A new riboflavin solution for trans-epithelial cross-linking: a study of corneal pharmacokinetic

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We describe in this letter the results of an experimental study aimed to determine the corneal penetration and concentration of a solution of riboflavin 0.1%, added with enhancers, as occurs during trans-epithelial cross-linking (TE-CXL).

The study adhered to the tenets of the Declaration of Helsinki, informed consent was obtained for each cornea; Institutional Review Board (IRB)/Ethics Committee, and Animal Care and Use committee approvals were obtained (authorization n. 1269).

We tested 15 warm-stored human corneas not suitable for transplant, due to contamination of the storage solution. Only corneas with a good transparency, thickness between 500 and 600 microns, intact epithelium and healthy endothelial mosaic were used.

The corneas, mounted on an artificial anterior chamber, were soaked for 30 minutes with a solution of riboflavin and enhancers; every five minutes, the solution present in the artificial anterior chamber was removed and placed in a test tube for spectrophotometric analysis.

Figure 1 – The artificial anterior chamber used for the experiment

The absorbance of riboflavin was correlated with its concentration, according to a calibration curve. Corneas were then sectioned and analyzed with optical (400X) and fluorescence microscopy.

A similar experiment was conducted with 12 porcine corneas, using a Franz cell. Every 5 minutes for a period of 30 minutes, 5 ml were removed from the receiver compartment to be analyzed with high-performance liquid chromatography (HPLC), and replaced with the same amount of saline solution.

After 30 minutes, the porcine corneas were washed with 2 ml of saline solution to remove the donor solution. A 5 mm diameter corneal area, which corresponds to 17.3 mg of tissue was extracted in 5 ml of anhydrous methanol for 20 hours in absence of light. The extracting solution, filtered, vacuum dehydrated, and rehydrated with a 0.2% acetic acid water solution, was analyzed with HPLC. Complete experimental parameters and results are available at http://aaojournal.org (Figures 1-8, Tables 1-6).

The proposed solution promoted the penetration of riboflavin through epithelialized corneas. Similar results were obtained with human and porcine corneas. The amount of riboflavin released by the corneas increased during the first 15 minutes, peaked at 15 minutes (average of 0.048 mg in human and 0.130 mg in porcine corneas), then sharply dropped after 20 minutes (average of 0.023 mg in human and 0.047 mg in porcine corneas).

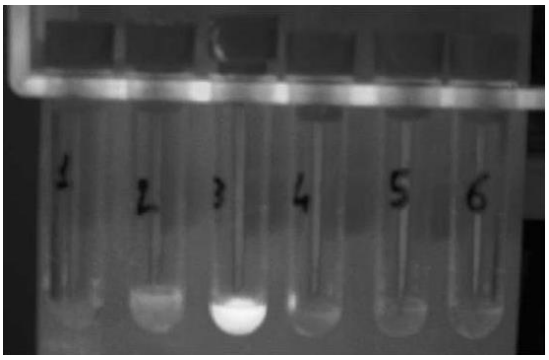

Figure 2 – Test tubes illuminated by wood light showing the peak of riboflavin fluorescence at 15 min.

The mean riboflavin concentration in corneal tissue at 30 minutes from application was 308 µg/g in porcine corneas, equal to 5.3247 µg with a standard error of mean of 1.7081, comparable to that of other solutions used for ultraviolet-A induced CXL[1], and superior to a theoretical concentration of 15 µg/g for safe and effective ultraviolet-A induced CXL treatment[1]. Optical microscopy showed that the riboflavin epithelial penetration occurred paracellularly. Fluorescence microscopy showed the complete dye diffusion in the corneal tissue.

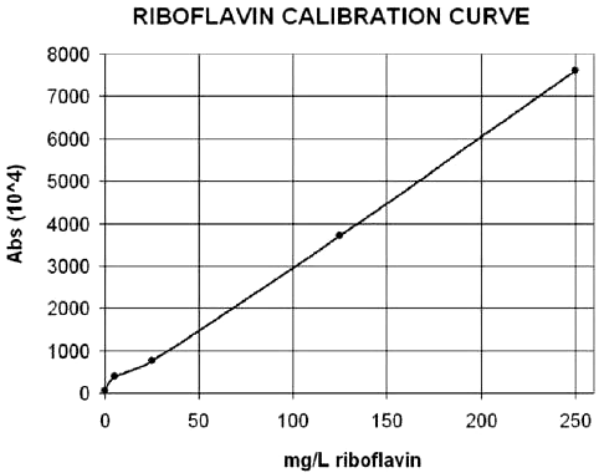

Figure 3 – Riboflavin absorbance calibration curve.

Figure 4 – Optical microscopy image (400X) showing corneal soaking after 30 min of the proposed solution topical instillation. The yellow areas surrounding epithelial cells suggest paracellular passage of riboflavin.

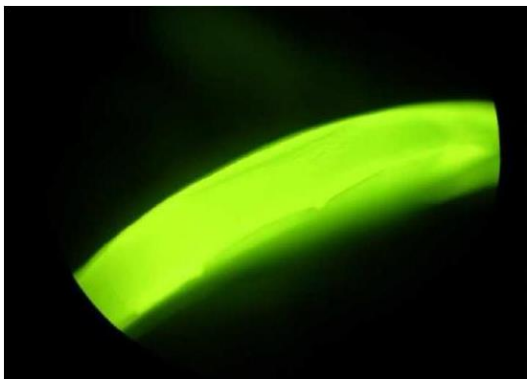

Figure 5 – Fluoroscopic image of a corneal section showing the complete diffusion of the fluorescent dye after 30 min of solution topical instillation.

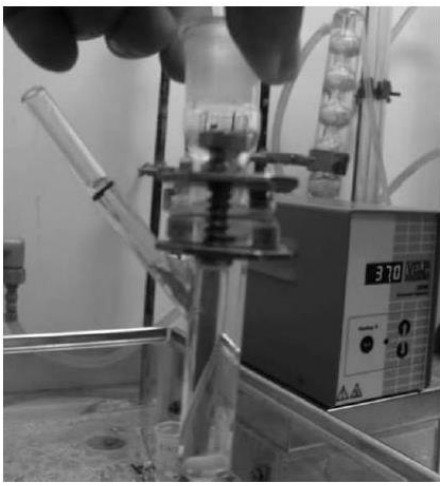

Figure 6 – The Franz-cell used for experimentation.

Our decision is to evaluate the proposed substances as penetration enhancers was based on their capability to penetrate through the corneal epithelium[2] and to protect the corneal structures agaist the toxic effects of UV rays and oxidant injuries[3-5].

We propose a mathematical model for corneal diffusion:

$$\frac{\partial w(z,t)}{\partial t} = \alpha \cdot r(z,t) - \beta \cdot w(z,t) \qquad (1)$$

$$\frac{\partial r(z,t)}{\partial t} = \nabla \cdot ((\gamma - \delta \cdot w(z,t)) \cdot \nabla r(z,t)) \qquad (2)$$

Wherein r is the mean concentration of riboflavin solution in a control volume centered at depth z and at time t, and w is the mean concentration of water in excess in the cell contained in the control volume centered at depth z and at time t, and α, β, γ, δ being four constants to be fixed.

Equation (2) differs from classic diffusion equations because it contains two unknown concentrations, r and w, instead of only one, and contemplates a stop condition:

$$w = \frac{\gamma}{\delta} \qquad (3)$$

When the above condition is satisfied, the riboflavin solution cannot penetrate further through the cornea.

With greater values of γ, riboflavin solution diffuses more and faster (r decreases rapidly) and w does not increase sufficiently to stop the diffusion of the solution. By contrast, if γ is small, the concentration r remains high for a sufficiently long time to enable w reaching the stop condition.

At the rear corneal surface it is expected that the riboflavin concentration r increases soon after topical instillation because w is initially null. As long as the concentration r increases, it closes to the stop condition w. Therefore, from this moment on, the dropping out of the riboflavin solution would be expected to decrease.

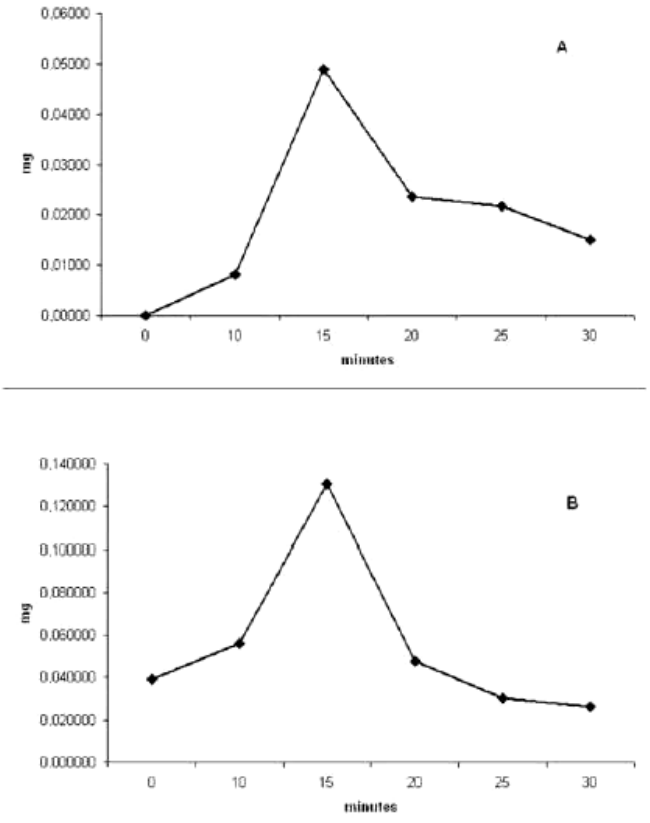

Figure 7 – Comparison of riboflavin passage through human and porcine corneas after topical application of the proposed solution: A) amount in artificial chamber with human warm-stored eye-bank corneas (15 samples). B) amount in receiver compartment with Franz-cell mounted porcine corneas (12 samples).

Table 1 – Description of the experimental parameters for the human corneas group

PARAMETER	DEVICE/VALUES
Artificial anterior chamber	MORIA™, Antony, France
Volume of the anterior chamber	0.8 ml
Composition of the riboflavin solution	Riboflavin-dextran 0.1 g/ 100 g, D-alpha tocopheryl polyethylene-glycol 1000 succinate (vitamin E TPGS) 500 mg/ 100 ml, coenzyme Q 100 mg/ 100 ml, L-proline 0.1 mg/ 100 ml, glycine 0.1 mg/ 100 ml, lysine hydrochloride 0.05 mg/ 100 ml and L-leucine 0.08 mg/ 100 ml (Sinergie Pharmaceuticals, italy)
Solution filling the anterior chamber	Balanced salt solution
Spectrophotometer	AU 600 Biochemistry Analyzer Automated, Olympus, NJ, USA
Wavelength analysis	340 nanometers

Table 2 – Description of the experimental parameters for the porcine corneas group

PARAMETER	DEVICE/VALUES
Franz Cell	SES GmbH, Bechenheim, Germany
Capacity of the donor compartment	2 ml
Composition of the riboflavin solution	Riboflavin-dextran 0.1 g/ 100 g, D-alpha tocopheryl polyethylene-glycol 1000 succinate (vitamin E TPGS) 500 mg/ 100 ml, coenzyme Q 100 mg/ 100 ml, L-proline 0.1 mg/ 100 ml, glycine 0.1 mg/ 100 ml, lysine hydrochloride 0.05 mg/ 100 ml and L-leucine 0.08 mg/ 100 ml (Sinergie Pharmaceuticals, italy)
Receiver compartment	5 ml of pH 7.4 saline solution , at 37°C, under electromagnetic stirring, shielded from light
High-pressure liquid chromatography	Jasco LC-200, Jasco Europe, Cremella, Italy

Table 3 – Description of the HPLC method adopted

Stationary phase	Mobile phase	Gradient	λ	Flux
Supelco C16 RP-amide 150 x 4.6 mm, 5 µm	Solution A: Acetonitrile Solution B: H_2PO_4 0.3% in water	From 100% of A to 100% of B in 8 minutes	268 nm	1.2 ml/min

Table 4 – HPLC validation parameters

	Tr (min) [1]	R^2 [2]	LOD (µg/ml) [3]	LOQ(µg/ml) [4]
Riboflavin	5.03	0.9732	0.037	0.045

Legend:
1)Retention time
2)Linear regression coefficient
3)Limit of detection
4)Limit of quantification

Table 5 – Riboflavin concentrations in artificial chamber after topical application of the proposed solution on human warm-stored eye-bank corneas (15 samples).

	(a)	(b)	(c)		(a)	(b)	(c)		(a)	(b)	(c)
Cornea 1	5 min.	0	0	Cornea 2	5 min.	0	0	Cornea 3	5 min.	0	0
	10 min.	0.008	67		10 min.	0.009	64		10 min.	0.008	62
	15 min.	0.048	135		15 min.	0.049	137		15 min.	0.048	134
	20 min.	0.025	22		20 min.	0.020	22		20 min.	0.035	25
	25 min.	0.030	23		25 min.	0.020	21		25 min.	0.010	13
	30 min.	0.025	20		30 min.	0.010	7		30 min.	0.010	7
	(a)	(b)	(c)		(a)	(b)	(c)		(a)	(b)	(c)
Cornea 4	5 min.	0	0	Cornea 5	5 min.	0	0	Cornea 6	5 min.	0	0
	10 min.	0.008	65		10 min.	0.009	61		10 min.	0.007	66
	15 min.	0.048	138		15 min.	0.049	136		15 min.	0.049	137
	20 min.	0.010	14		20 min.	0.015	14		20 min.	0.020	22
	25 min.	0.015	15		25 min.	0.025	22		25 min.	0.015	15
	30 min.	0.020	17		30 min.	0.010	8		30 min.	0.005	7
	(a)	(b)	(c)		(a)	(b)	(c)		(a)	(b)	(c)
Cornea 7	5 min.	0	0	Cornea 8	5 min.	0	0	Cornea 9	5 min.	0	0
	10 min.	0.008	68		10 min.	0.006	59		10 min.	0.005	57
	15 min.	0.048	135		15 min.	0.0495	137		15 min.	0.048	135
	20 min.	0.010	8		20 min.	0.03	27		20 min.	0.015	15
	25 min.	0.020	21		25 min.	0.01	7		25 min.	0.025	24
	30 min.	0.010	10		30 min.	0.015	11		30 min.	0.030	20
	(a)	(b)	(c)		(a)	(b)	(c)		(a)	(b)	(c)
Cornea 10	5 min.	0	0	Cornea 11	5 min.	0	0	Cornea 12	5 min.	0	0
	10 min.	0.005	60		10 min.	0.0105	67		10 min.	0.009	64
	15 min.	0.048	135		15 min.	0.0505	135		15 min.	0.049	137
	20 min.	0.025	21		20 min.	0.03	22		20 min.	0.040	22
	25 min.	0.030	27		25 min.	0.02	23		25 min.	0.025	21
	30 min.	0.015	11		30 min.	0.01	20		30 min.	0.015	7
	(a)	(b)	(c)		(a)	(b)	(c)		(a)	(b)	(c)
Cornea 13	5 min.	0	0	Cornea 14	5 min.	0	0	Cornea 15	5 min.	0	0
	10 min.	0.009	62		10 min.	0.0085	65		10 min.	0.010	61
	15 min.	0.050	134		15 min.	0.0485	138		15 min.	0.049	136
	20 min.	0.020	25		20 min.	0.025	14		20 min.	0.035	14
	25 min.	0.025	13		25 min.	0.03	15		25 min.	0.025	22
	30 min.	0.015	7		30 min.	0.02	17		30 min.	0.015	8

Average	(a)	(b)	(c)
	5 min.	0	0.00
	10 min.	0.008	63.20
	15 min.	0.048	135.93
	20 min.	0.023	19.13
	25 min.	0.021	18.80
	30 min.	0.015	11.80

Legend:
(a): minutes of topical application of the proposed solution
(b): mg of riboflavin released in the artificial chamber
(c): absorbance (10^4)

Table 6 – Mean riboflavin concentrations in receiver compartment after topical application of the proposed solution on Franz-cell mounted porcine corneas (12 samples).

minutes	mg of riboflavin released in the receiver compartment	standard error mean(mg)
5 min.	0.0394	0.0176
10 min.	0.0560	0.0235
15 min.	0.1305	0.0229
20 min.	0.0476	0.0112
25 min.	0.0302	0.0074
30 min.	0.0261	0.0019

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