

Cationic liposomes as promising vehicles for Timolol/Brimonidine combination ocular delivery in glaucoma: Formulation development and in vitro/in vivo evaluation

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Abstract

Glaucoma is a chronic eye disease in which the pressure inside the eye increases and leads to damage to the optic nerve, and eventually causes blindness. In this disease, it is often necessary to use a multi-drug treatment system. There is a fixed combination of Timolol maleate and Brimonidine tartrate among the combination drugs in glaucoma treatment. Liposomes are one of the most important targeted drug delivery systems to eye tissue, which leads to improved drug permeability and durability in ocular tissue. In this study, Thin Layer Hydration was used to make liposomal formulations containing Timolol maleate (TM) and Brimonidine tartrate (BT). After the necessary evaluations, one of the eight initial formulations was selected as an optimization formulation. Then, characteristics such as drug loading percentage, particle size, pH, zeta potential, and drug release were performed on the optimized formulation. The study of reducing intraocular pressure was performed on the optimized formulation. This study in total was performed on 18 Rabbits in three groups. Hydroxypropyl methylcellulose (HPMC) polymer was injected into the anterior chamber to Experimentally induce glaucoma. The selected formulation was within the acceptable range of ocular products in terms of physical properties. HPMC polymer injection successfully induced glaucoma in the animal model, resulting in a 79% increase in intraocular pressure. The results showed that the liposomal formulation significantly reduced the intraocular pressure compared to the simple formulation of the aqueous solution, and both formulations were able to significantly reduce the intraocular pressure compared to the control group ($p < 0.001$). The results also showed that liposomal formulation has a therapeutic effect in reducing intraocular pressure and has a preventive effect and intraocular pressure return to normal value after 90 h. It seems that selected liposomal formulation made by Thin Layer Hydration can act as a suitable drug carrier to increase the effectiveness of the fixed combination of Timolol maleate and Brimonidine tartrate and be proposed as a new drug formulation for targeted and controlled drug delivery in the treatment of glaucoma.

1. Introduction

Glaucoma is a chronic eye disease that is associated by progressive optic neuropathies and degeneration of retina ganglion cells. During this disease, the intraocular pressure (IOP) becomes so high that the optic nerve is damaged and eventually leads to ganglion cells' death [1]. Pathophysiology of glaucoma involves structures in both the anterior and posterior segments of the eye. The main and important pathophysiological results of glaucoma includes: loss of neuronal and axonal architecture, activation of glial cells, changes in ocular blood flow and tissue remodeling. According to global statistics, there are about 37 million blind people globally, 15–20% of whom have lost their sight due to glaucoma. Studies show that in 2010, nearly 60.5 million people worldwide were diagnosed with glaucoma [2]. It is estimated that by 2040 this number will reach 100 million people [3]. Visual damage caused by glaucoma is not reversible, but with timely diagnosis and effective treatment, the disease's progressive damage and destruction caused by the disease can be prevented and thus lead to preserving the patient's remaining vision [4]. For ocular diseases, ophthalmic drug delivery by the topical route is considered to be the most appropriate treatment route because the blood–ocular barrier does not allow effective drug delivery to ocular tissue in systemic administration [5]. However, eye drops alone have $< 5\%$ corneal bioavailability due to non-productive absorption in the conjunctiva and the nasal cavity, rapid tear turnover, a rapid clearance mechanism, and low permeability of corneal epithelium [6]. An important factor in maintaining adequate IOP control is the patient's adherence to the prescribed treatment. Low compliance greatly diminishes drug efficacy, increases healthcare costs, and often aggravates health problems [7]. According to studies, the initial monotherapy fails to control IOP within the first two years of treatment in about 50% of glaucoma patients. Thus in order to reach the target IOP, it is often necessary to

use multiple drugs [8–10]. Studies show that patients using two concomitant drugs often skip one drug in a few days.

Furthermore, patients do not always allow adequate time to absorb their first drop instillation before the second drug is administered [11–12]. For patients who need multiple drug regimens to control their IOP, using a fixed combination of two medications may improve compliance and better follow the protocols of multiple drug treatment prescribed by reducing the time required to administer drops, the number of drops, and the frequency of drug administration. Additionally, it reduces the concentration of preservatives in the eye by administering eye products [13–14]. Combigan® is the brand name of a combination of Brimonidine/Timolol in solution eye drop form in the market [15]. Clinical studies found that Brimonidine/Timolol fixed combination is more effective in lowering IOP than any of its components [16–17] and better therapeutic efficacy than other combinations, such as dorzolamide/Timolol [18]. Timolol maleate (TM) is a non-selective β -adrenergic receptor antagonist that inhibits this receptor in the epithelium ciliary body and caused decreased secretion of Aqueous humor. Brimonidine tartrate (BT) is an α_2 -agonist that caused decreased aqueous humor production and increasing its exiting. Decreasing the secretion of aqueous humor or increasing its exiting for the canal leads to a reduction in IOP [19–21]. Topical ophthalmic drug delivery has always been an important challenge in the pharmaceutical industry due to the eye's physiological barrier (different layers of the cornea, Sclera, and retina). In recent years, research has focused on developing new dosage forms to achieve higher drug levels in intraocular tissues. Recent advances in nanotechnology, especially liposomes, offer a great opportunity to increase drug release duration in the eye [22–23]. Liposomes are vesicles with a phospholipid bilayer membrane that are among the Nano-carriers in ocular drug delivery. Due to their phospholipid membrane, liposomes can adhere to the surface of tissue such as the cornea and gradually release the drug on the eye's surface, thus increasing the drug's shelf life in the eye. Other benefits of liposomes include low side effects, biocompatibility, and drug protection against degradation [23]. In the present study, liposomal vesicles were designed to sustain delivery of a beta-blocker (Timolol) and a α_2 -adrenergic agonist (Brimonidine) simultaneously and achieve the desired IOP-lowering better effect. Both Brimonidine and Timolol loaded liposomal vesicles were prepared by the thin-film hydration method. To our knowledge, this is the first study of simultaneous delivery of Brimonidine and Timolol from liposomal eye drop for the treatment of glaucoma.

2. Materials And Methods

2.1. Materials

The following materials were used in this study: Timolol maleate and Brimonidine tartrate obtained from SinaDarou pharmaceutical company (Iran). Cholesterol and stearylamine were obtained from Sigma-Aldrich (Germany). Chloroform, methanol, and egg lecithin PC (phospholipid 90G®) were obtained from Merck (Germany). Hydroxypropyl methylcellulose (K 15000) was obtained from the Oxichemistry company (Iran). A dialysis tubing cellulose membrane was obtained from Sigma-Aldrich (Germany). All other chemicals were of the highest grade commercially available.

2.2. Method

2.2.1. Preparation of TM/BT-Liposomes

In the present study, eight initial formulations of Timolol maleate and Brimonidine tartrate were based on three variables (Table 1) prepared by the film hydration method [24]. Variables were chosen based on preliminary study.

All the lipid compounds (including cholesterol and egg lecithin) and stearylamin of the formulation (as per the formula given in Table 2) were taken in a round-bottom flask dissolved in 100 mL chloroform-methanol solution (3:1 v/v). The organic solution was then evaporated in a rotary evaporator (EV311, Lab-Tech, Germany) at 55 °C under reduced pressure at 120 rpm, forming a thin lipid film on the inside of a round bottom evaporator flask. The thin lipid film obtained was maintained in a desiccator for 24h to ensure complete removal of solvents. The dried lipid film obtained was hydrated with an aqueous phase of phosphate buffer (pH 6.4) containing the 0.5%(w/v) TM, 0.2%(w/v) BT, and 0.25% (w/v) polymer HPMC. The flask was shaken for 5 min to get liposomal formulations. Liposomal formulations were named LF1 to LF8. After that, the stable colloidal suspension was then sonicated at 80% amplitude with a pulse of 0.5 cycles per second for 5 min using a bath sonicator (T710, Elma, Germany) to form smaller vesicles and make the extrusion process that followed be more straightforward. The size of the vesicles was analyzed by Nano Zeta-sizer (Malvern, Nano ZS, UK) after sonication. After reviewing the results, a selected liposomal formulation (LFs) was designed and created.

Table 1
variables in 2³ full factorial design for formulation development of TM/BT-loaded LV.

Independent variable (predicators)	Investigated levels	
	High(+)	Low(-)
X ₁ : lecithin: cholesterol	1:1	1:0.5
X ₂ : aqueous phase volume (cc)	10	5
X ₃ : Stearylamine concentration (μmole)	0.35	0.21
Dependent variables (response)	Unit	Goal
Particle size (PS)	nm	minimize
Polydispersity index (PDI)		minimize
Entrapment efficiency (EE)	%w/w	maximize

Table 2

Composition of liposomal formulations corresponding to 2³ full factorial design with their result.

Formulations	Level of independent variable			Dependent variables			
	X ₁	X ₂	X ₃	PS(nm)±SD	PDI±SD	EE(%w/w) OF TM	EE(%w/w) OF BT
LF1	+	+	+	264.13±3.75	0.443±0.002	24.50±3.40	29.30±3.80
LF2	-	-	-	127.54±4.25	0.459±0.015	16.80±2.53	19.50±2.20
LF3	+	-	+	123.30±9.70	0.447±0.011	17.50±2.10	21.50±1.90
LF4	-	+	+	187.00±3.63	0.446±0.012	21.50±1.60	25.20±2.70
LF5	+	+	-	199.8±5.18	0.505±0.063	23.29±1.90	26.30±3.30
LF6	-	-	+	86.67±5.25	0.457±0.029	18.70±2.20	18.10±1.60
LF7	-	+	-	178.33±6.60	0.461±0.039	27.50±2.50	27.50±3.70
LF8	+	-	-	143.77±2.73	0.471±0.027	16.50±1.70	6.85±2.33

2.2.2. Chromatographic conditions

Simultaneous analysis of Brimonidine tartrate and Timolol maleate was performed by HPLC technique connected to UV/Visible detector. 50 µl of sample solutions were analyzed at two fixed wavelengths (254 nm for Brimonidine tartrate and 300 nm for timolol maleate). Mobile phase composed of Ammonium acetate (pH 5)-methanol (40:60 v/v) with flow rate 1.3 ml/min. Cyano column (250×4.6 mm, 5µl) was used for drugs separation [25].

2.2.3. Atomic Force microscopy (AFM)

First, the sample of selective liposomal formula (LFs) was diluted 1: 400 with deionized water, and after being placed on the slide, it was dried for 24 hours at room temperature; then, the morphology of LFs were studied using nano wizard® II AFM (JPK, Germany).

2.2.4. Determination of size and shape

The following parameters were measured: The mean particle size, polydispersity index (PDI), and zeta potential of the liposomes by Nano Zeta-Sizer (Nano ZS, Malvern, UK).

2.2.5. Entrapment efficiency of TM/BT-Liposomal

Entrapment efficiency (EE%) of Timolol maleate and Brimonidine tartrate in the liposomal vesicles was determined as follows: After sonication, 1 mL of the vesicle suspension was taken in a 1 mL microcentrifuge tube, and centrifuged at 18,000 rpm for 30 min at 4°C in a cold centrifuge (Clements 2000, Germany) to get a yellow sediment deposited. To the sediment, 500 µL of chloroform (the vesicles liposomal is broken in organic solvent) was added and vortexed thoroughly for 5 min to ensure that the vesicles were lysed completely. 1 mL phosphate buffer (pH 6.4) was added and vortexed for 5 min (the drugs are highly soluble in water). Afterward, samples were centrifuged at 15,000 rpm for 15 min at 25°C (To separate the organic and aqueous phases). A clear solution containing the drug is obtained after separating the aqueous phase and passing it through a syringe filter

(0.45µm). Then, dilute 500 µl of clear solution 1: 5 with phosphate buffer and the was analyzed for free TM and BT using UV spectrophotometer (Biochorm, England) Respectively at 297 and 320 nm. EE% was calculated as follows [26]:

$$EE\% = \frac{\text{Entrapped drug} \left(\frac{\text{mg}}{\text{ml}} \right)}{\text{total drug added} \left(\frac{\text{mg}}{\text{ml}} \right)} \times 100$$

2.2.6. In vitro drugs release study

3mL of the liposomal suspension was placed on one side of the dialysis membrane in a Franz diffusion cell. The other side of the membrane was in continuous contact with the dissolution medium included 37mL phosphate buffer (pH 6.4). The entire dissolution assembly was placed on a magnetic stirrer and stirred at 100 rpm at a temperature of 37 °C. Aliquots (2 mL) of dissolution medium were withdrawn at different time intervals (30 min, 60 min, 2h, 4h, 6h, 9h, 12h) and replaced with fresh dissolution medium. Whenever the sample was withdrawn, an equal volume fresh dissolution medium was added to maintain the constant volume. Drug concentration in the dissolution medium was determined by UV spectrophotometric method at 297nm (for TM) and 320nm (for BT) [27]. Then to found the mechanism of drugs release from optimal liposomal formulation, the data were evaluated according to zero-order (cumulative amount of drug released VS time), first-order (log cumulative percentage of drug remaining VS time), and Higuchi's (cumulative percentage of drug released VS square root of time) pattern [28].

2.2.7. Animal Studies

A total of 36 adult male rabbits (weight, 2.5–3 kg) were used for the study. All animals were housed at controlled temperature (22 ± 2°C), and humidity (55 ± 5%), with a 12/12h light-dark cycle, were given access to food and water. The animal experiments were conducted in full compliance with regulatory principles of the ethics committee of Ahvaz Jundishapur University of Medical Sciences.

2.2.7. 1. In-vivo Glaucoma induction method

For this purpose, after anesthesia of rabbits by intramuscular ketamine (35mg/kg) and xylazine (5 mg/kg), and also corneal anesthesia was performed by using topical 0.5% Tetracaine eye drops, 0.2 mL of the solution of HPMC 2%(W/V) was injected under sterile conditions into the anterior chamber. Injection in the anterior chamber was performed only in the right eye of every rabbit in this study. A single dose of topical 0.3% ciprofloxacin eye drop was used to prevent eye infection after injection of HPMC 2% in the eye. IOP was measured before HPMC 2% injection and 24 hours after injection. A significant increase in IOP after injection was considered high ocular pressure and acute glaucoma [29].

2.2.7. 2. In vivo intraocular pressure lowering activity

Intraocular pressure (IOP) of both eyes was measured using IOPEN® tonometer every day at 8 am [30]. All IOP measurements were carried out by the ophthalmologist using the same tonometer. Rabbits that showed a consistent difference in IOP between the left and the right eye during baseline measurements, or any sign of eye stimulation, were excluded from the study. 36 rabbits were divided in 6 groups. The samples (TM/BT loaded liposomes(, TM loaded liposomes (TM control), BT loaded liposomes (BT control), aqueous solutions of TM/BT (aqueous control), Drug-free liposomal formulation (Blank control), and group without any treatment (negative

control) were instilled into the right eyes (One drop, per turn). Then, the eye was kept closed for 1 minute (to prevent wasting medicine). During the study, the left eye did not receive any intervention (considered as negative control). All formulations were used twice a day. We measured IOP before injection in the AC, 24h after and two hours after each intervention (27, 41, 51, 65, 75, 89, 99, 113, 123, and 137h after starting the injection in the right eye AC). The end of the study in each group was when eye pressure returned to baseline. It should be noted that the interval between pressure measurements in the morning to evening was ten hours and from evening to morning was 16h.

Data were expressed as mean (mean \pm SD) up to two decimal places.

Change in IOP (Δ IOP) is expressed as IOP right eye – IOP left eye.

2.2.8. Stability study

In the present work, a stability study was carried out for selected formulations LFs. Three formulations of LFs were stored at room temperature and under refrigeration (2–8°C) for 1, 3, and 5 months, and the formulations were then evaluated for the drug content and particle size [31].

2.2.9. Statistical analysis

All the experimental data were subjected to statistical analysis, using the IBM® SPSS® Statistics 26 followed by Turkey's test. $P < 0.001$ was considered to be statistically significant. All tests were carried out in triplicate, and data were reported as mean \pm SDs.

3. Results

3.1 Characterization of TM/BT-Liposomes

The particle size for TM/BT-liposomes was less than 214.53 ± 4.43 nm, which is a suitable size considering that the recommended particle size in ophthalmic products is less than ten μ m. The size of a particle, in ophthalmic, plays a vital role in the irritation potential of the formulation and bioavailability. Hence, it is recommended that the particle size of ophthalmic preparation be less than ten μ m to minimize irritation to the eye [32, 33]. The results showed that the EE% of TM and BT in TM/BT-liposomes were less than $34.36 \pm 2.04\%$ and $39.36 \pm 1.66\%$, respectively. Also, the EE% of TM in TM-liposomes and BT in BT-liposomes was less than 48.33% and 56.8%, respectively. pH and zeta potential values of TM/BT-liposomes were 6.22 ± 0.02 and $+12.3 \pm 0.12$ mv, respectively.

3.2. Characterization of selected liposomal formulation for animal studies

The optimal TM/BT-liposome was selected based on the lowest particle size, suitable zeta potential, and highest drug entrapment and contained a lipid molar ratio of 0.75:1 (Cholesterol: lecithin), 7.5mL phosphate buffer, and 0.275 μ mol stearyl amine (Table 3). The median values of variables amounts were selected to prepare optimized formulation. Regression analysis showed that there is a significant and direct relationship between the variables of X_1 ($P < 0.045$) and X_2 ($P < 0.012$) with particle size. Given that the optimized formulation should have a minimum particle size for better penetration into the cornea and a maximum drug loading, and given that the effect of X_2 on the particle size and drug loading is an increasing effect, so the average between the upper and lower volume levels of the aqueous phase volume was selected as the volume of the aqueous phase in the optimized formulation. Also, considering that the molar ratio of X_1 on the particle size is an increasing effect and no effect was observed

on the drug loading, so the amount of this variable in the optimize formulation was selected as the average between the high and low levels of this variable. Although we could use the low level of this variable in making the optimal formulation, we considered that increasing the ratio of cholesterol to lecithin is effective in the stability of the formulation and drug release, so it was decided to use the average of this variable. The effect of X_3 on particle size and drug loading was not significant, but it was important in terms of creating a positive electric charge and interaction with the cornea, so the mean of this variable was used in the selected formulation.

Table 3
Composition of TM/BT-LF_S corresponding factorial design with its result.

Formulation	Independent variable			Dependent variable					
	X_1	X_2	X_3	PS(nm) ± SD	PDI	EE(%w/w) of TM	EE(%w/w) of BT	Zeta potential(mV)	PH
LF _S	1:0.75	7.5	0.275	214.51 ± 4.43	0.403 ± 0.004	22.36 ± 2.04	41.36 ± 1.66	+ 12.3 ± 0.12	6.22 ± 0.02

3.2. AFM image

The AFM images show that all the vesicles were spherical, multilamellar, and have a distinct spherical shape surrounded by thin, evenly distributed coats (Fig. 1).

3.3. In vitro Release of TM/BT-liposomes and Determination of drug release kinetics

The comparative in vitro drug release profile is summarized in Fig. 2 for TM/BT-liposomes and TM/BT-aqueous solution formulation. It was observed that aqueous solution released > 95% of drugs within two hours, while LFs showed $70.67 \pm 5.87\%$ of TM and $58.52 \pm 4.9\%$ of BT release in 12h. The results of in vitro drug release profile of formulations showed that TM/BT-liposomes provide the prolonged release of drugs compared to aqueous solution formulation (12h VS 2h). Results also showed a slow and prolonged release of both drugs from TM/BT-liposomes and for TM followed first-order kinetics and for BT followed zero-order kinetics (r^2 values were nearer to 1 with the first order for TM and zero-order for BT compared to another release kinetic). Therefore, our result showed that TM and BT's release profile was dependent and independent of the concentration of drug entrapped, respectively (Fig. 2).

3.4. In-vivo Glaucoma induction

Mean IOP of the right eye in a total of 36 rabbits studied before injection of HPMC polymer into the anterior chamber (AC) was 16.8 ± 0.6 mmHg, and 24h after polymer injection was equal to 21.8 ± 0.53 mmHg ($P < 0.001$). Therefore, the intervention started 24h after polymer injection when the intraocular pressure was significantly increased. In the negative control group, before injection of HPMC polymer into the AC space, the mean right eye pressure was 16.73 ± 0.75 mmHg and at its highest value (79h after polymer injection) was 29.9 ± 0.48 mmHg arrived (Table 4). Therefore, it can be concluded that 0.2 ml injection of HPMC 2% (w / v) in AC space due to a 79% increase in IOP for 4.5-5 days is a suitable method for glaucoma induction (Table 4).

Table 4

Results of measurement of intraocular pressure in rabbits in different groups before and after polymer injection in the right eye and after using the eye drops in each group

Time(h)	Studied groups IOP(mmHg)						
		TM/BT liposomes	TM liposomes	BT liposomes	TM/BT Aqueous control	Negative control (without treatment)	Placebo liposomes
0 (Before polymer injection)	R	16.52 ± 0.79	16.54 ± 0.72	16.49 ± 0.66	16.97 ± 0.32	16.73 ± 0.75	17.1 ± 0.26
	L	16.4 ± 0.54	16.45 ± 0.51	16.52 ± 0.58	17.02 ± 0.66	16.93 ± 0.57	17.13 ± 0.41
(24 h after polymer injection)	R	21.65 ± 0.75	21.77 ± 0.32	21.85 ± 0.40	21.97 ± 0.22	21.8 ± 0.66	22.13 ± 0.2
	L	16.52 ± 0.66	16.46 ± 0.53	16.49 ± 0.48	17.2 ± 0.41	17.11 ± 0.49	17.4 ± 0.1
27 (2 h after the 1st intervention)	R	18.77 ± 0.55	20.57 ± 0.29	20.77 ± 0.19	19.95 ± 0.31	22.13 ± 0.61	22.43 ± 0.11
	L	16.62 ± 0.67	16.41 ± 0.50	16.46 ± 0.48	17.35 ± 0.34	17.08 ± 0.51	17.46 ± 0.05
41 (2 h after the 2nd intervention)	R	20.3 ± 0.64	21.67 ± 0.22	21.90 ± 0.28	22.47 ± 0.17	24.7 ± 0.54	25 ± 0.26
	L	16.57 ± 0.66	16.47 ± 0.44	16.50 ± 0.51	17.42 ± 0.15	17.06 ± 0.38	17.46 ± 0.05
51 (2 h after the 3rd intervention)	R	20.75 ± 0.69	22.47 ± 0.33	22.81 ± 0.41	23.6 ± 0.18	26.33 ± 0.52	26.66 ± 0.32
	L	16.62 ± 0.74	16.50 ± 0.51	16.49 ± 0.48	17.2 ± 0.21	17.16 ± 0.3	17.43 ± 0.2
65 (2 h after the 4th intervention)	R	21.82 ± 0.73	23.65 ± 0.18	24.10 ± 0.25	25.3 ± 0.18	28.48 ± 0.52	28.83 ± 0.37
	L	16.65 ± 0.77	16.43 ± 0.41	16.50 ± 0.53	17.25 ± 0.2	17.16 ± 0.36	17.3 ± 0.2
75 (2 h after the 5th intervention)	R	22.07 ± 0.63	25.52 ± 0.37	25.93 ± 0.35	26 ± 0.18	29.9 ± 0.48	30.13 ± 0.35
	L	16.72 ± 0.87	16.45 ± 0.59	16.54 ± 0.52	17.42 ± 0.33	17.2 ± 0.43	17.26 ± 0.05
89 (2 h after the 6th intervention)	R	18.42 ± 0.63	22.15 ± 0.14	22.35 ± 0.12	22.95 ± 0.19	26.6 ± 0.52	26.93 ± 0.4
	L	16.82 ± 0.8	16.44 ± 0.44	16.47 ± 0.51	17.35 ± 0.7	17.13 ± 0.32	17.43 ± 0.11
99 (2 h after the 7th intervention)	R	16 ± 0.57	18.75 ± 0.22	18.93 ± 0.20	20.32 ± 0.25	24.4 ± 0.52	24.63 ± 0.47

R: Right eye of rabbits, L: Left eye of rabbits.

Time(h)	Studied groupsIOP(mmHg)						
	TM/BT liposomes	TM liposomes	BT liposomes	TM/BT Aqueous control	Negative control (without treatment)	Placebo liposomes	
113 (2 h after the 8th intervention)	L	16.87 ± 0.77	16.50 ± 0.56	16.46 ± 0.55	17.35 ± 0.23	17.21 ± 0.5	17.3 ± 0.2
	R	16.11 ± 0.17	16.45 ± 0.28	16.65 ± 0.22	16.82 ± 0.29	21.3 ± 0.5	21.46 ± 0.35
	L	16.18 ± 0.15	16.49 ± 0.25	16.52 ± 0.24	16.62 ± 0.22	17.16 ± 0.47	17.26 ± 0.25
123 (2 h after the 9th intervention)	R	-	-	-	-	19.85 ± 0.45	19.8 ± 0.3
	L	-	-	-	-	17.16 ± 0.46	17.6 ± 0.2
137 (2 h after the 10th intervention)	R	-	-	-	-	17.3 ± 0.46	17.06 ± 0.37
	L	-	-	-	-	17.16 ± 0.42	17.16 ± 0.15
R: Right eye of rabbits, L: Left eye of rabbits.							

Table 5

Mean difference of IOP of right eye and P-Value measurement between the group receiving liposomal formulation and control and between the group receiving aqueous solution formulation and control

Time (h)	P-Value		Mean difference \pm SD	
	Loaded TM/BT - Liposome vs Control	Aqueous solution vs Control	Loaded Liposome vs Control	Aqueous solution vs Control
0	P = 0.687	P = 0.569	0.2 \pm 0.49	0.24 \pm 0.4
24	P = 0.748	P = 0.630	0.15 \pm 0.45	0.17 \pm 0.34
27	P < 0.001	P < 0.001	-3.35 \pm 0.38	-2.18 \pm 0.33
41	P < 0.001	P < 0.001	-4.4 \pm 0.37	-2.22 \pm 0.28
51	P < 0.001	P < 0.001	-5.58 \pm 0.38	-2.73 \pm 0.27
65	P < 0.001	P < 0.001	-6.65 \pm 0.39	-3.18 \pm 0.27
75	P < 0.001	P < 0.001	-7.82 \pm 0.35	-3.9 \pm 0.26
88	P < 0.001	P < 0.001	-8.17 \pm 0.36	-3.65 \pm 0.27
99	P < 0.001	P < 0.001	-8.4 \pm 0.35	-4.4 \pm 0.29
113	-	P < 0.001	-	-5.3 \pm 0.28

3.5. Magnitude and Duration of IOP Reduction by TM/BT-liposomes

The results of this study showed that in the group receiving TM/BT-liposomes compared with the control group, two hours after the first intervention (27h after injection of polymer in right eye) to 2 hours after the seventh intervention (105h after injection of polymer in right eye), the IOP of right eye decreased significantly ($P < 0.001$). The highest reduction in IOP in the group receiving TM/BT-liposomes compared to the control group was two hours after the seventh intervention (-8.4 ± 0.35 mmHg). In the group receiving the aqueous solution formulation of TM/BT compared to the control group, from 2h after the first intervention (27h after polymer injection in right eye) to 2h after the eighth intervention (121h after polymer injection in right eye), the IOP of right eye decreased significantly ($P < 0.001$). The highest reduction in right eye IOP between these two groups was two hours after the eighth intervention (-5.3 ± 0.28 mmHg). In the group receiving TM/BT-liposomes compared to the group receiving an aqueous solution of drugs, two hours after the first intervention, the difference in IOP in both groups was meaningful ($P < 0.05$). Groups TM-liposomes and BT-liposomes indicated similar pattern such as TM/BT-liposomes but with lower decreasing in IOP significantly ($P < 0.05$). Groups receiving TM-liposomes and BT-liposomes decreased IOP more than group receiving the aqueous solution formulation of TM/BT from 2h after the first intervention (27h after polymer injection in right eye) to 2h after the eighth intervention (121h after polymer injection in right eye). However, after the third intervention and until the IOP returned to baseline in the TM/BT-liposomes group, this group's IOP reduction rate was significantly more significant than the group receiving the aqueous solution formulation ($P < 0.001$). Finally, by comparing the blank and control groups, it was found that

there was no significant difference between the two groups during the study ($P > 0.05$). This indicates that the components of the selected liposomal formulation (except for the active ingredient) do not independently play a role in reducing IOP (Table 4–6) (Figur3-4).

Table 6
Mean difference of IOP of left eye and P-Value measurement between the group receiving liposomal formulation and aqueous solution formulation and between the group receiving control and blank

Time(h)	<i>P-Value</i>		Mean difference \pm SD	
	Loaded TM/BT -Liposome	Control	Loaded Liposome	Control
	vs	vs	vs	vs
	Aqueous solution	Unloaded Liposome	Aqueous solution	Unloaded Liposome
0	P = 0.333	P = 0.455	0.45 \pm 0.42	0.36 \pm 0.46
24	P = 0.438	P = 0.437	0.32 \pm 0.39	0.33 \pm 0.4
27	P < 0.05	P = 0.442	-1.17 \pm 0.31	0.3 \pm 0.36
41	P < 0.01	P = 0.410	-2.17 \pm 0.33	0.3 \pm 0.34
51	P < 0.01	P = 0.354	-2.85 \pm 0.35	0.33 \pm 0.33
65	P < 0.001	P = 0.345	-3.47 \pm 0.37	0.35 \pm 0.34
75	P < 0.001	P = 0.491	-3.92 \pm 0.33	0.23 \pm 0.32
89	P < 0.001	P = 0.372	-4.52 \pm 0.33	0.33 \pm 0.34
99	P < 0.001	P = 0.545	-4 \pm 0.31	0.23 \pm 0.36
113	-	P = 0.627	-	0.16 \pm 0.28
123	-	P = 0.869	-	0.05 \pm 0.25
137	-	P = 0.483	-	0.23 \pm 0.21

3.6. Adverse Effect

Eye examination revealed mild redness of the eyes in one of the rabbits treated with TM/BT-liposomes, and one of them in the group receiving the blank formulation 24h after application. Nevertheless, this condition was transient. Mild redness of the eyes disappeared at the end of the day. Other local adverse effects, including corneal erosion, corneal edema, and inflammatory signs in the anterior chamber were not observed throughout the experiment.

3.7. Stability

The reduction rate of EE% at 4–8 ° C after five months was 7.78% for TM and 3.79% for BT (Table 7). Also, the reduction of EE% after five months of storage at 25 ° C was 28.37% for TM and 17.16% for BT. Therefore, the stability of liposomal formulation in terms of drug leakage at 4–8 ° C is better than 25 ° C ($P < 0.001$). The particle size of liposome vesicles (LV) also increased during five months of storage at 4–8 ° C but was still within acceptable limits for ocular topical formulation [34]. This increase in particle size was meaningless compared to the increase in particle size following the formulations' storage at 25 ° C ($P < 0.05$) (Table 7).

Table 7
Effect of storage condition and time on PS, PDI and EE% of TM/BT-LF_s (mean ± SD, n = 3)

Storage condition	PS(nm) ± SD		PDI ± SD		EE(%w/w) of TM		EE(%w/w) of BT	
	4–8°C	25°C	4–8°C	25°C	4–8°C	25°C	4–8°C	25°C
Time(month)								
0	215.25 ± 3.50	208.33 ± 4.76	0.387 ± 0.012	0.393 ± 0.018	22.86 ± 1.41	23.33 ± 2.01	42.13 ± 2.10	42.53 ± 2.31
1	223.10 ± 4.12	220.32 ± 3.13	0.395 ± 0.017	0.401 ± 0.015	22.67 ± 1.53	21.47 ± 1.96	42.02 ± 2.16	40.63 ± 2.16
3	230.52 ± 3.72	234.20 ± 3.94	0.399 ± 0.024	0.409 ± 0.016	21.89 ± 1.48	18.23 ± 1.93	41.24 ± 1.98	38.47 ± 2.18
5	238.36 ± 4.22	243.57 ± 3.23	0.402 ± 0.019	0.424 ± 0.015	21.08 ± 0.96	16.71 ± 1.68	40.53 ± 2.07	35.23 ± 2.04

4. Discussion

This study aimed to develop long-acting and sustained release of combination eye drops for the prolonged management of glaucoma. This was accomplished by preparing Liposomal vesicles (LV) including 0.5% (w/v) TM, 0.2% (w/v) BT, and bioadhesive and biodegradable polymer (HPMC). Prepared liposomal formulation provided extended release of Timolol/Brimonidine and an extended IOP-lowering effect compared to aqueous solution formulation of Timolol/Brimonidine eye drops and TM and BT each alone loaded in liposomal formulations. Optimal liposomal formulation possessed ideal pH values that can be easily tolerated by the eye. The pH of tears is 7.4, and due to its natural buffering capacity, the eye is in the shadow of this unique feature can tolerate ophthalmic formulation within a wide pH range (3.5 to 8.5). Due to insufficient buffering, pH values outside this range can irritate, increased blinking, and tears of eyes, the sum of these factors leading to reduce the bioavailability of the drug in the eye [35].

Atomic force microscopy images showed that LV has a distinct shape and multilamellar structure. LVs were consistently small and about ranged 214.5 ± 19.43nm. Although there was an increase in the mean particle size of TM/BT-liposomes after five months storage at 2–8 °C, that it still was in the reasonable range. Previous studies showed that even particles smaller than 200nm are considered acceptable for passive drug targeting [36, 37].

Particle size demonstrated a key role in drug release and permeability through biological membranes in this manner that with lower particle size and higher surface area, the higher release and permeability rates were provided. This is due to the drug's increased contact surface and its better permeability to the target tissue [38].

TM and BT's release profiles from optimal formulation confirmed that it was possible to prepare sustained-release combination eye drops containing LV. TM/BT-liposomes possessed a sustained drug release rate free of any burst release that may cause a toxic effect. This topic may be due to two factors. The first is that the drugs are trapped in aqueous core because of hydrophilic natures of both drugs. The second factor is the structure of multilamellarmembrane of LV that was confirmed by AFM data. These layers around the liposomal vesicles prevent the burst release. Many studies show that coating around the nanoparticles using various materials effectively prevent the burst release of the drug [37, 39]. Many bioadhesive polymers have drug release retarding properties and are being used in ophthalmic preparations[40–41]. HPMC was chosen as a bioadhesive polymer in

our study. HPMC is GRAS (generally recognized as a safe) listed ingredient and used to manufacture various dosage forms available commercially [42]. This polymer is a hydrophilic polymer with many polar functional groups. Upon hydration, the polymeric chains of HPMC are entangled with glycoprotein chains of target tissue resulting in bioadhesion [43]. Different opinions have been proposed for polymers' adhesion behavior, such as hydrogen bonding, electronic interaction, electronic theory, wettability theory, adsorption theory, and diffusion and interlocking theory [44].

In our study, the EE% of TM and BT in the optimized formulation were 41.36% and 22.36%, respectively, which are relatively desirable rates. In previous study the EE% of BT in the optimal liposomal formulation, which was made by the thin layer hydration method, was equal to 42.43% [45]. These results were consistent with our findings. Also, in another study that focused on the production of TM liposomal hydrogels, it was found that the EE% of TM in LV is a function of the pH of the environment. They reported that the maximum EE% of TM was provided at pH 9.2 [46]. Therefore, according to the pH equal to 6.22 in our liposomal formulation, one of the important factors in the low loading of TM in our study can be considered the liposomal formulation's acidic pH.

As expected, the zeta potential, which represents the surface charge of LV, was positive and equal to +12.3. The positive surface charge was provided by using a positive agent called stearylamin in the formulation. The purpose of using stearylamin was to induce a positive surface charge in the LV and to increase the electrostatic interaction between the negative charge at the corneal epithelial level and the positive charge at the surface of the LV. In another study niosomal formulation prepared by stearylamin, and they found that the niosomal formulation of acetazolamide, reduced the IOP more than the suspension formulation of acetazolamide and niosomal formulation without a positive agent [40]. Besides, another study has been shown that liposomes with a positive surface charge have the greatest ability to penetrate the cornea [47]. Although various methods have been used to establish animal models of glaucoma. Due to elevated IOP being well recognized as the sole modifiable risk factor for the development of glaucoma in the majority of cases, the establishment of animal models with chronic and acute elevated IOP is favorable for simulating the pathogenesis of glaucoma and evaluation of the effectiveness of drugs [48–52]. One of these methods is the polymer's injection into the anterior chamber, which depends on the amount, viscosity, and structure of the polymer, leading to the induction of acute or chronic glaucoma [49–52 and 29]. One of our study goals is to investigate the effectiveness of HPMC injections in an animal glaucoma model. In our method, only one injection was performed per sample to reduce the risk of intraocular tissue damage and variation between tests that repeated injections might cause. HPMC increases aqueous humor outflow resistance by blocking the trabecular network. Therefore IOP increase. Our results showed HPMC significantly increased IOP. The elevated IOP levels were maintained for 4.5-5 days. 24h after polymer injection, the mean IOP increased by 29.76%. In the control group before injection of HPMC, the mean right eye pressure was 16.73 ± 0.75 mmHg, and at its highest value (79h after polymer injection) was 29.9 ± 0.48 mmHg. In another study injection of HPMC + microbead in an anterior chamber significantly increased IOP compared with the microbead receiving and control groups in mice [52]. Researchers in another study found that HPMC in an anterior chamber could increase IOP for four days in rabbits [29]. Our evaluation showed the difference between the duration of high IOP in our study compared with the studies mentioned in the injection method (no removal of aqueous humor before injection HPMC VS the removal of aqueous humor before injection HPMC), was injection volume (0.2 mL VS 0.25mL) and the number of samples (17 rabbits VS 3 rabbits). Our method kept the high IOP longer. The rate of reduction of IOP in liposomal, aqueous solution, and control groups in the first 24h after HPMC injection in the anterior chamber was $21.65 + 0.75$, $21.97 + 0.22$, and $21.8 + 0.66$ mmHg, respectively. Also, at the IOP peak, 79h after HPMC injection, the IOP was $22.07 + 0.63$, $26 + 0.18$, and $29.9 + 0.48$ mmHg, respectively. According to the mentioned results, the

formulation of the aqueous solution of Timolol/Brimonidine, despite the appropriate IOP reduction, did not prevent its increase. Therefore, it can be said that this formulation has not been effective in preventing increased IOP (IOP at peak time was 26 ± 0.18 mmHg VS before the first intervention was 21.97 ± 0.22 mmHg, $P < 0.001$). However, TM/BT-liposomes, TM-liposomes, and BT-liposomes despite being more effective in reducing IOP compared to aqueous solution formulation, were able to prevent the process of increasing intraocular pressure (IOP at peak time was 22.07 ± 0.63 mmHg VS before the first intervention was 21.65 ± 0.75 mmHg, $P > 0.05$). In other words, our results showed TM/BT-liposomes has been much more effective than aqueous solution formulation in both controlling and preventing increased IOP. One study found that single-dose intraocular injection of latanoprost nano liposomes in patients with glaucoma could significantly reduce IOP within the first hour after injection and up to 3 months later. This conjunctival injection was well tolerated in all patients [53]. In our study, in contrast to this study, a non-invasive method was used for drug delivery. From both studies, it can be concluded that using liposomes as a drug carrier, by an invasive or non-invasive method, can significantly improve the effectiveness of ophthalmic drugs with minimal side effects. In another study, the Liposomal formulation of Brimonidine reduced IOP by 39% and simple formulation of Brimonidine by 59%, but this effect was more stable in the liposomal group with a significant difference [45]. In the study mentioned above, contrary to our study, no positive agent and polymer were used. Also, in our study, a liposomal formulation containing a fixed combination of Brimonidine and Timolol was used, and the treatment period and number of times the intervention were longer. The sum of the mentioned factors can be considered as the factor of more stable and longer control of IOP in our study. According to studies, the greatest reduction in IOP in topical anti-glaucoma drugs dosage form aqueous solution is approximately when the most drug release is done [54–56 and 30]. our method was very appropriate for IOP measurement time because pressure measurement was performed when drug release from aqueous solution formulation was at its highest (2h after taking the drug).

Therefore, in our study more accurate comparison of IOP reduction between liposomal formulation and the aqueous solution was obtained. The result of the in vitro drug release profile of formulations showed that TM/BT-liposomes provide the prolonged release of drugs compared to aqueous solution formulation (12h VS 2h). Our results showed a slow and prolonged release of both drugs from TM/BT-LIPOSOMESs, and for TM followed first-order kinetics, and BT followed zero-order kinetics. Therefore, TM and BT's drug release was dependent and independent of the concentration of drug entrapped, respectively. This would mean that TM/BT-LIPOSOMESs can release TM/BT drug content in a sustained-release system. Therefore, it has expected to keep the drug concentration in the eye constant for a longer period. Which is consistent with the results of other studies [45]. In a study aimed at achieving a suitable liposomal formulation of the fixed Timolol/latanoprost combination, it was found that the selected liposomal formulation was able to release 72% of Timolol and 55% of latanoprost within 6 hours. It was also found that the release kinetics for both drugs follow a zero-order model. Animal studies also showed that the liposomal formulation of Timolol/latanoprost compared with the marketing formulation of Timolol/latanoprost reduces IOP equally after four days, and before that, the pressure reduction slope was in favor of the commercial formulation [57]. Our study had a longer release (12h VS 6h), lower EE%, and different release kinetics for Timolol (first-order VS zero-order) compared to this study. Another important difference between the two studies is that the difference between the amount of IOP between the liposomal formulation and the aqueous solution formulation was clear from the first intervention (from the first intervention onwards $P < 0.05$ and the fourth intervention onwards $P < 0.001$). This difference between the two studies could be due to the positive surface charge (+ 12.3mv VS -17.3mv) and the longer release profile in our study, which leads to better penetration and longer shelf life of the drug in the eye. In two other studies that aimed at achieving the Timolol/Brimonidine hydrogel formulation to increase the shelf life of the drugs in the eye, the researchers found that the hydrogel

formulation released the drugs almost completely after 8 hours [58–59]. In both studies, the release profile showed a burst release; for example, in one of these studies, more than 60% of the Timolol and Brimonidine were released in the first 1 hour [59]. Our study lacked a burst release and a longer release for TM and BT than these two studies. Also, in our study, unlike the two mentioned studies, the release kinetics of drugs were determined. Other research to improve retention time of TM/BT combination in the eye is confined to the development of an intraocular implant with significant IOP-lowering efficacy over 90 days in vivo [15]. In the light of what has been said and due to patients' better compliance and ease of administration of eye drops than marketed ophthalmic formulations [60]. It seems that the use of liposomal eye drops compared to hydrogels is an efficient method for increase the retention time of combination drugs in the eye, and it can be a bright future in the development of ocular drug delivery methods, especially for the treatment of glaucoma.

5. Conclusion

The development of TM/BT eye drop liposomal formulation containing stearylamin and HPMC as positive agent and bio-adhesive polymer, respectively, resulted in successful TM and BT delivery to eye tissue and significant therapeutic effectiveness in terms of IOP reduction. In vitro release study showed that the TM/BT-LIPOSOMESs had no burst release and could provide sustained release for 12 hours. Also, the small particle size (nm) of the LV leads to better permeability of the drug to the epithelial tissue of the cornea. It seems that optimize formulation has the potential of being developed into a eye drop formulation of TM/BT leading to better control IOP.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Behzad Sharif Makhmal Zaddeh], [Ali Bigdeli], [Mostafa Feghhi], and [Emad Soleimani Biatiani]. The first draft of the manuscript was written by [Behzad Sharif Makhmal Zadeh] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Ahvaz Jundishapur University of Medical Sciences (2020/02/04./ IR.AJUMS.ABHC.REC.1399.070.)”

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Figures

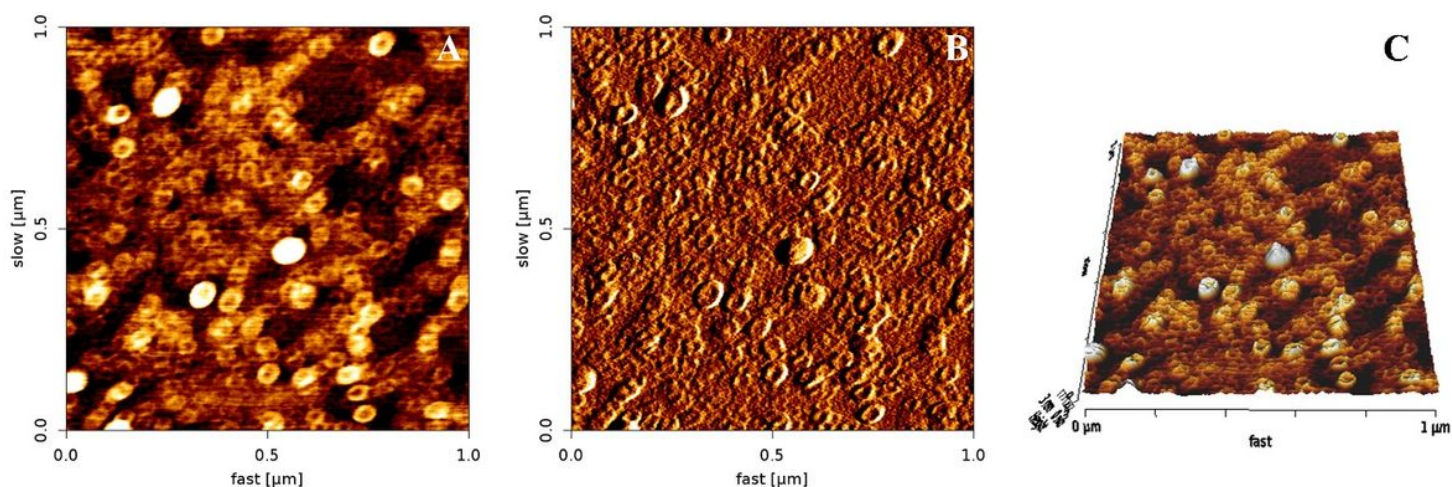


Figure 1

AFM image of the selected formulation. Topographic model (A), 2D model (B) and

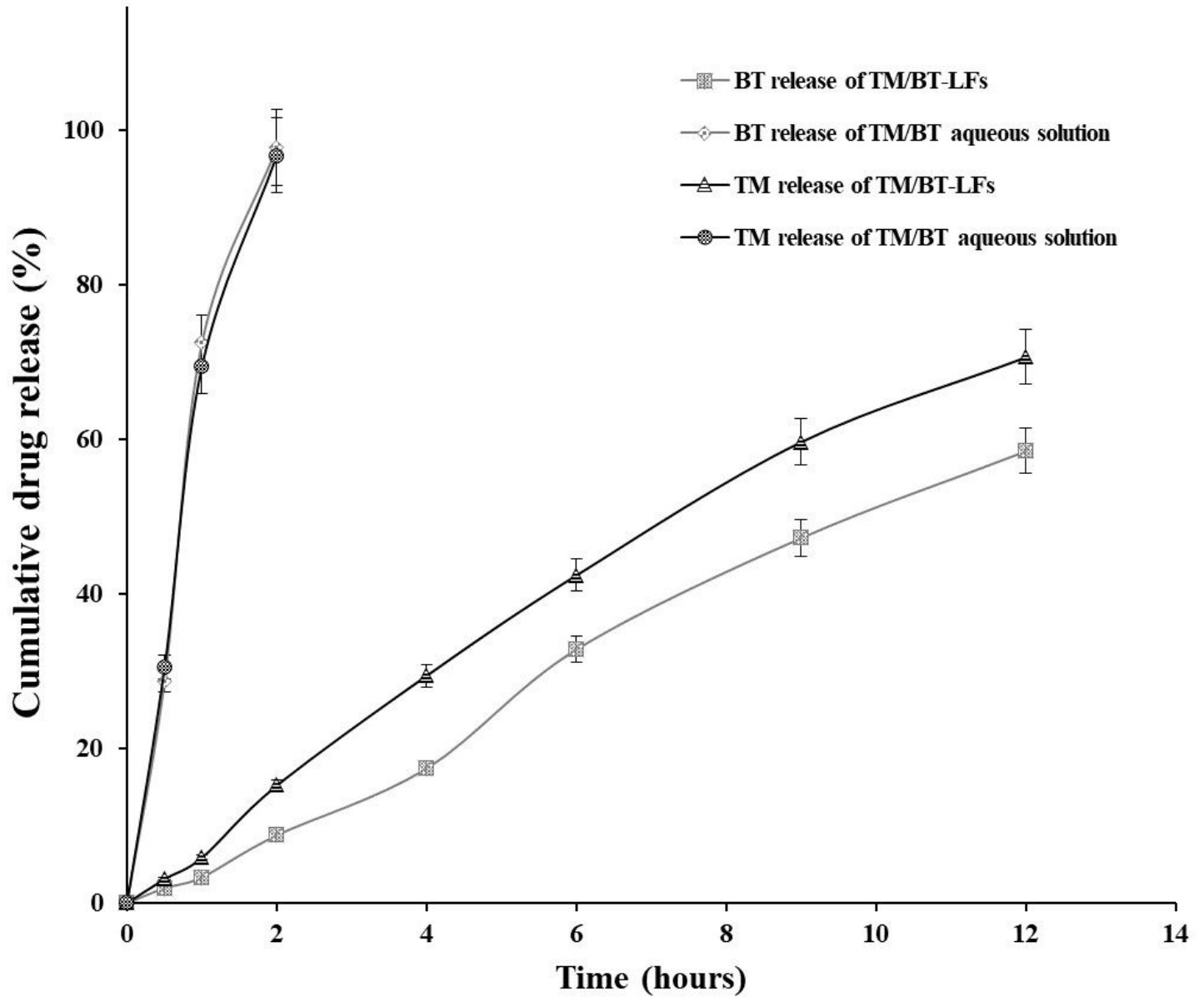


Figure 2

Release profile of TM and BT from TM/BT-LFs and TM/BT solution (mean \pm S.D, n=3)

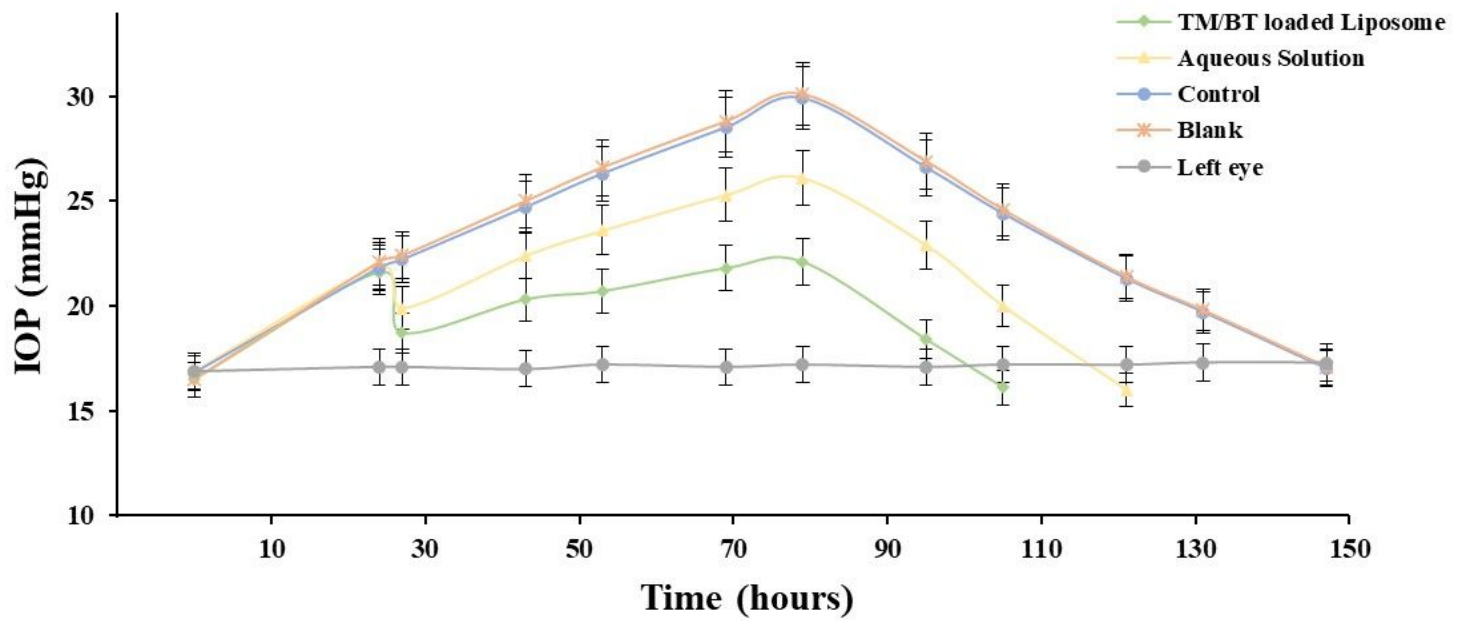


Figure 3

IOP reduction after administration of TM/BT-LFs, TM/BT solution and Blank (mean \pm S.D, n=3)

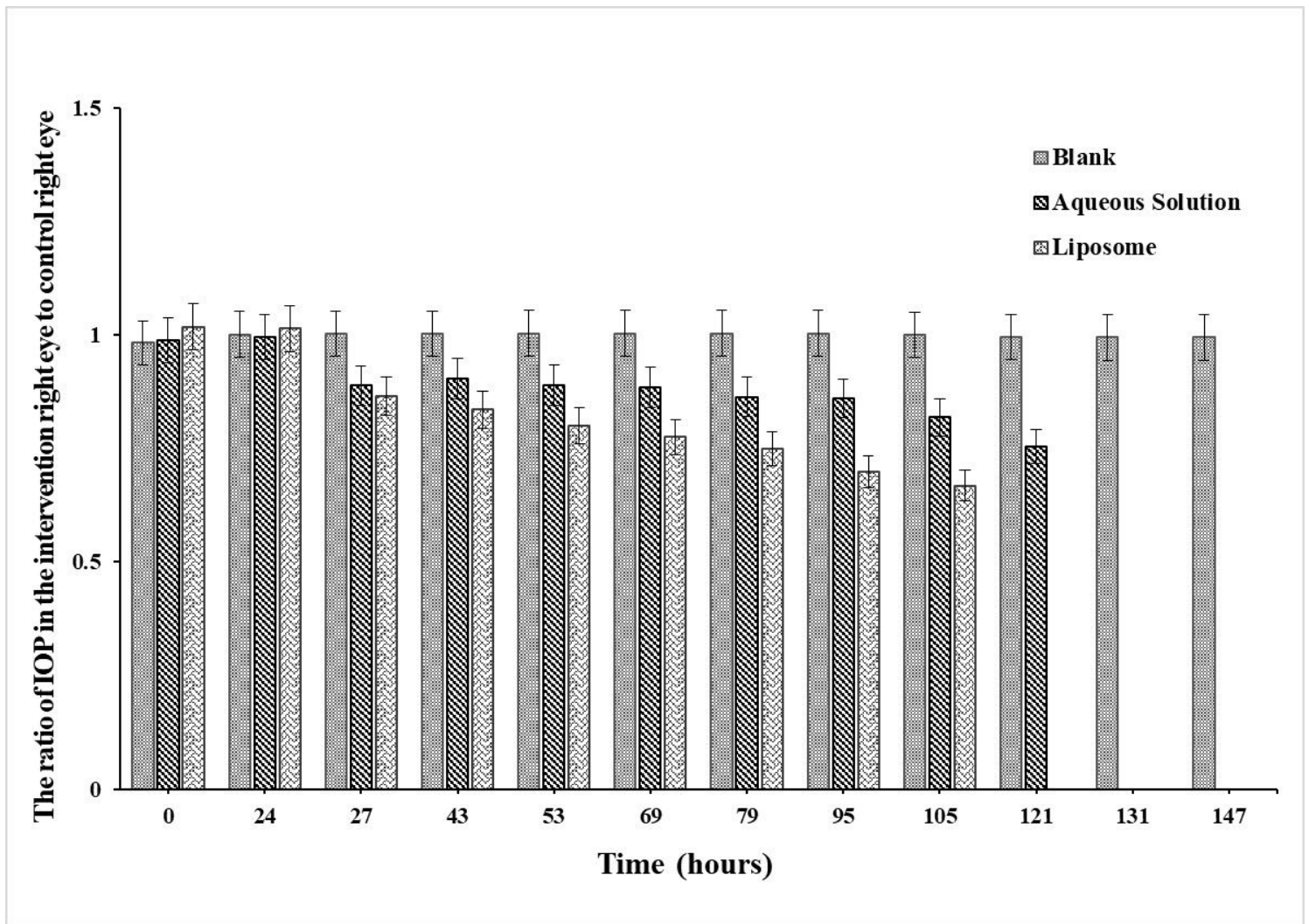


Figure 4

The ratio of IOP in the right eye of the Intervention groups to the right eye is control group(mean \pm S.D, n=3)

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