Quantitative analysis of corneal stromal riboflavin concentration without epithelial removal

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Purpose: To compare the corneal stromal riboflavin concentration and distribution using 2 transepithelial corneal crosslinking (CXL) systems.

Setting: Absorption Systems, San Diego, California, USA.

Design: Experimental study.

Methods: The stromal riboflavin concentration of 2 transepithelial CXL systems was compared in rabbit eyes in vivo. The systems were the Paracel/Vibex Xtra, comprising riboflavin 0.25% solution containing TRIS and ethylenediaminetetraacetic acid and an isotonic solution of riboflavin 0.25%, (Group 1) and the CXLO system (Group 2). Manufacturers’ Instructions For Use were followed. The intensity of riboflavin fluorescence by slitlamp observation 10, 15, and 20 minutes after instillation was graded on a scale of 0 to 5. The animals were humanely killed and the corneal stromal samples analyzed with liquid chromatography and mass spectrometry.

Results: The mean riboflavin fluorescence intensity grades in Group 1 (4 eyes) were 3.8, 4.8, and 4.8 at 10, 15, and 20 minutes, respectively. The mean grades in Group 2 (3 eyes) were 2.0, 2.3, and 2.0, respectively. The riboflavin distribution was uniform in Group 1 but not in Group 2. The mean riboflavin concentration by liquid chromatography and mass spectrometry was 27.0 µg/g stromal tissue in Group 1 and 6.7 µg/g in Group 2. A stromal riboflavin concentration theoretically adequate for CXL, 15 µg/g, was achieved in all eyes in Group 1 and no eyes in Group 2. Slitlamp grading correlated well with liquid chromatography and mass spectrometry concentration ($R^2 = 0.940$).

Conclusions: The system used in Group 1 produced corneal riboflavin concentrations that were theoretically adequate for effective transepithelial CXL, while the system in Group 2 did not. Slitlamp grading successfully estimated the stromal riboflavin concentration and can be used to ensure an adequate concentration of riboflavin in the cornea for transepithelial CXL.

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tiffening of the cornea by crosslinking collagen and other corneal stromal molecules was first reported in animals by Spoerl et al. in 1998 and in humans by Wollensak et al. in 2003. This original Dresden protocol requires the mechanical removal of the corneal epithelium because the intact epithelium prevents passage of topically applied riboflavin into the stroma. Epithelial debridement creates prolonged pain, delays visual recovery, and can lead to a variety of complications.

Strategies used to saturate the corneal stroma with riboflavin through an intact epithelium have included mechanical disruption of the epithelium, increased exposure time, the addition of excipients (eg, benzalkonium chloride, vitamin E), and the use of iontophoresis or ultrasound to transport riboflavin across the epithelium. These strategies have increased the absorption of topical riboflavin but have not produced stromal concentrations equal to those obtained with epithelial debridement.

The use of transepithelial riboflavin formulations, commercially available outside the United States, in patients with keratoconus produced encouraging early clinical results followed by reports of keratoconus progression between 1 year and 2 years after treatment. Overall, the effectiveness of transepithelial techniques and products has been disappointing.
We compared the transepithelial penetration of riboflavin into the corneal stroma using a new transepithelial corneal crosslinking (CXL) system and a commercially available transepithelial CXL system in vivo in rabbit eyes.

**MATERIALS AND METHODS**

**Animals**

Seven New Zealand White rabbits (Western Oregon Rabbit Co.) weighing 3.02 to 4.34 kg were housed under a 12/12 hour light/dark cycle with food and water provided ad libitum. All experimental protocols complied with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research and the U.S. Department of Agriculture Animal Welfare Act and Public Health Service approved Animal Welfare Assurance (A4282-01). The study protocol was approved by an Institutional Animal Care and Use Committee (Absorption Systems, Inc., San Diego, California, USA).

**Surgical Technique**

Seven animals with normal corneas and anterior ocular segments on slitlamp examination were divided into 2 groups. After an intramuscular injection of ketamine (30 mg/kg) and xylazine (5 mg/kg), 1 drop of topical proparacaine hydrochloride (0.5%) was instilled into 1 eye of each animal.

Animals in Group 1 were treated with a commercially available transepithelial CXL system (Paracel/Vibex Xtra, Avedro, Inc.) according to the manufacturer’s Instructions For Use. This system consists of 2 solutions, a riboflavin 0.25% solution containing TRIS and ethylenediaminetetraacetic acid (EDTA) (Paracel) and an isotonic solution of riboflavin 0.25% (Vibex Xtra) used sequentially. One drop of the solution containing TRIS and EDTA was applied to the cornea every 90 seconds for approximately 4 minutes. The cornea was rinsed with the riboflavin 0.25%, and 1 drop of riboflavin 0.25% was then applied every 90 seconds for 6 minutes, for a total riboflavin exposure time of 10 minutes.

Group 2 animals were treated with the test transepithelial riboflavin system (CXLO, CXL Ophthalmics LLC). This system consisted of (1) a new transepithelial riboflavin formulation without dextran and with a sodium iodide excipient in which the riboflavin concentration, pH, and osmolarity were designed to enhance absorption and (2) 2 sterile proprietary applicators designed to maximize contact between the riboflavin solution and the corneal surface. First, eyes were gently brushed with minimal pressure in a circular motion over the entire cornea for 30 to 40 seconds with a patent-pending specially-designed sterile applicator that had been fully saturated with proparacaine. This applicator (Figures 1 and 2) is constructed of a nonabrasive porous material with patent-pending shapes, pore size, flexibility, and hydration properties specifically designed to enhance penetration of the new transepithelial riboflavin solution into the corneal stroma without disrupting the corneal epithelium (Figures 3 and 4). Unlike previously described techniques and devices, this device does not use the principle of inducing epithelial disruption to improve epithelial permeability. The intact nature of the epithelium was confirmed on slitlamp examination by an independent laboratory researcher (G.G.G.). Next, a sponge-like loading device, shaped to conform to the cornea’s curvature (including steep, cone-shaped corneas) and maximize contact with the cornea was saturated with the test transepithelial riboflavin solution and placed over the entire cornea (Figure 5). Continuous exposure to the new transepithelial riboflavin solution was ensured by the addition of 1 to 2 drops of the solution to the sponge every 1 to 3 minutes for 10 minutes.

![Figure 1. Proprietary sterile delivery device before hydration.](image1)

![Figure 2. Proprietary device after hydration with no sharp edges to induce epithelial disruption.](image2)

![Figure 3. Epithelial defects typical of those induced by standard ophthalmic sponges with sharp edges in institutional review board–approved human studies.](image3)

![Figure 4. Undisrupted epithelium after treatment with the proprietary device shown in Figures 1 and 2. Human eye treated in an institutional review board–approved clinical trial.](image4)
using an Xevo TQ-S spectrometer (Waters Corp.).

formed using an Acquity Ultra Performance Liquid Chromatography. The intensity of riboflavin fluorescence in the corneal stroma was graded on a scale of 0 to 5 by a masked observer using standardized photographs (Table 1 and Figure 6).

Clinical Examination
Before the application of riboflavin, each eye had a baseline examination consisting of indirect ophthalmoscopy and corneal slitlamp examination (BP 900, Haag-Streit AG) with photography to ensure that only normal corneas were used for subsequent experiments. There were no epithelial defects or abnormalities visible on slitlamp examination before or after any of the treatments. Ten, 15, and 20 minutes after the treatments described above, each eye had slitlamp examination and slitlamp photography. The intensity of riboflavin fluorescence in the corneal stroma was graded on a scale of 0 to 5 by a masked observer using standardized photographs (Table 1 and Figure 6).

Liquid Chromatography and Mass Spectrometry
After the 20-minute slitlamp assessment, animals were humanely killed by intravenous injection of barbiturate (20 to 25 minutes after treatment). Treated eyes and untreated fellow eyes were harvested, the corneas were removed, and ethanol 20% was used to remove the corneal epithelium from the stroma. Corneal stromal samples from individual eyes were frozen at −60°C to −80°C until analyzed with liquid chromatography and mass spectrometry.

Samples were weighed, and a solution of 20:80 methanol:water was added to bring the final concentration to 4 mL/g sample. Samples were homogenized (Virsonic 100 ultrasonic homogenizer, Virtis, SP Scientific) and stored frozen until analyzed.

For liquid chromatography and mass spectrometry analysis, 50 μL aliquots of the corneal homogenate were mixed with 10 μL 50:50 acetonitrile:water. Proteins were precipitated in 150 μL of acetonitrile with 100 ng/mL warfarin, after which samples were centrifuged at 13 000 rpm for 10 minutes. Supernatant was aliquoted into 96-well plates for analysis. Standards consisted of untreated rabbit cornea processed in the same way as treated corneas. High-performance liquid chromatography was performed using an Acquity Ultra Performance Liquid Chromatography system (Waters Corp.). Mass spectrometry was performed using an Xevo TQ-S spectrometer (Waters Corp.).

Table 1. Riboflavin grading scale.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
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<tbody>
<tr>
<td>0</td>
<td>No green visible</td>
</tr>
<tr>
<td>1</td>
<td>Mild green tint just visible</td>
</tr>
<tr>
<td>2</td>
<td>Substantial green visible</td>
</tr>
<tr>
<td>3</td>
<td>Obvious green color</td>
</tr>
<tr>
<td>4</td>
<td>Bright green appearance</td>
</tr>
<tr>
<td>5</td>
<td>Strong bright green color</td>
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RESULTS
At baseline, all ophthalmic observations were normal. No evidence of epithelial disruption was seen before or after treatment of animals in either group. In Group 1, riboflavin fluorescence in the corneal stroma was patchy (not uniform). In some animals, no riboflavin staining was seen in the corneal stroma. In other animals, there was partial penetration of riboflavin into the stroma. Stromal penetration of riboflavin tended to be better superiorly than inferiorly (Figure 7). Group 2 corneas had uniform riboflavin fluorescence throughout the depth of the stroma and across the entire cornea. Figure 7 shows representative slitlamp images.

Figure 8 shows the slitlamp grading of the riboflavin fluorescence in individual corneas. Figure 9 shows the concentration of riboflavin in the corneal stroma measured by liquid chromatography and mass spectrometry analysis. The stromal riboflavin concentrations in Group 2 corneas were approximately 4 times greater than in Group 1 corneas.

To determine whether slitlamp grading of riboflavin fluorescence correlates with the concentration of riboflavin measured quantitatively by liquid chromatography and mass spectrometry, slitlamp grading scores were plotted against measured riboflavin concentrations (Figure 10). There was good correlation between the slitlamp grade and riboflavin concentration measured by liquid chromatography and mass spectrometry ($R^2 = 0.940$). A slitlamp grade of 3 corresponds to a riboflavin concentration of approximately 15 μg/g.

DISCUSSION
Corneal crosslinking has been shown to halt the progression of ectatic corneal disease, including keratoconus and ectasia after laser in situ keratomileusis (LASIK). It requires an adequate concentration of riboflavin, ultraviolet light, and oxygen in the corneal stroma. The initial Dresden protocol for CXL included removal of the epithelium, leading to posttreatment pain, delayed visual recovery, and increased complications. Attempts to load the corneal stroma with riboflavin without removing the epithelium have had little success.

Results in the current study suggest that the test CXLO transepithelial CXL system (Group 2) provides sufficient stromal riboflavin for effective CXL. An in vivo application of the standard epithelium-off CXL (Dresden) protocol resulted in stromal riboflavin concentrations ranging from 14.42 μg/g after 5 minutes of riboflavin exposure to 24.06 μg/g after a 30-minute exposure. In the current study, the transepithelial riboflavin concentration in the stroma in Group 2 eyes was 23.2 μg/g to 31.0 μg/g after a 10-minute exposure. This is well within the range of stromal riboflavin concentration achieved by the standard Dresden CXL protocol. Conversely, the range of stromal riboflavin concentrations with the commercially available transepithelial CXL Paracel/Vibex Xtra system (Group 1) (5.1 μg/g to 9.65 μg/g) was less than that produced by even a 5-minute application of riboflavin to human eyes.
under the Dresden protocol.\textsuperscript{4} It has been proposed that a concentration of 15 $\mu$g/g is necessary for successful CXL.\textsuperscript{4} This value was not achieved in any Group 1 eye but was achieved in all Group 2 eyes. Although the current study used rabbit eyes, this result suggests that the test system allows sufficient riboflavin transport across the corneal epithelium to support successful CXL in patients with keratoconus.

Slitlamp biomicroscopy of the treated eyes showed that the uniformity, extent, and concentration of riboflavin in corneas treated in Group 2 were greater than corneas treated in Group 1. The stromal riboflavin distribution in
Group 1 corneas was patchy and was not present in many areas of the cornea. In contrast, the stromal riboflavin distribution in Group 2 corneas was uniform, reaching all sectors and depths of the cornea. The distribution and intensity of riboflavin fluorescence were summarized by a loading grade score. The scores in Group 1 were tightly clustered (≤3), as were those in the Group 2 (≥3), suggesting that the loading grades represented the amount of riboflavin present in each eye. Despite the small number of eyes, the current study also found a strong linear correlation between the riboflavin fluorescence in the corneal stroma based on slitlamp examination and the concentration of riboflavin determined by liquid chromatography and mass spectrometry analysis, further supporting the use of the loading grade scale to estimate riboflavin concentration in vivo. The grading scale could allow clinicians to verify whether sufficient riboflavin has reached the corneal stroma for CXL. This grading scale has been used in a large, multicenter, institutional review board–approved clinical study, which found the same consistent, rapid, and homogeneous loading without epithelial disruption.

Group 1 and Group 2 differed in the method of riboflavin application and the composition of the riboflavin solution. Riboflavin was dropped onto the corneas in Group 1. In contrast, Group 2 corneas were first swabbed with a proprietary wand designed to improve penetration without disrupting the epithelium. Riboflavin was then applied via a patent-pending corneal sponge designed to maximize contact between the riboflavin solution and the surface of the cornea. Although the CXLO application system was devised to present more riboflavin to the cornea, it is not sufficient to account for the higher levels of stromal riboflavin in eyes in which that system was used. Previous research found that the use of a CXLO sponge-loading system with other riboflavin formulations in concentrations similar to the commercial riboflavin formulation tested in this analysis does not yield the same rapid and consistent transepithelial stromal riboflavin concentrations seen with the test riboflavin solution plus the applicators. The excellent transepithelial stromal loading in Group 2 was likely the result of the combination of the new application devices and the enhanced riboflavin formulation.

In summary, this study confirms that a new transepithelial CXL system can achieve corneal stromal riboflavin concentrations that are 4 times greater than those achieved with a commercially available system and theoretically adequate for effective treatment of ectatic corneal disease. The efficacy of transepithelial CXL for the treatment of keratoconus and corneal ectasia after LASIK using the new transepithelial CXL system has been studied in a large patient series and is currently under review. The data also support the use of a standardized slitlamp grading system to estimate the stromal riboflavin concentration.

WHAT WAS KNOWN
- The corneal epithelium restricts penetration of riboflavin into the corneal stroma.
- Epithelial debridement is necessary to permit sufficient absorption of riboflavin by the corneal stroma and effective CXL for the treatment of keratoconus and post-LASIK ectasia.
- Previously published results of CXL without epithelial removal have resulted in progression of the disease between 1 year and 2 years after treatment.

WHAT THIS PAPER ADDS
- The test transepithelial CXL system achieved corneal stromal concentrations of riboflavin that were 4-fold higher than those achieved with a commercially available transepithelial CXL system and that are theoretically adequate for effective CXL.
- A standardized slitlamp grading system can be used to estimate the riboflavin concentration in the corneal stroma.
REFERENCES


OTHER CITED MATERIAL


Disclosures: Drs. Rubinfeld, Stulting, and Talamo have a financial interest in the CXL0 system. Dr. Rubinfeld also has a financial interest in CXLUSA, LLC. Dr. Gum has no financial or proprietary interest in any material or method mentioned.

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