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Disclosing long-term tolerance, efficacy and penetration properties of hyaluronic acid-coated latanoprost-loaded liposomes as chronic glaucoma therapy

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ABSTRACT

Frequent topical administration of hypotensive eye drops in glaucoma patients may lead to the development of dry eye disease (DED) symptoms, because of tear film destabilization and the adverse effects associated with antiglaucoma formulations. To address all this, in the current study preservative-free latanoprost-loaded (0.005 % *w/v*) synthetic phosphatidylcholine (1,2-dioleoyl-sn-glycero-3-phosphocholine 0.75 % *w/v*, 1,2-dimyristoyl-sn-glycero-3-phosphocholine 0.25 % *w/v*) liposomes dispersed in the mucoadhesive polymer hyaluronic acid (0.2 % *w/v*), containing the osmoprotective ingredients betaine (0.40 % *w/v*) and leucine (0.90 % *w/v*) (LAT-HA-LIP), have been prepared and further characterised. Permeation and retention evaluations on a validated *ex vivo* porcine eye model revealed that the active metabolite latanoprost acid was quantified only starting from LAT-HA-LIP once passing conjunctiva, sclera and choroid compared to the marketed latanoprost (0.005 % *w/v*) benchmark (MF). The liposomal formulation outperformed MF when applied to the corneal tissue. Additionally, distribution and interactions within corneal and scleral tissues were investigated by means of two-photon microscopy with liposomal formulations containing coumarin-6. Furthermore, acute and chronic tolerance studies on rabbits revealed no signs of discomfort or ocular damage. Schirmer's test, tear osmolarity, tear breakup time (TBUT) and fluorescence staining evaluated through the Oxford grading scale, were assessed as DED diagnostic parameters over a 25-day monitoring period; LAT-HA-LIP consistently maintained levels comparable to physiological solution (0.9 % *w/v* NaCl) used as control, with a slight increase of TBUT values from day 15 (6.00 ± 0.63 s for control, 7.00 ± 0.78 s for LAT-HA-LIP at day 15, $p = 0.0066$). A daily topical application of LAT-HA-LIP for 15 consecutive days, effectively lowered IOP in a sustained way (2.51–3.88 mmHg mean IOP reduction over the 5–15-day period). These results highlight the clinical relevance of the proposed technological platform, able to provide IOP reduction during the simulated long-term administration and simultaneous ocular surface protection with potential for the treatment of glaucoma.

1. Introduction

Since its launch in 1996, latanoprost (LAT), a pentene-ring small hydrophobic molecule with an isopropyl ester terminal, demonstrated having a groundbreaking impact in reducing intraocular pressure (IOP) [1]. This molecule is a prostaglandin $F_{2\alpha}$ analogue, which increases

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uveoscleral outflow of aqueous humour through mechanisms involving relaxation of ciliary smooth muscles and remodelling the extracellular matrix *via* amplified metalloproteinases activity, among others [2,3]. LAT is an esterified prodrug with poor water solubility, which undergoes hydrolysis to its pharmacologically active form latanoprost acid (LAT ACID) once penetrated the cornea [4]. LAT is widely prescribed in eye drop formulations as a first-line treatment for ocular hypertension in primary open-angle glaucoma [5,6].

One of the main challenges of the topical ophthalmic administration is the low ocular bioavailability associated, estimated to be around 5 % [7]. After its application, the active compound must overcome physiological barriers such as nasolacrimal drainage, blinking, dilution in tears, and anatomical barriers such as the cornea, conjunctiva and sclera [8]. Considering these circumstances, enhancing LAT uptake close to the target site and its penetration across the ocular surface are primary objectives, as this prodrug requires ocular esterase's metabolism to be activated and, consequently, therapeutically effective [9].

LAT significantly revolutionised the landscape of glaucoma treatment due to its remarkable effectiveness and safety profile. However, while systemic side effects are uncommon after its topical ocular administration, LAT may still lead to ocular adverse events including conjunctival hyperaemia, pigmentation changes in the iris, periocular skin, eyelashes hypertrichosis, and ocular surface irritation among others [10]. In addition, the adverse effects reported are not only related to the drug. In fact, it is well known that chronic applications of preserved topical hypotensive medicines induce a progressive instability and disruption of the preocular tear film, which results in increased tear hypertonicity and desiccation of the ocular surface, inducing the development of dry eye disease (DED) [11]. Additionally, it has been reported that a hyperosmolar environment triggers inflammation and damage to the ocular surface, initiating a vicious cycle that exacerbates these effects. It is important to remark that simply removing preservatives from formulations is not sufficient to prevent these risks. Collectively, these circumstances can significantly diminish the quality of life of glaucoma patients [12]. Considering all these factors, new strategies need to be undertaken aimed at improving long-term tolerance and efficacy of antiglaucoma medications without compromising the integrity of the ocular surface.

To address these issues, three different technological approaches have been implemented in this research article with the aim to design a formulation that can improve LAT long-term safety and efficacy, while at the same time can prevent ocular surface alterations that trigger DED induction and/or progression. The first approach was the inclusion of LAT in liposomes, widely used as vehicles for topical ophthalmic drug delivery in which lipophilic drugs, such as LAT, are entrapped inside the vesicles' bilayers [13]. Liposomes have shown promising results in enhancing drug transport to the anterior segment of the eye, due to their ability in improving the solubility and penetration of active substances across cornea, conjunctiva, and sclera [14–17]. Most liposomal formulations for topical ophthalmic use have been based on soybean of egg phosphatidylcholine. Phosphatidylcholine constitutes a principal component of the lipid layer in the preocular tear film, accounting for nearly 60 % of the phospholipid content in meibomian secretions [18]. Liposomes composed of this amphiphilic molecule results of great interest due to their reported ability to enhance the lipid layer's thickness, interact with lipocalin, and help maintain tear film homeostasis [11]. However, the variable composition of natural-sourced phosphatidylcholine can ultimately affect the tolerance and stability of the formulation. In this sense, a blend of saturated and unsaturated synthetic phosphatidylcholine derivatives, primary excipients of the liposomes employed in this article, offer advantages over soy or egg phosphatidylcholine in terms of standardisation, characterisation, and potential large-scale manufacturing [13,19].

The second technological approach proposed is the use of mucoadhesive agents in combination with liposomes to enhance the formulation ocular surface retention time and stability. Mucoadhesive polymers improve the contact time thanks to the interaction with mucins of the preocular tear film. By incorporating polymers with viscosifying, gelling, and healing properties, such as polysaccharide-derived ones, a viscous protective layer can be formed, covering the entire ocular surface and protecting part of the drug from tear drainage [20–24]. In this context, hyaluronic acid (HA) was the mucoadhesive agent chosen for the current research. HA is an anionic polymer naturally found in the extracellular matrix of animal tissues, serving as one of the primary components within our bodies. HA exhibits biocompatibility and biodegradability with low toxicity [25], and it has the ability to retain water, forming a viscous polymeric chain dispersion with mucoadhesive properties. Furthermore, HA lubricates the ocular surface, shielding it from damage due to desiccation stress, and enhancing the density and thickness of the preocular tear film. Indeed, HA has been extensively utilised as an excipient in artificial tears to alleviate irritation and provide relief to patients suffering from DED [26–28].

The third technological advancement entailed incorporating osmoprotective agents, specifically betaine (BET) and leucine (LEU), into the aqueous dispersion of the liposomal formulation. This integration aimed to enhance protection against DED. Osmoprotectants are biocompatible compounds osmotically active; their function is stabilising cell membranes and preserving cellular hydration, consequently reducing inflammation and enhancing the stability of the tear film [29]. In addition, as widely described in literature, BET avoids protein misfolding and inhibits apoptosis, while LEU decreases inflammation and oxidative processes [30–32]. Ensuring long-term safety and protection against DED conditions is vital for medications administered over extended periods, especially for chronic conditions like glaucoma.

As there is a strong rationale for the combination of the three aforementioned technological approaches to improve LAT ocular bioavailability while preserving ocular surface in chronic treatments, in the present work a deeper evaluation of the selected formulation has been performed at different levels, as a continuation of a piece of research previously published [33]. *Ex vivo* permeation studies using porcine ocular tissues were carried out to quantify the retention and permeation of LAT and LAT ACID. For these studies, Monoprost®, a commercially available preservative-free ophthalmic solution containing 0.005 % *w/v* LAT (MF), was used as a reference. Furthermore, liposomes tolerance was *in vivo* evaluated under simulated acute and chronic conditions (after multiple administrations), evaluating the potential protection of the system proposed against DED development. Finally, long-term hypotensive studies were performed for a comprehensive and extended evaluation of the proposed formulation in (i) lowering IOP, (ii) understanding its long-term safety profile, (iii) providing insights into the optimal dosing regimen and frequency of administration and (iv) demonstrating its efficacy and security for future regulatory submission and approval.

2. Materials and methods

2.1. Chemicals

Latanoprost (LAT) (propan-2-yl (Z)-7-[(1*R*,2*R*,3*R*,5*S*)-3,5-dihydroxy-2-[(3*R*)-3-hydroxy-5-phenylpentyl]cyclopentyl]hept-5-enoate, CAS 130203–82-4, molecular weight 432.59 g mol^{-1}, pK_a 14.84, logP 4.19, water solubility 12.9 μg mL^{-1} [34]) was obtained by MedChemExpress (Monmouth Junction, New Jersey, United States of America). Latanoprost acid (LAT ACID) ((Z)-7-[(1*R*,2*R*,3*R*,5*S*)-3,5-dihydroxy-2-[(3*R*)-3-hydroxy-5-phenylpentyl]cyclopentyl]hept-5-enoic acid, molecular weight 390.51 g mol^{-1}, pK_a 4.31, logP 0.52 [35], water solubility 0.0385 mg mL^{-1} [36]) was prepared as described in Supplementary Information. Monoprost® (MF) was the benchmark employed (Laboratoires Théa, Clermont-Ferrand, France). 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (LIPOID PC 18:1/18:1, CAS 4235-95-4) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (LIPOID PC 14:0/14:0, CAS 18194–24-6) were purchased from Lipoid Gmbh

(Ludwigshafen, Germany). Cholesterol (CHOL) (CAS 57–88-5, ≥ 99 %), α-tocopherol acetate (VE) (≥ 96 %), coumarin 6 (COU) (CAS 38215–36-0, ≥ 99 %), L-leucine (LEU) (CAS 61–90-5, ≥ 98 %), boric acid (H_3BO_3) (CAS 10043–35-3, ≥ 99.5 %) sodium tetraborate decahydrate ($Na_2B_4O_7{\cdot}10H_2O$) (CAS 1303-96-4, ≥ 99.5 %), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) (≥ 99.5 %) were provided by Sigma-Aldrich (Madrid, Spain). Betaine anhydrous (BET) (≥ 98 %) and D-(+)-trehalose dihydrate (TREH) (CAS 6138-23-4) were purchased from Fisher Scientific (Geel, Belgium). Sodium hyaluronate ophthalmic grade (HA) (molecular weight 400–800 kDa) was supplied by Abaran Materias Primas S.L. (Villaviciosa de Odon, Spain). HEPES 25 mM buffered saline composition: 5.96 g L^{-1} HEPES, 9.0 g L^{-1} NaCl pH 7.4 with NaOH 5 N (ionic strength: 0.154 M). All chemicals used were of analytical grade.

2.2. Liposomes preparation and characterisation

Liposomes were elaborated according to the lipid film hydration protocol explained by Bangham et al., although with some modifications [37,38]. Components were chosen based on their mechanical characteristics and *in vitro* tolerance results documented in previous studies [39,40]. The aqueous dispersion was composed by a borate buffer (0.84 % *w/v* H_3BO_3, 0.14 % *w/v* $Na_2B_4O_7{\cdot}10H_2O$), 1.04 % *w/v* TREH, 0.40 % *w/v* BET and 0.90 % *w/v* LEU. DOPC (0.75 % *w/v*), DMPC (0.25 % *w/v*), CHOL (0.125 % *w/v*) and VE (0.01 % *w/v*) were the constituents of the lipid bilayer. This lipid mixture was first dissolved in chloroform, which was lately removed in a rotary evaporator (Buchi R-205, Massó Analítica S.A., Spain) at reduced pressure (100 mPa and 50 mPa for 30 min, at 150 rpm) at 33 °C. Afterwards, nitrogen flow was employed to guarantee solvent traces removal. Therefore, the aqueous dispersion was added to hydrate the lipid film at 185 rpm for 15 min. After allowing it to sit for two hours at room temperature, the mixture underwent 15 min of sonication in an ultrasonic bath (Bandelin® Sonorex Digiplus, DL 510H, Berlin, Germany), leading to multilamellar vesicles formation. Unilamellar vesicles were obtained by extrusion (high-pressure extruder, Lipex Biomembrane™, Vancouver, BC, Canada) by filtering the liposomes through 0.8 μm (Nuclepore™ Track-Etch Membrane, Whatman™, Cytiva Europe Gmbh, Freiburg, Germany) and then 0.2 μm polycarbonate membranes (Nuclepore™ Track-Etch Membrane, Whatman™, Cytiva Europe Gmbh, Freiburg, Germany) for ten times each at 25 °C under fume hood. At the end of the extrusion process, liposomes were diluted 1:1 with the aforementioned aqueous dispersion to achieve a final phospholipids' concentration of 1 % *w/v* (B-LIP). To produce liposomes dispersed in 0.2 % *w/v* HA (B-HA-LIP), 0.4 % *w/v* HA was incorporated into the aqueous dispersion used to dilute the liposomes following the extrusion process. In the case of the LAT-loaded liposomes, LAT was included in the lipid phase to obtain a final concentration of 0.005 % *w/v*. To assess the impact of mucoadhesive HA, two formulations were prepared: one without (LAT-LIP) and one with a final concentration of 0.2 % *w/v* HA (LAT-HA-LIP). For two-photon microscopy studies, COU-loaded liposomes with (COU-HA-LIP) and without (COU-LIP) 0.2 % *w/v* HA were produced. COU was dissolved in chloroform and included into the lipid mixture to a final concentration of 0.005 ‰ *w/v*.

Physicochemical characterisation of the formulations, including their morphology, was fully carried out in a recently published study [33]. The main parameters such as pH, ζ-potential, surface tension, osmolarity and viscosity have been measured following the same methodology and included in Supplementary Information (Table S1).

2.3. Latanoprost and latanoprost acid stability tests

The stability and potential degradation of the hypotensive active and its metabolite were assessed under conditions simulating those of the *ex vivo* permeation studies, reported in 2.4. Section. Briefly, working stock solutions of both LAT and LAT ACID at a concentration of 10 μg mL^{-1} in HEPES were placed in a heating water bath (Memmert® Shaking Bath WNB 29, Memmert GmbH, Schwabach, Germany) at 37 °C (n = 3). Samples were withdrawn at time zero, 5 h and 24 h, and analysed to determine the recovery percentages (Recovery %) relative to the control at time zero.

2.4. Ex vivo studies

Fresh porcine ocular bulbs (*Sus scrofa domestica*, female and male animals, age 10–11 months, weight 145–190 kg) were obtained from a slaughterhouse (Macello Annoni S.p.a., Busseto, Italy), transferred under refrigeration in physiological solution (0.9 % *w/v* NaCl) and manipulated within 4 h from the enucleation. Muscular and connective adnexa around the ocular bulbs were meticulously removed with scissors. In the case of trans-corneal permeation and two-photon microscopy investigations, only eyes with macroscopically intact corneas were used. For trans-conjunctival studies, conjunctiva was chosen from the inferior part of the eyeball and mounted on the Franz-type diffusion cell when still attached to the bulb [41]. For permeation and retention tests across sclera and choroid, the mentioned tissues were isolated by circumferentially cutting the sclera at the rear of the limbus to remove the anterior segment of the eye. Once the vitreous body was removed, the so-obtained eyecup was bisected along the line of the ciliary arteries and the retina and retinal pigmented epithelium were gently detached using filter paper.

2.4.1. Validation of latanoprost and latanoprost acid extraction method from porcine ocular tissues

Circular sections of fresh conjunctiva (with an area of 0.2 cm^2), cornea, sclera, and choroid (0.6 cm^2 area) were carefully punched out and accurately weighed into polypropylene tubes. Subsequently, working stock solutions of LAT at 2 mg mL^{-1} and LAT ACID at 0.5 mg mL^{-1} in ethanol and Milli-Q® water, respectively, were prepared. These solutions were carefully applied (10 μL) onto the isolated tissues using a Hamilton® syringe, allowing for solvent evaporation. After 30 min, 0.5 mL of an extracting mixture containing acetonitrile and Milli-Q® water (70:30, *v/v*) were added to each tube containing the tissues. Conjunctiva, cornea, sclera, and choroid (n = 3 each) were left in contact with the extracting mixture for 2 h at room temperature, with occasional sample vortexing. Subsequently, supernatants were collected, to quantify actives as described in 2.6. Section. The same protocol was followed both for working stock solutions of the two compounds without the presence of the tissues, which were used as control (n = 3), and for circular sections of the mentioned tissues without LAT and LAT ACID solutions, which were employed for checking any interferences in the chromatographic analyses due to tissues components (n = 3).

2.4.2. Permeation and retention experiments across cornea, conjunctiva, and sclera/choroid

Trans-corneal experiments were performed on whole eye bulbs using a 3D-printed customized applicator secured on the corneal surface thanks to a weak suction vacuum system; the eye was maintained at a temperature of 37 °C. Formulations MF or LAT-HA-LIP (0.005 % *w/v* LAT, 400 μL) were applied to the cornea for 5 h. The corneal surface was then washed with physiological solution, the applicator was removed, and the ocular surface was carefully dried. Then, the eye was kept at −20 °C for at least 24 h and, while still frozen, was dissected for isolating the cornea (that is, the area previously in contact with the formulation, corresponding to 0.6 cm^2) and the aqueous humour.

Conjunctiva (with a permeation area of 0.2 cm^2) or isolated sclera with attached choroid (with a permeation area of 0.6 cm^2) were mounted on Franz-type diffusion cells. The donor chamber was separately filled (0.005 % *w/v* LAT, 400 μL) with of MF (n = 4) or LAT-HA-LIP (n = 4) and covered with parafilm to prevent evaporation. The receptor chamber contained a defined volume (approximately 4 mL) of HEPES. Subsequently, 300 μL aliquots were taken at time zero, replaced with fresh medium, and at the end of the experiment (5 h for conjunctiva

and 24 h for sclera jointly with choroid). Sink conditions were maintained throughout the study. After these periods of time, the formulation was removed, and the tissue was meticulously cleaned with physiological solution and dried. Circular sections of conjunctiva (5 mm diameter) or sclera with attached choroid (9 mm diameter), corresponding to the permeation area, were cut, weighted, placed in extractive mixture for 2 h, to finally quantify LAT and LAT ACID following the procedure outlined in 2.6. Section.

2.5. Two-photon microscopy studies

Absorption, emission and excitation spectra of COU solutions in water, ethanol and toluene, and COU entrapped in the liposomal formulations (COU-LIP) were registered. Absorption spectra were recorded with a PerkinElmer Lambda 650 spectrophotometers, in 1 cm path length cuvettes. Fluorescence emission spectra were measured using an Edinburgh FLS1000 fluorometer on solutions with absorbance lower than 0.1 in 1 cm path length cuvettes. Measurements on liposomal formulations were performed using bandpass filter at 455 nm to avoid scattering signal (Fig. S1). For the two-photon microscopy analyses, COU-LIP and COU-HA-LIP were prepared and separately evaluated. The tissue (cornea or sclera with attached choroid) was mounted on a Franz cell with a 0.6 cm^2 permeation area. The donor chamber was filled with 400 μL of fluorescent formulation, whereas HEPES was gently pipetted in the receptor chamber and kept under magnetic stirring. After two hours, COU-LIP or COU-HA-LIP were removed, the tissue was washed, dismantled from the Franz-type cell, punched to obtain 9 mm diameter discs, corresponding to the contact area, and placed in a dedicated plexiglass holder to be analysed by two-photon microscope. Saline solution was used to dip the objective and to avoid dehydration.

Measurements were carried out on a Nikon A1R MP+ multiphoton upright microscope equipped with a Coherent Chameleon Discovery femtosecond pulsed laser (≈ 100 fs pulse duration with 80 MHz repetition rate and a tuneable excitation range of 700–1300 nm). A 25× water dipping objective with a numerical aperture of 1.1 mm and 2 mm working distance was employed for focusing the excitation beam and for collecting the two-photon excited fluorescence (TPEF) and the second harmonic generation (SHG) signals. TPEF and SHG signals were directed by a dichroic mirror to two non-descanned, high sensitivity photomultiplier GaAsP detectors allowing fast image acquisition. The two detectors are preceded by optical filters allowing the simultaneous acquisition of two separated channels: blue channel (415–485 nm) and green channel (506–593 nm). Imaging overlay of the two channels and processing was performed by the operation software of the microscope. A third GaAsP photomultiplier detector, connected to the microscope through an optical fiber and preceded by a dispersive element, was used to acquire the emission spectrum of the samples (wavelength range 430 to 650 nm with a bandpass of 10 nm). Laser power and detector gains have been regulated for different samples and different depths, to optimise the detected signal for images and spectra.

2.6. Quantification of latanoprost and latanoprost acid

A high-performance liquid chromatography coupled with ultraviolet detection (HPLC-UV) reversed phase method was developed to enable the simultaneous quantification of LAT and LAT ACID. This method was built upon a previously validated one [42]. Analyses were conducted with an Agilent 1260 Infinity apparatus (Agilent Technologies, Santa Clara, CA, USA). Separation occurred in an Ascentis® C18 HPLC Column (10 cm × 4.6 mm, 3 μm) (265458–04, Supelco®, Sigma-Aldrich, Madrid, Spain) column paired with a SecurityGuard™ C18 Cartridge (4 × 3.0 mm) (Phenomenex, Castel Maggiore, Italy), both thermostated at 30.0 ± 0.2 °C. Throughout the study, various isocratic mobile phase compositions and injection volumes were employed to ensure correct quantification of the active compounds. Specifically, for the validation of LAT extraction from ocular tissues, the mobile phase consisted of acetonitrile – 0.1 % *v/v* trifluoroacetic acid (TFA) in Milli-Q® water at a ratio of 70:30 % *v/v*, with an injection volume of 10 μL. For the validation of LAT ACID extraction, the mobile phase composition was acetonitrile – 0.1 % *v/v* TFA in Milli-Q® water, at a ratio of 60:40 % *v/v*, with an injection volume of 100 μL. For all other analyses described in 2.4. Section, the mobile phase composition was acetonitrile – 0.1 % *v/v* TFA in Milli-Q® water, at a ratio of 55:45 % *v/v*, with an injection volume of 100 μL. The flow rate and the detection wavelength were respectively set at 1 mL min^{-1} and at 210 nm. Retention times of 1.5 min for LAT ACID and 4.7 min for LAT were observed. Two calibration curves were employed for the determination of the hypotensive substance and its metabolite: one for the extraction samples in the validated extracting mixture (acetonitrile - Milli-Q® water, 70:30 % *v/v*) and one for permeation samples in HEPES. Both LAT and LAT ACID expressed linearity in the 0.25–10 μg mL^{-1} concentration range whether in the extracting mixture or HEPES. The limit of quantification (LOQ) for LAT ACID in HEPES was 0.1 μg mL^{-1}, while it was experimentally established at 0.25 μg mL^{-1} for LAT ACID in acetonitrile - Milli-Q® water, 70:30 % *v/v*, as well as for LAT in both conditions. The limit of detection (LOD) for both LAT and LAT ACID, using the two calibration curves employed, were ≥ 0.075 μg mL^{-1}. Precision (expressed as relative standard deviation percentage) was consistently lower than 5 % for all the concentrations evaluated, while the relative error, indicative of method accuracy, remained below 10 % in all cases.

2.7. In vivo studies

In vivo experiments were performed fulfilling the Spanish Regulation on Experimental Studies with Animals (RD 53/2013 of 1 February 2013, modified by RD 118/2021 of 23 February 2021), the European Council Directive (86/609/EEC) and the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic Vision Research. The Animal Experimentation Ethics Committee of the Complutense University of Madrid granted approvals for the procedures under the codes PROEX 091.5/21, February 24, 2021, and PROEX 114.4/21, March 16, 2021. The studies were conducted at the animal facility of the Complutense University of Madrid, in normotensive albino male New Zealand rabbits (n = 10; 3.67 ± 0.54 kg) from Granja San Bernardo (Tulebras, Spain). The sample size was determined by adhering to the principles of the 3Rs principle. The animals were individually housed in cages, provided with rabbit pellet and water *ad libitum*, and kept under 12-h light-dark cycles (with lights on from 8:00 pm to 8:00 am). The housing conditions maintained a room temperature of 22 °C in a controlled atmosphere. Prior to the tolerance and hypotensive studies described below, the biocompatibility of liposomal formulations was extensively assessed and validated in different cell cultures at different exposure times [33].

2.7.1. Acute tolerance studies

Acute tolerance studies were conducted as previously described [38,43,44]. Briefly, the tolerance of the developed vehicle (B-HA-LIP), filtered through 0.22 μm cellulose acetate filters (FILTER-LAB® CA Syringe Filter, Filtros Anoia S.A., Barcelona, Spain), was assessed by applying repeated topical instillations of 25 μL every 30 min for a total of 6 h to the animals' right eye (n = 6 eyes). As control, the left eye was instilled with single-dose Lusan® (0.9 % *w/v* NaCl) following the same procedure (n = 6 eyes). Signs of discomfort, secretions, swelling, and changes in both the cornea and conjunctiva were macroscopically assessed right before the beginning of the assay and at intervals of 3, 6, and 24 h following the initial administration, and classified according to the Organisation for Economic Cooperation and Development (OECD) Guidelines [45]. The scoring system is based on a modified protocol previously described in literature (Table 1) [46].

2.7.2. Chronic tolerance studies

Chronic tolerance studies were conducted according to the EMA/

Table 1
Scoring system for the *in vivo* acute tolerance studies of the proposed liposomal formulation.

Grade	Discomfort	Cornea	Conjunctiva	Discharge	Lids
0	No reaction	No alteration	No alteration	No discharge	No swelling
1	Blinking	Mild opacity	Mild hyperaemia / mild oedema	Mild discharge without moistened hair	Mild swelling
2	Enhanced blinking / intense tearing / vocalisations	Intense opacity	Intense hyperaemia / intense oedema / haemorrhage	Intense discharge with moistened hair and lids	Obvious swelling

CHMP/SWP/2145/2000 guidelines in order to check the long-term local tolerance of the proposed liposomal formulation, which is intended to be repeatedly administered to the eye [47]. Briefly, LAT-HA-LIP, previously filtered through 0.22 μm cellulose acetate filters, was topically instilled (25 μL) once per day for 15 consecutive days at the same time each day (9 am) to avoid diurnal fluctuations of the estimated parameters ($n = 14$ eyes). Ocular surface status and animals' discomfort were evaluated by the Draize test before the beginning of the study and each day before LAT-HA-LIP administration until the end of the follow-up period. As control, 0.9 % *w/v* NaCl was utilised ($n = 6$ eyes). Inflammation indicators were classified according to the scoring system of the Draize test with some modifications (ISO 10993-10:2021) (Table 2). Prior to initiating the study, the authors set a threshold of a cumulative score of 10, including the 'discomfort' factor, to define good tolerance of LAT-HA-LIP [48,49].

2.7.3. Evaluation of dry eye disease diagnostic parameters

As the liposomal formulations proposed have been designed with the inclusion of osmoprotective agents, the onset, evolution, or reduction of DED have been evaluated for LAT-HA-LIP throughout the study concomitantly with chronic tolerance studies. Tear osmolarity and secretion, tear film stability and eventual ocular surface damage were the DED diagnostic parameters evaluated throughout the whole study [50]. A TearLab® Osmolarity System (100000REV D, TearLab Corporation, San Diego, California, USA) coupled with TearLab® Pen and TearLab® Test Cards (T-100001, L'acuite, Madrid, Spain) was the device employed to test tear osmolarity (TOSM). It utilizes microfluidic lab-on-a-chip technology to measure electrical impedance [51]. Prior to measurements, TearLab® Control Solutions (T-100013, L'acuite, Madrid, Spain) have been used for a quality control. The reader collected and analysed 50 nL of tear sample gathered from the lower tear meniscus near the lateral canthus of the animal [52]. The manufacturers of the TearLab® Osmolarity System assert that the instrument maintains a coefficient of variation of around 1.5 %, leading to an analytical standard variation of 5 mOsm L^{-1} [53].

Tear secretion was assessed through the Schirmer's test (SCHT), without anaesthesia. Sterile Schirmer's test strips (Contacare Ophthalmics and Diagnostics, Gujarat, India) were positioned on the temporal tarsal conjunctiva of the lower lid for 60 s with closed eyes. The volume of tears, measured as millimetres of the moistened strip, was then registered [54,55]. A 5-min washout period was allowed between one SCHT and the following assays to account for potential interactions.

Afterwards, sterile fluorescein sodium ophthalmic strips (Contacare Ophthalmics and Diagnostics, Gujarat, India) were moistened with 0.9 % *w/v* NaCl and gently applied on animal inferior conjunctival fornix; the rabbit was allowed to blink to spread the dye onto the ocular surface evenly. After instillation of the dye, the eye was examined with a handheld slit-lamp microscope (SL-15 L, Kowa Optimed Deutschland GmbH, Düsseldorf, Germany) with Cobalt blue filter at 16× magnification. Fluorescent staining was described by the Oxford grading scale (OXGS) for evaluating the amount of corneal and conjunctival epithelial surface damage. OXGS divides corneal staining into six levels of severity ranging from 0 (indicating absence) to 5 (indicating severe). During the evaluation, rabbits' ocular surface staining was intuitively selected by visually comparing it to standard reference images, as described in literature [56].

Table 2
Scoring system for the Draize test, employed for determining the ocular signs severity during *in vivo* chronic tolerance studies of the proposed liposomal formulation.

CORNEA Score = A × B × 5 (range 0–80)	A. Opacity/Degree of density (most dense area taken for reading) - Scattered or diffuse area; details of iris clearly visible = 1 - Easily discernible translucent areas; details of iris slightly obscured = 2 - Opalescent areas; no details of iris visible, size of pupil barely discernible = 3 - Opaque; iris invisible = 4 B. Area of cornea involved - One quarter (or less), but not zero = 1 - Greater than one quarter, less than one half = 2 - Greater than one half, less than three quarters = 3 - Greater than three quarters, up to whole area = 4
IRIS Score = A × 5 (range 0–10)	A. Values Folds above normal, congestion, swelling, and/or circumcorneal injection; iris still reacting to light (sluggish reaction is positive) = 1 No reaction to light, haemorrhage, and/or gross destruction = 2
CONJUNCTIVA Score = (A + B + C) × 2 (range 0–20)	A. Redness of palpebral conjunctiva - Vessels definitely injected above normal = 1 - More diffuse, deeper crimson red; individual vessels not easily discernible = 2 Diffuse beefy red = 3 B. Chemosis - Any swelling above normal (includes nictitating membrane) = 1 - Obvious swelling with partial eversion of the lids = 2 - Swelling with lids about half closed = 3 - Swelling with lids about half closed to completely closed = 4 C. Discharge - Any amount different from normal = 1 - Discharge with moistening of the lids and hairs adjacent to the lids = 2 - Discharge with moistening of the lids and considerable area around the eye = 3
DISCOMFORT Score = A × 5 (range 0–10)	A. Values - No reaction = 0 - Blinking = 1 - Enhanced blinking; vocalisations = 2
Total score	= Sum of all scores obtained for the cornea, iris, conjunctiva, and discomfort

Moreover, the stability of the tear film was contextually assessed under a broad beam of Cobalt blue light by tear breakup time (TBUT) test. Briefly, TBUT was measured using a stopwatch by determining the duration in seconds from the moment of the last blink to the appearance of the first dry area within the tear film [57,58]. Evaluations of DED diagnostic parameters were performed following the same order as they have been described at the following timepoints: right before the first instillation and at 5, 10, 15, 20 (5 days after the last LAT-HA-LIP administration) and 25 days (10 days after the last LAT-HA-LIP administration) after the beginning of the studies ($n = 14$ eyes). TOSM measurements were obtained at time zero, after 8 and 15 days from the first instillation. The same procedures were followed for 0.9 % *w/v* NaCl, which was used as control ($n = 6$ eyes).

2.7.4. Long-term hypotensive studies

The efficacy of the proposed liposomal formulation (LAT-HA-LIP) in

reducing IOP simulating a chronic administration was assessed (n = 14 eyes). IOP measurements were conducted using an iCare® TonoVet TV01 rebound tonometer equipped with iCare® tonometer probes (iCare Finland Oy, Vantaa, Finland). Each measurement was averaged from six correct single readings, taken with the probe tip positioned 4–8 mm from the center of the cornea. To establish baseline IOP, two consecutive readings were taken at 30 min and immediately before the beginning of the study. Subsequently, 25 μL of LAT-HA-LIP, previously filtered through 0.22 μm cellulose acetate filter, were gently instilled onto both eyes of each rabbit every day for 15 consecutive days at the same time (9 am) to avoid biases. Following the first application, IOP readings were recorded every hour for a total of 11 h and at 24 h for the first day. After the first 24-h evaluation, tonometric readings were recorded each day during the 15-day follow-up prior to the LAT-HA-LIP administration and 3 h post-instillation, and then once daily until baseline IOP values were fully restored. The parameters analysed to assess the long-term hypotensive effect included the percentage reduction in IOP prior to the beginning of the study (IOP_{t0}) and maximum percentage reduction in IOP (IOP_{max}). Following the same protocol, 0.9 % *w/v* NaCl ($n = 6$ eyes), was used as control to monitor IOP fluctuations throughout the follow-up period due to circadian rhythms [59,60]. The hypotensive efficacy of LAT-HA-LIP after one single eye drop (25 μL) was previously assessed and discussed [33].

2.8. Statistical analysis

All data presented in text and tables are reported as mean value ± SD. *In vivo* chronic tolerance measurements were reported as the average value ± SEM. Each liposomal formulation was prepared in three distinct batches and each batch was analysed in triplicate. The significance of the differences in the *ex vivo* results and in the evaluation of DED diagnostic parameters was assessed using two-tailed Student's *t*-test. GraphPad Prism® software (version 9.5.0, GraphPad Software LLC) was used for the statistical determinations. A probability value lower than 0.05 (*p*-value <0.05) was considered statistically significant.

3. Results

3.1. Latanoprost and latanoprost acid stability tests

These studies were conducted to check and estimate any eventual degradation of the two actives at 37 °C, the same temperature at which *ex vivo* permeation experiments were performed. Both the prodrug and its active metabolite showed high Recovery % values at both 5 h (100.9 ± 0.6 % LAT ACID, 96.0 ± 0.6 % LAT) and 24 h (100.4 ± 0.4 % LAT ACID, 93.3 ± 0.8 % LAT) against control solutions at time zero. Additionally, the degradation of the ester prodrug did not lead to the formation of the acid metabolite, as no LAT ACID peak was detected in chromatograms.

3.2. Ex vivo studies

3.2.1. Validation of latanoprost and latanoprost acid extraction method from porcine ocular tissues

Table S2 collects Recovery % of LAT and LAT ACID from ocular porcine tissues (cornea, conjunctiva, sclera and choroid); these values resulted satisfactory compared to control solutions (*i.e.* ≥ 90 %), thus meaning that the protocol adopted resulted to be adequate for extracting the two compounds. No interfering peaks were detected when the extraction was performed in tissue sections without LAT and LAT ACID.

3.2.2. Permeation and retention experiments across ocular tissues

Prior to permeation and retention experiments, to confirm the esterase activity of the ocular tissues, fresh circular corneal sections (with an area of 0.6 cm^2) were immersed in a 12.5 μg mL^{-1} LAT working stock solution in HEPES and placed in a heated water bath. After 5 h, LAT and LAT ACID were quantified by HPLC-UV. The total LAT amount was found to be below the limit of quantification (LOQ), indicating complete metabolism to LAT ACID. While all the *ex vivo* experiments in this study were conducted using freshly excised ocular tissues, this preliminary test was included to demonstrate that esterase activity (essential for the metabolism of LAT and halted in frozen tissues) is fully retained in thawed ocular tissues. This finding is particularly relevant in scenarios where thawed tissues may need to be employed due to limited availability of fresh swine tissues.

To estimate the potential of the formulation candidate to deliver LAT and to explain its hypotensive effect, *ex vivo* trans-corneal, trans-conjunctival, and trans-scleral permeation experiments were conducted and compared to MF, used as a reference. Results are listed in Table 3. Given the very low titre of the formulations (0.005 % *w/v* LAT), and consequently the low amount of drug applied, several of the analysed samples had a drug concentration lower than the LOQ or equal to zero. It is however possible to draw some general conclusions: i) concerning sclera/choroid, only starting from LAT-HA-LIP it was possible to quantify LAT ACID in the receptor compartment, and ii) the liposomal formulation overperformed MF when applied to the corneal tissue. In fact, despite a similar amount accumulated, the permeation resulted higher.

Two-photon microscopy is a powerful fluorescence imaging technique used for in-depth tissue investigation [61]. Previous studies on ocular tissues have demonstrated the efficacy of this technique for permeation studies and for investigating the fate of nanocarriers [62–65]. To achieve this, the formulation must be made fluorescent, and COU-LIP/COU-HA-LIP formulations were prepared following the procedure described in section 2.2. Corneal and scleral tissues were exposed to either COU-LIP or COU-HA-LIP for a duration of 2 h under controlled conditions. Images and spectra were acquired by exciting the sample at 900 nm, in order to minimise tissue autofluorescence (that increases exciting at lower wavelengths) while simultaneously maximizing the COU signal. Images and 3D renderings collected from corneas are reported in Fig. 1A: the green signal is attributed to the two-photon excited fluorescence of COU, as confirmed by the emission spectra collected from tissues and reported in Fig. S2, whereas the blue one represented the second harmonic generation of collagen fibres (Fig. 1) [66]. In the cornea exposed to COU-HA-LIP, fluorescence signals indicative of COU were observed primarily within the epithelial layer. The penetration depth into the stroma was limited: in the images shown in Fig. 1A, COU signal was observed up to 150 μm below the surface, while with the spectral detector we were able to detect the signal up to 270 μm (Fig. S2). However, corneas exposed to COU-LIP exhibited fluorescent signal throughout the tissue (Fig. 1A), which was registered up to 360 μm (Fig. S2). The fluorescence intensity within the stromal layer was notably higher compared to samples with 0.2 % *w/v* HA in the vehicle, indicating deeper penetration. Similar results were obtained with sclera (Fig. 1B): no signal was detected for COU-HA-LIP-treated sclera at a depth of 90 μm, whereas fluorescence was registered even at 120 μm for COU-LIP (emission spectra collected from sclera are reported in Fig. S3). In sclera samples, there is no overlap between the green and blue signals, proving that the fluorescent dye arranges in the interfibrillar spaces of collagen.

3.3. In vivo studies

3.3.1. Acute tolerance studies

Before administering any formulations (time zero), a macroscopic evaluation of the rabbits' eyes revealed healthy conjunctiva and corneas; moreover, there was no evidence of discharge, eyelid swelling, or discomfort reported. At the timepoints evaluated, animals' ocular surface showed no alterations or inflammation (Fig. 2). Except for a few isolated cases in both test sample and control where a Grade 1 level of 'conjunctiva' was observed, most of the macroscopical observations disclosed that B-HA-LIP showed a Grade 0 throughout the experiment

Table 3
Permeation and retention studies results of porcine conjunctiva, sclera and choroids treated with MF or LAT-HA-ACID.

			Conjunctiva [μg $(cm^2)^{-1}$]	Sclera + choroid [μg $(cm^2)^{-1}$]		Cornea [μg $(cm^2)^{-1}$]
Permeation	MF	LAT	0	0		0
		LAT ACID	0	0		0.66 ± 0.28
	LAT-HA-LIP	LAT	0	0		0
		LAT ACID	0	1.55 ± 0.18		1.43 ± 0.62
			Conjunctiva [μg $(cm^2)^{-1}$]	Sclera [μg $(cm^2)^{-1}$]	Choroid [μg $(cm^2)^{-1}$]	Cornea [μg $(cm^2)^{-1}$]
Retention	MF	LAT	0	< LOQ	0	< LOQ
		LAT ACID	0	< LOQ	0	1.40 ± 0.50
	LAT-HA-LIP	LAT	0	< LOQ	0	< LOQ
		LAT ACID	0	< LOQ	0	1.86 ± 0.38

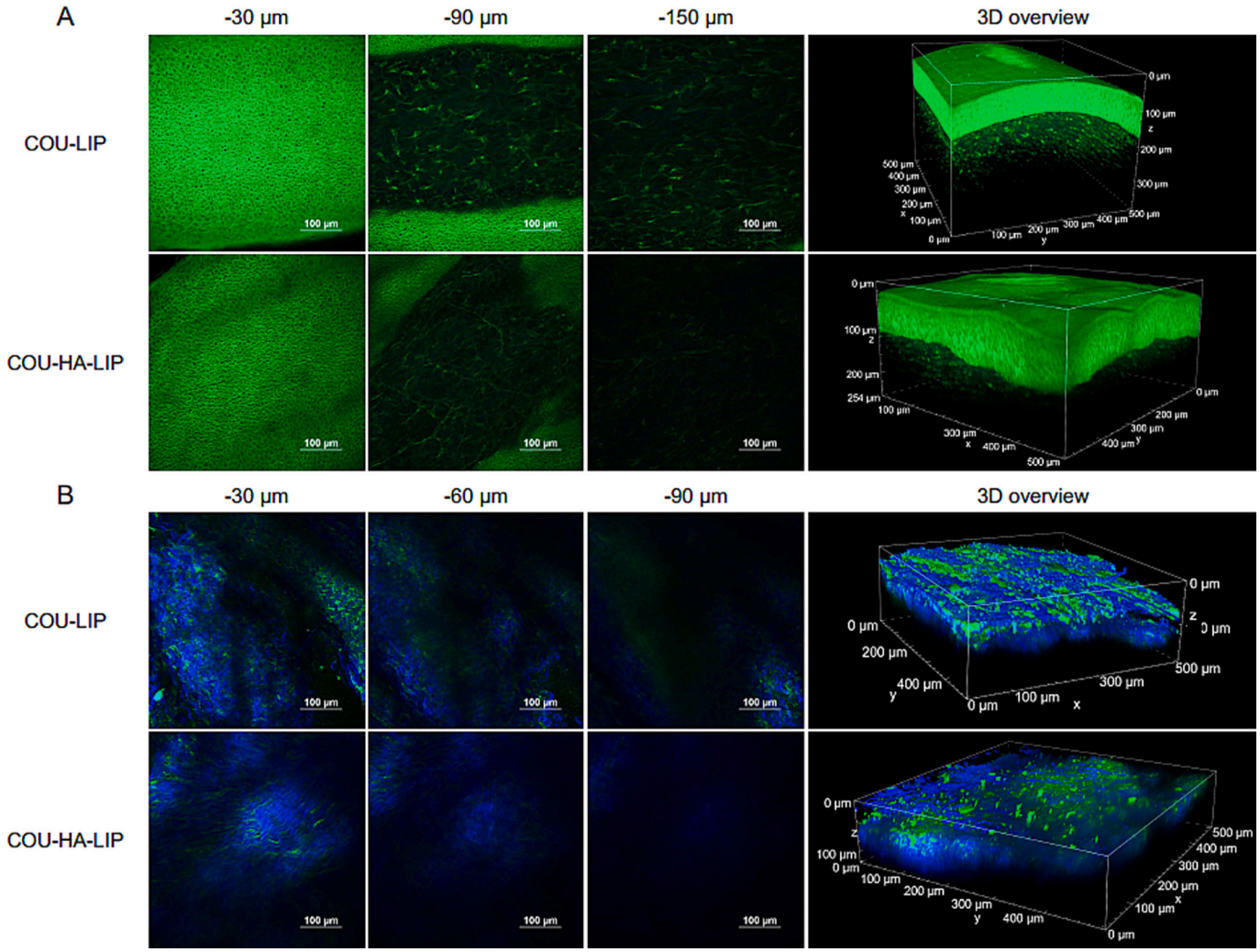

Fig. 1. (A) Two-photon images taken at different depths of cornea treated with either COU-LIP or COU-HA-LIP; excitation wavelength 900 nm, laser power images 2 %, detectors gain 70, laser power spectra 7 %. (B) Two-photon images taken at different depths of sclera treated with either COU-LIP or COU-HA-LIP; excitation wavelength 900 nm, laser power images 3 %, detectors gain 55 (blue detector) and 90 (green detector), laser power spectra 10 %. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and after 24 h, as per the criteria outlined in Table 1. Comparative analysis with 0.9 % *w/v* NaCl revealed no significant differences. Individual evaluations at each timepoint are reported in Table S3.

3.3.2. *Chronic tolerance studies*

Following the assessment of acute tolerance, further evaluations were conducted to determine the chronic tolerance of LAT-HA-LIP under conditions that closely mimic the everyday scenarios of glaucoma patients. Initial Draize test scores (time zero) showed minimal to no signs of irritation or inflammation in the ocular surfaces of the rabbits (0.17 ± 0.17 for 0.9 % w/v NaCl, 0.29 ± 0.13 for LAT-HA-LIP at time zero). Throughout the study period, ocular surface evaluations revealed no significant alterations or adverse effects from the initial status (0.11 ± 0.10 for 0.9 % w/v NaCl, 0.07 ± 0.07 for LAT-HA-LIP at day 16). Daily assessments maintained a consistent tolerance trend, with subjects that exhibited an average score below the predefined threshold one (0.11 ±

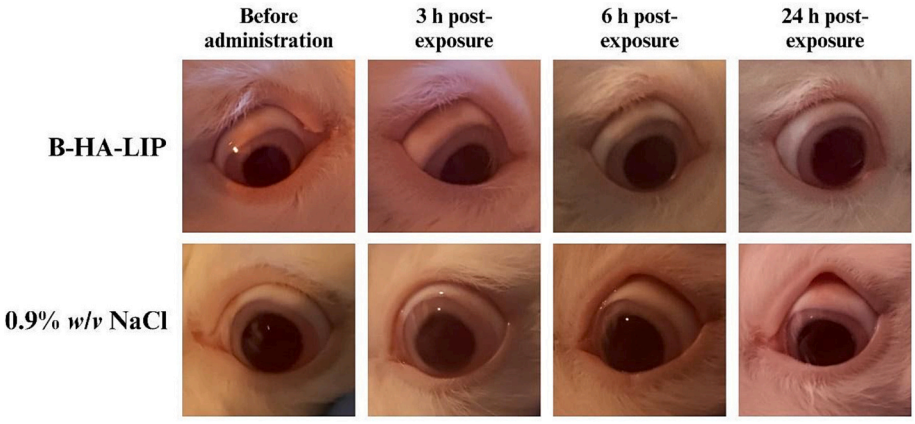

Fig. 2. Representative pictures of the acute *in vivo* tolerance studies of B-HA-LIP in comparison with 0.9 % *w/v* NaCl in albino male New Zealand rabbits ($n = 6$) after different exposure times.

0.10 for 0.9 % w/v NaCl, 0.14 ± 0.09 for LAT-HA-LIP average scores). Discomfort levels, as assessed by the modified Draize test criteria (Table 2), remained absent across the study duration. No statistical significance ($p > 0.05$) was observed between the control and the formulation candidate over the period tested, thus confirming the good LAT-HA-LIP chronic tolerance.

3.3.3. Evaluation of dry eye disease diagnostic parameters

The formulation candidate was designed not only to represent an innovative tool for managing elevated IOP levels in glaucoma patients, but also for effectively addressing the complications commonly arising from chronic administration of antiglaucoma medications. To this end, DED diagnostic parameters were systematically evaluated over a period of 25 days, thus being monitored for an additional period of 10 days following the interruption of LAT-HA-LIP instillations. Findings are represented in Fig. 3. Regarding the results obtained from SCHT, the average tear volume measured by the length of the moistened strip was comparable with the control ($p > 0.05$) both in pre-treatment (9.00 ± 1.79 mm for 0.9 % *w/v* NaCl, 10.21 ± 2.64 mm for LAT-HA-LIP at time zero) and post-treatment (9.83 ± 2.64 mm for 0.9 % *w/v* NaCl, 10.29 ± 3.00 mm for LAT-HA-LIP at day 25). Similar results were obtained when TOSM was measured using the TearLab® Osmolarity System ($p > 0.05$) (331.50 ± 29.53 mOsm L^{-1} for 0.9 % *w/v* NaCl, 329.86 ± 14.46 mOsm L^{-1} for LAT-HA-LIP at time zero and 320.33 ± 10.56 mOsm L^{-1} for 0.9 % *w/v* NaCl, 325.07 ± 15.22 mOsm L^{-1} for LAT-HA-LIP at day 15). Following the application of the fluorescent dye, the assessment of ocular surface damage using OXGS revealed absent to minimal signs both after LAT-HA-LIP and 0.9 % *w/v* NaCl long-term topical administrations in accordance with the protocol criteria throughout the study (1.33 ± 0.52 for 0.9 % *w/v* NaCl, 1.79 ± 1.12 for LAT-HA-LIP at time zero and 1.33 ± 0.52 for 0.9 % *w/v* NaCl, 1.86 ± 0.86 for LAT-HA-LIP at day 25). Representative pictures are shown in Fig. 4. Remarkably, when TBUT was evaluated, a statistical difference emerged after 15 consecutive days of administering the liposomal formulation compared to the sterile saline solution ($p = 0.0066$) (6.00 ± 0.63 s for 0.9 % *w/v* NaCl, 7.00 ± 0.78 s for LAT-HA-LIP). Moreover, comparing the LAT-HA-LIP

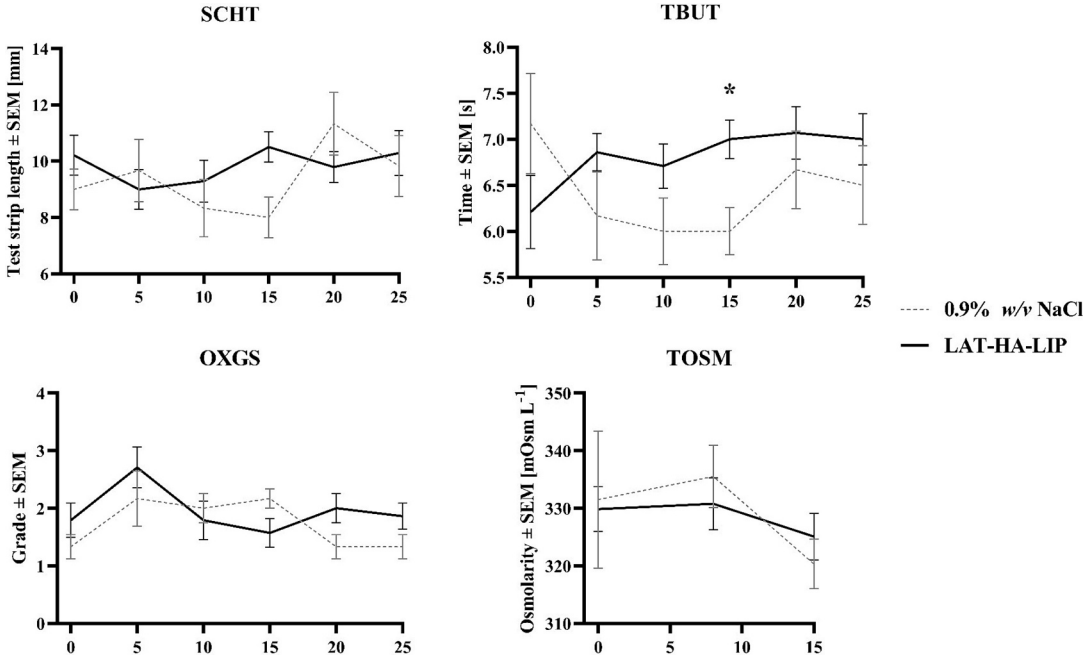

Fig. 3. DED diagnostic parameters measurements overtime (from time zero to day 25) regarding Schirmer's test (SCHT), tear breakup time (TBUT), fluorescence staining evaluated through the Oxford grading scale (OXGS) and tear osmolarity (TOSM) after 15 days of consecutive daily instillations of LAT-HA-LIP ($n = 14$, black line) or 0.9 % *w/v* NaCl ($n = 6$, grey dotted line) (* indicated statistical significance compared to control $p < 0.05$).

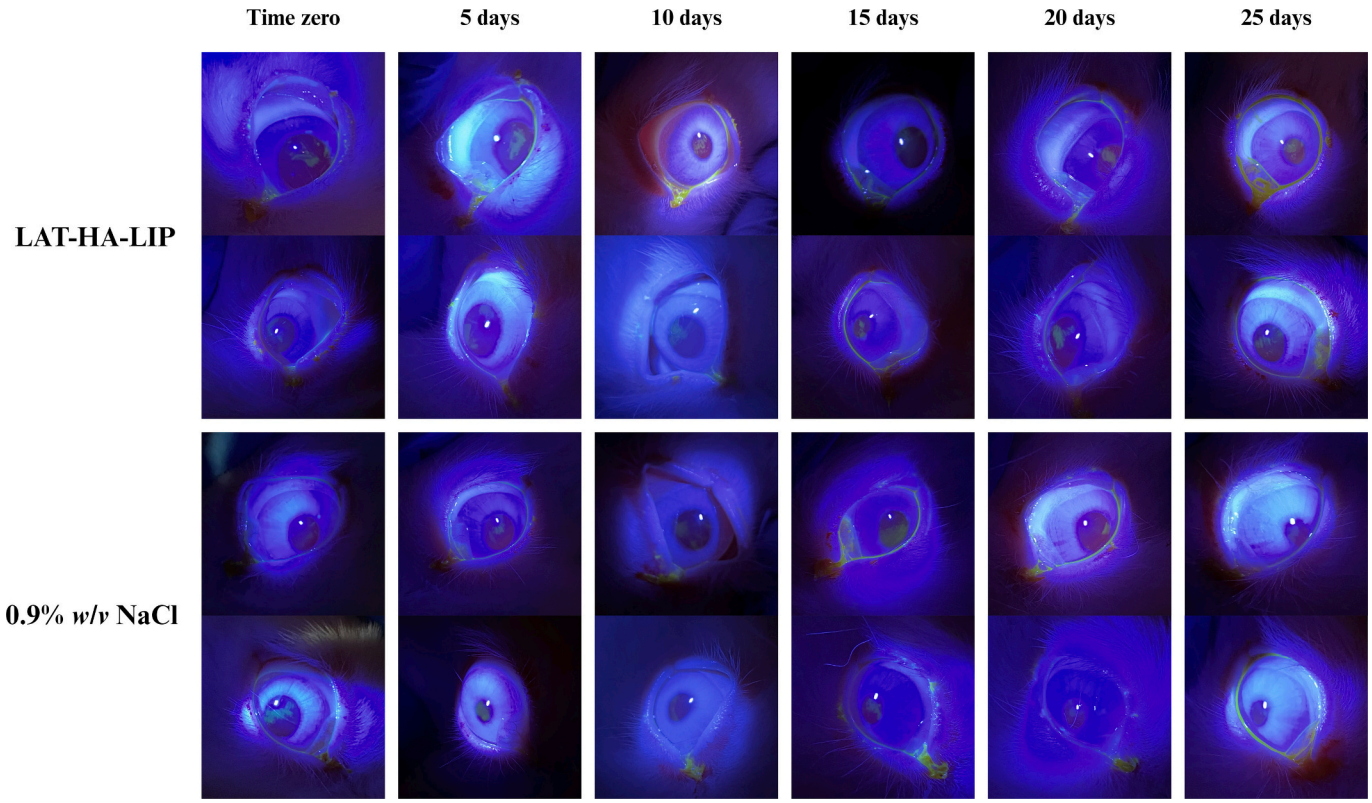

Fig. 4. Slit-lamp representative pictures of rabbits' left (upper row) and right (lower row) eyes after either LAT-HA-LIP (n = 14) or 0.9 % w/v NaCl (n = 6) daily administrations under Cobalt blue filter utilised for OXGS evaluation and where corneal and conjunctival fluorescence staining can be observed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

values before the study started (6.21 ± 1.48 s at time zero), to those during the study revealed a progressive increase in TBUT from day 15 onwards ($p = 0.0453$ at day 15) until the end of the study (p = 0.045, 7.07 ± 1.07 s at day 20 and $p = 0.0577$, 7.00 ± 1.04 s at day 25), indicating a potential improvement in preocular tear film stability.

3.3.4. *Long-term hypotensive studies*

This experiment evaluated the efficacy of the designed liposomes dispersed in the mucoadhesive polymer (LAT-HA-LIP) in reducing IOP simulating chronic administration conditions (Fig. 5). Baseline IOP values did not differ ($p > 0.05$) between the test sample and the control, ensuring a uniform starting point for establishing the liposomal formulation's impact (IOP_{t0} = 13.25 ± 1.76 mmHg for 0.9 % *w/v* NaCl, IOP_{t0} = 14.29 ± 1.67 mmHg for LAT-HA-LIP). Following the initial application of LAT-HA-LIP, IOP_{max} was recorded at 4 h (IOP_{max} = 21.33 ± 9.85 %), highlighting the rapid onset of the hypotensive effect and confirming the findings obtained previously [33]. During the 15-day follow-up, daily IOP measurements prior to LAT-HA-LIP administration and 3 h post-instillation revealed a sustained reduction in IOP levels. IOP_{max} across all administrations was recorded 3 h post-administration at day 14 (IOP_{max} = 27.24 ± 8.89 %). Considering the 5–15-day interval, average IOP decrease values before each daily administration was 13.98

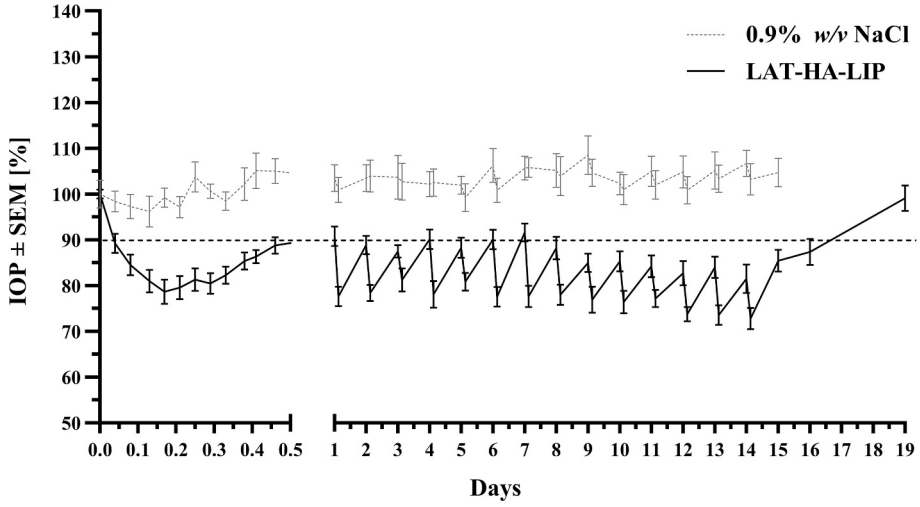

Fig. 5. Long-term hypotensive efficacy of LAT-HA-LIP ($n = 14$) when daily administered for 15 consecutive days. 0.9 % w/v NaCl ($n = 6$) was represented as control. IOP values are expressed in decreasing percentage *versus* baseline, set at 100 %.

± 8.80 %, whereas the mean IOP registered 3 h after each eye drop corresponded to 23.54 ± 8.21 %. During the second week of evaluations (days 8 to 15), no observed IOP fell below 10 % when compared to baseline IOP values. Baseline IOP values were fully restored 4 days after the end of the treatment, hence indicating that the hypotensive effect was extended for more than 24 h post-application.

4. Discussion

Liposomal formulations have been described and currently hold great promise in enhancing drug bioavailability to the anterior segment of the eye, its penetration across ocular tissues and for reducing the toxic side effects associated to the active substance [13,67]. Several authors have previously investigated the advantages of including antihypertensive agents in liposomes meant for topical ophthalmic administration – such as acetazolamide, brinzolamide, timolol maleate, brimonidine, travoprost – in terms of *in vitro* tolerance and efficacy [68–72]. Nevertheless, none of the cited studies have explored the possibility of the developed medications inducing adverse effects or exhibiting diminished efficacy over time, both of which are significant considerations for ensuring treatment success. Long-term adverse effects could potentially result in treatment failure or discontinuation, progression of pathological conditions, and the successive need for additional medications to mitigate these effects. [73]. The advantages of the present study are that it highlights the importance of addressing the challenge of achieving long-term tolerance to avoid the detrimental consequences associated with DED development and to ensure adherence among glaucoma patients to chronic topical ophthalmic treatments.

In a previous work published by the same research group, the manufacturing and physicochemical characterisation of the formulations used in the current article were presented. Furthermore, it was also demonstrated the enhanced hypotensive effect of the formulation candidate compared to the commercial one after a single application [33]. In the present work, the first part was dedicated to assessing whether that enhanced hypotensive activity may be attributed to the superior penetration of the drug delivery systems across ocular tissues. With this objective, first, to better understand the behaviour of liposomes when they interact with biological barriers, the authors measured the concentrations of the hypotensive drug once passed the ocular tissues. The extraction procedure utilised to recover both LAT and LAT ACID was effective, ensuring a satisfactory retrieval of the two drugs retained in all the evaluated ocular tissues. Then, LAT-HA-LIP proved to outperform MF regarding trans-scleral and trans-corneal permeation, thus confirming the enhanced ability of this drug delivery system to penetrate across tissue due to their flexibility and abilities to increase drug solubility and to engage a close contact with ocular tissues, aligning with findings previously reported in the literature [69,74–76]. As a matter of fact, liposomes and lipophilic drugs, such as LAT and the fluorescent probe COU, enter the cornea primarily through the transcellular pathway, where their nature allows them to permeate the corneal epithelium, bypassing the tight junctions. When reached the stroma, the hydrophilic environment poses a challenge for lipophilic molecules, requiring them to maintain sufficient concentration gradients for effective diffusion according to Fick's law. On the other hand, the sclera, characterised as a hydrophilic dense and fibrous tissue, allows liposomes and hydrophobic actives to permeate through passive diffusion, where the tissue's avascularity and collagen network influence the transport efficiency of drugs [77]. In addition to the barriers, the presence of drug-metabolising enzymes, particularly esterase, is also recognised as a limiting factor. However, considering prodrugs as LAT, enzymes are responsible for the conversion into the active ingredient [78,79].

Liposomes facilitates drug release through various mechanisms such as specific or non-specific adsorption, fusion with the cell membrane, lipid exchange, or endocytosis, and this could explain their penetration behaviour observed by two-photon microscopy, a technique that facilitates the non-invasive 3D visualization of biological tissues with minimal sample preparation. Interestingly, the results demonstrated that both formulations (COU-LIP and COU-HA-LIP) achieved significant penetration depths within both upper stroma and scleral tissues, with fluorescence probe signals detected at a deeper distance from the surface of both tissues for COU-LIP compared to COU-HA-LIP. The slower corneal permeability of COU-HA-LIP compared to COU-LIP can be explained by the increased vesicles size, the 3-fold higher viscosity of the vehicle, the interaction with mucin layer and by the ζ-potential of the first formulation, whose slightly negative charge can be repelled by the negative one of the corneal epithelium [33,80].

Regardless of all this, it is also important to consider the role of HA as an excipient in eye drops in healing and maintaining a hydrated and lubricated ocular surface, and in stabilising the precorneal tear film [81]. The authors observed these properties during the *in vivo* tolerance experiments and in the evaluation of the parameters linked to the development of DED. In the acute tolerance studies, the designed liposomal formulation demonstrated excellent tolerance. Chronic tolerance studies have been performed to replicate the potential variability in daily application times, dosing accuracy and environmental factors that patients might encounter. These good chronic tolerance results might be attributed to (i) the high purity of synthetic phosphatidylcholine derivatives (neutral unsaturated DOPC and saturated DMPC mixed in a 3:1 ratio), as revealed in previous studies conducted by our research group (ii) the inclusion of HA, (iii) and also to the presence of BET and LEU as osmoprotectants, the key components that collectively contribute to the innovative design of this hypotensive eye-drop medication [38,72]. Incorporating a combination of osmoprotective substances jointly with HA, for instance, has been recently explored in a clinical study (ClinicalTrials.gov identifier: NCT02117687) to offer an effective defence mechanism for preserving the ocular surface. An eye drop including osmoprotectants, 0.18 % *w/v* HA and carboxymethylcellulose demonstrated to have positive outcomes in reducing stinging and sandiness in patients previously diagnosed with moderate to severe symptomatic DED [82].

Moreover, the excellent tolerance exhibited by LAT-HA-LIP may offer a strategic advantage in addressing the safety concerns commonly linked to prostaglandin analogues. In fact, as mentioned in the introduction, conventional LAT topical medications have been associated with several local adverse effects, and systemic side effects such as respiratory infections or myalgia [10,83]. These observations underscore the critical need for a better 'recipe' capable of preventing the progression of glaucomatous damage. For instance, despite its preservative-free nature, MF itself demonstrated pronounced corneal toxicity when administered to simulate chronic treatment in a validated *ex vivo* eye irritation test system, contrasting with the findings observed with a 0.18 % *w/v* HA control, the same component and at a similar concentration as the one present in LAT-HA-LIP [84].

Regarding the evaluation of DED diagnostic parameters, assays conducted over a 25-day monitoring period demonstrated that LAT-HA-LIP consistently maintained levels comparable to those observed with a standard 0.9 % *w/v* NaCl solution, both prior to and following the treatment period. This outcome suggests it did not negatively impact tear secretion, tear osmolarity, or the ocular surface's condition, thus exhibiting a safety profile equivalent to the well-known sterile saline solution. Furthermore, the progressive increase in TBUT values from day 15 onwards highlights a potential long-term benefit in enhancing tear film stability; this is a key factor in the comfort and ocular health of glaucoma patients undergoing chronic treatment [85,86]. These findings are supported by previous research, which demonstrated that the combination of LAT and high molecular weight HA in eye drops resulted in reduced inflammation, diminished corneal vital staining, and in an improved epithelial barrier status in C57BL/6 mice, compared to the use of non-preserved commercial LAT eye drops containing polyoxyethylene castor oil derivative, such as MF; this improvement has been attributed to the stabilising effect of the mucoadhesive polymer [87].

Concerning hypotensive studies, the results confirmed the efficacy of LAT-HA-LIP in reducing IOP over both immediate and prolonged periods, with a significant hypotensive effect evident from the first day of administration and sustained throughout the 15-day study period. The potential clinical significance of these findings deserves particular attention; LAT-HA-LIP demonstrated consistency in inducing a hypotensive (*e.g.* average IOP reduction over the 5–15-day period included inside 2.51–3.88 mmHg range) without significant fluctuations that could compromise its efficacy and with a prolonged effect of 3–4 days after the last application. As previously corroborated by existing literature and the permeation studies conducted in this research, this improved effect is directly attributed to the encapsulation of LAT in liposomal vesicles and to the inclusion of HA, which significantly increases the retention time of the formulation onto the ocular surface; both these technological approaches, when combined, proved to increase the drug's relative ocular bioavailability [33,88].

Despite the promising findings, this study has some limitations that warrant discussion. First, the low initial concentration of LAT in both LAT-HA-LIP and MF formulations (0.005 % *w/v*) constrained the scope of the *ex vivo* permeation and retention experiments. These limitations restricted sampling to only two timepoints: the initial time (time zero) and the conclusion of the experiment. Future studies should address this limitation by employing higher LAT concentrations loaded in the vesicles' bilayer to enable a complete characterisation of the permeation profile across ocular tissues over time. Second, the *in vivo* evaluations were conducted using normotensive rabbits, which, while providing valuable insights, do not fully replicate the pathophysiological conditions of glaucoma. Although the tolerance and hypotensive effects of LAT-HA-LIP were assessed over a 15-day period in accordance with regulatory guidelines (EMA/CHMP/SWP/2145/2000), longer-term studies (*e.g.*, over 6 months or 2 years) are necessary to evaluate the prolonged safety and efficacy of the formulation. These extended studies should also include a broader and more diverse population, alongside parallel evaluations of the MF formulation. This approach would provide a more robust validation of the preclinical findings obtained and allow for a more meaningful comparison with the currently available medication. Future research should further assess the formulation under real-world conditions, using animal models that better replicate chronic glaucoma and associated ocular surface damage. Additionally, exploring alternative, industry-scalable manufacturing methods, such as microfluidics, will be significant for enhancing the reproducibility and scalability of this liposomal formulation for clinical applications.

5. Conclusions

The current investigation aims to elucidate and deepen the potential of an innovative topical ophthalmic formulation as possible eye-drop medication for glaucoma treatment, particularly regarding its long-term safety and efficacy profile. Permeation and retention experiments demonstrated an enhanced increase in the amount of latanoprost acid, the active metabolite of latanoprost, that penetrated across the cornea, conjunctiva and sclera plus choroid with the liposomal formulation compared to a marketed latanoprost one used as the benchmark. The formulation candidate proved excellent tolerance under multiple applications. This was also evidenced by the evaluation of dry eye disease diagnostic parameters, which remained within normal ranges after 15 consecutive days of instillation. Furthermore, the latanoprost-loaded liposomal formulation demonstrated its potential for clinical translation for effectively lowering intraocular pressure and maintaining it during a simulated chronic administration. These results highlight the clinical relevance of the technological platform proposed, able to provide with long-term ocular protective and hypotensive properties for the treatment of glaucoma.

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Ethics approval

All the experimental protocols regarding the *in vivo* studies were approved by the Animal Experimentation Ethics Committee of the Complutense University of Madrid (approval codes PROEX 091.5/21 and PROEX 114.4/21). All institutional and national guidelines on the care and use of laboratory animals were followed.

CRediT authorship contribution statement

Marco Brugnera: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Marta Vicario-de-la-Torre:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Miriam Ana González-Cela-Casamayor:** Methodology, Investigation, Formal analysis. **Felipe M. González-Fernández:** Validation, Formal analysis. **Ilaria Ferraboschi:** Investigation, Formal analysis. **Vanessa Andrés-Guerrero:** Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Formal analysis. **Sara Nicoli:** Writing – review & editing, Supervision. **Cristina Sissa:** Writing – review & editing, Supervision. **Silvia Pescina:** Writing – review & editing, Supervision. **Rocío Herrero-Vanrell:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Irene Bravo-Osuna:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jconrel.2025.01.041.

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