Koranappallil S. Aswathy*
Department of Pharmaceutics,
University College of Pharmacy,
M.G. University, Cheruvandoor-
68631, Kerala, India

Rose mol K John
Department of Pharmaceutics,
University College of Pharmacy,
M.G. University, Cheruvandoor-
68631, Kerala, India

Correspondence:
Koranappallil S. Aswathy
Department of Pharmaceutics,
University College of Pharmacy,
M.G. University, Cheruvandoor-
68631, Kerala, India
E-mail:
aswathyks2007@gmail.com

Design, characterization and evaluation of latanoprost niosomes for glaucoma treatment
Koranappallil S. Aswathy*, Rose mol K John

Abstract

Eye being a most delicate organ, ocular drug delivery is a challenge for the formulator. A drop of an aqueous solution, irrespective of instilled volume is eliminated completely from the eye within 5 to 6 minutes of its application and only a small amount (1-3%) actually penetrates the cornea and reaches the intraocular tissue. This problem can be addressed by use of suitable carrier systems. Niosomal vesicular system is one of the potential approaches, which can be suitably used. The aim of the present investigation was to prepare, characterize and optimize the latanoprost niosomes using 2-factor 3-level full factorial design and carry out stability studies. Niosomes of latanoprost were prepared by heating method of mozafari and optimized using 2-factor 3-level full factorial design. The independent levels selected were cholesterol:span 60 ratio and mixing time, and the response optimized were entrapment efficiency and particle size. Contour and response surface plots were constructed to further elucidate the relationship between the independent and dependent variables. The formulated latanoprost niosomes were evaluated for their particle size, zeta potential, surface morphology, entrapment efficiency, in vitro drug release and in vivo corneal residence time studies. The niosomes showed a vesicle size of 2.58-3.13 μm and zeta potential of -9 to -13 mV. SEM revealed that niosomes were unilamellar and smooth in texture. The in vitro release studies showed 95.98% of latanoprost released in sustained manner following Higuchi model kinetics for 12 hours. The n value of 0.865 suggested non fickian diffusion from niosomes. The stability study confirmed that latanoprost niosomes were stable. In vivo corneal residence time studies showed the levels of drug concentration in tear fluid were maintained for 12 hr while for eye drops concentration was very less after 6 hr. The designed latanoprost niosomes with span 60 showed good physicochemical properties, good stability, prolonged action and improved bioavailability than the commercially available conventional dosage form which might be a potential carrier system to improve the patient compliance and reduce the side effects.

Keywords: Niosomes, Latanoprost, 2-factor 3-level full factorial design, Entrapment efficiency.

Introduction

Intraocular pressure (IOP) remains the key modifiable risk factor in glaucoma –a progressive optic neuropathy resulting in irreversible visual field loss that affects more than 60 million worldwide and is the second leading cause of blindness after cataract. The first line treatment of glaucoma is topical medications to lower the IOP, thereby delaying damage to the optic nerve from elevated IOP. However, daily application, due to poor ocular bioavailability and other long-term side effects such as allergy and intolerance to medications, have negative effects on patient compliance, which leads to disease progression from suboptimal medical management of the disease resulting in poor IOP control. Latanoprost, a lipophilic drug usually delivered in the form of an oil/water emulsion, is very effective in reducing IOP in glaucoma. However, latanoprost acid, the hydrolyzed active product, is more hydrophilic and experiences a higher penetration resistance through the epithelium and endothelium of the cornea and hence lower bioavailability.

Niosomes, which have been shown to be biocompatible nanocarriers for ocular use, allows for
delivery of both the lipophilic drug molecule as well as its hydrophilic active products, due to its physical structure of a polar core and lipophilic bilayer. Niosomal encapsulation protects drug molecules from enzymatic hydrolysis in the physiological environment while in circulation, and thus increases stability. The design of carriers for sustained delivery in the eye necessitates the use of a formulation that has the required optical clarity with sufficient drug loading efficiency for administration. Also, drug-loaded carriers should be stable under testing conditions in vitro and in vivo. The size of the niosomes might also facilitate the delivery of the drug through the various anatomical structures of the eye (conjunctiva and sclera) to reach the targeted site (ciliary body) more efficiently, with increased bioavailability.

Thus the challenge is to formulate latanoprost into a niosomal vehicle such that its delivery is sustained after single application. In this study, we developed and optimized a niosomal formulation that provides prolonged delivery, and confirmed its safety and efficacy in the rabbit eye.

Materials and Methods

Materials

Latanoprost was a gift sample from Sun Pharmaceuticals, Gujarat. Span 60 were purchased from CDH chemicals, New Delhi, India. Cholesterol was obtained from Nice chemicals, Kochi, India and Glycerin from Nice chemicals, Kochi, India. The water used in all the experiments was from Milili purification system with a resistivity of at least 18.2 ± 0.2 mΩ cm. All other chemicals used throughout the study were of analytical grade.

Methods

2-factor 3-level full factorial design: Traditionally pharmaceutical formulations are developed by changing one variable at a time. By this method it is difficult to develop an optimized formulation, as it does not give an idea about the interactions among the variables. Hence, 2-factor 3-level full factorial design with 2 factors, 3 levels and 9 runs was selected for the optimization study. Independent variables with their levels selected are listed in Table 1.

<table>
<thead>
<tr>
<th>Table 1: Independent variables with their levels</th>
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<tbody>
<tr>
<td><strong>Factors</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td>Cholesterol :Span 60 (mol/mol)</td>
</tr>
<tr>
<td>Mixing time(min)</td>
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Preparation of niosomes Niosomes were prepared by the heating method. This is a non-toxic scalable and one step method and is based on the patented procedure of Mozafari (2005). Mixture of non- ionic surfactant (Span 60), cholesterol and or charge inducing molecules are added to an aqueous medium (eg: buffer/ distilled water) in the presence of polyol such as glycerol (3% v/v of final concentration). To this latanoprost (10 mg) was added. The mixture is heated while stirring at low shear force (100 rpm) until vesicles are formed. After removing the unentrapped drug by centrifugation at 15000 rpm, niosome suspension was lyophilized and stored in desiccators until use. All the batches were prepared according to the experimental design.

Evaluation of niosomes

Optical microscopy: A small droplet of the vesicle suspension was placed on a glass microscope slide, diluted with a few drops of distilled water and covered with a glass cover slip. The samples were examined for vesicle shape and crystal formation using a calibrated eyepiece micrometer.

Characterization of vesicles by scanning electron microscopy (SEM): The particle shape and size was observed by scanning electron microscopy (model: JEOL JSM-6390). The freeze dried niosomes were mounted on a platinum ribbon supported on a disc and was coated with platinum using platinum sputter module (JFC-1100, JEOL Ltd) in a higher vacuum evaporator for 5 min at 20 mA.

Determination of un-entrapped and entrapped drug: The niosome suspension was ultra centrifuged at 5000 rpm for 15 minutes at 4°C temperature by using remi cooling centrifuge to separate the free drug. A supernatant containing niosomes in suspended stage and free drug at the wall of centrifugation tube was obtained. The supernatant was collected and again centrifuged at 15000 rpm at 4°C temperature for 30 minutes. A clear solution of supernatant and pellets of niosomes were obtained. The pellet containing only niosomes was resuspended in distilled water until further processing. The niosome free from unentrapped drug were soaked in 1 ml of isopropyl alcohol and made up to 10 ml with STF pH 7.4 and then sonicated for 10 min. The vesicles were broken to release the drug, which was then estimated for the drug content. The absorbance of the drug was noted at 210 nm.

\[ \text{Entrapment efficiency} = \frac{\text{Amount of drug entrapped (mg)}}{\text{Amount of drug added}} \times 100 \]

Zeta potential measurement: Zeta potential of suitably diluted niosome dispersion was determined using zeta potential analyzer based on electrophoretic light scattering and laser Doppler velocimetry method using Zetasizer (Malvern instruments). The temperature was set at 25°C.

Particle size measurement: The z-average diameter of sonicated vesicles was determined by dynamic light scattering.
using a Zetasizer. For the measurement, 100 μl of the formulation was diluted with an appropriate volume of STF pH 7.4 and the vesicle diameter and polydispersity index were determined.

**In vitro release rate studies and kinetics model fitting**

*In vitro* release profile of latanoprost niosomes was performed in an open ended cylindrical tube (20 mm diameter) with one end tied with cellophane membrane. Latanoprost niosomes placed in an open ended cylinder and was suspended in a 50 ml of STF pH 7.4 receptor medium in a beaker placed on a thermostat magnetic stirrer and constantly stirred at 20 rpm and 37±1°C temperature. Aliquots (5 ml) of samples were withdrawn from the receptor compartment at predetermined time intervals of 1, 2, 4, 6, up to 24 hours and after each withdrawal same volume of fresh medium was replenished. The withdrawn samples were made up with STF pH 7.4 and latanoprost content in the withdrawn samples was estimated spectrophotometrically at 210 nm. *In vitro* release of latanoprost in free drug solution and suitable ratio of latanoprost niosomes were compared.

The *in vitro* release data were plotted according to the four different kinetic models, zero order, first order, Higuchi’s and Korsmeyer-peppas release model to know the release mechanisms. The order of kinetics and mechanism of the release were confirmed based on linearity of the graphical expression from *in vitro* release data.

**Statistical analysis:** Each experiment was carried out in triplicate and the results are mean ±SD.

**Sterilization:** Sterilization of niosomes was done by gamma radiation method before *in vivo* examination.

**Sterility testing:** Testing was done in petriplates containing 20ml of Nutrient agar and Sabouraud dextrose agar. The sample was swabbed on both Nutrient agar plate and Sabouraud dextrose agar and incubated for 72 hours and observed for the evidence of microbial contamination daily for 14 days.

**In vivo studies:** Approval for the use of animals in the study was obtained from the Institutional Animal Ethics Committee (IAEC No: 018/MPH/UCP/CVR/14). New Zealand female rabbits weighing 0.879 kg to 1.5 kg were used for *in vivo* studies. The rabbits were housed singly in restraining cages during the experiment and allowed food and water ad libitum. Free lag and eye movement was allowed. No ocular abnormalities were found on external and slit-lamp examination prior to beginning of the study.

**Ocular safety study:** The ocular safety of administered delivery system was observed based on the Draize Irritancy Test.

**Corneal residence time evaluation:** Precorneal residence time of the drug from the formulated delivery system has been assessed by a non-invasive method based on HPLC technique. Tear samples equivalent to 1 μL were collected from the left eye after application of test delivery system at 1, 2, 4, 6, 10, 22 and 24 hr, post dosing. Glass capillary tubes having 320 μm internal diameter and 1 μL premark were placed near the canthus of the eye without applying pressure. Tear fluid was drained into the tubes due to capillary action. Samples equivalent to 1 μL were mixed with 50 μL of mobile phase, a mixture of acetonitrile: water in the ratio of 70:30 (v/v) and injected into HPLC chamber.³

**Stability studies:** The accelerated stability study was carried out according to ICH Q1A guidelines with optimized ocuserts. Sealed vials of freshly prepared niosomes loaded with latanoprost were placed in stability chamber maintained at 25 °C ± 2 °C / 60 % RH ± 5 % RH. The samples subjected to stability tests were analyzed over 3 month’s period for physical appearance, and particle size.

**Result and Discussion**

**Evaluation of niosomes:** The photomicrographs (×40) of latanoprost niosomes are shown in Figure 1. Niosomes were observed as spherical large unilamellar vesicles. Aggregation/fusion of the vesicles could be occasionally observed. From the SEM morphology images Figure 2, the prepared niosomes were found to be spherical in shape with smooth surface and vesicles were discrete and separate with no aggregation or agglomeration.

**Particle size:** The particle size of the optimized formulation was found to be 2.58-3 μm and the curve obtained is given in Figure 3 suggesting the solution remained stable throughout the run.

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**Figure 1:** Photomicrograph of niosomes

**Figure 2:** SEM image of niosomes
Zeta potential measurement: Zeta potential is an important parameter for prediction of the stability of colloidal carrier system and fate of vesicles in vivo. The zeta potential value of latanoprost niosomes was found to be in the region of -13 mV (Figure 4) and this relatively small value indicated that there is little electrostatic repulsion between these vesicles. The near neutral charge is advantageous for in vivo use, as large positive or negative charges can lead to rapid aqueous humour clearance.

In vitro release rate studies and kinetics model fitting: The in vitro release profiles of latanoprost niosomes were obtained by representing the percentage of latanoprost release with respect to the amount of latanoprost loaded in the niosomes as shown in the Table 2 and Figure 5.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>% Cumulative release of niosomes</th>
<th>Eye drop</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>11.32±1.782</td>
<td>22.14±1.201</td>
</tr>
<tr>
<td>2</td>
<td>19.54±1.848</td>
<td>84.53±2.844</td>
</tr>
<tr>
<td>4</td>
<td>29.62±2.416</td>
<td>99.87±1.154</td>
</tr>
<tr>
<td>6</td>
<td>43.18±6.566</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>72.84±4.673</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>86.72±4.001</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>95.98±3.062</td>
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</table>

Figure 4: Zeta potential curve of optimized niosomes

The release behaviour of latanoprost from the developed niosomes exhibited a biphasic pattern characterized by an initial rapid release during the first hr, followed by a slower and continuous release up to 12 hours compared to eye drops. A high initial burst release can be attributed to the immediate dissolution and release of latanoprost adhered on the surface and located near the surface of the niosomes. Cholesterol favoured for the extended release up to 12 hours by abolishing the gel to liquid phase transition and promoting the formation of a less ordered liquid-crystalline state as vesicles. Thus leakage of content from the niosomes could be prevented.

Drug release mechanism of optimized formulation was determined by fitting its drug release data to various the kinetic models. The release of drugs from latanoprost niosomes were diffusion controlled as indicated by the higher $r^2$ values in Higuchi model and the n value of less than 1 suggests that, the mechanism of drug release from the niosomes were Fickian diffusion.

Sterility testing: The ocular niosomes were sterilized by gamma radiation and sterility testing was carried out under aseptic conditions. The growth of microorganism was observed in positive control, showing that the media was suitable for test conditions. No growth of microorganisms was observed in the negative control test, which confirmed that all the apparatus used for the test were sterile and aseptic conditions were maintained. There was no growth of microorganisms in the sample under test, confirming the sterility of niosomes; making it suitable for in vivo studies.

In vivo studies: Institutional Animal ethical committee, University College of pharmacy M G University, Kottayam has given permission for the conduct of the study with ethical committee number (IAEC No: 018/MPH/UCP/CVR/14).

Ocular safety studies: The ocular safety score of the optimized formulation F5 was found to be 1 at the end of 24 hr respectively and therefore, considered as non -irritating. Thus, it can be concluded that they were safe for ocular administration.

Corneal residence time evaluation: The precorneal residence of latanoprost after application of noisome in rabbit eyes is shown in Table III.
There was a significant improvement in precorneal residence of latanoprost after application of the formulated niosomes as compared to eye drops and maintained for 12 hrs. The increase in corneal residence may be attributed to the sustained release of drug from the niosomes as proved by in vitro studies.

**Stability studies:** The lyophilized latanoprost loaded niosomes were analyzed for 3 months for variations in particle size. On storage the particle size of the lyophilized niosomes does not show significant change. The particles still retain potency to penetrate ocular tissues to enhance bioavailability.

**Conclusion**

Latanoprost containing niosomes were prepared using Span 60 and cholesterol and evaluated for in vitro and in vivo tests. Morphological studies revealed that all the formulations were spherical in shape and existed as separate particles. Drug entrapment was higher enough to incorporate required dose of drug in minimum possible concentrated niosomal suspension. The release of drug from niosomes was controlled by diffusion for a prolonged period of time. The optimized formulation showed better release and bioavailability over eye drops, indicating that niosomes can be a choice of drug delivery for the treatment of glaucoma as a sustained ocular drug delivery system.

**Acknowledgement**

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**References**


