer buffer in a container held on ice until application. Transport and excision were performed within 2 hours after death of animals. Excised corneas were placed into

vertical diffusion chambers (Standard Vertical Ussing/Diffusion Chambers, made of acrylic with 0.64 cm^2 diffusion surface, Harvard Apparatus, Holliston, MA, USA). Epithelial side of the cornea was faced to donor phase of the system. Donor and acceptor phase volume were equally 3.5 mL. After 30 min incubation of corneas (Ringer buffer, 37 °C), the donor compartment was removed, and 3.5 mL sample was injected. 500 µL samples were collected from acceptor compartment at defined time intervals (0 min, 30 min, 1 h, 1.5 h, 2 h, 2.5 h, 3 h, 3.5 h, 4 h, 4.5 h, 5 h) and refilled with 500 µL Ringer-buffer at each sample collection. Continuous oxygenation was ensured during the experiment for mixing of the system and mimicking physiological circumstances for the tissue. 6 parallels were measured for each type of formulation (F1-F5). PR content of samples was analysed by HPLC. Apparent permeability was calculated at each sample by using Equation 2.

5.14 TEER measurement in *ex vivo* model

During the *ex vivo* permeability assay, the integrity of porcine corneas was monitored by TEER measurement, to check whether the compounds affect the barrier properties of the model. Ag/AgCl electrodes were used connected with Millicell® ERS-2 Epithelial Volt-Ohm Meter (EMD Millipore Corporation, Billerica, MA, USA). The resistance was measured at 0 min, 15 min, 150 min and 300 min in each cornea containing vertical diffusion chambers. Blank resistance was measured and subtracted to obtain exactly the TEER of *ex vivo* cornea-based model.

5.15 Quantification by High Performance Liquid Chromatography

The quantitative measurement of PR was performed by HPLC using Agilent Infinity 1260 (Agilent, Santa Clara, CA, USA). Phenomenex Gemini NX C18 column (150x4.6 mm, 5 µm) was used with the official method of European Pharmacopoeia [82]. The following conditions were applied during the analysis: highly purified and filtered water in channel A, HPLC grade acetonitrile/HPLC grade methanol 50/50 V/V% in channel B, 1 mL/min flow rate, 25 °C temperature. Gradient elution was used for the separation. Samples were collected from *in vitro* HCE-T and *ex vivo* cornea models. 20 µL volume of samples was injected and analysed on 254.4 nm wavelength.

5.16 Statistical analysis

All data presented are means ± SD. The values were compared using the one-way ANOVA followed by Dunett's test by GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, USA). Changes were considered statistically significant at $P < 0.05$.

6. Results and discussion

6.1 Characterization of PR-cyclodextrin inclusion complex

6.1.1 Phase solubility test

CD complex was formed in aqueous liquid environment; thus the characterization of PR-CD complex was performed via phase solubility method. The above mentioned analytical techniques (XRPD, DSC, FTIR) can be applied in the case of solid-state characterization. The phase solubility of PR in HPBCD and HPGCD containing aqueous solutions (0-150 mM) is shown in Figure 7.

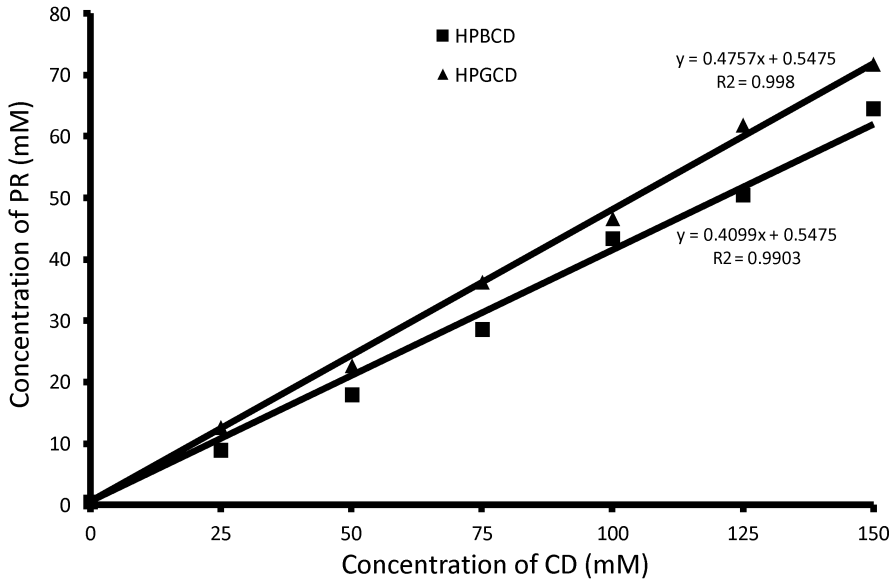

Fig. 7. Phase solubility diagrams of PR in aqueous HPBCD (■) and HPGCD (▲) solutions at 25 °C

The solubility of PR was increased linearly by increasing the concentration of HPBCD or HPGCD. The diagrams are Higuchi A_L type for both CDs, therefore the formation of 1:1 complexes can be assumed. In case of HPBCD, the apparent stability constant of the complex is 1286.4 M^{-1}, the constant of the PR-HPGCD complex was measured to be 1778.5 M^{-1}.

It is stated that PR has greater affinity for complex-formation with HPGCD. With the equation of regression lines, the concentration of CD needed to solubilize the required amount of API can be determined. These calculated concentrations are the centres of intervals which were used in the drug diffusion study in case of both types of CD derivatives.

6.2 Study of diffusion through dialysis membrane

Eye drops with or without mucoadhesive polymer were formulated and examined. The concentrations of CD derivatives for the penetration of PR were optimized. The penetrated PR as a function of the concentration of CD is shown in Figure 8.

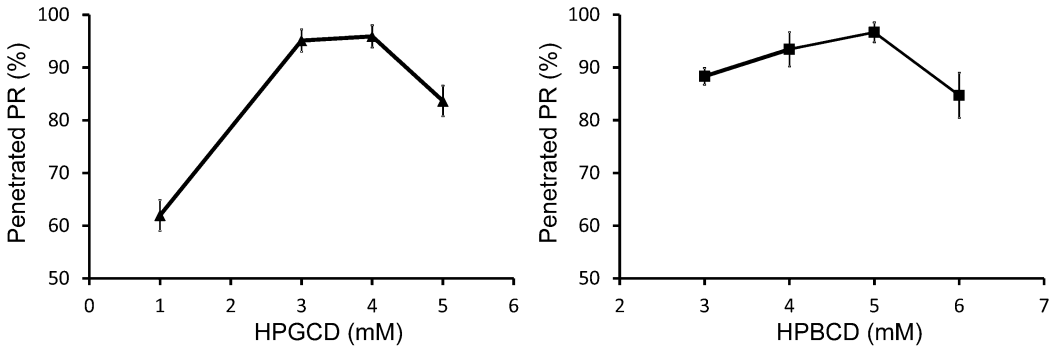

Fig. 8. PR penetration through semi-permeable membrane as a function of the concentration of HPGCD and HPBCD after 240 min

The results show that 4 mM HPGCD and 5 mM HPBCD induce the highest diffusion of PR through the dialysis membrane. Under the optimal CD concentration, a part of free, hydrophobic drug remained in the pouches. Above the optimal CD level, the excess amount of CD keeps the free PR in complex, therefore less amount of free API is detectable in the acceptor phase.

Thereafter, mucoadhesive ZnHA–ZnGlu additives were added to the eye drops, which ensure antimicrobial, preservative effect in the formulations. It was found that the application of biopolymer has no effect on the diffusion of PR. The same amount of drug penetrated through the dialysis membrane at each period in case of both compositions (Figure 9.).

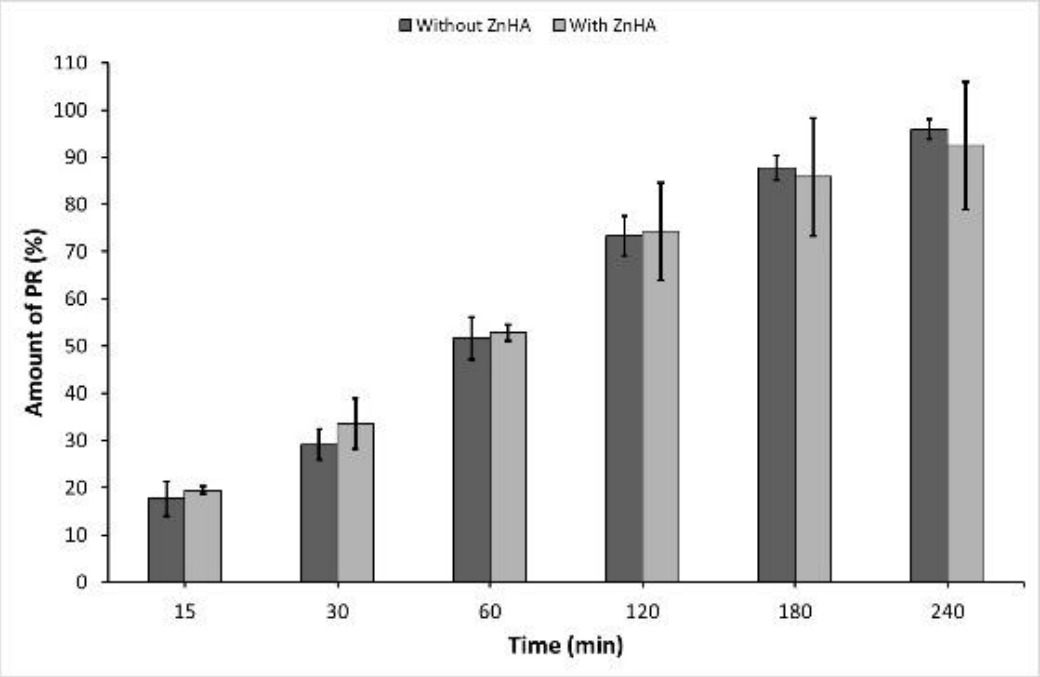

Fig. 9. Effect of ZnHA–ZnGlu on the diffusion of PR through dialysis membrane (light column: with ZnHA–ZnGlu; dark column: without ZnHA–ZnGlu)

6.3 Viscosity

The viscosity of ZnHA and PR-CD complex containing ophthalmic formulations was measured. The results are shown in Table 5.

Table 5. Viscosity values of ZnHA-PR-HPGCD (I.) and Zn-HA-PR-HPBCD (II.) containing products

	Concentration of CD (mM)	Viscosity (mPa s)	Standard Deviation
I.	3	22.8	4.5
	4	9.2	3.3
	5	19.7	1.2
II.	3	24.2	3.4
	4	18.4	2.8
	5	18.1	0.7
	6	22.1	3.7

According to previous reports, the viscosity should be under 30 mPa s [93,94]. Above this level blurred vision and discomfort appear, which result the faster elimination due to reflex mechanisms of the eye. The results show that the viscosity of our formulations is appropriate, in the range of 9.2-24.2 mPa s.

6.4 Surface tension

The surface tension of normal tear is about 43 mN/m [95]. It is not optimal if the surface tension of products is much higher than that of the lacrimal fluids because it has an impact on the therapeutic effect of pharmaceutics applied on the eye through affecting the spreading of the eye drops on the ocular surface, although no regular critical parameter was found for the surface tension of eye drops in the EP. The surface tension of formulations preserved with ZnHA was measured by using an OCA 20 contact angle system. The results are shown in Table 6.

Table 6. Surface tension of ZnHA-PR-HPGCD (I.) and ZnHA-PR-HPBCD (II.) containing formulations

	Concentration of CD (mM)	Surface tension (mN/m)	Standard Deviation
I.	3	61.12	0.24
	4	61.65	0.31
	5	61.20	0.24
II.	3	59.31	0.15
	4	59.46	0.23
	5	58.65	0.22
	6	59.06	0.37

No significant difference was found between the values. The surface tensions of the eye drops are about 60 mN/m, which is higher than the surface tension of lacrimal fluid. Ophthalmic products were investigated by *Han K. et al.*, and the range of the surface tension values was between 34.3 and 70.9 mN/m [96–98]. According to this study, the surface tension of formulations meets the requirements for ophthalmic products.

6.5 Efficacy of antimicrobial preservation

In Sample I. (with HPBCD) and II. (with HPGCD) ZnHA–ZnGlu were used as preservative compounds. The results can be seen in Table 7.

Table 7. Preservative effectiveness against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* in Samples I-II. (n.d. not detectable; n.i. no increase)

		Microbial log reduction				
		6 hours	24 hours	7 days	14 days	28 days
Staphylococcus aureus	Sample I.	1.5	2	n.d.	n.d.	n.d.
	Sample II.	1.5	2	n.d.	n.d.	n.d.
	Criteria (EP-B)	-	1	3	-	n.i.
Pseudomonas aeruginosa	Sample I.	2	3	-	4	-
	Sample II.	2	-	3	4	-
	Criteria (EP-B)	-	1	3	-	n.i.
Candida albicans	Sample I.	1.5	-	-	-	n.d.
	Sample II.	1	-	-	-	n.d.
	Criteria (EP-B)	-	-	-	1	n.i.

The logarithmic decrease of *Staphylococcus aureus* was 1.5 after 6 hours, 2 after 24 hours, and no bacteria were detected in the samples after 7 days. In case of *Pseudomonas aeruginosa*, the logarithmic decrease is higher at the earlier period, but the bacterium appeared in every sample. The logarithmic decrease of *Candida albicans* was 1 after 6 hours, and no fungi were detected later. In summary, the preservative effect of ZnHA–ZnGlu containing samples meets the EP-B criteria. The antimicrobial effectiveness of ZnHA–ZnGlu is lower against the Pseudomonas aeruginosa, compared with the other microorganisms.

The microbiological stability of Sample III. (with HPBCD) and IV. (with HPGCD), which contained BK as preservative agent, was tested (Table 8.).

Table 8. Preservative effectiveness in Samples III-IV. against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* (n.d. not detectable; n.i. no increase)

		Microbial log reduction				
		6 hours	24 hours	7 days	14 days	28 days
Staphylococcus aureus	Sample III.	1	2	n.d.	n.d.	n.d.
	Sample IV.	n.d.	n.d.	n.d.	n.d.	n.d.
	Criteria (EP-B)	-	1	3	-	n.i.
Pseudomonas aeruginosa	Sample III.	2	-	-	-	-
	Sample IV.	3	2	-	3	-
	Criteria (EP-B)	-	1	3	-	n.i.
Candida albicans	Sample III.	1	-	n.d.	n.d.	n.d.
	Sample IV.	n.d.	n.d.	n.d.	n.d.	n.d.
	Criteria (EP-A)	-	-	2	-	n.i.

The logarithmic decrease of *Staphylococcus aureus* was 1 after 6 hours, 2 after 24 hours, no bacteria were detected later in Sample III. For *Pseudomonas aeruginosa* the logarithmic decrease was 2 and no change was detected later. In *Candida albicans* containing samples, no fungi were found after 7 days. In Sample IV., for *Staphylococcus aureus* and *Candida albicans*, the number of CFU was zero after 6 hours. In case of *Pseudomonas aeruginosa* the antimicrobial effect was lower due to the known resistance of the bacterium against BK. The microbiological stability of Samples III-IV. meets the requirements of the EP. In Sample III., the antimicrobial effect of BK is lower than in Sample IV. It can be assumed that there is a competition between PR and BK for the cavity of HPBCD, so the preservative effect of BK is decreased by the inclusion complex formation [99].

According to the EP-B criteria, 0.5% ZnHA–ZnGlu compounds ensure the proper microbiological stability of eye drop formulations. In case of *Pseudomonas aeruginosa* the antimicrobial effect of ZnHA–ZnGlu system is higher than the effect of BK. Considering these results with the irritant attribute of BK, application of ZnHA–ZnGlu as a preservative can be favorable in ophthalmic products.

6.6 Mucoadhesion

The measured adhesive force values are shown in Figure 10. Lachrymal fluid was used as blank.

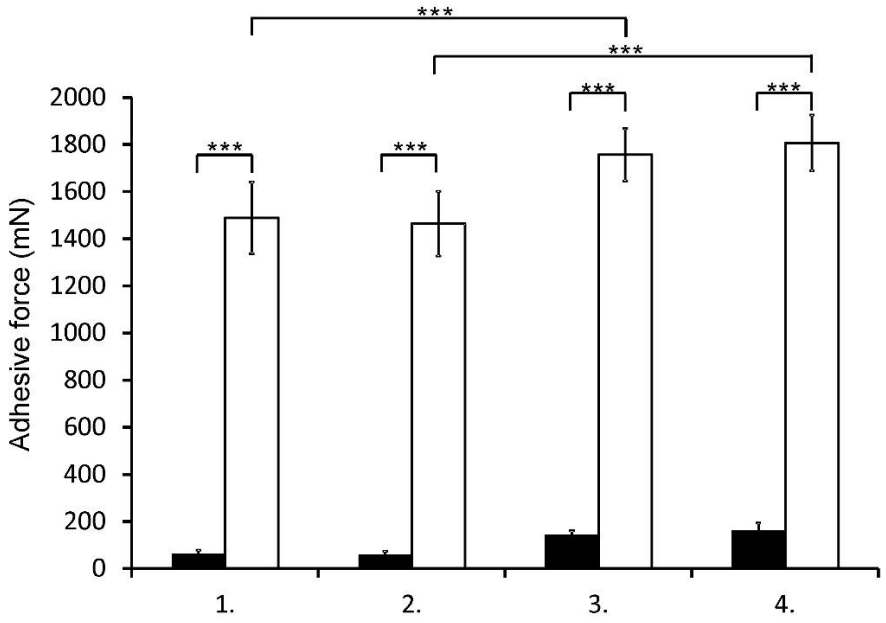

Figure 10. Adhesive force of eye drops; with (white columns) and without mucin (black columns) (1. with HPBCD; 2. with HPGCD; 3. with HPBCD and ZnHA–ZnGlu; 4. with HPGCD and ZnHA–ZnGlu) ***p<0.001

There is a large difference in the measured force between the blank and the mucin dispersion. All the samples show mucoadhesivity, samples prepared with ZnHA have significantly higher values than samples prepared without it. This proves the importance of the presence of ZnHA because the interpenetration between the ZnHA chains and mucin can be assumed. These samples can have better mucoadhesive properties and cause decreased administration frequency and a lower active ingredient concentration.

The type of CD does not play an important role in mucoadhesion because there is no significant difference between the samples prepared with HPBCD and HPGCD. According to the results, it can be established that ZnHA plays an important role in mucoadhesion.

6.7 Barrier properties of the cornea epithelial cell culture model

HCE-T cell layers showed good barrier properties as reflected by the TEER values (246 ± 7 Ω × cm^2, n = 3) after 6 days of air-liquid interface condition (Fig. 11A). The permeability of HCE-T cell layers was low (Fig. 11B) for the hydrophilic marker molecules fluorescein (P_{app}: 1.05 ± 0.11 × 10^{-6} cm/s) and the large biomolecule albumin (P_{app}: 0.10 ± 0.04 × 10^{-6} cm/s).

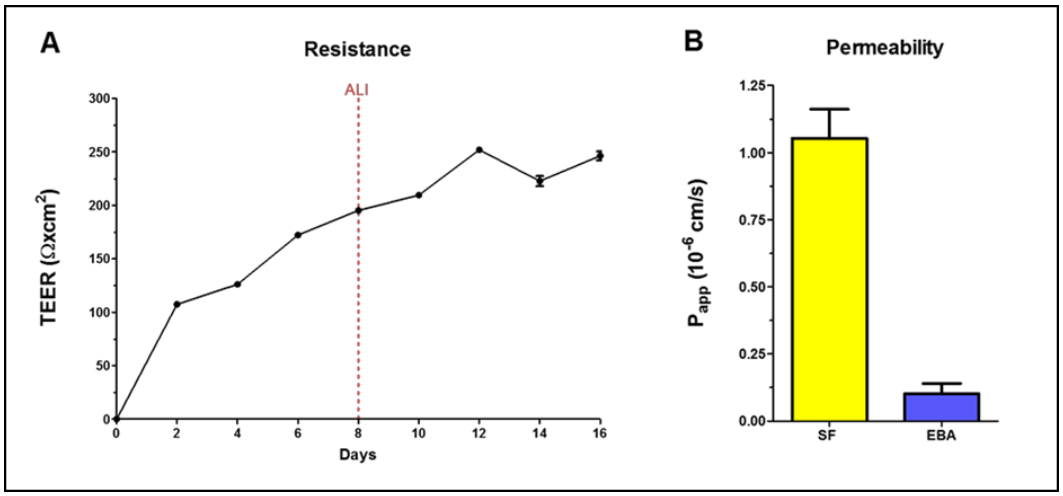

Figure 11. (A) Electrical resistance values of the HCE-T cornea epithelial cell layers cultured for 8 days at liquid-liquid interface and for an additional 8 days at air-liquid interface (ALI). (B) Permeability of HCE-T epithelial cell layers for fluorescein (SF) and Evans blue labeled albumin (EBA) marker molecules. Values are presented as means ± SD, n = 4

6.8 Cell viability assay

Impedance measurement, as a sensitive method to detect cellular effects, showed significant cell damage after treatment with all three formulations containing BK (III, IV, VI). PR containing formulations with HPBCD, HPGCD, ZnHA and ZnGlu did not show any cytotoxic effect. Normalized cell index was significantly higher in ZnHA–ZnGlu containing sample (V), than in formulation with BK (VI). As a comparison, maximal toxicity was detected in cells treated with the reference damaging agent Triton X-100 detergent (Fig. 12).

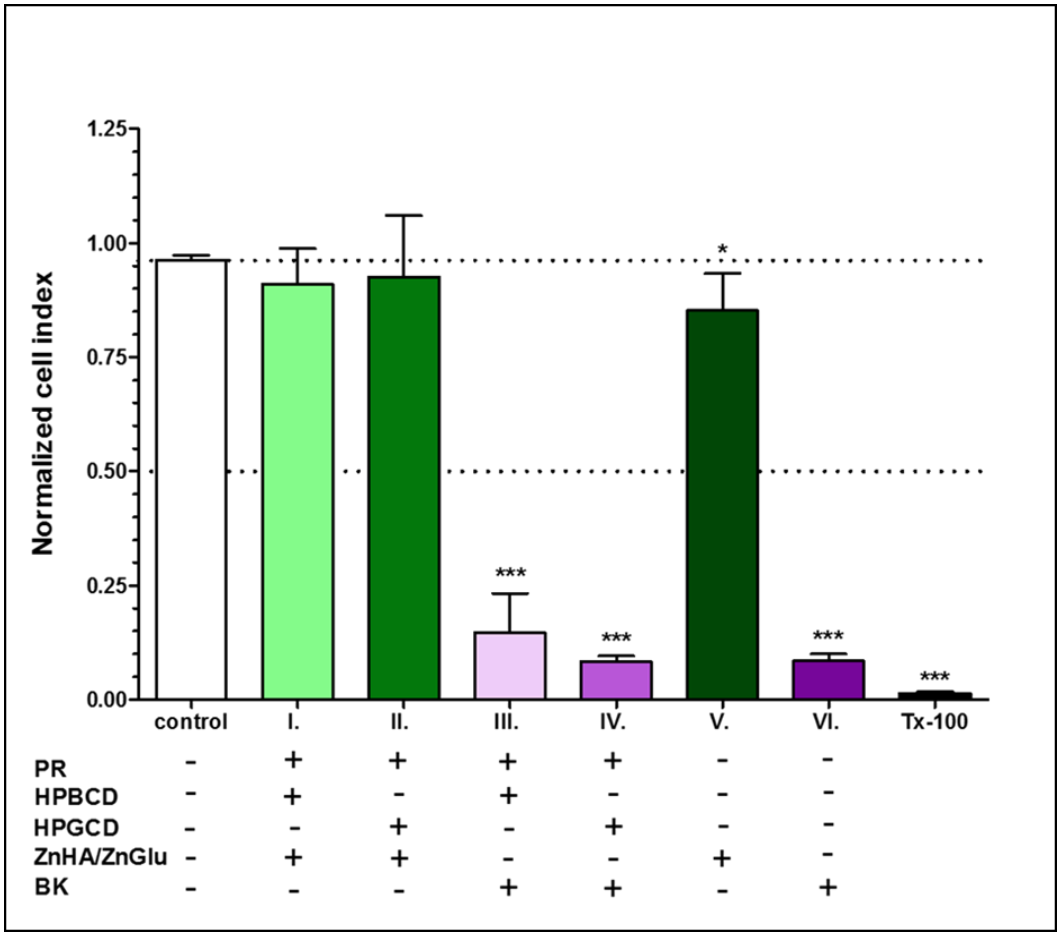

Figure 12. Cell viability of HCE-T corneal epithelial cells after 1-hour treatment with formulations measured by impedance. Values are presented as means ± SD, n = 6-12. Statistical analysis: ANOVA followed by Dunett's test. (*p<0.05; ***p<0.001 compared to control), Triton X-100 (Tx-100).

6.9 Immunohistochemistry

The cornea epithelial cells formed tight paracellular barrier visualized by the localization of the junctional proteins ZO-1, β-catenin, E-cadherin and occludin. The cells were tightly apposed, and all junctional proteins were localized at the intercellular connections forming pericellular belts in the control groups (Fig. 13). Morphological change can be observed in the case of BK containing sample (VI) by the localization of E-cadherin protein. No major morphological change was seen for the treatment of other groups. Immortalized human corneal epithelial cell line was used by *in vitro* toxicity and permeability tests. The cell culture was established by *Araki-Sasaki et al*., whose optimal grown attributes are favorable in the studies of ophthalmic formulations [91,100]. The toxicity was investigated by impedance measurement after the treatment of several types of formulations. According to the calculated normalized cell index,

ZnHA–ZnGlu containing samples are not toxic, meanwhile BK containing samples show significantly lower values and toxic effect can be observed on the HCE-T cells.

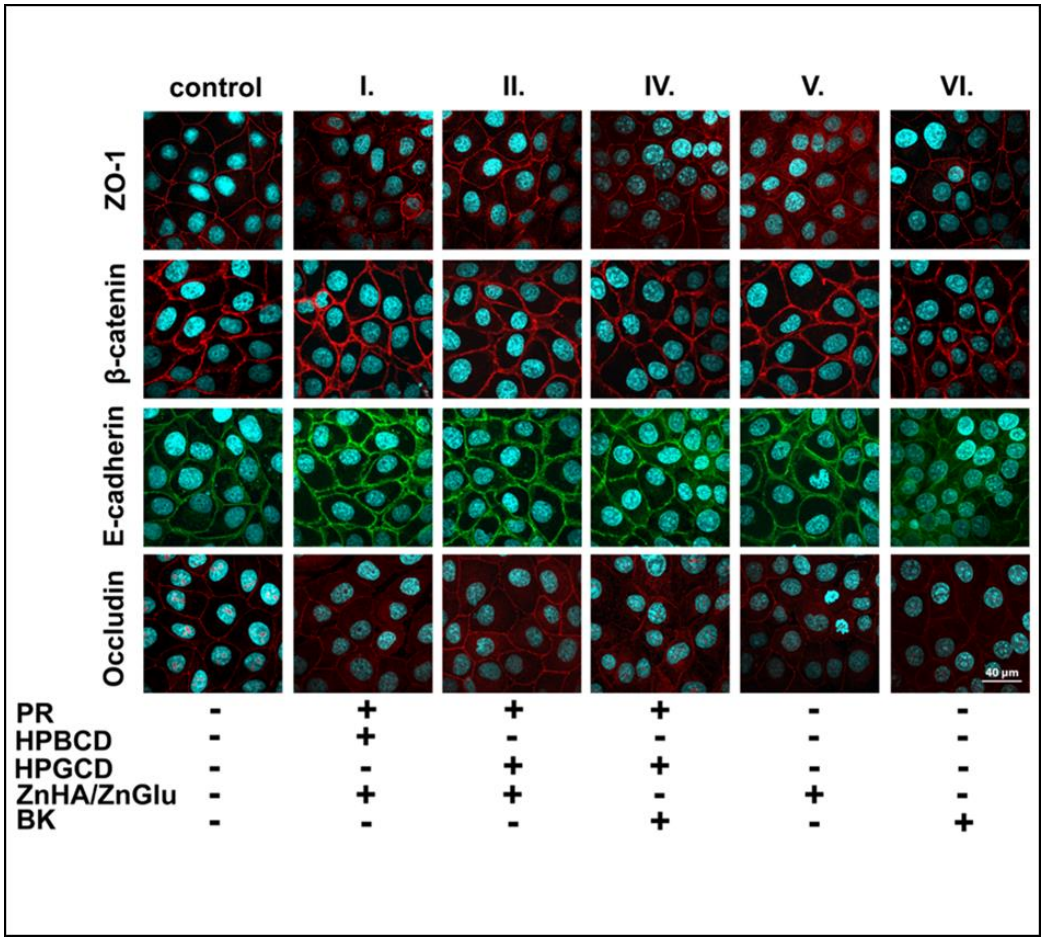

Figure 13. Effects of PR and pharmaceutical excipients containing formulations on junctional morphology of HCE-T corneal epithelial cells. Immunostaining for zonula occludens-1 (ZO-1), occludin tight junction proteins and β-catenin, E-cadherin adherens junction proteins after 1-hour treatment. Red and green color: immunostaining for junctional proteins. Blue color: staining of cell nuclei. Bar: 40 µm

The toxicity of BK containing formulations was also demonstrated by immunohistochemistry. Major morphological changes were seen on E-cadherin junctional protein by the cells treated by BK containing formulations. In the case of target eye drops formulated with ZnHA–ZnGlu, no morphological change and no toxic effect were detected on the cell culture. It can be stated that ZnHA–ZnGlu combination is non-toxic alternative preservative system, which is tolerable on HCE-T cells in the applied concentration. The results of BK containing samples confirmed the previously published toxic and expected irritant effect on the eye surface.

6.10 Permeability study on HCE-T cell culture model

The permeability of PR given in different formulations was tested on the HCE-T cell model (Fig. 14). After 30-minute treatment only dissolved PR-CD complex containing formulation 2 and formulation 3 showed significantly higher P_{app} values (F2: 5.97 × 10^{-6} cm/s, F3: 5.97 × 10^{-6} cm/s) compared with PR suspension (F1: 5.02 × 10^{-6} cm/s). P_{app} values were minimally lower in ZnHA–ZnGlu containing formulations (F4: 5.59 × 10^{-6} cm/s, F5: 5.31 × 10^{-6} cm/s). The model showed low P_{app} values for the two hydrophilic paracellular marker molecules indicating a good barrier.

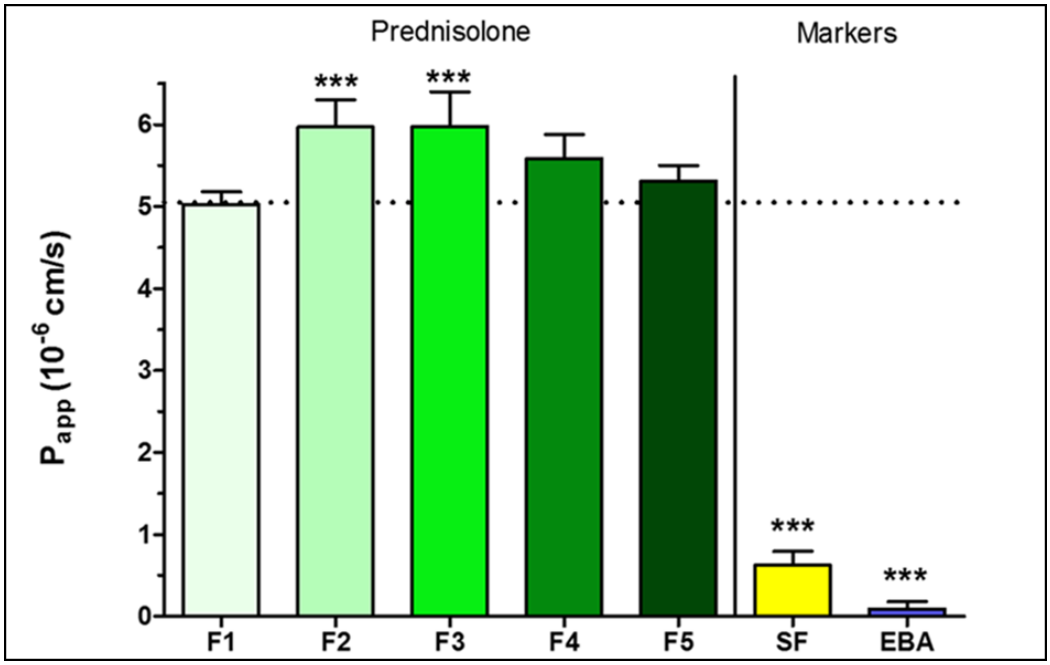

Figure 14. Permeability of PR (100 µg/mL in each formulations) (F1-F5) across HCE-T epithelial cell layers (30-minute assay). Values for paracellular permeability markers fluorescein (SF) and Evans blue labeled albumin (EBA) are also shown. Statistical analysis: ANOVA followed by Dunett's test. ***p<0.001. Values are presented as means ± SD, n = 4. p<0.001 compared to F1.

The applied *in vitro* model is suitable for the prediction of drug absorption through the lipophilic epithelial cell layer of cornea. It needs to be mentioned, that this *in vitro* model only shows the permeation through the epithelial layer, for prediction of transcorneal permeation, *ex vivo* cornea model is preferred. Meanwhile, testing the samples on cell culture, the absorption through the first obstacle of corneal barrier can be simulated. PR-CD complex containing solutions and PR-CD-ZnHA-ZnGlu containing target formulations were studied compared with sample which contains the

same amount of PR in suspension form. The electric resistance was monitored, and it confirmed the barrier integrity of cell layers. The permeability was significantly higher in samples containing the dissolved PR-CD complex compared with the suspension. Because the concentration of dissolved API is higher in PR-CD solutions, and higher towards the corneal epithelial cell layer, faster drug permeation is expected through the epithelial layer. In the target formulations with biopolymer, the permeability is minimally lower, due to the diffusion restrictive effect of the polymer structure.

6.11 *Ex vivo* permeability assay

Permeability of PR was tested on *ex vivo* porcine cornea model in the case of previously mentioned formulations (F1-F5) (Fig. 15.). In the case of PR-HPBCD (F2), permeability was higher (2.05×10^{-7} cm/s) compared with PR containing suspension (F1, 1.79×10^{-7} cm/s) Permeability in PR-HPGCD (F3) complex containing samples was lower (1.31×10^{-7} cm/s). Considering the relatively high standard deviations in F1-F3, no significant difference can be stated between their P_{app} values. In the presence of ZnHA–ZnGlu (F4-F5) significantly lower permeability values were measured (F4: 1.05×10^{-7} cm/s, F5: 0.98×10^{-7} cm/s). The monitored TEER values were in the range of 1052-2818 $\Omega \times cm^2$.

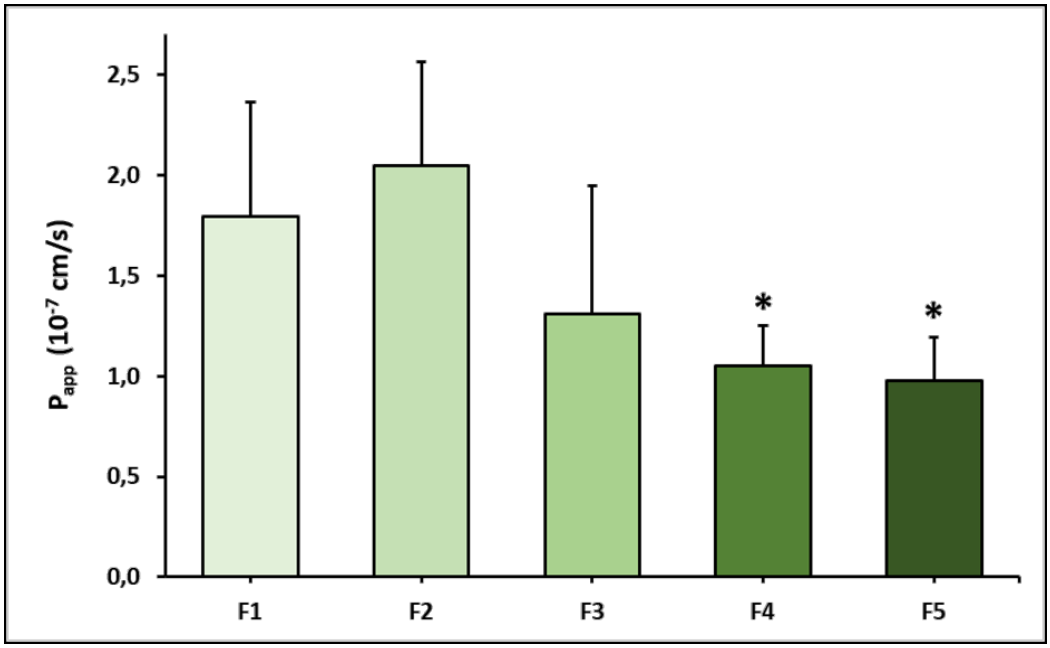

Figure 15. Permeability of PR in different formulations (F1-F5) across in *ex vivo* porcine cornea model. Statistical analysis: ANOVA followed by Dunett's test. *p<0.05 compared to F1. Values are presented as means ± SD, n = 6.

Freshly resected corneas were put into vertical diffusion chambers. O_2 was continuously circulated in the chambers to ensure the respiration of cornea, and the donor and receptor phases are mixed during the experiment. Same compositions were tested as in the HCE-T model. The monitored TEER was increased during the experiment, the integrity of cornea was acceptable, samples did not cause damage and toxic effects on the barrier, which can affect the permeability of PR. Compared with published results, these values meet the requirement of barrier properties [92,101]. According to the results, no significant difference was found between the eye drop suspension and solutions. Permeability values were significantly lower in ZnHA–ZnGlu containing formulations in comparison with the suspension form. Considering the continuous mixing, vertical position and complexity of porcine cornea, the optimal attributes are not observed in this model in solution-based samples. The permeability is lower in target formulations because polymer structure affects the drug diffusion negatively, however, the retention time is increased on the eye surface due to the increased viscosity and mucoadhesion. In the case of *ex vivo* study the whole porcine cornea is used formed by lipophilic and hydrophilic layers. Therefore, difference shall be observed in the permeability on *in vitro* HCE-T and *ex vivo* porcine cornea models.

The formulations can be optimal *in vivo*, because less irritation and lachrymal secretion are expected due to the not irritant solution form of eye drops.

As the eye drop contacts for longer time, reflex lachrymal secretion and eye blinking are limited, prolonged drug absorption is expected after administration, which results in less frequent application. We assume that the permeability of PR is optimal in the target formulations, which may result in enhanced therapeutic effect.

7. Conclusions and novelties

In conclusion, the development of optimal ocular drug delivery systems is a major challenge in the pharmaceutical field. Ensuring proper therapeutic effect with acceptable patient-compliance are key considerations in the research of innovative formulations. In this work PR-containing aqueous solutions were formulated by CD inclusion complex and addition of mucoadhesive, antimicrobial ZnHA and ZnGlu. The following results have been achieved:

- Aqueous solutions of PR were formulated by inclusion complexation with HPBCD and HPGCD.
- The investigation of the diffusion of PR through the dialysis membrane revealed that 5 mM of HPBCD, and 4 mM of HPGCD caused the highest penetration of API in *in vitro* circumstances. The addition of ZnHA and ZnGlu had no effect on the penetration properties.
- The measurement of viscosity showed that the 5 mM HPBCD- and 4 mM HPGCD-containing products had the lowest viscosity values, meeting the requirements of the EP.
- It was found that the concentration of CDs has no effect on the surface tension of the eye drops, and these values are optimal compared with previously investigated ophthalmic products.
- The mucoadhesive properties of ZnHA-containing formulations were proved with the tensile test, resulting in a higher retention time of the eye drop on the surface. The type of CD derivative has no influence on mucoadhesivity.
- During the preservative effectiveness test, the applicability of ZnHA–ZnGlu combination was proven as an antimicrobial, preservative system. The microbiological stability of ZnHA-containing products meets the requirements of the EP in case of *S. aureus*, *P. aeruginosa* and *C. albicans*. It can be stated that BK with unfavorable toxicity properties for the corneal epithelial cells can be replaced with the more biocompatible ZnHA–ZnGlu as alternative preservative compound.
- The formulations are non-toxic *in vitro* according to the immunohistochemistry and impedance measurement on HCE-T model
- Eye drops had optimal permeability according to the results of *in vitro* HCE-T and *ex vivo* porcine cornea models.

In summary, novel ophthalmic formulations were developed, where PR is dissolved in a water-based environment as CD inclusion complex. Eye drops contain a mucoadhesive and preservative ZnHA–ZnGlu system. These novel compositions are promising for overcoming the challenges of ocular drug delivery by optimal permeability, ensured microbiological stability, minimal irritation, and acceptable patient compliance.

8. References

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NYILATKOZAT SAJÁT MUNKÁRÓL

Név: Dr. Bíró Tivadar

A doktori értekezés címe: Application of cyclodextrins and mucoadhesive preservative system in ophthalmic formulations

Én, Dr. Bíró Tivadar teljes felelősségem tudatában kijelentem, hogy a Szegedi Tudományegyetem Gyógyszertudományok Doktori Iskolában elkészített doktori (Ph.D.) disszertációm saját kutatási eredményeimen alapulnak. Kutatómunkám, eredményeim publikálása, valamint disszertációm megírása során a Magyar Tudományos Akadémia Tudományetikai Kódexében lefektetett alapelvek és ajánlások szerint jártam el.

Szeged, 2021. 02. 23.

dr. [illegible]

ANNEX – Related articles

Drug Design, Development and Therapy

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ORIGINAL RESEARCH

Development of prednisolone-containing eye drop formulations by cyclodextrin complexation and antimicrobial, mucoadhesive biopolymer

This article was published in the following Dove Press journal:
Drug Design, Development and Therapy

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Purpose: The formulation of topical ophthalmic products with appropriate therapeutic effect and patient compliance is a major challenge. To increase the efficiency of the ocular delivery of the drug, the enhancement of water solubility and the contact time of the drug on the surface of the cornea are necessary. In this work, prednisolone (PR)-containing eye drops were formulated with antimicrobial, mucoadhesive biopolymer and PR–cyclodextrin inclusion complex. This approach can be used for the development of innovative ophthalmic formulations.

Materials and methods: After adjusting the optimal physiological parameters, the amount of the required cyclodextrin for the highest penetration of PR was determined by dialysis membrane diffusion study. The viscosity, surface tension and mucoadhesion of the eye drops were measured. The microbiological effectiveness of zinc-hyaluronate (ZnHA) was investigated by a standard method of the European Pharmacopoeia.

Results: In this case, no significant difference of surface tension was measured in products with different amounts of cyclodextrin. According to the results of the tensile test, ZnHA as a mucoadhesive biopolymer improves the mucoadhesion of ophthalmic products. The antimicrobial stability of formulations preserved by ZnHA meets requirement B of the European Pharmacopoeia.

Conclusion: It can be stated that the innovative PR-containing compositions are suitable for producing mucoadhesive, properly preserved aqueous ophthalmic solutions with increased bioavailability attributes.

Keywords: pharmaceutical formulation, zinc-hyaluronate, ocular drug delivery, microbiological stability, membrane diffusion

Introduction

Topical ocular drug delivery is restricted by barriers, such as eye blinking and lachrymal secretion, which result in low bioavailability after application. Solving this problem is a major challenge in the field of research and development and necessary because topically applied formulations have the highest patient adherence in the treatment of eye diseases.[1]

In order to reach an optimal efficiency, the transcorneal penetration of the drug is important. The cornea is composed of five layers: the lipophilic epithelium, the hydrophilic stroma between Descemet's membrane and Bowman's layer, and the lipophilic endothelium. To complete the optimal transcorneal penetration, a balance in the hydrophilicity and lipophilicity of the drug and the vehicle is needed. The continuous secretion of tear fluid rapidly dilutes and washes out the applied eye drop and limits the contact time of drugs on the eye surface.[2]

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In ophthalmic surgery, glucocorticoid derivatives like prednisolone (PR), dexamethasone and fluorometholone are widely used for postoperative inflammation prophylaxis. Due to their low aqueous solubility, they are present on the market primarily in suspension formulations. When there is a risk of severe inflammation, especially after cornea transplantation, anti-inflammatory steroid therapy is needed by giving a subconjunctival or subretinal injection.[2,3] Instead of these complicated and uncomfortable invasive applications, it would be more acceptable to use noninvasive, topical, water-soluble corticosteroid-containing eye drop formulations with higher efficiency.

One of the ways to increase efficiency on the eye surface is to solubilize the active pharmaceutical ingredient (API) in aqueous system, thereby ensuring that the optimal concentration of the drug appears near the epithelium of the cornea. Cyclodextrins (CDs) can solubilize lipophilic, water-insoluble drugs with suitable molecular size and structure, so they can be formulated in aqueous solutions. These cyclic oligosaccharides consist of α-(1,4) linked α-D-glucopyranose units. As a result of the lipophilic cavity of CD, an inclusion complex forms with the lipophilic drug molecules, due to thermodynamic interactions.[4–7] This complex is soluble in water because of the hydrophilic external surface of CD and can be dissociated in the aqueous tear fluid. As a result, equilibrium is achieved between the complexed and non-complexed components. The determination of the optimal concentration of CD is important. With the proper amount of CD, the API can be in aqueous solution permanently, so a sufficient amount of drug molecules appears at the surface of the cornea, which induces the increase of drug permeation. If the concentration of CD is too low, it is not able to bring about the required water solubility of the drug, whereas too much CD decreases the amount of free, permeable drug molecules at the cornea.[8] The cytotoxicity of β-CD derivatives was investigated by several research groups on different cell lines. In ophthalmology, the hydroxypropyl derivatives of β- and γ-CDs, the randomly methylated β-CD and sulfobutyl-ether-β-cyclodextrin are tolerated because of the preferential cytotoxic properties against cornea epithelial cells.[4,9,10] Now, licensed CD-containing products are widely used in medical practice.[5,6]

The reflex mechanisms of the eye, such as blinking and lachrymal secretion, result in rapid drug elimination from the surface. To maintain the optimal, therapeutic drug level, more frequent application is needed, which can induce many side effects and decrease patient compliance. This problem can be solved by increasing the contact time of API on the surface of the cornea by increasing the viscosity with inserts, microspheres or mucoadhesive polymers. With longer residence, the penetration rate of API can be increased.[11] At a needlessly high viscosity level, the reflex mechanisms of the eye, blinking and lachrymation, are induced until the physiological viscosity of the tear is regained. Some viscosity increasing compounds have a mucoadhesive effect. With these materials optimal residual time can be achieved, without increasing viscosity to an unnecessarily high level. The mechanism of mucoadhesion involves tight contact and interpenetration between the mucoadhesive component and the proteoglycan chains of the mucin. Bioavailability can be improved through this mechanism.[12,13] Hyaluronic acid is a linear anionic polysaccharide, a main component in the extracellular matrix of connective tissue. This biocompatible polymer can aid tissue manipulation and protect the corneal endothelium due to its proliferative effect. It interacts with mucin covering the conjunctival and corneal surfaces of the eye, and as a result ocular mucoadhesion is achieved.[14–16]

Benzalkonium chloride (BK) is a cationic surfactant additive, which is widely used as a microbiological preservative agent in eye drop formulations. BK may destroy the cell membrane of microorganisms, which results in an antimicrobial effect. Toxicity for corneal and conjunctival epithelial cells and incompatibility with contact lenses were reported earlier.[17–19] BK causes DNA single- and double-strand breaks in corneal epithelial cells, so the barrier of the eye surface may be damaged. Allergic reaction, eye irritation and increased tear secretion may be caused by application.[17–19] It is also known that resistance of *Pseudomonas aeruginosa* against BK appears due to decreasing the permeation through the cell wall.[20] The antimicrobial properties of Zn^{2+} ion-containing compounds are favorable in pharmaceutical formulations. Marketed products, like Ophylosa® (Gedeon Richter Plc, Budapest, Hungary) contain zinc-hyaluronate (ZnHA) and zinc-gluconate (ZnGlu) for replacing BK. The antimicrobial effect depends on the reactive oxygen species generating mechanism, the cell wall destabilizing effect of cytotoxic, dissolved Zn^{2+} ion in a water-based environment.[21] Zinc-containing polymers like ZnHA could be acceptable, combined with a zinc salt of gluconic acid, ZnGlu, to reach the suitable antimicrobial stability. Further investigation is needed to confirm the capability of these compounds as replacements of the unfavorable BK. ZnHA could be a useful antimicrobial and mucoadhesive additive in ophthalmic formulations.[22]

The aim of this study was to develop an innovative, water-soluble PR-containing eye drop formulation with

infrequent application, adequate microbiological stability and acceptable physiological (surface tension, pH, osmolality), rheological and mucoadhesive parameters using a CD inclusion complex, and preservative, mucoadhesive ZnHA and ZnGlu.

Material and methods

Materials

PR was purchased from Henan Lihua Pharmaceutical Company (Henan, China). Hydroxypropyl-β-cyclodextrin (HPBCD) was obtained from Wacker-Chemie GmbH (Munich, Germany), hydroxypropyl-γ-cyclodextrin (HPGCD) was kindly donated by Cyclolab Ltd. (Budapest, Hungary), ZnHA and ZnGlu from Gedeon Richter Plc (Budapest, Hungary). BK, sodium chloride (NaCl), boric acid and borax (for borate buffer) were obtained from Molar Chemical Ltd. (Halásztelek, Hungary). Mucin (porcine gastric mucin type II) was purchased from Sigma-Aldrich (St Louis, MO, USA). Lachrymal fluid of pH=7.4 was prepared by dissolving 2.2 g L^{-1} $NaHCO_3$, 6.26 g L^{-1} NaCl, 1.79 g L^{-1} KCl, 96.4 mg L^{-1} $MgCl_2 \cdot 6H_2O$ and 73.5 mg L^{-1} $CaCl_2 \cdot H_2O$ in distilled water, the pH being adjusted with 1 M HCl.[23]

Methods

Phase solubility test

The phase solubility of PR was measured by adding it in excess amount to HPBCD- and HPGCD-containing solutions (purified water was used as a solvent) with different concentrations (0–150 mM) and allowing it to be intermixed for 48 hours. Thereafter, the solutions were filtered with a 0.45 μm membrane filter (Millex-HV Syringe Driven Filter Unit, 0.45 μm, EMD Millipore, Billerica, MA, USA) and analyzed with UV spectrophotometry (wavelength: 248 nm, Unicam UV/Vis Spectrometer, ATI Unicam, Cambridge, UK).

The type of diagrams and the ratio of complexes were determined and the stability constants of complexes (K_s) were calculated by Equation (1):[24,25]

$$K_s = \text{Slope}/\{\text{Intercept } (1-\text{Slope})\} \quad (1)$$

Preparation of products

The eye drops in the market contain 1% or 0.12% PR-acetate in suspension formulations. According to these products, we use 0.1% PR as API. Considering the probable improvement of the bioavailability, this amount is suitable and proper therapeutic effect can be expected. Defined amounts of HPGCD or HPBCD was dissolved in borate buffer and 0.5% ZnHA–ZnGlu-containing aqueous solutions. According to Horvát et al, this amount of ZnHA could not create a viscous, gel-formulation, therefore the unfavorable attributes of high viscosity are not expected.[26] PR was dissolved in these solutions. Products were put into an ultrasonic bath for 10 minutes. Osmolality was set with NaCl to about 300 mOsm kg^{-1}; the pH was about 6.20 in every product. Every eye drop was prepared in aseptic environment.

Study of diffusion through dialysis membrane

The penetration of API depends on the concentration of CD. Overly high or low amounts of CD can cause a decreased absorption of API, therefore its determination is important. With the investigation of the diffusion of PR through a dialysis membrane, the optimal CD quantity as a function of drug penetration can be adjusted.

The amount of CD for the optimal penetration of API was determined by drug diffusion monitoring. Zellutrans/Roth cellulose dialysis membrane tube (10 mm wide, 6.4 mm diameter, MWCO: 12,000–14,000 D) was used for the experiment. The membrane pouches were closed with Spectra/Por Closures. The sample (2.00 mL) was injected into the pouches and put into 25 mL of borate buffer-containing aqueous acceptor phase (pH=7.4) tempered at 35°C. At various time intervals (15, 30, 60, 120, 180, and 240 minutes), 1.00 mL of the sample was removed from the acceptor phase and refilled with the buffered solution. The length of the measurement is reasonable, due to the possible increased retention on the surface of the eye. Four samples were measured parallel at the same time. The PR content was analyzed with UV spectrophotometry.

Viscosity

A Physica MCR 101 rheometer with cone-plate measuring device (Anton Paar, Graz, Austria, CP25-1, cone angle 0.997°, 25 mm diameter) was used for the measurement. The formulations were investigated at 25°C; the shear rate was increased from 0.1 s^{-1} to 100 s^{-1}, the means of the data at 100 s^{-1} shear rate were calculated at the evaluation. The viscosity values were illustrated as a function of the concentration of CD derivatives.

Surface tension

The surface tension of the samples was measured with OCA 20 contact angle system (Dataphysics Instruments GmbH, Filderstadt, Germany) by analyzing the shape of pendant drop. The values of surface tension were determined with SCA 20/22 software module using the Young-Laplace equation.[27]

Efficacy of antimicrobial preservation

The applicability of ZnHA–ZnGlu as ophthalmic preservative system was investigated vs BK, because the other components in the formulation could affect its antimicrobial effect. The antimicrobial effectiveness of the ophthalmic samples was determined according to the standards of the European Pharmacopoeia (EP). ZnHA–ZnGlu and BK as preservatives were tested on control strains, *Staphylococcus aureus* (ATCC 6538), *P. aeruginosa* (ATCC 9027) and *Candida albicans* (ATCC 10231). Inoculum suspensions of the microorganisms were prepared by using a sterile suspending fluid containing 9 gL^{-1} NaCl. The number of colony-forming units (CFU) was determined with plate count. The microbial count was about 10^8 CFU per milliliter. Preserved samples were inoculated with the suspensions of bacteria and fungus by adding 10^6 CFU per milliliter. The volume of the inoculated suspensions of microorganisms did not exceed 1% of the volume of the product. According to the standard method, three parallel samples were removed at zero hours and at appropriate intervals (6 hours, 24 hours, 7 d, 14 d, 28 d), and plated to Sabouraud-dextrose fluid agar (fungus) or tryptic soy fluid agar (bacteria). Bacteria-containing samples were incubated at 30°C–35°C for 24 hours and fungus-containing samples at 20°C–25°C for 48 hours. The reduction of these values was converted to $\log_{10}$ and compared with requirements A and B of the EP (EP-A, EP-B). The requirement of preservative is determined by the EP as the logarithmic reduction of CFU. The effectiveness needed against bacteria and fungus is managed separately. The decrease of CFU needs to be in accordance with the EP-A criteria. In cases when adverse drug reaction can appear with the A criteria, the EP-B criteria are acceptable.[28] The aim was to determine whether the preservative effect of ZnHA meets the requirements of EP in the presence of CD derivatives.

Mucoadhesion

The mucoadhesion of CD-containing eye drops was determined by the tensile test method, based on the measurement of the forces of detachment and the total work of adhesion needed to separate the surfaces, resulting from the area under the force–distance curve.[29,30] Samples contained two types of CDs (HPBCD and HPGCD) prepared with and without ZnHA–ZnGlu. The purpose was to determine the effect of ZnHA on mucoadhesion and to establish if the presence of the type of CD has an effect in mucoadhesion. The measurement was performed with a TA.XT Plus Texture analyzer (ENCO, Spinea, Italy) instrument equipped with a 1 kg load cell and a cylinder probe with a diameter of 1 cm. The sample (20 µL) was attached to the cylinder probe and placed in contact with a filter paper disc wetted with 50 µL of an 8% w/w mucin dispersion or simulated lachrymal fluid (blank, pH=7.4). The mucin dispersion was made with simulated lachrymal fluid.[26,31]

A 2,500 mN preload was used for 3 minutes. The cylinder probe was moved upward to separate the sample from the substrate at a prefixed speed of 2.5 mm min^{-1}.

Statistical analysis

One-way and two-way analysis of variances were used to compare the mean values. Statistical analysis was performed by GraphPad Prism five statistical software (GraphPad Software, Inc., La Jolla, CA, USA). The level of significance was set to $P<0.05$.

Results and discussion

Phase solubility test

The phase solubility of PR in HPBCD- and HPGCD-containing aqueous solutions (0–150 mM) is shown in Figure 1.

The solubility of PR was increased linearly by increasing the concentration of HPBCD or HPGCD. The diagrams are Higuchi A_L type for both CDs, therefore the formation of 1:1 complexes can be assumed. In case of HPBCD, the apparent stability constant of the complex is 1,286.4 M^{-1}, and the constant of the PR–HPGCD complex was measured to be 1,778.5 M^{-1}.

It is stated that PR has greater affinity for complex-formation with HPGCD. With the equation of regression lines, the concentration of CD needed to solubilize the required amount of API can be determined. These calculated concentrations are the centers of intervals that were used in the drug diffusion study in case of both types of CD derivatives.

Study of diffusion through dialysis membrane

Eye drops with or without mucoadhesive polymer were formulated and examined. The concentrations of CD derivatives for the penetration of PR were optimized. The penetrated PR as a function of the concentration of CD is shown in Figure 2.

The results show that 4 mM HPGCD and 5 mM HPBCD induce the highest diffusion of PR through the dialysis membrane. Under the optimal CD concentration a part of free, hydrophobic drug remained in the pouches. Above the optimal CD level, the excess amount of CD keep the free PR

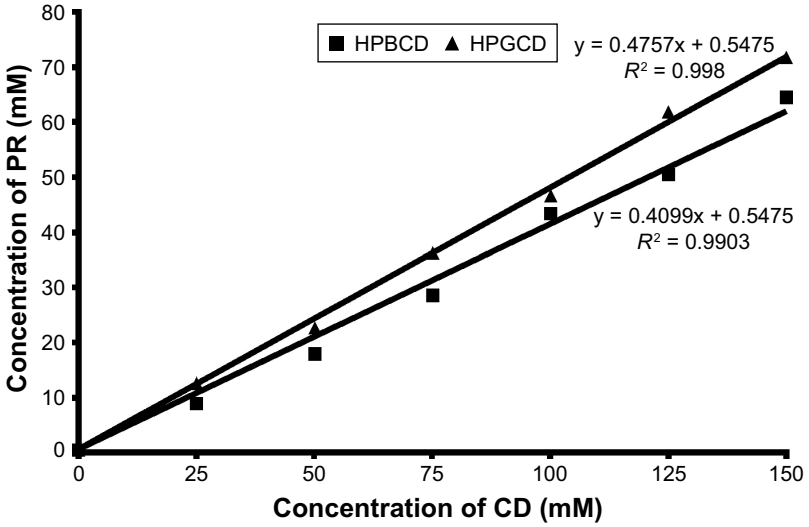

Figure 1 Phase solubility diagrams of PR in aqueous HPBCD (■) and HPGCD (▲) solutions at 25°C.
Abbreviations: CD, cyclodextrin; HPBCD, hydroxypropyl-β-cyclodextrin; HPGCD, hydroxypropyl-γ-cyclodextrin; PR, prednisolone.

in complex, therefore less amount of free API is detectable in the acceptor phase.

Thereafter, mucoadhesive ZnHA–ZnGlu additives were added to the eye drops, which ensure antimicrobial, preservative effect in the formulations. It was found that the application of biopolymer has no effect on the diffusion of PR. The same amount of drug penetrated through the dialysis membrane at each period in case of both compositions (Figure 3).

Viscosity

The viscosity of ZnHA and PR-CD complex-containing ophthalmic formulations was measured. The results are shown in Table 1.

According to previous reports, the viscosity should be under 30 mPa s.[32,33] Above this level, blurred vision and discomfort appear, which result in faster elimination due to reflex mechanisms of the eye. The results show that the viscosity of our formulations is appropriate in the range of 9.2–24.2 mPa s.

Surface tension

The surface tension of normal tear is about 43 mN m^{-1}.[34] It is not optimal if the surface tension of products is much higher than that of the lacrimal fluids because it has an impact on the therapeutic effect of pharmaceutics applied on the eye, through affecting the spreading of the eye drops on the ocular surface, although no regular critical parameter was found for the surface tension of eye drops in the EP. The surface tension of formulations preserved with ZnHA was measured by using an OCA 20 contact angle system. The results are shown in Table 2.

No significant difference was found between the values. The surface tensions of the eye drops are about 60 mN m^{-1},

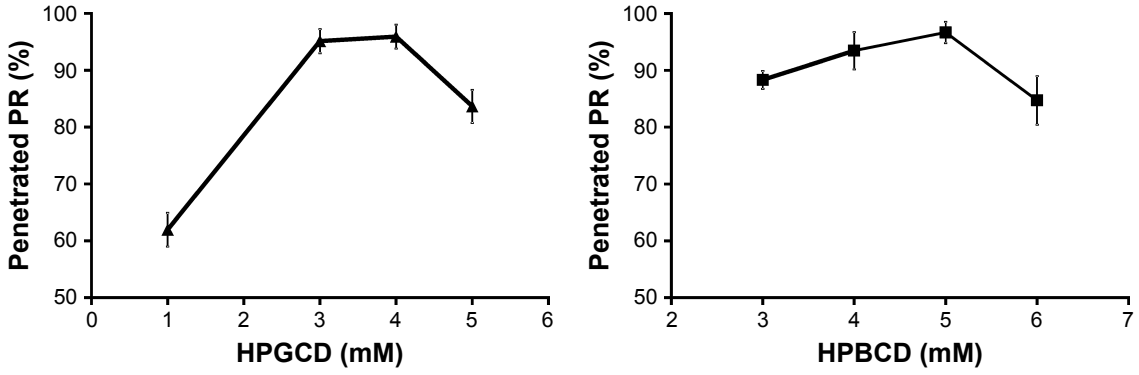

Figure 2 PR diffusion through dialysis membrane as a function of the concentration of HPGCD and HPBCD after 240 minutes.
Abbreviations: HPBCD, hydroxypropyl-β-cyclodextrin; HPGCD, hydroxypropyl-γ-cyclodextrin; PR, prednisolone.

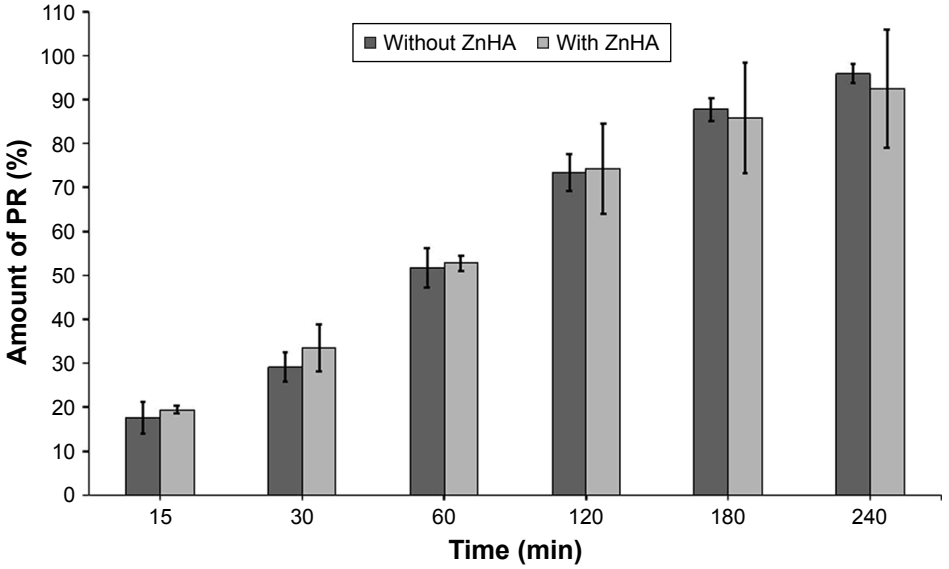

Figure 3 Effect of ZnHA–ZnGlu on the diffusion of PR through dialysis membrane (light column: with ZnHA–ZnGlu; dark column: without ZnHA–ZnGlu).
Abbreviations: PR, prednisolone; ZnGlu, zinc-gluconate; ZnHA, zinc-hyaluronate.

which is higher than the surface tension of lacrimal fluid. Ophthalmic products were investigated by Han et al, and the range of the surface tension values was between 34.3 mN m^{-1} and 70.9 mN m^{-1}.[35–37] According to this study, the surface tension of formulations meets the requirements for ophthalmic products.

Efficacy of antimicrobial preservation

In Samples I (with HPBCD) and II (with HPGCD), ZnHA–ZnGlu were used as preservative compounds. The results are shown in Table 3.

The logarithmic decrease of *S. aureus* was 1.5 after 6 hours, 2 after 24 hours, and no bacteria were detected in the samples after 7 days. In case of *P. aeruginosa*, the logarithmic decrease is higher at the earlier period, but the bacterium appeared in every sample. The logarithmic decrease of *C. albicans* was 1 after 6 hours, and no fungi were detected later. In summary, the preservative effect of ZnHA–ZnGlu-containing samples meets the EP-B criteria. The antimicrobial effectiveness of ZnHA–ZnGlu is lower against *P. aeruginosa* compared with the other microorganisms.

The microbiological stability of Samples III (with HPBCD) and IV (with HPGCD), which contained BK as a preservative agent, was tested (Table 4).

The logarithmic decrease of *S. aureus* was 1 after 6 hours, 2 after 24 hours, and no bacteria were detected later in Sample III. For *P. aeruginosa* the logarithmic decrease was two and no change was detected later. In *C. albicans*-containing samples, no fungi were found after 7 days. In Sample IV, for *S. aureus* and *C. albicans*, the number of CFU was zero after 6 hours. In case of *P. aeruginosa*, the antimicrobial effect was lower due to the known resistance of the bacterium against BK. The microbiological stability of Samples III and IV meets the requirements of the EP. In Sample III, the

Table 1 Viscosity values of ZnHA-PR-HPGCD (I)- and ZnHA-PR-HPBCD (II)-containing products

	Concentration of CD (mM)	Viscosity (mPa s)	SD
I	3	22.8	4.5
	4	9.2	3.3
	5	19.7	1.2
II	3	24.2	3.4
	4	18.4	2.8
	5	18.1	0.7
	6	22.1	3.7

Abbreviations: CD, cyclodextrin; HPBCD, hydroxypropyl-β-cyclodextrin; HPGCD, hydroxypropyl-γ-cyclodextrin; PR, prednisolone; ZnHA, zinc-hyaluronate.

Table 2 Surface tension of ZnHA-PR-HPGCD (I)- and ZnHA-PR-HPBCD (II)-containing formulations

	Concentration of CD (mM)	Surface tension (mNm^{-1})	SD
I	3	61.12	0.24
	4	61.65	0.31
	5	61.20	0.24
II	3	59.31	0.15
	4	59.46	0.23
	5	58.65	0.22
	6	59.06	0.37

Abbreviations: CD, cyclodextrin; HPBCD, hydroxypropyl-β-cyclodextrin; HPGCD, hydroxypropyl-γ-cyclodextrin; PR, prednisolone; ZnHA, zinc-hyaluronate.

Table 3 Preservative effectiveness against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans* in Samples I and II

Microbial log reduction					
	6 hours	24 hours	7 days	14 days	28 days
S. aureus					
Sample I	1.5	2	nd	nd	nd
Sample II	1.5	2	nd	nd	nd
Criteria (B)	–	1	3	–	ni
P. aeruginosa					
Sample I	2	3	–	4	–
Sample II	2	–	3	4	–
Criteria (B)	–	1	3	–	ni
C. albicans					
Sample I	1.5	–	–	–	nd
Sample II	1	–	–	–	nd
Criteria (B)	–	–	–	1	ni

Abbreviations: nd, not detectable; ni, no increase.

antimicrobial effect of BK is lower than in Sample IV. It can be assumed that there is a competition between PR and BK for the cavity of HPBCD, so the preservative effect of BK is decreased by the inclusion complex formation.[38]

According to the EP-B criteria, 0.5% ZnHA–ZnGlu compounds ensure the proper microbiological stability of eye drop formulations. In case of *P. aeruginosa*, the antimicrobial effect of the ZnHA–ZnGlu system is higher than the effect of BK. Considering these results with the irritative attribute of BK, application of ZnHA–ZnGlu as a preservative can be favorable in ophthalmic products.

Mucoadhesion

The measured adhesive force values are shown in Figure 4. Lachrymal fluid was used as blank.

Table 4 Preservative effectiveness in Samples III and IV against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*

Microbial log reduction					
	6 hours	24 hours	7 days	14 days	28 days
S. aureus					
Sample III	1	2	nd	nd	nd
Sample IV	nd	nd	nd	nd	nd
Criteria (B)	–	1	3	–	ni
P. aeruginosa					
Sample III	2	–	–	–	–
Sample IV	3	2	–	3	–
Criteria (B)	–	1	3	–	ni
C. albicans					
Sample III	1	–	nd	nd	nd
Sample IV	nd	nd	nd	nd	nd
Criteria (A)	–	–	2	–	ni

Abbreviations: nd, not detectable; ni, no increase.

There is a large difference in the measured force between the blank and the mucin dispersion. All the samples show mucoadhesivity; samples prepared with ZnHA have significantly higher values than samples prepared without it. This proves the importance of the presence of ZnHA because the interpenetration between the ZnHA chains and mucin can be assumed. These samples can have better mucoadhesive properties and cause decreased administration frequency and a lower active ingredient concentration.

The type of CD does not play an important role in mucoadhesion because there is no significant difference between the samples prepared with HPBCD and HPGCD. According to the results, it can be established that ZnHA plays an important role in mucoadhesion.

Conclusion

To increase the bioavailability of steroid-containing ophthalmic products is a great challenge. Over the years, there have been numerous attempts to enhance the efficacy of eye drops. Our aim was to formulate PR-containing aqueous solutions by CD inclusion complex and to create mucoadhesive eye drops using antimicrobial ZnHA and ZnGlu, with suitable physiological parameters.

Aqueous solutions of PR were formulated by inclusion complexation with HPBCD and HPGCD. The investigation of the diffusion of PR through the dialysis membrane revealed that 5 mM of HPBCD, and 4 mM of HPGCD caused the highest penetration of API in in vitro circumstances. The addition of ZnHA and ZnGlu had no effect on the penetration properties. The measurement of viscosity showed that the 5-mM HPBCD- and 4-mM HPGCD-containing products had the lowest viscosity values, meeting the requirements of the EP. It was found that the concentration of CDs has no effect on the surface tension of the eye drops, and these values are optimal compared with previously investigated ophthalmic products. The mucoadhesive properties of ZnHA-containing formulations were proved with the tensile test, resulting in a higher retention time of the eye drop on the surface. The type of CD derivative has no influence on mucoadhesivity. During the preservative effectiveness test, it was proven that the ZnHA–ZnGlu combination is applicable as an antimicrobial, preservative compound. The microbiological stability of ZnHA-containing products meets the requirements of the EP in case of *S. aureus*, *P. aeruginosa* and *C. albicans*. It can be stated that BK with unfavorable toxicity properties for the corneal epithelial cells can be replaced with the more biocompatible ZnHA.

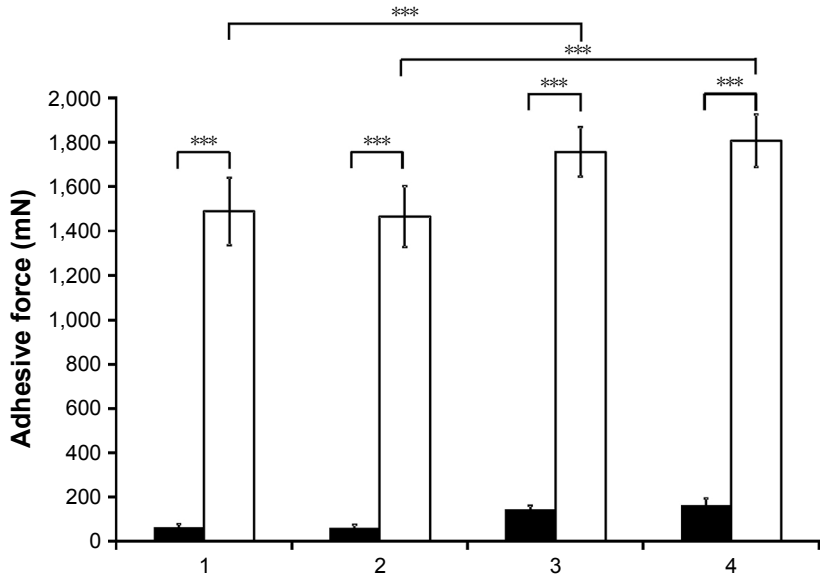

Figure 4 Adhesive force of eye drops with (white columns) and without mucin (black columns) (1, with HPBCD; 2, with HPGCD; 3, with HPBCD and ZnHA–ZnGlu; 4, with HPGCD and ZnHA–ZnGlu). The asterisks mark the significant difference between the results. The analysis was performed by the written statistical software and method.
Abbreviations: HPBCD, hydroxypropyl-β-cyclodextrin; HPGCD, hydroxypropyl-γ-cyclodextrin; ZnGlu, zinc-gluconate; ZnHA, zinc-hyaluronate.

In summary, anti-inflammatory ophthalmic products containing PR with enhanced bioavailability were formulated in aqueous solutions by means of CD inclusion complex formation with optimal mucoadhesion and antimicrobial properties. Although further experiments such as toxicity and permeation studies are needed, these results are promising for the formulation of innovative eye drops with high therapeutic effect and sufficient patient compliance.

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Disclosure

The author reports no conflicts of interest in this work.

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Review

Current Approaches to Use Cyclodextrins and Mucoadhesive Polymers in Ocular Drug Delivery—A Mini-Review

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Abstract: Ocular drug delivery provides a challenging opportunity to develop optimal formulations with proper therapeutic effects and acceptable patient compliance because there are many restricting factors involved, such as complex anatomical structures, defensive mechanisms, rapid drainage, and applicability issues. Fortunately, recent advances in the field mean that these problems can be overcome through the formulation of innovative ophthalmic products. Through the addition of solubility enhancer cyclodextrin derivatives and mucoadhesive polymers, the permeability of active ingredients is improved, and retention time is increased in the ocular surface. Therefore, preferable efficacy and bioavailability can be achieved. In this short review, the authors describe the theoretical background, technological possibilities, and the current approaches in the field of ophthalmology.

Keywords: ocular drug delivery; pharmaceutical technology; ophthalmic formulation; cyclodextrin; mucoadhesion; polymers

1. Introduction

Eyes are one of the most important organs of the human body and in the case of any dysfunction in vision, serious drawbacks can appear in daily activities. Ocular drug delivery is a major challenge in the pharmaceutical research and development field because of restrictions caused by many factors. When considering patient-oriented therapy, patient compliance is a key factor, thus the mission of many researchers is to find an optimal administration method that is self-applicable for the patients, and to find the optimal formulation with accomplished therapeutic effect and zero irritation.

1.1. Anatomical and Physiological Perspectives

The complex anatomy of the eye limits the amount of therapy options for different diseases, especially when deeper drug permeation is needed. The eye has two main parts; the anterior segment, which includes the cornea, aqueous humor, iris, and lens, and the posterior segment, which includes from the lens to the deeper tissues (vitreous humor, retina, sclera, optic nerve) (Figure 1). The cornea consists of five layers; the lipophilic epithelium with tight junctions, Descemet's membrane, the hydrophilic stroma (which is the thickest part of the cornea), Bowman's layer, and the lipophilic endothelium [1–4]. When considering the optimal drug penetration through the cornea, a balance between the hydrophilicity and lipophilicity of the drug and the delivery system is necessary. Due to its complex anatomical structure, formed physiological barriers protect the eye from surrounding exposures. The first barrier is built by the tear film and includes a lipid layer, mucins, and water. It protects the cornea and conjunctiva. The composition of the corneal barrier was mentioned before. It

mainly restricts drug permeation to the anterior tissues. The conjunctival barrier consists of epithelial layers and connective tissue with blood and lymphatic vessels. The blood–aqueous barrier (BAB) contains tight junctions of the capillary endothelium of the iris, and ciliary epithelium. It is mildly permeable for low-weight molecules. The drug permeation is restricted from systemic circulation to the posterior segment of the eye by the blood–retinal barrier (BRB) due to the tight junctions of retinal pigment epithelium and the endothelial membrane of retinal blood vessels [5–7]. After any stimulus reflex mechanisms, like lachrymal secretion and eye blinking, are induced, thus eliminating the irritative agents in minutes from the eye surface. If the drug is passed through the cornea, the opposite flow of aqueous humor also limits penetration to the posterior direction [8]. Therefore, these mechanisms also limit the therapy by blocking drug permeation into the targeted tissues.

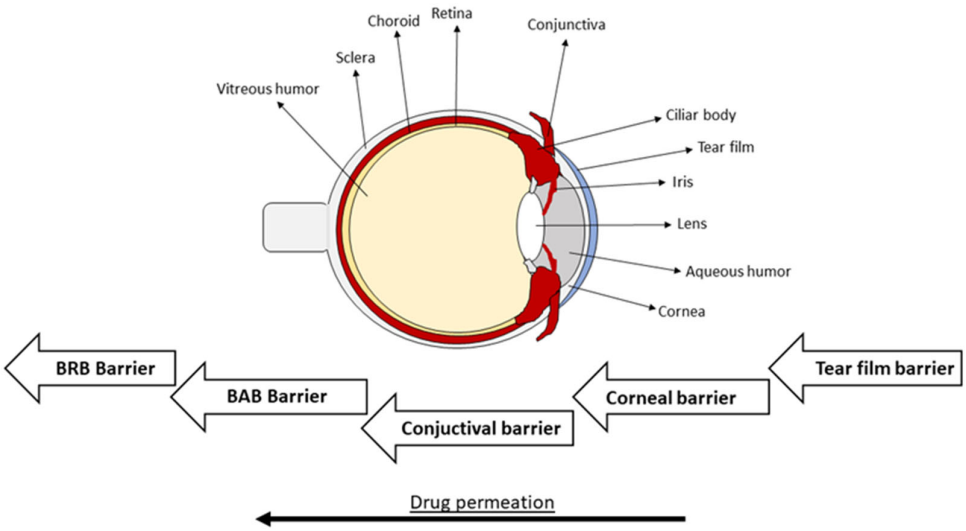

Figure 1. Structure of eye and physiological barriers (BRB: Blood–retinal barrier, BAB: Blood–aqueous barrier).

1.2. Conventional Routes of Administration

Considering the above-mentioned defensive blockade, the process of ensuring the required therapy is excessively difficult in ophthalmology. In clinical practice, there are invasive and non-invasive methods for administration of the formulation to reach the targeted site (Table 1). Non-invasive routes, also known as topical formulations, are mainly for reaching the anterior segment. The indications at this site are cataract, glaucoma, dry eye, inflammatory diseases, trauma or surgery induced diseases, injury, and tumor. Topical administration is the easiest and most commonly applied non-invasive method which is self-applicable for the patient. Mostly eye-drops, semisolid formulations, inserts, and contact lenses containing the active pharmaceutical ingredient (API) are used [1,9,10]. The requirements are exact for these products. Sterile, isotonic, and microbiologically stable formulations must be prepared with acceptable pH and viscosity. If any of the parameters differ from the optimal range, defensive mechanisms are induced in the eye, therefore the expected efficacy would be much worse. After application, the tear film barrier is the first blockade. For optimal drug permeation, sufficient concentration of drug must present at the cornea. The second obstacle is the corneal barrier, where firstly the drug meets the corneal epithelial multilayer. Because of the tight junction proteins between the epithelial cells, penetration of hydrophilic molecules are restricted, and lipophilic drugs can permeate transcellular by passive diffusion. The second part of the cornea is the stroma, which is a hydrophilic environment, therefore the penetration of lipophilic drugs is restricted there. The lipophilic endothelial monolayer is more transparent for macromolecules than the

epithelium. The non-corneal pathway is also known as conjunctival–scleral route, where the permeation mostly depends on the molecular weight. Through the corneal and non-corneal pathway, the anterior tissues are partly reachable for the active ingredients. From the precorneal area (tear film), the applied formulation is eliminated through the tear turnover and nasolacrimal drainage to the systemic circulation [11–13].

The invasive administration methods like intravitreal, subconjunctival injections, and inserts have both advantages and limitations. With these methods, the target tissues are directly reachable, although these invasive administrations are limited because of the required expertise, proper dosage, and possible side-effects, like toxic reactions of the cornea. The subconjunctival application is less invasive, although the elimination is decently fast through the conjunctival blood and lymphatic vessels.

Oral and intravenous administration are rather unfavored because of the presence of the BAB and the first pass metabolism. To overcome the barrier, a high concentration of the drug needs to be used, which is difficult because of the possible side effects and poor solubility of the most active ingredients [14,15].

Table 1. Routes of administration to the eye with advantages and limitations.

Route of Administration	Advantages	Limitations
Topical	Patient-compliance, self-applicable, non-invasive, simple, no first-pass effect	Frequent administration needed, low bioavailability, short contact time on the eye surface, tear dilution
Subconjunctival	Barely invasive, high efficacy, no first pass effect	Fast clearance, expertise needed, not self-applicable
Intravitreal	High bioavailability, avoiding cornea, no first-pass effect	Critical dosing, very invasive method, expertise needed, not patient compliant, toxic side effects
Intravenous	Avoiding cornea, less frequent application	Invasive, expertise needed, not targeted exposure, large dose needed
Oral	Patent compliant, non-invasive	First pass effect, low ocular efficacy, not targeted exposure, large dose needed

2. Novel Approaches in the Research of Ophthalmic Formulations

When considering the attributes of physiological obstacles of administration routes, an innovative solution is required, one that is acceptable in terms of patient compliance and efficient therapy. A topically self-administrable formulation would be optimal, with enhanced drug permeability into the anterior/posterior tissues and increased residence time on the surface of the eye. Enticing results are published on the impact of complex of drug-cyclodextrin derivatives, mucoadhesive polymers, and nanotechnology. This short review on recent ocular drug delivery approaches summarizes these innovations from recent years.

2.1. Cyclodextrins

To reach optimal penetration, the API needs to be dissolved in lachrymal fluid and pass the tear film barrier. If the concentration of API is going to be optimal near the corneal epithelium, a steady amount needs to be ensured for optimal permeation [16]. A major challenge is presented by the fact that the applied APIs in ophthalmology are mostly lipophilic molecules, with low water solubility. Application of solubility enhancer additives, like cyclodextrins (CD) could be the first step for the optimization of eye drop formulations. CDs are cyclic oligosaccharides with α-(1,4) linked α-D-glucopyranose units. In nature, three types are formed by bacterial digestion of starch; α-CD with 6, β-CD with 7, and γ-CD with 8 glucopyranose units. The external surface of these molecules is hydrophilic due to the orientation of hydroxyl groups, which form hydrogen bonds with surrounding water molecules. Inside the cavity of CDs, the environment is hydrophobic, therefore an inclusion complex can be formed with lipophilic agents by hydrogen bonds, van der Waals, and charge-transfer interactions. In aqueous solution dynamic equilibrium is created between the free CD and drug molecules and the complex. After application on the eye surface, only the free lipophilic

molecule can permeate through the cornea, the hydrophilic CD remains and is eventually eliminated through the nasolacrimal pathway (Figure 2).

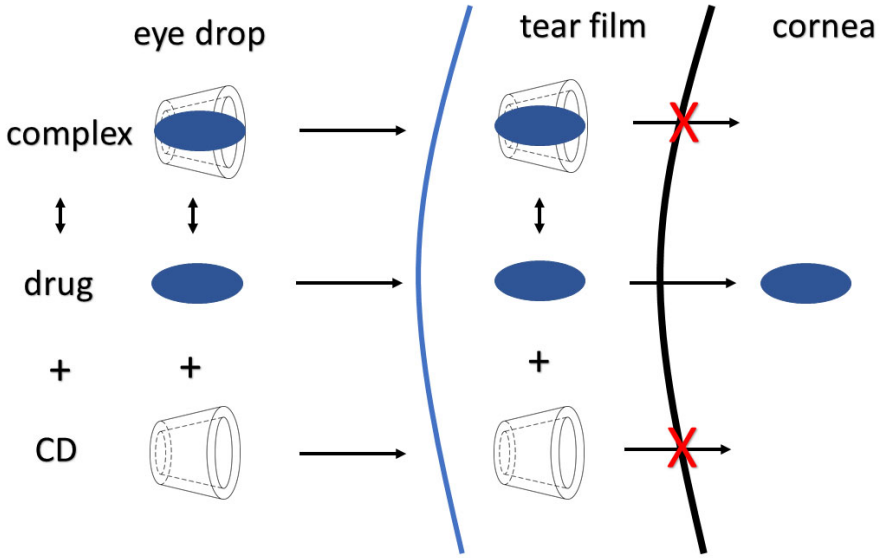

Figure 2. Schematic figure about the cyclodextrin drug permeability enhancer attributes.

With the formation of inclusion complexes, the APIs are dissolved in the tear and concentrated near the cornea epithelium. Low or unnecessarily high amounts of CD restrict the permeation of drug, thus the CD concentration needs to be optimized in the formulation [16–21]. To investigate the formation of the inclusion complex in solution, the phase solubility test is a well-known method, which has been described previously by Higuchi and Connors. The stability constant of the complex (K_s) is calculable from the slope of phase solubility diagram using Higuchi–Connors equation (Eq. 1.):

$$K_s = \text{Slope}/\{\text{Intercept } (1–\text{Slope})\} \quad (1)$$

The intensity of binding forces between the API and CD molecules can be established by the stability constant [22,23]. A phase–solubility diagram is shown on Figure 3, which was published before by our research group [24].

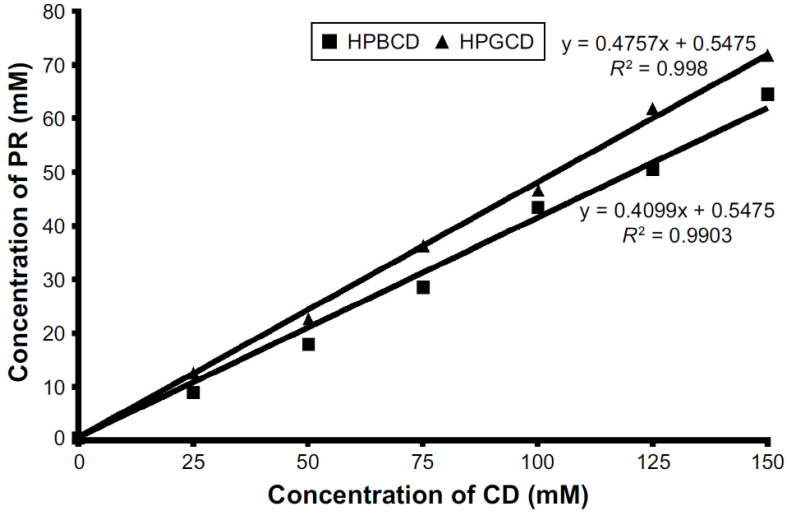

Figure 3. Phase–solubility diagram of prednisolone (PR)-hydroxypropyl-β-cyclodextrin (HPBCD) and PR-hydroxypropyl-γ-cyclodextrin (HPGCD) inclusion complexes. The solubility of PR increased linearly by increasing the concentration of cyclodextrins (CDs). The curve is A_L type, thus inclusion complexes with 1:1 molecular ratio are formed in the case of HPGCD and HPBCD [24].

In the solid state, the formation of drug–CD inclusion complexes can be investigated by differential scanning calorimetry (DSC), Fourier-transformed infrared spectroscopy (FTIR), X-Ray powder diffraction (XRPD) and C_{13}-NMR methods. Considering the changes in physicochemical attributes, crystallization, and the IR spectrum, the formation of complexes can be assumed [25–28]. CD derivatives have been developed with more favorable attributes like increased solubility, stability, and less toxicity. In ophthalmic formulations the hydroxypropyl-β-and γ-cyclodextrin and sulfobuthylaether-β-cyclodextrin are the most commonly applied derivatives, which are also official in European Pharmacopoeia. Studies on rabbit corneal epithelial cell-line showed non-toxic attributes after application of these types of CDs [29–32]. Recent approaches are shown on Table 2., where CDs are applied in ophthalmic formulations.

Table 2. Recent approaches to use CDs in ophthalmic formulations.

API	CD derivative	Formulation	Reference
Flurbiprofen	HPBCD	eye drop	[33]
Nepafenac	HPBCD HPGCD	eye drop	[34]
Amlodipine	HPBCD SBEBCD	eye drop	[35]
Dexamethasone acetate	HPBCD HPGCD	eye drop	[36]
Cyclosporine	HPBCD	insert	[37]

2.2. *Mucoadhesion*

Application of polymers for prolonged ocular drug delivery is a common strategy. When the viscosity is increased, necessarily not to a high level, the residence time of the eye drop on the surface of the eye is prolonged without any side-effect, such as visual disorder or irritation. Mucoadhesive polymers are especially useful, because of the possible adhesion due to the interaction of polymer chains and the mucin layer of the tear film. It is defined as bioadhesion if the polymer chains are attached to the biological surface. Several theories are associated with the mechanisms of mucoadhesion. The wetting theory describes the effect of drop spreadability and wettability on the eye surface. According to the electrosatic theory, electron transfer is the mechanism of mucoadhesion. Adsorption theory is about primary and secondary chemical bonds between the polymer and mucus. In the case of high molecular weight polymers, the diffusion of polymer chains and glycoproteins of mucus can interpenetrate into each other creating an intermolecular net and mucoadhesion. This mechanism is also known as mechanical theory (Figure 4) [38–42].

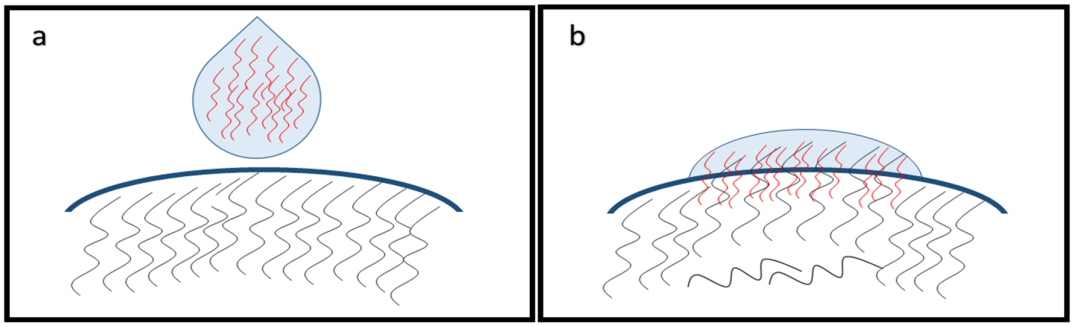

Figure 4. Mechanical theory of mucoadhesion. Before application of eye drop (**a**) and after interpenetration of polymer chains (**b**). After application, the polymer chains penetrate during the spreading of eye drop on the ocular surface.

The most commonly used mucoadhesive polymers are carbomers, alginates, methylcellulose, hydroxypropylmethylcellulose, hydroxyethylcellulose, chitosan, thiolated polymers and hyaluronic acid. Biopolymers like hyaluronic acid are favorable in ophthalmic formulations, because of the biocompatible, non-toxic and biodegradable attributes [40,43,44].

2.3. Cyclodextrins and Mucoadhesive Polymers as Ophthalmic Drug Delivery Systems

Patient-compliance and effective therapy is desired, where the medicine is self-applicable, safe, and economical. Topical eye drop formulations would be optimal if they matched all previously mentioned requirements. Nowadays, several research groups have published articles about approaches for improved ocular drug delivery systems, where the API was dissolved in the aqueous solvent by CD complex formation, and the residence time was increased by mucoadhesive polymers, and therefore expected to have higher therapeutic efficacy [19,43,45].

Our research group has developed a promising formulation, where the prednisolone(PR)–CD complex was dissolved in aqueous solution containing zinc-hyaluronate and zinc-gluconate. With the addition of zinc-hyaluronate–zinc-gluconate system (ZnHA–ZnGlu), the antimicrobial stability was ensured during storage, application, and due to mucoadhesive attributes, increased residence time is expected on the eye surface. The osmolality and pH were set to physiological parameters by the sodium–chloride and borate buffer. Optimal PR diffusion was investigated in vitro using dialysis cellulose membrane. Mucoadhesive properties were tested by tensile test on mucin impregnated surface. All of the samples show that the mucoadhesivity, formulations with ZnHA–ZnGlu have significantly higher adhesive force values (Figure 5). [24].

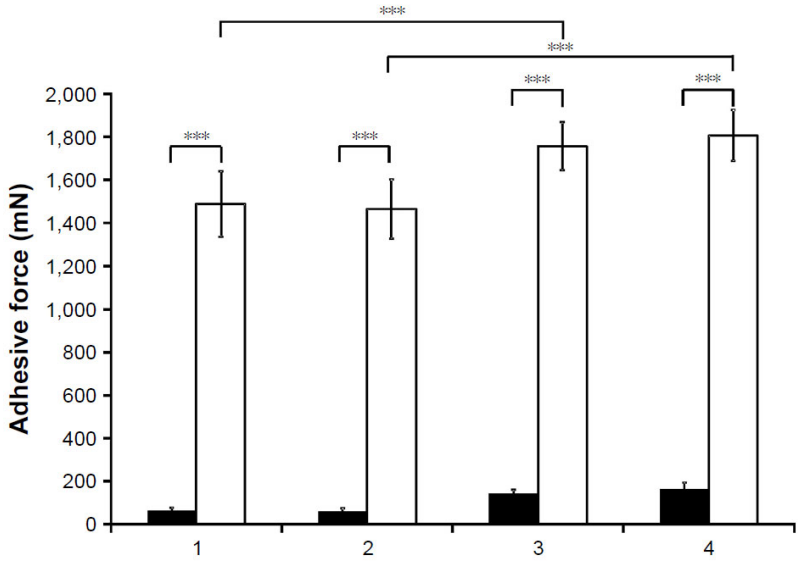

Figure 5. Results of tensile test with (white columns) and without (black columns) mucin. (1, with HPBCD; 2, with HPGCD; 3, with HPBCD and ZnHA–ZnGlu; 4, with HPGCD and ZnHA–ZnGlu) [24] (*** $p \leq 0.001$).

An innovative approach was published by Budai-Szűcs et al., where PR was incorporated into cyclodextrin-modified thiolated poly(aspartic acid) in aqueous in situ gelling solution. The complex formation was investigated by the XRPD method, as were the physicochemical attributes, rheology, and drug diffusion. In the drug diffusion study PR suspension was used as reference. The drug diffusion was tested over 24 h (Figure 6). In the case of the unbound PR cyclodextrin complex

containing thiolated polymer gel, the diffusion rate was similar to PR suspension, due to the increased solubility and the prolonged effect of the polymer. In the formulation where the CD was covalently bound to the thiolated polymer, the drug diffusion was slower and dependent on the dissociation of PR from the CD complex and the inhibition of the polymer matrix. With the combination of the two type of formulations, an intermediate diffusion rate was observed. In this case the free PR–CD complexes caused a rapid biological effect, meanwhile the bound complexes prolong the drug release on the eye surface, therefore a less frequent application is needed to reach the target therapy [46].

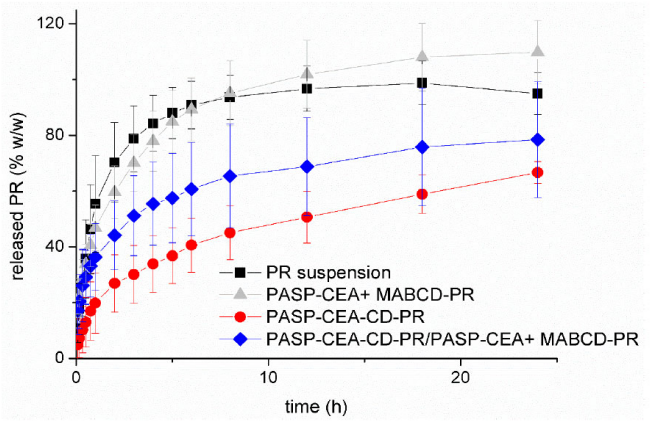

Figure 6. Drug release from the formulations containing prednisolone (PR). Cumulative mean values and standard deviations (SD) n = 3; PR: prednisolone, PASP-CEA: Thiolated poly(aspartic acid), MABCD: 6-monodeoxy-6-monoamino-beta-cyclodextrin hydrochloride [46].

Nanda et al. developed amlodipine containing mucoadhesive films through the addition of hydroxypropyl methylcellulose (HPMC) and CD derivatives (β-CD, HPBCD, sulfobuthylaether-β-cyclodextrin) using casting and solvent-evaporation methods. The authors investigated the swelling and erosive attributes, morphology, inclusion complex formation by DSC, FTIR and XRPD, in vitro and ex vivo diffusion of drug and anti-inflammatory effect on carrageenan induced rabbit model. As the result of the ex vivo permeations study shows, the applied CDs increased the permeability of drug on the excised sheep cornea, meanwhile the flux was dependent on the binding constants of the different CDs (Figure 7) [35].

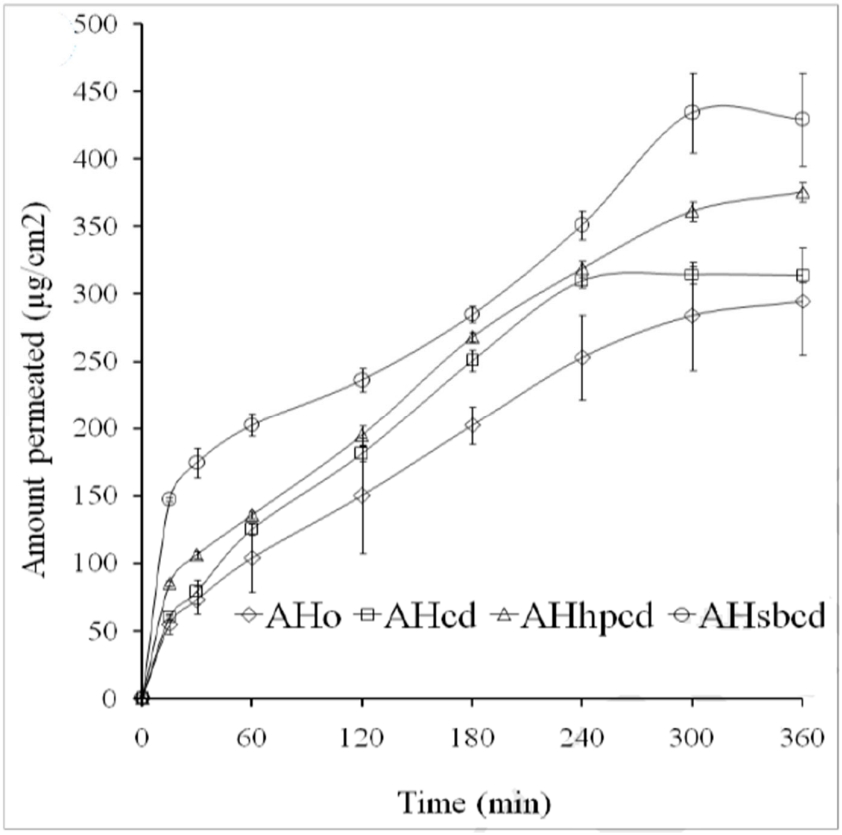

Figure 7. Ex vivo permeation study of amlodipine–CD containing HPMC films on excised sheep cornea. AHo: films without CD, AHcd: with β–CD, AHhpcd: With HPBCD, AHsbcd: With sulfobuthylaether-β-CD [35].

Shelley et al. published promising results about in situ gelling, nepafenac-HPBCD complex, sodium alginate containing ophthalmic formulation. Ex vivo permeability of API was tested using excised porcine cornea for 24 hours. The results showed that, the drug permeation from in situ gelling formulations was significantly higher compared with the official suspension formulation, Nevanac®, which was caused by the permeability enhancer effect of HPBCD. The highest permeation was observed in the case of composition F15 due to the low viscosity (Figure 8). This article also confirms the favorable effect of adding cyclodextrin and mucoadhesive polymers into the developed ocular drug delivery system [47].

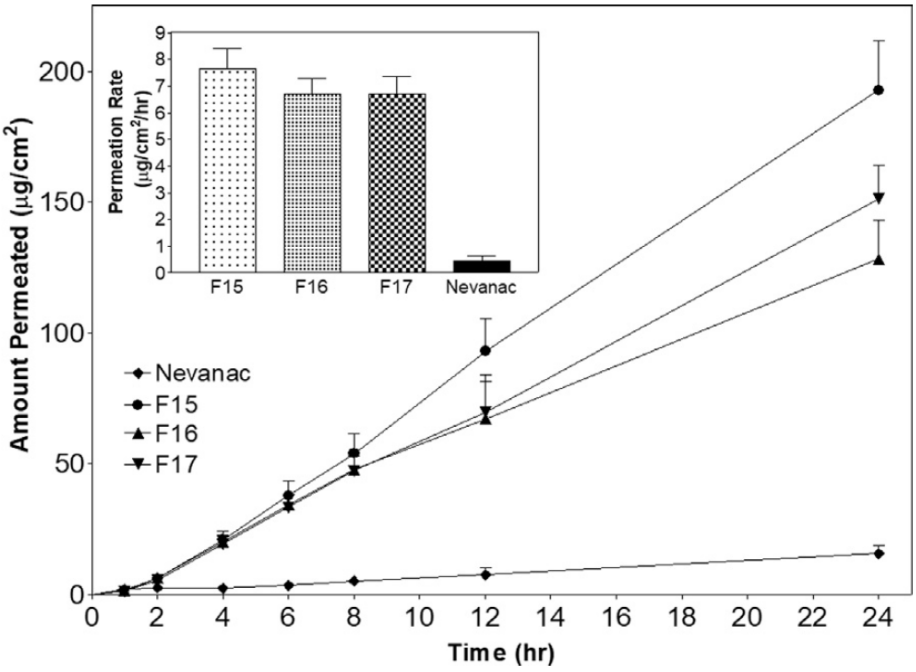

Figure 8. Ex vivo permeation test of nepafenac-HPBCD containing an in situ gelling system on porcine cornea. Nevanac: Official product used as reference, F15: 0.1 *w*/*v*% sodium-alginate, F16: 0.3 *w*/*v*% sodium-alginate, F17: 0.5 *w*/*v*% sodium-alginate [47].

3. Summary

In conclusion, the development of optimal ocular drug delivery systems is a major challenge in the pharmaceutical field. Ensuring proper therapeutic effect with acceptable patient-compliance are key considerations in the research of innovative formulations. Several promising approaches are described in the literature, where topical eye drops were characterized using cyclodextrins and mucoadhesive polymers together. Noticeably, there is lack of in vivo and clinical investigations, which are necessary to ensure innovative work is successful.

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ORIGINAL RESEARCH

New Approach in Ocular Drug Delivery: In vitro and ex vivo Investigation of Cyclodextrin-Containing, Mucoadhesive Eye Drop Formulations

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Background: Optimal transcorneal penetration is necessary for ocular therapy; meanwhile, it is limited by the complex structure and defensive mechanisms of the eye. Antimicrobial stability of topical ophthalmic formulations is especially important. According to previous studies, the mostly used preservative, benzalkonium-chloride is irritative and toxic on corneal epithelial cells; therefore, novel non-toxic, antimicrobial agents are required. In this study, prednisolone-containing ophthalmic formulations were developed with expected optimal permeation without toxic or irritative effects.

Methods: The toxicity and permeability of prednisolone-containing eye drops were studied on a human corneal epithelial cell line (HCE-T) and ex vivo cornea model. The lipophilic drug is dissolved by the formation of cyclodextrin inclusion complex. Zinc-containing mucoadhesive biopolymer was applied as an alternative preservative agent, whose toxicity was compared with benzalkonium-chloride.

Results: As the results show, benzalkonium-chloride-containing samples were toxic on HCE-T cells. The biopolymer caused no cell damage after the treatment. This was confirmed by immunohistochemistry assay. The in vitro permeability was significantly higher in formulations with prednisolone-cyclodextrin complex compared with suspension formulation. According to the ex vivo permeability study, the biopolymer-containing samples had significantly lower permeability.

Conclusion: Considering the mucoadhesive attribute of target formulations, prolonged absorption is expected after application with less frequent administration. It can be stated that the compositions are innovative approaches as novel non-toxic ophthalmic formulations with optimal drug permeability.

Keywords: prednisolone, cyclodextrin, ocular drug delivery, mucoadhesion, human corneal epithelial cell line, ex vivo cornea model

Introduction

Ocular drug delivery is a difficult challenging task in the field of pharmaceutical research and development. The main goal is to meet the requirements of patient-based therapy and technological formulation aspects. The special environment of the eye makes the optimization difficult. Several methods have been developed for enhanced ocular drug delivery. Mainly topical solutions are in the focus of research laboratories.[1–4] Besides the advantages of eye drop formulations (self-applicable, non-invasive, convenient, economical), many difficulties are known, which need to be overcome (short retention time, low drug absorption, and bioavailability and problematic microbiological stability).

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Drug delivery from eye drops is limited because of the special anatomical structure and reflex mechanisms, such as eye-blinking and lachrymal secretion.[5,6] Sufficient amounts of the active pharmaceutical ingredient (API) need to be ensured on the eye surface, therefore the API is able to pass the hydrophilic tear film. The corneal and non-corneal pathways for topically applied drug absorption are complex, and consist of lipophilic and hydrophilic layers. For optimal drug penetration the required physicochemical characteristics of the drug delivery system must be strictly designed. Optimally a lipophilic-hydrophilic balance is needed in the system to induce the required therapeutic effect without minimal precorneal drug elimination.[7–10]

In ocular postoperative therapy, glucocorticoids are applied for treatment and prevention of inflammation. These steroids are poorly soluble in water, commonly steroid-containing formulations are suspensions on the market. Eye drops with prednisolone (PR), prednisolone-acetate, dexamethasone, fluorometholone, and fluocinolone are licensed worldwide.[11–13] PR is mostly used in acetate-form as a suspension; however, it is more water-soluble than the base form. Problems of suspension formulations are well known. After application, increased tear secretion and reflex blinking are induced, causing rapid drug elimination from the surface of the eye. Increasing the efficiency could be possible with a selection of appropriate additives.[12]

The efficiency can be increased by optimization of the amount of dissolved API molecules at the site of drug absorption. By using cyclodextrin (CD) derivatives the solubility of drug can be increased, therefore a solution as a dosage form can be prepared with minimal irritative effect and optimal permeation of API.[14] CDs are cyclic oligosaccharides, which consist of α-(1,4) linked α-D-glucopyranose units, which form a water soluble inclusion complex with lipophilic molecules. Due to the hydrophilic external surface, the molecular complex is soluble in aqueous medium. Thermodynamic interactions are formed between the CD and API molecule, and equilibrium is created between the free and complexed molecules.[15–17] Due to complex formation, a more dissolved, lipophilic API molecule can permeate through the epithelium, meanwhile the hydrophilic cyclodextrin is eliminated.[4,16] Considering the toxicological toleration, hydroxypropyl-, sulfobutyl-ether-β-CD, and hydroxypropyl-γ-CD derivatives are mostly applied in ocular drug delivery. The amount of CD concentration must be set strictly to ensure the optimal drug permeation. Unnecessarily high amount restricts the drug permeation, meanwhile low concentration cannot keep the API in solution, which causes limited efficiency.[18,19]

Besides enhancement of permeation, increasing the residence time on the eye surface induces a higher therapeutic effect. Residence time is increasable by mucoadhesive polymers, which can ensure prolonged drug absorption; therefore, less frequent administration is needed during the therapy. By addition of these compounds, the viscosity is increased which is also favorable considering the increment of contact time on the eye surface.[20–22] However, if the viscosity of the applied formulation significantly differs from the tear, the defensive mechanisms of the eye are induced, thus the elimination rate of the API will be increased. Besides the increased viscosity, mucoadhesion also provides enhanced retention on the eye surface due to the interpenetration of proteoglycan and polymer chains.[23,24] Nowadays, mucoadhesive biopolymers, such as hyaluronic acid (HA), are widely used in ophthalmic formulations. As a biocompatible polymer, HA is tolerable, non-toxic, and protects the cornea with a proliferative effect.[25,26]

The microbiological stability is especially important in the case of ophthalmic formulations, because of the special sensitivity of the eye. Mostly, benzalkonium-chloride (BK) is applied as a preservative compound, meanwhile its destructive effect on corneal epithelial cells is proven. BK damages the eye barrier through breaking the DNA single- and double-strands in the epithelial cells. Application of BK-containing eye drops may induce irritation and high lachrymal secretion.[27,28] As an alternative preservative, Zn^{2+} ion-containing systems can be added. In aqueous environment, the dissolved Zn^{2+} ion destabilizes the cell wall of microbes. Therefore zinc-containing compounds like zinc-hyaluronate (ZnHA) and zinc-gluconate (ZnGlu) may ensure the antimicrobial stability in the formulation with less irritative and toxic effect in the corneal epithelial cells in comparison with BK.[20,29]

Previously, formulation and investigation of innovative eye drop were published by our research group, where innovative PR-containing ophthalmic solution was developed in aqueous medium by the addition of CD derivatives and ZnHA-ZnGlu system. The physiological parameters (surface tension, pH, osmolality) were set with optimized mucoadhesive, rheological, and preservative attributes.[12] The aim of this study was to continue and complete the

research by investigating the cytotoxicity and API permeability of the formulation on an in vitro human corneal cell line and ex vivo, porcine cornea model.

Materials and Methods

Materials

PR was purchased from Henan Lihua Pharmaceutical Company (Henan, China). hydroxypropyl-γ-cyclodextrin (HPGCD) was donated by Cyclolab Ltd. (Budapest, Hungary), ZnHA and ZnGlu from Gedeon Richter Plc (Budapest, Hungary). Hydroxypropyl-β-cyclodextrin (HPBCD) was obtained from Wacker-Chemie GmbH (Munich, Germany). Dimethyl sulfoxide (DMSO), BK, NaCl, boric acid, and borax (for borate buffer) were obtained from Molar Chemical Ltd. (Halásztelek, Hungary), KCl, $NaHCO_3$, D-glucose monohydrate from Kemika (Zagreb, Croatia), $CaCl_2 \times 2H_2O$ and $MgCl_2 \times 6H_2O$ from Sigma-Aldrich, Chemie GmbH (Steinheim, Germany). Ringer-buffer was used as medium during the experiments. The pH of Ringer buffer was set to 7.4.

Sample Preparation

0.1% PR was used as API. Defined amounts of HPGCD or HPBCD were dissolved in borate buffer (prepared by water for injection filtered on 0.22 µm membrane filter). After addition of PR products were sonicated for 10 minutes, until total dissolution of API. Then 0.5% ZnHA and 0.5% ZnGlu was added to the system. Osmolality was set with NaCl to about 300 mOsm/kg, the pH was about 6.20 in every formulation. Every eye drop was prepared in aseptic environment. The containers were stored in the fridge for at least 24 hours for completely wetting of ZnHA. Final composition is shown in Table 1.

Table 1 Composition of Target Eye Drop Formulation

Materials	Concentration
Prednisolone	0.1%
Hydroxypropyl-β-cyclodextrin or hydroxypropyl-γ-cyclodextrin	5 mM or 4 mM
Zinc-hyaluronate	0.5%
Zinc-gluconate	0.5%
Borate buffer	Quantum satis
Sodium-chloride	Quantum satis
Water for injection	Quantum satis

Preparation of Human Corneal Epithelial Cell Line (HCE-T) Model

Human corneal epithelial cells (HCE-T; RCB 2280; RIKEN BRC, Tsukuba, Japan) were immortalized by transfection with a recombinant SV40-adenovirus vector, established and characterized by Araki-Sasaki.[30] The cells were grown in Dulbecco's Modified Eagle's Medium/F-12 (Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco), 0.5% DMSO, 5 µg/mL recombinant human insulin, and 10 ng/mL recombinant human epidermal growth factor (EGF) in a humidified incubator with 5% CO_2 at 37°C. All plastic surfaces were coated with 0.05% rat tail collagen in sterile distilled water before cell seeding in culture dishes. The culture medium was changed every second day. The air–liquid interface is crucial for the development of a tight multilayer epithelium in HCE-T cells.[31] HCE-T cells were cultured first in liquid–liquid condition for 5–8 days. To create the air–liquid condition the medium from the upper compartment was removed and only 1 mL of medium was added to the lower compartment to keep the liquid level at the appropriate height for the next 5–8 days. To measure TEER cells were fed with 500 µL medium in the upper compartment every second day.

Treatment of Cultured Cells

The final concentration of the PR in the formulations for cell culture experiments was 100 µg/mL. The formulations were diluted in Ringer buffer (pH=7.4) (150 mM NaCl, 2.2 mM $CaCl_2$, 0.2 mM $MgCl_2$, 5.2 mM KCl, 5 mM glucose, 6 mM $NaHCO_3$). We tested the following samples (Table 2).

Cell Viability Measurement by Impedance

Impedance was measured at 10 kHz by an RTCA SP instrument (ACEA Biosciences, San Diego, CA, USA).

Table 2 Composition of Investigated Samples by Toxicity and Permeability Studies. Defined Amounts Were Completely Dissolved or Suspended in Ringer Buffer

For Cell Viability Measurements		For Permeability Assay	
I.	PR/HPBCD/ZnHA/ZnGlu	F1	PR
II.	PR/HPGCD/ZnHA/ZnGlu	F2	PR/HPBCD
III.	PR/HPBCD/BK	F3	PR/HPGCD
IV.	PR/HPGCD/BK	F4	PR/HPBCD/ZnHA/ZnGlu
V.	ZnHA/ZnGlu	F5	PR/HPGCD/ZnHA/ZnGlu
VI.	BK		

This method is label-free, non-invasive, and monitors cell adherence, growth, and viability real time. We have successfully tested the cellular effects of pharmaceutical excipients and peptides by impedance kinetics in our previous studies.[32–34] For background measurements 50 µL of cell culture medium was added to the wells, then cells were seeded at a density of 5×10^3 cells/well to 96-well plate with gold electrodes (E-plate 96, ACEA Biosciences) coated with collagen. Cells were cultured for 4–5 days in a CO_2 incubator at 37°C and monitored every 10 minutes until the end of experiments. Cells were treated at the beginning of the plateau phase of growth. The treatment solutions were dissolved in Ringer buffer. Triton X-100 (TX-100) detergent (1 mg/mL) was used as a reference compound to induce cell toxicity. Cell index was defined as R_n-R_b at each time point of measurement, where R_n is the cell-electrode impedance of the well when it contains cells and R_b is the background impedance of the well with the medium alone.

Immunohistochemistry

To evaluate morphological changes in HCE-T cells caused by the different formulations, cell viability assay was followed by immunostaining for junctional proteins zonula occludens protein-1 (ZO-1), occludin, β-catenin, and E-cadherin. Cells were grown on glass coverslips (Menzel-Glaser, Braunschweig, Germany) at a density of 4×10^4 cells/coverslips and treated with different formulations containing PR for 30 minutes. After the treatment, coverslips were washed with phosphate buffer (PBS) and the cells were fixed with 3% paraformaldehyde solution for 15 minutes at room temperature. The cells were permeabilized by 0.2% TX-100 solution for 10 minutes and the non-specific binding sites were blocked with 3% bovine serum albumin in PBS. Primary antibodies rabbit anti-ZO-1 (AB_138452, 1:400; Life Technologies, Carlsbad, CA, USA), rabbit anti-β-catenin (AB_476831, 1:400), rabbit anti-occludin (AB_2533977, 1:100; Life Technologies), and mouse anti-E-cadherin (AB_397580, 1:400; Life Technologies) were applied as overnight treatment. Incubation with secondary antibodies Alexa Fluor-488-labeled anti-mouse (AB_2534088, 1:400; Life Technologies) and anti-rabbit IgG Cy3 conjugated (AB_258792, 1:400) lasted for 1 hour. Hoechst dye 33,342 was used to stain cell nuclei. After mounting the samples (Fluoromount-G; Southern Biotech, Birmingham, IL, USA) staining was visualized by a Visitron spinning disk confocal system (Visitron Systems GmbH, Germany).

Permeability Study on HCE-T Cell Culture Model

Transepithelial electrical resistance (TEER) reflects the tightness of the intercellular junctions closing the paracellular cleft, therefore the overall tightness of cell layers of biological barriers. TEER was measured to check the barrier integrity by an EVOM volt-ohmmeter (World Precision Instruments, Sarasota, FL, USA) combined with STX-2 electrodes, and was expressed relative to the surface area of the monolayers as $\Omega \times cm^2$. TEER of cell-free inserts was subtracted from the measured data.

HCE-T cells were seeded at a density of 10^5 cells onto Transwell inserts (polycarbonate membrane, 0.4 µm pore size, 1.12 cm^2 surface area; 3401, Corning Life Sciences, Tewksbury, MA, USA) and cultured for 5–8 days at liquid–liquid and for 5–8 days at air–liquid interface. The culture medium was changed and TEER was checked every second day.

For the permeability experiments the inserts were transferred to 12-well plates containing 1.5 mL Ringer buffer in the acceptor (lower/basal) compartments. In the donor (upper/apical) compartments 0.5 mL buffer was pipetted containing different formulations (F1–F5) of PR for 30 minutes. To avoid unstirred water layer effect, the plates were kept on a horizontal shaker (120 rpm) during the assay. Samples from both compartments were collected and the PR concentration was detected by HPLC.

To determine the tightness of the cornea epithelial culture model two marker molecules were tested.[34] In the donor compartments 0.5 mL buffer containing fluorescein (10 µg/mL; M_w: 376 Da) and Evans blue labeled albumin (167.5 µg/mL Evans blue dye and 10 mg/mL bovine serum albumin; M_w: 67.5 kDa) was added. The inserts were kept in the multiwell plates on a horizontal shaker (120 rpm) for 30 minutes, then the concentrations of the marker molecules in the samples from the compartments were determined by a fluorescence multiwell plate reader (Fluostar Optima, BMG Labtechnologies, Germany; fluorescein: excitation wavelength: 485 nm, emission wavelength: 520 nm; Evans-blue labeled albumin: excitation wavelength: 584 nm, emission wavelength: 680 nm).

The apparent permeability coefficients (P_{app}) were calculated as described previously.[34] Briefly, cleared volume was calculated from the concentration difference of the tracer in the acceptor compartment ($\Delta[C]_A$) after 30 minutes and donor compartments at 0 hour ($[C]_D$), the volume of the acceptor compartment (V_A; 1.5 mL) and the surface

area available for permeability (A; 1.1 cm^2) using Equation 1:

$$P_{app}(cm/s) = \frac{\Delta[C]_A \times V_A}{A \times [C]_D \times \Delta t} \quad (1)$$

Ex vivo Permeability Assay

The ex vivo permeability model was published previously by Juretić et al.[35,36] Fresh porcine eyes were collected from Large White Pigs (weight 90–115 kg, male and female, 6–7 months) from a local slaughterhouse. Enucleated eyeballs were washed by isotonic saline solution (NaCl, 0.9%; B. Braun, Melsungen, Germany) and stored in Ringer buffer in a container held on ice until application. Transport and excision were performed within 2 hours after the death of animals. Excised corneas were placed into vertical diffusion chambers (Standard Vertical Ussing/Diffusion Chambers, made of acrylic with 0.64 cm^2 diffusion surface, Harvard Apparatus, Holliston, MA, USA). The epithelial side of the cornea was faced to the donor phase of the system. Donor and acceptor phase volume were equally 3.5 mL. After 30 minutes incubation of corneas (Ringer buffer, 37°C), the donor compartment was removed, and 3.5 mL sample was injected. Then 500 µL samples were collected from the acceptor compartment at defined time intervals (0 minutes, 30 minutes, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 hours) and refilled with 500 µL Ringer-buffer at each sample collection. Continuous oxygenation was ensured during the experiment for mixing of the system and mimicking physiological circumstances for the tissue. Six parallels were measured for each type of formulation (F1–F5). PR content of samples was analyzed by HPLC. Apparent permeability was calculated at each sample by using Equation 1.

TEER Measurement in ex vivo Model

During the ex vivo permeability assay, the integrity of porcine corneas was monitored by TEER measurement, to check whether the compounds affect the barrier properties of the model. Ag/AgCl electrodes were used connected with Millicell® ERS-2 Epithelial Volt-Ohm Meter (EMD Millipore Corporation, Billerica, MA, USA). The resistance was measured at 0, 15, 150, and 300 minutes in each cornea containing vertical diffusion chambers. Blank resistance was measured and subtracted to obtain exactly the TEER of ex vivo cornea-based model.

Quantification by High-Performance Liquid Chromatography

The quantitative measurement of PR was performed by high-performance liquid chromatography (HPLC) using Agilent Infinity 1260 (Agilent, Santa Clara, CA, USA). Phenomenex Gemini NX C18 column (150x4.6 mm, 5 µm) was used with the official method of European Pharmacopoeia.[37] The following conditions were applied during the analysis: highly purified and filtered water in channel A, HPLC grade acetonitrile/HPLC grade methanol 50%/50% in channel B, 1 mL/min flow rate, 25°C temperature. Gradient elution was used for the separation. Samples were collected from in vitro HCE-T and ex vivo cornea models; 20 µL volume of samples was injected and analyzed on 254.4 nm wavelength.

Statistical Analysis

All data presented are means±SD. The values were compared using the one-way ANOVA followed by Dunnett test by GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, USA). Changes were considered statistically significant at $P<0.05$.

Results

Barrier Properties of the Cornea Epithelial Cell Culture Model

HCE-T cell layers showed good barrier properties as reflected by the TEER values (246±7 Ω×cm^2, n=3) after 6 days of air-liquid interface condition (Figure 1A). The permeability of HCE-T cell layers was low (Figure 1B) for the hydrophilic marker molecules fluorescein (P_{app}: 1.05 ±0.11×10^{-6} cm/s) and the large biomolecule albumin (P_{app}: 0.10±0.04×10^{-6} cm/s).

Cell Viability Assay

Impedance measurement, as a sensitive method to detect cellular effects, showed significant cell damage after treatment with all three formulations containing BK (III, IV, VI). PR containing formulations with HPBCD, HPGCD, ZnHA, and ZnGlu did not show any cytotoxic effect. The normalized cell index was significantly higher in ZnHA/ZnGlu containing sample (V) than in formulation with BK (VI). As a comparison, maximal toxicity was detected in cells treated with the reference damaging agent Triton X-100 detergent (Figure 2).

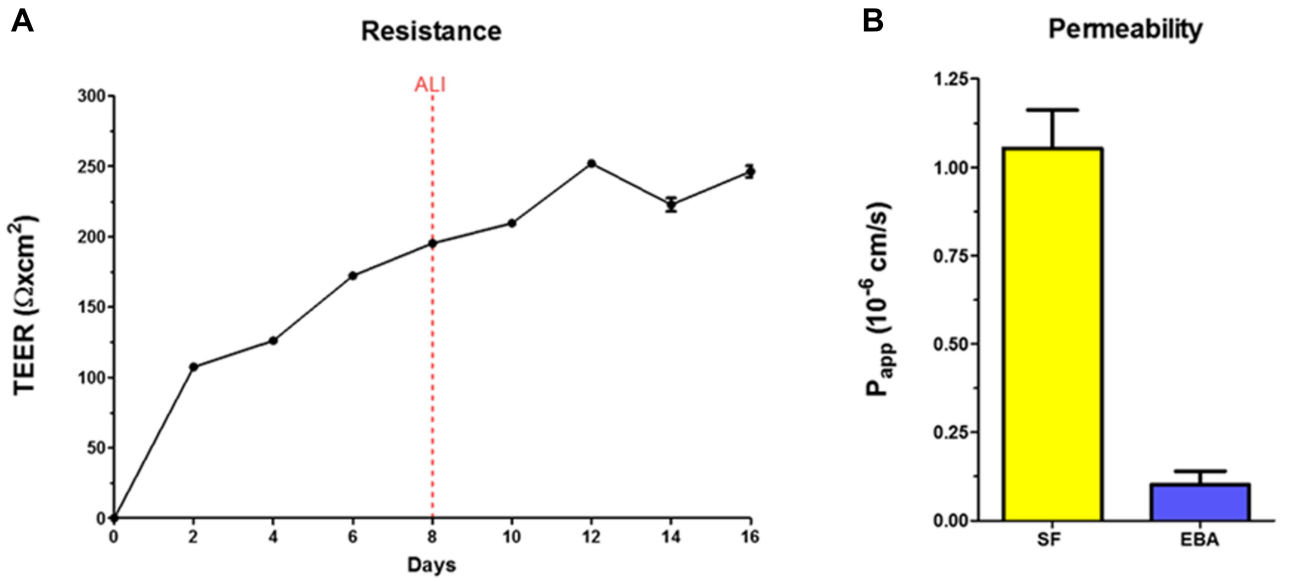

Figure 1 (**A**) Electrical resistance values of the HCE-T cornea epithelial cell layers cultured for 8 days at liquid–liquid interface and for an additional 8 days at air–liquid interface (ALI). (**B**) Permeability of HCE-T epithelial cell layers for fluorescein (SF) and Evans blue labeled albumin (EBA) marker molecules. Values are presented as means ±SD (n=4).

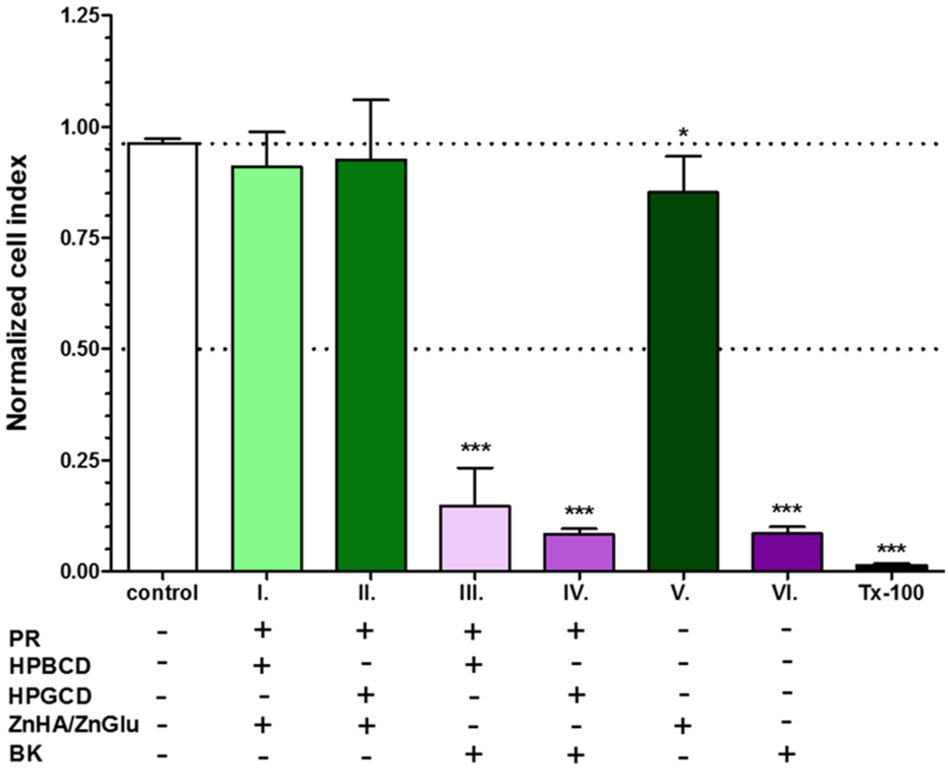

Figure 2 Cell viability of HCE-T corneal epithelial cells after 1-hour treatment with formulations measured by impedance. Values are presented as means±SD (n=6–12). Statistical analysis: ANOVA followed by Dunnett's test (*P<0.05; ***P<0.001 compared to control).
Abbreviation: Tx-100, Triton X-100.

Immunohistochemistry

The cornea epithelial cells formed a tight paracellular barrier visualized by the localization of the junctional proteins ZO-1, β-catenin, E-cadherin, and occludin. The cells were tightly apposed, and all junctional proteins were localized at the intercellular connections forming pericellular belts in the

control groups (Figure 3). Morphological change can be observed in the case of BK-containing sample (VI) by the localization of E-cadherin protein. No major morphological change was seen for the treatment of other groups.

Permeability Study on HCE-T Cell Culture Model

The permeability of PR given in different formulations was tested on the HCE-T cell model (Figure 4). After 30-minute treatment only dissolved PR-CD complex containing formulation 2 (F2) and formulation 3 (F3) showed significantly higher P_{app} values (F2: 5.97×10^{-6} cm/s, F3: 5.97×10^{-6} cm/s) compared with PR suspension (F1: 5.02×10^{-6} cm/s). P_{app} values were minimally lower in ZnHA/ZnGlu-containing formulations (F4: 5.59×10^{-6} cm/s, F5: 5.31×10^{-6} cm/s). The model showed low P_{app} values for the two hydrophilic paracellular marker molecules indicating a good barrier.

Ex vivo Permeability Assay

Permeability of PR was tested on an ex vivo porcine cornea model in the case of previously mentioned formulations (F1–F5) (Figure 5). In the case of PR-HPBCD (F2), permeability was higher (2.05×10^{-7} cm/s) compared with PR-containing suspension (F1, 1.79×10^{-7} cm/s). Permeability in PR-HPGCD (F3) complex-containing samples was lower (1.31×10^{-7} cm/s). Considering the relatively high standard deviations in F1–F3, no significant difference can be stated between their P_{app} values. In the presence of ZnHA/ZnGlu (F4–F5), significantly lower permeability values were measured (F4: 1.05×10^{-7} cm/s, F5: 0.98×10^{-7} cm/s). The monitored TEER values were in the range of 1052–2818 Ω cm^2.

Discussion

Glucocorticoids are widely used as anti-inflammatory drugs in ophthalmology, mostly applied in the therapy of diseases affecting the anterior segment of the eye. Due to the poor water solubility of steroids, they can be found in suspension formulations on the market. Disadvantageous attributes of suspensions like irritation on the eye may induce fast elimination of drug, therefore frequent application is needed to ensure the proper therapy.[4,11] Our aim was a pharmaceutic formulation development, where PR is in water dissolved form with expected increased therapeutic effect. In the early phase of the study, PR-CD inclusion complex was formed and dissolved in a borate-buffer-based environment. The formulations contain ZnHA and ZnGlu to increase the residence time by mucoadhesion and the antimicrobial

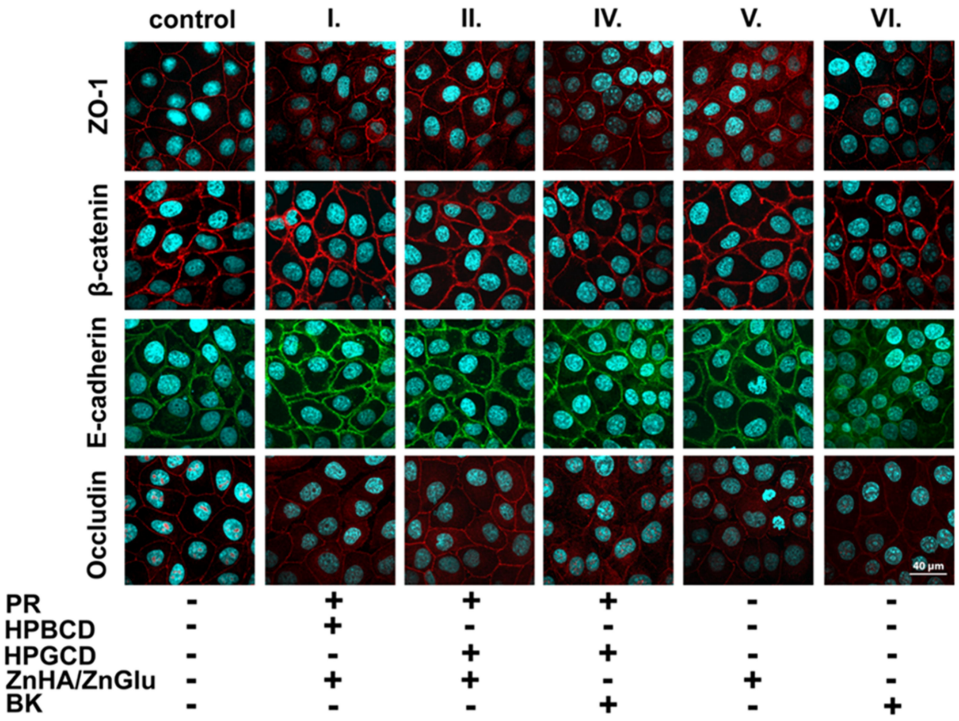

Figure 3 Effects of PR and pharmaceutical excipients containing formulations on junctional morphology of HCE-T corneal epithelial cells. Immunostaining for zonula occludens-1 (ZO-1), occludin tight junction proteins and β-catenin, E-cadherin adherens junction proteins after 1-hour treatment. Red and green color: immunostaining for junctional proteins. Blue color: staining of cell nuclei. Bar: 40 µm.

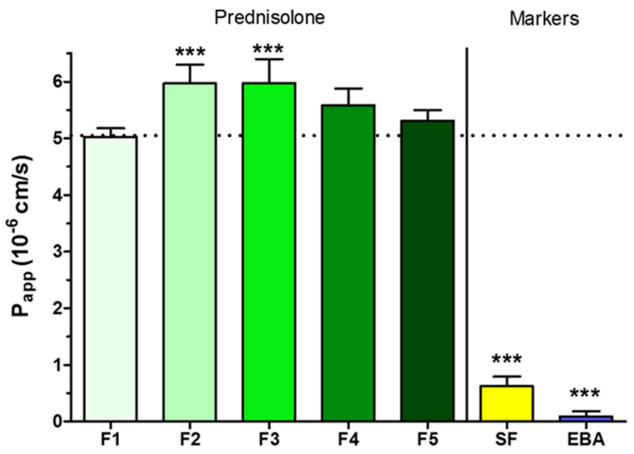

Figure 4 Permeability of PR (100 μg/mL in each formulations) (F1–F5) across HCE-T epithelial cell layers (30-minute assay). Values for paracellular permeability markers fluorescein (SF) and Evans blue labeled albumin (EBA) are also shown. Statistical analysis: ANOVA followed by Dunnett's test. ***P<0.001. Values are presented as means±SD (n=4). P<0.001 compared to F1.

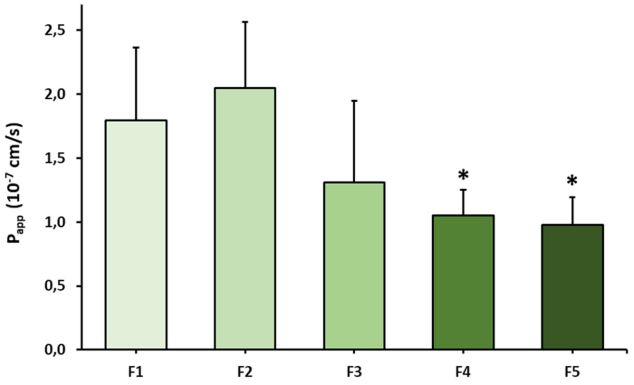

Figure 5 Permeability of PR in different formulations (F1–F5) across in ex vivo porcine cornea model. Statistical analysis: ANOVA followed by Dunnett's test. *P<0.05 compared to F1. Values are presented as means±SD (n=6).

stability by the preservative effect Zn^{2+} ion. In this ophthalmic solution, the physiological parameters were set. Previous investigations confirmed that the formulations have adequate mucoadhesiveness with suitable antimicrobial stability (Ph Eur. requirement B).[12] Continuing the project, our aim was to test the formulations for toxicity and permeability on an in vitro cell culture model and ex vivo cornea to predict the safety and expected absorption after topical application on the eye.

An immortalized human corneal epithelial cell line was used by in vitro toxicity and permeability tests. The cell culture was established by Araki-Sasaki, whose optimal grown attributes are favorable in the studies of ophthalmic formulations. The toxicity was investigated by impedance measurement after the treatment of several types of formulations. According to the calculated normalized cell index, ZnHA-ZnGlu-containing samples are not toxic, meanwhile BK-containing samples show significantly lower values and the toxic effect can be observed on the HCE-T cells. The toxicity of BK-containing formulations was also demonstrated by immunohistochemistry. Major morphological changes were seen on E-cadherin junctional protein by the cells treated by BK-containing formulations. In the case of target eye drops formulated with ZnHA-ZnGlu, no morphological change and no toxic effect were detected on the cell culture. It can be stated that the ZnHA-ZnGlu combination is a non-toxic alternative preservative system, which is tolerable on HCE-T cells in the applied concentration. The results of BK-containing samples confirmed the previously published toxic and expected irritative effect on the eye surface.

Furthermore, the permeability of PR was tested on HCE-T model in different formulations. The applied in vitro model is suitable for the prediction of drug absorption through the lipophilic epithelial cell layer of the cornea. It needs to be mentioned that this in vitro model only shows the permeation through the epithelial layer, for prediction of transcorneal permeation, ex vivo cornea model is preferred. Meanwhile, testing the samples on cell culture, the absorption through the first obstacle of corneal barrier can be simulated. PR-CD complex-containing solutions and PR-CD-ZnHA-ZnGlu-containing target formulations were studied compared with sample which contains the same amount of PR in suspension form. The electric resistance was monitored, and it confirmed the barrier integrity of cell layers. The permeability was significantly higher in samples containing the dissolved PR-CD complex compared with the suspension. Because the concentration of dissolved API is higher in PR-CD solutions, and higher towards the corneal epithelial cell layer, faster drug permeation is expected through the epithelial layer. In the target formulations with biopolymer, the permeability is minimally lower, due to the diffusion restrictive effect of the polymer structure.

Transcorneal permeation of PR was investigated by ex vivo porcine cornea model. Freshly resected corneas were put into vertical diffusion chambers. O_2 was continuously circulated in the chambers to ensure the respiration of cornea, and the donor and receptor phases are mixed during the experiment. The same compositions were tested as in the HCE-T model. The monitored TEER was increased during the experiment, the integrity of the cornea was acceptable, samples did not cause damage and toxic effects on the

barrier, which can affect the permeability of PR. Compared with published results, these values meet the requirement of barrier properties.[35,37] According to the results, no significant difference was found between the eye drop suspension and solutions. Permeability values were significantly lower in ZnHA-ZnGlu-containing formulations in comparison with the suspension form. Considering the continuous mixing, vertical position, and complexity of porcine cornea, the optimal attributes are not observed in this model in solution-based samples. The permeability is lower in target formulations because polymer structure affects the drug diffusion negatively; however, the retention time is increased on the eye surface due to the increased viscosity and mucoadhesion. In the case of ex vivo study the whole porcine cornea is used, which is complex, formed by lipophilic and hydrophilic layers. Therefore, the difference shall be observed in the permeability on in vitro HCE-T and ex vivo porcine cornea models. The formulations can be optimal in vivo, because less irritation and lachrymal secretion are expected due to the not irritative solution form of eye drops.

As the eye drop contacts for longer time, reflex lachrymal secretion and eye blinking are limited, prolonged drug absorption is expected after administration, which results in less frequent application. We assume that the permeability of PR is optimal in the target formulations, which may result in an enhanced therapeutic effect.

Conclusions

In summary, novel ophthalmic formulations were developed, where PR is dissolved in a water-based environment as CD inclusion complex. Eye drops contain a mucoadhesive and alternative preservative ZnHA-ZnGlu system. We found that the formulations are non-toxic with optimal permeability according to the results of in vitro and ex vivo models. These compositions are promising for overcoming the challenges of ocular drug delivery by increased bioavailability, ensured microbiological stability, minimal irritation, and acceptable patient compliance.

Author Contributions

All authors contributed to the data analysis, drafting, or revising of the article, have agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest.

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