The role of hyaluronan as a drug carrier to enhance the bioavailability of extended release ophthalmic formulations. Hyaluronan-timolol ionic complexes as a model case


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ABSTRACT

The aim of this work was to obtain information concerning the properties of ophthalmic formulations based on hyaluronan-drug ionic complexes, to identify the factors that determine the onset, intensity and duration of the pharmacotherapeutic effect.

Dispersions of a complex of 0.5% w/v of sodium hyaluronate (HyNa) loaded with 0.5% w/v of timolol maleate (TM) were obtained and presented a counterionic condensation higher than 75%. For comparison a similar complex obtained with hyaluronic acid (HyH) was also prepared. Although the viscosity of HyNa-TM was significantly higher than that of HyH-TM, in vitro release of TM from both complexes showed a similar extended drug release profile (20–31% over 5 h) controlled by diffusion and ionic exchange.

Ocular pharmacokinetic study performed in normotensive rabbits showed that HyNa-TM complex exhibited attractive bioavailability properties in the aqueous humor (AUC and Cmax significantly higher and later Tmax) compared to commercial TM eye-drops. Moreover, a more prolonged period of lowered intra-ocular pressure (10 h) and a more intense hypotensive activity was observed after instillation of a drop of HyNa-TM as compared to the eye-drops. Such behavior was related to the longer pre-corneal residence times (400%) observed with HyNa-TM complex. No significant changes in rabbit transcorneal permeation were detected upon complexation.

These results demonstrate that the ability of HyNa to modulate TM release, together with its mucoadhesive-ness related to the viscosity, affected both the pharmacokinetic and pharmacodynamic parameters. The HyNa-TM complex is a potentially useful carrier for ocular drug delivery, which could improve the TM efficacy and reduce the frequency of administration to improve patient compliance.

1. Introduction

It is well known that conventional liquid ophthalmic formulations designed for topical use exhibit low (1–10%) intracocular bioavailability due to their short pre-corneal residence time (Lee, 1993; Schoenwald, 1997), with > 75% of an ophthalmic formulation being lost through nasolacrimal drainage and absorbed systemically when applied locally into the eye. Compliance is also a problematic issue, particularly among patients who have chronic diseases such as glaucoma (Nordstrom et al., 2005). As a consequence, current research in drug delivery systems in ophthalmology is orientated to achieve: a) pre-corneal contact time lengthening; b) an increase in drug permeability; and c) a reduction in the rate of drug elimination.

The use of biodegradable polymers as drug carriers for ophthalmic formulations has been the subject of numerous studies. Among these, hyaluronan (Hy) was included as a component of ophthalmic formulations due to its unique physicochemical and intrinsic properties (Horvát et al., 2015; Rah, 2011). Hy is a glycosaminoglycan composed of alternating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine with beta (1 → 4) and beta (1 → 3) glycosidic bonds (Fig. 1a), which is distributed throughout the extracellular matrix of all higher animals (Leach and Schmidt, 2008). Moreover, Hy is a biodegradable, biocompatible, non-toxic, viscoelastic and is a bioadhesive linear polysaccharide having a wide molecular weight range (1000 to 10,000,000 Da), commercially available as sodium salt of hyaluronic acid (HyNa). Hy bearing a carboxylic group (pKa 3–4) in each glucuronic unit behaves as an acidic polyelectrolyte (PE) able to form complexes with drugs possessing an appropriate basic group (Brown...
and Jones, 2005). In previous studies, Doherty et al. (1995, 1996) obtained stable and reversible ionic complexes between lidocaine and medium molecular weight Hy, which allowed the prolongation of epidual analgesia when injected into the epidural space in rabbits. In addition, Saettone et al. (1989, 1991) reported some ionic complexes between Hy and pilocarpine able to prolong the residence time of the complex in the eye, and a patent covering ophthalmic formulations based on the so-called Hy-drug salts has also been registered (Della Valle et al., 1995). Other studies have demonstrated the carrier capacity of Hy through the formation of ionic complexes with basic model drugs (Battistini et al., 2014; Battistini et al., 2013).

The aim of this work was to obtain detailed information concerning the properties of ophthalmic formulations based on Hy-drug complexes, in order to identify the factors that determine the onset, intensity and duration of the pharmacotherapeutic effect. For this purpose the β-blocker timolol maleate (TM, Fig. 1b) was selected as a model drug since it has a basic group in its structure, presents high permeability and its pharmacodynamic effect can be monitored through the determination of the intra-ocular pressure (IOP).

2. Materials and Methods

2.1. Materials

Hy sodium salt (HyNa, from bacterial fermentation, molecular weight: 1,655,000 Da) and Timolol Maleate (TM, melting range: 201.5–202.5 °C) were obtained from Parafarm® (Buenos Aires, Argentina), with both HyNa and TM being of pharmaceutical grade. Sucrose was purchased from Sigma Aldrich.

Ringer buffer solution was prepared by dissolving the ingredients (all from Para farm®, Buenos Aires, Argentina) in water as follows: NaCl 0.44% w/v, H3NaPO4 0.37% w/v, Na2HPO4 1.51% w/v and NaNO3 6 × 10−3% w/v, adjusted to pH 7.4, filtered through 0.2 μm filter and stored at 4 °C.

The following commercial eye-drops were used: TM 0.5% (Zopirol®, Elea, Buenos Aires, Argentina), Fluorescein sodium salt 0.25%, Fluorescein*, Poen, Buenos Aires, Argentina) and proparacaine 0.5% (Anestalcon®, Alcon-Couvreur, Puurs, Belgium). Phenobarbital injectable solution (50 mg/mL, Fada Fenobarbital*) was from Fada Pharma, Buenos Aires, Argentina.

Milli-Q water was used in all the experiments. All other reactants used were of analytical grade.

2.2. Preparation and characterization of hyaluronan free acid form (HyH)

This methodology has been previously described by Tokita et al. (1995) and Battistini et al. (2013) which demonstrate that HyH obtained through this technique has a high ionic purity. Briefly, aqueous dispersions of HyNa (0.5% w/v pH = 6.5) were passed through a glass column (4.2 cm diameter and 21 cm high) containing Amberlite® IR 120 resin. The eluate (HyH, pH 2.8) was freeze-dried under a vacuum of 10 × 10−3 mbar after initial freezing with liquid air, and the solid product obtained was titrated with NaOH (0.05 M) to determine the equivalents per gram of the carboxylic acid groups (2.445 meq/g).

2.3. Rheological characterization of HyNa and HyH

Dispersions containing 0.5% w/v of HyNa were prepared using water or NaCl 0.9% w/v as solvent and subjected to rheological evaluation. For comparison, a 0.5% w/v HyH dispersion added with NaOH to neutralize the carboxylic acid groups and obtain the same pH value as that of HyNa (pH 6.5) was also prepared. All the assays were performed at 37 °C, over a range of shear rate 2–100 rpm in a Haake (Karlsruhe, Germany) viscometer VT500 provided with software VT500/VT 3.01 and an MV2 sensor.

2.4. Animals

White normotensive New Zealand rabbits weighing 2–2.5 kg were used. The rabbits were provided with food and water ad libitum in a temperature-controlled room (21 ± 5 °C) and exposed to 12 h light/12 h dark cycles. After a week of adaptation to the facilities, animals were admitted to the experimental sessions. Animal management procedures conformed to the Association for Research in Vision and Ophthalmology and the European Communities Council directive (86/ 609/EEC) and were approved by the Institutional Care and Use Committee (Res. 342/09, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba).

2.5. Preparation of hyaluronan dispersions

Dispersions of HyNa-TM complexes were prepared by the addition of water to a mixture of adequate amounts of TM and HyNa to obtain a 100% neutralization of their carboxylic groups with the amino groups of TM. Sucrose was added to attain physiological osmolarity (308 mOsm/L), while NaOH was used to obtain a pH of approximately 7 (final pH = 7.02). For comparison purposes, a HyH-TM dispersion was similarly prepared (pH = 6.52). Osmolarity measurements were made with a vapor pressure osmometer VALPRO model S600 (ELITEch Group Wescor Utah, USA), and the pH values were recorded at room temperature with an Ag/AgCl-reference electrode (Mettler Toledo). The clear dispersions obtained were stored overnight at 4 °C and then allowed to reach room temperature before use.

The dispersions used for in-vivo experiments were sterilized by filtration with a 0.2 μm filter (PES 0.2 μm membrane, GVS-Biopore), as reported by Doherty et al. (1995).

2.6. Species distribution at equilibrium

The proportions of the species (TM), (TMH+), and the complexed one ([R-COO−·TMH+]) were determined by dialysis equilibrium using a
K = (\[\text{RCOO}^+\text{TMH}^-\]) (H^+) / (\text{RCOOH}) K_d (\text{TMH}^+),
\]

in which (RCOOH) was calculated from the expression:

\[
(R - \text{COOH}) = [\text{RCOOH}] - [\text{RCOO}^+\text{TMH}^+] - [\text{RCOO}^-] = [\text{RCOOH}] - [\text{RCOO}^+\text{TMH}^+] - (\text{TMH}^+) \quad (1)
\]

Since the sum of the negative species ([RCOO]+) plus (OH−) equals (TMH+) plus (H+), and considering that (RCOO−) ⇔ (OH−) and (TMH+) ⇔ (H+), then (RCOO−) approaches (TMH+).

2.7. Release rate of TM from the HyNa-TM

The rate of in-vitro release of TM from 0.5% w/v aqueous dispersions of HyNa-TM was determined by comparison with the release rate of an aqueous solution of TM and the HyH-TM dispersion, both having a TM equivalent concentration. All experiments were performed in bicompartmental Franz cells maintained at 35.0 ± 0.1 °C. A semi-permeable acetate cellulose membrane (12,000 Da, Sigma) was placed between the donor and the receptor compartments while complex dispersions or TM aqueous solution were placed in the donor compartment.

The donor compartment was filled with an exactly weighed amount (close to 1 mL) of the dispersion assayed, and the receptor compartment was aerated with a teflon-coated magnetic stirring bar. Samples of 0.9 mL of the receptor medium were withdrawn at predetermined time intervals and immediately replaced with an equal volume of pre-warmed medium, with the data obtained being corrected for dilution. TM concentrations were determined by UV-spectroscopy. All the assays were run in triplicate and followed for 300 min.

2.8. Tolerability

The potential ocular irritancy or damaging effects of the HyNa-TM complex dispersions was evaluated using a slightly modified version of the Draize test (Palma et al., 2009). One drop of the dispersions was instilled in the conjunctival sac of the right eye; while a NaCl 0.9% solution was introduced in the left eye as a control. Pre- and post-exposure evaluations of the eyelids, conjunctiva, cornea and iris were performed by external observation with adequate illumination using a binocular indirect ophthalmoscope (Neitz IO-30 small pupils Tokyo, Japan) provided with a 20 diopter lens (Nikon, Tokyo, Japan). For each observation, one drop of fluorescein ophthalmic solution was instilled to contrast the potential corneal injury. Six rabbits were used for each formulation.

2.9. Pre-corneal residence time evaluation

One drop (50 μL) of fluorescein ophthalmic solution was instilled in the conjunctival fornix of the right eye of the rabbit, while 50 μL of HyNa (0.5% w/v) loaded with fluorescein, prepared by mixing equal parts of fluorescein ophthalmic solution and HyNa 0.5% dispersion, was instilled in the left eye. Six rabbits were used for each formulation.

The presence of fluorescein in the eyes was assessed with an ophthalmoscope, considering the remaining amount of fluorescein in the eye compartment with scores assigned according to Palma et al. (2009). In all cases, the highest value corresponded to the ocular matrix full of fluorescein, a zero value corresponded to the matrix without fluorescein and middle values indicated intermediate situations. The scores recorded were: in the cornea from 0 to 4; in the fornix from 0 to 3; in the meniscus tear from 0 to 3.

2.10. Intraocular pressure (IOP) measurements

IOP was measured in rabbits (N = 10) using a Perkins MK2 tonometer (HS Clement Clarke, England). An infant blepharostat was used to maintain the eyelids opened during IOP measurement. The experiments were always performed at the same time of the day.

Zopiril® (50 μL) was instilled into the right eye and the IOP was monitored at 0, 2, 4, 6, 8 and 10 h. Three weeks later, the same procedure was repeated but instilling a 50 μL aliquot of the HyNa-TM or HyH-TM ionic complex.

The basal IOP was measured two days before treatments application, so that the normal baseline of each animal could be established before the treatment. During treatments the IOP of the left eye was also measured as a control.

The results were expressed as the percentage of change in IOP from the basal level. In addition, the area over the curve (AOC) of IOP vs time from t = 0 to t = 10 h was calculated using Origin 6.0®.

2.11. Ex-vivo transcorneal permeation studies

Rabbits were anesthetized with phenobarbital before being euthanized with a mixture of 10% O2 and 90% CO2 in an acrylic hermetic chamber. Next, the corneas, together with a 2 mm ring of sclera, were immediately excised and mounted in the diffusion chamber.

The transcorneal permeation studies were performed, in triplicates, using a modified diffusion chamber, with the cell (made of acrylic), consisting of a donor and a receptor compartment (volumes of 1.0 and 5.0 mL, respectively) (Camber, 1985). Previous studies demonstrated that no significant adsorption of the tested formulations to the diffusion chamber was observed over the assayed period.

A 4 mL aliquot of the Ringer buffer was then added to the endothelial face (receptor compartment), whereas 1 mL of HyNa-TM or TM solution containing 0.5% of TM was added to the epithelial side face (donor compartment). The receptor compartment was aerated with a mixture of 95% O2 and 5% CO2 before use to maintain oxygenation of the cornea.

The temperature in the diffusion chamber was maintained at 35.5 ± 0.1 °C using a thermostatic water bath. Sample aliquots (1 mL) from the receptor chamber were withdrawn at pre-set times and immediately replaced by aerated fresh receptor medium. The amount of TM in the withdrawn samples was then determined by UV-spectroscopy.

The cumulative amount of TM permeated per unit area in the receptor compartment (μg/cm²) was plotted against time. The apparent permeability coefficient (Papp) was determined by the following equation:

\[
Papp = \frac{ΔQ}{Δt × C_o × A}
\]

where \(ΔQ/Δt\) is the permeation rate of TM across the cornea obtained from the slope of the straight line of corneal permeability vs time (min), A is the corneal surface area (0.758 cm²), and \(C_o\) is the initial TM concentration in the donor compartment.

The steady-state flux (Jss) at 2 h was calculated from the following equation:

\[
Jss = Papp × C_o
\]

The differences in flux values and total amount of drug permeated
were statistically analyzed by one-way ANOVA using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA. Post-hoc analysis was done using Tukey’s HSD (honest significant difference) test. The difference at P ≤ 0.05 was considered significant.

2.12. Ocular pharmacokinetics

For the comparative study, 16 rabbits were randomly divided into two treatment groups (n = 8) that received one drop of Zopirol® or the HyNa-TM complex into the lower cul-de-sac of both eyes. At pre-set times (0.5, 1, 2, and 4 h) one eye (2 rabbits per group at each time) was anesthetized with proparacaine and 200 μL of aqueous humor were withdrawn from the anterior chamber of the eye using an insulin syringe (Terumo®, 1 mL). The samples were collected and stored at −20 °C until analysis. Before quantification, the samples were thawed out and 150 μL were taken and added with 100 μL of methanol to eliminate the proteins. After centrifuging the samples for 8 min at 8000 rpm, the clear supernatant was removed, filtered and then injected into the HPLC for TM quantification.

2.13. UV quantification of TM

Quantification analyses were performed on a spectrophotometer at λ = 295 nm (Thermo-Electronic Corporation, Evolution 300 BB, England). Accompanying software (UV Probe 2.0; Shimadzu) was used for all the absorbance signals and treatment of data. The spectroscopic method was validated with respect to linearity, accuracy, and precision.

For the calibration curve (performed in triplicated), a stock solution of TM (121 μg/mL) was prepared in distilled water as well as in Ringer buffer. Linearity in both media was found over the concentration range of 5.6E−07 to 9.8E−05 M (R2 = 0.999) for water and in the range of 2.90E10−6 and 1.2E−04 M for the Ringer buffer. Precision and accuracy on the lower, medium and higher concentrations were also verified in both media with a RSD < 15% in all cases.

2.14. HPLC quantification of TM

The analytical quantification of TM in the aqueous humor was performed according to Boiero et al. (2015). Chromatography was performed using a Waters® HPLC system equipped with a 1500 HPLC pump, a 717 auto sampler and a Waters 2996 PDA detector at 276 nm, with data acquisition and processing being performed using Empower® system software. The temperature was maintained at 45 °C with a Waters 1500 series column heater. Chromatographic separations were carried out using a Phenomenex Luna® C18 reverse phase column (250 × 4.6 mm, 5 μm particle size) and a Phenomenex® guard column (C18 4 × 3 mm ID). Analysis was performed with 0.05% (v/v) trifluoroacetic acid in water/0.05% (v/v) trifluoroacetic acid in an acetonitrile (40:60, v/v) as the mobile phase at a flow-rate of 1 mL/min in the isocratic mode. For analysis, 20 μL of each sample were injected, and the run time was set at 7 min.

Stock solutions of TM (100 μg/mL) were prepared in water. Working solutions were prepared by diluting several milliliters of stock solution with aqueous humor and methanol.

All solutions prepared for HPLC were passed through a 0.45 μm cellulose acetate filter before use. Four-seven point calibration curves were prepared over a range of 0.5 to 50 μg/mL. The linearity, obtained from squared correlation coefficient, r2, was 0.9998 ± 0.0002. The lower quantification limit was 1 μg/mL. The maximum concentration (Cmax), the time at Cmax (Tmax), and the area under the curve between t = 0 h and t = 3 h (AUC0-3) were calculated using the software PK Solution 2.0. The statistical analysis of pharmacokinetics and tissue distribution parameters between complex and reference groups was carried out by Student’s t-test using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA. The results were expressed as mean ± standard deviation (SD) with the 95% confidence interval and the P-value ≤ 0.05.

3. Results and discussion

3.1. Preparation and characterization of HyH

As shown in Fig. 2, HyNa dispersions exhibited a pseudoplastic flux, and the addition of NaCl induces a reduction in the viscosity. This behavior is a consequence of the perturbation in the associative structure of HyNa, which is mediated in particular by electrostatic repulsion/attraction forces between the charged Hy polymer chains (Chytíl and Pekář, 2007). In contrast, dispersions of HyH have a very low viscosity. This behavior has been previously associated with some degree of depolymerization during lyophilization of HyH dispersion. The average molecular weight of the polymer so obtained, determined by gel permeation chromatography, was reported to be around 40,000 Da (Tokita et al., 1995). As the same procedure was followed in this article, it is reasonable to assume that the molecular weight of HyH obtained here is in the same order of magnitude. The addition of sufficient NaOH to neutralize the carboxylic groups did not increase the viscosity. Interestingly, the comparison between the complexes obtained from the highly viscous HyNa or the low viscosity HyH provides insight into drug release mechanism and allows differentiating the role of the viscosity and the ionic interaction in the release of TM.

3.2. Species distribution at equilibrium

A series of aqueous dispersions were obtained by the reaction of carboxylic groups (−COOH) of HyH and −COO− of HyNa with TM, according to Eqs. (3) and (4), respectively.

As can be seen in Table 1, all complexes presented a high counter-ionic condensation of >75%, with log Keq being between 6.0 and 6.3. The comparison between the HyH-TM and HyNa-TM complexes revealed that the former maintained the complexing capacity of the original salt form despite being partially depolymerized. Finally, the higher percentage of counterionic condensation and Keq, observed with HyH respect to HyNa could be attributed to the perturbation of the

<table>
<thead>
<tr>
<th>Complex</th>
<th>Species (%)</th>
<th>log Keq</th>
</tr>
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<tbody>
<tr>
<td>HyNa-TM</td>
<td>24.55 ± 0.92</td>
<td>75.40 ± 1.21</td>
</tr>
<tr>
<td>HyH-TM</td>
<td>23.31 ± 2.08</td>
<td>76.69 ± 0.87</td>
</tr>
</tbody>
</table>

Table 1

Species distribution after dialysis of the ionic complexes with a dialysis tube.
ionic equilibrium of complex formation due to the presence of Na\(^+\) in the HyNa-TM complexes.

### 3.3. Release rates of TM from the HyNa-TM dispersions

Fig. 3 shows the slow release of TM from HyNa-TM and HyH-TM at a pH value of approximately 7. Since TM is a strong base (pKa 9.2), under the experimental conditions assayed, the release to water as a receptor medium occurs essentially through Fickian diffusion of the neutral species, since diffusion of TMH\(^+\) is mainly prevented by the electrostatic gradient provided by the polyanion. Using NaCl 0.9% as the receptor medium instead of water resulted in a rise in the release rate of TM. This may have been associated with the diffusion of Cl\(^-\) and Na\(^+\) from the receptor compartment to the donor. Whereas Cl\(^-\) can promote the diffusion of TMH\(^+\) by acting as a counterion, the ionic exchange between Na\(^+\) and TMH\(^+\) can also promote drug release from the polyanion (Eq. (5)). Such interactions illustrate the expected ones between the complexes and biological fluids.

The carboxylic groups of HyNa or HyH had a high affinity towards amino groups of TM, and the HyH-TM ionic complexes behaved as a reservoir that released TM over the time and that TM release is delayed by electrostatic interaction with HyNa or HyH.

Horvát et al. (2015) reported HyNa-sodium diclofenac as a potential drug delivery system for the eye, and stated that the drug was rapidly released (75% of drug release in 5 h) though its diffusion from the polymer chains. The difference in the percentage of released drug between this system and the HyH-TM complex lies in the fact that there is no electrostatic interaction between HyNa and sodium diclofenac, which facilitates drug diffusion.

It has been reported the use of HyNa as a functional additive in contact lenses to modify the release of TM. In agreement with our results, Guidi et al. (2014) reported an increased loading capability of the lenses and a delay in TM release due to electrostatic interactions between HyNa and TM. Later, the same group of authors (Korogiannaki et al., 2015) evaluated the impact of releasable HyNa, on the release profile of TM from model silicone hydrogel contact lens materials. The release of TM was controlled by the release of HyNa from the lenses, likely due to electrostatic interactions between protonated TM and anionic Hy.

### 3.4. Pre-corneal residence time evaluation and tolerability

Fig. 4 shows the decrease in IOP in rabbits caused by HyNa-TM and Zopirol\(^\text{®}\) administration to rabbits versus time. The maximum activity (lowest IOP) produced by Zopirol\(^\text{®}\) is observed 2 h after administration with no hypotensive effects detected after 8 h. In contrast, HyNa-TM dispersion produced at 2 h a decrease of IOP equal to the maximum decrease of Zopirol\(^\text{®}\), but a still lower IOP is attained at t = 4 h. At t = 8 h, the reduction of IOP produced by HyNa-TM was maintained in the eye for all the assayed period (60 min). In contrast, it disappeared within 15 min when administered as a solution. Notice that HyH loaded with fluorescein was not assayed since it is well known that depolymerized HyH loses its mucoadhesive properties (Durrani et al., 1995).

The 400% longer residence time of fluorescein in the HyNa dispersion should not only be attributed to its mucoadhesivity (Durrani et al., 1995), but also to a contribution of the viscosity. HyNa is a mucoadhesive polymer that interacts with mucin through physical and chemical bonds (hydrogen bonds) (Horvát et al., 2015). The mucoadhesive nature of HyNa is very useful in ocular therapy, since the washing-out from the eye by lacrimation after instillation is greater in formulations without mucoadhesive polymers. Thus, the mucoadhesive and viscoelastic behavior of HyNa was able to prolong the residence time on the ocular surfaces. Considering the high association constant between HyNa and TM and the ability of HyNa to modulate TM release, it is reasonable to assume that the residence time of TM administered as HyNa-TM would be similar to that observed for HyNa and clearly superior to the solution of TM.

Dispersions of HyNa-TM or HyH-TM did not produce any conjunctival or corneal irritation, with no intraocular irritation being observed during 10 h, which correlates with the non-irritating and non-inflammatory properties of Hy.

### 3.5. Pharmacodynamic study

Fig. 5 shows the decrease in IOP in rabbits caused by HyNa-TM and Zopirol\(^\text{®}\) administration to rabbits versus time. The maximum activity (lowest IOP) produced by Zopirol\(^\text{®}\) is observed 2 h after administration with no hypotensive effects detected after 8 h. In contrast, HyNa-TM dispersion produced at 2 h a decrease of IOP equal to the maximum decrease of Zopirol\(^\text{®}\), but a still lower IOP is attained at t = 4 h. At t = 8 h, the reduction of IOP produced by HyNa-
TM is similar to the lowest value obtained with Zopirol® and 10 h after administration the hypotensive effect remained and disappeared approximately 12 h after application. The profile described for HyH-TM dispersion (not shown in the figure for simplicity) overlaps that of Zopirol® confirming that viscosity and mucoadhesiveness are essential to increase TM effectiveness.

Considering the areas over the curves (AOC) to be an indication of the system’s capacity to decrease IOP, the area of HyNa-TM (21.29) is approximately 2-fold the Zopirol's one (12.44). This effect may be attributed to the increased residence time of the formulation in the eye due the mucoadhesiveness of HyNa. In contrast, the HyH-TM dispersion did not reveal any differences with Zopirol® probably due to its low viscosity, which is closely related to its bio-adhesiveness properties. These results confirm that viscosity is the main factor that controls the prolonged pharmacodynamic effect in the HyNa-TM complex.

3.6. Ex vivo transcorneal permeation and pharmacokinetic studies

Concentrations of TM in the aqueous humor were determined at various intervals after instillation of a drop of Zopirol® or HyNa-TM. Fig. 6 shows the mean concentration–time profiles that were obtained. As can be seen there, the AUC_{0-24} of HyNa-TM complex (607.7 ± 53.4 μg·h/mL) was significantly higher than that of Zopirol® (409.5 ± 12.2 μg·h/mL) (P < 0.032), indicating an increase in the bioavailability of TM. In agreement with its slower release rate, a later Tmax (1 h) was observed from the HyNa-TM complex as compared to Zopirol® (0.5 h). The Cmax value for HyNa-TM complex (4.5 ± 0.3 μg/mL) was also significantly higher than that of Zopirol® (3.7 μg/mL) (P < 0.0006).

On the other hand, the transcorneal permeation parameters of TM (Table 2) revealed no significant differences when the TM cumulative amount permeated from HyNa-TM complex was compared with the TM solution (Fig. 7). Besides, the permeation profiles described are similar to those obtained by in vitro evaluation suggesting that permeation across the cornea is controlled by the release rate of TM.

The lack of enhancement of corneal permeation in HyNa-TM complex suggests that the increase in the TM ocular bioavailability is due to the enhanced pre-corneal residence time.

All together these results are in agreement with the higher efficacy observed in the pharmacodynamic study. Thus, since TM is a highly permeable drug (log P = 1.83), the ability of HyNa to modulate TM release, together with its mucoadhesiveness related to the viscosity, affected both the pharmacokinetic and pharmacodynamic parameters.

Two recent publications (Carvalho et al., 2015 and Cope et al., 2016) report the use of HyNa in contact lenses to extend the release of TM, with in vitro studies showing that TM release was sustained for approximately 2 days. Although these systems were not subjected to efficacy or pharmacokinetic studies, an increased bioavailability of TM can be advised with these systems. The use of contact lenses for therapeutic purposes has been postulated for about 50 years, however, the use of this technology is still fairly limited. In addition, contact lenses are not exempt of side effects. A recent survey indicate that approximately 41 million U.S. residents wear contact lenses, and in 2014, > 99% of contact lens wearers surveyed reported at least one behavior that puts them at risk for a contact lens-related eye infection.

It is worth to note that HyNa-TM hydrogel was able to increase ocular bioavailability and efficacy of TM without the need to use contact lenses. Such a simpler approach highlights the translational potential of HyNa-TM complexes. In addition, the presence of HyNa can help to control dry eye syndrome, which frequently develops in patients with prolonged use of antiglaucoma medications (Andrés-Guerrero et al., 2011). It has also been informed that increasing the viscosity of ophthalmic formulations not only enhances the ocular absorption of TM (due to the longer residence time) but reduces its systemic absorption by the slower spreading of the solution on the nasal mucosa (Kyrönén and Urtti, 1990) Therefore, systemic concentrations of TM after instillation of HyNa-TM could be possibly related with reduced side effects. However, the systemic absorption of these formulations needs to be studied further.
4. Conclusions

The advantageous rheological and mucoadhesive properties of HyNa-TM, together with its release behavior, ensure resistivity of the complex during blinking, and consequently ensure a prolongation of the treatment. In fact, the results demonstrated that HyNa-TM extended the pre-corneal TM residence time, contributing to a long-lasting action to increase both bioavailability and efficacy.

HyNa can be considered to be a viable alternative to the conventional ophthalmal dosage form due to its non-irritation, biodegradability and tolerability. HyNa was used without the addition of cross linkers and, on adding the basic drug TM, anionic complex capable of increasing and prolonging the hypotensive effect was produced. The higher efficacy of HyNa-TM can be explained by the microviscosity produced by the complex in which the drug is included, its bioadhesive properties and controlled release of TM. Interestingly, the improved efficacy observed with the HyNa-TM complex can reduce the frequency of administration to improve patient compliance.

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