www.sphinxsai.com
PharmTech
International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.6, No.3, pp 942-950, July-Aug 2014

Development and investigation of Niosomes of Brimonidine tartrate and Timolol maleate for the treatment of glaucoma

Akhilesh Dubey[1]* and Prabhakara Prabhu[2]

*[1]Bhagwant University, Ajmer-305001, Rajasthan, India
[2]Department of Pharmaceutics, Shree Devi college of Pharmacy, Airport Road, Mangalore-574142, Karnataka, India.

*Corres. Author: akhilesh_intas@rediffmail.com
Tel. 0824-2254105, Fax: 0824-2451108, Mobile: 9986893787

Abstract: Due to the drawback of conventional therapy for ocular delivery like poor bioavailability of drugs due to tear production, non productive absorption, transient residence time, impermeability of corneal epithelium. These problems can be minimized by the application of niosomal vesicular system as well as combination of drugs provides the additive effect on reduction of IOP. The aim of the present study was to develop niosomal combination formulation of timolol maleate and brimonidine tartrate for the treatment of glaucoma. Niosomal formulations of brimonidine tartrate and timolol maleate were prepared by film hydration method. Span 60 and cholesterol used as an excipient. White rabbits of both sexes, weighing between 2 to 3 kg were used for the study**.** The prepared vesicles were evaluated for photomicroscopic characteristics, entrapment efficiency, *in vitro, ex- in vitro* drug release, *in vivo* intra ocular pressure lowering activity. Methods employed for the preparation of vesicles were found to be simple and reproducible, produced vesicles of acceptable shape and size with unimodal frequency distribution pattern. 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 intra ocular pressure lowering action was sustained for longer period of time which provides additive effect with combination. Stability study data revealed that the formulations were found to be stable when stored at refrigerator temperature (2 °C to 8 °C) and at 25 °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 niosomal formulation which is patient compliance. IOP lowering activity of the combination of timolol maleate and brimonidine tartrate in niosomes was better as compared to alone medication, which shows the additive effect of combination medication.
Keywords: Niosomes, Brimonidine tartrate, Timolol maleate, treatment of glaucoma.

Introduction

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[1, 2]. Topical and localized applications are still an acceptable and preferred route, such dosage forms are no longer sufficient to overcome the various ocular diseases like glaucoma due to poor bioavailability, due to the efficient mechanism protecting the eye from harmful materials and agents. This includes reflex, blinking, lachrymation, 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. Vesicular

drug delivery systems allows the entrapment of drug molecule into lipid bilayer or surfactant vesicles and thus increase drug concentration at the 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[3]. 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 load. Niosomes can encapsulate both lipophilic and hydrophilic drugs and protect against acidic and enzymatic effects *in vivo*. They offer several advantages over liposomes such as higher chemical stability, intrinsic skin penetration enhancing properties and lower costs. However, there may be problems of physical instability of niosomes during the storage, which includes vesicles aggregation, fusion, leaking or hydrolysis of encapsulated drugs. This may affect the stability of niosomes. Thus, niosomes entrapped through *insitu* hydrogel system has been developed to increase precorneal residence time, to minimize interference with blinking, enhance ocular bioavailability, and reduce frequency of the administration of a drug[4, 5].

Glaucoma comprises a group of chronic conditions that is characterized by progressive deformation of the optic nerve head and elevated intraocular pressure (IOP), a risk factor. It affects primarily the middle aged and elderly, the glaucoma currently constitute second most common cause of treatable blindness worldwide[6]. Timolol maleate is a beta blocker which acts by reducing the synthesis of aqueous humour production through blockade of β receptors on ciliary epithelium has a half life of 2.5-5 h. Brimonidine tartrate is an α 2 agonist, acts by decreasing the synthesis of aqueous humour and increasing the amount that drains from the eyes through uveoscleral outflow, has a half life of 3 h. 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 μl. 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 systematically via nose or gut after drainage from eye. This excessive systemic absorption not only reduces ocular bioavailability but may also lead to unwanted side effects and toxicity. In the present investigation, two main strategies are employed for improving ocular absorption are increasing the corneal permeability and prolonging contact time on ocular surface as well as combined medications which provides additive effect on reducing IOP [7].

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

Material and Methods

Materials

Timolol maleate and Brimonidine tartarate were provided by FDC Ltd. Aurangabad (India). Span 60, Span 40, and Span 20 were recieved from National Chemicals, Gujarat. Cholesterol was purchased from Merck specialties Pvt. Ltd. Mumbai. All other reagents used were of analytical grade.

Preformulation studies

Pure drug of timolol maleate and brimonidine tartrate were analyzed by FTIR for drug purity.

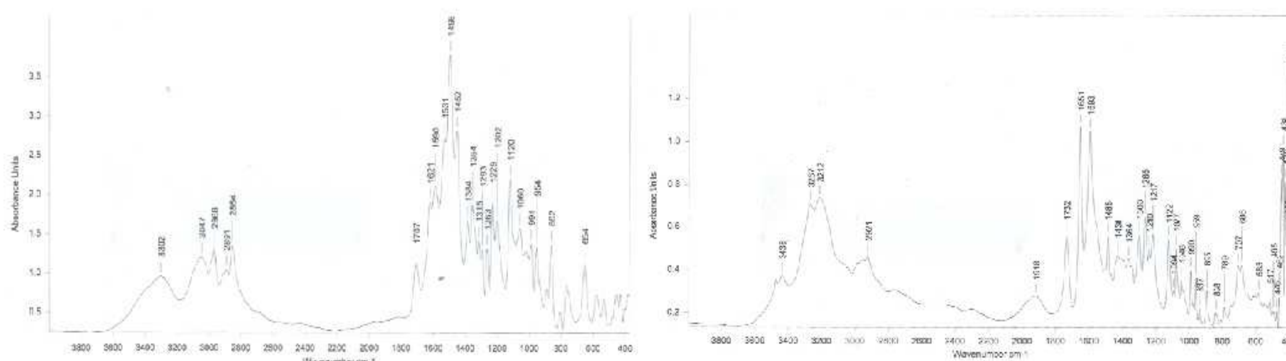

Figure. 1 FTIR Spectra of timolol maleate and brimonidine tartrate

Methods

Preparation of niosomes

In the present study six niosomal formulations of brimonidine tartrate and timolol maleate were prepared by film hydration method. All the lipid components including surfactant, span 60, as per the formula were taken in round bottom flask and dissolved into sufficient quantity (10 ml) of organic solvent (chloroform). Organic solvent was evaporated under reduced pressure, at a temperature about 60 °C, till the lipid film was formed. Dried lipid film obtained was hydrated with aqueous phase of phosphate buffer pH 7.4 (10 ml) containing drug. The flask was shaken for 1 h to get niosomal formulation. Niosomal formulations prepared were coded as F1, F2, F3, F4, F5 and F6. Once a stable 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 niosomal suspension was sonicated at 80% amplitude with a pulse of 0.5 cycles per second for a period of 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[8, 9].

Table.1 Composition of niosomal batches of TM and BT

Formulation Code	Ratio (μmol) (Surfactant : Cholesterol)	Surfactant (mg)	Cholesterol (mg)	TM (mg)	BT (mg)
F1	100:120			0.5	0.25
F2	120:100			0.5	-
F3	140:80			0.5	0.25
F4	160:60			-	0.25
F5	180:40			0.5	0.25
F6	200:20			0.5	0.25

TM-Timolol maleate, BT-Brimonidine Tartrate

Evaluation of niosomes

Microscopy

The niosomal suspensions was subjected to size analysis under a microscope (10×400 magnification) fitted with a calibrated ocular micrometer. The shape of prepared niosomes studied[10].

Drug entrapment efficiency determination

Entrapment efficiency of brimonidine tartrate and timolol maleate in the niosomes were determined as follows: After sonication, 1 ml of niosomal 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. 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 vertexed thoroughly for 3 min. After vertexing 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 vertexed in a vertex mixer for 2 min such that to ensure that the niosomes are lysed completely to release the drug. This solution (1 ml) was further diluted with methanol and the absorbance was determined using a UV spectrophotometer (Jasco V-530) [10,11].

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

In vitro drug release study

In vitro drug release study of niosomal formulations were studied by membrane diffusion technique. *In vitro* diffusion cell was made by using cellophane membrane as a semipermeable membrane. The diffusion cell consists of a beaker, magnetic stirrer with temperature control and test tube with both ends open. One end of test tube was closed using treated cellophane membrane as semi permeable membrane and other end was open to introduce the niosomal formulation. The diffusion medium was freshly prepared phosphate buffer pH 7.48 solution (100 ml) equilibrated at 37± 0.5°C temperature. The niosomal formulation (5 ml) was placed inside the diffusion cell through open end of test tube on the cellophane membrane. The diffusion medium of freshly prepared phosphate buffer pH 7.4 solution (100 ml) was placed inside the beaker such way that the lower surface of cellophane membrane makes contact with the buffer. The temperature of buffer solution was maintained at 37± 0.5 °C and stirred with magnetic stirrer throughout the study period. Aliquots (5 ml) of the medium was withdrawn every hour and replaced with fresh diffusion medium of phosphate buffer pH 7.4, to maintain constant volume (sink condition). The withdrawn samples were analysed spectrophotometrically at 292 nm and 272.2 nm for TM and BT respectively by using Shimazdu Double beam UV-Visible spectro photometer [10].

Ex in vitro release study

Ex- in vitro drug release studies of prepared niosomes were studied by membrane diffusion technique. In this study *in vitro* diffusion cell was made using porcine cornea as semipermeable membrane. All the procedures followed were similar to that explained under *in vitro* drug release study, except the cellophane membrane was replaced by fresh porcine cornea [10].

In vivo intra ocular pressure lowering activity

This study was conducted in accordance with CPCSEA (guidelines and the experimental protocol was approved by Institutional Animal Ethics Committee. The animals were housed under well controlled conditions of temperature (22± 2 °C), humidity (55±5%) and 12/12 – h, light-dark cycle, were given access to food and water. The protocol of the experiment was approved by the Institutional Animal Ethics Committee. Glaucoma was induced in rabbits by instilling prednisolone eye drops (1% w/v) upto 3-4 weeks. The study was performed on 9 white rabbits weighing 2 to 3 kg divided into three groups. First group received the F2 second group received received F6 and the third group received marketed combination eye drops in right eye and the other eye was untreated. IOP was measured using a Schiötz tonometer after instilling a drop of procaine hydrochloride local anaesthetic (1% w/v). The left eye was used as control and treatment was carried out on the right eye [11]. All the formulations were instilled into the lower conjunctival sac. At regular intervals, the IOP was measured. Change in IOP was expressed as follows:

Δ IOP= IOP untreated eye- IOP treated eye

Stability study

For stability testing, the sonicated niosomal suspension was stored away from light in sealed 2 ml micro centrifuge eppendroff tubes in refrigerator (4-8 ºC) and at room temperature (25 ºC) for 3 months. Sampling was done by withdrawing 100 μl of the supernatant using a micro-pipette at different time intervals of 2nd day, 4th day, 10th day, 20th day, 40th day, 45th day, 60th day, 80th day and 90th day respectively. Suitable dilutions were made with PBS 7.4 whenever sample was withdrawn and UV absorbance was determined. The entrapment efficiency was calculated. In the present work, stability study was carried out for selected formulation F3, at room temperature and refrigerator (2 °C to 8 °C), for 3 months and evaluated for the drug content[12].

Result and Discussion

Drug purity studies were carried out by Infrared spectral analysis. Timolol maleate 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. Brimonidine tartrate IR spectra obtained was elucidated for important groups. –NH stretching was obtained at 3438 cm−1 with a shoulder at 3437 cm−1, -CN stretching at 1300 cm−1, 1732 cm−1 indicates presence of –C=O stretching.

Niosomes were prepared by thin film hydration method as per the method described by Bangham *et al.,* 1965. The molar ratios of surfactant 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 niosomes to prevent too much oxygen to get dissolved. Gradually the nitrogen pressure was raised at the cylinder head until there was no pressure difference between the inside and outside 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. These formulations were characterized in Table 2. The size of niosomal formulations ranged from 8.00 μ to 10 μ and showed unimodal normal symmetrical frequency distribution patterns. All the vesicles were found to be spherical in shape Figure 2. Further, the sonication, resulted in much smaller vesicles, which is very essential in avoiding the irritation to the eye. The size of particles in ophthalmic dosage forms 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. Further, the size of sonicated niosomes was found to be 245. The results shows that the amount of drug entrapped in niosomes ranged between 45.10 % to 55.4 %.

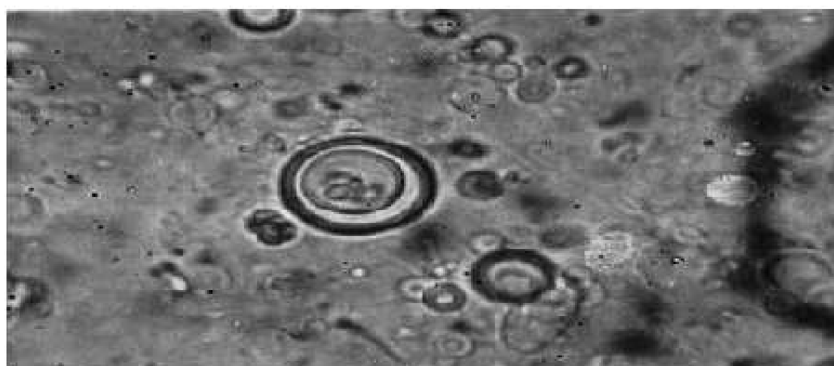

Figure.2 Microscopic view of niosomes

Table.2 Average particle size and entrapment efficiency of niosomal formulations

Formulation Code	Average Particle size μ (micron)*	Percentage drug entrapment efficiency TM	BT
F1	9.34±2.31	45.10±2.31	43.10±2.31
F2	8.23±3.21	49.85±2.45	-
F3	9.24±3.32	55.89±2.74	56.24±1.33
F4	10.23±2.45	-	50.20±2.67
F5	8.22±2.68	48.86±1.56	45.32±2.33
F6	10.73±2.45	44.20±1.56	39.13±1.38

TM-Timolol maleate, BT-Brimonidine tartrate, Data are represented as mean±SD (*n=3*)

The *in vitro* drug release profile summarized in Table 3 and Figure.3. It was observed that pure drug solution released approximately 78% of drug within 2 h, while niosomal formulations F1, F2, F3, F4, F5, and F6 showed approximately 24 to 33% drug release for TM and 20 to 31% drug release for BT respectively in 8 h. The result of *in vitro* drug release profile of formulations showed that niosomal 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 4, 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 niosomal formulations showed approximately 18 % to 30 % drug release in 8 h for TM and BT. Hence, from *in vitro* and e*x-in vitro* drug release data of TM and BT niosomes, 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 e*x- in vitro* drug release studies showed that, there was slow and prolonged release of drug from all the formulations and followed zero order kinetics. This indicated that the drug release was independent of concentration of drug entrapped.

Table.3 *In vitro* drug release pattern of niosomal formulations

Drug release studies	Percentage drug release at the end of 8th h	
	TM	BT
F1	27.19±1.05	28.23±2.23
F2	33.85±0.98	-
F3	24.92±1.10	20.32±1.33
F4	-	26.98±2.33
F5	29.39±1.32	24.83±2.44
F6	30.31±1.40	31.56±2.38

TM-Timolol maleate, BT-Brimonidine tartrate, Data are represented as mean±SD (*n=3*)

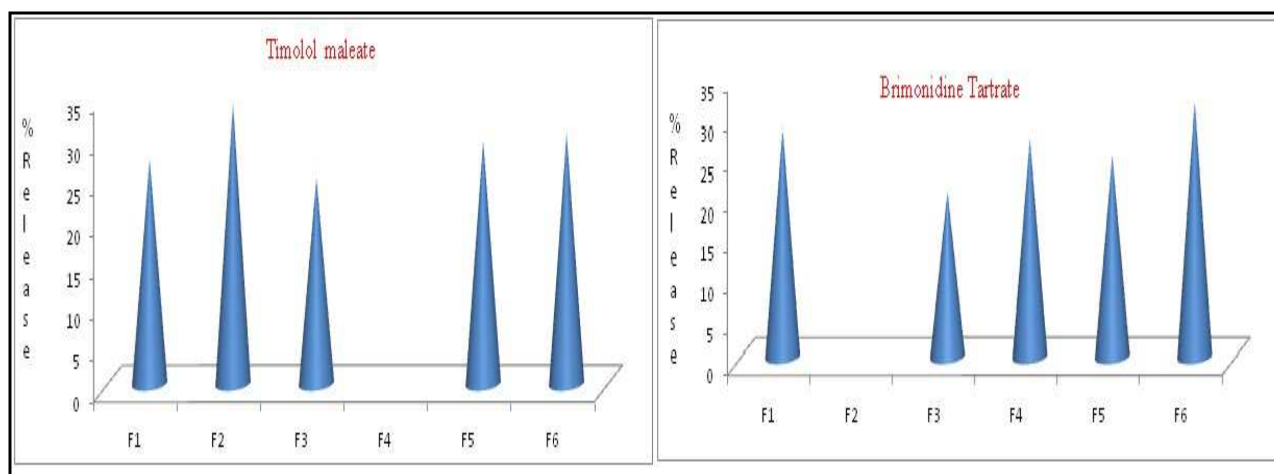

Figure 3. *In vitro* release pattern of timolol maleate and brimonidine tartrate

Table.4 *Ex in vitro* drug release pattern of niosomal formulations

Drug release studies	Percentage drug release at the end of 8th h	
	TM	BT
F1	30.81±1.07	27.23±1.29
F2	19.89±1.78	-
F3	26.92±0.10	24.92±0.93
F4	-	23.98±1.33
F5	21.39±1.11	21.83±1.10
F6	18.31±1.06	20.56±1.18

TM-Timolol maleate, BT-Brimonidine tartrate, Data are represented as mean±SD (*n=3*)

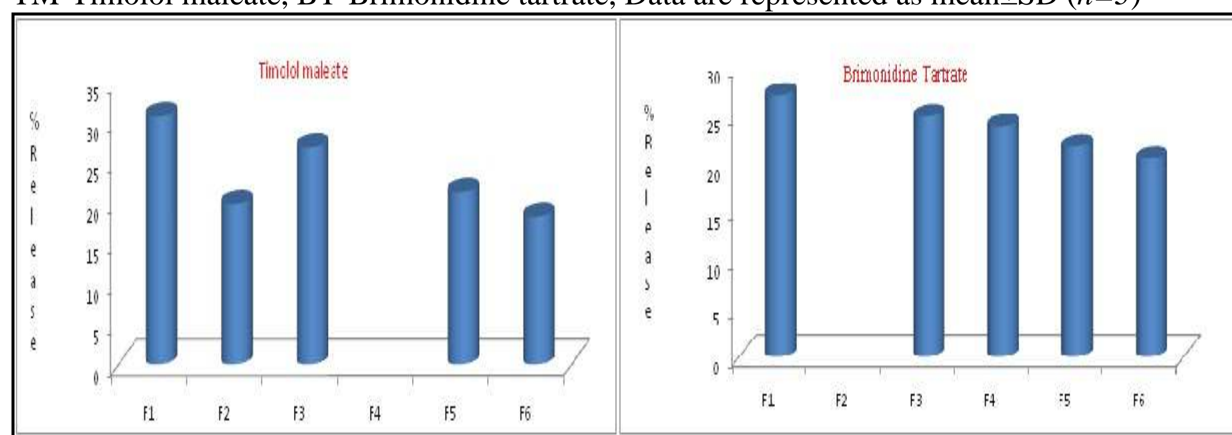

Figure 4. *Ex in vitro* drug release pattern of timolol maleate and brimonidine tartrate

The marketed eye drops suddenly lowered the intraocular pressure to a minimum and, afterwards, there was a sudden increase in the intraocular pressure whereas the niosomal formulation lowered the intraocular pressure slowly to the original and, thereafter, a gradual increase in the intraocular pressure was observed. Combigen (Marketed formulation) decreases IOP by 5mmHg whereas F1 decreases IOP by 2mmHg and F6 decreases 3mmHg at the end of 30 min. Marketed formulation showed a decrease in IOP upto 13mmHg at the end of 4hr but then there was increase in the IOP which may be due to the elimination of the drug from the site of action. Hence it was unable to sustain the activity for a long period of time which calls for frequent administration of the formulation. F2 decrease 12mmHg and F6 decrease 13mmHg at the end of 6h. The decrease in IOP was greater in combination niosomes, when compared to marketed combination. Hence the IOP lowering activity of the combination of timolol maleate and brimonidine tartrate in niosomes was better as compared to marketed formulation**.**

Table 5: ΔIOP at various time intervals

Formulation	ΔIOP(mm of Hg) at various time intervals (h) (IOP treated eye-IOP untreated eye)						
	0.5	1	2	3	4	5	6
Combigen	5.59±0.0841	7.875±0.010	11.32±0.058	12.41±0.037	13.20±0.052	12.48±0.045	11.17±0.029
F2	2.171±0.0841	3.465±0.017	5.280±0.013	6.340±0.029	9.852±0.0841	11.12±0.024	12.44±0.048
F3	3.182±0.0841	4.777±0.024	6.487±0.013	7.405±0.029	10.713±0.076	12.17±0.032	3.44±0.050

IOP-Intra ocular pressure, Data are represented as mean±SD (*n=1*)

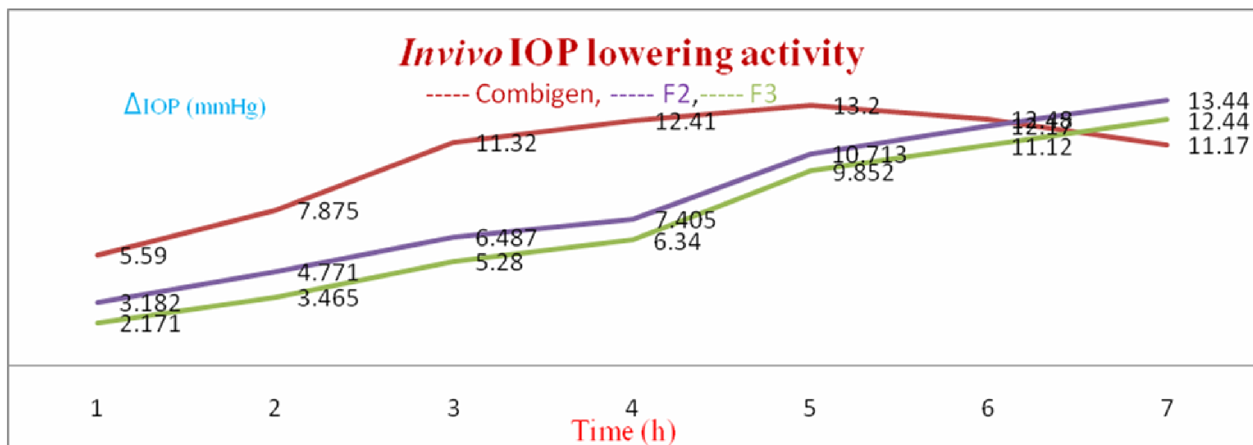

Figure 5. Effect of formulation on IOP

However, the better reduction in IOP with niosomes may probably due to the better partitioning of drug between vesicle and eye corneal surface. Further, it is believed that the release of drug from niosome will increase the local concentration 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 niosome acts as drug carrier, which changes rate and extent of absorption resulting in reduction of IOP for prolonged period of time.

Result of stability study was found to be satisfactory and acceptable. The niosomes stored at refrigerator (2 °C to 8 °C), and room temperature, found to be sufficiently stable with no change in shape and no significant difference in drug content.

Conclusion

Maintaining an adequate concentration of the medications in the eye has remained a serious practical problem to the ophthalmologist since they exhibit many disadvantages which include poor bioavailability because of rapid precorneal elimination, conjunctival adsorption, solution drainage due to induced lacrimation, tear evaporation, tear turn over, metabolism, limited corneal area and poor corneal permeability, binding of lachrymal proteins etc. To enhance the amount of active substance reaching the target tissue or exerting a local effect in the cul-de-sac the residence time of the film should be lengthened. Moreover, combination medication provides additive effect for lowering IOP. Hence a once a day combination formulation of niosomes was formulated. Timolol maleate (β-blocker) and brimonidine tartrate (α-agonist) were chosen as drug candidates for lowering the IOP.

Niosome was successfully formulated and evaluated for microscopic studies, drug entrapment studies, *In vitro*, *ex-vivo* drug release studies, *In vivo* studies and stability studies. The size of niosomal formulations ranged from 8 µ to 10 µ and showed unimodal normal symmetrical frequency distribution patterns. The result of *in vitro* drug release profile of formulations showed that niosomal formulations provides the prolonged release of drug when compared to pure drug solution. IOP lowering activity of the combination of timolol maleate and brimonidine tartrate in niosomes was better as compared to alone medication, which shows the additive effect of combination medication.

Hence, niosomal formulations offer a promising avenue to fulfill the need for an ophthalmic drug delivery system that can localize and maintain drug activity at the site of action for a longer period of time thus allowing a sustained action; minimizing frequency of drug administration with patient compliance.

Acknowledgement

The authors wish to acknowledge the Shree devi college of Pharmacy, Mangalore (Karnataka) India for providing necessary facilities and financial support to carry out this project. The authors are also thankful to FDC Ltd., Aurangabad, Maharashtra (India) for providing free gift sample of drugs.

Conflicts of interest

There are no conflicts of interest.

Abbreviation

IOP-Intra ocular pressure**, TM-**Timolol maleate**, BT-**Brimonidine tartrate**, FTIR-**Fourier transform infra red spectroscopy**, h-**Hours**,** μ-Micron, **%-** Percentage**, UV-**Ultra violet.

References

1. Prabhu P, Koland M, Harish NM, Vijaynarayan K,Dhondge G,Charyulu RN,Satyanarayan D. Preparation and evaluation of niosomes of brimonidine tartrate as ocular drug delivery system.J Pharm Res health care.2010;2:293-301.
2. Tamizharasi S, Dubey A, Rathi V, Rathi JC. Development and characterization of niosomal drug delivery of gliclazide. J Young Pharma. 2009;1:205-9
3. Prabhu P,Nitish KR, Koland M, Harish NM, Vijaynarayan K,Dhondge G,Charyulu RN.Preparation and evaluation of nanovesicles of brimonidine tartrate as an ocular drug delivery system.J Young Pharm.2010;3
4. Amaranth S, Uma S. Liposome in drug delivery: progress and limitation. Int J Pharma. 1997; 154:123-40.
5. Sandeep KS, Meenakshi C, Narayanapillay AK. Span-60 niosomal oral suspension of fluconazole: Formulation and *in vitro* evaluation. J Pharm Res Health Care. 2009; 1:142-56.
6. Anne L,Coleman, Kodjebacheva G. Risk factors for glaucoma needing more attention. Open Opthalmol J 2009; 3:38-42.
7. Syed MF,Loucks EKR.Update and optimal use of a brinzolamide-timolol fixed combination in open angle glaucoma and ocular hypertension. Clinical Ophthalmology 2011; 5:1291-96.
8. Deepika A, Alka G, Indupal K. Development of topical niosomal preparation of acetazolamide: preparation and evaluation. J Pharm Pharmacol. 2002; 56: 1509-17.
9. Bangham AD, Standish MM, Watkens JC. Liposomes by film hydration technique. J Mol Biol. 1965; 13: 238.
10. Kaur IP, Singh M, Kanwar M. Formulation and evaluation of ophthalmic preparation of acetazolamide. Int J Pharm. 2000; 199: 119-27.
11. Indu PK**,** Alka G, Anil K, Deepika A. Vesicular systems in ocular drug delivery: An overview. Int J Pharma. 2000; 269: 41-14.
12. Anuja B, George A, Michale E, Selim O, Lin W. Effect of brimonidine on optic nerve blood flow in rabbits. Ame J Ophtha. 1999 ; 128: 601-05.
13. Armengol X, Estelrich J. Physical stability of different liposome compositions obtained by extrusion method. J Microencapsul. 12; 1995:525-535.

*****