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Ophthalmic administration of a 10-fold-lower dose of conventional nanoliposome formulations caused levels of intraocular pressure similar to those induced by marketed eye drops

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ABSTRACT

The purpose of this study was to compare the *in vivo* efficacy of several timolol (TM)-loaded liposomal formulations with current TM antiglaucoma treatment (aqueous 0.5% w/v eye drops).

In this study, conventional liposomes (CL) and deformable liposomes, without (DL1) and with ethanol (DL2) were prepared and characterized. In addition, *in vitro* release and permeation studies, as well as *in vivo* lowering intraocular pressure (IOP) and biocompatibility studies were performed.

It was found that the quali and quantitative lipid bilayer composition played a significant role in modifying the physical properties of vesicles. The deformability study and electronic microscopy images revealed that membrane elasticity of DL1 and DL2 was much higher than CL. However, *in vitro* permeation results showed that the flux and permeability coefficient were significantly higher in CL compared to DL.

The IOP study revealed that TM-loaded CL showed the best pharmacological activity, in comparison to deformable vesicles. Compared to the eye drops, CL formulation could equally reduce the IOP but using a concentration 10-fold lower, whereas the effective time was significantly longer. In addition, the formulations showed no irritant effects after instillation on the ocular surface.

1. Introduction

Glaucoma is an ocular disease characterized by the increase of the intraocular pressure (IOP), leading to the degeneration of axons from the retinal ganglion cells and the progressive loss of vision (Yu et al., 2015). Current therapies decrease IOP by reducing aqueous humor formation, or by increasing outflow of fluid through the uveoscleral pathway (Aggarwal and Kaur, 2005; He et al., 2013), or increasing the fluid outflow through the trabecular meshwork cytoskeleton, a novel strategy for drug targeting (Rasmussen and Kaufman, 2014).

Timolol maleate (TM) is a non-selective beta-adrenergic receptor blocking agent which has demonstrated to be effective in lowering IOP by decreasing aqueous humor flow (Jung et al., 2013). The conventional eye drops, in monotherapy or in dual-therapy, account for nearly 90% of currently marketed formulations due to their safety, simplicity and acceptance by patients (Gan et al., 2013; Abdel-Maaboud et al., 2014; Hafez et al., 2014).

The effectiveness of the marketed ocular TM products is restricted by extremely low bioavailability of the drug. Ophthalmic TM delivery has certain restrictions due to the structure of the eyeball: some instilled amount is absorbed by the conjunctiva, passing into the systemic circulation, without exerting its therapeutic effect; also, the frequent instillation can generate an excessive loss of drug through nasolacrimal drainage causing respiratory and cardiovascular side effects (Diggory et al., 1995). In addition, the mucin present in the tear film has a protective effect preventing tear evaporation due to the formation of a hydrophilic gel layer, but also this glycoprotein limits the penetration of the drug.

In order to overcome these drawbacks, current trends in ocular therapy suggest replacing the conventional forms with delivery systems that extend the contact time with the ocular surface, improving bioavailability and decreasing systemic absorption (Shafaa et al., 2011; Tuomela et al., 2014). In this field, adequate carrier systems act by following two main strategies: promoting the passage of the drug through the cornea increasing corneal permeability and prolonging the contact time (Järvinen et al., 1995; Tártara et al., 2012). It is achieved

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Table 1
Composition of liposomes in 3 mL aqueous solution. CLs: conventional liposomes; DL1: deformable liposomes; DL2: deformable liposomes containing ethanol.

Batch	EPC (μmol)	Ch (μmol)	SDC (μmol)	TM (μmol)	Ethanol (μL)	Water (μL)	Total lipids
CLs	68.0	27.0	–	4.7	–	3000	95
DL1	95.45	81.2	7.24	4.7	–	3000	183.89
DL2	95.45	81.2	7.24	4.7	45	2955	188.59

by using colloidal systems including nanoparticles (Diebold and Calonge, 2010) and liposomes (Gan et al., 2013), emulsions (Gallarate et al., 2013), prodrugs and penetration enhancers (Moghimipour et al., 2015; Elnaggar, 2015) among others.

Lipid-based nanocarriers provide numerous potential advantages as delivery systems for ophthalmic administration (Gan et al., 2013; Yu et al., 2015) since they act as bionic tear films (have similar properties). In this sense, the lipid composition may interact with the lipid layer of the tear film, allowing the carriers to remain in the conjunctival sac for a long time, where they act as drug reservoirs. Also, the ocular residence of drug-loaded lipid carriers may be prolonged through the adhesion effect of the polymers (Tan et al., 2017); finally, the composition of lipid nanocarrier may increase the corneal permeability, for example, liposomes and derivatives could inhibit the activity of P-glycoprotein in epithelial cells, with the opening of narrow junctions to improve drug penetration.

Liposomes are colloidal vesicles that are composed of concentric bilayers formed from self-assembly of amphiphilic molecules, such as phospholipids and cholesterol. The composition of these vesicles influences their physicochemical characteristics such as size, charge, thermodynamic phase, lamellarity and bilayer elasticity (Maestrelli et al., 2006; González-Rodríguez et al., 2007; González-Rodríguez and Rabasco, 2011).

These physicochemical properties, as well, are determinant in the behavior of the vesicles and, therefore, on their efficacy in drug delivery potentiation (Hironaka et al., 2009).

Once administered onto the ocular surface, liposomes act as a reservoir of drug remaining in the surface layer (Yu et al., 2015). In some circumstances, this effect become undesirable and modified liposomes have been designed, elastic vesicles capable of traversing mucous membranes without being destroyed (Romero and Morilla, 2013; Ascenso et al., 2015). They are deformable liposomes and transfersomes, nanovesicles composed of phospholipids and edge activators that confers them flexibility. Because of the presence of these substances, they can change their shape and transverse the corneal barrier in response to mechanical stress by relocating the edge activator inside the vesicle to zones with smaller curvature, thus reducing the membrane elastic energy to a minimal level. These modified vesicles have been widely studied for transdermal drug delivery whereas for ophthalmic drug delivery, fewer approaches have been published (Maestrelli et al., 2010; Cevc, 2012; Song et al., 2012; Mahmood et al., 2014).

The aim of the current study was to analyze the *in vivo* effectiveness of TM-loaded liposomes and transfersomes when they were applied to rabbit corneal epithelium, in terms of IOP decrease. The difference in composition and physicochemical properties of these lipid vesicles may contribute in enhancing the drug permeability and minimizing the dosage to be administered.

2. Materials and methods

2.1. Materials

L-α phosphatidylcholine from egg-yolk (EPC), cholesterol (Ch) and sodium deoxycholate (SDC) were purchased from Sigma-BioChemika (Steinheim, Germany). Timolol maleate (TM) and dodecylsulfate were purchased from Acofarma (Barcelona, Spain). Acetonitrile (ACN), trichloromethane, methanol, ethanol and 2 - [4 (2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (Hepes) were obtained from Panreac Química (Barcelona, Spain). Polycarbonate membranes with 800, 200 and 100 nm pore-size were purchased from Millipore (County Cork, Ireland). All other chemicals were of analytical degree.

2.2. Preparation of lipid vesicles

TM-loaded conventional liposomes (CL) were prepared by the lipid film-hydration method, by following the procedure previously detailed (González-Rodríguez et al., 2016) with slight modifications. Prepared vesicles and their compositions are exhibited in Table 1. Narrowly, EPC (final concentration of 22.7 mM) and Ch (final concentration of 9 mM) were dissolved in methanol:trichloromethane (0.73:1). After removing the solvent under rotary evaporation (Büchi R-210 with Heating Bath Buchi B-491, Switzerland), the balloon was kept overnight into a desiccator to ensure that the solvents were totally removed. Then, the lipid film was hydrated and vortexed (Velp Scientifica Zx3) with 3 mL of Hepes buffer pH 7.4 containing 0.5 mg/mL of TM. The formulation was quickly sealed in glass containers and stored in the dark at 4 °C.

Similar procedure was carried out to prepare deformable liposomes (DL1), using EPC (final concentration 31.8 mM), Ch (final concentration 27.1 mM) and SDC as edge activator (final concentration 2.4 mM and 13.25 molar ratio in relation to EPC). Slight modifications were introduced in the procedure. First, SDC and TM were dissolved in methanol and EPC and Ch were dissolved in trichloromethane. Afterwards, these organic solutions were mixed. All the products were kept at 4 °C until used.

Similarly, deformable liposomes containing ethanol (DL2) formulations were prepared as previously. The lipid film was hydrated with 3 mL of Hepes buffer pH 7.4 containing absolute ethanol (1.5% v/v).

Unilamellar nanoliposomes (LUV) were obtained by extruding the above samples through 800 and 200 nm pore sized polycarbonate membrane filters equipped in the Lipex Thermobarrel extruder (Northern Lipids Inc., Canada) under air flow.

Control liposomal formulations without TM were obtained by following the same methodology.

2.3. Characterization of liposomes

2.3.1. Vesicle size and zeta potential

The average size and polydispersity index (PdI) of vesicles were determined by dynamic light scattering technique by using a Zetasizer Nano-S equipment at room temperature (Malvern Instruments, Malvern, UK). PdI < 0.2 indicates a homogeneous and monodisperse population whereas larger PdI (> 0.3) indicates heterogeneity.

Zeta potential was determined from electrophoretic mobility (μ) measurements. The mobility μ was converted to Z by the Smoluchowski equation:

$$Z = \mu\eta/\varepsilon$$

where η is the viscosity and ε is the permittivity of the solution.

For both measurements, 200 μL of CL and DL formulations were diluted with Hepes solution (1/20).

2.3.2. Morphology

CL and DL were visualized by transmission electron microscopy (TEM) (ZEISS LIBRA 120). All samples (10 μL) were previously diluted with 1 mL of Hepes solution. Then, a drop of the diluted sample was left to dry on a microscopic copper-coated grid (transmission electron microscopy grid support films of 300 mesh Cu). After drying completely, a drop of an aqueous solution of uranyl acetate (1% w/v) was added for negative staining. Ten minutes later, the excess solution was wiped with filter paper and washed with purified water. Then, the specimen was viewed under the microscope with an accelerating voltage of 75 kV at different magnifications.

2.3.3. Percentage of drug entrapment

TM concentrations were estimated using an HPLC system (Hitachi Elite LaChrom) equipped with an L-2130 isocratic pump, a diode array detector L-2455 and an L-2200 autosampler. For data collection and calculation, EZChromElite Data System Software Manager was used. The chromatographic separation was performed following the methodology already published, by using the same specifications (González-Rodríguez et al., 2016).

The percentage of TM entrapment (PDE%) was obtained after removing the unentrapped TM by centrifugation in a cooling centrifuge (Eppendorf Centrifuge 5804 R) at 10,000 rpm at 4 °C for 60 min. The whole supernatant was filtered and analyzed by HPLC for the drug content. In addition, the pellets of vesicles were disrupted with 0.5% w/v sodium dodecylsulfate aqueous solution and diluted for HPLC quantification after submitting the sample to a sonication process for 10 min and filtering (Bhardwaj and Burgess, 2010).

This entrapment parameter was calculated as follows:

$$\mathrm{PDE}(\%) = \frac{Q_i}{F_t} \cdot 100$$

where PDE is the percentage of drug entrapped, F_t is the total amount of TM in the sample and Q_i is the amount of drug retained into the vesicles.

The total recovery of TM in the prepared vesicle formulations was calculated by the following equation:

$$\mathrm{Recovery}(\%) = \frac{F_t}{F_i} \cdot 100$$

where F_i is the initial amount of TM.

2.3.4. Vesicle elasticity

The bilayer elasticity of the prepared vesicles was measured by the extrusion method as reported earlier (González-Rodríguez et al., 2016). Briefly, samples were extruded for 1 min through a 100 nm pore size cellulose membrane filter by applying a pressure of 5 bar. The elasticity of the vesicles was calculated from the following equation:

$$E = j \cdot \left(\frac{r_v}{r_p} \right)^2$$

where E is the elasticity index of the vesicle bilayer; j is the rate of penetration through a membrane filter (the volume of sample extruded in 1 min); r_v is vesicle size (after extrusion); and r_p is the size of membrane pore.

2.3.5. Estimation of TM partition coefficient

Based on theoretical physicochemical properties of TM (log P 1.34–1.44, pKa 9.21), it is predictable that molecules distribute between the lipid bilayer and the aqueous continuous phase, and the concentration ratio between these two phases determines the drug partition coefficient, as was reported by Natarayan et al. (2012). This parameter was estimated from samples before the extrusion step.

Briefly, known volumes of samples were centrifuged at 10,000 rpm for 30 min. The drug estimated from the supernatant is a measure of continuous (buffer) phase drug concentration. This amount of drug subtracted from the total drug concentration yields drug partitioned into the bilayer. Thus, the drug partition coefficient (PC_D) into liposomes was estimated using the following expression:

$$PC_D = \frac{TM_T - TM_B}{TM_B}$$

where TM_T is the total amount of TM and TM_B is the amount of drug in buffer.

2.4. Stability studies

The physical stability of vesicles and the ability of them to retain the drug (*i.e.* drug retentive behavior) were assessed for 1 month at 2–8 °C. Samples were withdrawn periodically and analyzed for PDE, vesicle size, PdI and zeta potential.

2.5. In vitro release studies

The release tests of TM from liposome systems were carried out by the dialysis method. In this study, 1 mL of liposome dispersion was placed in a dialysis bag (Spectra/Por 4, molecular cut-off 12–14 kD), previously rinsed and soaked for 1 h, sealing both borders with a dialysis clip. The device was then incubated in 50 mL of artificial tears ($NaHCO_3$ 0.200% w/v, NaCl 0.670% w/v and $CaCl_2$ 0.008% w/v), maintaining the stirring rate at 100 rpm and 37 °C (IKA® RT10), thus reproducing partially the biological conditions (Rathore et al., 2010; Bhowmik et al., 2011). At predetermined time intervals, aliquots of dissolution medium were collected and replaced with equal volume of fresh medium. The amount of drug dissolved with time was quantified using the HPLC method previously described.

2.6. In vitro permeation studies

Permeation studies have been performed using Franz diffusion cells (diffusion area of 3.14 cm^2) by using hydrophilic polysulfone membranes (Tuffryn®; Pall Corporation, Port Washington, NY, USA). This membrane was impregnated with a gelatin solution (12% w/v) mimicking the high protein content of the stroma (mainly collagen), the widest and the main membrane of the cornea (which supposes the 90% of the total content). We selected gelatin at this concentration as protein model as resulting from the partial hydrolisis of collagen able to generate a flexible gelled film (Lee et al., 2004). The modified membrane was then saturated with the receptor phase (RP) for 30 min. Membranes were mounted between clamped donor and receptor compartments. The receptor compartment was filled with 14 mL of Hepes buffer solution (pH 7.4) and was maintained at 32 °C–37 °C under continuous stirring (SES-GmbH Analysesystemes, Germany). A fixed volume of each formulation (1 mL) was applied in the donor compartment under non-occlusive conditions and the permeation study was continued for 24 h. Samples of 1 mL were collected after 15 and 30 min, 1, 2, 3, 4, 5, and 24 h according to International guidelines, being the same volume replaced with fresh RP kept at the same temperature. The samples were quantified by HPLC. In the reference cell, 1 mL of TM solution (0.5%) was used as control.

Data (n = 3) were expressed as cumulative amount of TM permeated through the membrane filter. The permeation (%) or *in vitro* ocular availability was calculated as follows (Krämer, 2016):

$$\mathrm{Permeation}(\%) = (\text{Amount of drug permeated in RP} / \text{Initial amount of drug in donor}) * 100$$

2.7. Hypotensive efficacy studies in vivo: IOP determination

In this experiment we have used 10 normotensive non sedated New

Zealand male white rabbits of 2–2.5 kg of weight each. Animal management procedures based on the resolution on the use of animals in research, established by the Association for Research in Vision and Ophthalmology (ARVO), the European Communities Council Directive (86/609/EEC) and the Institutional Committee of Care and Use of Animals for Research, from the Faculty of Chemistry in Córdoba National University, Argentina, were reviewed and the protocols were met. Rabbits were kept in individual cages, having free access to food and water, and were maintained in a controlled 12/12 h light/dark cycle. The formulations were applied in all 10 rabbits (n = 20 eyes) and each control was evaluated in 5 of them (n = 10 eyes). IOP was measured with a Tonovet rebound tonometer (Tiolat, Helsinki, Finland), and by using this technique, topical anesthesia was not required. For each eye, IOP was set at 100% with two basal readings taken 30 min before and immediately after the instillation. Then, a single dose of the formulation (50 μL) was applied to both eyes. IOP determinations were performed once every hour over the following 7 h. For control purposes, rabbits received the formulations without the hypotensive agent. The administration protocol included at least, a 48 h washout period between experiments.

2.8. Ocular irritation tests

2.8.1. Evaluation with the modified Draize method

The potential ocular irritancy and/or damaging effects of the developed formulation, in comparison to sodium dodecyl sulfate (SDS) solution in PBS 2% w/v (positive control), were evaluated using a slightly modified version of the Draize test (Draize et al., 1944). For this assay, twelve eyes of six male albino white rabbits of 2.0–2.5 kg of weight were used. A volume of 50 μL of test formulations was instilled into the conjunctiva sac of each eye (the rabbit's conjunctival sac capacity is ~30 μL). Another six rabbits were separated and used as control group, and they received a normal saline solution (NaCl 0.9% w/v) instilled in each eye. Pre- and post-exposure evaluations of the eyelids, conjunctiva, cornea and iris were performed by external observation with adequate illumination, and additional information was provided by examination using slit-lamp bio-microscopy (Kowa SL-14). To proceed with the observation, one drop of fluoresce in sodium salt (0.25% w/v) was instilled to contrast the potential corneal injury. The rating of ocular irritation or damage was scored (Table 2) for each observation at 30, 60, 120 and 180 min.

2.8.2. Histological examination

Based on the results described above and aiming to examine the effects on corneal structure and integrity, it was observed that the maximum irritation generated by SDS solution occurred 30 min after instillation. At that time, the animal was sacrificed, its corneas were removed and histological examination was performed. In order to get a point of reference, the effect of 0.9% w/v NaCl and SDS solutions in PBS 2% w/v were also evaluated in the same experimental conditions. After incubation, the corneas were washed with PBS and immediately fixed with a formalin solution 8% (w/w). The material was dehydrated with an alcohol gradient, put in melted paraffin and solidified in block form. Cross-sections (< 1 μm) were cut, stained with haematoxylin and eosin (H & E) and microscopically observed for any pathological modification (Baydoun et al., 2004).

Table 2
Score for potential corneal injury.

Score value	Formulation effect
0–8	No irritation
9–20	Mild irritation
21–40	Mild to moderate irritation
41–60	Moderate irritation
61–80	Severe injury
81–110	Very severe injury

2.9. Statistical analysis.

Student's *t*-test was used to compare the differences between groups. Results are given as mean ± SD and results with $p < 0.05$ were considered statistically significant. Reported as mean ± SD, the experiments were performed by triplicate.

The reduction was of an intraocular hypotensive kind, and it was expressed as means 6 standard error of the means (SEM). Also, other parameters as means 6 standard deviation (SD) were evaluated. Statistical differences between two mean values were evaluated by two-tailed student's *t*-test, and an analysis of variance (ANOVA) was applied when necessary. Results obtained were taken as significantly different at p-values, < 0.05.

3. Results

3.1. Characterization of vesicles

Uranyl acetate was used as negative stain for these samples, as it is an electron dense or opaque reagent for electrons. The reaction with the vesicles was suitably produced allowing visualization and differentiation of the vesicular membrane and lamellae. TEM study (Fig. 1) revealed that DL1 and DL2 exhibited irregular spherical shape, due to greater fluidity because the lipid bilayer was disturbed compared to other vesicles that were spherical in shape (CL). This occurs as an effect of the high deformability of the vesicular membranes allowing them to adapt their shape to the surrounding space.

The results of the analyzed parameters used to characterize all formulations were showed in Table 3. In optimizing vesicle formulations for ophthalmic application, these physicochemical properties are essential parameters, which constitute a decisive function in providing enhanced flux of drugs across the corneal membrane.

In relation to the vesicle size, two relevant factors have been evaluated to explain the obtained results. First, the difference in total lipids (mainly Ch) in these formulations has demonstrated that while the formulation CL contained 99.7 μmol (27 μmol of Ch), the formulations DL1 and DL2 were prepared with 183.89 (81.2 μmol of Ch) and 188.59 (81.2 μmol of Ch), respectively. This composition gives rise to smaller vesicles in the case of CL before extruding compared to deformable liposomes (data not shown). On the other hand, the presence of the edge activator into the lipid bilayer contributed to the recovery of vesicles after the extrusion process with higher diameter and lower PdI than CL. Our results were agreed with Duangjit et al. (2014), who concluded that the incorporation of the edge activator can reach higher curvature giving rise to deformable liposomes with larger vesicle size respect to conventional liposomes, despite 10–15% cholesterol was added. The vesicle size has been increased because of increasing the net repulsion force and reducing the van der Waals attraction forces between the lipid bilayers of liposomal systems (Liang et al., 2004).

The achievement of vesicle sizes > 250 nm in the case of DL2 can be attributed to the combined effect of the edge activator and the ethanol on deformability properties of the liposomes.

Concerning to the PdI, CL formulation showed values higher than 0.2 (heterogeneous population). When compared to DL1 (PdI = 0.199) and DL2 (PdI = 0.243), the crucial effect of edge activator on the recovery of more homogeneous vesicles was evident, mainly by adding the drug into the aqueous compartment, as DL1.

The surface charge was not significantly modified when the lipid bilayer was different. The slightly low negative values may be explained as a consequence of neutralizing all charged-compounds, such as EPC and SDC (negatively charged substances) with the drug (positively-charged compound), according to Shihui et al. (2015).

Concerning to the PDE parameter, the highest values were obtained in DL2 formulations. The vesicle size and the unilamellar structure of extruded liposomes contributed to the maintenance of TM entrapped into the aqueous compartment.

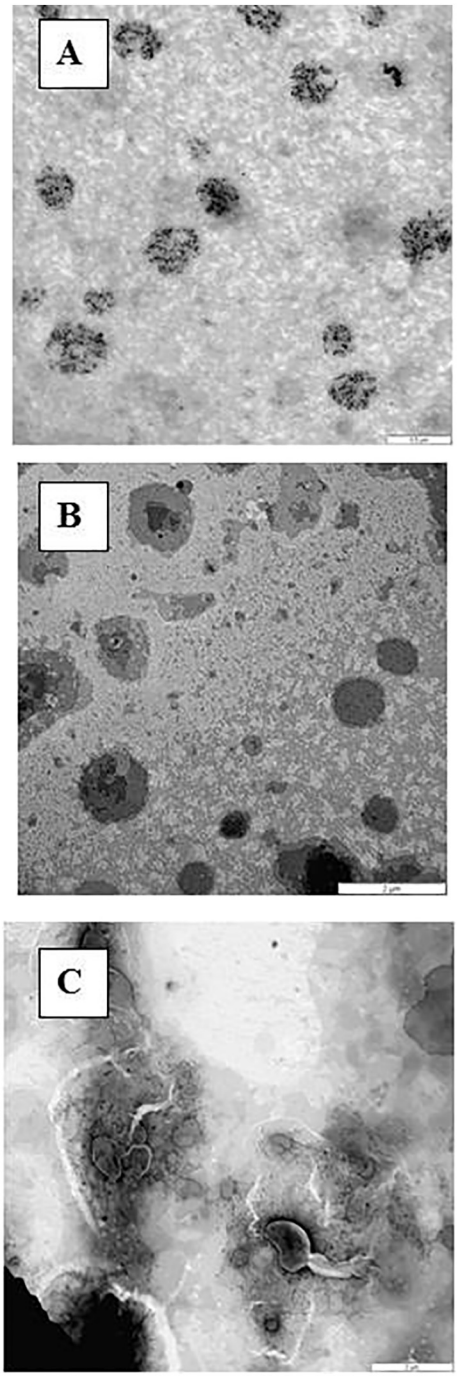

Fig. 1. TEM images of TM-loaded vesicles. A) Conventional liposomes (CL). B) Deformable liposomes (DL1). C) Deformable liposomes with ethanol (DL2).

3.2. Elasticity of vesicles

Elasticity is a key parameter for lipid vesicle penetration through the ocular barrier; therefore, it is critical that the nanocarrier system must be deformable to be able to pass easily through the corneal pores (Gupta et al., 2005; Song et al., 2012). The prepared formulations were subjected to the deformability study by following the extrusion method. The obtained values were expressed in terms of elasticity index using the equation reported in experimental section (Table 3). As logical, the CL showed the lower elasticity (0.936 $\mu L \cdot s \cdot cm^{-2}$) as compared to the flexible vesicles. The highest elasticity values showed in DL2 were according to other authors (Song et al., 2012). Although Ch increases packing density and rigidity of EPC molecules, which tends to reduce the elasticity of the vesicle bilayers (Elsayed et al., 2007; Gracià et al., 2010), the incorporation of edge activator SDC has a positive effect on the deformability properties because the higher radius of curvature increases this parameter. In addition, the presence of ethanol into the sample disturbs significantly the lipid bilayer, providing malleability and flexibility that allow liposomes to force more easily through the extruding filter (Ghanbarzadeh and Arami, 2013).

3.3. Drug partition coefficient

Partition coefficient (PC_D) is the ratio between the drug concentrations into the lipid phase to that in the continuous phase. A PC_D value of 4.22 ± 0.958 was estimated for conventional liposomes, in which TM was incorporated into the aqueous phase. This value was significantly higher than observed with DL1 (3.21 ± 0.741) and DL2 (3.19 ± 0.087), where a higher total lipid amount exists.

This result predicts the TM behavior concerning to the affinity of the drug to the lipid bilayer and the influence of lipid to aqueous phase ratio on this partition value.

3.4. Stability study

The stability studies of prepared liposomal formulations were carried out for 30 days.

Vesicle size of all extruded formulations increased by 25 nm approximately, and zeta potential changed in the range of about 5 mV over the storage period. These results are according to increased values of 20 nm presented by other authors (Meissner et al., 2015). TM was found to be stable among all the prepared liposomal formulations when analyzed in terms of vesicle size, PdI, zeta potential and drug entrapment in liposomes at 4 °C (Table 4).

3.5. In vitro release studies

The test was performed in artificial tears at 37 °C. As shown in Fig. 2, identical release kinetic of TM was obtained in all formulations: initially, a burst release phenomenon was observed, corresponding to the free molecules dissolved in the dissolution medium. Almost 90% of TM was released in 2 h. The faster release of drug was attributed to the free diffusion of drug from the core of liposomes to the release media and, despite the different composition, lipid vesicles are not the controlling factor for the drug release, but TM solubility.

3.6. In vitro permeation studies

In vitro permeation studies were performed for evaluating the influence of composition and drug disposition into the vesicles on drug permeation.

Table 3
Physicochemical properties of the prepared lipid vesicles.

Group	Size (nm)	PdI	Zeta potential (mV)	Partition coefficient	Elasticity index ($\mu L \cdot s \cdot cm^{-2}$)	PDE (%)
CL	151.0 ± 1.33	0.459 ± 0.02	−2.95 ± 0.27	4.22 ± 0.958	0.936 ± 0.020	17.652 ± 2.574
DL1	247.6 ± 4.73	0.199 ± 0.01	−2.03 ± 0.79	3.21 ± 0.741	2.296 ± 0.060	14.806 ± 5.471
DL2	354.9 ± 5.41	0.243 ± 0.19	−2.66 ± 0.29	3.19 ± 0.087	7.871 ± 0.258	26.690 ± 5.231

Table 4
Stability study realized for one month at 4 °C. Mean ± SD.

Days	Vesicle size (nm)	PdI	Zeta potential (mV)	PDE (%)
CLs				
0	151.0 ± 1.33	0.459 ± 0.02	− 2.95 ± 0.27	17.65 ± 2.57
7	152.0 ± 2.11	0.489 ± 0.01	− 2.95 ± 0.14	14.81 ± 3.12
14	150.8 ± 2.58	0.550 ± 0.02	− 3.05 ± 0.13	15.20 ± 2.58
21	155.5 ± 1.95	0.702 ± 0.03	− 3.29 ± 0.10	15.74 ± 3.69
30	149.4 ± 3.12	0.317 ± 0.01	− 6.72 ± 0.15	19.09 ± 1.47
DL1				
0	247.6 ± 4.73	0.199 ± 0.01	− 2.03 ± 0.79	14.81 ± 5.47
7	240.6 ± 3.58	0.189 ± 0.02	− 2.33 ± 0.25	17.65 ± 3.65
14	230.6 ± 1.47	0.184 ± 0.03	− 2.58 ± 0.36	16.55 ± 4.11
21	229.8 ± 3.69	0.218 ± 0.05	− 3.84 ± 0.41	15.47 ± 6.54
30	222.1 ± 2.58	0.199 ± 0.08	− 5.65 ± 0.25	12.48 ± 5.68
DL2				
0	354.9 ± 5.41	0.243 ± 0.19	− 2.66 ± 0.29	26.69 ± 5.23
7	355.9 ± 6.11	0.293 ± 0.12	− 2.86 ± 0.15	27.69 ± 6.11
14	353.8 ± 4.23	0.300 ± 0.13	− 3.12 ± 0.20	27.80 ± 5.41
21	354.6 ± 6.87	0.363 ± 0.11	− 3.62 ± 0.23	30.46 ± 6.23
30	356.9 ± 5.12	0.197 ± 0.08	− 8.53 ± 0.25	29.50 ± 7.11

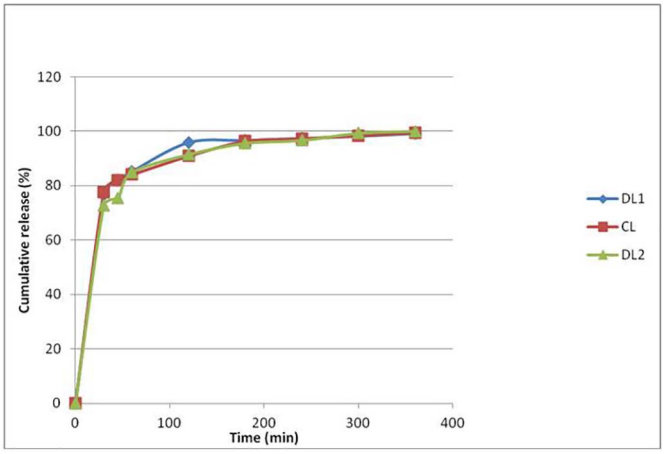

Fig. 2. The *in vitro* release profiles of TM from deformable liposomes (DL1 and DL2) and conventional liposomes (CL).

Equal amount of TM from different formulations, including solution, CL, DL1 and DL2 were tested on the artificial membrane surface in the donor compartment to evaluate their penetration ability through membrane. The amount of TM permeated over 24 h was plotted *versus* time.

Table 5 presents the permeation parameters of TM through membrane and accumulated amount of remained and permeated drug over 24 h from different formulations. The cumulative amount of drug permeated from vesicle formulations after 24 h was not significantly different than drug permeated from the aqueous solution ($p < 0.05$). However, the mean steady state flux (J_{ss}) and permeability coefficient (K_p) ranged from 0.3036 ± 0.008 (DL2) to 1.6467 ± 0.052 (control solution) μg/cm^2.min and 0.364 ± 0.009 (DL2) to 1.976 ± 0.001 (control solution) mm/h, respectively. Results indicated that the flux and permeability coefficient of TM solution and CL were 5- and 4-fold higher than DL2 formulation, respectively. Results also revealed that, compared to CL, the incorporation of edge activator and ethanol into the composition could significantly decrease the J_{ss} and K_{ps} up to 4 times. It can be concluded from the study that DL1 and DL2 could penetrate and deposit TM less than CL. High deposition percent indicated that these vesicles could provide a drug reservoir to prolong the effect of TM, according to other authors (Tsai et al., 2015).

Table 5
Ocular permeation parameters.

Formulation (Code)	Permeated amount at 24 h (μg/cm^2)	J_{ss} (μg/cm^2.min)	K_p (mm/h)
Control solution	144.78	1.647	1.976
CL	132.67	1.267	1.520
DL1	141.31	0.489	0.588
DL2	130.62	0.304	0.364

3.7. IOP measurement

Independent experiments were carried out in 10 rabbits (n = 10) to evaluate the hypotensive effect of each formulation. Two different concentrations of TM solutions (0.5 mg/mL and 5 mg/mL) were used as control samples. A single dose was tested on a single animal every day, and at least a 48 hour wash-out period between experiments was applied.

The effect of the TM formulations described in this paper was compared by using the maximum hypotensive effect of the drug (%), the area under the curve of the ΔIOP (%) *versus* time (h) from 0 to 7 h (AUC), and the mean time (h) in which the duration of the hypotensive effect was maintained.

All the vehicles containing TM were able to maintain the hypotensive effect of TM, providing different maximal effects and maximum percentage of IOP reduction, as it is shown in Table 6 and Fig. 3. Actually, the concentration of TM used commercially is 5 mg/mL but *in vivo* studies performed on rabbits, efficacy showed that the hypotensive effect of TM (0.5 mg/mL) was remarkably increased with the combination of CL or DL2. Taking the formulation TM 0.5 mg/mL as a solution reference (11.58% IOP reduction), significant differences in the maximal hypotensive effect were obtained with DL2 (20.06%, $p = 0.0072$) and CL (23.02%, $p = 0.0008$), as shown in Fig. 4. Thus, the efficacy was similar to TM concentration of 5 mg/mL (24.47% IOP reduction) when using a drug concentration ten-fold lower.

The AUC of TM 0.5 mg/mL solution was significantly lower than the values obtained with DL2 and CL ($p = 0.0072$ and 0.0008, respectively).

Concerning the mean time effect, the reference formulation 0.5 mg/mL lasted approximately 4 h. This effect was exceeded by the formulation which contained ultra-deformable and conventional liposomes, which effect lasted approximately 5 and 7 h, respectively.

3.8. Ocular irritation assays

3.8.1. Modified Draize method

Before the experiment was performed, all rabbits showed a normal ocular surface with a transparent cornea. Both formulations can be considered non-irritating because they presented a score lower than 10. After instillation, the SDS solution produced a noticeable irritation with a score higher than 60. On any of the tested formulations, conjunctival disorders can be appreciated, including hyperemia or edema, eyelid swelling, or intense blinking. However, animals showed no discomfort

Table 6
Pharmacokinetic and pharmacological parameters evaluated of the different formulations. [TM] = 0.5 mg/mL.

Formulation	Mean time (h)	Maximal IOP reduction (%)	AUC_0^{7h} (%.h)
TM solution (5 mg/mL)	5	24.47 ± 1.85	71.78 ± 7.08
TM solution (0.5 mg/mL)	4	11.58 ± 2.58	32.13 ± 7.70
DL2	5	20.06 ± 2.89*	63.80 ± 12.82*
CLs	7	23.02 ± 1.38*	86.14 ± 7.26*

* Significant differences with solution [TM] 0.5 mg/mL (p-value < 0.05).

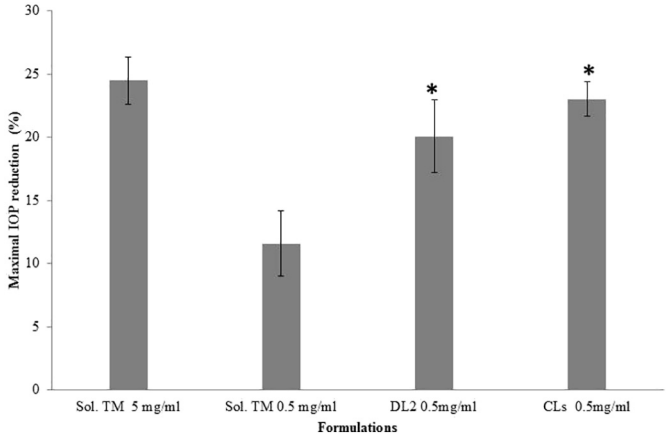

Fig. 3. Maximal hypotensive effect (% ± S.E.M) of solution [TM] 5 mg/mL and [TM] 0.5 mg/mL and DL2 and CL with [TM] 0.5 mg/mL. *Significant differences with solution [TM] 0.5 mg/mL (p-value < 0.05).

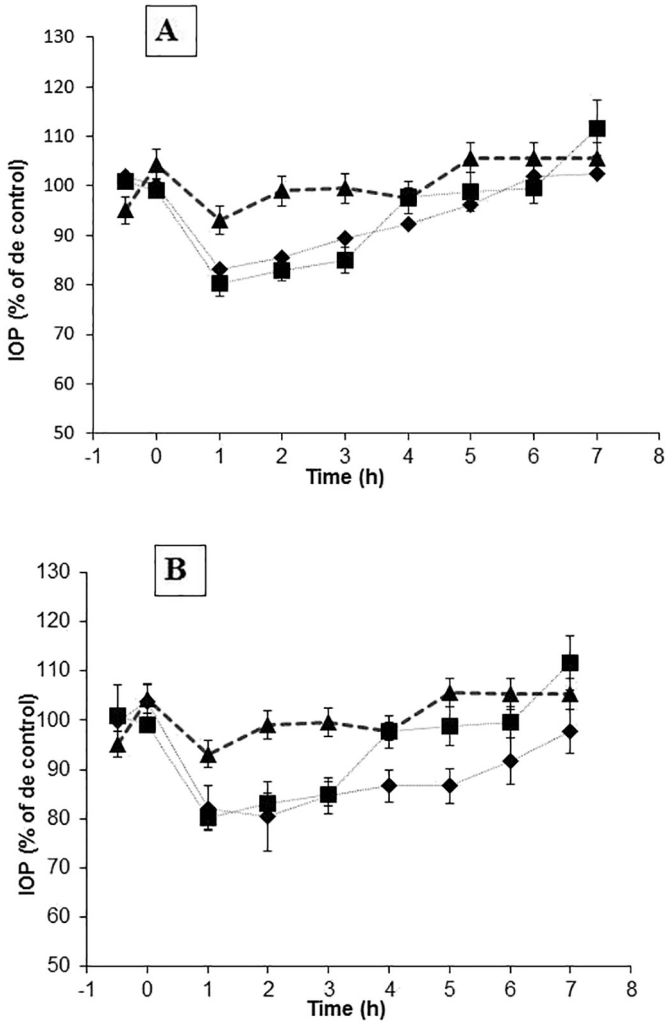

Fig. 4. IOP profiles of (▲) Solution [TM] 0.5 mg/mL; (■) Solution [TM] 5 mg/mL and **A)** (♦) DL2 [TM] 0.5 mg/mL. **B)** (♦) CL [TM] 0.5 mg/mL.

or irritation during the test. The cornea remained transparent (no vessels) throughout the assay and the coloration of the conjunctiva remained normal. No secretions of mucus were seen on any animal. On the contrary, the SDS solution produced a noticeable irritation with a score higher than 60, reaching its peak at 30 min after instillation, and then, the irritant effect decreased as time passed.

3.8.2. Histological examination

Fig. 5 shows the cross-sections of corneas after the administration of different formulations. In Fig. 5A, it is shown that, apparently, no changes appeared on the epithelium and stroma structures when NaCl solution was administered, but some typical stratified layers can be recognized, due to the rise of a bulge in the nuclei of the basal columnar cells, as well as the squamous on the surface of them. In Fig. 5B, it is shown the damage of the corneal epithelium structure when exposed to SDS solution, due to obvious changes in superficial cells. No morphological or structural changes could be seen in Fig. 5C and D, neither in the structure nor in the integrity of the corneas, even when they were visibly affected. These results revealed that the formulations of CL and DL2 had a high biocompatibility.

4. Discussion

Decreased IOP remains the key adjustable risk factor in glaucoma. The main treatment of glaucoma is based on topical medications for reducing the IOP, thus delaying damage to the optic nerve due to elevated IOP. However, periodic application, because of poor ocular bioavailability and other long-term side effects such as allergy and intolerance to medications, imply negative effects on patient compliance, leading to poor adherence of the patient to medication and to the progression of the disease resulting in poor IOP control.

In the present study, we investigated the *in vitro* permeation and *in vivo* ocular effect of TM-loaded from different types of lipid vesicles: conventional liposomes (CL) and deformable vesicles with either an edge activator (DL1) or an edge activator together with a permeation enhancer (DL2). The properties in terms of structure, vesicle size, surface charge, PdI, percentage of drug entrapped, elasticity, partition coefficient, release behavior, *in vitro* permeation studies and *in vivo* corneal studies, were examined.

Theoretically, DL2 composition (a permeation enhancer, ethanol, together with SDC as edge activator) make these vesicles to provide improved permeation characteristics compared to DL1 and CL. Ethanol interacts with lipid molecules in the polar head group region, resulting in a reduced phase transition temperature (TM) of the corneal lipids and higher fluidity which provides an increase in the membrane permeability. Ethanol may also provide malleability and flexibility to the vesicles, and it allow them forcing more easily and penetrating into the deeper layers of the eye. In addition, during permeation process, the edge activator (SDC) is demixed from the lipid bilayer and displaced to relocate in the zones of higher curvature/stress, whereas the more hydrophobic amphiphilic molecules enrich the bilayer regions with a smaller curvature. These rearrangements are the cause of the decrease of the membrane elastic energy and the formation of structures that are more deformable than CL by up to five orders of magnitude. In this sense, DL2 reported higher values, which imply improved elasticity compared to both CL and DL1, as revealed TEM images, could be due to the combined effect of ethanol content and SDC in these vesicles.

However, no correlation exists between the elasticity properties and the enhanced permeation behavior of DL. The influence of total lipid amount on the drug permeation and, therefore, the vesicle size, was evident.

The observed results of CL are correlative with *in vitro* permeation results, which showed a slow and sustained release of TM. Concerning the vesicle composition, the presence of Ch in different mol% can affect to the drug diffusion. Taking into account that the mol% was higher in DL2 (44.17 *versus* 27.08 mol%) and that the presence of this steroid to a lipid bilayer strongly influence its thermodynamic and mechanical properties by decreasing the passive permeability of the bilayer, we can explain why TM release was slower and incomplete as Ch concentration increased (Khajeh and Modarress, 2014). In addition, TM has a high partition coefficient (log P 1.34–1.44) and the solubility-diffusion

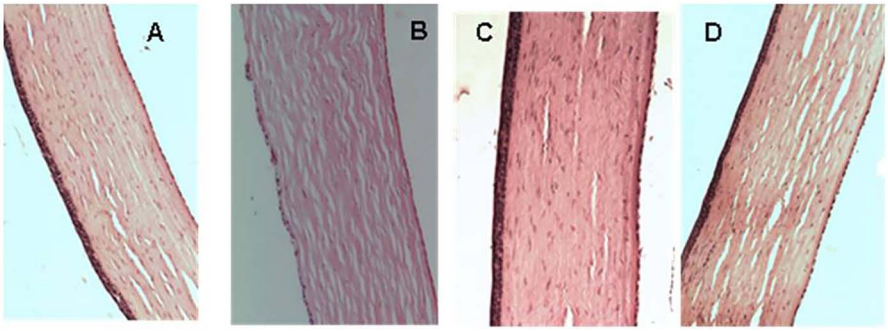

Fig. 5. Histological cross-sections of excised rabbit cornea showing epithelium and stroma stained with hematoxylin & eosin. (A) 0.9% w/v NaCl solution, (B) 2% w/w SDS solution, (C) CL and (D) DL2.

mechanism is probably a key factor in the permeation of TM molecules (Boggara and Krishnamoorti, 2010). The disposition of drug into the bilayer favours the diffusion to the dissolution medium meanwhile the drug entrapped into the aqueous phase is maintained as reservoir into the vesicle for a longtime, as occurs in CL.

The hypothesized effect of the ionization characteristics of TM in aqueous medium on the diffusion through lipid bilayers can be explained according the study proposed by Ulander and Haymet (2003) for valproic acid into DPPC vesicles. TM has pKa 9.21 and was mainly protonated at pH 7.4. As the charged timolol approached the bilayer, the headgroups polarize, and at the same time permit water fingers protruding into the bilayer to relax the electrostatic penalty from desolvation. Additional important contributions were proposed by Carrozzino and Khaledi (2005) who postulated the effect of pH on drug interactions and lipid bilayers. The electrostatic interactions due to the functional groups persisting in the TM have a key function in the partition coefficient, which is dependent on the extent of solute ionization as determined by the pH. At physiological pH (7.4), TM is positively charged and is electrostatically attracted by liposome lipids that carry a net negative charge. This fact could explain the practically neutral zeta potential values obtained in this study.

The *in vivo* results revealed that a single topical application of TM (0.5 mg/mL)-loaded EPC liposomes could effectively reduce the IOP in rabbit's eyes for at least 7 h. This IOP-lowering effect was significantly greater than topical administration of TM solution 0.5 mg/mL, and the same effect than the current standard treatment in glaucomatous patients (5 mg/mL).

The mechanism of permeation through cornea can explain the behavior of this formulation. A combined transcellular and paracellular mechanism might be attributable to TM as a function of its partitioning and water solubility properties. Permeability studies indicated that as the corneal epithelium is lipophilic, low in porosity and relatively high in tortuosity (Malhotra and Majumdar, 2001), a rapidly penetrating drug must possess log partition coefficient > 1 in order to assess to achieve a sufficient penetration rate. In this study, for hydrophilic drugs, such as TM, with log partition coefficient of 1.34–1.44, the epithelium and stroma contribute to a significant enhancement of permeation.

On the other hand, ethanol and SDC have been shown to improve transcorneal drug penetration. Certainly, their potential benefits in improving the poor topical bioavailability of ophthalmic drugs, has been reported. SDC, a bile salt, is an anionic surfactant. In general, these types of adjuvants, at low concentrations, are added into the lipid bilayer, with consequent changes on the physical properties of the cell membranes (Moghimipour et al., 2015). When the bilayer becomes saturated, mixed micelles are formed, causing the removal of phospholipids from the cell membranes and the membrane solubilization (Stojancevic et al., 2013). Also, SDC can increase the paracellular transport by disruption of the hemidesmosomes or by binding to Ca^{2+} in the regions of tight junctions (Shaikh et al., 2012). Simultaneously, ethanol may disorder the structure of the ocular lipid bilayer and enhance its lipid fluidity, as it has been widely reported in the literature (López-Pinto et al., 2005; Patra et al., 2006).

As expected, tested formulations were adequately tolerated on the ocular surface. However, their use should be considered with caution since these ocular penetration enhancers can cause ultrastructural changes in the corneal epithelium. Studies showed that SDC is intensively ciliotoxic and at a concentration of 1%, is irritant and causes corneal damage (Saettone et al., 1996). Similarly, ethanol can cause an irritant effect on cornea with repetitive use.

Regarding the reduction of IOP in rabbit eyes, the results obtained are encouraging. To date, no other studies show such a significant effect of TM sustained-release using liposomes in the animal eye, at a concentration of TM ten times lower than control solution. The size of these nanocarriers could also be a key choice in drug delivery through the various anatomical structures of the eye (conjunctiva and sclera) to reach the targeted site (ciliary body) more efficiently, with increased bioavailability.

Certainly, in the case of ophthalmic drug products, it is difficult to develop a reliable *in vitro* predictive model. Despite the existence of precorneal constraints of the eye, such as continuous clearance of dosage forms and the released drug from the cul-de-sac area through tear drainage, tear dilution and lacrimation, our study showed a good correlation in terms of *in vitro* permeation parameters and therapeutic effect obtained. This concordance makes the permeation protocol selected as appropriate for further studies by using this route.

In addition, data from *in vitro* experiments are useful for a rational extrapolation to *in vivo* prediction in order to minimize the use of animal testing, which is limited due to ethical, economical and technical reasons.

5. Conclusions

This study showed that the combinatory effect of penetration enhancer (ethanol) and surfactant (SDC) increased the elasticity of vesicles. However, according our *in vivo* results, the conventional liposomes showed an extended hypotensive effect compared to other liposomal formulations. The maintenance of depot system onto the corneal membrane becomes crucial for prolonging the IOP lowering.

The current work emphasizes that the use of lower dosage of TM (0.05% w/v) than marketed eye drops (0.5% w/v), strongly enhances the pharmacological and toxicological profile of TM from liposomal ophthalmic formulations.

Overall, these *in vivo* findings indicate that TM-loaded liposomes have great potential to deliver the drug through the corneal membrane. However, *in vivo* results demonstrated that in addition of *in vitro* characterization and evaluation, also the physiological and toxicological aspects of corneal epithelium must be taken into account in order to adequately predict the *in vivo* behavior.

References

Abdel-Maaboud, I.M., Abdel-Wadooda, H.M., Mousab, H.S., 2014. Simultaneous determination of dorzolamide and timolol in aqueous humor: a novel salting out

liquid–liquid microextraction combined with HPLC. Talanta 130, 495–505.
Aggarwal, D., Kaur, I.P., 2005. Improved pharmacodynamics of timolol maleate from a mucoadhesive niosomal ophthalmic drug delivery system. Int. J. Pharm. 290, 155–159.
Ascenso, A., Raposo, S., Batista, C., Cardoso, P., Mendes, T., Praça, F.G., Bentley, M.V., Simões, S., 2015. Development, characterization, and skin delivery studies of related ultradeformable vesicles: transfersomes, ethosomes, and transethosomes. Int. J. Nanomedicine 10, 5837–5851.
Baydoun, L., Furrer, P., Gurny, R., Muller-Goymann, C.C., 2004. New surface-active polymers for ophthalmic formulations: evaluation of ocular tolerance. Eur. J. Pharm. Biopharm. 58, 169–175.
Bhardwaj, U., Burgess, D.J., 2010. Physicochemical properties of extruded and non-extruded liposomes containing the hydrophobic drug dexamethasone. Int. J. Pharm. 388, 181–189.
Bhowmik, M., Das, S., Chattopadhyay, D., Ghosh, L.K., 2011. Study of thermo-sensitive in-situ gels for ocular delivery. Sci. Pharm. 79 (2), 351–358.
Boggara, M.B., Krishnamoorti, R., 2010. Partitioning of nonsteroidal antiinflammatory drugs in lipid membranes: a molecular dynamics simulation study. Biophys. J. 98, 586–595.
Carrozzino, J.M., Khaledi, M.G., 2005. pH effects on drug interactions with lipid bilayers by liposome electrokinetic chromatography. J. Chromatogr. A 1079, 307–316.
Cevc, G., 2012. Rational design of new product candidates: the next generation of highly deformable bilayer vesicles for noninvasive, targeted therapy. J. Control. Release 160, 135–146.
Diebold, Y., Calonge, M., 2010. Applications of nanoparticles in ophthalmology. Prog. Retin. Eye Res. 29, 596–609.
Diggory, P., Cassels-Brown, A., Vail, A., Abbey, L.A., Hillman, J.S., 1995. Avoiding unsuspected respiratory side-effects of topical timolol by using cardioselective or sympathomimetic agents. Lancet 345, 1604–1606.
Draize, J.H., Woodard, G., Calvery, H.O., 1944. Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J. Pharmacol. Exp. Ther. 82, 377–390.
Duangjit, S., Obata, Y., Sano, H., Onuki, Y., Opanasopit, P., Ngawhirunpat, T., Takayama, K., 2014. Comparative study of novel ultradeformable liposomes: menthosomes, transfersomes and liposomes for enhancing skin permeation of meloxicam. Biol. Pharm. Bull. 37 (2), 239–247.
Elnaggar, Y.S., 2015. Multifaceted applications of bile salts in pharmacy: an emphasis on nanomedicine. Int. J. Nanomedicine 10, 3955–3971.
Elsayed, M.M.A., Abdallah, O.Y., Naggar, V.F., Khalafallah, N.M., 2007. Lipid vesicles for skin delivery of drugs: reviewing three decades of research. Int. J. Pharm. 332, 1–16.
Gallarate, M., Chirio, D., Bussano, R., Peira, E., Battaglia, L., Baratta, F., Trotta, M., 2013. Developmentof O/W nanoemulsions for ophthalmic administration of timolol. Int. J. Pharm. 440, 126–134.
Gan, L., Wang, J., Jiang, M., Bartlett, H., Ouyang, D., Eperjesi, F., Liu, J., Gan, Y., 2013. Recent advances in topical ophthalmic drug delivery with lipid-based nanocarriers. Drug Discov. Today 18 (5/6), 290–297.
Ghanbarzadeh, S., Arami, S., 2013. Enhanced transdermal delivery of diclofenac sodium via conventional liposomes, ethosomes, and transfersomes. Biomed. Res. Int. 2013, 616810. http://dx.doi.org/10.1155/2013/616810.
González-Rodríguez, M.L., Rabasco, A.M., 2011. Charged liposomes as carriers to enhance the permeation through the skin. Expert Opin. Drug Deliv. 8 (7), 1–15.
González-Rodríguez, M.L., Barros, L.B., Palma, J., González-Rodríguez, P.L., Rabasco, A.M., 2007. Application of statistical design to study the formulation variables influencing the coating process of lidocaine liposomes. Int. J. Pharm. 337, 336–345.
González-Rodríguez, M.L., Arroyo, C.M., Cózar-Bernal, M.J., González-R, P.L., León, J.M., Calle, M., Canca, D., Rabasco, A.M., 2016. Deformability properties of timolol-loaded transfersomes based on the extrusion mechanism. Statistical optimization of the process. Drug Dev. Ind. Pharm. 42 (10), 1683–1694.
Gracià, R.S., Bezlyepkina, N., Knorr, R.L., Lipowsky, R., Dimova, R., 2010. Effect of cholesterol on the rigidity of saturated and unsaturated membranes: fluctuation and electrodeformation analysis of giant vesicles. Soft Matter 6, 1472–1482.
Gupta, P.N., Mishra, V., Rawat, A., Dubey, P., Mahor, S., Jain, S., Vyas, S.P., 2005. Non-invasive vaccine delivery in transfersomes, niosomes and liposomes: a comparative study. Int. J. Pharm. 293 (1), 73–82.
Hafez, H.M., Elshanawane, A.A., Abdelaziz, L.M., Mohram, M.S., 2014. Development and validation of HPLC method for simultaneous estimation of brimonidine tartrate and timolol maleate in bulk and pharmaceutical dosage form. J. Appl. Pharmacol. 6 (4), 398–407.
He, W., Guo, X., Feng, M., Mao, N., 2013. In vitro and in vivo studies on ocular vitamin A palmitate cationic liposomal in situ gels. Int. J. Pharm. 458, 305–314.
Hironaka, K., Inokuchi, Y., Tozuka, Y., Shimazawa, M., Hara, H., Takeuchi, H., 2009. Design and evaluation of a liposomal delivery system targeting the posterior segment of the eye. J. Control. Release 136, 247–253.
Järvinen, K., Järvinen, T., Urtti, A., 1995. Ocular absorption following topical delivery. Adv. Drug Deliv. Rev. 16, 3–19.
Jung, J.H., Abou-Jaoude, M., Carbia, B.E., Plummer, C., Chauhan, A., 2013. Glaucoma therapy by extended release of timolol from nanoparticle loaded silicone–hydrogel contact lenses. J. Control. Release 165, 82–89.
Khajeh, A., Modarress, H., 2014. The influence of cholesterol on interactions and dynamics of ibuprofen in a lipid bilayer. Biochim. Biophys. Acta 1838, 2431–2438.
Krämer, S.D., 2016. Quantitative aspects of drug permeation across in vitro and in vivo barriers. Eur. J. Pharm. Sci. 87, 30–46.
Lee, K., Shim, J., Lee, H., 2004. Mechanical properties of gellan and gelatin composite films. Carbohydr. Polym. 56, 251–254.
Liang, X., Mao, G., Ng, K.Y.S., 2004. Mechanical properties and stability measurement of cholesterol-containing liposome on mica by atomic force microscopy. J. Colloid Interface Sci. 278, 53–62.
López-Pinto, J.M., González-Rodríguez, M.L., Rabasco, A.M., 2005. Effect of cholesterol and ethanol on dermal delivery from DPPC liposomes. Int. J. Pharm. 298, 1–12.
Maestrelli, F., González-Rodríguez, M.L., Rabasco, A.M., Mura, P., 2006. Effect of preparation technique on the properties of liposomes encapsulating ketoprofen–cyclodextrin complexes aimed for transdermal delivery. Int. J. Pharm. 312, 53–60.
Maestrelli, F., González-Rodríguez, M.L., Rabasco, A.M., Ghelardini, C., Mura, P., 2010. New “drug-in cyclodextrin-in deformable liposomes” formulations to improve the therapeutic efficacy of local anaesthetics. Int. J. Pharm. 395, 222–231.
Mahmood, S., Taher, M., Mandal, U.K., 2014. Experimental design and optimization of raloxifene hydrochloride loaded nanotransfersomes for transdermal application. Int. J. Nanomedicine 9, 4331–4346.
Malhotra, M., Majumdar, D.K., 2001. Permeation through cornea. Indian J. Exp. Biol. 39, 11–24.
Meissner, J.M., Toporkiewicz, M., Czogalla, A., Matusewicz, L., Kuliczkowski, K., Sikorski, A.F., 2015. Novel antisense therapeutics delivery systems: in vitro and in vivo studies of liposomes targeted with anti-CD20 antibody. J. Control. Release 220, 515–528.
Moghimipour, E., Ameri, A., Handali, S., 2015. Absorption-enhancing effects of bile salts. Molecules 20, 14451–14473.
Natarayan, J.V., Ang, M., Darwitan, A., Chattopadhyay, S., Wong, T.T., Venkatraman, S.S., 2012. Nanomedicine for glaucoma: liposomes provide sustained release of latanoprost in the eye. Int. J. Nanomedicine 7, 123–131.
Patra, M., Salonen, E., Terama, E., Vattulainen, I., Faller, R., Lee, B.W., Holopainen, J., Karttunen, M., 2006. Under the influence of alcohol: the effect of ethanol and methanol on lipid bilayers. Biophys. J. 90, 1121–1135.
Rasmussen, C.A., Kaufman, P.L., 2014. Exciting directions in glaucoma. Can. J. Ophthalmol. 49, 534–543.
Rathore, K.S., Nema, R.K., Sisodia, S.S., 2010. Preparation and characterization of timolol maleate ocular films. Int. J. Pharm. Technol. Res. 2 (3), 1995–2000.
Romero, E.L., Morilla, M.J., 2013. Highly deformable and highly fluid vesicles as potential drug delivery systems: theoretical and practical considerations. Int. J. Nanomedicine 8, 3171–3186.
Saettone, M.F., Chetoni, P., Cerbai, R., Mazzanti, G., Braghiroli, L., 1996. Evaluation of ocular permeation enhancers: *In vitro* effects on corneal transport of fourβ-blockers, and *in vitro/in vivo* toxic activity. Int. J. Pharm. 142, 103–113.
Shafaa, M.W., Sabra, N.M., Fouad, R.A., 2011. The extended ocular hypotensive effect of positive liposomal cholesterol bound timolol maleate in glaucomatous rabbits. Biopharm. Drug Dispos. 32 (9), 507–517.
Shaikh, M.S.I., Derle, N.D., Bhamber, R., 2012. Permeability enhancement techniques for poorly permeable drugs: a review. J. Appl. Pharm. Sci. 2, 34–39.
Song, C.K., Balakrishnan, P., Shim, C.K., Chung, S.J., Chong, S., Kim, D.D., 2012. A novel vesicular carrier, transethosome, for enhanced skin delivery of voriconazole: characterization and in vitro/in vivo evaluation. Colloids Surf. B: Biointerfaces 92, 299–304.
Stojancevic, M., Pavlovic, N., Golocorbin-Kon, S., Mikov, M., 2013. Application of bile acids in drug formulation and delivery. Front. Life Sci. 7, 112–122.
Tan, G., Yu, S., Pan, H., Li, J., Liu, D., Yuan, K., Yang, X., Pan, W., 2017. Bioadhesive chitosan-loaded liposomes: a more efficient and higher permeable ocular delivery platform for timolol maleate. Int. J. Biol. Macromol. 94, 355–363.
Tártara, L.I., Quinteros, D.A., Saino, V., Allemandi, D.A., Palma, S.D., 2012. Improvement of acetazolamide ocular permeation using ascorbyl laurate nanostructures as drug delivery system. J. Ocul. Pharmacol. Ther. 28 (2), 102–109.
Tsai, M.J., Huang, Y.B., Fang, J.W., Fu, Y.S., Wu, P.C., 2015. Preparation and characterization of naringenin-loaded elastic liposomes for topical application. PLoS One 10 (7), e0131026.
Tuomela, A., Liu, P., Puranen, J., Rönkkö, S., Laaksonen, T., Kalesnykas, G., Oksala, O., Ilkka, J., Laru, J., Järvinen, K., Hirvonen, J., Peltonen, L., 2014. Brinzolamide nanocrystal formulations for ophthalmic delivery: reduction of elevated intraocular pressure in vivo. Int. J. Pharm. 467, 34–41.
Ulander, J., Haymet, A.D.J., 2003. Permeation across hydrated DPPC lipid bilayers: simulation of the titrable amphiphilic drug valproic acid. Biophys. J. 85, 3475–3484.
Yu, S., Wang, Q.M., Wang, X., Liu, D., Zhang, W., Ye, T., Yang, X., Pan, W., 2015. Liposome incorporated ion sensitive in situ gels for opthalmic delivery of timolol maleate. Int. J. Pharm. 480, 128–136.