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Research Article

Preparation and evaluation of liposomes of brimonidine tartrate as an ocular drug delivery system

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ABSTRACT

Liposomes of brimonidine tartrate were prepared by film hydration method. The prepared vesicles were evaluated for photomicroscopic characteristics, entrapment efficiency, *in vitro, ex- in vitro* drug release, *in vivo* intraocular pressure lowering activity. Method employed for preparation of vesicles was found to be simple and reproducible, produced vesicles of acceptable shape and size with unimodal frequency distribution pattern. The size of sonicated liposomes was found to be approximately 210 nm and drug loading was approximately 30- 40%. The *in vitro, ex- in vitro* drug release studies showed that there was a slow and prolonged release of drug which followed zero order kinetics. The intra ocular pressure lowering activity of prepared formulations were determined and compared with pure drug solution. It was found that intraocular pressure lowering action was sustained for longer period of time than the pure drug solution. Stability studies data revealed that the formulations were found to be stable when stored at refrigerator temperature (2 °C to 8 °C) with no change in shape and drug content. Results of the study indicated that it is possible to develop a safe and physiological effective topical liposomal formulation which is patient compliance.

Keywords: liposome; film hydration; brimonidine tartrate; intraocular pressure.

INTRODUCTION

The main objective of drug delivery system in ocular therapeutics is to improve existing ocular dosage forms and exploit newer drug delivery system for improving the bioavailability of existing drug molecule. The most common method of administering drugs to the eye in the treatment of ocular diseases is the topical application of eye drops. Although the topical and localized applications are still an acceptable and preferred route to achieve therapeutic level of the drug at the site of action in ocular drug delivery system, such dosage forms are no longer sufficient to combat various ocular diseases like glaucoma due to poor bioavailability, which is a result of efficient mechanism protecting the eye from harmful materials and agents. Such protecting mechanism 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 incompliance. The development of various vesicular drug delivery systems allows the entrapment of drug molecule into lipid bilayer or surfactant vesicles and thus increased drug concentration at site of application with sustained drug delivery of medicament which results in improved bioavailability. Such vesicles (liposome and niosome) acts as carrier for controlled ocular drug delivery by preventing metabolism of drug from enzymes present at the corneal epithelial surface. Vesicle entrapped drug can be easily administered in liquid dosage forms such as eye drops with patient compliance, modulated drug release profile and high drug pay load1 (Amaranth S & Uma S, 1997 and Kaur IP *et al* 2004). Brimonidine tartrate is α_2-adrenergic agonist indicated in open angle glaucoma, which is common form of glaucoma. Glaucoma is group of diseases of optic nerve involving loss of retinal ganglion cells. Increased intra ocular pressure (IOP) is significant risk factor for the development of glaucoma. At present the eye drops (0.2%) of the said drug is available in the market all over the world. However, the drug has to be instilled into the eye 3-4 times a day (Dong H S *et al* 1997 and Anuja Bhandari *et al* 1997). To avoid such frequent administration of the drug, in the present study an attempt was made to develop a liposomal drug delivery system of brimonidine tartrate for ocular administration and investigated its intraocular pressure lowering activity.

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MATERIALS AND METHODS

Materials

Brimonidine tartrate was a gift sample from Indoco Remedies Ltd Goa. 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.

Preparation of liposomes

Liposomes were prepared by passive loading technique- thin film hydration method as per the method described by Bangham *et al*., 1965. 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 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 brimonidine tartrate 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 (multilamellar 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.

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, and LF3.

Photo microscopic study of liposomes

The liposomal suspensions was subjected to size analysis under a microscope (10×400 magnification) fitted with a calibrated ocular micrometer. The shape of prepared liposomes was also studied (Law SL & Shih CL, 2001).

Table 1: Composition of formulations and their characterization

Formulation code	Formulation Ratio (Drug: Cholesterol:DPCC)	Average particle size (µ)	Percentage drug entrapment efficiency
LF 1	1:1:1	5.00	32.34
LF 2	1:1:2	6.97	39.78
LF 3	1:1:3	7.37	42.43

Drug entrapment efficiency determination

Entrapment efficiency of brimonidine tartrate in the liposomes was determined as follows: After sonication, 1 ml of liposomal suspension (SUVs) was taken in a 1 ml micro-centrifuge tube. Centrifuged at 20,000 rpm for 1 h at 4 ºC in a cold centrifuge to get a white pellet of SUVs this was settled at the bottom of the centrifuge tube. Supernatant was separated as it contains unentrapped drug which is highly soluble in PBS 7.4 using a micro-pipette. To the remaining pellet in the centrifuge tube 500 µl of 0.1 N NaOH (as drug is highly soluble in 0.1N NaOH) was added and vortexed thoroughly for 3 min. After vortexing a white suspension was obtained and 1 ml of this suspension was taken in a micro-pipette and transferred to a test tube. To this 5 ml methanol was added which resulted in a clear solution, this was further vortexed in a vortex mixer for 2 min (such that to ensure that the liposomes are lysed completely to release the drug). This solution (1 ml) was further diluted with methanol and the absorbance

was determined using a Shimadzu UV spectrophotometer (Deepika Aggarwal *et al* 2002).

The entrapment efficiency (EE) was calculated using the following formula:

$$\text{Percentage entrapment (\%EE)} = \frac{\text{Entrapped drug (mg)}}{\text{Total drug added (mg)}} \times 100$$

***In vitro* drug release study**

In vitro drug release study for liposomal formulations was studied by membrane diffusion technique as reported in the literature (Deepika Aggarwal *et al* 2002). *In vitro* diffusion cell was made using cellophane membrane as a semipermeable membrane. The diffusion medium was 19 ml of freshly prepared glutathione bicarbonated ringer (GBR) equilibrated at 37± 0.5 °C temperature. The pH of the medium was maintained at 7.2-7.4 by passing CO_2. This medium closely resembles to simulated tear fluid (STF). The samples were analyzed spectrophotometrically for concentration of brimonidine tartrate at 320 nm. The experimental data was subjected to statistical analysis, using one-way ANOVA. P< 0.05, was considered to be statistically significant.

***Ex- in vitro* drug release study**

Ex- in vitro drug release study for prepared liposomes was studied by membrane diffusion technique. In this study porcine cornea was used as diffusion semipermeable membrane. All the procedures followed were similar to that explained under i*n vitro* drug release study except the cellophane membrane was replaced by fresh porcine cornea (Deepika Aggarwal *et al* 2002).

***In vivo* intra ocular pressure lowering activity**

In vivo intra ocular pressure lowering activity of selected liposomal preparation (LF3) of brimonidine tartrate was studied in normotensive male albino rabbits weighing 1.2 to 2.5 Kg. The animals were housed under well controlled conditions of temperature (22± 2 °C), humidity (55±5%) and 12/12 – h, light-dark cycle, was given access to food and water. The rabbits were divided into three groups, each containing of single male albino rabbit. The protocol of the experiment was approved by the Institutional Animal Ethics Committee. To induce acute glaucoma, 5% dextrose solution (15 ml/kg) was intravenously infused through marginal ear vein. The basal intraocular pressure was measured by tonometer. The drug formulations (20 µl, drug equivalent to pure drug solution 0.2%) were administered to rabbits in different sequence. In sequence 1: Drug formulations were administered 30 min before the administration of dextrose solution. In sequence 2: Drug formulations and dextrose solution were administered together. In sequence 3: Drug formulations were administered 30 min after the administration of dextrose solution. The intraocular pressure (IOP) changes were recorded every 30 min till the pressure became normal. Formulation was instilled on to corneal surface of one eye and contra lateral eye was remaining as control. Intraocular pressure (IOP) was measured by tonometry method with the help of Schiotz tonometer and mean was taken at three times fixed interval. All IOP measurements were carried out by the same operator, using same tonometer. Each rabbit was given washout period of three days after every treatment. The ocular hypotensive activity was expressed as the average difference in IOP between the treated and control eye of the same rabbit, according to equation Δ IOP = IOP of Treated Eye – IOP of control eye (Kaur IP *et al* 2000). The experimental data was subjected to statistical analysis, using one-way ANOVA. P< 0.05, was considered to be statistically significant.

Stability study

For stability testing, the sonicated liposomal suspension of SUVs was stored away from light in sealed 2 ml micro centrifuge eppendroff tubes in refrigerator (4-8 ºC) for 3 months. Sampling was done by withdrawing 100 µl of the supernatant using a micro-pipette at different time intervals of 2^{nd} day, 4^{th} day, 10^{th} day, 20^{th} day, 40^{th} day, 45^{th} day, 60^{th} day, 80^{th} day and 90^{th} day respectively. Suitable dilutions were made with PBS 7.4 whenever sample was withdrawn and UV absorbance was determined. The entrapment efficiency was calculated. from the regression equation In the present work, stability study was carried out for selected formulations LF3 and LE3, at room temperature, refrigerator (2 °C to 8 °C) and accelerated temperature (40 °C to 50 °C) for 8 weeks and evaluated for the drug content (Armengol X & Estelrich J 1995).

Determination of drug release kinetics

To know the mechanism of drug release from these formulations, the data were treated according to first-order (log cumulative percentage of drug remaining vs. time), Higuchi's (cumulative percentage of drug released vs. square root of time), and zero order (cumulative amount of drug released vs. time) pattern (Higuchi T, 1963).

RESULTS AND DISCUSSION

Liposomes were prepared by thin film hydration method as per the method described by Bangham *et al.,* 1965. The molar ratios of lipids and cholesterol were dissolved in 2 ml of a mixture of choloroform: methanol (2:1) in a 250 ml round bottom flask. The powder particles of lipid mixture don't seem to dissolve readily in the chloroform: methanol solution. So the flask was rotated for 15 min over the water bath (at a temperature above the transition temperature of the lipids) before starting the vacuum pump. A very low nitrogen flux (Through a nitrogen cylinder connected to the evaporator by an inlet rubber pipe) was set up during the preparation of liposomes to prevent too much oxygen to get dissolved. Gradually the nitrogen pressure was raised at the cylinder head until there was no pressure differential between the inside and the out-

side of flask. The pressure release valve between the cylinder and the evaporator prevents the buildup of pressure inside the apparatus. If this flux is too high the solvent may evaporate. Some of the solvent evaporates inevitably during this period, but the solution thermalizes and the lipids get dissolved. The vacuum pump was turned on after the dissolution of the lipids, together with the cooling coil which allows cold water to flow through the coil (passing cold water through the cooling coil before turning on the vacuum results in water condensating inside the evaporator). The vacuum was set appropriately (too high vacuum may create high pressure drop, resulting in violent boiling of the lipid solution leading to foaming/ bumping of the product, where as too low vacuum lead to the formation of small air bubbles). This can further be controlled by the nitrogen flux. Again, a high nitrogen flux may result in a too rapid evaporation of the solvent, resulting in the formation of a non-uniform film deposited on the walls of the flask, which is undesirable. The flask was rotated for about 1 h with intermittent vacuum and nitrogen flux, to obtain completely dried thin lipid film with minimal chloroform residue (if the chloroform residue was found to be present, the flask containing the thin film must be placed inside the vacuum desiccator for few hours).These formulations were characterized (Table 1). The average size of liposomal formulations (MLVs) ranged from 5.00 µ to 7.37 µ and showed unimodal normal symmetrical frequency distribution patterns. All the vesicles were found to be spherical in shape (Fig 1). Further, the sonication of the MLVs has resulted in much smaller vesicles, which is very essential in avoiding the irritation to the eye. The size of particles in ophthalmic apart from influencing bioavailability, plays important role in the irritation potential of formulation, hence it is recommended that particles of ophthalmic solution should be less than 10 µ to minimize irritation to the eye (Kaur IP *et al* 2000). Further, the size of sonicated liposomes was found to be approximately 210 nm.

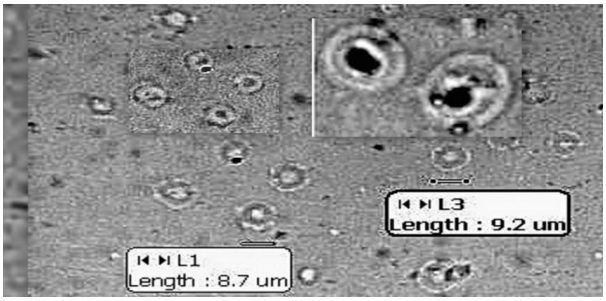

Figure 1: Photomicrograph of liposomes (10×400 magnification)

The results of drug entrapment study showed that the amount of drug entrapped in liposomes ranged between 32.33 % to 43.4 %. Entrapment efficiency increased with increase in D/L ratio as shown in the calculation above. Increase in size of the liposomes also increased the entrapment efficiency.

The comparative *in vitro* drug release profile summarized in Table-3, for pure drug solution and for each formulation. It was observed that pure drug solution released approximately 75% of drug within 2 h, while liposomal formulations LF3 and LF2 showed 18.92 % and 22.50 % drug release respectively in 8 h. The result of *in vitro* drug release profile of formulations showed that liposomal formulations provides the prolonged release of drug when compared to pure drug solution. Similarly, the comparative *ex-in vitro* drug release profile was summarized in Table 3, for pure drug solution and for each formulation. It was observed that pure drug solution released major amount of drug within 1 h, while the liposomal formulations LF3 and LF2 showed 18.89 % and 22.56 % drug release respectively in 8 h. Hence, from comparative *in vitro* and *ex-in vitro* drug release data of brimonidine tartrate from liposomes and pure drug solution, it has been observed that the amount of drug release remained similar. Further the delayed drug release rate may be attributed largely to the drug transport by diffusion controlled mechanism resulting in prolonged drug release profile. The *in vitro* and *ex- in vitro* drug release studies showed that, there was slow and prolonged release of drug from all the formulations and followed zero order kinetics (r^2 values were nearer to 1 with zero order compared to other release kinetic). This indicated that the drug release was independent of concentration of drug entrapped.

To study the *in vivo* performance of prepared formulations, intra ocular pressure lowering activity was determined. It was found that in sequence 1, where drug formulations were administered 30 min before the administration of dextrose solution (Fig 2), intraocular pressure lowering activity with liposomal formulation was sustained for longer period (3-4 h). However marketed product though showed activity within 30 min, but could not sustain for more than 60 min. Further, there was no increment in the intraocular pressure was observed with the eye treated with liposomes. In sequence 2, formulation and dextrose solution were administered together; intraocular pressure lowering activity with liposomal formulation was observed after 60 min considering that 30-60 min time is required to produce the effect (glaucoma). Whereas for marketed product, the effect was observed immediately. The duration of intraocular pressure lowering activity remained more or less similar to that of sequence 1 (Fig 3). In sequence 3, Dextrose solution was administered before the administration of liposomal formulation and marketed product and intraocular pressure lowering activity with liposomal was observed after approximately 2 h. The effect of marketed product nearly abolished after 1-2 h, while effect of liposomal formulation was found to be more longer period of time and sustained for 3-4 h (Fig 4). Probably it may be due to the better partitioning of drug between vesicle and eye corneal surface. Further, it is believed that the release of drug from liposome will increase the local concen-

Table 2: Comparative In vitro dissolution profile of different formulations

Time (h)	Percentage amount of drug release*			
	Pure Drug Solution	Formulation LF1	Formulation LF2	Formulation LE3
0	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00
1	68.00 ± 1.40	03.26 ± 0.45	04.46 ± 0.99	05.81 ± 0.99
2	77.40 ± 1.20	10.86 ± 0.37	12.86 ± 0.98	12.75 ± 0.98
3	78.80 ± 1.30	13.42 ± 0.56	14.40 ± 0.99	13.76 ± 1.00
4	78.30 ± 1.20	14.38 ± 0.91	14.58 ± 0.98	16.55 ± 1.10
5	80.00 ± 1.30	15.67 ± 0.35	16.67 ± 0.97	19.05 ± 0.99
6	79.64 ± 1.40	16.23 ± 0.45	16.83 ± 0.98	19.40 ± 0.98
7	81.54 ± 1.60	16.92 ± 1.12	16.98 ± 1.00	21.11 ± 0.99
8	83.42 ± 1.10	18.12 ± 1.23	18.92 ± 1.10	22.51 ± 0.98

* Average of three readings.

Table 3: Comparative Ex-in vitro dissolution profile of different formulations

Time (h)	Percentage amount of drug release*			
	Pure Drug Solution	Formulation LF1	Formulation LF2	Formulation LF3
1	67.56±1.60	6.81±0.89	7.89±0.99	06.37± 0.99
2	78.31±1.10	12.12±0.45	13.04±0.98	13.12± 0.98
3	78.80±1.40	13.17±0.67	13.36±0.99	14.31± 1.00
4	78.98±1.30	13.98±0.28	14.00±0.98	15.87± 1.10
5	80.00±1.30	14.69±0.57	15.64±0.97	16.59± 0.99
6	80.64±1.25	16.34±0.78	17.56±0.99	18.57± 0.98
7	81.74±1.80	17.26±0.98	17.76±1.00	20.55± 0.99
8	83.12±1.30	18.39±1.05	18.89±1.10	22.56± 0.98

* Average of three readings.

tration at corneal surface, after the release from vesicle depending on passive diffusion of drug molecule across the corneal barrier. The longer contacts time of vesicles at corneal surface, leads to higher bioavailability of drug. Thus the liposome acts as drug carrier, which changes rate and extent of absorption resulting in reduction of IOP for prolonged period of time.

Further, the onset of action, duration of action, percentage IOP lowering activity and peak effective time were measured (Table 4). The IOP was recorded maximum between 1.5 h – 2.5 h (30.4 mmHg) and considered as 100% glaucoma induction. The reduction in IOP observed with liposomal formulation during the same period considered as percentage reduction in IOP. Marketed formulation showed maximum percentage reduction between 1.0 h - 1.5 h and the extent of reduction was found to be 59%, whereas, liposomes showed 39% of reduction in IOP between 2 h - 2.5 h. Though, the higher percentage reduction of IOP with marketed product observed, it could not sustain for longer period of time. However, liposomal formulations sustain the action of lowering IOP for longer period of time (3 h, Table 4).

The stability of the liposomes is a major consideration in all steps of their production and administration: from process steps to storage to delivery. A stable dosage form maintains its physical integrity and does not adversely influence the chemical integrity of the active ingredient during its life on the shelf. As the liposomes are thermodynamically unstable systems, they tend to fuse, grow into bigger vesicles resulting in breakage of the liposomes on storage which poses a problem of drug leakage from the vesicles. Unsaturated phospholipids undergo oxidation easily. Hence, in the present work only saturated phospholipid like DPPC was used to formulate the liposomes, to avoid oxidation. Therefore no any antioxidant like α-tocopherol was used. Since the saturated phospholipids have a high T_m they showed a good physical stability.

The physical stability of sonicated liposomes on storage was studied by monitoring the amount of leaked drug from liposomes into the supernatant and by the size of liposomes. The liposome size could not be measured due to the aggregation. The presence of divalent metal ions such as calcium and magnesium in the aqueous buffer causes the aggregation of liposomes particularly those with negative charge. Therefore, aqueous buffer used for the preparation of liposomes was made using double distilled water. The liposomes stored at refrigerator (2°C to 8°C), found to be sufficiently stable with no change in shape and no significant difference in drug content. At accelerated temperature, they were found not to be stable as structural changes took place because of partial hydrolysis of lipid components and

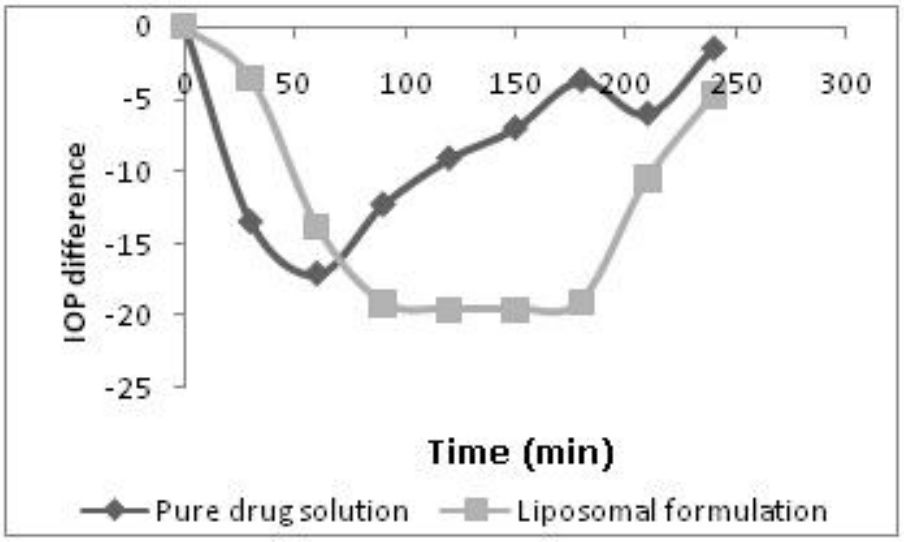

Figure 2: Change in IOP. Sequence-1 Drug formulation was administered 30 min before the administration of dextrose solution

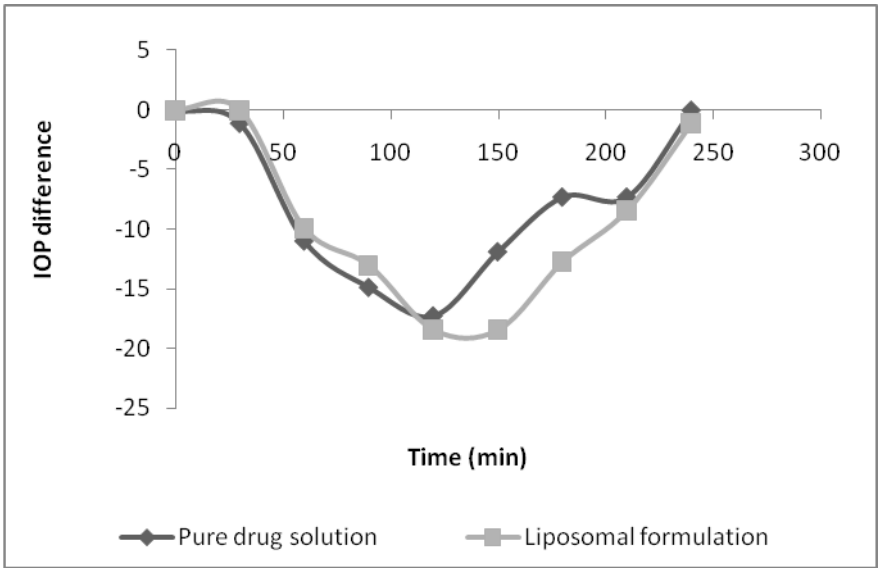

Figure 3: Change in IOP. Sequence-2. Formulation and dextrose solution were administered together

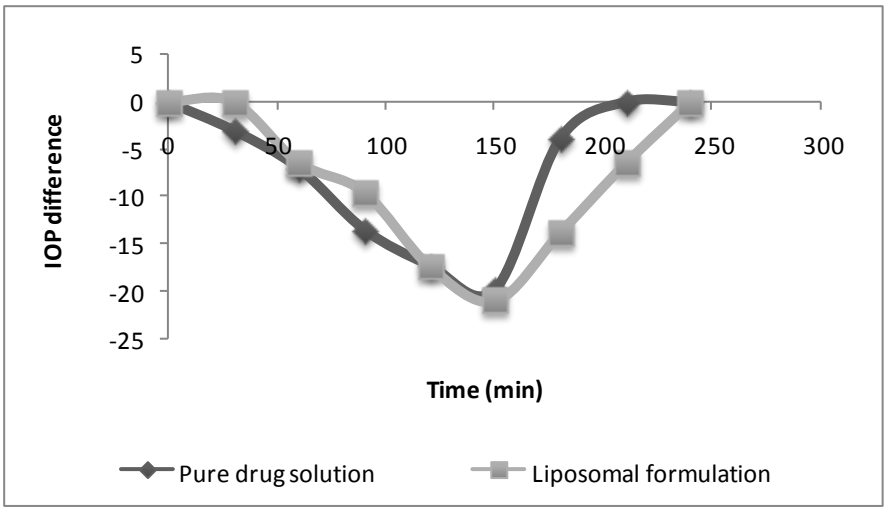

Figure 4: Change in IOP. Sequence-3. Dextrose solution was administered before formulation

Table 4: Change in IOP. Activity parameters

Formulation	Onset of time (h)	Peak effective time (h)	Duration of sustainability of action	% IOP lowering activity
Marketed formulation	0.5± 0.2	1.5± 0.2	0.5± 0.4	59
LF3	0.5± 0.4	1.1± 0.3	3.0± 0.5	39

the drug content was also found to be lower due to leakage (more amount of drug was found in supernatant).

CONCLUSION

Liposomes of brimonidine tartrate allowed a significant vesicular carrier system for therapeutic effectiveness in terms of duration of action and decrease in dose frequency. The *in vitro* and *ex- in vitro* drug release studies showed that, there was slow and prolonged release of drug from all the formulation and followed zero order kinetics. The *in vivo* intraocular pressure lowering activity of liposome formulation was found to be significant and sustained for longer period of time which encourages its physiological effectiveness. Thus liposomes offer a promising avenue to fulfil the need for an ophthalmic drug delivery system that not only has the convenience of a drop, but that can localize and maintain drug activity at its site of action for a longer period of time thus allowing for a sustained action; minimizing frequency of drug administration with patient compliance.

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