Current Eye Research

ISSN: 0271-3683 (Print) 1460-2202 (Online) Journal homepage: http://www.tandfonline.com/loi/icey20

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To cite this article: Arthur Hammer, Serge Rudaz, Sylvie Guinchard, Sabine Kling, Olivier Richoz & Farhad Hafezi (2016): Analysis of Riboflavin Compounds in the Rabbit Cornea In Vivo, Current Eye Research, DOI: 10.3109/02713683.2015.1101141

To link to this article: http://dx.doi.org/10.3109/02713683.2015.1101141

CURRENT EYE RESEARCH
http://dx.doi.org/10.3109/02713683.2015.1101141

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Analysis of Riboflavin Compounds in the Rabbit Cornea *In Vivo*

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ABSTRACT

Purpose: To investigate the composition and concentration of individual riboflavin compounds in the corneal stroma *in vivo* after soaking with various commercially available riboflavin formulations.

Methods: Experiments were performed in 26 rabbit corneas *in vivo*: 24 corneas were soaked with riboflavin formulations for 30 minutes or with 0.9% NaCl for control (n = 2). After treatment, corneas were excised and prepared for ultra-high-pressure liquid chromatography (UHPLC) analysis. Additionally, computational chemical analysis of riboflavin compounds and keratan sulfate were performed.

Results: The amount of riboflavin and riboflavin phosphate isomers in cornea decreased by a factor of 10 to 100, when compared to the amount in riboflavin formulations. In particular, we found an inverse relationship in the ratio of riboflavin to riboflavin phosphate isomer concentration between formulations and cornea. The electronegativity and ionization potential of riboflavin and phosphate isomers are different.

Conclusions: The inverse relationship observed might be explained by a stronger electronegativity of the phosphate isomers, leading to a stronger repulsion by corneal proteoglycans. Indicating the individual concentration of riboflavin compounds in formulations is more representative than the total riboflavin concentration. Riboflavin formulations and CXL protocols might be improved considering the differences in diffusion and ionization potentials of the different riboflavin compounds.

ARTICLE HISTORY
Received 19 July 2015
Revised 31 August 2015
Accepted 20 September 2015

KEYWORDS
Corneal collagen cross-linking; CXL; riboflavin; UHPLC

Introduction

Corneal cross-linking with riboflavin and UV-A light (CXL) has initially been developed in *ex vivo* porcine[1,2] and *in vivo* rabbit eyes,[3] before it was applied in humans as a treatment for corneal ectasia.[4–7] With a high success rate in stabilizing corneal shape in long-term follow-up, CXL has since become the golden standard to stop keratoconus progression.[8–13]

Current CXL protocols are based on empirical protocols, which showed clinical efficiency in stopping corneal ectatic disorder evolution. The standard CXL protocol consists of corneal de-epithelialization ("epi-off"), followed by instillation of 0.1% riboflavin solution for 30 minutes and UV-A irradiation at 365 nm with an irradiance of 3 mW/cm^2 for 30 minutes (total energy of 5.4 J/cm^2). Recently, modifications of this original protocol have been proposed to increase patient comfort during and after treatment: accelerated CXL uses a higher irradiance[14–17] to deliver the same total energy within less time, while transepihtelial ("epi-on") CXL[18–20] aims at reducing postoperative pain and the risk of infection by trying to preserve an intact corneal epithelium.

The concentration of riboflavin in the corneal stroma is crucial for a successful CXL procedure: on one hand, it is needed for the photochemical process that induces cross-links to stiffen the cornea; on the other hand it "shields" the corneal endothelium from excessive UV irradiation.

A number of studies to determine total stromal riboflavin concentration have been published in the past years, using high-pressure liquid chromatography (HPLC),[21,22] confocal fluorescence microscopy,[23] two-photon fluorescence microscopy,[24,25] and imaging mass spectrometry by matrix-assisted laser desorption/ionization.[26] However, total riboflavin is composed of a number of chemically distinct riboflavin compounds, and little is known about the concentration and composition of these compounds in the corneal stroma.

In this study, we used ultra-high pressure liquid chromatography (UHPLC) to identify the various riboflavin compounds and their concentration in the corneal stroma after applying different commercially available riboflavin formulations. We also used computational chemical analysis to determine the electronegativity and ionization potentials of these molecules for a better understanding of UHPLC results.

Materials and methods

Riboflavin applications

Thirteen male New-Zealand white Crl:KBL (NZW) rabbits (Charles River Laboratories, Chatillon-sur-Chalaronne, France), aged 12 weeks, were used in this study. Prior to the experiments, animals were anesthetized with 35 mg/kg ketamine (Ketalar Pfizer AG, Zurich, Switzerland) and 5 mg/kg

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xylazine (Rompun, Bayer AG, Leverkusen, Germany) intramuscularly. All procedures concerning animals in this study adhered to the ARVO resolution for the care and use of animals in vision research and have been approved by the local animal experimentation committee (G04/3902, Republique et Canton de Genève, Domaine de l'expérimentation animale) as in accordance with Art.18 of the "Loi fédérale sur la protection des animaux" (LPA), Art.141 of the "Ordonance sur la protection des animaux" (OPAn) and Art.30 of the "Ordonance sur l'expérimentation animale."

Corneas were divided into different experimental groups. In groups 1 to 3, corneas were de-epithelialized under an operating microscope using a hockey knife, and a suction cup filled with epi-off riboflavin formulations was applied on the stromal surface for 30 minutes. In group 4 (n = 8), the epithelium of the corneas was left intact and a suction cup filled with an epi-on riboflavin formulation was applied on the epithelial surface for 30 minutes. Corneas in group 5 (n = 2) were de-epithelialized under an operating microscope using a hockey knife and a suction cup filled with 0.9% NaCl was applied on the stromal surface for 30 minutes. These corneas served as controls. Immediately after riboflavin formulation soaking, all corneas were washed with NaCl 0.9%. Corneas in group 1 (n = 5) were treated with Mediocross D riboflavin formulation, corneas in group 2 (n = 5) with Mediocross M riboflavin formulation (both Avedro, Inc., MA, formerly PeschkeMed GmbH, Waldshut-Tiengen, Germany), corneas in group 3 (n = 6) were treated with 0.1% vitamin B2 formulation (Streuli Pharma AG, Uznach, Switzerland), and corneas in group 4 (n = 8) were treated with Mediocross TE riboflavin formulation (Avedro, Inc.).

Mediocross D consisted of a 0.1% riboflavin formulation with 20% dextran, Mediocross M of a 0.1% riboflavin formulation with 1.1% hydroxypropylmethylcellulose (HPMC), 0.1% vitamin B2 consisted of a 0.1% riboflavin formulation in NaCl 0.9%, and Mediocross TE consisted of a 0.25% riboflavin formulation with 1.2% HPMC and 0.01% benzalkonium chloride.

Immediately following the corneal washing with 0.9% NaCl, rabbits were sacrificed using an intravenous injection of 120 mg/kg of thiopental in the ear vein (Pentothal® Ospedalia AG, Hünenberg, Switzerland). Corneas were excised using a manual trepan of 7 mm diameter (Katena Products Inc, Denville, NJ) and prepared for UHPLC analysis as described below.

UHPLC measurements

UHPLC allows determining the amount of riboflavin and riboflavin phosphate isomers that are present in riboflavin formulations and hence that have been absorbed by the corneal stroma. For the measurements, riboflavin was extracted from corneal samples using 400 µl methanol. For reference, standard riboflavin (50 µl) (Fluka 83810, riboflavin 5′-monophosphate sodium salt dehydrate) was diluted in 350 µl methanol. Pharmaceutical riboflavin formulations (Mediocross D, Mediocross M, Mediocross TE, and 0.1% vitamin B2) and standard were diluted in ammonium acetate 10 mM pH 5.5 and placed in 2 mL tubes for centrifugation (Eppendorf AG, Hamburg, Germany). Tubes were incubated for 12 hours under stirring at 4°C and then centrifuged for 2 minutes at 12 000 rpm. The supernatant was evaporated under a stream of nitrogen at room temperature until completely dry. The dry extracts were then recovered with 50 µl of ammonium acetate, 10 mM pH 5.5, and methanol with 80:20 respective proportions, vortexed and centrifuged for 2 minutes at 12 000 rpm. 40 µl was transferred into a UHPLC vial for quantification.

UHPLC analysis was performed as described previously.[27] Briefly, the liquid chromatography system consisted of a Water Acuity UPLC® (Milford, MA) equipped with a photodiode array detector Acquity QDa detector® (Waters, Milford, MA). Samples were injected through an autosampler injection system with an injection volume of 5 µl at a temperature of 4°C. The mobile phase flow rate was 0.5 ml/minutes. Mobile phases consisted of ammonium acetate 10 mM (pH 5.5) and methanol in a gradient flow mode. The gain parameter was fixed at 1. The instrument control, data acquisition, and processing were performed using the Empower v.4.1® software (Waters). As flavins are light sensitive, the preparation of samples and standard was performed under restrained light. The limit of detection (LOD), estimated from a signal-to-noise ratio of 3:1, and the limit of quantification (LOQ) were determined at 25 ng/ml and 75 ng/ml, respectively. These values were assumed respectively for undetectable compounds and when present in amounts inferior to the LOQ.

Data analysis

UHPLC raw data were processed using Empower v.4.1® software (Waters). The statistical analysis was done with SPSS version 23® (IBM corp., Armonk, NY) and consisted of two analysis at different levels: (i) to test for differences of riboflavin compound concentrations and (ii) to test for differences of the ratio riboflavin/riboflavin 5′-monophosphate concentrations.

(i) Four individual MANOVAs (one for each pharmaceutical formulation) were realized to test for the global differences of the four riboflavin compound concentrations. Each MANOVA was completed with individual t-tests, testing for differences in single-compound concentrations. Both the MANOVAs and the t-tests analyzed, for a given riboflavin pharmaceutical formulation, the differences between the cornea and the formulation they were soaked with. The MANOVAs correct for multiple two by two testing of the four riboflavin compound concentrations differences. The t-tests are not subject to multiple testing.

(ii) Individual t-tests were realized to test for difference of the ratio riboflavin/riboflavin 5′-monophosphate concentrations for a given riboflavin formulation between the cornea and the formulation they were soaked with. Again, these t-tests are not subject to multiple testing.

Computational model

A computational analysis of the chemical structures of riboflavin, riboflavin 5′-monophosphate, and keratan sulfate was

performed using *Molecular Operating Environment*® (MOE 2012.10, CCG Inc., Montreal, Canada). The protonation plugin *Protonate 3D*® was used to determine the protomers at pH = 7.4, which were then used to compute the electrostatic potential maps of these molecules using the Hartree–Fock method and basis-set 6/31G** in quantum chemistry software *Spartan'14*® (Wavefunction, Inc, Irvine, CA). The ionization potential maps of riboflavin and riboflavin 5′-monophosphate were also determined with the Hartree–Fock method and basis-set 6/31G** in *Spartan'14*®. The ionization potential represents the necessary energy in eV (electronVolt) to ionize the molecule and hence participate in photochemical reactions.

Results

Figure 1 shows the distribution of the different riboflavin compounds in a laboratory standard riboflavin (*Fluka 83810, riboflavin 5′-monophosphate sodium salt dehydrate*). Four different compounds were identified: riboflavin 3′-monophosphate, riboflavin 4′-monophosphate, riboflavin 5′-monophosphate, and riboflavin. Thereby, riboflavin 5′-monophosphate showed the highest concentration in formulation.

The concentration of riboflavin compounds is shown in Table 1 for different pharmaceutical formulations and their respective corneal concentrations.

As shown in Figure 2(A)–(D), the concentrations of riboflavin and riboflavin phosphate isomer compounds decreased by a factor of 10 to 100 after diffusion into the cornea.

In particular, an inverse relationship was found between the concentrations of riboflavin and riboflavin phosphate isomer compounds. As shown in Table 1, riboflavin 5′-monophostphate was found to be the compound with the highest concentration in all formulations, but was a minor component in the cornea. In contrast, riboflavin was a minor component in formulations but always showed the highest corneal concentration.

The group-specific MANOVAs showed significant global differences of the four riboflavin compound concentrations between the cornea and the respective riboflavin formulations: group 1 versus Mediocross D formulation ($p < 0.01$), group 2 versus Mediocross M formulation ($p < 0.01$), group 3 versus 0.1% vitamin B2 formulation ($p < 0.01$), and group 4 versus Mediocross TE formulation ($p < 0.01$). Table 1 shows the p-values of the corresponding individual t-tests. Significant differences were found for all comparisons of single-compound concentrations between the cornea of groups 1–4 and their respective formulation.

Table 2 and Figure 3 show the p-values of the individual t-tests analyzing the differences of the ratio riboflavin/riboflavin 5′-monophosphate between the cornea of groups 1–4

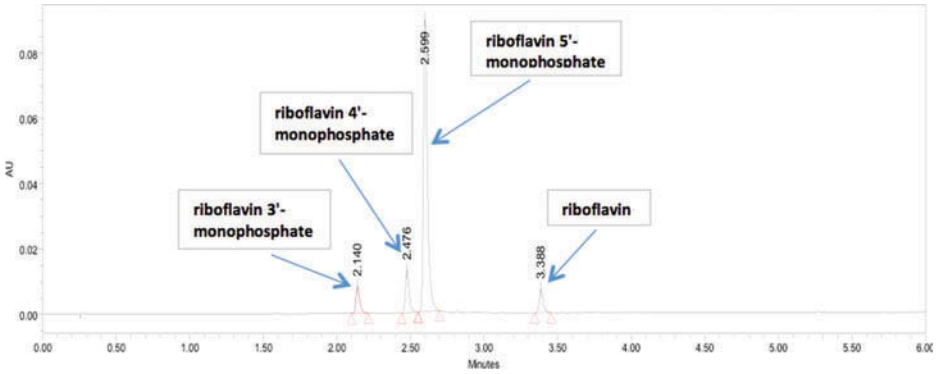

Figure 1. Distribution of the different riboflavin compounds in a standard riboflavin formulation (Fluka 83810, *riboflavin 5′-monophosphate sodium salt dehydrate*) (AU: arbitrary unit).

Table 1. Concentration (µg/ml) of riboflavin compounds in various riboflavin pharmaceutical formulations and in the cornea after soaking with respective riboflavin formulations.

Compound / Condition	Riboflavin 3′-monophosphate	Riboflavin 4′-monophosphate	Riboflavin 5′-monophosphate	Riboflavin
In formulation				
Mediocross D	190.65 (6.58)	229.00 (7.64)	1499.05 (49.57)	146.95 (5.02)
Mediocross M	135.8 (1.41)	165.25 (1.48)	1092.85 (10.11)	97.705 (0.78)
0.1 % vitamin B2	70.79 (0.32)	121.58 (0.85)	856.32 (4.81)	156.23 (3.83)
Mediocross TE	258.3 (0.42)	332.05 (0.21)	2378.1 (1.84)	302.3 (0.42)
In cornea				
Mediocross D (group 1)	0.29 (0.41)	0.11 (0.14)	0.034 (0.024)	8.72 (6.58)
Mediocross M (group 2)	2.71 (1.69)	1.60 (1.11)	0.62 (0.45)	36.77 (22.03)
0.1% vitamin B2 (group 3)	1.33 (1.50)	0.83 (1.10)	0.40 (0.68)	29.16 (21.32)
Mediocross TE (group 4)	0.87 (0.71)	0.52 (0.72)	0.40 (0.83)	17.36 (5.13)
p-values				
Group 1 versus Mediocross D	0.015	0.015	0.015	<0.01
Group 2 versus Mediocross M	<0.01	<0.01	<0.01	<0.01
Group 3 versus 0.1% Vitamin B2	<0.01	<0.01	<0.01	<0.01
Group 4 versus Mediocross TE	<0.01	<0.01	<0.01	<0.01

Indicated are the standard deviations in parenthesis and the p-values of the t-tests, testing the differences of concentrations of riboflavin compounds between the cornea of groups 1–4 and the formulations they were soaked with.

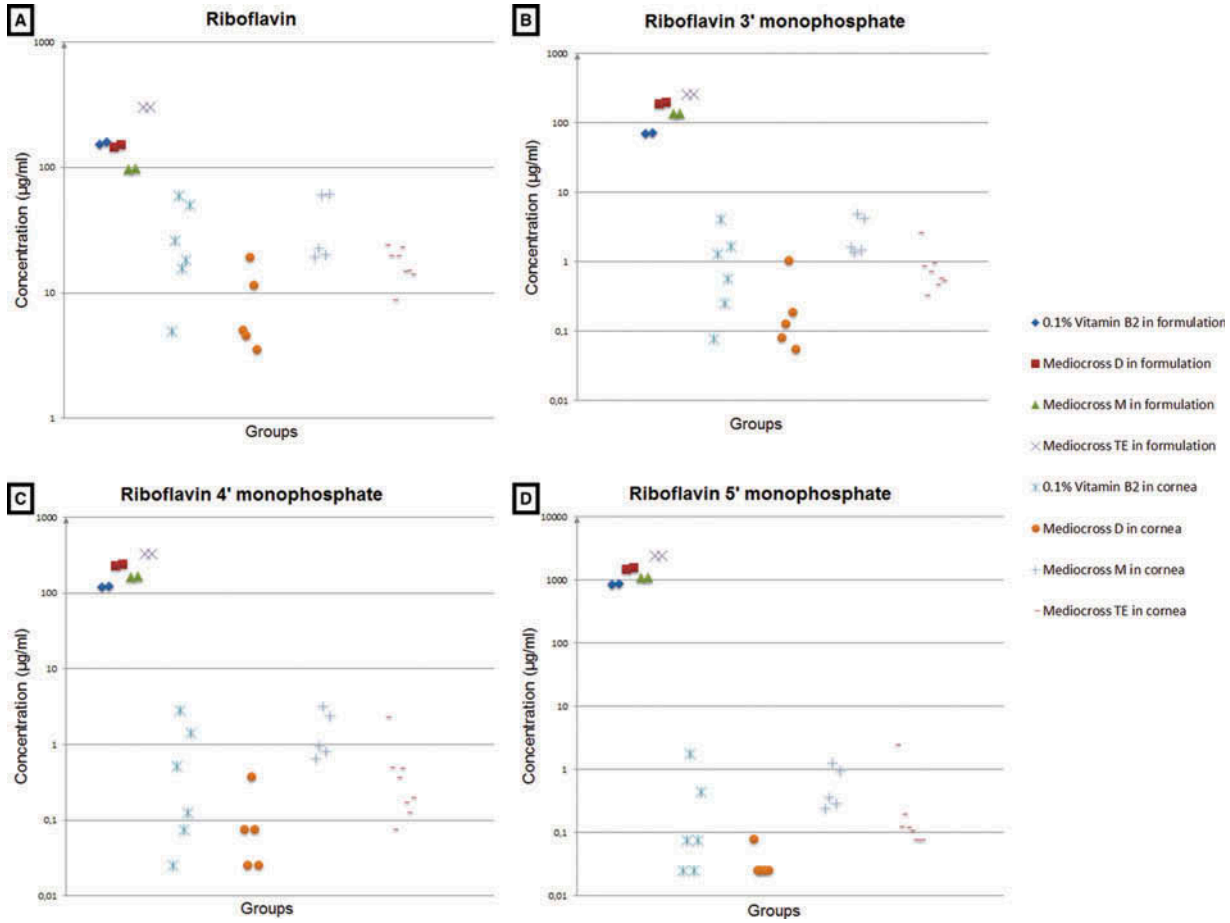

Figure 2. Concentrations (µg/ml) of various riboflavin compounds in the formulations and extracted from corneas: riboflavin (A), riboflavin 3′-monophosphate (B), riboflavin 4′-monophosphate (C), and riboflavin 5′-monophosphate (D) (legend: blue squares = 0.1% vitamin B formulation, red squares = Mediocross D formulation, green triangles = Mediocross M formulation, purple crosses = Mediocross TE formulation, blue stars = corneal stroma soaked with 0.1% riboflavin, orange dots = corneal stroma soaked with Mediocross D, blue crosses = corneal stroma soaked with Meidocross M, and red dash = cornea soaked with Mediocross TE) (logarithmic scale).

Table 2. Mean ratios riboflavin/riboflavin 5′-monophosphate concentrations in the cornea of groups 1–4 and in their respective formulations with standard deviations and *p*-values (*indicate significance).

Condition \ Localization	Cornea	Pharmaceutical formulation	*p*-values
Mediocross D	321.37 (288.38)	0.098 (0.0001)	0.067
Mediocross M	64.65 (11.09)	0.089 (0.0001)	<0.01*
0.1 % vitamin B2	260.22 (208.38)	0.18 (0.0034)	0.028*
Mediocross TE	146.95 (76.39)	0.13 (8.01E-05)	<0.01*

and their respective formulation. Significant differences were observed between group 2 and Mediocross M formulation ($p < 0.01$), group 3 and 0.1% vitamin B2 formulation ($p = 0.028$), and group 4 and Mediocross TE formulation ($p < 0.01$), but not between group 1 and Mediocross D formulation ($p = 0.067$).

Using MOE® software, we determined the major protomers at a pH of 7.4 (physiological pH of the corneal stroma[28]) for riboflavin, riboflavin 5′-monophosphate, and keratan sulfate (one of the major proteoglycan in the corneal stroma[29]). These protomers showed neutral, −2, and −4 global charges, respectively. In addition, the electrostatic potential maps of these protomers predicted with Spartan'14® showed a more negative electrostatic potential for riboflavin 5′-monophosphate (−106 to − 862 kJ/mol) than for riboflavin (+211 to −205 kJ/mol) as shown in Figure 4(A) and (B). The electrostatic potential of keratan sulfate was also observed to be negative (−349 to −903 kJ/mol) as shown in Figure 4(C). In Figure 4(A)–(C), the red areas indicate the predicted electronegative zones of the molecules.

As shown in Figure 5(A) and (B) the ionization potential maps indicate a lower ionization energy for riboflavin 5′-monophosphate (5.48 eV) compared to riboflavin (13.18 eV); the red areas indicate the predicted ionization zones.

Discussion

In the past few years, a number of studies determining the total stromal riboflavin concentration have been published.[21–26] However, none of these described the distribution of the different riboflavin compounds or their relative concentrations in the formulations and the cornea.

In this study, we analyzed the absorption of riboflavin and its various compounds in the cornea for different riboflavin formulations independently. Figure 2(A)–(D) and Table 1 show the significant drop observed in the concentrations of the riboflavin compounds present in the cornea compared to the formulations they were exposed to.

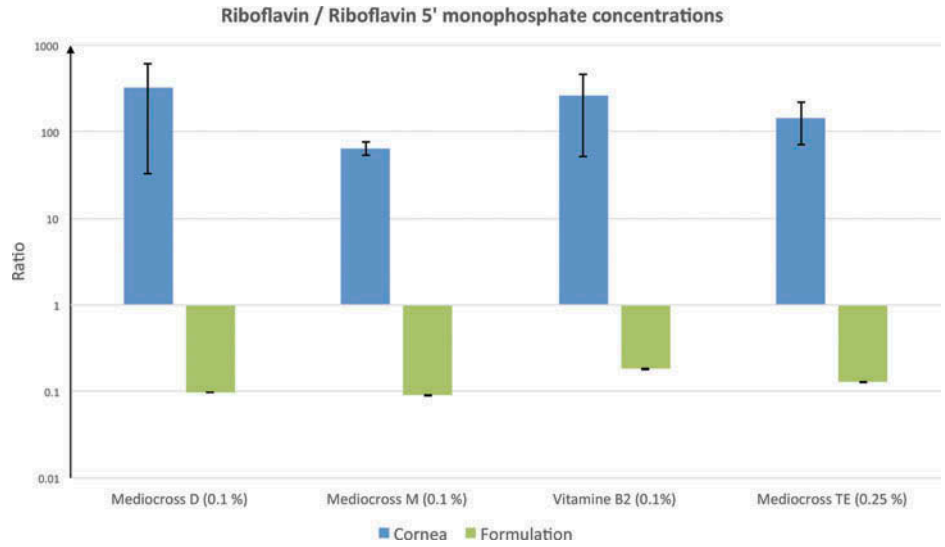

Figure 3. Mean ratios riboflavin/riboflavin 5′-monophosphate concentrations in the cornea and in the respective formulations, with standard deviations (blue bars correspond to cornea and green bar to formulation) (logarithmic scale).

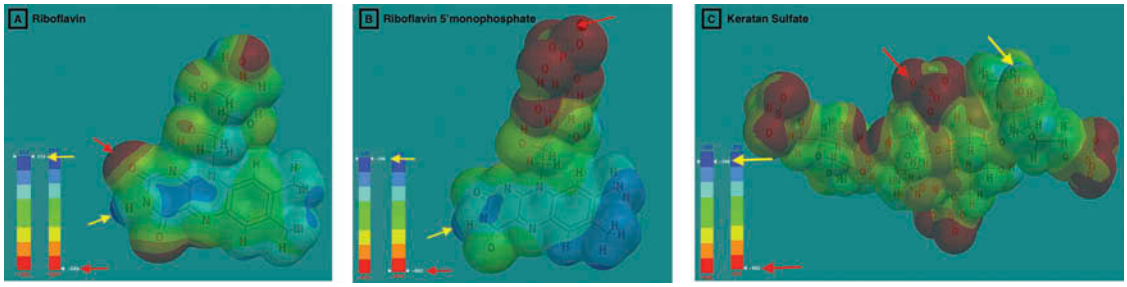

Figure 4. Electrostatic potential maps of various riboflavin compounds and keratan sulfate. A, riboflavin (extremes −205 kJ/mol and 211 kJ/mol). B, riboflavin 5′-monophosphate (extremes −862 kJ/mol and −106 kJ/mol). C, keratan sulfate (extremes −903 kJ/mol and −349 kJ/mol). Arrows indicate the maximum (red) and minimum (yellow) electrostatic potentials of the molecules. (Computed using the Hartree–Fock method and basis-set 6/31G** in quantum chemistry software *Spartan'14®*.)

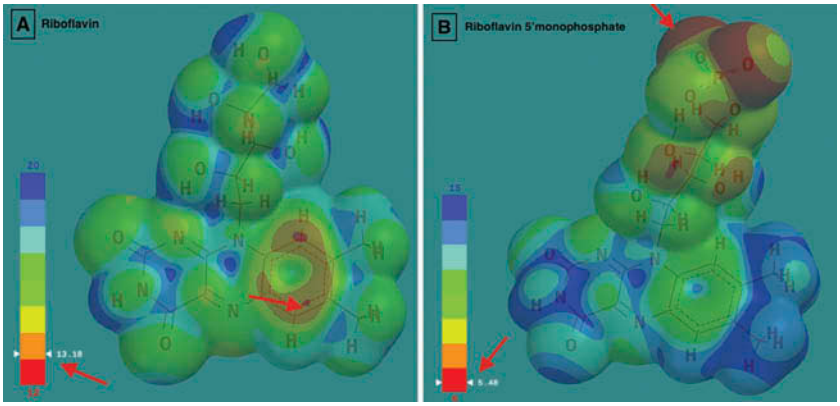

Figure 5. Ionization potential maps of (A) riboflavin, showing ionization energy of 13.18 eV (electronVolt) and (B) riboflavin 5′-monophosphate showing ionization energy of 5.48 eV. The red zones indicate the predicted ionization areas of the molecules. Red arrows indicate the ionization energy of the red areas. (Computed using the Hartree–Fock method and basis-set 6/31G** in quantum chemistry software *Spartan'14®*.)

Interestingly, the relative concentrations of riboflavin and riboflavin phosphate isomer compounds changed drastically between formulations and cornea, showing an inverse relationship: Figure 3 and Tables 1 and 2 show a significantly higher concentration of riboflavin 5′-monophosphate in the pharmaceutical formulations Mediocross M, Mediocross TE, and 0.1% vitamin B2 where it is the major riboflavin compound, whereas in the cornea it is the riboflavin which shows a significantly higher concentration and is the major riboflavin compound. To ensure that this phenomenon was not limited to a single riboflavin formulation or to a particular soaking method, we tested a number of commercially

available formulations under epi-on and epi-off conditions. The trend of inverse relationship was observed for all formulations tested, both under epi-on and epi-off conditions, while only three out of four independent comparisons were significant. A limitation of this study was the small number of samples per group, which could explain why we were not able to find significance between the corneas of group 1 and the formulation Mediocross D regarding the riboflavin/riboflavin 5′-monophosphate concentration ratio. A further limitation was that the epithelium was not removed after epi-on soaking of Mediocross TE. This means it is possible that a significant portion of riboflavin compounds found with UHPLC in this condition came from the epithelium and not from the stroma. However, as we analyzed the difference of riboflavin concentrations between solution and cornea independently for each formulation, it did not affect our observation of a strong decrease of riboflavin compounds between the formulation and the cornea, as well as the inverse relationship between riboflavin and riboflavin 5′-monophosphate.

Experimental studies with a higher number of eyes as well as studies on human corneas and testing other commercially available riboflavin formulations are needed to better understand our results.

Our results indicate that the main compound in the riboflavin formulations only plays a minor role in the actual riboflavin concentration present in the corneal stroma. This might be explained by the fact that, as shown in Figure 4(A) and (B), the phosphate increases the electronegativity of the riboflavin molecule. This in turn decreases the capacity of the riboflavin phosphate isomers to penetrate the cornea, where negatively charged proteoglycans shown in Figure 4 (C) might repel any other negatively charged molecule. Riboflavin formulations mainly contain monophosphate isomers as they show higher solubility compared to riboflavin. Also, as shown in Figure 5(A) and (B), the ionization potential of the riboflavin 5′-monophosphate is lower than the one of riboflavin, indicating a lower threshold of energy needed for the riboflavin 5′-monophosphate to participate in photochemical reactions. The electronegativity and ionization potentials of riboflavin and riboflavin 5'-monophosphate indicate that the latter is essential for photochemical reactions of CXL but that it is mainly the former that diffuses into the cornea. This means that increasing the concentration of riboflavin 5′-monophosphate into the cornea could improve biomechanical and clinical efficiency of CXL. Using iontophoresis in epi-off protocols might be a possibility to increase especially riboflavin phosphate isomer concentration in the cornea, as it specifically drives negatively charged as opposed to neutral molecules into the cornea.

In conclusion, our results imply that not only the total riboflavin concentration alone, but also the concentration, ionization energy, and diffusion capability of individual compounds should be considered for a representative description of riboflavin pharmaceutical formulations. This potentially provides a better understanding for CXL treatment assessment and might improve clinical efficiency. Considering these characteristics suggests that increasing the diffusion potential of riboflavin 5′-monophosphate may help develop more efficient riboflavin formulations and CXL protocols in the future.

Acknowledgments

We thank Dr Alessandra Nurisso (PhD) from the pharmacochemistry laboratory of the pharmaceutical sciences of the University of Geneva for the protomer determination with MOE® (MOE 2012.10, CCG Inc., Montreal, Canada). Also, we thank Dr Marc-Olivier Boldi (PhD) from the Research Center for Statistics of the University of Geneva for the statistical analysis counseling.

Funding

This study supported by the Swiss National Science Foundation (A.H. and O.R.).

Declaration of interest

Arthur Hammer, None; Serge Rudaz, none; Sylvie Guinchard, none; Sabine Kling, None; Olivier Richoz, named co-inventor of PCT/CH2014/000075 application; Farhad Hafezi, named co-inventor of PCT/CH2014/000075 application.

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