

Research Article**Development and characterization of prolonged release Timolol maleate cubosomal gel for ocular drug delivery**

Ankit Acharya*, Prakash Goudanavar, Vedamurthy Joshi

Department of Pharmaceutics, Sri Adichunchanagiri College of Pharmacy, B.G. Nagara-571448, Karnataka, India

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Abstract

Objective: The aim of the present study was to formulate and evaluate an ocular effective prolonged-release cubogel formulation of Timolol maleate for the treatment of glaucoma. **Material and Methods:** Timolol loaded cubosome was prepared by top down technique using glycerol monooleate and poloxamer 407 in different concentrations. Optimized batch of cubosome was selected based on the results of entrapment efficiency and *in-vitro* release results. Timolol maleate cubogel was prepared by dispersing cubosome in to *in-situ* gelling system. Interaction studies were confirmed by Fourier transforms infrared spectroscopy (FTIR) studies. The effect of surfactant and additives on particle size, entrapment efficiency and *in-vitro* drug release behaviour of cubosome was evaluated. Mucoadhesive strength was determined by ultracentrifugation and turbidity method. Optimized batch of cubogel was selected for short term stability studies. **Results:** FTIR results showed no sign of interaction drug and excipients, hence compatible. Formulation A6, CG1 and CG2 showed 78.84%, 54.77% and 51.45% of drug release at the end of 12 hours and follow Higuchi's release kinetic model, whereas marketed formulation completes its drug release within 2 hours. Cubogel formulation CG1 and CG2 exhibited a 2.51 and 2.376-fold increase in permeability (Papp) compared to marketed formulation, suggesting that higher amount of Timolol maleate-cubogel were taken up by the goat cornea than that of marketed eye drops. **Conclusion:** From the results obtained, it can be concluded that cubosome might a good alternative to conventional eye drops as it showed higher permeability and sustained release behaviour.

Keywords: Timolol maleate, cubosome, top down technique, permeability, glycerol monooleate, poloxamer 407

Introduction

The main objective of ocular drug delivery is to improve the currently available conventional dosage form and exploit newer drug delivery system, with the view to improve their therapeutic efficiency. Topical application of eye drops is quite common among the patients suffered from the eye diseases. Commonly used conventional eye drops shows low ocular bioavailability due to constant lachrymal secretion, tear turnover, blinking, reflex and rapid nasolachrymal drainage. Normal drainage of an instilled dose commences immediately on instillation and is essentially completed within 5 min (Achouri et al., 2013). Typically less than

5% of ocular bioavailability is achieved for eye drops due to the short precorneal residence times. Consequently, there is a need for frequent instillation of concentrated solutions to achieve the desired therapeutic effect. To overcome these problems various ophthalmic formulations, such as viscous solutions, ointments, gels, nanoparticles or polymeric inserts have been investigated (Patel et al., 2013). The corneal contact time has been increased to varying degrees by these vehicles. But, they have not been unanimously accepted, because of blurred vision (e.g. ointments) or lack of patient compliance (e.g. inserts). As a result, good ocular bioavailability following topical delivery of a drug to the eye remains a challenge and yet to be resolved (Li et al., 2010).

In order to improve ocular bioavailability many research on drug reservoir system have been made. These reservoir systems are carrier of drug molecules and the controller of the drug release pattern. Cubosome is recently developed drug reservoir system and successfully used for ocular drug

***Address for Corresponding Author:**

Mr. Ankit Acharya,
Department of Pharmaceutics,
Sri Adichunchanagiri College of Pharmacy, B.G. Nagar, Karnataka,
India
E. mail: ankitbaba99@gmail.com

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delivery system. Cubosome is cubic shape liquid crystals dispersion (Baranowski et al., 2014). Cubosome have unique nanostructure of a highly twisted, uninterrupted lipid bilayer and two congruent non-transecting water channels. Furthermore, the microstructure of cubosome is similar to that of biological membranes. This enables the lipid carriers to fuse with the lipid bilayers of the corneal epithelial cells. Therefore, cubosome is outstanding candidates for the ocular drug delivery system according to its physicochemical features (Liu et al., 2010).

However, cubosome is low viscosity liquid dispersion and its particle is susceptible to aggregation. Due to low viscosity of cubosome, the contact time of the drug in the eye is not sufficient, so the method needs to be improved (Chen et al., 2012). Usually one strategy is used to increase the precorneal residence time of the drug-loaded vesicular system is dispersing them into the *in-situ* gel system. *In-situ* gel consists of polymers that can easily undergo sol-to-gel phase transitions due to a change in environmental condition like pH, temperature and specific ions (Hartnett et al., 2015). Such cubosome dispersed in *in-situ* gel is called as cubogel. These cubogel formulation shows dual mechanism, where *in-situ* gel helps to prolong the contact time of the formulations on the cul-de-sac region and cubosome increases the corneal permeability of the drugs Boyd (BJ et al., 2003).

In this study Timolol maleate (TM) was selected as model drug. TM is a non-selective beta-blocker and most commonly used drug in the treatment of glaucoma. Glaucoma constitutes second most common cause of blindness in the world. It affects more than 70 million people worldwide (Wu et al., 2013). TM was selected because of more rapid onset, better tolerance, and fewer side effects than other drugs in the same class. This drug lowers intra ocular pressure (IOP) by reducing the production of the aqueous humor through blocking the sympathetic nerve endings in the ciliary epithelium. The conventional eye drops of TM are available at low-cost, convenient to use, and it significantly lower IOP (Dubey et al., 2014). However, due to the unique anatomical structure of the eye, the corneal epithelial barrier function limits drug absorption from the lacrimal fluid into the anterior chamber after eye drop administration. Low bioavailability (<5%) of TM eye drops can lead to ineffective therapy. Topically administrated TM eye drops have poor corneal permeability, and most of the eye drops drain through the nasolacrimal duct and causes systemic side effects like hypotension, bradycardia, and bronchospasm. In order to minimize such problems mucoadhesive cubogel formulation of TM was formulated and evaluated for its mucoadhesive property (Yu et al., 2005; Gupta et al., 2010). In this study, an attempt has been made to find a preparation method that dispersed cubosome containing TM into *in-situ* gel to enhance the mucoadhesive as

well as sustain release behaviour of the cubogel formulation.

Material and Methods

Pure drug Timolol maleate (TM), poloxamer 407 and glycerol monooleate (GMO) were purchase from Yarrow Chem products, Mumbai, India. Carbopol p934, gellan gum and chitosan were procured from Himedia, Mumbai. All other reagents used were analytical grade.

Determination of λ_{\max} of Timolol maleate

Accurately weighed drug TM (100 mg) was transferred into the 100 ml volumetric flask. Drug was then dissolved in stimulated tear fluid (STF) and diluted up to 100 ml mark with STF to get the stock solution-I of 1mg/ml i.e. 1000 $\mu\text{g/ml}$ concentration. 10 ml of stock solution-I was taken in 100 ml volumetric flask and making the volume to the mark with STF to make 100 $\mu\text{g/ml}$ of TM. Serial dilutions with concentrations 5, 10, 15, 20, 25 and 30 $\mu\text{g/ml}$ were prepared by transferring 0.5, 1, 1.5, 2.0, 2.5 and 3.0 ml of the stock solution in 10 ml volumetric flask and makeup the volume with STF. As the sample solution was colourless, the solution was scanned between 200 and 400 nm using UV-visible spectrophotometer (UV 1800, Shimadzu, Japan). The composition of pH 7.4 STF was NaCl 6.8 g, NaHCO_3 2.2 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.08 g, KCl 1.4 g, and water up to 100 ml (Liu et al., 2016).

Preparation of Timolol maleate cubosome

TM-loaded cubosome was prepared by top down techniques using GMO and poloxamer 407 as lipid and surfactants, respectively. The composition of cubosome is showed in table 1. In brief, GMO and poloxamer 407 were accurately weigh and melted at 60°C in a hot water bath. Drug (0.5%) dissolved in 3 ml of Millipore water was gradually added to above dispersion using an 18-gauge needle and vortex mixed for 2 minute. After equilibration for 10 hours at room temperature, the cubic phase gel was formed. In some formulations additives like glycerol and oleic acid, were added just before addition of drug solution to the lipid dispersion. Remaining quantity of water was added to cubic gel and gel was fragmented for 10 minutes by intermittent probe sonication (Fisherbrand™ Q500 Sonicator). Afterward, the resulting milky coarse dispersion of cubosome was formed and stored in well closed glass vials at room temperature and later subjected to evaluation parameters (Liu et al., 2016)

Characterization studies of cubosome

Compatibility study using FT-IR

Infrared spectroscopy was conducted using a Thermo

Table 1. Formulation design of Timolol maleate loaded cubosome

Formulation Code	Timolol maleate (%)	GMO (%)	Poloxamer 407 (%)	Glycerol (%)	Oleic acid (%)	Millipore water (%)
Blank	--	6	0.6	--	--	93.4
A1	0.5	6	0.4	--	--	93.1
A2	0.5	6	0.6	--	--	92.9
A3	0.5	6	0.8	--	--	92.7
A4	0.5	6	1.0	--	--	92.5
A5	0.5	6	0.6	2	--	90.9
A6	0.5	6	0.6	4	--	88.9
A7	0.5	6	0.6	--	2	90.9
A8	0.5	6	0.6	--	4	88.9

Nicolet FTIR and the spectrum was recorded in the region of 4000 to 400 cm^{-1} . The interaction between drug-excipients was observed from IR-Spectral studies by observing any shift in the peaks of drug in the spectrum of physical mixture of drug (Ammar et al., 2009).

Compatibility studies by DSC

Pure drug TM and mixture of TM with excipients were subjected to DSC study (Shimadzu DSC-60). Indium/Zinc standards were used to calibrate the DSC temperature and enthalpy scale. The samples (Pure drug, and mixture of drug with excipients) were sealed in aluminium pans and heated at a constant rate of 10°C/min over a temperature range of 20-300°C. Inert atmosphere was maintained by purging nitrogen gas at a flow rate of 10 ml/min (Ammar et al., 2009).

Visual inspection

Prepared cubosome dispersion was visually inspected for appearance and phase separation (Liu et al., 2016).

Particle size and zeta potential

Particle size, polydispersity index, and zeta potential were determined by using Zetasizer Nano ZS (Malvern Instruments, UK). The instrument was equipped with dynamic light scattering particle size analyzer at a wavelength of 635 nm and a fixed scattering angle of 90°. The values of z-average diameters were used. Before analysis, samples were diluted with water to 2% before measurement and measured at 25°C (Liu et al., 2016).

Optical microscopy and scanning electron microscopy

Surface morphology of cubosome was determined by optical microscopy and scanning electron microscopy (SEM). An optimized batch of the cubosome was selected and observed under optical microscope (Olympus Microscope, Japan) under the magnification of 10× and scanning electron microscopy (JSM-T330A, JEOL) (Liu et al., 2016).

Confirmation of cubosomes containing TM

Encapsulation efficiency

In order to determine Timolol maleate content encapsulated in cubosomes after production,

1ml of cubosome dispersion was taken and cubosomes were first separated by centrifugation. The separation of the free drugs from the entrapped drug in the cubosome dispersion was achieved by centrifugation at 4500 rpm for 20 minute. The supernatant liquid was separated and diluted. The amount of free drug in the dispersion was then analyzed spectrophotometrically at 294 nm which was then subtracted from the total amount of drug initially added. The % entrapment efficiency (EE) was calculated by the following equation (Thimmasetty et al., 2012).

$$EE = \frac{(C_{\text{totalcon}} - C_{\text{freecon}})}{C_{\text{totalcon}}} \times 100\%$$

UV spectroscopic examination

The spectra of TM in different samples, which included TM solution, TM cubosome and blank cubosomes, all at the concentration of 5 mg/ml, were investigated by a UV-visible spectrometer at the wavelength of 200 nm to 400 nm. The ratio of the absorbance of the first peak (I) to the last peak (III) was used to monitor the aggregation state of Timolol maleate (Thimmasetty et al., 2012).

Drug content estimation

Drug content was determined by suitably diluting TM cubosomal formulation with STF and analyzed by UV spectroscopy at 294 nm. Measurement was done in triplicate (Shun et al., 2010).

Preparation of TM cubogel

The composition of TM cubogel is depicted in table 2. TM cubogel was prepared by selecting optimized batch of cubosome. Selection of optimized batch was done based on

Table 2. Composition of TM *in-situ* gel and cubogel formulation

Ingredients (%w/w)	IG1	IG2	A6	CG1	CG2
TM	0.5	0.5	0.5	0.5	0.5
Poloxamer 407	--	--	0.6	0.6	0.6
Gellan gum	0.5	--	--	0.5	--
Chitosan	--	0.5	--	--	0.5
Carbopol p934	0.5	0.4	--	0.5	0.4
Methyl paraben	0.1	0.1	0.1	0.1	0.1
NaCl	0.45	0.45	0.45	0.45	0.45
Distilled water	Qs	Qs	Qs	Qs	Qs

TM is Timolol maleate, IG1 and IG2 are *in-situ* gel formulations, A6 is cubosome; CG1 and CG2 are cubogel formulations

entrapment efficiency of cubosome. Cubosome was then dispersed in previously prepared *in-situ* gel. *In-situ* gel was prepared by cold method using various concentrations of gellan gum and chitosan as polymers and carbopol p934 as co-polymer. Briefly, cubosome dispersion was sprinkled into previously prepared *in-situ* gel and stirred for half an hour using magnetic stirrer. Thus the obtained product is considered as cubogel. Methylparaben (0.1%) and NaCl (0.45%) were used as preservatives and tonicity adjusting agents, respectively (Yoncheva et al., 2011).

Characterization studies of cubogel

In-vitro mucoadhesion studies of cubogel

Two *in-vitro* methods were used to evaluate the mucoadhesive properties of cubogel.

Centrifugation method

In this method, the mucoadhesive properties of cubogel formulations were evaluated by calculating binding efficiency of mucin with the prepared formulation. The pig mucin was procured from Himedia, Mumbai, India. The mucin suspension (0.1%) was prepared in 0.05 M saline phosphate buffer (pH 7.4). Then 5 ml of cubogel or cubosome formulation was mixed with 5 ml of mucin suspension and the dispersion was incubated at 37 °C±2 for 1 hour and kept aside for 24 hour at room temperature. The samples were centrifuged (12,000 rpm) in cooling centrifuge (Remi, R-8C, laboratory centrifuge) for 30 min. Then supernatant liquid was collected and quantified free pig mucin by UV spectrophotometer at 251 nm. Finally, the binding efficiency of mucin with prepared formulation was calculated by following equation (Yoncheva et al., 2011):

E Mucoadhesion

$$= \frac{\text{Total mucin concentration} - \text{Mucin concentration in supernatant}}{\text{Total mucin concentration}} \times 100$$

Turbidimetric measurements

Turbidimetric measurements of prepared formulations were compared with mucin dispersion at 251 nm by ultraviolet-visible spectrophotometer. The accurately measured formulation (5 ml) were added to 5 ml aqueous pig mucin dispersion and stirred at 200 rpm. The turbidity of the formulation mucin dispersion was measured at certain time intervals and compared to the turbidity of the mucin dispersion. The increase in turbidity of mucin dispersion indicated mucoadhesive property (Rencher et al., 2016).

In-vitro drug release study

The release of TM from the cubosome dispersion was evaluated by using Franz diffusion cell (supplier; Techno scientific products, Bangalore). The cell consisted of two compartments i.e., the donor compartment and the receptor compartment. A previously soaked cellophane membrane (molecular cut off 12,000-14,000 D, Sigma, India) was placed between these two compartments. Briefly, 1 ml of TM-loaded cubosomal dispersion was placed in donor compartment above the membrane. The receptor compartment contained 25 ml of stimulated tear fluid (STF) as receptor medium. The diffusion cell was placed over a magnetic stirrer (Techno scientific products, India) maintained at 50 rpm at 37±0.5°C. At predetermined time intervals, aliquots of the release medium were withdrawn and were diluted with the receptor medium and receptor compartment was compensated with an equal volume of fresh receptor medium. The drug concentrations in the release medium at various time intervals were analyzed spectrophotometrically at 294 nm (Gadad et al., 2016).

Release kinetics studies

To understand the kinetics of drug release from TM cubosome, data obtained from *in-vitro* drug release study were fitted into a Zero-Order (cumulative percentage of drug released vs time), First Order (log cumulative percentage of drug remaining vs time), Higuchi's model (cumulative percentage of drug released vs square root of time, SQRT) and Korsmeyer–Peppas model (log percentage cumulative drug release vs log time). By comparing the r^2 values obtained, the best fit model was selected (Jain et al., 2015).

In-vitro corneal permeation studies

The procedure followed for corneal permeation studies was same as *in-vitro* drug release studies. Only difference is

cellophane membrane used in *in-vitro* release study was replaced with goat cornea. Data, obtained from *in-vitro* corneal permeability study was used to calculate cumulative percentage of drug permeated, flux (J), and apparent permeability coefficient (Papp). Apparent permeability coefficient was also calculated using the following equation:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0 \times 3600}$$

Where, dQ/dt ($\mu\text{g}/\text{cm}^2 \cdot \text{h}$) is the flux across the corneal tissue. A is the area of diffusion (cm^2), C_0 is the initial concentration of drug in donor compartment ($\mu\text{g}/\text{ml}$) and 3600 is taken as the factor to convert hour into second. The flux across the cornea was obtained from the slope of the regression line obtained from the linear part of the curve between the amount permeated (Q) versus time (t) plot and lag time (T_{lag}) was extrapolation of the linear portion to the x-axis (Dave et al., 2015).

Evaluation of corneal hydration level (HL)

Corneal hydration level was determined by gravimetric analysis. First, immediately after the completion of *in-vitro* corneal permeation study, wet cornea was carefully removed and weighed (W_w). Then it was soaked in 1 ml of methanol and each corneal sample was then desiccated at 70°C for 12 hours to give the corresponding dry corneal weight (W_r) (Dave et al., 2015). The corneal hydration level (HL %) can be calculated by the following equation:

$$\text{HL\%} = \frac{W_w - W_r}{W_w} \times 100 \%$$

Stability studies

Stability testing of prepared formulation was done to ensure the efficacy, safety and quality of active drug substance in dosage forms during the storage. The stability study was carried out on cubogel containing Timolol maleate. Cubogel formulations (CG1 and CG2) were stored in tightly closed amber colored glass containers sealed with aluminum foil at refrigeration temperature ($4.0^\circ\text{C} \pm 1.0^\circ\text{C}$), room temperature ($25^\circ\text{C} \pm 2^\circ\text{C}/60\% \text{RH} \pm 5\%$), and accelerated temperature ($40^\circ\text{C} \pm 2^\circ\text{C}/75\% \text{RH} \pm 5\%$), respectively, for the period of 6 months in stability chamber (Lab Top, stability chamber, Mumbai, India). At the end of the first, second, third and six month, the samples were withdrawn and evaluated for pH, entrapment efficiency, drug content and *in-vitro* drug release (Morsi et al., 2015).

Statistical analysis

All experiments were performed in triplicate and data were reported as a mean \pm SD. Student's *t*-test was performed on the data sets using SPSS 16.0 for Windows®. Differences were

considered significant for *P* values < 0.05 .

Results and Discussion

For the preparation of TM cubosome, GMO and poloxamer 407 was used as lipid and non-ionic surfactants, respectively. In all formulation concentration of GMO was kept constant and concentration of poloxamer 407 was changed in order to determine the effect of surfactant on particle size, entrapment efficiency and *in-vitro* drug release profile of the drug. Then optimized batch of cubosome was selected based on entrapment efficiency and *in-vitro* drug release and finally dispersed in *in-situ* gel to form TM cubogel.

Determination of λ_{max} and calibration curve

TM showed absorption maximum (λ_{max}) at 294 nm by UV method. The concentration range over which the drugs obeyed Beer-Lambert's law was found to be between 5-30 $\mu\text{g}/\text{ml}$ for Timolol. The UV spectrum of TM is shown in figure 1A. Calibration curve was obtained by plotting absorbance against drug concentrations (figure 1B) and the correlation coefficient obtained was found to be 0.997.

Compatibility study using FT-IR

Compatibility study was conducted by using Fourier spectrophotometer to get FTIR spectrum. FTIR spectrum so obtained deals about characteristic of entire molecule and provides structural information by referring to peaks associated with characteristics groups. Timolol maleate

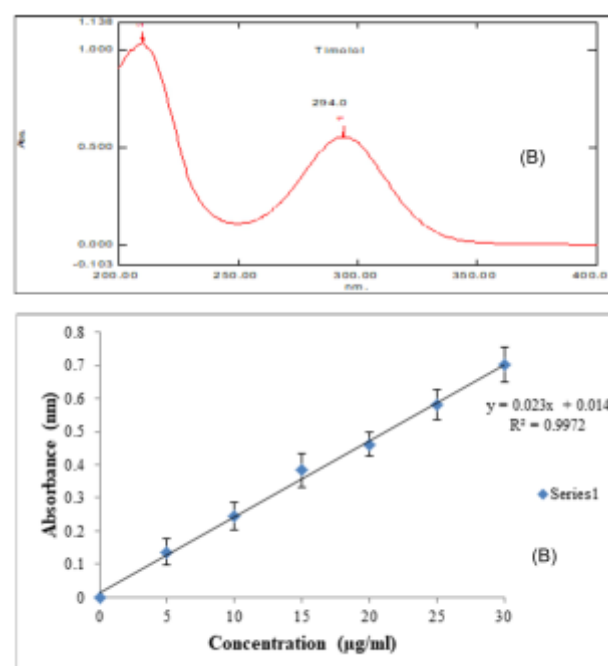


Figure 1. UV spectrum of Timolol maleate in STF (A) Calibration curve of Timolol maleate in STF (B)

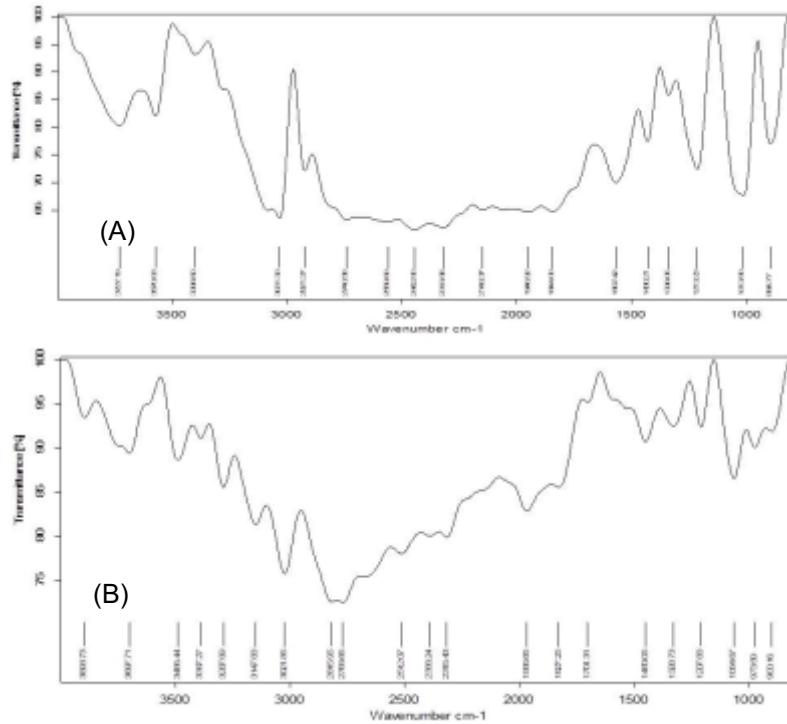


Figure 2. (A) Infrared spectrum of Timolol maleate (B) Infrared spectrum of Timolol maleate and GMO

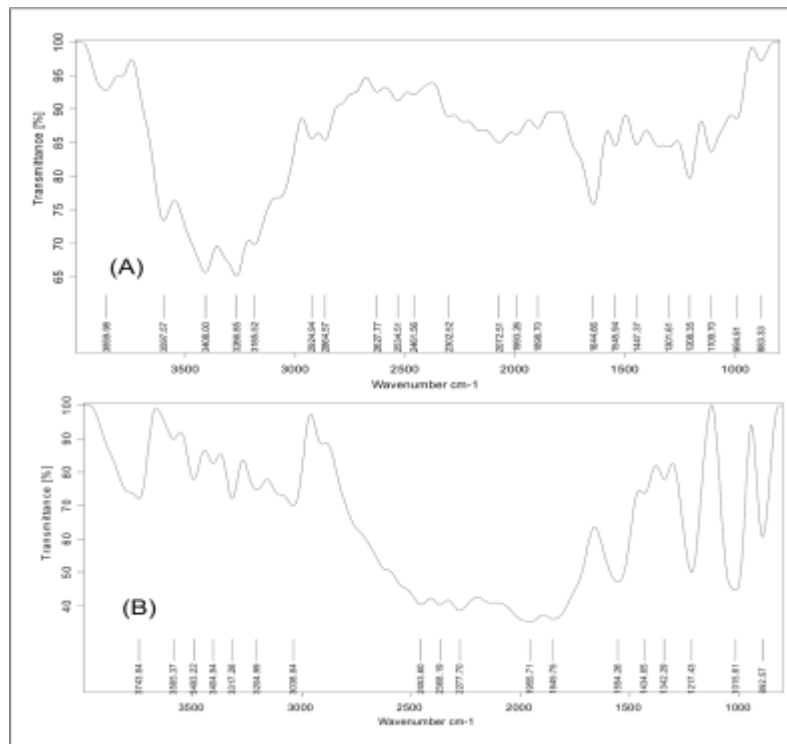


Figure 3. (A) Infrared spectrum of Timolol maleate and poloxamer 407 (B) Infrared spectrum of Timolol maleate and its physical mixtures

pure drug showed a broad band spectrum at 3570.83 cm^{-1} due to O-H stretching, bands at 3398.80 cm^{-1} was due to N-H stretching bond, bands at 2921.27 cm^{-1} was due to aliphatic C-H stretching vibration, while band at 1844.0 cm^{-1} and 1430.21 cm^{-1} was due to acid carbonyl group of maleic acid and N-H bending vibration respectively. Bands at 1567.42 cm^{-1} was due to C=N stretching

vibrations, whereas band at 894.77 cm^{-1} are due to hydroxyl C-O stretching vibrations. Presence of all characteristics peak of Timolol maleate in its physical mixture and cubosome formulation indicated the absence of any interaction between the drug and excipients.

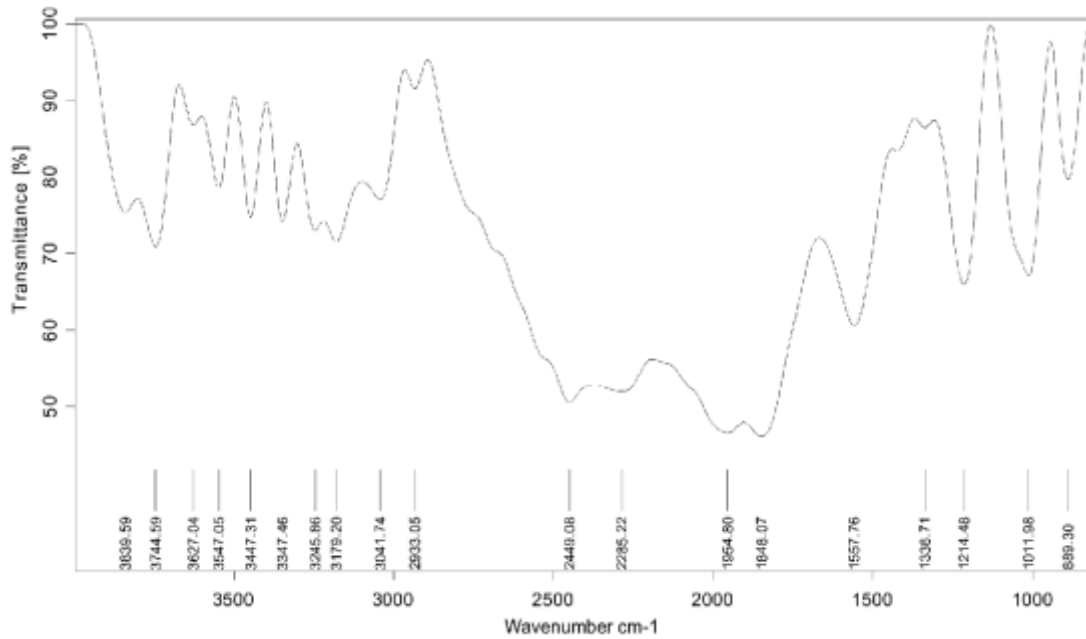


Figure 4. Infrared spectrum of Timolol maleate loaded cubosome

Compatibility studies by DSC

The thermal behaviour of TM and its physical mixture was determined using DSC studies. The results of DSC studies are shown in figure 8A (pure drug) and figure 8B (drug excipients

mixture). The thermogram of pure drug exhibited a single endothermic peak at temperature of 209.5°C corresponding to the melting point of the drug. This is a sharp significant and intense peak at melting point of the drug. The

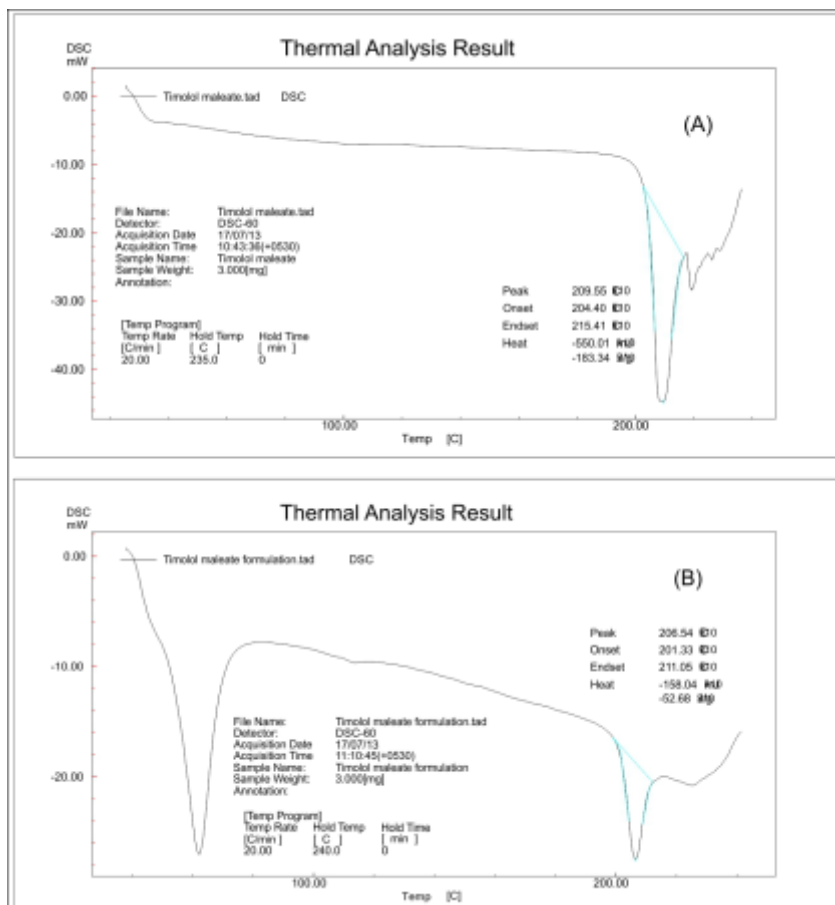


Figure 5. DSC thermogram of pure drug TM (A) and (B) drug physical mixture

thermogram of drug and excipients mixtures exhibited an endothermic broad peak at 206.5°C which is in the same reported melting point range of the drug and poloxamer 407 showed an endothermic peak at 53°C. There was no appearance and disappearance of one or more new peak corresponding to these of the individual components thus indicating the absence of drug polymer interaction.

Visual inspection

All cubosomal dispersions were milky white liquid in appearance. The formulation batches A3 and A4 showed phase separation whereas, batches A1, A2, A5, A6, A7, A8 and blank does not showed any sign of phase separation i.e. physically stable. The main purpose of visual inspection was to exclude the very poor dispersions from the further study. Hence, formulation batches with 0.8% and 1.0% poloxamer 407 were rejected whereas, other bathes of cubosomal dispersion were found to be stable and hence selected for further studies.

Particle size and zeta potential determination

Particle size of cubosomes has an important effect especially on the dispersion of ophthalmic formulations. Particle size is a vital parameter for absorption or transportation through the

ocular barriers and should not be more than 10 μm . The mean particle size of cubosome was found in the range of 3.174 ± 0.45 - 191.6 ± 8.96 nm. No significant difference in the particle size between formulations prepared with various concentration of poloxamer was observed in formulation batches A1–A4. The impact of additives on the particle size of the cubosome was also investigated. Addition of oleic acid showed no significant influence on particle size of cubosome size with the exception of glycerol. Addition of glycerol slightly increases the particle size of the cubosome (>150 nm). Thus, the Timolol maleate loading was found to only marginally increase the mean particle size of the cubosomes. The PDI values were lower than 0.4 for the formulations containing only poloxamer 407 as stabilizer, suggesting narrow size distribution and suitability of preparation method employed in this study. Whereas, in case of cubosomal dispersions A7-A8, PDI was increased due to the addition of additives and PDI value was remained in the range which indicates monodispersed dispersion. Surface charge of the cubosome was determined by zeta potential analysis. Zeta potential is another important index for the stability of the cubosomes. The zeta potential value was found in the range of -34.7 ± 2.68 mV to -9.92 ± 0.98 mV. The

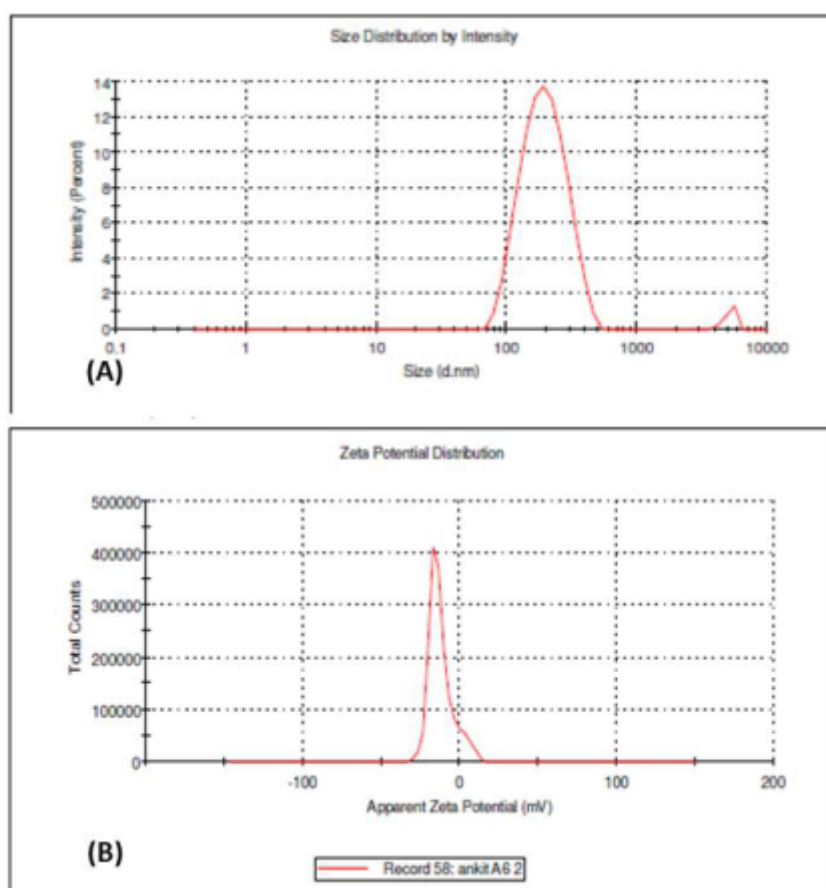


Figure 6. (A) Particle size distribution of cubosome (B) Zeta potential distribution of cubosome

Table 3. Results of particle size, PDI and zeta potential of drug loaded cubosomes

Formulation	Particle size (nm)	PDI	Zeta potential (mV)	Entrapment efficiency (%)
Blank	3.174±0.45	0.228	-15.9±1.35	--
A1	5.320±1.73	0.229	-10.0±0.99	90.09
A2	7.799±0.47	0.389	-9.92±0.98	91.50
A3	7.814±0.82	0.391	-11.9±1.33	89.45
A4	24.32±0.35	0.388	-11.4±1.64	91.73
A5	178.8 ±7.68	0.268	-21.4±1.12	98.77
A6	191.6±8.96	0.279	-12.3±1.36	98.91
A7	7.792±1.27	0.403	-34.7±2.68	92.28
A8	24.48±2.34	0.417	-24.8±2.76	92.66

lower zeta potential value of the cubosomes in this study might be presence of non-surfactants, as it stabilizes the cubosomal dispersion by a steric hindrance to avoid aggregation during storage. The zeta potential was found to be lower in the cubosomal dispersion A5, A8, which in turn indicates high negative surface charge on the cubosomes which in turn indicates higher stability because of the anticipated surface repulsion between similar charged particle. Table 3 and figure 6A and 6B showed the results of particle size, PDI and zeta potential.

Determination of entrapment efficiency (EE)

Drug entrapped into the cubosome was found in the range of 89.45-92.66% for the formulation batch A1, A2, A3, A4, A7 and A8 and in these batches EE was not dependent on the concentration of surfactant (Poloxamer 407) used. Whereas, formulation batches A5 and A6 containing glycerol showed more than 98% of EE, this might be due to the fact that glycerol lends drug to be easily incorporated in the hydrophobic region of the cubosome. Table 3 showed the EE of all batches of TM cubosome.

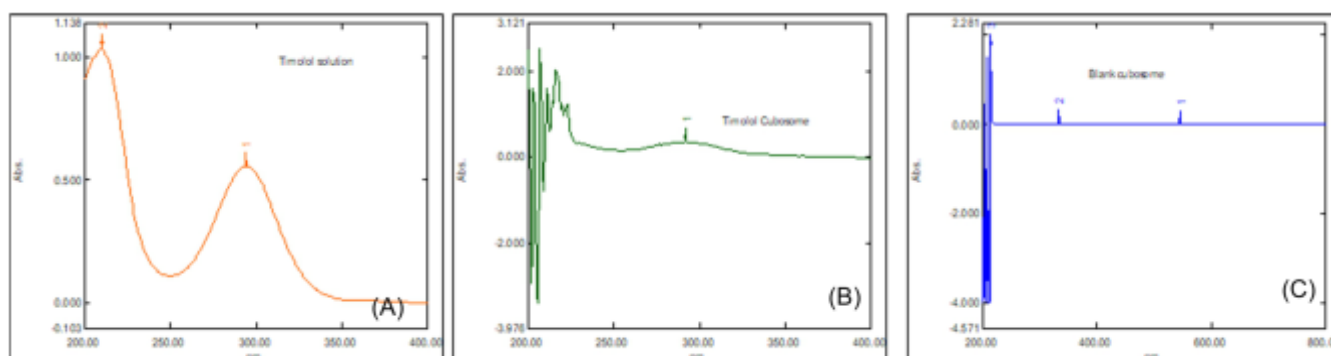
UV Spectroscopic examination

The UV spectroscopic examination was performed to determine the aggregation state of Timolol maleate. Generally,

in aqueous solution, Timolol maleate is aggregated and showed absorption maximum at 294 nm. In this study, the absorption spectra of Timolol maleate in three samples, including Timolol maleate solution, cubosomes containing Timolol maleate, and a blank cubosomes which all contained 5 mg/ml of Timolol maleate as well as blank cubosomes, were compared to demonstrate the successful entrapment of Timolol maleate in cubosomes. Timolol maleate in solution form showed broad absorption band at 294 nm whereas, the spectroscopy of cubosomes containing Timolol maleate showed the absorption peak at different position with very lower intensity. These findings suggested that Timolol maleate was highly encapsulated in the cubosomes. Hence, UV spectroscopy study showed that when the drug was incorporated in the cubosome, its absorption peak position and intensity were dramatically influenced. The UV spectra of Timolol maleate were showed in the figure 7.

Particle morphology by optical microscopy and SEM

Surface morphology of prepared cubosomes was examined under optical microscope. When the samples were visualized using optical microscope, cubosomes were bilayer in shape with smooth surfaces. Photomicrograph also showed that cubosomes are well separated from each

**Figure 7.** UV spectra of TM (A) TM solution, (B) TM cubosomes, (C) blank cubosomes

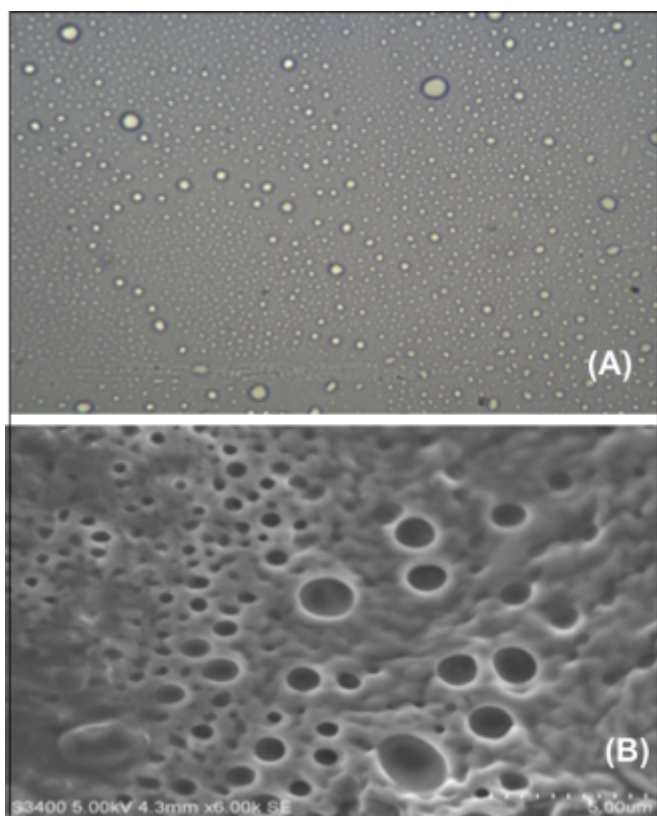


Figure 8. (A) Optical microscopy image of cubosome (B) SEM images of TM- loaded cubosome

other. The surface morphology of TM-loaded cubosome was also determined by SEM. The SEM image revealed that the drug-loaded cubosomal nanoparticles were bilayer in shape, with no sign of aggregation. Results of optical microscopy and SEM studies are showed in figure 8 A and 8B, respectively.

Drug content estimation

Drug content was determined using UV method. The drug content was found in the range of 97.78 ± 0.034 - 99.26 ± 0.055 %. Hence, drug distribution was found to be uniform.

Characterization studies of cubogel

In-vitro mucoadhesion studies of cubogel

Mucoadhesive strength of cubogel formulation was

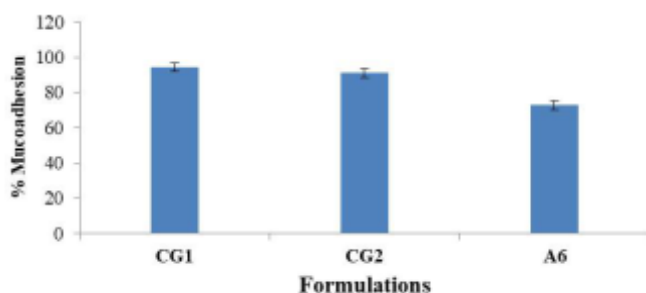


Figure 9. Comparative mucoadhesive strength of cubogel and cubosome by centrifugation method

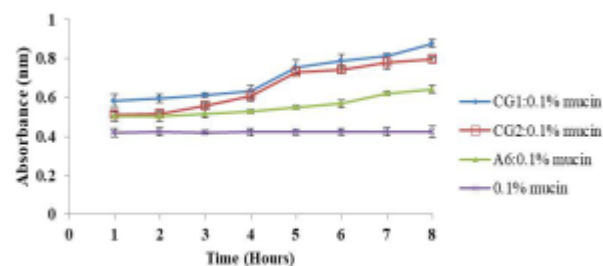


Figure 10. Estimation of the interaction between cubogel formulation and 0.1% mucin dispersion by turbidimetric method

determined by two methods. In first method, mucoadhesive strength was determined by centrifugation method, where cubogel formulation CG1 ($94.44 \pm 2.180\%$) and CG2 ($91.20 \pm 2.590\%$) showed excellent mucoadhesive strength compared to cubosome ($72.79 \pm 2.720\%$). Results of centrifugation method are showed in figure 9. In another method, turbidity of drug loaded cubogel-mucin dispersions were examined to obtain information about the mucoadhesive property of prepared formulation. The absorbance was taken up to 8 hours. The absorbance of the mucin-free aqueous cubosomal dispersions of Timolol maleate did not significantly deviated absorbance from zero (0.045 - 0.057). Therefore, some changes occurred in the turbidity of cubogel-mucin dispersions was considered as an indication for an eventual interaction occurred between mucin and cubogel, and not due to the motion of particles. Cubogel-mucin dispersions showed higher turbidity after 5 hours suggested that the interaction between particles and mucin increases. The turbidity of cubogel-mucin dispersions was higher than that of mucin dispersion alone (figure 10). This phenomenon might be due to the higher thickness of gellan gum (CG1) and chitosan (CG2) around liquid crystalline nano particles (cubosome). These findings were in agreement with another study conducted by Yoncheva et al., 2011 and Kesavan et al., 2010, where the authors reported higher mucoadhesive property of chitosan and gellan gum respectively in ophthalmic formulations.

Cubogel gel CG1 showed highest mucoadhesive strength; this might be due to presence of hydrophobic groups on mucoadhesive polymers (gellan gum and carbopol p934) and the large amount of water associated with mucin (Yoncheva et al., 2011). In this two possible adhesion mechanisms might occurs, hydrogen bonding and/or interpenetration of a swollen gel network with hydrated mucin, would seem to be possible gellan gum with carbopol p934.

The increase in mucoadhesive strength of cubogel CG2 formulation might be due to interaction of negatively

charged sialic groups of mucin with positively charged surface layer of chitosan based cubogel. Another possible mechanism might be due to the formation of hydrogen bond between carbopol p934 and oligosaccharide chains of mucin (Kesavan et al., 2010). Results of mucoadhesive studies were further correlated with the *in-vitro* corneal permeation studies.

In-vitro drug release studies

In-vitro drug release profile of prepared formulation is shown in figure 11. The cubogel formulations of TM were solutions at room temperature and turned into stiff gels at body temperature and pH. The release of TM from cubogel formulations over a period of 12 hours was compared with the TM release from the marketed formulation, cubosome and *in-situ* gelling system. The optimized batch of cubosomal dispersion (A6) exhibited a non-linear, albeit sustained release of TM over a period of 12 hour. At the end of 12 hour, almost 80% of drug was released from the cubosomal dispersion. The cubogel formulations CG1 and CG2 showed an initial burst release of 14.47% and 12.21% respectively, for the 1st hour and prolonged the release of TM for over 12 hour. At the end of 12 hour, the TM release ranged from 54.77% and 51.45% from these cubogel formulations. Whereas TM-loaded *in-situ* gel formulations, IG1 and IG2 showed more than 95% of TM was released within 10 hours. This might be due to lower drug loading capacity of *in-situ* gel formulations compared to TM-cubosome and TM-cubogel formulation.

In-vitro release kinetics studies

Mathematical models like zero-order, first-order, Higuchi model, Hixson-Crowell and Korsmeyer-Peppas models are important tool to understand the drug release kinetics of prepared formulations. The *in-vitro* release data were fitted to zero-order, first-order, Higuchi model and Hixson-Crowell kinetics models to predict the drug release mechanism from the prepared *in-situ* gel, cubosomal dispersion and cubogel formulations. The results showed that all the formulations were best explained by Higuchi release kinetics model, as the plots

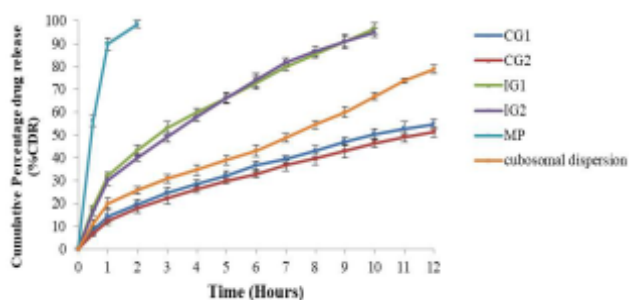


Figure 11. Comparative drug release profile of TM from cubogel, *in-situ* gel, marketed (MP) and cubosome formulation

shows high linearity ($r^2 = 0.986-0.998$) in comparison to zero order ($r^2 = 0.931-0.977$), first order ($r^2 = 0.922-0.991$) and Hixson-Crowell model ($r^2 = 0.588-0.664$). Other researchers (Shun et al., 2010; Boyd et al., 2003) have also found that *in-vitro* drug release profiles of cubosomes followed the Higuchi square root model. Hence, it can be concluded that all prepared formulation followed Higuchi's release pattern, where the drug diffuses at a slower rate as the distance for diffusion increases, referred to the square root kinetics.

To confirm the drug release mechanism, the drug release data were further analyzed using Korsmeyer-Peppas release kinetics model. For the *in-situ* gel formulations IG1 and IG2, the release exponent (n) values were found in the range of 0.90-0.93, which indicate that prepared *in-situ* gel formulation followed Super-case II transport mechanism as their 'n' values are higher than 0.89. Cubosomal dispersion and cubogel formulation showed 'n' value ranged from 0.84 to 0.87, which indicate a coupling of diffusion and erosion mechanism so-called anomalous release mechanism. This may indicate that the drug release was controlled by more than one process.

In-vitro corneal permeation studies

Ability of the substance to permeate the corneal barrier depends on various factors that is chemical nature of substance, size and conformation, lipid/water partition co-efficient, and degree of ionization, etc. Epithelium, which is lipidic in nature, is main barrier for hydrophilic drug whereas the aqueous stroma, which constitutes bulk of cornea, is the major rate limiting barrier for hydrophobic agent. Transcorneal permeation of Timolol maleate cubogel, *in-situ* gel and cubosomal dispersion was compared with marketed formulation (table 4 and figure 12). The drug permeated from marketed formulation was 19.48%, whereas cubogel formulations CG1 and CG2 released 50.01% and 47.94% in 12 hours. Cubogel and cubosomal dispersion showed a significantly higher permeation capability as

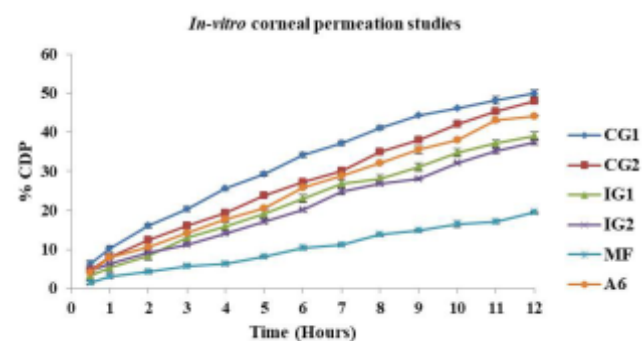


Figure 12. Comparative %CDP of prepared Timolol maleate cubogel, *in-situ* gel, cubosome and marketed formulations

compared to marketed formulation ($P < 0.05$). As showed in table 4, apparent permeability coefficient (P_{app}) of Timolol maleate in cubogel formulation CG1, CG2 and marketed formulation was 1.88×10^{-5} , 1.78×10^{-5} and 0.749×10^{-5} cm/s, respectively. Compared with marketed formulation, TM cubogel CG1 and CG2 exhibited a 2.51 and 2.376-fold increase in P_{app} , suggesting that higher amount of Timolol maleate-cubogel were taken up by the goat cornea than that of marketed eye drops.

The increase in drug permeation through these liquid crystalline nanoparticles might be due to agglomeration of cubosome as depot near the cornea from which the drug is slowly delivered to the precorneal area. Another possible mechanism might be due to higher mucoadhesive nature of the polymer used in the cubogel formulation.

Cubogel formulation CG1 contains gellan gum as gelling agent. Gellan gum form network with hydrated mucin. Gellan gum forms a clear gel in the presence of mono- or divalent cations (Na^+ , Ca^{2+} and Mg^{2+}) present in the tear fluid. These cations are particularly suited to initiate gelation of the polymer when instilled as a liquid solution into the cul-de-sac. Once gelled, the formulation resists the natural drainage process from the precorneal area. Residence at the site of drug absorption is prolonged and, subsequently, the bioavailability of the drug is increased (Yoncheva et al., 2011).

Cubogel formulation CG2 contains chitosan as gelling agent. The increase in mucoadhesive strength of cubogel CG2 formulation might be due to interaction of negatively charged sialic groups of mucin with positively charged surface layer of chitosan based cubogel (Yoncheva et al., 2011). Another possible mechanism might be due to the formation of hydrogen bond between carbopol p934 and oligosaccharide chains of mucin.

Corneal hydration level (%HL)

The percent corneal hydration is frequently used to evaluate the *in-vitro* corneal damage. According to Saettone et al., the

normal cornea has a hydration level of 76-80%. When a hydration level exceeds more than 83%, it indicates the damage of the corneal epithelium and/or endothelium (Rencher et al., 2016). In this study corneas treated with marketed formulation was considered as control and its results were compared with hydration level of test formulation. The average hydration level of control corneas was $80.77\% \pm 1.23$. Almost same values of percentage hydration level are obtained for cornea exposed to the test formulations. Both control corneas and corneas treated with test formulations showed percentage hydration level in the range of $80.77 \pm 1.23\%$ to $82.04 \pm 0.92\%$. The percentage corneal hydration was less than 83%, which indicated the integrity of the corneas was maintained throughout the experiments. Hence, there was no damage to the corneal epithelium or endothelium had occurred during experiments. Results of corneal hydration level are shown in table 4.

Stability studies

The cubogel formulations CG1 and CG2 were subjected to stability studies at $4.0^\circ\text{C} \pm 1.0^\circ\text{C}$, $25^\circ\text{C} \pm 1^\circ\text{C}$, and $40^\circ\text{C} \pm 2^\circ\text{C}$ for the period of 6 months. The samples were withdrawn at 0, 1, 2, 3, and 6 months and analysed for their physical appearance, entrapment efficiency, drug content, and the *in-vitro* release profile. The results showed that there were no significant changes in physical appearance of cubogel (milky white in appearance) stored at different storage conditions. Furthermore, there was no significant ($P > 0.05$) change in entrapment efficiency EE (%), drug content and *in-vitro* drug release behaviour (data not shown), indicating good stability behaviour cubogel formulation.

Conclusion

During this study cubosomal dispersion of TM was prepared by top down technique. Form all prepared cubosomal dispersion, formulation A6 was considered as optimum batch based up on entrapment efficiency and *in-vitro* drug release

Table 4. Steady state fluxes (J), apparent permeability coefficient (P_{app}), lag time (min) and corneal hydration levels (HL) of various formulations

Formulation code	J ($\mu\text{g}/\text{cm}^2 \cdot \text{h}$)	$P_{app} \times 10^{-5}$ (cm/s)	Lag time (min)	Hydration levels (HL) (%)
MF	10.12	0.749	5.95 ± 0.25	80.77 ± 1.23
CG1	25.38	1.88	56.07 ± 1.22	81.75 ± 1.15
CG2	24.84	1.78	29.22 ± 0.32	82.04 ± 0.92
IG1	21.08	1.56	18.47 ± 0.51	80.96 ± 1.66
IG2	19.24	1.41	20.40 ± 0.48	81.34 ± 0.98
A6	23.31	1.72	24.56 ± 0.37	80.82 ± 1.16

profile. TM cubogel formulation was prepared by dispersing optimum batch of cubosome in to *in-situ* gel. *In-vitro* corneal permeation efficacy of cubogel formulation was significantly higher than marketed formulation and plain *in-situ* gel. Six months of stability study showed no change in drug content and percentage drug release when stored at refrigerator, room and accelerated. Finally it can be concluded that TM cubogel could be a good alternative for conventional eye drops, as it sustained the drug release for longer duration and may also reduce the number of application of drug. Further animal studies will be performed to understand *in-vivo* efficacy.

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Conflict of Interests

The authors declare no conflict of interests regarding the publication of this article.

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