Cyclodextrin Mediated Enhancement of Riboflavin Solubility and Corneal Permeability

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Abstract: Cyclodextrins are water-soluble cyclic oligosaccharides consisting of six, seven and eight α-(1,4)-linked glucopyranose subunits. This study reports the use of different cyclodextrins in eye drop formulations to improve the aqueous solubility and corneal permeability of riboflavin. Riboflavin is a poorly water-soluble drug, whose solubility is up to ~0.08 mg mL⁻¹ in deionized water. It is used as a drug topically administered to the eye to mediate UV-induced corneal cross-linking in the treatment of keratoconus. Aqueous solutions of β-cyclodextrin (10-30 mg mL⁻¹) can enhance the solubility of riboflavin up to 0.12 – 0.19 mg mL⁻¹, whereas the higher concentration of α-cyclodextrin (100 mg mL⁻¹) achieved a lower level of enhancement of 0.11 mg mL⁻¹. The other oligosaccharides were found to be inefficient for this purpose. In vitro diffusion experiments performed with fresh and cryopreserved bovine cornea have demonstrated that β-cyclodextrin enhances riboflavin permeability. The mechanism of this enhancement was examined through microscopic histological analysis of the cornea and is discussed in this paper.
Keywords: Ocular drug delivery, cornea, corneal epithelium, cyclodextrin, Franz diffusion cell, corneal cross-linking, permeability, riboflavin, trans-corneal.

Introduction

Despite easy accessibility of the eye for drug administration, ophthalmic delivery is a very challenging area of pharmaceutics; there is the issue of poor permeability due to several ocular barriers. Drug retention is impeded by blinking, tear reflex and nasolacrimal drainage. Typically less than 5% of the topically applied drug penetrates the cornea and reaches intraocular tissues, while a major fraction of the instilled dose is often absorbed systemically via the conjunctiva and nasolacrimal duct. The cornea comprises of five layers, starting from the outermost epithelium, Bowman’s membrane, stroma, Descemet’s membrane and endothelium. The epithelium is a 50-100 μm lipophilic layer that contributes around 90% resistance to hydrophilic drugs and 10 % to hydrophobic drugs. Immediately underneath the epithelium is the Bowman’s membrane, a thin homogenous layer forming a transition towards the stroma, and it is not considered to be a barrier to drugs diffusion. The stroma forms the main section at around 90% of the total corneal thickness. It has a hydrophilic gel structure comprised of collagen, other proteins and mucopolysaccharides. The stroma may act as the main barrier to very lipophilic drugs. Descemet’s membrane is a tough, homogenous band supporting the endothelium, a single layer of cells important in keeping the hydration of the stroma constant. Mammalian eyes typically share many of these features and are often used as models representing human eyes. Bovine eyes are established models for drug studies and were used in this work. Figure 1 shows a micrograph of bovine cornea cross-section highlighting its multilayered structure.
Keratoconus is a degenerative disorder of the eye affecting ~1 in 2000 of the population. This debilitating condition results in the cornea becoming more conical leading to serious vision distortion. Around 20% of sufferers ultimately require corneal transplantation (penetrating keratoplasty). In 2003 Wollensak et al published details of a novel treatment for keratoconus employing riboflavin as a photosensitive compound to initiate ultraviolet induced collagen cross-linking in the diseased cornea. The procedure developed by Wollensak relies on physical removal of the epithelium to allow riboflavin to enter the corneal stroma. Although it is a fully established and widely used clinical method to facilitate the riboflavin-mediated corneal cross-linking, it is an invasive technique that has to be performed under local anesthesia. There is a clear need to develop novel formulations that could potentially enhance the penetration of riboflavin into the cornea without recourse to surgical removal of the epithelium.

In the present work we investigated the effects of various cyclodextrins on riboflavin solubility and permeability through bovine cornea. We also explored corneal integrity after exposure to
cyclodextrin solutions and established that they are able to extract lipids from epithelial membranes. Further work also investigated differences in drug permeability between fresh and cryopreserved ocular tissue.

**Materials and Methods**

**Materials.** Riboflavin, β-cyclodextrin, sodium hexane-1-sulfonate monohydrate and glacial acetic acid were purchased from Sigma-Aldrich (Gillingham, UK). α-cyclodextrin, γ-cyclodextrin, hydroxypropyl-β-cyclodextrin and cholesterol were obtained from TCI Ltd (Oxford, UK). Sodium chloride, potassium chloride, sodium phosphate, potassium dihydrogen phosphate, sodium hydroxide, Minisart syringe filters (0.2 μm), optimal cutting temperature compound (OCT) and HPLC grade acetonitrile, chloroform, isopropanol and methanol were obtained from Fischer Scientific (Hemel Hempstead, UK). Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) obtained from Vector Laboratories Ltd (Peterborough, UK). MilliQ ultrapure water (18 mΩ cm-1) was used for all aqueous solutions. All materials were used as supplied without modification.

**Preparation of solutions.** PBS was prepared in-house and was adjusted to pH 7.4 using 0.1M solution NaOH.\(^8\) Ion-pair buffer was prepared using sodium hexane-1-sulfonate monohydrate adjusted to pH 3.0 using glacial acetic acid.\(^9\)

**HPLC analysis.** HPLC analysis was conducted using a manual injection HPLC system (Perkin Elmer Inc, UK) comprising of Flexar UV-Vis detector (FXUVDET), Flexar binary pump (FxBPump2), Flexar solvent manager (3CH Degasser), Ascentis C\(_{18}\) column, 150 x 4.6
mm, 5μm, (part number: 581324-U) and data acquisition software (Chromera, version 3.2.0.4847).

Analysis of riboflavin was achieved with a run time of 4 minutes using the method adapted from Anyakora et al.\textsuperscript{9} Isocratic conditions were used at ambient temperature with the mobile phase comprising of 40% methanol and 60% ion-pair buffer, flow rate 1.2 mL min\textsuperscript{-1}, 10 μL injection volume, UV detector at 267 nm corresponding to riboflavin absorption $\lambda_{\text{max}}$, a retention time of 2.4 minutes. Quantitation was achieved by reference to a calibration curve produced from riboflavin standards at concentrations ranging from 0.00001 to 0.01 mg mL\textsuperscript{-1} ($r^2 = 0.9996$).

HPLC method for cholesterol analysis was adapted from the protocols reported by Hoving\textsuperscript{10} and Osman and Yap.\textsuperscript{11} Isocratic conditions were used at ambient temperature with the mobile phase comprising of 60% acetonitrile and 40% isopropanol at 1 mL min\textsuperscript{-1} flow rate, injection volume 10 μL, with 10 minutes runtime, UV detector at 210 nm. Solutions of cholesterol in isopropanol were prepared at 0.005, 0.05 and 0.5 mg mL\textsuperscript{-1}, and used to identify its retention time at 7.9 minutes.

### Preparation of animal tissues.

Bovine eyes were provided by P C Turners abattoirs (Farnborough, UK) and stored on ice during transport. The eyes were carefully handled and used whole or cornea dissected depending on the experiment. Dissection took place within four hours of slaughter. Using a sharp blade, the cornea with 2-3 mm of sclera attached was carefully removed, quickly rinsed with PBS and wrapped in cling film to prevent dehydration. Fresh tissues were stored at 4°C in a refrigerator and used within 48 hours prior to experiments (fresh cornea), or were stored at -18°C until use (cryopreserved cornea).
Cornea sections from experiments were prepared by setting the cornea segment in OCT, quick freezing on dry ice and subsequent microtome sectioning. Specimens were prepared for microscopy using a microtome (Bright, model 5040) within a cryostat (Bright, model OTF). Sections were cut at 7 μm, placed in groups of four on 75mm x 25mm glass slides, dried gently using a hot air blower on low power for ten minutes from a distance of ~50 cm. The specimens were stained using Vectashield with DAPI mounting medium and a glass coverslip placed over the specimens. All cornea sections were examined within 48 hours of preparation using an AXIOCAM MRm1.3 MP digital camera attached to a Zeiss AXIO Imager A1 fluorescent microscope, using AXIO Vs 40 V.4.8.2.0 software (Zeiss, Oberkochen, Germany). A 10 x magnification eyepiece together with a 5 x magnification objective lens was employed, and a light filter for DAPI fluorescence was selected.

**Effect of cyclodextrins on the solubility of riboflavin.** Solutions of α-, γ- and hydroxypropyl-β-cyclodextrins in ultrapure water were prepared at 10, 20, 30, 50 and 100 mg mL⁻¹. β-cyclodextrin is known to have lower aqueous solubility (18.5 mg mL⁻¹) at room temperature, increasing with higher temperature,¹²⁻¹⁴ therefore solutions were prepared at 10, 20 and 30 mg mL⁻¹. β-cyclodextrin was heated to 60°C to dissolve, all other solutions were stirred at 35°C until dissolved. β-cyclodextrin slowly crystallizes from solution upon storing at room temperature, however this re-dissolved quickly when heated to 60°C, then cooled to physiological temperature. Cyclodextrin solutions were saturated with riboflavin by adding an excess at 1 mg mL⁻¹ and stirring overnight. Riboflavin saturated water was prepared as a control. Our preliminary observations have shown that riboflavin is photodegradable in solution (data not shown); this is also in agreement with the data reported by Terekhova et al.¹⁵ Therefore in all
experiments riboflavin preparations were wrapped in aluminum foil to prevent its photo-degradation. HPLC analysis was carried out on the riboflavin saturated solutions to determine the effect of cyclodextrin on its solubility after passing them through 0.2 μm syringe filters. Phase-solubility analysis was used to determine association constants the complexation.\textsuperscript{16}

**Corneal permeability.** The effect of cyclodextrin on corneal permeability of riboflavin was studied \textit{in vitro} using Franz diffusion cells. Solutions of α-, β-, γ- and hydroxypropyl-β-cyclodextrins in ultrapure water were prepared at 30 mg mL\textsuperscript{-1} which was subsequently dosed with riboflavin at 0.08 mg mL\textsuperscript{-1}; an aqueous drug solution at the same concentration was also prepared as a control. Bovine corneas were mounted between donor and receiver compartments of standard Franz diffusion cells, epithelium side facing uppermost. The receiver compartment was filled with pre-warmed PBS (16.5 mL), ensuring no air bubbles were trapped under the membrane. Experiments were conducted in a water bath, stirred at 34°C ± 1°C to mimic physiological temperature at the corneal surface.\textsuperscript{17,18} 1 mL of riboflavin in water or in cyclodextrin solution was added to the donor compartment, and then sealed with cling film to prevent evaporation. All apparatus was entirely covered with aluminum foil to exclude light. HPLC analysis was carried out at the time of sampling to avoid drug degradation; sink conditions were not employed because low volume (0.2 mL) aliquots were taken every 30 minutes for 180 minutes and this did not significantly reduce the cell volume. Experiments were carried out in triplicate using different corneas for each repeat.

**Corneal integrity.** The effect of cyclodextrin on corneal integrity was investigated using fresh, whole bovine eyes each placed in individual 150 mL beakers, cornea facing uppermost. A
Franz diffusion cell donor compartment was placed on the cornea and held in place using cling film ensuring a good seal at the cornea surface; the beakers were placed in a water bath at 37°C, and allowed to equilibrate for 60 minutes after which 1 mL of cyclodextrin solution was added to the donor compartment by syringe (experimental set up is shown in Figure 1S in Supplementary Information). Corneas were exposed to cyclodextrin solutions for 15, 45 and 75 minutes. The rationale for using whole eyes during this experiment was to avoid swelling of the stroma that is observed when using dissected cornea in Franz diffusion cells. Immediately after exposure time had elapsed, the experimental set up was disassembled and cornea dissected, mounted in OCT on dry ice, and examined using microscopy.

A further experiment was designed to investigate the ability of cyclodextrin to extract cholesterol from corneal epithelia. Corneas of whole bovine eyes were exposed to α, β, γ and HP-β-cyclodextrin solution (30 mg mL⁻¹) for 90 minutes, then the cyclodextrin solution was carefully recovered using a 1 mL syringe without a needle; 5 eyes were used for each cyclodextrin and recovered doses were combined giving ~5 mL of cornea exposed solution for each cyclodextrin. These were placed in glass vials to which 10 mL of chloroform was added, capped and shaken, then left to stand at room temperature for 1 hour, 50 mL ultrapure water was added. The mixture was filtered, allowed to settle into aqueous (upper) and organic (lower) phases, and then separated. The solvent was allowed to evaporate off from the organic phase, and 0.5 mL of isopropanol was added to dissolve the residue and analyzed by HPLC.

**Results and discussion**
**Effect of cyclodextrins on solubility of riboflavin.** Cyclodextrins are toroid shaped oligosaccharides with hydrophilic external surfaces and a lipophilic internal cavity. They are able to form guest-host inclusion complexes with hydrophobic compounds rendering them more soluble.\(^{12,19}\) Studies have shown that pre-treatment using cyclodextrin solutions can enhance corneal permeability of some drugs such as pilocarpine.\(^{20}\) Sultana et al reported their use in formulations containing corticosteroids, chloramphenicol, diclofenac and cyclosporine.\(^{21}\) Roy et al\(^{22}\) and Terekhova et al\(^{15}\) have demonstrated that riboflavin can form complexes with cyclodextrins; however, their studies were limited to the dynamics of complexation using α- and β-cyclodextrins. To the best of our knowledge no study has been reported on the effects of cyclodextrins on corneal permeability of riboflavin.

Our experiments have shown that riboflavin has relatively low aqueous solubility at \(~0.079\) mg mL\(^{-1}\) (0.209 mM L\(^{-1}\)) in deionized water and it is in agreement with the literature.\(^{23,24}\) Drug permeability through biological membranes is typically expected to increase when higher concentration formulations are employed, therefore cyclodextrins ability to enhance the aqueous solubility of riboflavin was investigated. It was found that α-cyclodextrin at 100 mg mL\(^{-1}\) and β-cyclodextrin 20 and 30 mg mL\(^{-1}\) offered a significant (p < 0.05) enhancement in the aqueous solubility of riboflavin, but γ- and hydroxypropyl-β-cyclodextrin failed to enhance drug solubility to any significant level (p > 0.05), compared with its solubility in water.

The cavity of cyclodextrin determines which molecules are able to form inclusion complexes. When the cavity is small, as with α-cyclodextrin (4.7 – 5.3 Å), many drug molecules will be too large to fit and will not be able to form inclusion complexes. When the cavity is too large drug molecules may be bound only loosely.\(^{12}\) β-cyclodextrin proves suitable for many pharmaceutical applications, with a cavity size of 6.0 – 6.5 Å that can accommodate a wide range of drugs.\(^{13}\)
The size of riboflavin molecule determined using Jmol software was found to be 10 x 12 Å (Figure 2). This is too large to completely fit within any cyclodextrin cavity; however, the aromatic ring with attached methyl groups would suitably accommodate within β-cyclodextrin’s cavity and bind through weak hydrophobic effects. The cavity of α-cyclodextrin would be too small to accommodate the aromatic ring of riboflavin although partial complexation via the methyl groups could occur. Involvement of other parts of the riboflavin molecule, such as the ribitol chain and pyrimidine ring could be ruled out as these are highly polar, hydrophilic and would be unlikely to participate in the formation of an inclusion complex. The fit would be too loose in the case of γ-cyclodextrin.

Figure 2. Computer generated image of riboflavin showing dimensions.

Complexation of riboflavin with cyclodextrins was determined by phase-solubility analysis, Figure 3, from which the association constants \( (K_{CD:RF}) \) were determined using the methods
reported Jarho et al.\textsuperscript{26} Table 1. Size considerations and association constants give reasonable explanation for the superior performance of $\beta$-cyclodextrin in solubilization of riboflavin.

![Figure 3. Phase solubility diagrams of $\alpha$, $\beta$, $\gamma$ and hydroxypropyl-$\beta$-cyclodextrin](image)

**Table 1.** Riboflavin association constant and cyclodextrin enhanced solubility compared to riboflavin in de-ionized water

<table>
<thead>
<tr>
<th>Cyclodextrin</th>
<th>Riboflavin $30$ mg mL$^{-1}$</th>
<th>Association constant $K_{CD:RF}$ mol$^{-1}$</th>
<th>Riboflavin solubility enhancement %</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-CD</td>
<td>0.081</td>
<td>4.32</td>
<td>3.2</td>
</tr>
<tr>
<td>$\beta$-CD</td>
<td>0.191</td>
<td>57.62</td>
<td>142.8</td>
</tr>
<tr>
<td>$\gamma$-CD</td>
<td>0.08</td>
<td>5.27</td>
<td>1.7</td>
</tr>
<tr>
<td>HP-$\beta$-CD</td>
<td>0.084</td>
<td>5.75</td>
<td>7.3</td>
</tr>
</tbody>
</table>
Corneal permeability of riboflavin. Drug permeability studies often rely on the regular access to suitable biological tissues, and fresh material is not always available. Therefore cryopreservation offers a convenient means to store biological samples until needed. Human amniotic membrane is an example of material that can be cryopreserved until required. It is used for wound protection, ocular surface reconstruction and drug delivery.\textsuperscript{27-30} Cryopreserved samples are also often employed in drug permeability studies; however, there is little known on the effect of cryopreservation on the barrier function of the cornea.

In the present study we investigated the effect of cyclodextrins (30 mg mL\textsuperscript{-1}) on the permeability of riboflavin (0.08 mg mL\textsuperscript{-1}) through fresh and cryopreserved bovine cornea. The permeation of riboflavin from cyclodextrin-free formulation into the receiver solution is observed within \sim 75 minutes in experiments with fresh bovine cornea, whilst this lag time reduced to \sim 40 minutes in the case of cryopreserved tissue (Figure 4). The existence of this lag time is related to the initial penetration of the drug into the cornea before it starts to permeate. Levels of riboflavin in receiver solution recorded at 180 minutes of the diffusion experiment were 0.0054 \, \text{\mu g mL\textsuperscript{-1}} and 0.0515 \, \text{\mu g mL\textsuperscript{-1}} for fresh and cryopreserved cornea, respectively.
Comparing cyclodextrin enhanced permeability of riboflavin at 180 minutes it was found that β- and HP-β-cyclodextrins significantly enhanced riboflavin permeability to $0.0159 \pm 0.0010 \, \mu g \, mL^{-1}$ and $0.0094 \pm 0.0016 \, \mu g \, mL^{-1}$ respectively for fresh cornea ($p < 0.05$), but α- and γ-cyclodextrin offered no significant improvement in drug permeation. For cryopreserved corneas β-cyclodextrin significantly enhanced riboflavin permeability to $0.1132 \pm 0.0143 \, \mu g \, mL^{-1}$ ($p < 0.001$), whilst α-, γ- and hydroxypropyl-β-cyclodextrins offered no enhancement.

Statistically significant differences ($p < 0.001$) were observed for riboflavin permeability between cryopreserved and fresh corneas.
Further interpretation was achieved by calculating steady-state flux and apparent permeability coefficients \( (P_{\text{app}}) \) from phase solubility diagrams comparing aqueous riboflavin solution and riboflavin/cyclodextrin solutions, Table 2. Overall an order of magnitude increase in drug permeability through cryopreserved tissues was observed compared to fresh corneas.

Table 2. Steady-state flux and apparent permeability coefficient \( (P_{\text{app}}) \) of riboflavin through bovine cornea in the presence and absence of cyclodextrin

<table>
<thead>
<tr>
<th>Solution</th>
<th>Fresh</th>
<th></th>
<th></th>
<th>Cryopreserved</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Steady state flux</td>
<td>( P_{\text{app}} ) ( \text{cm} \text{s}^{-1} \times 10^8 )</td>
<td></td>
<td>Steady state flux</td>
<td>( P_{\text{app}} ) ( \text{cm} \text{s}^{-1} \times 10^8 )</td>
</tr>
<tr>
<td>De-ionised water</td>
<td>0.0002</td>
<td>0.49</td>
<td></td>
<td>0.0014</td>
<td>8.57</td>
<td></td>
</tr>
<tr>
<td>( \alpha )-cyclodextrin</td>
<td>0.0003</td>
<td>1.72</td>
<td></td>
<td>0.0014</td>
<td>10.1</td>
<td></td>
</tr>
<tr>
<td>( \beta )-cyclodextrin</td>
<td>0.0006</td>
<td>2.69</td>
<td></td>
<td>0.0024</td>
<td>12.8</td>
<td></td>
</tr>
<tr>
<td>( \gamma )-cyclodextrin</td>
<td>0.0006</td>
<td>0.42</td>
<td></td>
<td>0.0011</td>
<td>5.10</td>
<td></td>
</tr>
<tr>
<td>HP-( \beta )-cyclodextrin</td>
<td>0.0015</td>
<td>3.35</td>
<td></td>
<td>0.0011</td>
<td>6.34</td>
<td></td>
</tr>
</tbody>
</table>

To provide a further insight into the effect on cryopreservation of bovine cornea on its permeability we have evaluated the integrity of corneal tissues using microscopy. Microscope slides were prepared using fresh, one week and six weeks cryopreserved bovine cornea (-18°C) to investigate any morphological differences between these tissues (Figure 5). It can be seen from the micrographs that epithelium of fresh cornea is intact and adhered to underlying stroma of fresh tissue. In one week frozen cornea, integrity of epithelium showed signs of degradation and at six weeks, there was evidence of significant degradation to the epithelium.
Figure 5. Micrographs of bovine cornea cross-sections: (a) Fresh cornea; (b) one week cryopreserved cornea; (c) six weeks cryopreserved cornea. Scale bar = 100 μm.

It appears that epithelium degradation increases with increased time when stored at -18°C and this is likely to be due to the formation of ice crystals developing in the cellular matrix, and possibly from ‘freeze drying’ effect of stored tissue. For all samples there was no visual evidence of degradation to the stroma, however, Fullwood and Meek\textsuperscript{31} and Yi-fei et al\textsuperscript{32} report structural changes in the stroma due to cryopreservation when investigated using synchrotron X-ray diffraction techniques.

Further microscopy experiments were conducted to establish the effect of cyclodextrins on the integrity of fresh bovine cornea. Figure 6 compares images of fresh bovine cornea treated with 30 mg mL\textsuperscript{-1} β-cyclodextrin solution with control samples. In each figure, images to the left are from unexposed regions of cornea, images to the right are from cyclodextrin solution exposed regions, with increasing exposure at 15, 45, 75 minutes. Samples treated with β-cyclodextrin show some epithelial disruption, which becomes more noticeable with longer exposure time. At 15 minutes exposure we begin to see what looks like a loosening of the cell structure in the surface of the epithelium; at 45 minutes this phenomenon becomes more pronounced and at 75 minutes the changes are apparent throughout the whole epithelial layer.
Micrographs for α-, γ- and hydroxypropyl-β-cyclodextrin exposed corneas are given in Supporting Information (Figures 2S, 3S and 4S) and they show a similar trend of increasing epithelium disruption with increasing exposure time to cyclodextrin solutions. All images shown are representative examples, considerable variation was observed between samples of the same exposure time due to natural tissue variability.

![Micrographs of bovine cornea exposed to 1 mL β-cyclodextrin (30 mg mL⁻¹) (b, d and f) against non-exposed regions (a, c and e). Exposure time: 15 minutes (a and b), 45 minutes (c and d) and 75 minutes (e and f). Scale bar = 100 μm.](image)

**Figure 6.** Micrographs of bovine cornea exposed to 1 mL β-cyclodextrin (30 mg mL⁻¹) (b, d and f) against non-exposed regions (a, c and e). Exposure time: 15 minutes (a and b), 45 minutes (c and d) and 75 minutes (e and f). Scale bar = 100 μm.

**Cyclodextrin-mediated cholesterol extraction.** Cellular membranes are known to consist of lipid bi-layers interspersed with cholesterol-rich lipid rafts, and corneal epithelial cells also consist of similar membranes. Cyclodextrins have previously been reported to extract cholesterol and other lipids from cell membranes. Zaidi *et al* investigated the effect of β-cyclodextrin on laboratory cultured human corneal cells in vitro and murine corneal tissue in vivo. They
demonstrated the disruption of lipid rafts and hypothesized that it is related to extraction of cholesterol from the plasma membrane of epithelial cells. Ohtani et al studied the influence of α-, β- and γ-cyclodextrins on human erythrocytes and demonstrated that β-cyclodextrin extracted cholesterol from cell membranes.\textsuperscript{34}

To prove the extraction of cholesterol from bovine cornea under experimental conditions used in this work, cyclodextrin solutions that had been in contact with biological tissues together with positive and negative controls, were analyzed using HPLC. Figure 7 shows evidence of cholesterol extraction employing β-cyclodextrin and hydroxypropyl-β-cyclodextrin, no such evidence was seen for corneas exposed to water, α- and γ-cyclodextrin.

![Figure 7. HPLC traces from analysis of cyclodextrin mediated cholesterol extraction.](image)

**Conclusions**

This study has shown that the solubility of riboflavin can be enhanced using α-cyclodextrin at 100 mg mL\textsuperscript{-1} and β-cyclodextrin at 20 - 30 mg mL\textsuperscript{-1}, thereby increasing its availability when used in ocular drug formulations. Permeability of riboflavin through fresh bovine cornea has
been shown to improve in the presence of β- and hydroxypropyl-β-cyclodextrins; however, only β-cyclodextrin enhanced permeability through cryopreserved cornea. The study has shown that there is an order of magnitude difference in riboflavin permeability between fresh and cryopreserved bovine corneas and the lag time reduced from ~75 minutes for fresh corneas to ~40 minutes for cryopreserved materials. Microscopy has highlighted morphological differences between fresh and cryopreserved ocular tissue which should be taken into consideration when using cryopreserved corneas as permeability models.

Aqueous cyclodextrin solutions have been shown to disrupt the epithelium integrity at the cornea surface and might offer a means to render this lipophilic barrier less resistive to permeation of aqueous solubilized compounds. Our study has shown that cyclodextrins have the ability to extract cholesterol and other lipids from ocular cellular membranes; this could provide an explanation for the observed epithelium disruption and enhancement to permeability.

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Supporting information

The supporting information provided here shows an experimental set up for corneal exposure to drug formulations using a ‘whole eye’ method (Figure 1S).

Figure 1S Schematic of a whole eye experimental set up developed during the course of this study, this method allows the cornea to be exposed to drugs without prior dissection.

Micrographs of cornea cross-sections from cyclodextrin exposure experiments, Figure 2S shows micrographs from α-cyclodextrin experiments, Figure 3S from γ-cyclodextrin and Figure 4S from hydroxypropyl-β-cyclodextrin. Images to the left of the frame are non-exposed sections and images to the right are cyclodextrin exposed sections. Moving down the frame show images of progressively longer duration of exposure at 15, 45 and 75 minutes.
Figure 2S. Micrographs of bovine cornea exposed to α-cyclodextrin (30 mg ml⁻¹). Images a and b, 15 minutes, c and d, 45 minutes, e and f, 75 minutes exposure. Images a, c and e from non-exposed regions, b, d and f from cyclodextrin exposed regions.
Figure 3S  Micrograph of bovine cornea exposed to $\gamma$-cyclodextrin (30 mg mL$^{-1}$). Images a and b, 15 minutes, c and d 45 minutes, e and f, 75 minutes exposure. Images a, c and e from non-exposed regions, b, d and f from cyclodextrin exposed regions.

Figure 4S  Micrograph of bovine cornea exposed to hydroxypropyl-\(\beta\)-cyclodextrin (30 mg mL$^{-1}$).

Images a and b, 15 minutes, c and d 45 minutes, e and f, 75 minutes exposure. Images a, c and e from non-exposed regions, b, d and f from cyclodextrin exposed regions.