



Research Article

DEVELOPMENT AND INVESTIGATION OF TIMOLOL MALEATE AND LATANOPROST COMBINATION LIPOSOMES FOR THE TREATMENT OF GLAUCOMAAkhilesh Dubey¹, Prabhakara Prabhu², Ketan Beladiya², Nitin Nair², Vivek Ghate²¹Research Scholar, Department of Pharmaceutics, Institute of Pharmaceutical sciences, Bhagwant University, Ajmer, Rajasthan, India²Department of Pharmaceutics, Shree Devi College of Pharmacy, Airport Road, Mangalore, Karnataka, India

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ABSTRACT

In this study liposomes were developed and evaluated for timolol maleate (TM) and latanoprost (LP) by lipid film hydration technique for the treatment of glaucoma. A 3² factorial design was utilized to study the effect of the molar ratio of 1, 2 – Dipalmitoyl-sn-glycero-3-phosphocholine (DPCC) (X₁) and cholesterol (X₂) on vesicle size, drug entrapment efficiency and *in-vitro* release study. Fourier transform infrared spectroscopy (FT-IR) studies were performed to investigate and predict any physiological interactions between components in the formulation. The liposomal vesicles were found to be uniform in size and shape. The drug entrapment efficiency values for the seven batches showed a variation from a minimum of 56.65 ± 1.39 % to maximum of 70.24 ± 1.14 %. This clearly indicates that the drug entrapment value is strongly dependent on the variables selected. The Zeta potential, average particle size and poly dispersibility index was found to be -17.3 mV, 240 nm and 0.114 respectively. Most of the formulations were found to have a linear release and the formulations were found to provide approximately 72 % - 55 % of drug release for TM and LP respectively within a period of 6 h. Kinetic study reveals that release mechanism depends on swelling, diffusion and relaxation. Best formulation (F2) was found to be sterile and stable and free from microorganisms and passes the isotonicity test. Reduction in intra ocular pressure (IOP) was greater in liposomes as compared to the marketed product of alone and combination medication. A latanoprost-timolol maleate fixed combination, has the advantage of a more convenient delivery system as well as providing a simple summing effect of the two constituents.

Key words: Liposomes, DPCC, Cholesterol, Zeta potential, IOP**INTRODUCTION**

Drug delivery in ocular therapeutics is a challenging problem and a difficult task to scientists working in the multidisciplinary areas pertaining to the eye. Current trends in ocular therapeutics and drug delivery suggest that the existing dosage forms should be replaced by novel drug delivery systems that offer novelty and improved therapy. The main objective of drug delivery system to the eye is to improve existing ocular dosage forms and exploit newer drug delivery system for improving the therapeutic efficiency. Topical application of eye drops is the most common method of administering drugs to the eye in the treatment of ocular diseases. Topical and localized applications are still an acceptable and preferred route, such dosage forms are no longer sufficient to overcome the various ocular diseases such as glaucoma due to poor bioavailability, due to the efficient mechanism protecting the eye from harmful materials and agents. This includes reflex, blinking, lacrymation, tear turnover, and drainage of tear results in the rapid removal of the drug from eye surface. Similarly, frequent instillation of concentrated medication is required at the site of action which is patient in compliance.¹

Glaucoma is an eye disease in which the optic nerve is damaged in a characteristic pattern. This can permanently damage vision in the affected eyes and lead to blindness if left untreated. It is normally associated with increased fluid pressure in the eye (aqueous humour). The term 'ocular hypertension' is used for people with consistently raised intraocular pressure (IOP) without associated optic nerve damage. Conversely, the term 'normal tension' or 'low

tension' glaucoma is used for those with optic nerve damage and associated visual field loss but normal or low IOP. The nerve damage involves loss of retinal ganglion cells in a characteristic pattern. There are many subtypes of glaucoma, but they can all be considered to be a type of optic neuropathy. Raised intraocular pressure (above 21 mmHg or 2.8 kPa) is the most important and only modifiable risk factor for glaucoma. However, some may have high eye pressure for years and never develop damage, while others can develop nerve damage at a relatively low pressure. Untreated glaucoma can lead to permanent damage of the optic nerve and resultant visual field loss, which over time can progress to blindness.²

Ophthalmic drug delivery is the challenging and an interesting area facing the pharmaceutical scientists. The conventional ocular delivery systems like solutions, suspensions and ointments show drawbacks such as increased precorneal elimination, high variability in efficiency and blurred vision, respectively. The major problem encountered with solution is the rapid and extensive elimination of drugs from the precorneal lachrymal fluid by solution drainage, lacrymation, and nonproductive absorption by the conjunctiva, which may lead to undesirable side-effects. It must be noted that this high drainage rate is due to the tendency of the eye to maintain its residence volume at 7-10 µl permanently, whereas volumes topically instilled range from 20 to 50 µl. Ointments increase the contact time, minimize the dilution by tears and resist nasolachrymal drainage, however are responsible for blurring of vision. Suspensions show high variability due to inadequate dosing, mainly due to lack of patient compliance inadequate shaking before use.

Ophthalmic inserts constitutes a psychological and physiological barrier to user acceptance and compliance.^{3,4}

Due to the various disadvantages associated with conventional drug delivery systems, attention has been focused on developing controlled and sustained drug delivery system in order to reduce the frequency of dosing or to increase the effectiveness of the drug by localization at its site of action, decreasing the dose required or providing uniform drug delivery. This problem can be overcome by using vesicular system. Vesicular drug delivery system using colloidal particulate carriers (liposomes or niosomes) have distinct advantages over conventional dosage forms because colloidal particles can act as drug containing reservoirs. Modification of the particle composition or surface can adjust the affinity for the target site and/or the drug release rate. The slow drug release from the carrier system may reduce the toxicity of the drug and hence these carriers play an important role in drug delivery. Vesicular systems not only provide sustained and controlled release of the medication at the corneal surface but also prevent metabolism of the drug at tear/corneal epithelium surface by various enzymes including esterases, oxido reductases.⁵

Liposomes are the microscopic vesicles composed of one or more concentric lipid bilayers, separated by water or aqueous buffer compartments. Liposomes possess the ability to have an intimate contact with the corneal and conjunctival surfaces, which increases the probability of ocular drug absorption. This ability is especially desirable for drugs that are poorly absorbed, the drugs with low partition coefficient, poor solubility, or those with medium to high molecular weights. The behavior of liposomes as an ocular drug delivery system has been observed to be, in part, due to their surface charge. Positively charged liposomes seem to be preferentially captured at the negatively charged corneal surface as compared with neutral or negatively charged liposomes. It is droppable, biocompatible, and biodegradable in nature. It reduced the toxicity of the drug.⁶

Timolol maleate is a beta blocker, which acts by reducing the synthesis of aqueous humor production through the blockade of β receptors on ciliary epithelium. Latanoprost works by relaxing muscles in the eye's interior structure to allow better outflow of fluids, thus reducing build up of eye pressure. A latanoprost and timolol fixed combination, has the advantage of a more convenient delivery system as well as providing a simple summing effect of the two constituents. Two studies shows that this fixed combination of 0.005% latanoprost and 0.5% timolol, administered once daily in the morning for 6 months, is more effective in reducing IOP than the individual components alone. The above combination is marketed in the form of eye drops; however, due to problems such as rapid tear turnover, lachrymal drainage rate and drug dilution by tears, it has been demonstrated that 90% of the administered dose was cleared off within 2 min for an instilled volume of 50 μ l. The ocular residence time of conventional solution is limited to few minutes, and the overall absorption is limited to 1-10%. Consequently most drugs get absorbed systemically through the nose or gut after drainage from the eye. This excessive systemic absorption not only reduces ocular bioavailability, but may also lead to unwanted side-effects and toxicity. The two main strategies for improving ocular absorption are increasing the corneal permeability and prolonging contact time on the ocular surface as well as combined medications, which provide additive effect of reducing IOP.⁷

With all the above aspects in mind the present work was aimed at investigating the potential of liposomes containing a combination of timolol maleate and latanoprost as ocular drug delivery system for the treatment of glaucoma so as to increase the contact time of the drug with the eye, reduce systemic side-effects and toxicity, reduce the number of application, and better patient compliance.

In the present work, liposomes of timolol maleate and latanoprost were prepared and evaluated for glaucoma treatment.

MATERIALS AND METHODS

Timolol maleate was provided by FDC Ltd., Aurangabad, Latanoprost were purchased from Unimed Technologies Ltd., Ahmedabad, Cholesterol was obtained from CDH Laboratories Ltd, New Delhi. DPPC (1, 2 - Dipalmitoyl-sn-glycero-3-phosphocholine) was procured from Genzyme Pharmaceuticals, Switzerland. Diethyl ether, chloroform, methanol, potassium dihydrogen phosphate, disodium hydrogen phosphate were obtained from E- Merck India Ltd, Mumbai.

Preformulation studies

Drug excipients interaction studies

The successful formulation of a stable and effective dosage form depends on the careful selection of the excipients that are added to facilitate administration, promote the consistent release, improved bioavailability and protects from degradation. FT-IR studies were followed to investigate and predict any physiological interactions between components in the formulation and therefore can be applied to the selection of suitably chemically compatible excipients.

Preparation of liposomes

A 3² full factorial design was used to study the effect of independent variables on quality attributes of liposomes. Independent variables have been selected on basis of highest entrapment and desired *in-vitro* drug release. In present study, thin film hydration method was used to prepare liposomes containing timolol maleate (TM) and latanoprost (LP) as it gives better entrapment supported by percent entrapment efficiency study. For optimization of DPPC (1, 2 - Dipalmitoyl-sn-glycero-3-phosphocholine) and cholesterol ratio, 3² full factorial design was used and liposomal batches (F1-F7) were prepared as per runs obtained in design using Design Expert 8.0.7.1 trial version software.⁸ The independent variables selected were amount of DPCC (X_1) and amount of cholesterol (X_2) as it is mentioned in Table.1.

The empirical second order equation for applied 3² full factorial designs is as given below.

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2 + \dots \dots \dots (1)$$

Where, Y = Dependent variable, X_1 and X_2 = Independent variables,
 β_0 = Overall coefficient

$\beta_1, \beta_2, \beta_{11}, \beta_{22}, \beta_{12}$ = The coefficients from the response of the formulation in design.

Liposomes were prepared by passive loading technique thin film hydration method as per the method described by Bangham *et al.*, 1965.⁹ The molar ratios of lipid (DPPC and cholesterol) was accurately weighed and dissolved in minimum quantity (about 2 ml) of a mixture of chloroform: methanol (2:1) in a 250 ml round bottom flask having a ground glass neck to obtain a clear solution (typically lipid solutions were prepared at 10-20 mg lipid / ml of the organic solvent). Round bottom flask was then attached to a rotary flash evaporator by means of a elastic rubber band, was evacuated with vacuum for few minutes through a vacuum pump connected to the rotary evaporator, and rotated at 60 rpm with the round bottom flask being immersed in a water bath with a thermostat set at a temperature above the phase transition temperature (T_m of DPCC is 42°C) of the phospholipid to obtain a thin dry lipid film. Hydration of the dry lipid film was accomplished by adding the timolol maleate and latanoprost solution in PBS 7.4 (prepared using sterile water for injection IP) at a concentration of 1 mg/ml, and the temperature of the hydrating medium (52°C) was maintained above

the gel-liquid crystal transition temperature (T_m) of the phospholipid, before adding to the dry lipid. After the addition of hydrating medium, the lipid suspension was maintained at a temperature above the T_m of the phospholipid used during the hydration period with the vacuum pump switched off. In the present work high transition lipid DPPC was used, and lipid suspension was transferred to a 250 ml round bottom flask and placing the flask on a rotary evaporation system for a hydration period of one hour (hydration time) without vacuum, at a temperature higher than the T_m of the phospholipid used (which was maintained using a thermostat water bath) which produced a homogenous milky yellowish white suspension of MLVs (multi lamellar vesicles) free of visible particles. Once a stable MLV suspension was produced subjected to ultra probe sonication by transferring the colloidal suspension on to a glass vial. The probe tip of the ultra sonicator was just dipped into the suspension (care should be taken such that the probe tip does not touch the bottom of the glass vial during sonication). Sonication was done in 2 cycles. First the liposomal suspension was sonicated at 80 % amplitude with a pulse of 0.5 cycles per second for a period for 3 min, followed by 3 min rest (excess heat may be generated during probe sonication, which may damage the lipids). After 3 min, second cycle was processed for 3 min at 80 % amplitude with 0.5 sec pulse for another 3 min.^{10,11} After sonication the heterogeneous liposomal suspension of SUVs (small uni-lamellar vesicles) were converted to homogenous suspension of SUVs by passing through 0.22 μ syringe filter which further improves the polydispersibility index and also achieved the sterilization of the liposomal suspension which can be administered by intravenous route. Liposomal formulations prepared by film hydration method were coded as LF1, LF2, LF3, LF4, LF5, LF6, and LF7.

EVALUATION OF LIPOSOMES

Microscopy

The vesicle formation was confirmed by optical microscopy (Medilux-207R (II), Kyowa-Getner, India) in 1200 X resolution.¹²

Entrapment efficiency

The drug entrapment efficiency of liposomes was determined by dialysis bag method. The dialysis was carried out by adding the liposomal dispersion to a dialysis tube (donor compartment) and then dipping the tube into a beaker containing 400 ml of PBS pH 7.4 (receptor compartment) on a magnetic stirrer, rotated at a speed of 80-120 rpm and continued for 4 h. After 4 h, the solution in the receptor compartment was estimated for entrapped drug at λ_{max} of 293.8 nm and 204.8 nm of timolol maleate and latanoprost respectively using an UV spectrophotometer (Shimadzu-1800, Kyoto, Japan).^{12,15} The entrapment study (EE) was calculated using the formula:

$$\text{Entrapment efficiency (\%)} = \frac{\text{Entrapped amount of drug} \times 100}{\text{Total amount of drug added}}$$

Drug content

The drug content and uniformity of drug content was determined by assaying liposomal formulation dissolved in 50 ml PBS pH 7.4. The volumetric flask containing liposomal formulation was shaken for 2 hours on a mechanical shaker in order to get complete solubility of drug. This solution was filtered and estimated spectrophotometrically. The absorption was calculated at 293.8 nm and 204.8 nm for TM and LP respectively.

pH and clarity

pH of the formulation was determined using digital pH tutor. Clarity

of the formulation was inspected under light against white and black background in a well-lit cabinet for appearance and clarity.

Zeta potential and particle size distribution analysis

Measurement of zeta potential of the niosomal formulation was done by using a Malvern nano zeta sizer instrument. Average particle size (in nanometers) and size distribution (as the poly dispersibility index) of the liposomal suspension (SUVs) was measured using a Malvern nano zeta sizer instrument.

In-vitro drug release study

The liposomal suspension (1 ml) was placed on one side of the dialysis membrane in a Franz diffusion cell. Other side of the membrane was in continuous contact with the dissolution medium. Entire dissolution assembly was placed on a magnetic stirrer at temperature of 37°C. The dissolution medium was 20 ml of the STF pH 7.4. Aliquots (3 ml) of dissolution medium were withdrawn at different time intervals (15 min, 30 min, 45 min, 60 min, 2h, 3h, 4h, 5h and 6 h). Whenever the sample was withdrawn, equal volume fresh dissolution medium was added to maintain the constant volume. Drug concentration in the dissolution medium was determined by UV spectrophotometric method.¹⁴

Kinetic data analysis

To analyze the *in-vitro* release data various kinetic models including zero order, first order, Higuchi's and Korsmeyer-Peppas's models were used to describe the release kinetics.^{15,16}

Test for sterility

The sterility testing of the liposomes was performed for the aerobic, anaerobic bacteria and fungi by using alternative thioglycolate medium and soyabean casein digest medium. The medium was prepared by dissolving 500 mg of peptic digest of animal tissue (such as bacteriological peptone) or its equivalent in water to make 100 ml, and the pH was adjusted to 7.1 ± 0.2 . The medium was filtered or centrifuged to clarify and dispensed into flasks in 10 ml quantities and was sterilized at 121°C for 20 min. The positive control (growth promotion) and negative control (sterility) test were also carried out. *Bacillus subtilis*, *Bacteriodes vulgatus*, and *Candida albicans* were used as test organisms in the aerobic bacteria, anaerobic bacteria and fungi test, respectively. Incubation was carried out in all cases and growth was observed.^{17,18}

Isotonicity Test

Isotonicity was the important characteristic of the ophthalmic formulations. Isotonicity has to be maintained to prevent tissue damage or irritation of eye. The best formulation was subjected to isotonicity testing, where formulation was mixed with few drops of blood and observed under microscope at 45X magnification and compared with standard marketed ophthalmic formulation. The shape of blood cell (bulging or shrinkage) was compared with standard marketed ophthalmic formulation containing TM and LP.¹⁹

In-vivo intraocular pressure lowering activity

Glaucoma was induced in rabbits by instilling prednisolone eye drops (1% w/v) up to 2-3 weeks. The study was performed on 12 white Albino rabbits with weighing 2-3 kg divided into three groups. First group received the best formulation, second group received conventional marketed product containing same formulation and also third group received conventional marketed product containing only timolol maleate in the right eye and the

other eye was untreated. IOP was measured using a Schiötz tonometer (Rudolf Riester, GmbH & Co., K.G. Postfach 35, Jungingen, Germany) after instilling a drop of procaine hydrochloride local anesthetic (1% w/v). The left eye was used as control and treatment was carried out on the right eye. All the formulations were instilled into the lower conjunctival sac. At regular intervals, the IOP was measured. Change in IOP was expressed as follows:

$$\Delta\text{IOP} = \text{IOP untreated eye} - \text{IOP treated eye.}$$

Results are reported as mean ($\pm$ standard error) ANOVA one way statistical test was used to identify statistically significance at $P < 0.05$. [18, 20]

This study was conducted in accordance with CPCSEA guidelines and experimental protocol was approved by Institutional Animal Ethics Committee (SDCP/IAEC-24/2012-13).

Ocular irritation study

Ocular irritation study was performed on 6 rabbits weighing 2-3 kg. The best formulation was selected for the study. The formulation was applied into the cul-de-sac region once a day for a period of 7 days and rabbits were observed periodically for irritation, inflammation etc by naked eye or by means of a pen torch. The test may be considered positive if there are one or more positive reactions at any observation period. One eye was used as test and the other as control. Rabbits were divided into two groups (3 rabbits in each group). For first group containing three rabbits, the best formulation was applied to one eye and other eye was kept as control. For second group containing three rabbits, the marketed formulation was instilled into one eye and the other eye was kept as control. During the time of observation period each rabbit was scored for ocular reaction. The guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India were followed and prior permission was sought from the Institutional Animal Ethics Committee for conducting the study (SDCP/IAEC-24/2012-13).²⁰

Stability Study

Stability study was carried out to investigate the leaching of drug from liposomes (in a suspension) during storage. The best formulation was kept at refrigerated temperature (2-8°C), room temperature and 40°C (75 % RH) as three different groups. Stability chamber was used for the third group. The best niosomal suspension composed of span 60 and cholesterol, sealed in glass vials and stored in refrigerated temperature (2-8°C) for a period of 90 days. Samples from each batch were withdrawn after definite time intervals and the residual amount of drug in the vesicle was determined by UV spectrophotometric method.²¹

Statistical analysis

Statistical analysis included descriptive statistics, where the mean and standard deviation (SD) was calculated for the continuous variables and the data are expressed as mean of three experiments along with the standard deviation (SD). ANOVA one way statistical test was used to identify statistically significance at $P < 0.05$.

RESULTS AND DISCUSSION

Fourier transform infrared spectroscopy (FTIR) is the most applied technique in the chemical and pharmaceutical sciences. Its scope includes many applications especially to find out the compatibility between active and inactive pharmaceutical ingredients, which is

prerequisite for the designing of formulation. The IR spectra of liposomes raw material, physical mixtures and liposomal formulations were taken and interpreted to find any interaction between the components. The main goal of these experiments was to ascertain the compatibility of timolol maleate and latanoprost with formulation components i.e. DPCC and cholesterol. The FTIR peaks of timolol maleate (TM), latanoprost (LP), DPCC, cholesterol and the final formulation with TM and LP are shown in Figure 1(a), (b) and (c).

TM showed a broad band appearing at 3302 cm^{-1} due to O-H/N-H stretching vibrations. The bands at 2968 cm^{-1} , 2891 cm^{-1} , and 2854 cm^{-1} are due to aliphatic C-H stretching vibrations. Acid carbonyl group of maleic acid and N-H bending vibrations gave band at 1707 cm^{-1} and 1496 cm^{-1} . The C=N stretching vibrations appears at 1621 cm^{-1} . Bands at 1263 cm^{-1} and 1120 cm^{-1} are due to the=C-O-C and morpholino C-O-C stretching vibrations, respectively, while the bands at 1229 cm^{-1} and 954 cm^{-1} are due to O-H bending and hydroxyl C-O stretching vibrations, respectively. The principal peaks for timolol maleate as per literature were 1497 cm^{-1} , 1527 cm^{-1} , 1120 cm^{-1} , 1230 cm^{-1} , 1590 cm^{-1} , 1620 cm^{-1} . The principal peaks for latanoprost as per literature were 3473 cm^{-1} , 3438 cm^{-1} , 1300 cm^{-1} , 2362 cm^{-1} , 2341 cm^{-1} , 1718 cm^{-1} . DPCC showed characteristic peaks at 2816.75 cm^{-1} (-OH str, broad), 2837.58 (-OH str, broad), 1775.65 cm^{-1} (cyclic 5-membered ring), 1467.56 cm^{-1} (-CH₃), and small peaks 1000 cm^{-1} to 1200 cm^{-1} (aliphatic). Cholesterol has key role in stabilizing the unilamellar or multilamellar bilayers. The IR spectrum of cholesterol shows major peaks at 2931.41 cm^{-1} (Acetyl groups), 2866.83 cm^{-1} (symmetric -CH₃), 1770.20 cm^{-1} (vinyl group), and 1055.17 cm^{-1} (R-O strong).

Physical mixtures of TM and LP with DPCC and cholesterol were prepared by simple mixing in equimolar ratios and were scanned. The interaction between drug and carrier may lead to identifiable changes in FTIR profile. These findings of present study revealed that TM and LP with its physical mixture of nonionic surfactant and cholesterol possessed the same peaks and TM and LP showed the peaks at the same points as in individual spectrum. The spectra of physical blends of TM and LP with selected nonionic amphiphiles did not depict identifiable shift of peaks indicating no physical interactions and arguing the stability of physical mixture.

DPCC and cholesterol were selected, for the preparation of liposomes based on the basis of preformulation studies. The process variables like temperature, vacuum applied and hydration time were kept constant. Seven batches of liposomes were prepared by thin film hydration method by using 3² factorial design varying two independent variables molar ratio of DPCC (X_1) and cholesterol (X_2).

The liposomes prepared using TM and LP was studied under 1200X magnifications to observe the formation of liposomal vesicles. The liposomal vesicles were found to be uniform in size and shape. (Figure 2)

The drug entrapment efficiency values for the seven batches showed a variation from a minimum of 56.65 $\pm$ 1.39 % to maximum of 70.24 $\pm$ 1.14 %. This clearly indicates that the drug entrapment value is strongly dependent on the variables selected. The second order polynomial equation relating the response drug entrapment and independent variables was obtained as equation 2. This equation represents the quantitative effect of independent variables and their interaction on the drug entrapment efficiency.

$$\text{Drug entrapment efficiency} = +70.65 - 63.25X_1 + 15.12X_2 - 15.5X_1X_2 - 28.6X_1^2$$

$$+ 4.93X_2^2 \dots \dots \dots (2)$$

The high value of correlation coefficient ($r^2 = 0.99$) for drug entrapment indicates a good fit. The polynomial equation was used to draw conclusions after considering the magnitude of coefficient

and the mathematical sign it carries. When the coefficient values of the independent variables and their interaction in equation 2 were compared, the value for variable X_1 ($b_1 = -64.45$) was found to be maximum and hence, the variable X_1 was considered to be a major contributing variable for predicting the drug entrapment of TM and LP liposomes. Drug content was found between 76% - 87% and maximum was found in the formulation LF2. The observed pH of the liposomal formulation was found within the range of 5.65-5.85. Liposomal formulation appeared turbid when observed visually which might be due to the dispersion of liposomes into the buffer solution. (Table 2).

The Zeta potential was found to be -17.3 mV after analyzing the best formulation F2 by malvern nano zeta potential analyser. The average particles size of liposomal formulation was found to be 240 nm and Poly dispersibility index value was found to be 0.114. All the found values are in the range which shows the stability and homogeneity of the formulation.

The release study was conducted for all the seven formulations. Most of the formulations were found to have a linear release and the formulations were found to provide approximately 72 % - 55 % of drug release for TM and LP respectively within a period of 6 h. The slower release of the drug from the multilamellar vesicles may be attributed to the fact that multilamellar vesicles consist of several concentric sphere of bilayer separated by aqueous compartment. Studies showed that rate of drug release also depends on the percentage of drug entrapment as it clearly shows that maximum release of 72.76 % was found in F2 of which entrapment efficiency was 70.24%. Similarly, minimum release of drug was found in F6 which clearly shows the impact of the ratio of DPCC and cholesterol. It has been found that optimum ratio of DPCC and cholesterol is required to get maximum drug entrapment and efficient release of drug. (Figure 3)

The result of the kinetic data revealed that both the drugs followed zero order release. According to the "n" value found from Peppas's equation, TM followed anomalous transport while LP followed super case II transport. It indicates that release mechanism of release for TM and LP depends swelling, diffusion and relaxation mechanism. The release kinetics was evaluated considering four different models including zero order, first order, Higuchi's equation and Peppas's equation. From the release kinetics of all the formulations, it was found that zero order kinetics and Korsmeyer-Peppas's model were best fitted for the liposomal formulations of TM and LP. The correlation coefficient (r) was used as an indicator for the best fitting for each of the models considered. The release pattern of all the developed formulations followed zero order kinetics and Korsmeyer-Peppas's model. The mechanism of drug release was followed Anomalous transport in case of TM with "n" values greater than 0.5 to less than 1 in Korsmeyer-Peppas's model. The mechanism of drug release was followed super case II transport in case of LP with "n" values greater than 1 in Korsmeyer-Peppas's model. This indicated that the drug release depends on swelling, diffusion and relaxation mechanism of release for TM and LP. (Table 3)

The sterility testing was performed for seven days for the best formulation F2. The test tube (negative control) in which only the growth medium was used, showed no signs of precipitates indicating no growth of microorganisms. The test tube (positive control) in which the microorganisms were inoculated in the growth medium showed significant growth in the form of milky white precipitates.

The test tube containing the formulation F2 and the growth medium was found to be sterile and free from microorganisms as there were no such precipitates found in the test tube. (Table 4)

Isotonicity testing of formulation F2 exhibited no change in the shape of blood cells (bulging or shrinkage), which reveals the isotonic nature of the formulation and compared with that of standard marketed ophthalmic preparation.

After induction of glaucoma in rabbits, the treatment was carried out to check and compare the IOP lowering activity of NIF1 formulation and marketed formulation containing combination of same drugs as NIF1 formulation and also the marketed formulation containing timolol maleate (IOTIM) was compared. IOP was measured using Schiotz tonometer. For *in-vivo* study the dose was selected in such a way that drug concentrations when administered into the eye comparable with the eye drops i.e each drop of marketed formulation contains latanoprost 0.005 % and timolol maleate 0.5 % hence in the present study each drop of formulation (F2) contains the same concentration as in the marketed drops. Comparison between the best formulation (F2) and marketed formulation (Xalacom, IOTIM) was carried out and the result indicated that the F2 lowers IOP very consistently to normal IOP within a period of 7 days where as Xalacom lowers IOP within 9 days. When the best formulation (F2) was compared with marketed formulation of alone medication (IOTIM) which lowers the IOP within 11days. So it indicates that the reduction in IOP was greater in liposomes as compared to the marketed product of alone and combination medication, which also proves that the combination therapy is more effective in reducing IOP. It was also observed that sustained effect was maintained for more time in the liposomes as compared to marketed preparations. The marketed eye drops suddenly lowered the IOP to a minimum and afterwards, there was a sudden increase in the IOP, whereas liposomes lowered the IOP slowly to the original and thereafter, a gradual increase in the IOP was observed. (Figure 4)

The ANOVA test was carried out by using Tukey-Kramer multiple comparison test between the IOP of best formulation (F2) and marketed formulation containing TM and LP (Xalacom), and also between the (F2) and marketed formulation containing TM (IOTIM) and the "p" values was found to be < 0.001. It indicates that the F2 is extremely significant in comparison with the marketed formulation of Xalacom and IOTIM.

The ocular irritation study was done on six rabbits for 7 days. The result of the ocular irritation study indicates that the formulations are non-irritant to the eye. Excellent ocular tolerance was noted. No ocular damage or abnormal signs to the cornea, iris and the conjunctiva was visible.

The best formulation was subjected to stability study as described in methodology and was checked for any change in the physical appearance, pH, entrapment efficiency and *in-vitro* release study. The stability study was done in a stability chamber for 60 days for the best formulation F2. This formulation showed good stability at 2-8°C. Therefore it can be concluded that liposomes can be stored at 2-8°C in tightly closed container however, there was no significant change in the physical appearance and pH of the formulation. (Table 5)

Table 1: Composition of liposomes as per 3² full factorial designs

Batch code	X ₁	X ₂	Ratio(μ mol) DPCC:cholesterol	Lipid (mg)	cholesterol (mg)
F1	-1	-1	100:50	73	19
F2	-1	0	100:100	73	38
F3	-1	1	100:150	73	58
F4	0	-1	50:50	36	19
F5	0	0	50:100	36	38
F6	0	1	50:150	36	58
F7	1	1	150:150	110	58

LF; Liposomal formulation, X₁; Indicates the amount of lipid (mg), X₂; Indicates the amount of cholesterol (mg), Each batch contains 0.5% and 0.005% of Timolol maleate and Latanoprost respectively.

Table 2: Characterization of liposomes

Formulation Code	pH	Clarity	Drug content (%)		Entrapment efficiency (%)	
			TM	LP	TM	LP
F1	5.65	Turbid	80.55±1.55	81.55±1.14	60.58±1.34	59.58±1.81
F2	5.85	Turbid	84.67±1.34	85.65±1.36	70.24±1.14	69.58±1.59
F3	5.75	Turbid	82.33±1.64	81.80±1.74	65.28±1.24	66.58±1.83
F4	5.72	Turbid	83.74±1.91	82.55±1.94	68.57±1.35	67.58±1.80
F5	5.73	Turbid	81.07±1.54	82.45±1.44	61.48±1.64	60.58±1.19
F6	5.67	Turbid	76.30±1.74	87.55±1.64	56.65±1.39	58.58±1.24
F7	5.78	Turbid	83.88±1.34	82.55±1.34	69.18±1.31	67.58±1.14

Data are represented as mean±SD (n=2), TM-Timolol maleate, LP- Latanoprost

Table 3: Slope and correlation values of TM and LP

Formulation Code	Zero order		First order		Higuchi's model		Peppas's model	
	Slope	R ²	Slope	R ²	Slope	R ²	Slope	'n'
F1	0.210	0.998	0.210	0.945	4.990	0.922	0.897	0.999
F2	0.216	0.997	0.218	0.957	4.920	0.901	0.899	0.997
F3	0.230	0.993	0.224	0.970	4.981	0.882	0.910	0.992
F4	0.245	0.984	0.229	0.956	4.885	0.899	0.925	0.993
F5	0.213	0.994	0.213	0.994	4.922	0.987	0.899	0.991
F6	0.220	0.996	0.232	0.955	4.870	0.902	0.902	0.994
F7	0.211	0.991	0.242	0.975	4.910	0.930	0.908	0.995

Formulation No.	Zero order		First order		Higuchi's model		Peppas's model	
	Slope	R ²	Slope	R ²	Slope	R ²	Slope	'n'
F1	0.190	0.998	0.171	0.937	4.450	0.950	1.059	0.997
F2	0.187	0.999	0.182	0.947	4.473	0.959	1.050	0.996
F3	0.182	0.994	0.187	0.960	4.483	0.945	1.063	0.999
F4	0.189	0.995	0.170	0.971	4.459	0.943	1.051	0.993
F5	0.195	0.997	0.195	0.997	4.450	0.973	1.053	0.991
F6	0.192	0.992	0.190	0.982	4.453	0.967	1.049	0.994
F7	0.198	0.996	0.189	0.960	4.472	0.976	1.054	0.991

Table 4 Results of test for sterility

Sterility Tests	Results Obtained		
	Negative Control	Test	Positive Control
Test For Aerobic Bacteria	-	-	+
Test For Anaerobic Bacteria	-	-	+
Test For Fungi	-	-	+

Table 5: Stability studies for F4 formulation

Parameters evaluated	Before storage		At the end of 8 weeks					
			2-8°C		25-30°C		45-50°C	
	TM	LP	TM	LP	TM	LP	TM	LP
Entrapment efficiency	88.78	87.86	86.32	86.84	86.10	86.22	85.30	85.50
Surface pH	5.85		5.95		5.91		5.88	
Maximum <i>in vitro</i> release	81.30	75.36	79.42	73.21	76.48	71.56	77.49	73.78
Physical appearance	Turbid		Turbid		Turbid		Turbid	

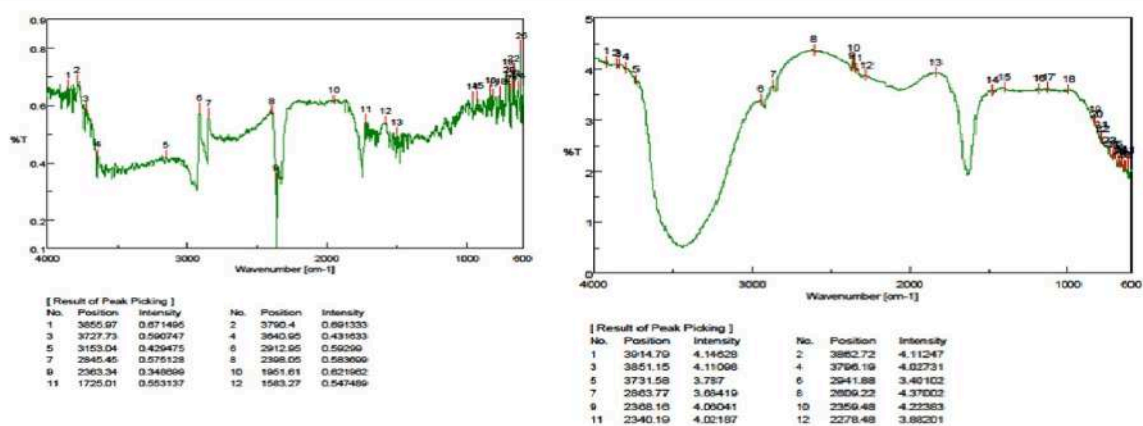
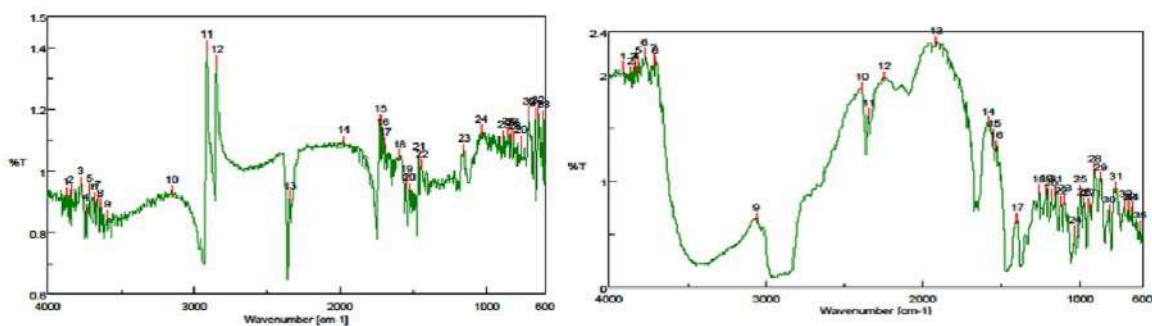
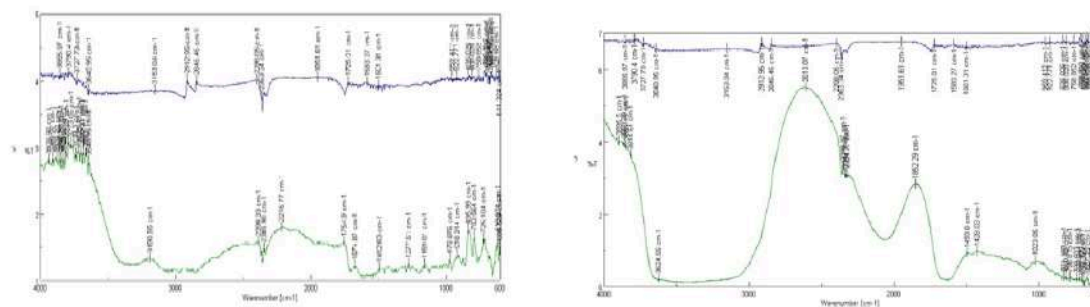


Figure (a): FTIR Spectra of timolol maleate and latanoprost



(b) FTIR Spectra of DPCC and Cholesterol



(c) Formulation overlay structure

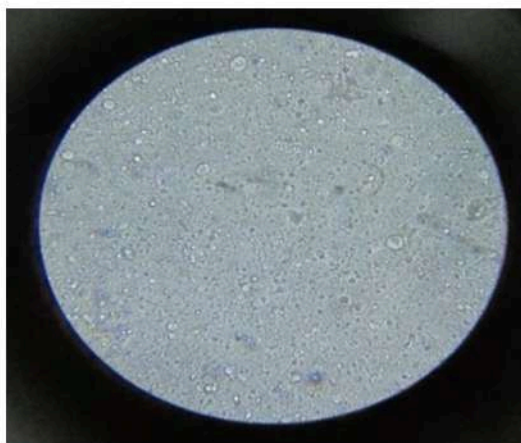


Figure 2: Microphotograph of liposome

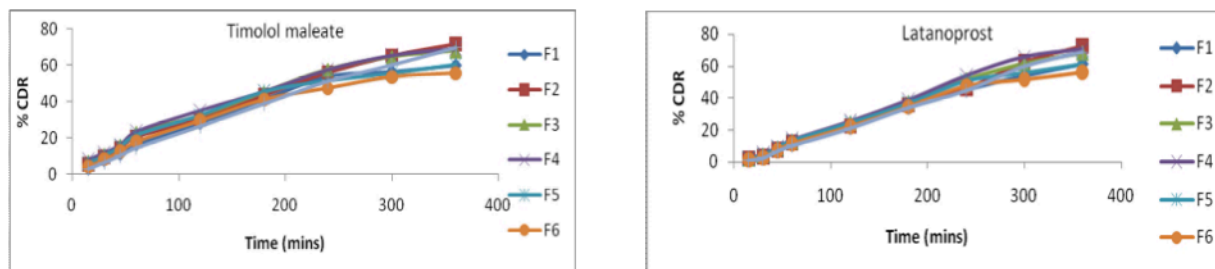
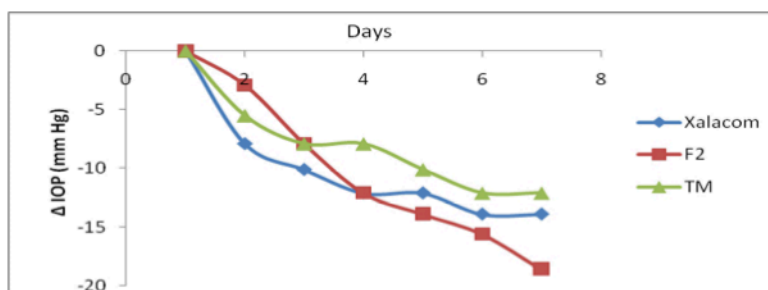
Figure 3: *In vitro* drug release of Timolol maleate and Latanoprost liposomes

Figure 4: Effect of formulations on intraocular pressure

CONCLUSION

In this study, we have investigated liposomes of timolol maleate and latanoprost topically for the management of glaucoma. The objective of the present investigations has been achieved by the evaluation of the liposomes. 3^2 factorial designs showed that the molar ratio of DPCC and cholesterol significantly affect the drug entrapment, *in-vitro* release and size of the liposomes. Reduction in IOP was greater in liposomes as compared to the conventional marketed product of alone and combination medication, which also proves that the combination therapy is more effective in reducing IOP. This study reveals that fixed combination of 0.005% latanoprost and 0.5% timolol maleate, administered once daily, is more effective in reducing IOP than the individual components alone. Finally, it can be concluded that liposomes offer a promising drug carriers to fulfill the need of an ophthalmic drug delivery system with increased patient compliance.

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