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Research Article

Niosomal gel containing dipivefrin hydrochloride for glaucoma treatment: Development, characterization and *In vitro-In vivo* assessment

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Abstract
The present study was aimed on developing and characterizing niosomal gels loaded with adrenergic agonist; dipivefrin HCl for prolonging precorneal residence time and improving bioavailability of drug for glaucoma treatment. Dipivefrin HCl niosomes were prepared using various non-ionic surfactants (span 20, span 60 and span 80) in the presence of cholesterol in different molar ratios by ether injection method. The selected formulations were incorporated into carbopol 934 and locust bean gum-based gels. TEM studies confirmed that niosomes formed were white and spherical in shape and has a definite internal aqueous space with uniform particle size. Formulation F4 composed of span 60 and cholesterol (1:1) gave the highest entrapment (92.16±0.25%) and slower release results after 8 hours (Q8h=61.05±2.87%) among other formulations. The *in-vitro* drug permeation studies showed that there was a slow and prolonged release of drug from niosomal gel formulations as compared to niosomes itself. Considering the *in-vitro* release, niosomal gel formulation G2 were the best among the studied formulations. Gel formulation G2 showed higher spreadability (2.21±1.05 g.cm/s), higher bioadhesive strength (2314±1.29 dynes/cm^2) but slower drug release (Q8h=52.13±1.81%) due to high gelling capacity. No sign of redness, inflammation, swelling or increased tear production was observed by Draize test. The IOP lowering activity of selected formulation was detected and compared with marketed Pilopine HS® gel. G2 formulation showed relative bioavailability 2.64 times more than bioavailability of marketed Pilopine HS® gel. These results suggest that the niosomal gels containing dipivefrin HCl are promising carriers for glaucoma treatment.

Keywords
Niosomes, Dipivefrin HCl, Niosomal gel, Draize test, IOP, Antiglaucomatic activity.

Introduction

Glaucoma is a prevalent neurodegenerative disorder of the eye. Increased intraocular pressure (IOP) and subsequent retinal ganglion cell (RGC) death leading to the loss of visual field characterizes the pathology of primary open angle glaucoma (POAG), which is the most common form. The disease affects over 66 million people worldwide, causing bilateral blindness in 6.8 million [1, 2]. Patients with POAG typically exhibit increased resistance to the outflow of aqueous humor through the trabecular meshwork, which can result in an increase in IOP and subsequent cell death from compression of the optic nerve axons [3]. However, IOP is the primary risk factor causing the loss of RGCs, the strategies of treatment mostly involve lowering IOP[4]. Current treatment options primarily aim at decreasing IOP by utilizing pharmacological agents, laser therapy and surgery. The method of reducing IOP is by enhancing the outflow of humor from the eyes using muscarinic

acetylcholine receptor agonists [5, 6].

Dipivefrin (DV) HCl, a prodrug of epinephrine (EP), is an adrenergic agonist and direct acting sympathomimetic agent that is used to reduce IOP in patients suffering from chronic open angle glaucoma[7]. This drug acts through decreasing production and increasing the outflow of aqueous humor from the eye[8]. A controlled study proved the usefulness of topically applied DV (0.1%, w/v) over EP (2%, w/v) in reducing the IOP in the patients who were intolerant to topically applied EP [9]. In terms of safety, DV is associated with less systemic adverse effects (e.g., cardiovascular side effects) compared to EP, since it is only needed in very small dose. Thus, DV is considered more suitable for ocular application as compared to EP, especially in patients with cardiovascular disorders [10]. In addition to the clinical benefits, DV has favorable physicochemical properties compared to EP. DV has an ideal lipophilicity and diffusivity across the lipophilic ocular dynamic and static barriers, due to the esterification of the two hydroxide (-OH) functional groups of EP, yielding dipivaloyl-EP. This chemical modification allows DV to avoid the unfavorable physicochemical and biopharmaceutical characteristics of the EP [11]. Therefore, using DV in an ocular formulation will resolve the lipophilicity issue associated with EP and would provide a site-specific delivery with a 10-fold enhanced therapeutic efficacy compared to EP [12]. Delivering drugs via the ocular route is challenging due to the immediate tear-turnover rate and corneal impermeability, which restricts the ocular bioavailability of conventional topical eye drops or solutions [13]. Therefore, there is a need for an appropriate ocular delivery system to achieve high trans corneal permeation, sustained and controlled delivery while providing sufficient ocular bioavailability [14]. These problems can be minimized using niosomal vesicular system.

Niosomes are formed from the self-assembly of non-ionic amphiphiles in aqueous media resulting in closed bilayer structures [15], which can entrap both hydrophilic and lipophilic drugs either in an aqueous layer or in vesicular membrane[16]. Niosomes in topical ocular delivery are preferred over other vesicular systems because of the chemical stability; low toxicity due to their non-ionic nature; handling surfactants with no special precautions or conditions; the ability to improve the performance of the drug via better availability and controlled delivery at a particular site and being biodegradable, biocompatible and non-immunogenic [17]. Some researchers reported that there was approximately 2.5 times increase in the ocular bioavailability of timolol maleate (a water-soluble drug) encapsulated in niosomes as compared to timolol maleate solution [18].

Niosomes, administered as an ophthalmic gel, are capable of localizing and maintaining drug activity at its site of action due to their easy transition through ocular barrier with reduced drug frequency and toxicity. Moreover, niosomes based ocular gel containing bioadhesive polymer helps the drug to remain adhered to corneal surface for a long period of time. Hence, precorneal residence time is increased, resulting in significant enhancement of ocular bioavailability.

Therefore, the present study aims to develop and evaluate niosomal gel formulations containing dipivefrin HCl to achieve prolonged precorneal residence time and improved bioavailability. Niosomes were prepared using various non-ionic surfactants (span 20, span 60 and span 80) in the presence of cholesterol in different molar ratios by ether injection method. Selected formulations of niosomes were incorporated into carbopol 934 (1% w/w) and locust bean gum (3% w/w) gels.

Materials and methods

The dipivefrin HCl was kindly received as a gift sample by M/s Piramal Enterprises Ltd. (Digwal AP, India). Sorbitan monolaurate (span 20), sorbitan monosterate (span 60), sorbitan monooleate (span 80), cholesterol, locust bean gum and carbopol 934 were procured from Loba Chemie Pvt. Ltd. (Mumbai, India). Double distilled water was used throughout the study.[1]

Preparation of dipivefrin hydrochloride loaded niosomes

Dipivefrin hydrochloride loaded niosomes were prepared by ether injection technique [43]using non-ionic surfactants (span 20, span 60 and span 80) and cholesterol in different ratios as shown in Table 1. For each ratio, non-ionic surfactant

Table 1. Composition for niosomes

Formulation Code	Surfactant	Amount of surfactant (mg)	Cholesterol (mg)	Drug (mg)	Ratio (Surfactant: Cholesterol)
F1	Span 20	100	100	10	1:1
F2	Span 20	200	100	10	2:1
F3	Span 20	100	200	10	1:2
F4	Span 60	100	100	10	1:1
F5	Span 60	200	100	10	2:1
F6	Span 60	100	200	10	1:2
F7	Span 80	100	100	10	1:1
F8	Span 80	200	100	10	2:1
F9	Span 80	100	200	10	1:2

and cholesterol were weighed accurately and dissolved in 20 ml of diethyl ether. Dipivefrin hydrochloride (10 mg) was then dissolved in this lipid solution. The resulting solution was taken in a syringe and injected slowly through a 16 gauge needle into 10 ml of aqueous phase (phosphate buffer saline pH 7.4) held in a beaker maintained at 60^0C to 65^0C and agitated slowly. As the lipid solution was injected slowly into the aqueous phase, vaporization of diethyl ether resulted in the formation of niosomes. The prepared niosomes were separated by ultracentrifugation (Remi C-24, Mumbai, India) at 4^0C.

Evaluation of dipivefrin hydrochloride loaded niosomes

Drug entrapment efficiency (% EE)

The proportion of encapsulated dipivefrin hydrochloride was obtained by ultracentrifugating 1 ml of the niosomal suspension at 15,000 rpm for 1 hr using a cooling centrifuge at 4°C (Remi C-24, Mumbai, India). The niosomes were separated from the supernatant and were washed twice, each time with 1 ml phosphate buffered saline, and recentrifuged again for 1 hr. The amount of entrapped dipivefrin hydrochloride was determined by lysis of the separated vesicles with isopropanol. A 100 µl sample of niosomes was mixed with 1 ml of isopropanol; the volume was completed to 10 ml with phosphate buffered saline and covered with parafilm to prevent evaporation. The concentration of the drug was determined by UV spectrophotometer (UV 1700 Pharm Spec, Shimadzu, Japan) at 254 nm. Study was done in triplicate and % drug entrapment efficiency can be calculated by using following formula:

$$\% \text{ EE} = \frac{\text{Actual drug content}}{\text{Theoretical drug content}} x \ 100$$

Vesicle size and zeta potential measurements

Vesicle size of different niosomal formulations were observed under an optical microscope (Olympus Model BX 41, Japan) at suitable magnification. The measurements were done in triplicate and vesicle size was recorded. The zeta potential of the prepared niosomal formulations was determined by Zetasizer Nano ZS-90 (Malvern Instruments Ltd., UK) using 0.1 M KCl buffer in demineralized water at 25°C [19].

Transmission electron microscopy (TEM)

The prepared niosomal formulations were characterized for their shape using transmission electron microscope (JEM-200 CX, JEOL, Tokyo, Japan) at 80 KV, after being stained and TEM micrograph was taken at suitable magnification [20].

Differential scanning calorimetric (DSC) study

Differential scanning calorimetric analysis was performed on the drug loaded niosomes. Initially, the moisture was removed by heating the samples and then, each sample (about 3-7 mg)

was accurately weighed into platinum crucible 40 µl aluminum pan in hermetically sealed condition, where alpha alumina powder used as a reference. Thermogram was recorded from 50°C to 300°C at the heating rate of 20°C/min under a constant flow of an inert nitrogen gas atmosphere with the flow rate of 20 ml/min. These analyses were done on Perkin-Elmer instrument (Pyris-1, Osaka, Japan) available at Department of Textile Technology, Indian Institute of Technology, New Delhi, India.

In-vitro drug release from niosomes

The *in-vitro* release of entrapped drug within niosomes was determined using membrane diffusion technique. The niosomal formulation equivalent to 1 mg of dipivefrin HCl was placed in a glass tube that was previously covered with presoaked cellulose membrane, which acts as a donor compartment. The glass tube was placed in a beaker containing 50 ml of simulated lachrymal fluid (pH 7.4), which acted as receptor compartment. The whole assembly was fixed in such a way that the lower end of the tube containing suspension was just touching (1-2 mm deep) the surface of diffusion medium. The temperature of receptor medium was maintained at 37±100^{0}C and agitated at 100 rpm speed using magnetic stirrer. Aliquots of 5 ml sample were withdrawn periodically and after each withdrawal same volume of medium was replaced. The collected samples were analyzed spectrophotometrically at 254 nm using simulated lachrymal fluid (pH 7.4) as blank.

Preparation of gels containing dipivefrin hydrochloride niosomes

Selected drug loaded niosomes (equivalent to 0.1% w/w drug) were incorporated into different gel bases as shown in Table 2. The polymers used were locust bean gum and carbopol 934. The required quantity of these polymers was weighed and dispersed in a small amount of phosphate buffer saline pH 7.4 to prepare an aqueous dispersion and sterile in hot air oven at 160^{0}C for 1 hr. The aqueous dispersion was allowed to hydrate for 4-5 hrs. The pH was adjusted to 6 by addition of triethanolamine solution. The final weight of the gel was adjusted with phosphate buffer saline pH 7.4. Niosomal suspension containing drug was separated from aqueous medium by ultracentrifugation at 15000 rpm at 4^{0}C and was added gently by vortex in the sterile blank gel under laminar air flow cabinet. The solution was made isotonic with sodium chloride (0.9% w/v). Then, benzalkonium chloride (0.001% v/v) was added as a preservative. The gel was made consistent with glycerin (10% w/v). Vortexing was continued until a homogenous niosomal gel was obtained and the gel was then sonicated to become bubble-free. The prepared gels were filled in amber colored glass vials refrigerated at 4 to 8ºC.

Evaluation of gels containing Dipivefrin hydrochloride niosomes

Rheological studies

The viscosity of different gel formulations using Brookfield DV-II+Pro model LV viscometer equipped with a helipath stand and T bar spindles. Viscosity measurements were made at variable shear rate. It was carried out at constant temperature of 37±1^{0}C, but varying the rotation speed of the spindle from 10 to 100 rpm in a small sample adaptor. Evaluations were done in triplicate[21].

Spreadability

The therapeutic efficacy of a formulation also depends on its spreading value. Spreadability is

Table 2. Composition for niosomal gels

Gel formulation	Niosomes loaded	Locust bean gum (% w/w)	Carbopol 934 (% w/w)
G1	F1	3%	1%
G2	F4	3%	1%
G3	F7	3%	1%

expressed in terms of time in seconds taken by two slides to slip off from the formulation, placed in between, under the application of a certain load. Lesser the time is taken for the separation of the two, better the spreadability. Two glass slides of standard dimensions were selected. 1 g gel was placed over one of the slides. The other slide was placed on top of the formulations and was sandwiched between the two slides across the length of 5 cm along the slide. 100 g weight was placed upon the upper slide so that the formulation between the two slides was pressed uniformly to form a thin layer. The weight was removed and the excess of formulation adhering to the slides was scrapped off. One of the slides was fixed on which the formulation was placed. The second movable slide was placed over it, with one end tied to a string to which load could be applied by the help a simple pulley and a pan. A 30 g weight was put on the pan and the time taken for the upper slide to travel the distance of 5.0 cm and separate away from the lower slide under the direction of the weight was noted. Spreadability was then calculated by using the formula[22]:

$S = M.L / T$

Where, S = Spreadability, M = Weight tide to upper slide (gm), L = Length of glass slide (cm), T = Time taken to separate the slide completely from each other (sec).

Ex-vivo bioadhesive strength

Freshly excised conjunctiva of an adult goat was used as model membrane for the measurement of bioadhesive strength. The conjunctiva was placed in an aerated saline at 4°C and later washed with isotonic phosphate buffer, pH 7.4 before use. Bioadhesive strength of ocular gel was measured on a modified two-arm physical balance. The pan at the left arm of the balance was detached and a vertical thread was hung to the lever of the left arm which had a rubber stopper tied to its end, hanging downward. The formulation to be tested was adhered to the downward facing side of the rubber stopper. Conjunctival membrane was tied onto the open mouth of a glass vial which was filled with isotonic phosphate buffer. The vial was fitted in the center of a glass beaker filled with simulated tear fluid (pH 7.4) maintained at 37°C. The apparatus was set such that the vial (conjunctival membrane tied on it, facing upward) lies exactly below the rubber stopper (insert tied on it, facing downward). The rubber stopper was lowered so as to make the formulation come in contact with the membrane. After facilitating the contact between the two, weight was put on the right limb of balance (gram force) required to detach the formulation from the conjunctival surface[23]. The detachment stress (dynes/cm^2) was then calculated by using formula:

Detachment stress = mg / A

Where, m = Weight required for detachment (gm), g = Acceleration due to gravity (980 cm/s^2), A = Area of mucosa exposed (cm^2).

In-vitro drug permeation from niosomal gels

The *in-vitro* drug permeation of niosomal gels was studied through cellophane membrane using a diffusion cell, as in case of niosomal formulations.

Release kinetics modeling

To investigate the release mechanism of drug from niosomal gel preparations, the *in-vitro* release data were fitted with the following mathematical models[24]:

Zero-order kinetics equation:

$Q_t = k_0.t$

Where, Q_t is the amount of drug released at time t, k_0 is the zero-order release rate constant, t is the time.

First-order kinetics equation:

$\ln Q_t = \ln Q_0 - k_1 t$

Where, Q_t is the amount of drug released at time t, Q_0 is the initial amount of drug in the solution, k_1 is the first-order release rate constant.

Higuchi model kinetics equation[25]:

$Q_t = k_H . t^{1/2}$

Where, Q_t is the amount of drug released at time t, k_H is the Higuchi release rate constant.

Hixson-Crowell model kinetics equation[26]:

$Q_0^{1/3} - Q_t^{1/3} = K_{HC}.t$

Where, Q_0 is the initial amount of the drug in the dosage form, Q_t is the remaining amount of drug in the dosage form at time t, K_{HC} is the Hixson-Crowell release rate constant.

Korsmeyer-Peppas model kinetics equation[27]:

$M_t/M_\infty = K_{KP}.t^n$

Where, M_t is the fraction of drug released at time t, M_∞ is the fraction of drug released at infinite time, K_{KP} is the Korsmeyer-Peppas release rate constant, n is the release exponent. The value of exponent (*n*) indicates the mechanism of drug release.

Isotonicity study

Isotonicity should be maintained to restrict tissue damage or irritation of eye. Three different concentrations of NaCl solution were prepared to obtain hypertonic (3% w/v), hypotonic (0.2% w/v) and isotonic (0.9% w/v) concentrations. Four clean slides were taken. They were labeled as hypertonic (HT), hypotonic (HP), isotonic (IS) and test (T). A small drop of blood was applied to the center of each slide along with a drop of heparin solution (1% w/v) to prevent coagulation of blood. A drop of each solution under test (G1, G2 and G3) was placed on the respective slides. Using the edge of the cover slip, the contents were mixed and put under microscope at 45X magnifications to observe the morphology of RBCs. If a preparation is isotonic, the structure of the cell will not be disturbed by either ingress of water from the instilled solution (hypotonic) or egress of water of the cell (hypertonic) [28].

Stability studies

Adequate samples of each of the selected niosomal formulations (niosome and niosomal gel) from formulation F4 and G2 were sealed into 10 ml ambered glass vials and stored at temperature 4±1 °C in a refrigerator and 37±1 °C in a thermostat controlled hot air oven for 28 days. After every 7 days the formulations were evaluated for % dipivefrin HCl retained in gel formulation. The initial drug content was considered as 100% [29].

Ex-vivo drug permeation study

Freshly excised whole cow eyeball was procured from a slaughter house and transported to the laboratory in cold condition. They were maintained in normal saline at 4°C. The cornea was then carefully removed along with a 5-6 mm of surrounding scleral tissue and washed with cold saline. The washed cornea was preserved in freshly prepared phosphate buffer (pH 7.4) and stored under refrigeration until the time of the study. The preserved cornea was mounted on Franz diffusion cell by sandwiching between the donor and receptor compartment. It was positioned on the donor half-cell such that the epithelial surface was facing the donor solution. The receptor half-cell was positioned symmetrically opposing the donor half-cell. The half-cells were secured together with a clamp. This procedure prevents any leaks [30].

One gram of selected niosomal gel formulation (G2) was placed inside the donor half-cell over the corneal membrane. The entire surface of the cornea was in contact with the receptor compartment that contained 50 ml of simulated tear fluid (pH 7.4), which was stirred continuously using a magnetic stirrer at 100 rpm to simulate blinking action. At predetermined time intervals for up to 8 hr, 5 ml aliquots of the release medium were withdrawn for analysis and were replaced with equal volume of release medium at the same temperature to maintain constant volume. *Ex-vivo* drug permeation through cornea from niosomal gel was analyzed spectrophotometrically using UV-spectrophotometer at 254 nm and compared with marketed formulation (Pilopine HS® gel). Results were tabulated and graph was plotted as cumulative percentage of drug permeated versus time for niosomal gel formulation (G2). Study was done in triplicate.

Ocular irritancy test (Draize's test)

Rabbits were divided into two groups (four rabbits in each group). G2 niosomal gel formulation and marketed Pilopine HS® gel were applied to Group I and Group II of rabbit's eyes respectively. The untreated eye serves as control. 20 µl of the representative formulation was instilled into the lower conjunctival sac of the rabbit's right eye, while the left was kept as a control. The solutions were instilled periodically twice a day. The test eyes were examined for any abnormality (irritation signs) that were recorded before treatment and 30 min, 1 h, 24 h, 48 h, 72 h, 7 days, 14 days and 21 days after treatment [31]. The common irritation signs are expected to

be conjunctival redness, swelling and discharge scoring 0 (absence) to 4 (highest).

In-vivo antiglaucoma activity by measurement of intraocular pressure (IOP)

Rabbits were randomly divided into three groups (six rabbits in each group). Group I served as control while Group II and Group III were treated with G2 niosomal gel formulation and marketed Pilopine HS® gel respectively [32]. Glaucoma disease was induced by Bonomi *et al.*, 1978 method [33]. Rabbits were treated with subconjunctival injections of 0.25 ml Betamethasone injection (Betamethasone sodium 4 mg/ml) every week for three successive weeks in left and right eyes. Local anesthetic eye drops (Benox®) were used prior to subconjunctival injection. The activity was confirmed by noticing a bulge formation at the site of injection. The right eye of each rabbit was kept as control and the left eye was treated for glaucoma using 10 µl (equivalent to 10 µg of dipivefrin HCl) of selected formulation (G2) and marketed Pilopine HS® gel for group II and group III respectively. The intraocular pressure (IOP) readings were measured using Schiotz Tonometer, before drug administration and 2 h, 4 h, 6 h, 7 h, 8 h, 9 h, 10 h, 11 h and 24 h after drug administration. IOP was measured three times at each time interval and the means were recorded.

The change in IOP (ΔIOP) is expressed as follows:

ΔIOP = IOP dosed eye – IOP control

The pharmacokinetic parameters taken into consideration such as the maximum percentage decrease in IOP (% IOP_{max}) and the time of maximum response (T_{max}) were estimated through constructing % ΔIOP versus time curves.

% Maximum reduction of IOP (% IOP_{max}) = IOP_o - IOPmax / IOP_o *x* 100

Where, IOP_o is the intra-ocular pressure at 0 time, IOP_{max} is the intra-ocular pressure at $T_{max.}$

The area under the curve (AUC) was calculated according to trapezoidal rule. Relative bioavailability for formulations was determined through the equation below:

Relative bioavailability = AUC_t / AUC_S

Where, AUCt and AUCs are the AUC estimated for test formulation and standard formulation (marketed Pilopine HS® gel).

Results and discussion

Evaluation of dipivefrin hydrochloride loaded niosomes

Drug entrapment efficiency in niosomes

Effect of surfactant type

From the results in Table 3, It was observed that the entrapment efficiency of niosomes composed of span 60 were superior as compared to those prepared from span 20. The formulation containing span 80 showed the lowest entrapment efficiency. This can be due to:

(a) The hydration temperature used to make niosomes should usually be above the gel to liquid phase transition temperature of the system that results in niosomes that are less leaky and have high entrapment efficiency. Span 60 has highest phase transition temperature (50^0C) as compared to span 20 (16^0C) and span 80 (-12^0C) and hence high entrapment efficiency.

(b) The length of alkyl chain of surfactant has a prominent effect on permeability of prepared niosomes. As the length of surfactant increases, entrapment efficiency also increases. Span 60 has a longer saturated alkyl chain (C18) compared to span 20 (C12), so it produces niosomes with higher entrapment efficiency. Span 60 and span 80 have the same head group but span 80 has an unsaturated alkyl chain which results in enhanced permeability and decreased entrapment.

Effect of cholesterol weight ratio

The entrapment efficiency decreased with increasing cholesterol concentration for span 60 formulations. This may be due to higher amounts of cholesterol competing with the drug for packing space within the bilayer, hence excluding the drug as the amphiphiles assemble into vesicles. Another explanation may be that the increasing cholesterol beyond a certain concentration can disrupt the regular linear structure of the vesicular membranes. Formulation F4 shows the maximum % EE among all the formulations.

Table 3. Evaluation of niosomes

Formulation Code	Entrapment efficiency (%)	Vesicle size (µm)	Zeta potential (mV)	Q8h (%)
F1	81.43±2.09	1.33±1.76	-31.04±0.25	78.81±4.82
F2	86.17±3.07	1.56±1.59	-29.54±0.72	73.15±6.32
F3	78.56±0.99	2.71±0.57	-28.84±0.27	68.74±0.78
F4	92.16±0.25	6.13±0.31	-15.04±0.45	61.05±2.87
F5	90.66±3.80	5.16±0.27	-22.21±1.28	63.54±4.42
F6	84.06±9.36	7.12±2.80	-24.64±0.53	66.98±3.83
F7	79.11±3.96	3.68±0.69	-30.04±0.72	74.04±2.25
F8	83.47±2.65	3.09±1.32	-28.84±0.47	75.33±0.89
F9	72.33±2.03	4.24±0.41	-27.32±0.34	71.05±0.93

Vesicle size and zeta potential measurements

The size of particles in ophthalmic dosage forms plays an important role in the irritation potential of formulation; hence it is recommended that the particles of ophthalmic solution should be less than 10 µ to minimize irritation to the eye.[43] Vesicle size of all formulations was ranges between 1.44±2.76 µm to 7.12±2.80 µm as shown in Table 3. These sizes are well acceptable for ocular administration. From Table 3, it was observed that the niosomes prepared using span 60 is larger in size than those prepared using span 20 and span 80. Span 60 has a longer saturated alkyl chain and it was reported that surfactants with longer alkyl chains generally give larger vesicles.

The zeta values for niosomal formulations were found to be in range of -15.04±0.45 mV to -31.04±0.25 mV as shown in Table 3. The zeta potential of the niosomal formulation F1 composed of Span 20 was found to be -31.04±0.25 mV as shown in Figure 1. The results revealed that the zeta values of the vesicles increase towards negative with increasing the HLB values of the surfactants. The effect of HLB values of surfactants on zeta potential could be explained in terms of surface energy, which tends to increase with increase in HLB values towards the hydrophilicity. Increase in surface energy of the vesicles leads to increase the values of zeta

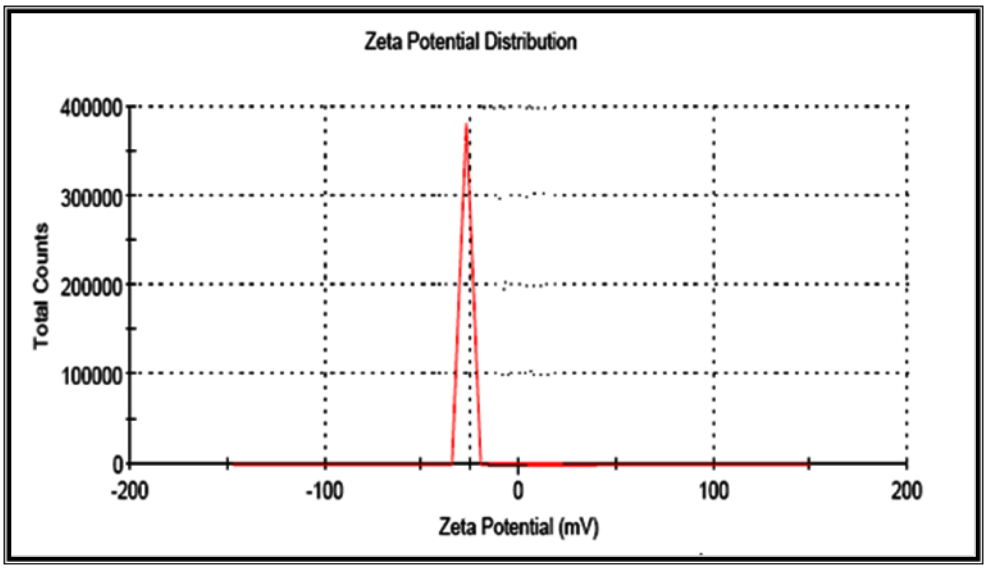


Figure 1. Zeta potential of niosomal formulation

potential towards negative[34]. The high negative surface charge on niosomes indicates higher stability because of the anticipated surface repulsion between similarly charged particles therefore, inhibiting aggregation of the colloidal niosomal particles. It was observed that all the formulations were sufficient to keep the particles stable.

Transmission electron microscopy

Transmission electron micrographs of selected Dipivefrin HCl loaded niosomal formulation was shown in Figure 2. It was demonstrated that the vesicles are well identified and present in a nearly perfect sphere-like shape with a smooth surface and having a definite internal aqueous space.

Differential scanning calorimetric (DSC) study

DSC thermogram of drug loaded niosomal formulation (F4) was displayed in Figure 3. Endotherm at 131.202°C (area=1726.267 mJ,

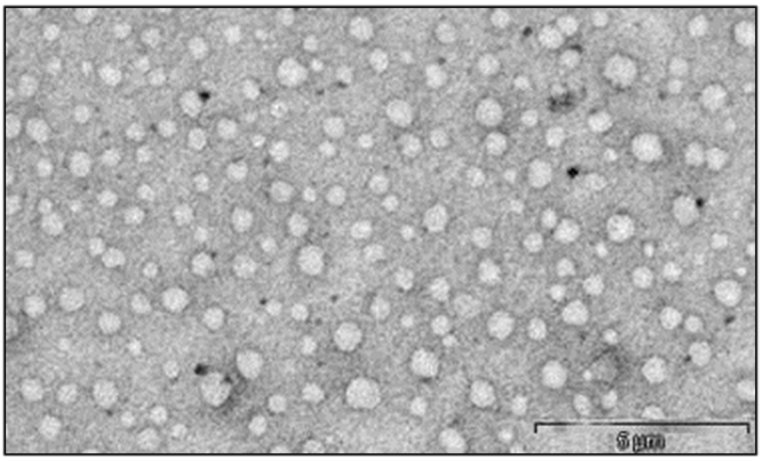


Figure 2. Transmission electron micrograph of niosomal formulation at 22000X

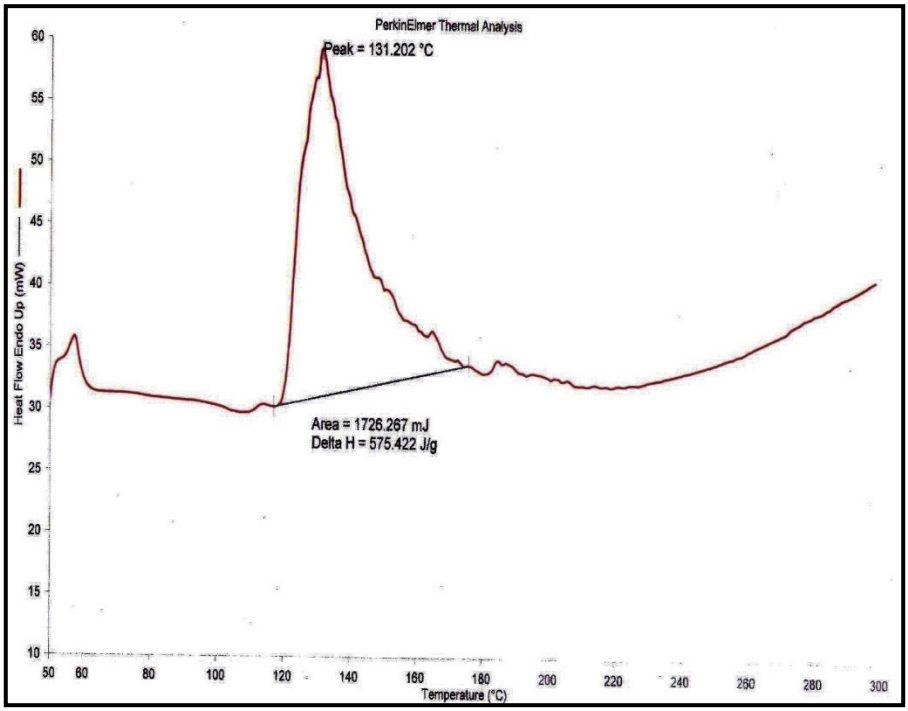


Figure 3. DSC thermogram tracings of drug loaded niosomes

delta H=575.422 J/g) indicated the increase in phase transition temperature of niosomes upon loading with drug. Absence of the melting endotherm of dipivefrin HCl suggested that drug changed from crystalline to amorphous state. These results suggest significant interaction of drug with the bilayer structure and can account for the enhanced entrapment of drug into niosomal formulations and sustained drug release.

In-vitro drug release from niosomes

The percentage of drug released after 8 hr from the prepared niosomal vesicles at simulated lachrymal fluid of pH 7.4 varied from 60.35±3.83% to 78.81±4.82% as shown in Figure 4 and the data was presented in Table 3. By inspection of the data, it could be concluded that niosomal formulations prepared using span 60 (1:1) yielded a lower rate of release compared to span 20 and span 80 niosomes. This can be explained by the fact that niosomes exhibit an alkyl chain length dependent release and the higher the chain length, the lower the release rate[35]. By reviewing the data, it has been revealed that release after 8 hours for the niosomal formulations can be arranged in the following decreasing order: F1 > F8 > F7 > F2 > F9 > F3 > F6 > F5 > F4. From results, it is obvious that the increase of cholesterol molar ratio reduced the efflux of the drug from niosomal preparations, which is in accordance with its membrane stabilizing ability. Cholesterol is known to abolish the gel to liquid phase transition of niosomes systems, resulting in niosomes that are less leaky. Therefore, the diffusion of dipivefrin HCl entrapped in the hydrophobic regions of the vesicles would be expected to occur over a prolonged period[36].

Evaluation of gels containing Dipivefrin hydrochloride niosomes

Rheological studies

The viscosity of the all-gel formulations ranged from 135-1900 cps as shown in Table 4. A shear thinning formulation with a high viscosity at low shear rate and lower viscosity at higher shear rates will be preferable. All the formulations showed pseudoplastic rheological flow, as evidenced by shear thinning and increase in shear stress with increased angular velocity. From Figure 5, it was observed that viscosity of all the formulations was decreasing with the increase in shear rate. The non-Newtonian formulations with pseudoplastic properties can acquire a viscosity decrease with increasing shear rate, creating blinking and ocular movement. Pseudoplasticity is thus interesting because it offers significantly less resistance to

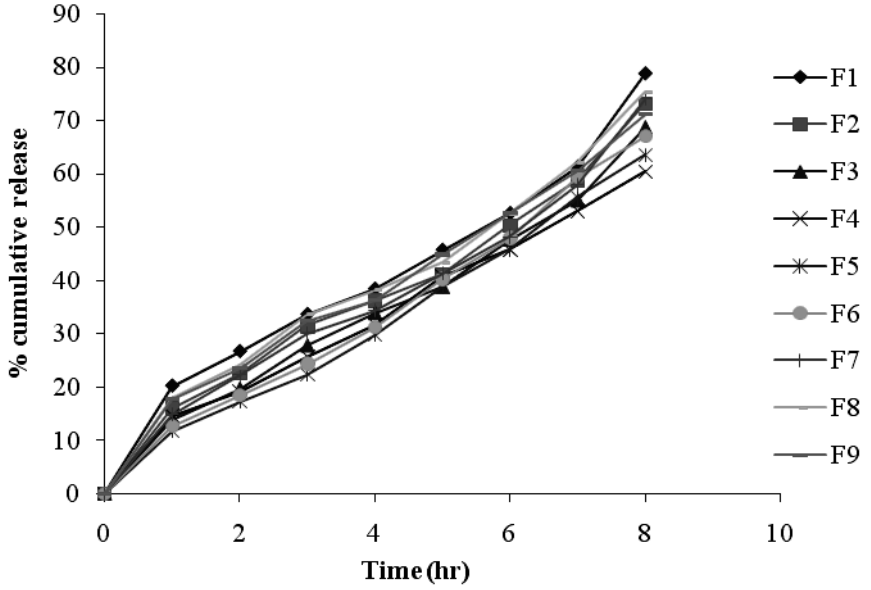


Figure 4. *In-vitro* release profile of different niosomal formulations

Table 4. Viscosity of the niosomal gel formulations (Mean ± SD, n=3)

Code	Viscosity in cps					
	10 rpm	20 rpm	40 rpm	60 rpm	80 rpm	100 rpm
G1	1900±1.70	1457±0.40	986±0.05	675±0.03	453±0.01	320±0.03
G2	1700±0.05	1267±0.60	689±0.20	394±0.10	264±0.05	135±0.03
G3	1890±0.68	1342±0.68	880±0.34	568±0.12	385±0.08	260±0.05

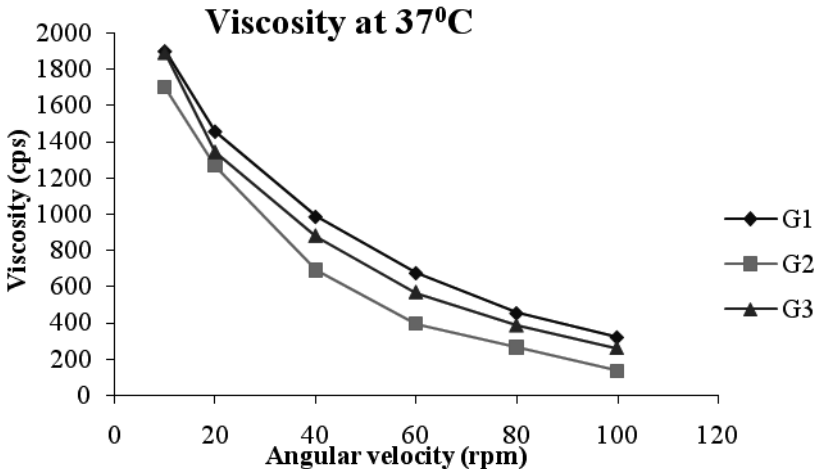


Figure 5. Viscosity of niosomal gel formulations

blinking and shows much greater acceptance than viscous Newtonian formulation[37].

Spreadability

The spreadability of the prepared niosomal gel (G1, G2 and G3) was ranges from 1.41±0.72 to 2.21±1.05 g.cm/s as shown in Table 5. It was observed that with increase in polymer concentration, the spreadability decreased due to the increase in viscosity of the formulation [38]. Formulation G2 has higher spreadability (2.21±1.05 g.cm/s) with low viscosity as compared to formulation G1 and G3. One of the criteria for a gel to meet the ideal quality is that it should possess good spreadability. It is the term expressed to denote the extent of area, to which gel readily spreads on application site. Lesser the time is taken for separation of two slides, better the spreadability.

Ex-vivo bioadhesive strength

The bioadhesive strength of the niosomal gel formulations was ranges from 1968±0.23 to 2314±1.29 dynes/cm^2 respectively as shown in Table 5. The bioadhesive values show considerable potential of sustaining the residence and enhancing contact with ocular tissue. Formulation G3 showed least bioadhesive detachment force (1968±0.23 dynes/cm^2) as compared to G1 (2036±0.16 dynes/cm^2). The highest bioadhesive detachment force of formulation G2 (2314±1.29 dynes/cm^2) could be attributed to the fact that an anionic polymer carbopol 934 is a polyacrylic acid derivative. Its mucoadhesive property is due to hydrogen bonding with mucin, resulting in good adhesion [39]. The adhesive behavior of locust bean gum is due to increased viscosity in polymer solution, resulting in effective bioadhesion.

In-vitro drug permeation from niosomal gels

The cumulative percentage of drug permeated from niosomal gel formulation was ranges between 52.13±0.81% to 62.89±2.21% after 8 hrs as shown in Figure 6 and the data was presented in Table 5. The *in-vitro* release data

Table 5. Some characteristics of niosomal gels

Gel formulation	Spreadability (g.cm/s)	Bio adhesive strength (dynes/cm2)	Q8h (%)
G1	1.41±0.72	2036±0.16	62.89±2.21
G2	2.21±1.05	2314±1.29	52.13±0.81
G3	2.16±0.27	1968±0.23	56.94±1.48

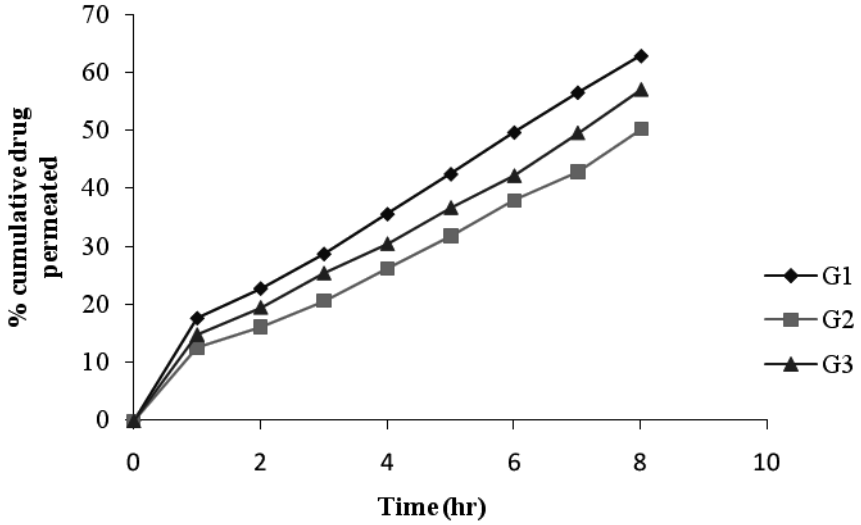


Figure 6. *In-vitro* drug permeation of niosomal gels

of gel formulation G2 shows a lower percentage of drug release as compared with niosomal formulation F4 (Figure 7). These results can be attributed to the presence of bioadhesive polymer which retains the formulation in contact with the eye for a long period of time. Among all the formulations, G2 showed slower drug release (52.13±0.81%) due to high gelling capacity.

Release kinetics modeling

Kinetics for drug release was studied for zero order kinetics, first order kinetics, Higuchi's model kinetics, Hixson-Crowell model kinetics and Korsmeyer-Peppas model kinetics with interpretation of diffusional release mechanism and the results were shown in Table 6 and Table 7. The co-relation coefficient (R^2) and K values are obtained through various graphs of these above-mentioned release kinetics models of all niosomal gel formulations (G1 to G3) which were graphically shown in Figure 8, 9, 10, 11 and 12 respectively. The determination of the co-relation coefficient (R^2) value indicated that drug release has followed zero order kinetics in case of gel formulation G2 and Korsmeyer-Peppas kinetics in case of formulation G1 and G3 which predicts the release may be diffusion-controlled mechanism from the niosomal formulations. The 'n' value could be used to characterize different drug transport mechanisms and were in the range of 0.5851 to 0.7234 ($0.5<n<1.0$). This indicates that the release of gel formulations follows non-Fickian diffusion transport mechanism.

Isotonicity study

The selected formulation G2 did not show any change in the morphology (swelling or shrinkage) of blood cells. This indicates that these formulations were isotonic (Figure 13).

Stability studies

The stability studies revealed that the selected formulation (F4 and G2) met the pharmacopeial requirements of drug content (80–110%) as

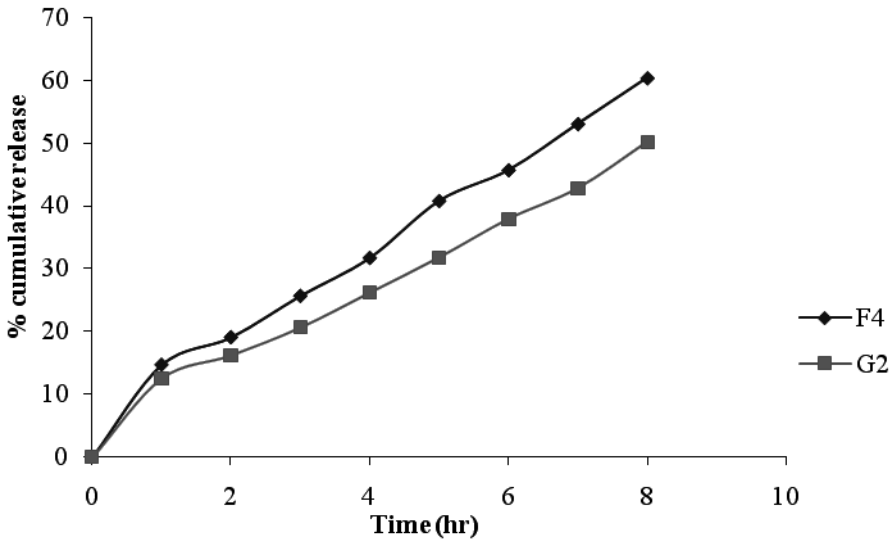


Figure 7. Comparative study for *in-vitro* drug release from niosome (F4) and niosomal gel (G2)

Table 6. Release kinetics of niosomal gels

Gel Formulation	Zero order R2	K0	First order R2	K1	Higuchi R2	KH	Hixson-Crowell R2	KHC	Korsmeyer-Peppas R2	n
G1	0.980	7.262	0.980	0.049	0.965	21.96	0.980	0.160	0.974	0.58
G2	0.987	5.764	0.977	0.034	0.944	17.18	0.985	0.154	0.986	0.67
G3	0.983	6.471	0.972	0.041	0.954	19.43	0.980	0.148	0.985	0.72

Table 7. Interpretation of diffusional release mechanisms

Diffusion exponent (n)	Drug transport mechanism
0.5	Fickian diffusion
0.5<n<1.0	Anomalous (non-Fickian) diffusion
1.0	Case-II transport
n>1.0	Super Case-II transport

shown in Figure 14 and 15 respectively. From Table 8, it was observed that at 4±1°C, the % of drug retained in G2 was 86.76±1.25% and at 37±1°C, the % remained was 80.58±1.07%. The % Dipivefrin HCl retained in F4 was 80.36±1.05% at 4±1°C and 76.45±2.67% at 37±1°C. From these results it was concluded that the incorporation of niosomes in gel increased their stability than the niosome itself. This may be due to the rigidity of gel structure which resists the leakage of drug.

Ex-vivo drug permeation study

The *ex-vivo* permeation of dipivefrin HCl from selected formulation G2 and marketed formulation (Pilopine HS gel) through bovine cornea was determined in triplicate and their mean values with standard deviation are shown in Table 9 and the plot of cumulative percent drug permeated as a function of time was shown in Figure 16. The results showed slow and sustained release of drug through the corneal membrane for prolonged period in case of

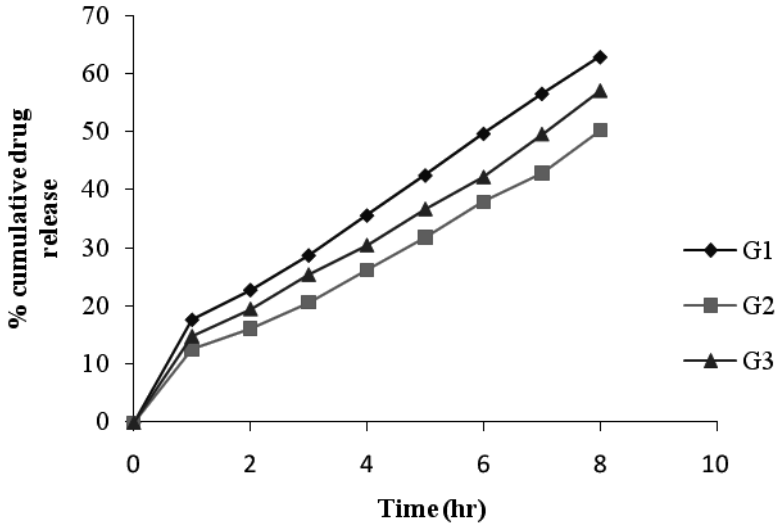


Figure 8. Zero-order release kinetics for gel formulations

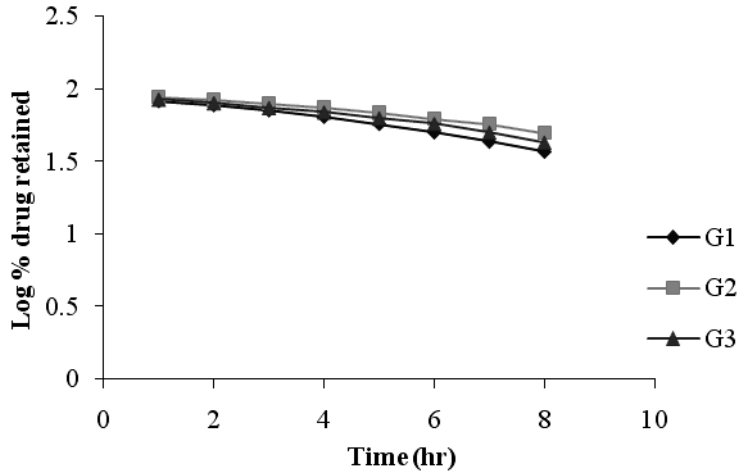


Figure 9. First-order release kinetics for gel formulations

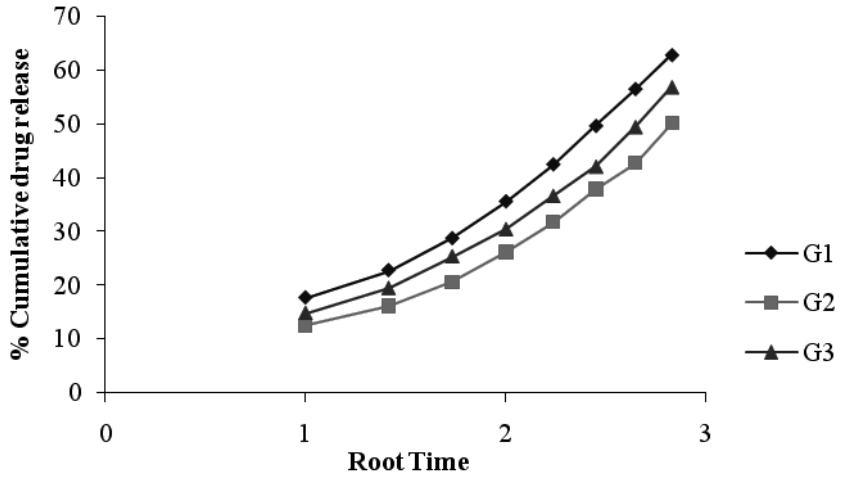


Figure 10. Higuchi model kinetics for gel formulations

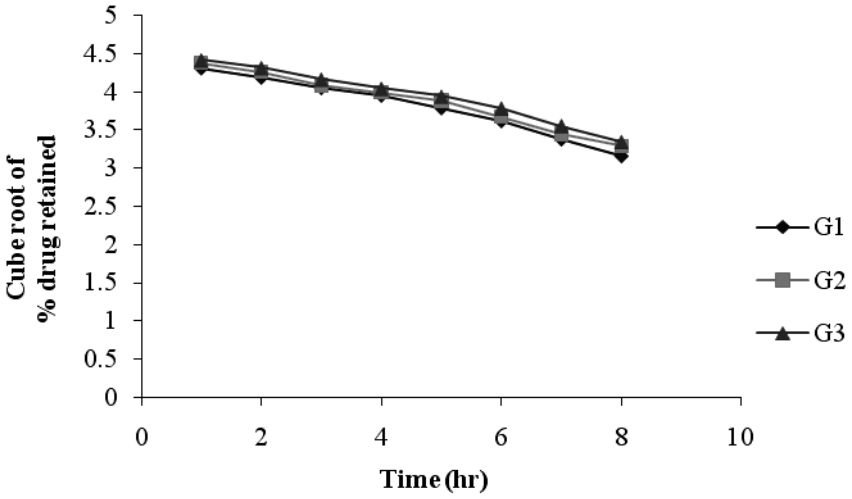


Figure 11. Hixson-Crowell kinetics for gel formulations

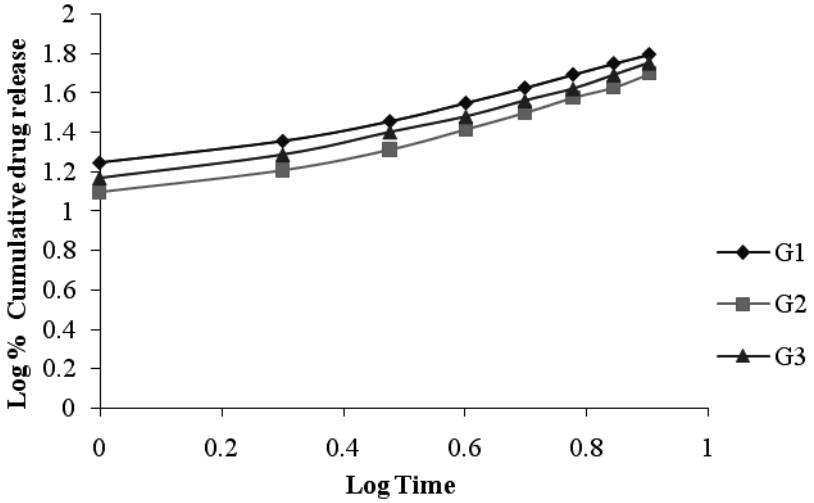
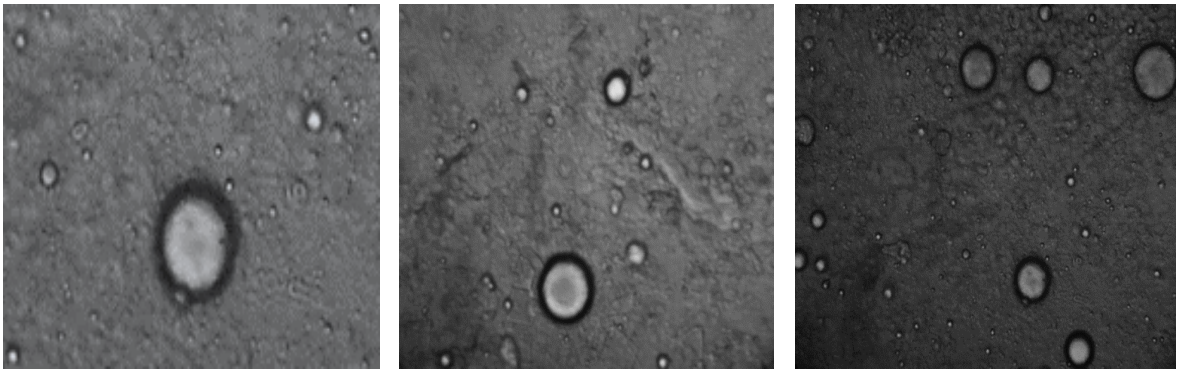


Figure 12. Korsmeyer-Peppas model kinetics for gel formulations



Hypertonic **Hypotonic** **Isotonic**

Figure 13. Photomicroscopy of gel formulation (G2) after isotonicity testing

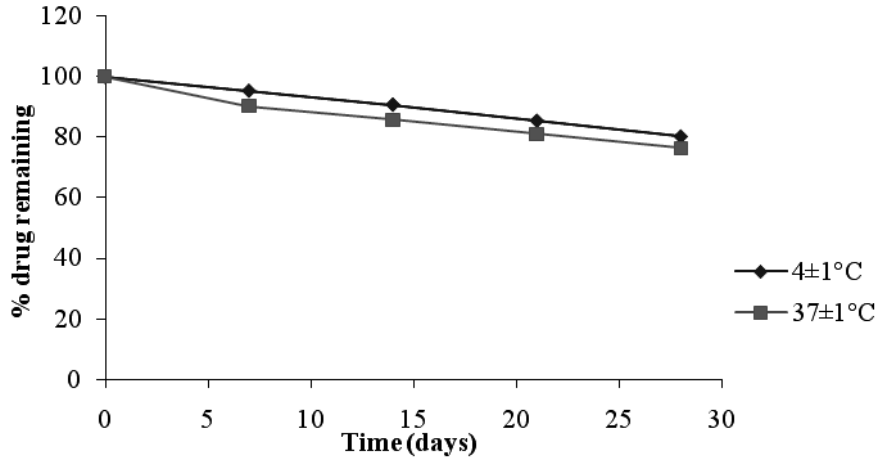


Figure 14. Stability studies of niosome formulation (F4) at different temperatures

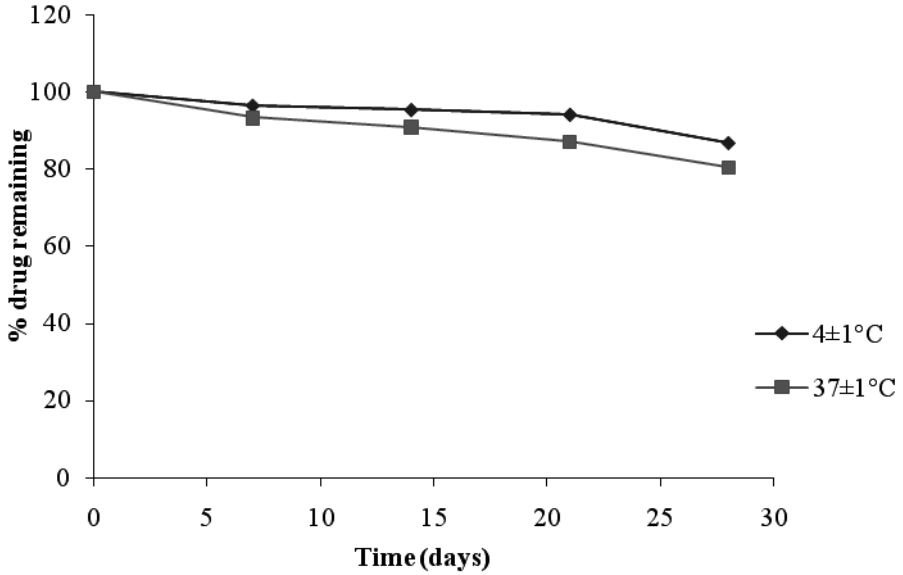


Figure 15. Stability studies of niosomal gel formulation (G2) at different temperatures

Table 8. Percent dipivefrin HCl remained from niosomal formulation F4 and niosomal gel formulation G2 stored at 4±1 °C and 37±1°C

Time (days)	Percent of dipivefrin HCl remained ± SD			
	F4		G2	
	4±1oC	37±1oC	4±1oC	37±1oC
0	100	100	100	100
7	95.37±1.42	90.36±2.09	96.48±1.23	93.27±3.19
14	90.79±2.32	85.75±1.87	95.35±0.17	90.92±1.17
21	85.54±0.37	80.97±1.51	94.12±0.62	87.19±2.11
28	80.36±1.05	76.45±2.67	86.76±1.25	80.58±1.07

Table 9. Results of percent cumulative drug permeated from niosomal gel formulation (G2) and marketed formulation in STF of pH 7.4

Time (hr)	Average percent cumulative drug permeated (Mean ± SD, n=3)	
	G2	Marketed formulation
0	0	0
1	06.97±1.23	10.95±0.34
2	12.65±0.86	14.15±0.76
3	20.81±1.98	23.97±0.43
4	32.46±1.17	38.64±1.45
5	42.12±0.24	47.71±1.21
6	52.75±1.65	57.94±0.97
7	65.51±0.67	69.29±0.56
8	74.21±1.05	81.47±0.24

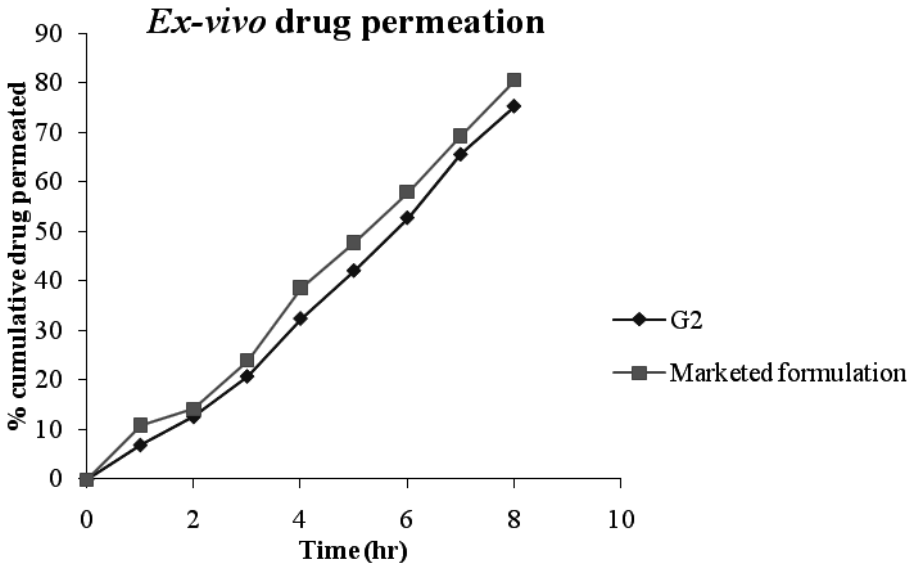


Figure 16. *Ex-vivo* permeation study of selected formulation (G2) and marketed formulation

niosomal gel formulation (G2) i.e. 74.21±1.05% of drug release in 8 h as compared to marketed formulation i.e. 81.47±0.24% of drug release in 8 h. Faster release of dipivefrin HCl from the marketed gel may be due to the free drug being present in gel structure as compared to niosomal gel in which the drug was entrapped into niosomal vesicular structure. These results were in accordance to Asthana *et al.*, 2016 [40]. It has been stated that more hydrophobic span surfactants form more compact niosomes when hydrated in presence of cholesterol [41].

Ocular irritancy test (Draize's test)

The possibility of eye irritation due to selected niosomal gel formulation (G2) and marketed formulation instillation were evaluated in rabbits. At the point of instillation, rabbit showed slight eye irritation but no redness or any other sign of inflammation was observed in the eyes. No signs of redness, inflammation, swelling or increased tear production were observed over the study period for tested formulation. No ophthalmic damage or abnormal clinical signs to the cornea, iris or conjunctivae were visible. This indicated

that the non-ionic surfactants namely span 60 as well as cholesterol used in the niosome formulations were non-irritant to the eye and could be used safely [42]. By instillation of marketed Pilopine gel, the rabbit's eye showed irritation, redness and inflammation at conjunctiva which may be due to its large molecular size, indicating a problem in the conjunctival absorption of drug.

In-vivo antiglaucoma activity by measurement of intraocular pressure (IOP)

As shown in Table 10, it was observed that marketed formulation showed a decrease in IOP up to 17.42±1.12 mmHg at the end of 7 h, but then there was an 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. G2 decreased IOP by 16.99±1.25 mmHg at the end of 9 h. G2 maintain the sustained effect up to 24 h. This control of IOP for prolonged periods may be attributed to the increased corneal residence and sustained drug release of the formulated ophthalmic niosomal gel compared to marketed Pilopine gel. It was also observed that upon administration of dipivefrin HCl ocular niosomal gel, no effect on IOP in the control eye, this may be an indication that no systemic absorption occurred. It was revealed that the sustained effect was maintained for more time in the niosomal gel as compared to the marketed formulation. The marketed Pilopine gel lowered the IOP to minimum (16.32±1.11 mmHg) and afterwards, there was a sudden increase in the IOP upto 40.01±0.43 mmHg in 24 h whereas, niosomal gel lowered the IOP slowly to the minimum and thereafter, a gradual increase in the IOP upto 21.93±1.24 mmHg in 24 h was observed.

The mean percentage decrease in IOP after installation of single dose of either niosomal gel formulations (G2) or marketed formulation was calculated and the data was shown in Figure 17. The mean pharmacokinetic parameters for niosomal gel G2 and marketed Pilopine gel was listed in Table 11. The ΔIOP_{max} values for G2 and marketed formulation were 22.49±0.06% and 22.65±1.12% respectively. T_{max} values were 9 h and 7 h respectively. Greater AUC values were observed for G2 (596.99 mmHg.h) compared with marketed Pilopine gel (225.34 mmHg.h). The relative bioavailability for G2 to marketed formulation was 2.64.

Higher bioavailability in case of G2 confirmed that the niosomal gel formulation had a prolonged duration of its anti-glaucomatic effect in comparison to the marketed Pilopine gel.

Table 10. IOP lowering effects of G2 and marketed formulation after treatment

Time (hr)	IOP (mmHg) lowering effects of G2 and marketed formulation			
	Group II (G2)		Group III (marketed Pilopine HS gel)	
	R	L	R	L
0	40.47±0.42	40.47±0.04	40.07±0.19	40.07±0.02
2	40.47±0.42	35.12±0.17	40.07±0.19	30.95±1.68
4	40.47±0.42	27.89±0.28	40.07±0.19	27.37±2.32
6	40.47±0.42	23.85±0.31	40.07±0.19	21.69±0.45
7	40.47±0.42	21.20±0.53	40.07±0.19	16.32±1.11
8	40.47±0.42	19.28±0.06	40.07±0.19	20.95±3.21
9	40.47±0.42	16.99±1.25	40.07±0.19	21.69±0.57
10	40.47±0.42	19.54±2.31	40.07±0.19	24.23±1.12
11	40.47±0.42	20.94±0.96	40.07±0.19	28.96±4.96
24	40.47±0.42	21.93±1.24	40.07±0.19	40.01±0.43

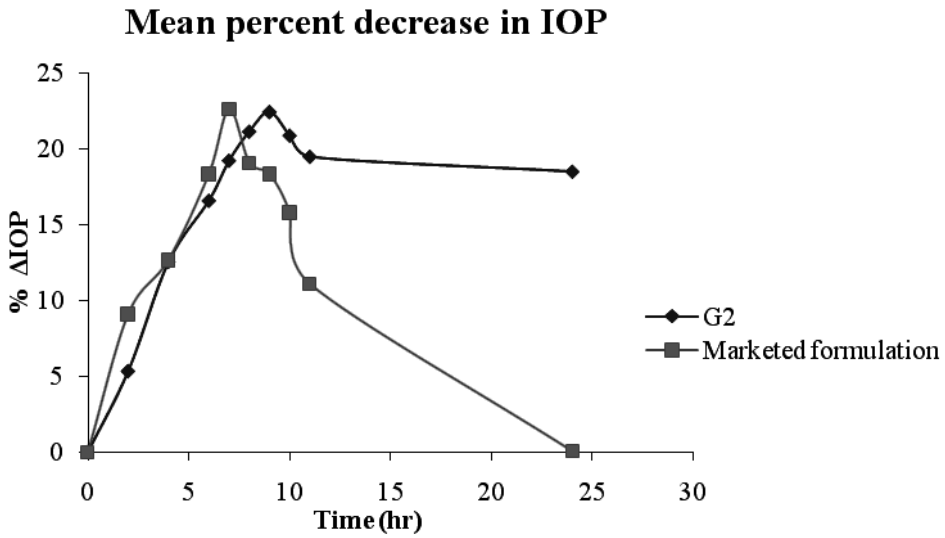


Figure 17. Mean percentage decrease in intraocular pressure (% ΔIOP) versus time

Table 11. Pharmacokinetic parameters for G2 and marketed Pilopine gel

Formulations	IOPmax (%)	Tmax (hr)	AUC0-24 h (mmHg.h)
G2	22.49±0.06	9	596.99
Marketed Pilopine gel	22.65±1.12	7	225.34

Conclusion

The results of this study indicate that cholesterol content and type of surfactant altered the % EE, vesicle size and release rate from dipivefrin HCl niosomes. Formulation F4 composed of span 60 and cholesterol (1:1) gave the most advantageous entrapment (92.16±0.25%) and release results after 8 hrs (Q8h=61.05±2.87%) as compared to other compositions. The *in-vitro* release data of gel formulations shows a lower percentage of drug release as compared with niosomes itself. The release data were fitted to an empirical equation to estimate the diffusion parameters, which indicated that the release follows non-Fickian diffusion mechanism. Among all formulations, G2 showed higher bioadhesive strength (2314±1.29 dynes/cm^2), higher stability while slower drug release in 8 hr due to high gelling capacity. Niosomal gel formulation gave higher AUC than that given by marketed gel and increased the bioavailability of dipivefrin hydrochloride by 2.64 times than marketed gel. These results suggest that the niosomes containing gels are promising ocular carriers for the controlled delivery of dipivefrin HCl in glaucoma treatment.

List of abbreviations

IOP, Intraocular pressure; **RGC,** Retinal ganglion cell; **POAG,** Primary open angle glaucoma; **FTIR,** Fourier transform infrared; **TEM,** Transmission electron microscopy; **UV,** Ultraviolet; **EE,** Entrapment efficiency; **SD,** Standard deviation; **STF,** Simulated tear fluid; **AUC**, Area under curve; **T_{max}**, Maximum time; **Q8h,** *In-vitro* release after 8 hours.

Ethics approval and consent to participate

This protocol was approved by the Institutional Animal Ethical committee of Department of Pharmacy, B.R. Nahata College of Pharmacy, Mandsaur (M.P.)

Human and animal rights

This article does not contain any studies with human subjects performed by any of the authors;

all institutional and national guidelines for the care and use of laboratory animals were followed.

Conflict of interest

The authors report no conflicts of interest.

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