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Enhancement in corneal permeability of riboflavin using cyclodextrin derivates complexes as a previous step to transepithelial cross-linking

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A R T I C L E I N F O

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A B S T R A C T

Corneal cross-linking has been described as an effective treatment to slow the progression of keratoconus. The standard protocol entails corneal epithelial removal to allow the diffusion of riboflavin into the stroma. Although, de-epithelization can generate risks or complications that transepithelial cross-linking tries to solve or avoid. Different formulations were developed after verifying that hydroxypropyl-β-cyclodextrin (HPβCD) and sulfobuthylether-β-cyclodextrin (SBEβCD) in a 20% concentration, increased the solubility of practically insoluble in water drugs such as riboflavin from 0.12 mg/mL to 0.35 mg/mL and 0.29 mg/mL respectively. These values were higher when chitosan and arginine were added to the formulation, showing solubility of 0.78 mg/mL when HPβCD concentration was not modified. *Ex vivo* corneal permeability was measured after having kept in contact bovine corneas with intact epithelium for 5 h with the 0.1 mg/mL riboflavin solution, the formulations developed and a reproduced nanoemulsion from another work. Riboflavin's permeability was increased when cyclodextrins, chitosan, and arginine were part of the formulations, compared to the control drug solution. The best permeability coefficient was reached when riboflavin was combined with 40% (w/v) HPβCD, 0.5% (w/w) arginine, and 0.5% (w/w) chitosan. After having carried out toxicity studies as bovine corneal opacity and permeability (BCOP) and Heńs Egg Test - Chorioallantoic Membrane Test (HET-CAM) it was verified that both, the active ingredients and the excipients of the different formulations were not harmful without generating irritation, loss of transparency or corneal permeability alterations.

The results show the great potential of the ocular developed solution for their use in transepithelial cross-linking for keratoconus treatment.

1. Introduction

Keratoconus is a corneal ectasia that results in a cone-shaped deformation, an irregular surface, and a progressive thinning of the cornea with the consequent vision problems, which cannot be corrected with spectacles or contact lenses. The origin is in biomechanical alterations of stromal collagen, whose specific cause is still unknown [1].

The current goal is to slow the progression of keratoconus to prevent symptoms from worsening and prevent more serious situations requiring a corneal transplant since keratoconus was the principal cause of keratoplasty in the last years [2].

Abbreviations: HPβCD, hydroxypropyl-β-cyclodextrin; SBEβCD, sulfobuthylether-β-cyclodextrin; BCOP, bovine corneal opacity and permeability; HET-CAM, Heńs Egg Test - Chorioallantoic Membrane Test; PBS, phosphate-buffered saline; α-CD, α-cyclodextrin; HPMC, hydroxypropyl methylcellulose; PVP, polyvinylpyrrolidone; CE, complexing efficiency; D:CD, drug: cyclodextrin ratio; HPLC, high-performance liquid chromatography; CAM, chorioallantoic membrane; IS, Irritation score; OD_{490}, optical density at 490 nm; CD, cyclodextrin; Zeta potential, ζ.

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Corneal cross-linking concept was introduced by Spoerl and Seiler in 1998 and until now is the most effective method to stop or delay keratoconus progression [3]. The first treatment based on corneal collagen crosslinking was carried out by Wollensak et al. for the keratoconus treatment, although its usefulness in other pathologies such as microbial keratitis, corneal ulcers, bullous keratopathy, pellucid marginal degeneration, or post-Lasik ectasia has also been studied [4]. The procedure involves the use of UVA and a riboflavin solution (base or phosphate) as a photosensitizing molecule.

Riboflavin as photosensitizer is excited into its single or triplet state with the help of UVA radiation at 370 nm generating reactive oxygen species that interact with stromal collagen and proteoglycans, which will be subjected to a photo-oxidation, making it possible to generate links between them. That is why oxygen is essential to drive the process in the early phase through a type II photochemical reaction. It exists an anaerobic second phase in which corneal stroma interacts with radical ions [3,5,6].

The standard Dresden epithelial-off corneal cross-linking (epi-off CXL) protocol, which was developed by Wollensak et al., entails epithelial removal before applying the 0.1% (w/v) riboflavin-5-phosphate in 20% (w/v) dextran solution, to allow the diffusion of riboflavin into the stroma. The solution is administered every 2–3 min for 30 min. After this time, the stromal thickness must be verified before irradiating corneas with UVA (370 nm) at 3 mW/cm^2 for 30 min, which amounts to an energy of 5.4 J/cm^2 [7]. In this protocol, riboflavin not only acts as a photosensitizer but also like photoprotector since it absorbs an extensive amount of UVA irradiation [2,8,9], protecting structures as endothelium, lens, or retina. For this reason, the riboflavin phosphate solution is also applied every 2–3 min at the same time as UVA irradiation, ensuring an adequate riboflavin concentration in the stroma but also limiting the crosslinking effect to the anterior 200–300 μm of the cornea.

Despite epi-off CXL are an effective and very extended method, these methods cause severe pain, discomfort, and some other visual serious impairments as a consequence of epithelial removal. Endothelium and intraocular structures could be damaged when the UVA dosimetry, riboflavin concentration, or corneal thickness are not adequate [8]. Besides, the quantity of corneal infections is increased due to the de-epithelialization before the procedure, as well as infiltrates, scarring, pain, or recovery time, not to mention the time it takes to carry out the procedure [10].

To solve these risks or complications, some variations of the initial protocol have emerged which does not require the epithelial removal denominates epithelium-on corneal cross-linking (epi-on CXL) such as the use of a hypo-osmolar solution of riboflavin base or phosphate in thin corneas, pulsed cross-linking, accelerated cross-linking with a higher UVA power, improving the diffusion of riboflavin through the epithelium by iontophoresis or absorption enhancers, or by introducing it directly into the stroma [11].

Transepithelial cross-linking has been studied to solve the risks and discomforts that epithelial debridement entails. With the standard riboflavin solution, the effectiveness of transepithelial cross-linking is reduced. Although the epithelium does not block the passage of UVA light, it prevents the penetration of an adequate amount of riboflavin into the stroma [12].

Riboflavin is a practically insoluble in water drug with a molecular weight of 376 Da. Both, its phosphate salt form and riboflavin base will have difficulties in reaching the stroma due to their physicochemical properties.

The standard solution consists of 0.1% (w/v) riboflavin-5-phosphate in 20% (w/v) dextran solution [7], with hydrophilic characteristics, which are going to be an impediment to permeate the lipophilic epithelium and a molecular weight greater than 180 Da, which is going to prevent the passage through the paracellular pathway by intercellular tight junctions. Besides, there will be repulsive forces between the negative charges of the anion and the negatively charged corneal surface.

On the other hand, if a riboflavin-base solution is used, its lipophilic behavior will cause a low solubility in the aqueous tear film and the mucus, composed of water and mucins, that line the surface of the epithelium with which riboflavin will interact, preventing its diffusion to the stroma[13–15].

Lately, some strategies such as the use of benzalkonium chloride [16], edetate disodium or tromethamine [17], to enhance riboflavin penetration through the intact epithelium had been carried out. However, *in vivo* and clinical studies show doubts about the effectiveness of any of these epi-on CXL proposed strategies. For example, clinical studies developed using 0.1% (w/v) riboflavin-5-phosphate in 20% (w/v) dextran solution containing benzalkonium chloride/edetate disodium as promoters did not show effective improvements. The most probable cause of these negative results was the hypertonicity of the ocular solution and that high molecular weight dextran can inhibit the penetration of riboflavin phosphate in the cornea [18,19].

Better results in clinical studies have been obtained using d-α-tocopheryl poly(ethylene glycol) 1000 succinate [20,21] or sodium iodide based formulation [22,23]. Both studies have shown an improvement of the riboflavin corneal penetration, a stoppage of cone progression and, a visual acuity improvement, showing the great potential of these two epi-on CXL techniques.

Research has also tested epi-on CXL approaches based on colloidal drug delivery systems. Liposomes [24] microemulsions [25,26] and nanoemulsions [27] have been studied to promote the corneal penetration of riboflavin. The use of riboflavin-5′-monophosphate encapsulated in conventional and propylene glycol–containing liposomes did not provide any improvement of riboflavin penetration into the cornea [24]. However, microemulsions and nanoemulsions have shown an improvement of the riboflavin corneal incorporation. Nanoemulsions containing riboflavin-5-phosphate or riboflavin base induced greater *in vitro* changes in corneal transmittance and absorption coefficients of rabbit corneas (riboflavin-5-phosphate > riboflavin base) due to the riboflavin penetration compared with 1% (w/w) riboflavin-dextran standard solutions [26]. For his part, *ex vivo* preliminary studies developed in corneas of freshly enucleated New Zealand White rabbit treated with water-dilutable microemulsions containing riboflavin phosphate shows an increase in biomechanical stiffness of eye rabbits but with less intensity than classical epi-off CXL treatment [27].

Cyclodextrins [28] has also been proposed to improve the riboflavin base solubility and corneal permeability. Preliminary studies using 30 mg/mL cyclodextrin aqueous solutions containing 0.08 mg/mL of riboflavin showed the ability of natural β-cyclodextrin and its hydroxypropylated derivative to improve both riboflavin base solubility and apparent corneal permeability coefficient. However, natural β-cyclodextrin has low water solubility (1.9% (w/v) at 25 °C and slightly more than 3% (w/v) to 37 °C) which limits its ability to solubilize riboflavin, so hydroxypropyl-β-cyclodextrin (HPβCD) has greater potential for the preparation of eye drops for epi-off CXL. The combination of HPβCD, EDTA and trometamol has also been studied [29,30]. The soaking of human sclerocorneal tissues with a solution containing 0.1% (w/v) riboflavin base, 2.6% (w/v) HPβCD, 0.2% (w/v) EDTA and 0.268% (w/v) trometamol produces an improvement in the riboflavin penetration and the biomechanical strength of the anterior corneal stroma.

This work aimed to carry out a comparative study between different cyclodextrin formulations of riboflavin base to verify which of them provides a greater solubility to get a greater concentration in the aqueous tear film and thus facilitate the diffusion, as well as a greater permeability through the cornea, exploring whether corneal properties as transparency or permeability are modified after exposure to formulations as well as identify their irritating potential. The riboflavin base-Nanostructured System proposed by Bottos et al. [27] was used as a reference. These researches developed a new riboflavin nanoemulsion to achieve riboflavin penetration through the corneal epithelium and thus carry out transepithelial crosslinking. This formulation brings together

the characteristics that allow riboflavin to cross the corneal epithelium and to be retained in the stroma due to the amphiphilic character of the formulation. Despite this, the time needed for riboflavin to reach the stroma at concentrations that allow safe treatment remains too long.

The comparative study previously mentioned was carried out with formulations containing different cyclodextrins derivatives and concentrations (β-cyclodextrin derivates and α-cyclodextrin) used as biocompatible compounds, drug release modulators, solubility and permeability enhancers of poorly water-soluble drugs like riboflavin [15] and polymers such as chitosan alone or combined forming binary or ternary compounds respectively.

Chitosan is a polysaccharide biocompatible and biodegradable which is also appropriate in the case we are dealing with because of its positive charge that can facilitate negative corneal surface interaction, bioadhesive and penetration enhancer behavior [31]. Besides, it is has been reported that chitosan can improve corneal wound healing [32] and change collagen microfibrillar structure providing a greater corneal strength [33].

2. Materials and methods

2.1. Materials

The bovine eyes were obtained from the local slaughterhouse (Compostelana de Carnes S.L, Santiago de Compostela, Spain) and transported in phosphate-buffered saline (PBS) in cold conditions to the laboratory where the corneas with 2–4 mm of the surrounding sclera were excised with a scalpel.

The phosphate-buffered saline (PBS) was prepared according to the 9th edition of the European Pharmacopoeia, from potassium dihydrogen phosphate (GPR Rectapur, Spain), sodium chloride (Labkem, Spain) and di-sodium hydrogen phosphate dodecahydrate (Scharlau, Spain) and adjusted to pH 7.4 using 0.1 M solution of sodium hydroxide (Merck, Spain).

Components of the formulations included riboflavin-base (Acofarma, Spain), hydroxypropyl-β-cyclodextrin (HPβCD, Kleptose HPB® parenteral grade with a 0.65 M substitution ratio and MW 1399 Da, Roquette, France), sulfobutyl ether-β-cyclodextrin (SBEβCD, average degree of substitution of 6.5 and MW 2163, Captisol®, Cydex USA), α-CD (α-cyclodextrin, Ashland Switzerland), arginin-L chlorhydrate (Acofarma, Spain), lysin-L monochlorhydrate (Acofarma, Spain), low substitution hydroxypropyl methylcellulose (HPMC) Methocel® 4KM (Dow Chemical, USA), polyvinylpyrrolidone (PVP) (Acofarma, Spain) MW = 111.1, caprylic/capric triglyceride Captex® 200P (Abitec corporation, USA), fluid soy lecithin (Acofarma, Spain), polysorbate 80 (Guinama, Spain), octadecylamine (Aldrich, Spain), poloxamer 407 (Kolliphor® P 407, Sigma, Spain), chitosan hydrochloride Seacure® 210Cl MW < 50 kDa, DD 87, stearyl amine (Merck, Spain).

2.2. Methods

Since riboflavin is photodegradable, all experiments were carried out protecting the solutions of the light, and concentration analysis were carried out at the time of sampling to prevent riboflavin from degradation.

2.2.1. Phase solubility test

Different solutions without and with cyclodextrins (SBEβCD, HPβCD, and α-CD) with increasing concentrations were prepared with PBS as the solvent and added to an excess amount of riboflavin-base (0.24 mg/mL) to get a saturated solution. After five days of incubation to get a balance between free riboflavin and riboflavin associated with cyclodextrins, solutions were incubated under constant temperature and horizontal stirring conditions (100 rpm, 25 °C) with VWR incubating Mini Shaker (Spain), taking care that the solid does not run out. Solutions were centrifugated for 20 min at 10,000 rpm and 25 °C (Eppendorf Centrifuge 5804 R). The amount of riboflavin solubilized was measured by spectrophotometry (Agilent Technologies Cary 60 UV–vis, USA) at 267 nm corresponding to riboflavin absorption λ_{max}, after filtering the solutions with nylon syringe filter (BIOFIL, Spain) of 0.45 μm and making the necessary dilutions. Besides, different concentrations of amino acids and polymers like arginine (0.25%, 0.5% and 1% (w/v)), lysine (0.25%, 0.5% and 1% (w/v)), HPMC (0.125% and 0.25%(w/v)), PVP (0.25%(w/v)) or chitosan (0.5% (w/v)) were added to these cyclodextrin solutions, in order to find out their effect on the solubility. Each experiment was repeated six times.

Solubility diagrams were elaborated according to the Higuchi and Connors method [34] and the apparent stability constant (k) assuming the formation of inclusion complexes of cyclodextrin with 1:1 stoichiometry, was calculated from the slope of the diagrams according to the equation:

$$k_{1:1} = slope/S_0(1 - slope) \tag{1}$$

The complexing efficiency (CE) and the Drug:Cyclodextrin (D:CD) ratio were calculated according to the equations [35]:

$$CE = \frac{[D/CD]}{[CD]} = S_0 \times K_{1:1} = slope/(1 - slope) \tag{2}$$

$$D : CD = 1/\left(1 + \frac{1}{CE}\right) \tag{3}$$

2.2.2. Formulations preparation

2.2.2.1. Cyclodextrins-riboflavin complex solutions. Different cyclodextrins solutions were prepared with PBS as the solvent. The cyclodextrin used was HPβCD (20% (w/v) and 40% (w/v)) single or combined with 0.5% (w/v) arginine or/and chitosan (see Table 1). Riboflavin-base was added in excess to get a saturated solution and stirred for 12 h. Finally, the suspensions were filtered (0.22 μm cellulose filters) and the actual concentration of riboflavin was measured.

2.2.2.2. Riboflavin nanoemulsion. To get the adapted 0.5% (w/v) riboflavin nanoemulsion (*E)* proposed by Bottos et al [27], 5% (w/w) Captex® as oil phase, stearyl amine 0.3% (w/w) as cationic agent, polysorbate 80 12% (w/v), kolliphor® P 407 2% (w/v) as surfactant and soy phosphatidylcholine 12% (w/v) were completely homogenized applying some heating. Riboflavin-base was incorporated into the oil phase. The PBS as aqueous phase 68.2% (w/v) was added to the lipid phase after having been submitted to the same temperature while stirring.

Nanodroplet size was achieved by sonication 15 min, using 1-minute pulses and 1-minute intervals with a 20% amplitude using a probe tip sonicator (Branson Digital Sonifier) taking care not to increase the temperature with an ice bath. Particle size and Zeta potential (ζ) of nanoemulsions were determined using a Malvern Instruments Zetasizer Nano (Malvern, UK).

2.2.3. In vitro riboflavin release assay

Riboflavin release of different formulations was studied *in vitro* using Franz diffusion cells with an effective diffusion surface of 0.784 cm^2. The assay was carried out at 35 °C for 8 h by triplicate. The receiver compartment was filled with 6 mL of PBS (ensuring that there were no air bubbles in the compartment) and the donor compartment with 1 mL of formulation. The donor compartment was sealing with Parafilm M™ to prevent evaporation. Visking dialysis tubings of a Cutoff of 12–14 KDa (Medicell membranes Ltd) were used as a diffusion membrane. Aliquots (1 mL) were taken at stipulated time intervals and replaced with 1 mL of PBS. The assay was carried out for 24 h. Riboflavin measurements were taken by spectrophotometry at 267 nm after filtering samples (0.45 μm nylon filter).

The amount of riboflavin released versus time was analyzed with the

Table 1
Composition of the solutions used in this work. (% w/v for 10 mL in PBS).

Components/Formulations	R	20	40	20A	20AQ	40A	40AQ	Q
Riboflavin	Saturation							
HPβCD	–	20%	40%	20%	20%	40%	40%	–
Arginine	–	–	–	0.5%	0.5%	0.5%	0.5%	–
Chitosan	–	–	–	–	0.5%	–	0.5%	0.5%

software GraphPad Prism 6.01 (GraphPad Software, La Jolla, CA, USA).

Various kinetic models were used to determine the release kinetics according to the following equations:

$$\text{Zero}-\text{order}: \textit{Released amount} = kt \quad (4)$$

$$\text{First}-\text{order}: \ln(\textit{released amount}) = \ln(\textit{initial amount})kt \quad (5)$$

$$\text{Higuchi}: \textit{Released amount} = kt^{1/2} \quad (6)$$

$$\text{Korsmeyer}-\text{Peppas}: \textit{Fraction released} = kt^{n} \quad (7)$$

2.2.4. *Ex-vivo corneal permeability assay*

Bovine corneas have been used as a substitute for human corneas by different authors due to the difficulty of supplying them [36–39]. In this study, bovine corneas were used as a model to carry out the permeation study. Similar conditions to the *in vitro* release assay were used, but instead of artificial membranes, bovine corneas were placed between donor and receiver compartments of Franz diffusion cells. The corneal epithelium was oriented to the donor chamber. Especial care was taken to avoid damaging in the corneal epithelium before mounting the cornea in the Franz diffusion cells. The corneal surface was meticulously visually inspected to ensure no alterations occurred during handling. Riboflavin measurements were taken with liquid chromatography.

The fact of using bovine corneas limits the duration of the assay to 6 h. Permeability constants were calculated according to the equation:

$$P_{app} = J/C_d \quad (8)$$

where J is the flux of riboflavin across the cornea calculated from the linear portion of the plot that represents the accumulated amount of permeabilized drug versus time, and C_d is the riboflavin concentration in the donor compartment.

The corneal integrity was also tested after the end of the assay. For this, once the formulation was removed, a dextran-Blue solution (2000 KDa) was incorporated, observing that its penetration did not occur in the cornea.

2.2.5. *Liquid chromatography analysis*

High-performance liquid chromatography (HPLC) analysis was performed with Merck Hitachi equipment and a column Waters Symmetry C18 (3.9 × 150 mm, 5 µm). Chromatographic method consisted in a mobile phase 30:70 (v/v) methanol HPLC grade (Fisher): ion-pair buffer (methanesulphonic acid (VWR chemicals) 0.05% (w/v) / phosphoric acid 85% (Panreac) 0.1%), a flow rate of 0.8 mL/min, a 50 µL injection volume and Merck Hitachi L-4500 Diode Array UV detector at 267 nm.

2.2.6. *Modified bovine corneal opacity and permeability (BCOP) assay*

BCOP assay was carried out following the INVITTOX N°127 protocol with some variations [40–43]. The next paragraph shows the protocol and modifications used to made transmittance, opacity, and fluorescein permeability measurements.

Transmittance and opacity: Transparency of removed corneas was measured in transmittance values with the Cary 60 UV–vis spectrophotometer from Agilent technologies (USA) taking measurements from 800 nm to 200 nm with the corneas placed in a solid sample holder between the lamp and the detector (Fig. 1). Corneal opacity was measured by determining the light intensity passing through the cornea with a lux meter (Gossen Mavolux lux meter 5032c) by applying a light source (Olympus Europe Highlight 2000 Fiber Optic Light Source, Spain). Corneas were placed between two 3D printed cylindrical black holders adapted to the cornea with a hole that allows the passage of light from the source to the lux meter (Fig. 2) and the difference of light intensity without and with cornea was calculated ($\%T_{initial}$).

Fig. 1. Bovine cornea placed in the solid sample holder of the Agilent Cary 60 UV–vis spectrophotometer.

After the first opacity readings of untreated corneas, they were incubated in Franz cells for 60 min with 6 mL of PBS in the receptor compartment and 1 mL of PBS in the donor at 35 °C before re-measuring their transmittance and opacity. Then, 1 mL of the formulations to be tested, positive control (ethanol absolute VWR chemicals, Spain) and negative control (PBS) were placed in the donor compartment and were incubated for 10 min. After this, products on the donor chamber were removed to add 1 mL of PBS. After 120 min of incubation, transmittance and opacity were measured again ($T\%_{10min}$). Three corneas were used for each formulation.

Fluorescein permeability: After incubation for opacity and transmittance studies, 1 mL of a 0.4% (w/v) fluorescein aqueous solution was added to the donor compartment and incubated for 90 min keeping 6 mL

Fig. 2. Bovine cornea placed in the cylindrical holder between the light source and the lux meter.

of PBS in the receptor compartment. Samples were collected at 30, 60, and 90 min and the fluorescein concentration in the receptor chamber was determined using the spectrophotometer and a calibration curve at 490 nm.

2.2.7. Heńs egg test - Chorioallantoic membrane test method

The Hen's Egg Test-Chorioallantoic Membrane Test Method (HET-CAM TEST) is an organotypic method that uses the chorioallantoic membrane (CAM) of embryonated chicken eggs to detect the irritant potential of chemicals during research. The test was used to obtain toxicological information about the studied formulations.

Broiler fertilized eggs were incubated in a YZ-56S incubator for 9 days at 38 °C and 70% relative humidity, after which eggs were opened by cutting the shell on the side where the air cell is located with a tiny drill (Dremel®), the inner shell membrane was removed with a forceps, and the CAM was left exposed. 300 μL of each formulation object of study was deposited into each egg by triplicate, as well as 300 μL of positive (NaOH 0.1 N) and negative (NaCl 0.9%) control.

The formulations tested were R, 40, and 40AQ to analyze the differences between the formulation that does not contain cyclodextrin, chitosan, or arginine, with those that contain them in greater concentration.

Blood vessel behavior (hemorrhage, lysis, coagulation) after 300 s was analyzed and compared with positive control. Irritation score (IS) was calculated according to the equation:

$$IS = [((301 - haemorrhage\ \ time)/300) \times 5] + [(301 - lysis\ \ time)/300) \times 7] + [((301 - coagulation\ \ time)/300) \times 9] \quad (9)$$

Values between 0 and 0.9, 1–4.9, 5–9.9, 10–21 of IS are indicative of non-irritating substances, slightly irritating substances, moderately irritating substances, and severely irritating substances respectively [44].

2.2.8. Molecular modelling

Molecular modelling of the riboflavin base or flavin/HPβCD 1:1 inclusion complex was carried out using Chem3D® (PerkinElmer) software. For this chemical structure and 3D model of riboflavin-base was obtained from PubChem (ID 23666409). The ribityl substituent was eliminated from the riboflavin to obtain the flavin. The initial geometry of complexes was obtained by manual docking of riboflavin-base or flavin molecules into the HPβCD cavity. The conformational energy of the geometry was minimized using the MM2 force field to minimum RMS gradient of 0.01.

3. Results and discussion

3.1. Phase solubility test

Cyclodextrins have been used to solubilize lipophilic drugs in aqueous solutions [15,35]. In the case that concerns us, we can expect that they will enhance the riboflavin solubility in the aqueous tear film and thus reducing the resistance of the aqueous eye layer improving the penetration to the corneal stroma [45,46].

Riboflavin solubility obtained experimentally shows low aqueous solubility in PBS ($S_0 = 0.12$ mg/mL (± 0.014)) providing slightly higher values than the literature [28,47,48].

Phase solubility diagram (Fig. 3) shows the formation of A_L type inclusion complex with all cyclodextrins, indicative of the formation of soluble complexes. Regarding αCD, due to its limited aqueous solubility, the experiment could only be conducted at 15% (w/v). The values of apparent stability constants of inclusion complexes were calculated assuming the formation of a stoichiometry 1:1 based on the linear increase of the riboflavin solubility with cyclodextrin concentration with a slope less than unity (Table 2). HPβCD and SBEβCD show similar

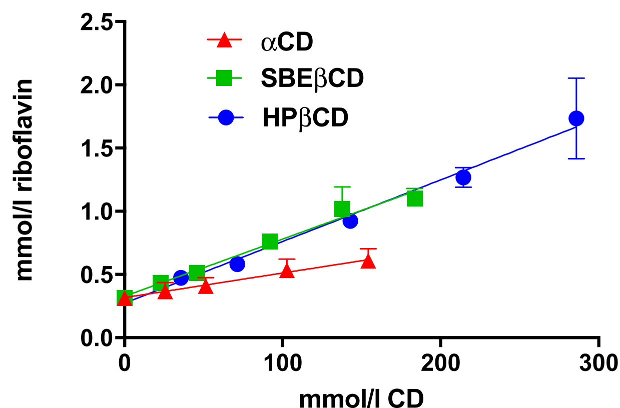

Fig. 3. Phase Solubility diagrams of riboflavin in different cyclodextrin solutions.

riboflavin complexation parameters with equivalent values of stability constant, CE, and D:CD and in both cases significantly higher than αCD. The values of $K_{1:1}$ of HPβCD are in accordance with the 26.6 M^{-1}calculated by Bispo de Jesus et al in 2012 in water at 25 °C [49]. In all cases, results show a weak interaction between cyclodextrins and riboflavin, which is in agreement with previous studies [49,50].

The riboflavin molecule is d-ribitol in which the hydroxy group at position 5 is substituted by a 7,8-dimethyl-2,4-dioxo-3,4-dihydrobenzo [g]pteridin-10(2H)-yl moiety (Fig. 4). βCD derivatives have a cavity size well adapted to include molecules with aromatic moieties. However, the ribityl substituent and the presence of oxo groups in 2,4 and the methyl residues in 7,8 can hinder the incorporation of the molecule to the cyclodextrin cavity. In this line, it has been previously reported that the ribityl substituent prevents deeper insertion of riboflavin into the CD cavity. Zielenkiewicz et al [51] suggest a partial insertion of the benzene ring of riboflavin into the HPβCD based on ^1H NMR and molecular mechanics calculations. Nevertheless, in 2012 Bispo de Jesus et al [49], studying the interaction of the βCD and HPβCD and the complex topology using differential scanning calorimetry, different NMR techniques, and molecular simulation, proposed that the increase in the solubility of riboflavin in presence of CDs is a result of a non-inclusion complex formation.

To evaluate the possible influence of the ribityl substituent in the complex formation a molecular modeling experiment was made using Chem3D software (PerkinElmer). For this, the Chemical structure and 3D model of riboflavin were obtained from PubChem (ID 23666409). Furthermore, the ribityl substituent was eliminated from the riboflavin to obtain the flavin. To study de molecular model of the HPβCD complexes with flavin and riboflavin manual docking of drug molecules into the cyclodextrin cavity was made and the energy was minimized by using MM2 force field. Results are shown in Fig. 4. Total energy obtained for complexes was 92.6705 kcal/mol or 95.3785 kcal/mol for flavin and riboflavin inclusion complexes. The elimination of the ribityl substituent increases the stability of the complex decreasing energy especially the torsion energy (24.4451 vs 31.4755 kcal/mol). Therefore, in accordance with this study, the presence of the ribityl substituent could decrease the affinity of riboflavin for HPβCD cavity.

Regardless of whether a true inclusion complex or a non-inclusion complex is formed the HPβCD is the most effective CD increasing the riboflavin solubility. At the concentration of 20% (w/v), HPβCD produces a riboflavin solubility increase up to 0.35 mg/mL in contrast to the 0.29 mg/mL of SBEβCD and the 0.22 mg/mL of 15% of αCD. These data show a higher solubilization capacity than the 0.12–0.19 mg/mL or the 0.11 mg/mL of riboflavin dissolved using solutions of 1% to 3% (w/v) βCD or 10% (w/v) αCD respectively reported by Morrison et al. [28].

HPβCD and SBEβCD complexes show similar apparent stability constant and complexation efficiency but due to the lower molecular

Table 2
Values of stability constant of the complex ($K_{1:1}$), encapsulation efficiency (CE), and the ratio between drug and cyclodextrin (D:CD) calculated with the slope and the initial solubility (S0) obtained from the solubility diagrams.

Cyclodextrin	S_0 (M)	S_0 (mg/mL)	Slope	$K_{1:1}$ (M^{-1})	CE	D:CD	R^2
HPβCD	0.0003	0.11	0.0049	16.41	0.0049	1:204	0.9909
SBEβCD	0.0003	0.11	0.0045	15.06	0.0045	1:222	0.9831

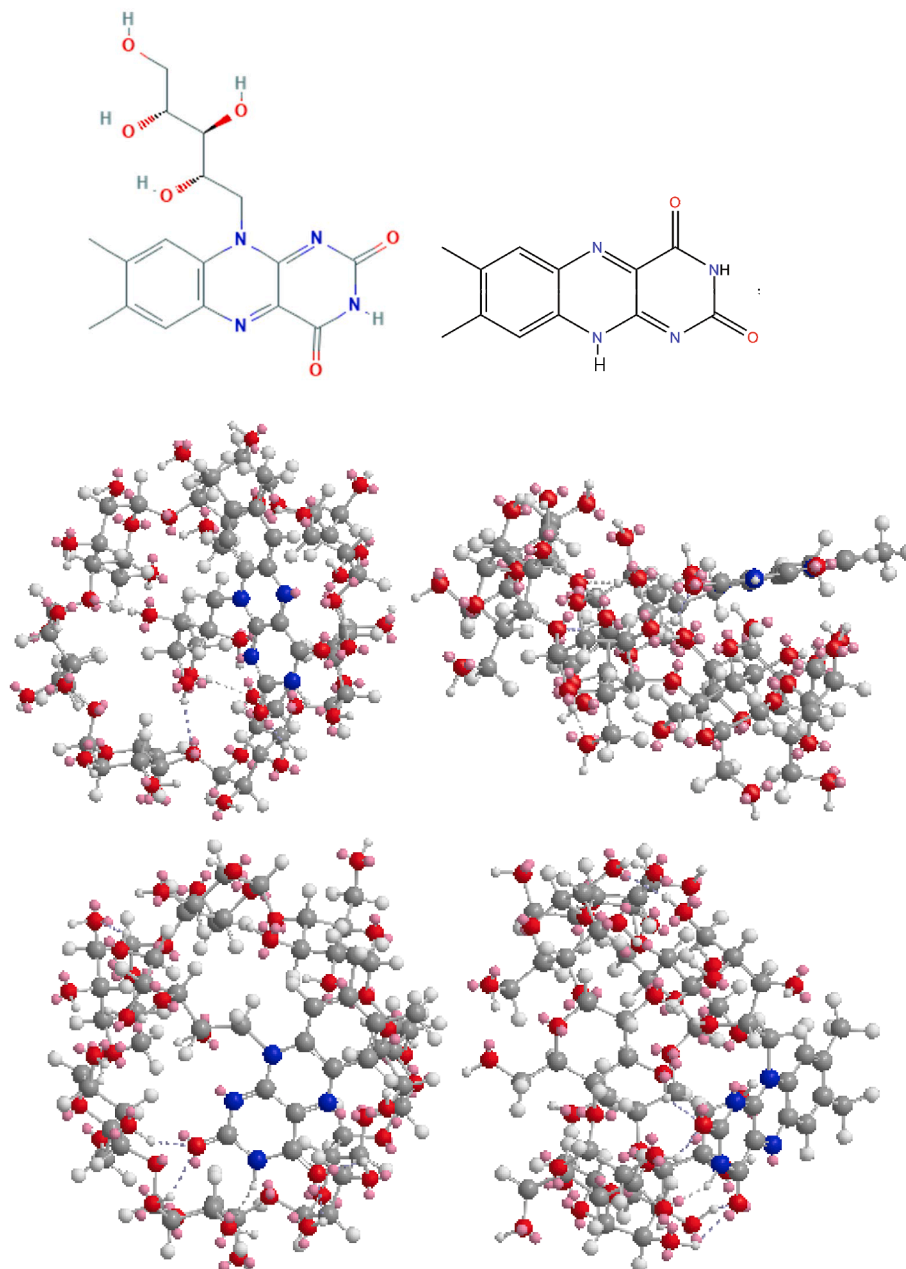

Fig. 4. Chemical structure of riboflavin and flavin (top) and 3D model of their inclusion complexes with HPβCD. Middle: riboflavin inclusion complex; Bottom: flavin inclusion complex.

weight of HPβCD compared SBEβCD (1399 Da vs 2163 Da) for the same weight in solution, HPβCD provides significantly higher riboflavin concentrations.

Nonetheless, in all cases, the magnitude of complexation is weak, and different alternatives have been proposed to increase the solubilizing power of cyclodextrin as well as their solubilization capacity. The formation of ternary complexes using amino acids [52,53] and water-soluble polymers [35,52,54,55] in many cases allows reducing formulation or toxicity problems by reducing the amount of cyclodextrin used. For all this, different amino acids and polymers concentrations were added to a 20% (w/v) HPβCD solution, to evaluate its effect on riboflavin solubility. Results in Fig. 5 show an increase in the solubility of riboflavin when chitosan is added or when arginine and chitosan are combined with the 20% (w/v) HPβCD solution, obtaining greater values

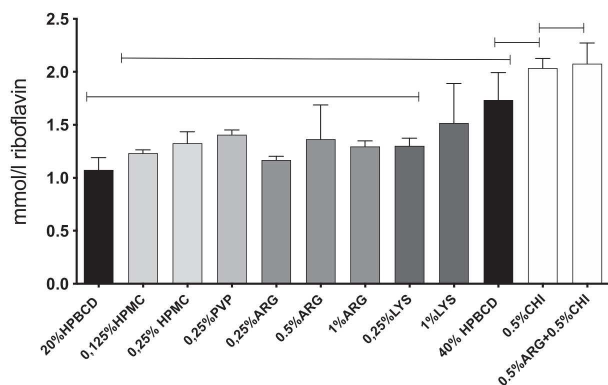

Fig. 5. Influence of arginine (ARG), lysine (LYS), hydroxypropyl methylcellulose (HPMC), polyvinylpyrrolidone (PVP) and chitosan (CHI) in the solubility of riboflavin when they are added to a saturated in riboflavin 20% (w/v) HPβCD solution in contrast with a saturated in riboflavin (w/v) 40% HPβCD solution. The lines group the homogeneous set of means.

than those reached with the 40% (w/v) HPβCD solution.

Neither arginine nor low lysine concentrations appear to have any effect on riboflavin solubility, probably due to the impossibility to form a ternary complex caused for steric limitations. No effect was shown by HPMC or PVP. Solely the incorporation of chitosan seems to improve solubilization and to a lesser extent, the higher concentrations of lysine. Both molecules are positively charged at pH 7 and riboflavin is an amphoteric compound with an isoelectric point of 6 and consequently is negatively charged in PBS at pH 7.4. Therefore, the increase in solubility may be due to an electrostatic interaction between the drug and the cationic components.

3.2. Formulations preparation

In the light of the results of the solubility studies, we have prepared different formulations to evaluate the effect on the riboflavin corneal penetration and ocular toxicity. Riboflavin concentrations obtained in each of the formulations are summarized in Table 3. The amount of riboflavin base that can be loaded is determined by changes in formulations composition, which will also influence the release properties.

Riboflavin concentration is increased when a higher amount of cyclodextrin is used, and the effect is even more appreciable when it is combined with arginine and chitosan. It has been descrived that the formation of ternary complexes with aminoacid [52,53] and the presence of hydrophillic polymers [15] as chitosan can increase the drug solubilization by cyclodextrins.

For the 0.05% (w/v) riboflavin-base nanoemulsion (E) droplets of 34.9 nm of diameter with a polydispersity of 0.2 were obtained, which is indicative of a homogeneous population. Nanoemulsion shows a negative surface charge with a low value of ζ (-13 mV) which must be originated by the ion concentration of PBS used in the nanoemulsion elaboration. The ionic strength has an important influence on the ζ and PBS can induce an electric double layer compression causing a reduction in ζ. The negative ζ is probably due to the soy phosphatidylcholine and riboflavin negative charge at formulation pH.

Previous reports presented a higher diameter (82 nm) and a positive and higher ζ (30–40 mV) [27]. The negative charge obtained differs from the positive obtained by Bottos et al. [27] and, therefore, its bioadhesion is expected to be lower since the corneal surface is negatively charged [32].

3.3. Riboflavin release assay

Fig. 6 shows the *in vitro* release kinetics of the ocular formulations. The results of fitting the accumulative release to different kinetic models are reflected in Table 4. The best fit was obtained with zero-order and the Peppas and Korsmeyer models, in this latter case, “n” values are close to one, confirming that the release of the riboflavin from solutions is constant over time. The riboflavin solution and the nanostructured system show “n” values of 0.73 and 0.81 suggesting a limited diffusion process. The incorporation of CD and chitosan increases the “n” value and decreases the release rate, probably because the riboflavin establishes interactions with both components, hindering the diffusion across the artificial membrane. The membrane used has a molecular cut-off of 12000–14000 Da and consequently allows the diffusion of the free riboflavin molecules giving more rapid release rates in the riboflavin solution R and nanoemulsion E. The incorporation of HPβCD produces the formation of the inclusion complexes that have higher molecular volume and consequently less diffusion rate across artificial membranes producing a lower riboflavin release rate. Finally, positively charged chitosan probably interacts electrostatically with negatively charged riboflavin reducing the permeation rate through the artificial membrane.

The decrease observed in the release rate could stimulate a greater permanence of the drug on the ocular surface by avoiding a rapid dilution in the tear and clearance.

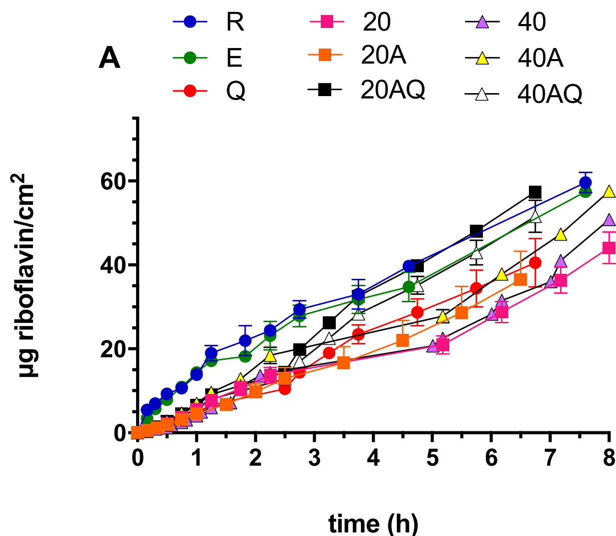

Fig. 6. In vitro release assays in vertical Franz diffusion cells at 35 °C for 24 h.

Table 3
Riboflavin formulations concentration obtained after 12 h of incubation and filtration of a saturated riboflavin suspensions.

Formulations	R[1]	20[2]	40[2]	20A[3]	20AQ[4]	40A[3]	40AQ[4]	Q[5]	E[6]
Riboflavin-base concentration (mg/mL)	0.11 ± 0.01	0.51 ± 0.15	0.65 ± 0.09	0.61 ± 0.2	0.78 ± 0.07	1.02 ± 0.1	1.04 ± 0.16	0.33 ± 0.1	0.55 ± 0.15

[1] Riboflavin aqueous saturated solution
[2] 20% and 40% (w/v) HPβCD solutions
[3] 20% or 40% (w/v) HPβCD and 0.5% (w/v) arginine solutions
[4] 20% or 40% (w/v) HPβCD, 0.5% (w/v) arginine and 0.5% (w/v) chitosan solutions
[5] 0.5% (w/v) chitosan solution
[6] 0.5% (w/v) riboflavin nanoemulsion

Table 4
Release kinetics parameters.

Formulations	Zero-order		Higuchi		Korsmeyer-Peppas		
	R^2	k	R^2	k	R^2	n	k
R	0.9752	7.42	0.9609	21.09	0.9941	0.73	13.12
E	0.9741	7.11	0.9513	20.12	0.9949	0.81	12.03
Q	0.9916	6.17	0.8823	15.74	0.9931	1.10	5.09
20	0.9762	4.98	0.9017	14.68	0.9770	1.11	3.91
20A	0.9936	5.42	0.8544	14.17	0.9969	1.16	3.99
20AQ	0.9910	8.65	0.8895	22.00	0.9937	1.14	6.68
40	0.9625	5.49	0.8756	15.46	0.9673	1.34	2.72
40A	0.9784	6.54	0.9085	19.26	0.9792	1.11	5.14
40AQ	0.9906	7.7	0.8808	19.55	0.9949	1.18	5.53

3.4. Corneal permeability

The results from the *ex vivo* corneal permeability study for ophthalmic solutions are shown in Fig. 7. Riboflavin shows a reduced corneal permeability, with a large lag time and low apparent permeability (Table 5). Fig. 7 shows that riboflavin corneal permeability is increased by all formulations studied compared with the solution. No drug permeation was observed from the solution but after a lag time of 3 h, riboflavin is able to permeate when HPβCD complex combined with arginine and chitosan or the nanoemulsion is used, with flux and apparent permeability coefficients reflected in Table 5. The extra sum of squares F test shows significant differences in the flux for $\alpha < 0.01$.

The nanostructured lipid system (E) induces a small increase in P_{app} and a significant reduction in the lag time compared to the riboflavin aqueous solution, but lower than formulations with chitosan or HPβCD. The best permeability coefficient is reached when riboflavin is combined with 40% (w/v) HPβCD, 0.5% (w/v) arginine, and 0.5% (w/v) chitosan.

Apparent permeability values concur with previous studies published by Morrison et al. [28]. These authors found a P_{app} of $0.49x10^{-8}$ and $3.35x10^{-8}$ cm/s for a 0.08 mg/mL riboflavin aqueous solution in deionized water or 3% (w/v) HPβCD respectively, which are in the same order as our results. In this work, slightly higher values of P_{app} in presence of HPβCD were observed, probably due to the higher concentrations of the oligosaccharide used. However, we found higher lag-times values for all formulations, which may be caused by a thickness variation of the bovine corneas used in the experiments. Cholesterol extraction from corneal epithelium membrane cells can be a possible mechanism of action whereby cyclodextrin facilitates drug penetration through the membrane [56–58]. Probably, the increase of the HPβCD concentration from 3% to 20–40% can cause a higher extraction of cholesterol, increasing drug permeability.

The incorporation of chitosan to the riboflavin aqueous solution increases drug permeability in the same magnitude as HPβCD (2–3 folds). It has been reported that chitosan is able to adhere to the ocular surface and open tight junctions between epithelial cells temporally, increasing drug permeability without apparent toxicity [32,59]. Additionally, Nemoto et al. reported that poly-L-arginine [60] can act as a paracellular modulator by affecting the localization of tight junction-associated proteins, increasing the drug permeability through surface ocular tissues [61]. We have observed that the combination of chitosan and arginine in riboflavin/HPβCD aqueous solutions provides the most significant increase of the P_{app} and a greater reduction in the lag time, resulting in the higher permeability increase of the drug. The combination with 20% and 40% HPβCD produces an increase of 5 and 8.3 folds in riboflavin P_{app} values respectively, suggesting a cooperative effect presumably caused by the increase in the drug solubility, the depletion of the lipids, and the alteration of the tight junction. Therefore, the combination of these excipients can be considered as a potential treatment for keratoconus, accordingly, their ocular toxicity studies are required.

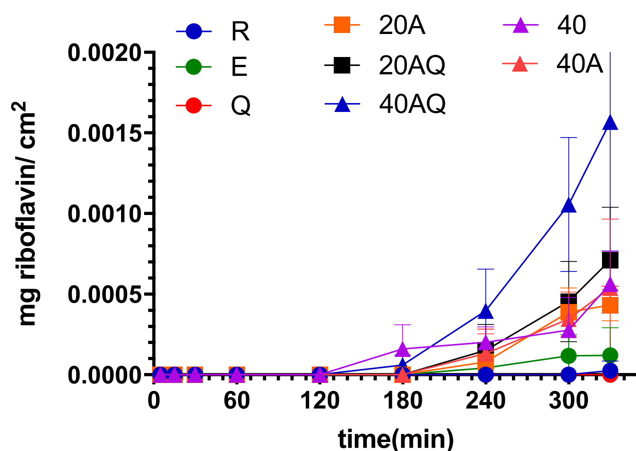

Fig. 7. Corneal permeability assay in vertical Franz diffusion cells at 35 °C during 6 h.

Table 5
Lag time, apparent permeability (P_{app}), and flux (J) of different formulations.

Formulations	Lag time (min)	J (mg/cm² min) ×106		P_{app} (cm/s) ×108	
		mean	SE	mean	SE
R	300	0.13	0.01	1.93	1.49
E	180	0.87	0.16	2.63	0.35
Q	180	1.02	0.31	5.13	1.58
20A	180	3.16	0.58	8.63	1.58
20AQ	180	4.64	0.76	9.92	1.62
40	120	2.31	1.03	5.92	2.64
40A	180	3.49	0.51	5.70	0.84
40AQ	120	9.91	1.49	15.87	2.68

3.5. Eye irritation and eye damage studies

The results of the modified bovine corneal opacity and permeability

Table 6
Bovine corneal opacity and permeability results.

Formulations	Transparency				Permeability	
	% T initial		% T 10 min		μg fluorescein/cm² after 90 min	
	Mean	SD	Mean	SD	Mean	SD
PBS	88.27	5.87	62.93	7.13	0.72	0.89
Ethanol	63.89	9.75	39.86	11.64	11.01	9.78
R	92.43	11.16	83.59	10.15	2.18	0.93
20A	93.95	5.12	86.99	2.94	0.48	0.17
20AQ	70.66	10.49	68.99	11.84	0.47	0.33
40A	80.72	7.30	71.29	6.43	0.59	0.54
40AQ	72.70	8.88	60.05	10.02	1.26	0.17
E	94.51	7.30	73.70	6.89	1.23	0.02
Q	86.39	5.83	54.36	3.51	0.03	0.00

assay shown in Table 6 and Fig. 8 suggest that there were no changes in either corneal permeability or transparency when the corneas were treated with the different riboflavin formulations since the transparency (%) and the OD_{490} (optical density at 490 nm) were similar in the formulations compared with the negative control and significantly different to the positive control.

The results of Hen's Egg Test – Chorioallantoic Membrane (HET-CAM) Test Method are shown in Fig. 9. These images exhibit no hemorrhage, lysis, or coagulation after having kept in touch for 300 s the chorioallantoic membrane with formulations, unlike the positive control (Fig. 9D). No difference was appreciated between the negative control (Fig. 9B) and the formulations (Fig. 9A, B, C, E, G, I), hence the irritation score obtained was null.

The results gained in both, the modified BCOP assay and HET-CAM test allow for classifying our formulations as non-irritating and eye-safe substances.

4. Discussion

Overcoming the impediments that transepithelial crosslinking entails, certain strategies have emerged including the use of benzalkonium chloride [16], edetate disodium, tromethamine [17], d-α-tocopheryl poly(ethylene glycol) 1000 succinate [20,21], sodium iodide based formulation [22,23], cyclodextrins [28–30] and nanosystems such as liposomes, nanoemulsions or microemulsions [24–27].

Some studies conducted with benzalkonium chloride have concluded that it causes damage to the ocular surface, corneal neurotoxicity, inflammation, and reduce aqueous tear production [16].

The results obtained after having performed transepithelial cross-linking with formulations containing edetate disodium and tromethamine show that the efficacy of cross-linking is reduced, probably due to limited penetration through the corneal epithelium [17]. Additionally, clinical studies combining 20% dextran solution with benzalkonium chloride/edetate disodium do not show effective improvements [18,19] probably due to the presence of high molecular weight dextran's.

In addition to absorption promoters such as benzalkonium chloride or edetate disodium, formulations with amphiphilic character and with some bioadhesion have been formulated in other works [27] to achieve the stroma improving penetration through the epithelium.

These formulations have reached a riboflavin higher concentration in the corneal stroma than conventional formulations used in the standard protocol, with the inconvenience that the exposure time is extended up to 240 min. The time needed for riboflavin to reach the stroma at concentrations that allow safe treatment remains too long. In this work, these formulations have been reproduced to evaluate their penetration through bovine corneas, as well as to establish a comparison with other formulations proposed for transepithelial crosslinking.

Given the lipophilic nature of the riboflavin-base molecule, it is essential for its application in the treatment of keratoconus by cross-

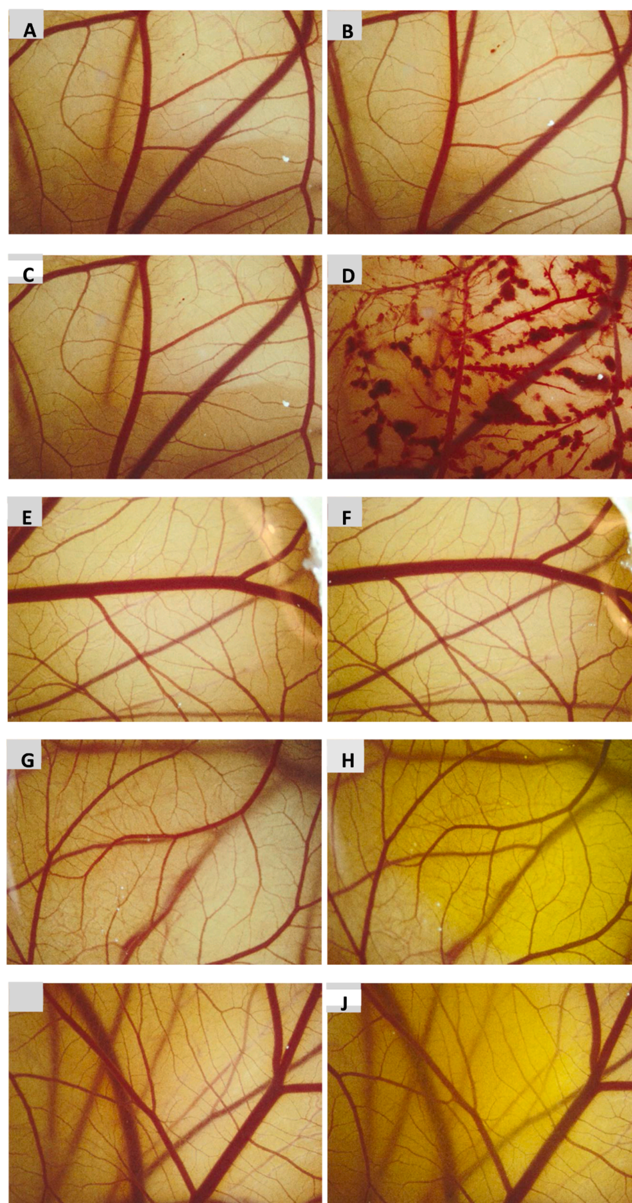

Fig. 9. Chorioallantoic Membrane before (A, C, E, G, I) and after 300 s of contact with negative control (B), positive control (D), and formulations R (F), 40 (H) and 40AQ (J).

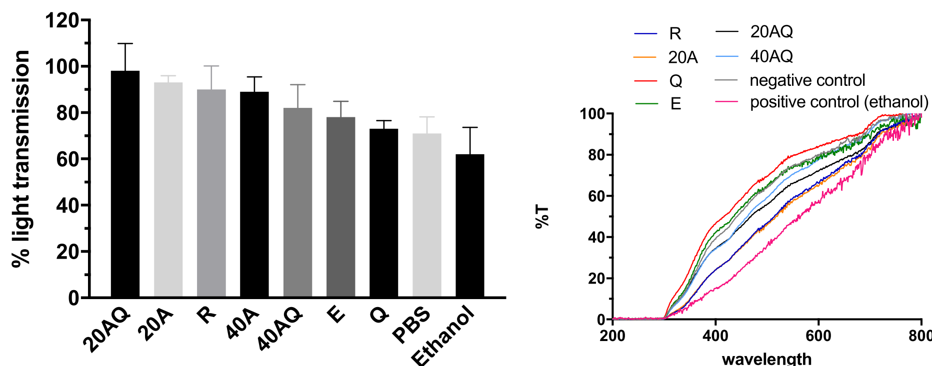

Fig. 8. Light transmission percentage (left) and corneal transmittance (right) of corneas incubated with the different formulations in relation to corneas incubated with PBS (negative control) for ten minutes.

linking, an improvement in the solubility in an aqueous medium such as the aqueous tear film and the mucus. For this purpose, studies with promoters of solubility such as cyclodextrins were carried out.

Riboflavin solubility in an aqueous medium is increased when cyclodextrins derivates are added to riboflavin solutions, getting the highest values when riboflavin-HPβCD and SBEβCD complex are formulated.

When HPβCD is combined with arginine and chitosan, the riboflavin solubility in an aqueous medium is even more increased, allowing smaller amounts of cyclodextrin to achieve the same amount of solubilized riboflavin.

Besides enhancing riboflavin solubility in the tear fluid it was found that these formulations also improve riboflavin permeability through the corneal epithelium finding higher concentrations of riboflavin permeating the entire bovine cornea in contrast with riboflavin solution or nanoemulsion reproduced from Bottos et Al. work [27].

However, these results must be carefully analyzed since the *ex vivo* permeation method used has some limitations. Riboflavin-base is unstable and better corneal fluxes do not guarantee a higher drug concentration in the stroma or greater efficiency in the crosslinking process. Additionally, the bovine cornea is larger than the human and has a much thicker corneal epithelium. Therefore, it will be necessary for future studies to check the accumulation of riboflavin in the stroma and its effect on the mechanical properties of the cornea after the crosslinking process.

Thus, cyclodextrins due to their solubilizer and penetration enhancer capacity, turn these proposals into potential formulations for their use as eye drops to increase the stromal concentration of riboflavin as a previous step to transepithelial crosslinking. Besides, chitosan presence facilitates negative corneal surface interaction, bioadhesion, and improves penetration enhancer behavior. Developing a new formulation for ophthalmic use, some factors generating toxicity or irritation must be taken into account. To verify that both the active ingredients and the excipients of the different formulations are not harmful, studies such as BCOP and HET-CAM were carried out. According to the results obtained, none of the formulations proposed in this work generate irritation, loss of transparency, or alterations in corneal permeability.

5. Conclusion

Riboflavin ophthalmic formulations were successfully developed that improved riboflavin corneal permeability and biocompatibility. The combination of HPβCD with chitosan and arginine has demonstrated a synergistic effect on the permeability of riboflavin with the absence of toxicity by the formulations. Thus, we can conclude that the developed formulations are options to avoid epithelium debridement in the corneal crosslinking procedure. Although subsequent studies are required to ascertain if an adequate riboflavin concentration is available in the corneal stroma in a shorter period as well as if an effective strengthening of the cornea after UVA light application is achieved.

References

[1] P. Fournié, D. Touboul, J.-L. Arné, J. Colin, F. Malecaze, Keratoconus, J. Fr. Ophtalmol. 36 (7) (2013 Sep) 618–626.
[2] F. Raiskup-Wolf, A. Hoyer, E. Spoerl, L.E. Pillunat, Collagen crosslinking with riboflavin and ultraviolet-A light in keratoconus: Long-term results, J. Cataract Refract. Surg. 34 (5) (2008) 796–801.
[3] G. Wollensak, Crosslinking treatment of progressive keratoconus: new hope, Curr. Opin. Ophthalmol. 17 (4) (2006) 356–360.
[4] G.R. Snibson, Collagen cross-linking: a new treatment paradigm in corneal disease - a review, Clin. Exp. Ophthalmol. 38 (2) (2010) 141–153.
[5] L. Mastropasqua, Collagen cross-linking: when and how? A review of the state of the art of the technique and new perspectives, Eye Vis. 29 (2) (2015) 19.
[6] P. Kamaev, M.D. Friedman, E. Sherr, D. Muller, Photochemical kinetics of corneal cross-linking with riboflavin, Invest. Ophthalmol. Vis. Sci. 53 (4) (2012) 2360–2367.
[7] G. Wollensak, E. Spoerl, T. Seiler, Riboflavin/ultraviolet-A-induced collagen crosslinking for the treatment of keratoconus, Am. J. Ophthalmol. 135 (5) (2003) 620–627.
[8] E. Chan, G.R. Snibson, Current status of corneal collagen cross-linking for keratoconus: a review, Clin Exp Optom. 96 (2) (2013) 155–164.
[9] G.R. Snibson, Collagen cross-linking: a new treatment paradigm in corneal disease – a review, Clin. Exp. Ophthalmol. 38 (2) (2010) 141–153.
[10] R.S. Rubinfeld, C. Caruso, C. Ostacolo, Corneal cross-linking: the science beyond the myths and misconceptions, Cornea 38 (6) (2019) 780–790.
[11] K.A. Beckman, P.K. Gupta, M. Farid, J.P. Berdahl, E. Yeu, B. Ayres, C.C. Chan, J.A. P. Gomes, E.J. Holland, T. Kim, C.E. Starr, F.S. Mah, Corneal crosslinking: Current protocols and clinical approach, J. Cataract Refract. Surg. 45 (11) (2019) 1670–1679.
[12] K.M. Bottós, P. Schor, J.L. Dreyfuss, H.B. Nader, W. Chamon, Effect of corneal epithelium on ultraviolet-A and riboflavin absorption, Arq. Bras. Oftalmol. 74 (5) (2011) 348–351.
[13] E. Mannermaa, K.-S. Vellonen, A. Urtti, Drug transport in corneal epithelium and blood–retina barrier: emerging role of transporters in ocular pharmacokinetics, Adv. Drug Deliv. Rev. 58 (11) (2006) 1136–1163.
[14] A.J. Huang, S.C. Tseng, K.R. Kenyon, Paracellular permeability of corneal and conjunctival epithelia, Invest. Ophthalmol. Vis. Sci. 30 (4) (1989) 684–689.
[15] T. Loftsson, E. Stefánsson, Cyclodextrins and topical drug delivery to the anterior and posterior segments of the eye, Int. J. Pharm. 531 (2) (2017) 413–423.
[16] J.R. Kim, T.H. Oh, H.S. Kim, Effects of benzalkonium chloride on the ocular surface of the rabbit, Jpn. J. Ophthalmol. 55 (3) (2011) 283–293.
[17] A. Caporossi, C. Mazzotta, S. Baiocchi, T. Caporossi, A.L. Paradiso, Transepithelial corneal collagen crosslinking for keratoconus: qualitative investigation by in vivo HRT II confocal analysis, Eur. J. Ophthalmol. 22 (7) (2012) 81–88.
[18] G. Wollensak, E. Iomdina, Biomechanical and histological changes after corneal crosslinking with and without epithelial debridement, J. Cataract Refract. Surg. 35 (2009) 540–546.
[19] N. Soeters, R.P.L. Wisse, D.A. Godefrooij, S.M. Imhof, N.G. Tahzib, Transepithelial versus epithelium-off corneal cross-linking for the treatment of progressive keratoconus: a randomized controlled trial, Am. J. Ophthalmol. 159 (2015) 821–823.
[20] C. Ostacolo, C. Caruso, D. Tronino, S. Troisi, S. Laneri, L. Pacente, A. Del Prete, A. Sacchi, Enhancement of corneal permeation of riboflavin-5'-phosphate through vitamin E TPGS: a promising approach in corneal trans-epithelial cross linking treatment, Int. J. Pharm. 440 (2) (2013) 148–153.
[21] C. Caruso, R.L. Epstein, P. Troiano, C. Ostacolo, G. Barbaro, L. Pacente, S. Bartollino, C. Costagliola, Topography and pachymetry guided, rapid epi-on corneal cross-linking for keratoconus: 7-year study results, Cornea 39 (1) (2020) 56–62.
[22] R.S. Rubinfeld, R.D. Stulting, G.G. Gum, J.H. Talamo, Quantitative analysis of corneal stromal riboflavin concentration without epithelial removal, J. Cataract Refract. Surg. 44 (2) (2018) 237–242.
[23] R.D. Stulting, W.B. Trattler, J.M. Woolfson, R.S. Rubinfeld, Corneal crosslinking without epithelial removal, J. Cataract Refract. Surg. 44 (11) (2018) 1363–1370.
[24] N. Kandzija, V. Khutoryanskiy, Delivery of riboflavin-5'-monophosphate into the cornea: can liposomes provide any enhancement effects? J. Pharml. Sci. 106 (10) (2017) 3041–3049.
[25] N. Lidich, E.J. Wachtel, A. Aserin, N. Garti, Water-dilutable microemulsions for transepithelial ocular delivery of riboflavin phosphate, J. Colloid Interface Sci. 463 (2016) 342–348.
[26] N. Lidich, S. Garti-Levy, A. Aserin, N. Garti, Potentiality of microemulsion systems in treatment of ophthalmic disorders: T Keratoconus and dry eye syndrome – in vivo study, Colloids Surf., B 173 (2019) 226–232.
[27] K.M. Bottos, A.G. Oliveira, P.A. Bersanetti, R.F. Nogueira, A.A.S. Lima-Filho, J. A. Cardillo, P. Schor, W. Chamon, Corneal absorption of a new riboflavin-nanostructured system for transepithelial collagen cross-linking, PLoS ONE 8 (6) (2013), e66408.
[28] P.W.J. Morrison, C.J. Connon, V.V. Khutoryanskiy, Cyclodextrin-mediated enhancement of riboflavin solubility and corneal permeability, Mol. Pharm. 10 (2) (2013) 756–762.
[29] C. Labate, M. Lombardo, G. Lombardo, M.P. De Santo, Biomechanical strengthening of the human cornea induced by nanoplatform-based transepithelial riboflavin/UV-A corneal cross-linking, Invest. Ophthalmol. Vis. Sci. 58 (1) (2017) 179–184.
[30] G. Lombardo, N.L. Micali, V. Villari, N. Leone, S. Serrao, D. Rusciano, M. Lombardo, Assessment of stromal riboflavin concentration-depth profile in nanotechnology-based transepithelial corneal crosslinking, J. Cataract Refract. Surg. 43 (5) (2017) 680–686.
[31] J. Berger, M. Reist, J.M. Mayer, O. Felt, N.A. Peppas, R. Gurny, Structure and interactions in covalently and ionically crosslinked chitosan hydrogels for biomedical applications, Eur. J. Pharm. Biopharm. 57 (1) (2004) 19–34.
[32] T. Irimia, M.V. Ghica, L. Popa, V. Anuța, A.-L. Arsene, C.-E. Dinu-Pîrvu, strategies for improving ocular drug bioavailability and corneal wound healing with chitosan-based delivery systems, Polymers 10 (11) (2018) 1221.
[33] U. Daood, K. Iqbal, L.I. Nitisusanta, A.S. Fawzy, Effect of chitosan/riboflavin modification on resin/dentin interface: spectroscopic and microscopic investigations, J. Biomed. Mater. Res. A 101A (7) (2013) 1846–1856.
[34] T. Higuchi, K.A. Connors, Phase-solubility techniques, in: Advances in analytical chemistry and instrumentation, Reilly In C. N., New York, 1965.
[35] S.V. Kurkov, T. Loftsson, Cyclodextrins, Int. J. Pharm. 453 (1) (2013) 167–180.
[36] C. Loch, S. Zakelj, A. Kristl, S. Nagel, R. Guthoff, W. Weitschies, A. Seidlitz, Determination of permeability coefficients of ophthalmic drugs through different layers of porcine, rabbit and bovine eyes, Eur. J. Pharm. Sci. 47 (1) (2012) 131–138.

[37] D. Myung, K. Derr, P. Huie, J. Noolandi, K.P. Ta, C.N. Ta, Glucose permeability of human, bovine, and porcine corneas in vitro, Ophthalmic Res. 38 (3) (2006) 158–163.
[38] I. Rodríguez, J.A. Vázquez, L. Pastrana, V.V. Khutoryanskiy, Enhancement and inhibition effects on the corneal permeability of timolol maleate: polymers, cyclodextrins and chelating agents, Int. J. Pharm. 529 (1–2) (2017) 168–177.
[39] P.W.J. Morrison, V.V. Khutoryanskiy, Enhancement in corneal permeability of riboflavin using calcium sequestering compounds, Int. J. Pharm. 472 (1–2) (2014) 56–64.
[40] ECVAM DB-ALM: INVITTOX protocol (primero). Bovine Corneal Opacity and Permeability (BCOP) Assay INVITTOX no127, 2009.
[41] OECD. Test No. 437: bovine corneal opacity and permeability test method for identifying i) chemicals inducing serious eye damage and ii) chemicals not requiring classification for eye irritation or serious eye damage [Internet]. 2017. Available from: https://www.oecd-ilibrary.org/content/publication/9789264203846-en.
[42] S.P. Srinivas, A. Goyal, D.P. Talele, S. Mahadik, R.R. Sudhir, P.P. Murthy, et al., Corneal epithelial permeability to fluorescein in humans by a multi-drop method, PLoS ONE 13 (6) (2018), e0198831.
[43] M. Parekh, S. Ferrari, A. Ruzza, M. Pugliese, D. Ponzin, G. Salvalaio, A portable device for measuring donor corneal transparency in eye banks, Cell Tissue Bank 15 (1) (2014) 7–13.
[44] A. Fernández-Ferreiro, M. González Barcia, M. Gil Martínez, J. Blanco Méndez, M. J. Lamas Díaz, F.J. Otero Espinar, Análisis de la toxicidad ocular de los colirios de voriconazol y fluconazol con HET-CAM [Analysis of ocular toxicity of fluconazole and voriconazole eyedrops using HET-CAM], Farm hosp. 38 (4) (2014) 300–304.
[45] T. Loftsson, Drug permeation through biomembranes: cyclodextrins and the unstirred water layer, Pharm 67 (5) (2012) 363–370.
[46] T. Loftsson, E. Stefánsson, Cyclodextrins in eye drop formulations: Enhanced topical delivery of corticosteroids to the eye, Acta Ophthalmol. Scand. 80 (2) (2002) 144–150.
[47] R.E. Coffman, D.O. Kildsig, Effect of nicotinamide and urea on the solubility of riboflavin in various solvents, J. Pharm. Sci. 85 (9) (1996) 951–954.
[48] P.W.J. Morrison, N.N. Porfiryeva, S. Chahal, I.A. Salakhov, C. Lacourt, I.I. Semina, et al., Moustafine RI, Khutoryanskiy VV. Crown ethers: novel permeability enhancers for ocular drug delivery? Mol. Pharm. 14 (10) (2017) 3528–3538.
[49] M.B. de Jesus, L.F. Fraceto, M.F. Martini, M. Pickholz, C.V. Ferreira, E. de Paula, Non-inclusion complexes between riboflavin and cyclodextrins, J. Pharm. Pharmacol. 64 (6) (2012) 832–842.
[50] I.V. Terekhova, M. Koźbiał, R.S. Kumeev, G.A. Alper, Inclusion complex formation between modified cyclodextrins and riboflavin and alloxazine in aqueous solution, J Solut Chem. 40 (8) (2011) 1435.
[51] W. Zielenkiewicz, I.V. Terekhova, M. Koźbiał, R.S. Kumeev, Thermodynamic study on inclusion complex formation of riboflavin with hydroxypropyl-β-cyclodextrin in water, J. Therm. Anal. Calorim. 101 (2) (2010) 595–600.
[52] V. Aiassa, A. Zoppi, I. Albesa, M.R. Longhi, Inclusion complexes of chloramphenicol with β-cyclodextrin and aminoacids as a way to increase drug solubility and modulate ROS production, Carbohydr. Polym. 5 (121) (2015) 320–327.
[53] S.G. Méndez, F.J. Otero Espinar, A.L. Alvarez, M.R. Longhi, M.A. Quevedo, A. Zoppi, Ternary complexation of benzoic acid with β-cyclodextrin and aminoacids. Experimental and theoretical studies, J. Incl. Phenom. Macro 85 (2016) 33–48.
[54] M.J. Mora, M.R. Longhi, G.E. Granero, Synthesis and characterization of binary and ternary complexes of diclofenac with a methyl-β-CD and monoethanolamine and in vitro transdermal evaluation, Eur. J. Med. Chem. 45 (9) (2010 Sep 1) 4079–4088.
[55] M. Ponce-Ponte, C. Aloisio, D.M. Romero-Guerra, S. Gracia-Vásquez, C. Garnero, M. Longhi, Binary and ternary complexes of norfloxacin to improve the solubility of the active pharmaceutical ingredient, Ther. Deliv. 9 (9) (2018 Sep 1) 639–652.
[56] R.V. Moiseev, P.W.J. Morrison, F. Steele, V.V. Khutoryanski, Penetration enhancers in ocular drug delivery, Pharmaceutics. 11 (7) (2019) 321.
[57] T. Zaidi, M. Bajmoczi, T. Zaidi, D.E. Golan, G.B. Pier, Disruption of CFTR-dependent lipid rafts reduces bacterial levels and corneal disease in a murine model of pseudomonas aeruginosa keratitis, Invest. Ophthalmol. Vis. Sci. 49 (3) (2008) 1000–1009.
[58] Y. Ohtani, T. Irie, K. Uekama, K. Fukunaga, J. Pitha, Differential effects of α-, β- and γ-cyclodextrins on human erythrocytes, Eur. J. Biochem. 186 (1–2) (1989) 17–22.
[59] T.-H. Yeh, L.-W. Hsu, M.T. Tseng, P.-L. Lee, K. Sonjae, Y.-C. Ho, H.W. Sung, Mechanism and consequence of chitosan-mediated reversible epithelial tight junction opening, Biomaterials 32 (26) (2011) 6164–6173.
[60] E. Nemoto, H. Takahashi, D. Kobayashi, H. Ueda, Y. Morimoto, Effects of poly-L-arginine on the permeation of hydrophilic compounds through surface ocular tissues, Biol. Pharm. Bull. 29 (1) (2006) 155–160.
[61] H. Sekijima, J. Ehara, Y. Hanabata, T. Suzuki, S. Kimura, V.H.L. Lee, Y. Morimooto, H. Ueda, Characterization of ocular iontophoretic drug transport of ionic and non-ionic compounds in isolated rabbit cornea and conjunctiva, Biol. Pharm. Bull. 39 (6) (2016) 959–968.