2.1 DRUG AND EXCIPIENTS PROFILES

Drug Profile

Drug selected for the study: Bimatoprost and Sparfloxacin

Bimatoprost

Bimatoprost is newly discovered prostaglandin derivative which is highly effective intraocular pressure (IOP) lowering agent used in the treatment of glaucoma.

Structural formula:

\[
\text{Chemical formula: } 7\text{-[3, 5-dihydroxy-2- (3-hydroxy-5-phenyl-pent-1-enyl)-}
\text{cyclopentyl]-N-ethyl-hept-5-enamide.}
\]

Physical properties:

- **Appearance**: White crystalline powder
- **Molecular Formula**: C_{25}H_{37}NO_{4}
- **Molecular Weight**: 415.56
- **Melting range**: 68-75 °C
- **Solubility**: Slightly soluble in water
- **Log P Value**: 3.2
- **Category**: Anti-Glaucoma

Fig 2: Chemical Structure of Bimatoprost.
Mechanism of action:

Bimatoprost reduces the intraocular pressure (IOP) by rising outflow of aqueous humor through both the uveoscleral routes and trabecular meshwork. Bimatoprost lowers IOP by imitating the action of a naturally occurring prostaglandin. Prostaglandins found many areas in the body. In eyes, they increase the outflow of the aqueous humor from the eyeball. Bimatoprost may also reduce the rate of aqueous humor formation in the eye. Both these effects decrease the pressure within the eye ball.

Pharmacokinetic parameters:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Half life</td>
<td>45 min</td>
</tr>
<tr>
<td>Plasma protein binding</td>
<td>88 %</td>
</tr>
</tbody>
</table>

Metabolism:

Bimatoprost undergoes oxidation, N-demethylation and glucuronidation to form a variety of metabolites.

Toxicity:

In oral mouse and rat studies, doses up to 100 mg/kg/day did not produce any toxicity. This dose expressed as mg/m² is at least 70 times more than the unintentional dose of one bottle of Bimatoprost for a 10 kg child.
Sparfloxacin is a fluoroquinolone antibiotic used in the management of bacterial infections. Sparfloxacin shows its antibacterial potential by inhibiting DNA gyrase, a bacterial topoisomerase.

Chemical structure:

![Chemical Structure of Sparfloxacin]

Chemical formula:

5-amino-1-cyclopropyl-7-[(3R,5S)-3,5-dimethylpiperazin-1-yl]-6,8-difluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid

Physical properties:

- Appearance: Yellow crystalline powder.
- Molecular Formula: C<sub>19</sub>H<sub>22</sub>F<sub>2</sub>N<sub>4</sub>O<sub>3</sub>
- Molecular Weight: 392.39
- Melting range: 265 °C
- Solubility: Practically insoluble in water.
- Log P Value: 2.5
- Category: Anti-Bacterial.
Mechanism of action:

The bactericidal action of sparfloxacin results from inhibition of the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV, which are required for bacterial DNA replication, transcription, repair, and recombination.

Pharmacokinetic parameters:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Half-life</td>
<td>16 hrs.</td>
</tr>
<tr>
<td>Plasma protein binding</td>
<td>45%</td>
</tr>
</tbody>
</table>

Metabolism:

Metabolized primarily by phase II glucuronidation to form a glucuronide conjugate in liver. Metabolism does not utilize or interfere with the cytochrome P450 enzyme system.

Toxicity:

The single dose of Sparfloxacin found non-toxic on oral administration in rats, mice and dogs. Not a single animal died within a 14-day post-treatment observation period at the maximum oral doses tested, up to 5000 mg/kg in both rodent species, or up to 600 mg/kg in the dog.
Carboxymethyl Chitosan

Carboxymethyl chitosan is made from the reaction of chitin and chloride, acetic acid in alkalinity circumstance. It has higher improved physical and chemical capability and is entirely 100% saluted in water having properties like Innocuous, flavorless, white or off-white powder; freely soluble in water, the solution is clear and character is stable.

Structural formula:

![Chemical Structure of n-Carboxymethyl chitosan](image)

**Fig 4: Chemical Structure of n-Carboxymethyl chitosan**

**Synonym:** Carboxymethyl chitosan, water soluble chitosan.

**Chemical name:** Carboxymethyl chitosan

**Molecular weight:** 40–405 g/mol

**Viscosity:** 20–40 Cps

**Technical Specification:**
- **Appearance:** Off white flakes
- **Solubility:** Soluble in cold water
CHAPTER 2 MATERIALS AND METHODS

pH: 7-9
Viscosity: 20-40 Cps
Heavy metals (as Pb): ≤ 0.005%

Application:

The product is the higher ramification of chitosan, which have 100% water solubility. It has been applied in advanced cosmetics, drug sustained release agent, plant growth regulator agent and waste water treatment and etc. Chitosan is used in cosmetics and also the research is going on with pharmaceutical formulations. The fitness and performance of chitosan as a component of pharmaceutical formulations for drug delivery applications has been invented in various studies. These contain controlled drug delivery applications, use as a part of mucoadhesive dosage forms, immediate release dosage forms, enhanced peptide delivery, colonic drug delivery systems and used for gene delivery. Chitosan has been processed into a number of pharmaceutical forms including gels, films, beads, microspheres, tablets and coatings for liposomes.

Stability and Storage condition:

Carboxymethyl Chitosan powder is a stable at room temperature, although after drying it is hygroscopic. Chitosan should be stored in a tightly closed container in a dry, cool place. The PhEur 6.5 specify that chitosan must be stored at a temperature of 2–8°C

Safety: Chitosan is being studied extensively for use as an excipient in oral and other pharmaceutical formulations and cosmetics. Chitosan is usually regarded as a nontoxic and nonirritant. It is biocompatible with healthy as well as infected skin.
Regulatory Status: Carboxymethyl Chitosan is registered as a food supplement in some countries.

HPMC E565

Nonproprietary Names

BP, JP, PhEur, USP: Hypromellose

Synonyms: Hydroxypropylmethylcellulose; HPMC; Methocel; Hypromellose; Metolose and Pharmacoat

Chemical Name and CAS Registry: Number Cellulose hydroxypropyl methyl ether [9004-65-3]

Empirical Formula and Molecular Weight:

Molecular weight: 10000–1500000

Empirical Formula: C_{56}H_{108}O_{30}

Fig 5: Chemical Structure of HPMC

Functional Category: Coating agent; Bioadhesive material, sustained-release agent granulation aid emulsifying agent stabilizing agent; controlled-release agent; dissolution enhancer; emulsion stabilizer; extended-release agent; film-forming agent; foaming agent; dispersing agent; mucoadhesive; release-modifying agent; solubilizing agent; suspending agent; modified-release agent, tablet binder; thickening agent.

Description: Hypromellose is an odorless and tasteless, white or creamy-white fibrous or granular powder.
Acidity/alkalinity: pH = 5.0–8.0 for a 2% w/v aqueous solution,

Cloud point: >100°C for a 1% w/v aqueous solution,

Melting point: 225–230°C

Moisture content: Hypromellose absorbs moisture from the environment; the quantity of water absorbed depends upon the primary moisture content, temperature and relative humidity of the contiguous air

Solubility: Freely soluble in cold water.

Viscosity: 5 mPa.s

Table 2: Pharmacopoeial Specifications for HPMC

<table>
<thead>
<tr>
<th>Test</th>
<th>PhEur 2005</th>
<th>USPNF 23</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identification</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Characters</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Appearance of solution</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Average Molecular Weight</td>
<td>9,840–14,600</td>
<td>9,840–14,600</td>
</tr>
<tr>
<td>pH (aqueous solution)</td>
<td>5.0–8.0</td>
<td>5.0–8.0</td>
</tr>
<tr>
<td>Total ash</td>
<td>≤0.4%</td>
<td>—</td>
</tr>
<tr>
<td>Heavy metals</td>
<td>≤20 ppm</td>
<td>≤20 ppm</td>
</tr>
</tbody>
</table>

Stability and Storage Condition:

Solutions are stable at pH 3-11. Hypromellose powder should be stored in a well-closed container, in a cool, dry place.
Safety:

Hypromellose is widely used as a raw material in oral, ophthalmic, topical and nasal, formulations. It is also used widely in food and cosmetics products. Hypromellose is usually regarded as a nontoxic and nonirritating material but the too much oral consumption may have a laxative effect.

Applications in Pharmaceutical Formulation or Technology

HPMC is widely used in pharmaceutical in oral, ophthalmic, nasal, and topical routes. In oral drug delivery HPMC is mostly used as a tablet binder in film-coating agent and as a matrix for use in extended release formulations. HPMC is also used in liquid oral dosage forms as a suspending and thickening agent. HPMC is also used as a suspending and thickening agent in topical formulations. Compared with methylcellulose, HPMC produces aqueous solutions of better clarity, with less undissolved fibers and is therefore favored in formulations for ocular use. HPMC at concentrations between 0.45–1.0% w/w may be used as thickening agent in ophthalmic formulations. It is also used commercially in liquid nasal formulations. HPMC is used as a suspending agent, emulsifying and stabilizing agent in topical ointments and gels. It is also widely used in cosmetics and food products.

Regulatory Status:

Accepted for use as a food additive in Europe ad included in the FDA Inactive Ingredients Databases of various countries.

Poloxamer 407

Nonproprietary Names

BP: Poloxamer; PhEur: Poloxamera; USPNF: Poloxamer
Synonyms
Pluronic, Lutrol, Supronic, poloxalkol, Synperonic.

Chemical Name and CAS Registry Number
α-Hydro-ω-hydroxypoly (oxyethylene) poly (oxypropylene) poly (oxyethylene) block copolymer
CAS NO: 9003-11-6

Empirical Formula and Molecular Weight:
HO(C₂H₄O)ₐ(C₃H₆O)ₗ(C₂H₄O)ₐH. For the Poloxamer 407 the corresponding values for a, b are 101 and 56 respectively.
The average molecular weight: 9840–14 600.

Structural Formula:

![Chemical Structure of Poloxamer-407](image)

Functional Category:
Emulsifying agent, dispersing agent, solubilizing agent, Coemulsifying agent, wetting agent and tablet Lubrictant.

Description:
Poloxamer 407 generally occurs as white, waxy, free-flowing granules. It is practically odorless and tasteless.
### Table 3: Pharmacopoeial Specifications for Poloxamer 407

<table>
<thead>
<tr>
<th>Test</th>
<th>PhEur 2005</th>
<th>USPNF 23</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identification</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Characters</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Appearance of solution</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Weight percent oxyethylene</td>
<td>71.5–74.9</td>
<td>73.2 ± 1.7</td>
</tr>
<tr>
<td>pH (aqueous solution)</td>
<td>5.0–7.5</td>
<td>5.0–7.5</td>
</tr>
<tr>
<td>Unsaturation (mEq/g)</td>
<td>—</td>
<td>0.048 ± 0.017</td>
</tr>
<tr>
<td>Oxypropylene:oxyethylene ratio</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Heavy metals</td>
<td>—</td>
<td>≤0.002%</td>
</tr>
<tr>
<td>Organic volatile impurities</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>Water</td>
<td>≤1.0%</td>
<td>—</td>
</tr>
<tr>
<td>Ethylene oxide</td>
<td>—</td>
<td>≤1 ppm</td>
</tr>
<tr>
<td>Propylene oxide</td>
<td>—</td>
<td>≤5 ppm</td>
</tr>
<tr>
<td>1,4-Dioxane</td>
<td>—</td>
<td>≤5 ppm</td>
</tr>
</tbody>
</table>

**Typical Properties:**

**Acidity/alkalinity:** pH = 5.0–7.4 for a 2.5% w/v aqueous solution,

**Cloud point** : >100°C for a 1% w/v aqueous solution,

**HLB** : 18-23

**Density** : 1.06 g/cm³ at 25°C,
Flash point : 260°C,

Flowability : Free flowing,

Melting point : 52–57°C

Moisture content: Poloxamer generally contain less than 0.5% w/w water.

Solubility : Freely soluble in Water and 95 % Ethanol.

Stability and Storage Conditions

Poloxamer 407 is a stable and the aqueous solutions are stable in the presence of acids, alkalis, and metal ions. The bulk material must be stored in a well-closed container in a cool and dry place.

Applications in Pharmaceutical Formulation or Technology

Poloxamer used as wetting agents in ointments and gels. Poloxamer are used solubilizing and stabilizing agents as may also be used as emulsifying agents in intravenous fat emulsions. Recently, Poloxamer have found use in drug-delivery systems. Poloxamer 407 is used in solutions for contact lens care.

Regulatory Status

Included in the FDA Inactive Ingredients Guide IV ophthalmic preparations; inhalations injections; oral powders, solutions, suspensions, and syrups; topical preparations.

Kolliphor P188

Nonproprietary Names

BP: Pluronic; PhEur: Lutrol ; USPNF: Kolliphor
Synonyms: Lutrol, Poloxamer, Pluronic F68

Chemical name and CAS registry no:

Poly (ethylene glycol)-block-poly (propylene glycol)-block-poly (ethylene glycol), CAS Number 9003-11-6

Empirical Formula and Molecular Weight:

Empirical Formula - C_{5}H_{10}O_{2}

Molecular weight – 102.133 g/mol

Structural Formula:

![Chemical Structure of Kolliphor P188](image)

Functional Category:

Emulsifying agent, Dispersing agent, Solubilizing agent, Coemulsifying agent, wetting agent and Tablet Lubricant.

Description:

Kolliphor generally occurs as white, waxy, free-flowing granules. It is practically fragrance-free and tasteless.
Table 4: Pharmacopoeial Specifications for Kolliphor P188

<table>
<thead>
<tr>
<th>Test</th>
<th>PhEur 2005</th>
<th>USPNF 23</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identification</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Characters</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Appearance of solution</td>
<td>+</td>
<td>—</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>102.133 g/mol</td>
<td>102.133 g/mol</td>
</tr>
<tr>
<td>Weight percent oxyethylene</td>
<td>—</td>
<td>102.133 g/mol</td>
</tr>
<tr>
<td>pH (aqueous solution)</td>
<td>5 – 7.5</td>
<td>5 – 7.5</td>
</tr>
<tr>
<td>Heavy metals</td>
<td>≤ 20 ppm</td>
<td>≤0.002%</td>
</tr>
<tr>
<td>Organic volatile impurities</td>
<td>—</td>
<td>+</td>
</tr>
<tr>
<td>Water</td>
<td>≤0.75%</td>
<td>—</td>
</tr>
<tr>
<td>Ethylene oxide</td>
<td>—</td>
<td>≤1 ppm</td>
</tr>
<tr>
<td>Propylene oxide</td>
<td>—</td>
<td>≤5 ppm</td>
</tr>
<tr>
<td>1,4-Dioxane</td>
<td>—</td>
<td>≤5 ppm</td>
</tr>
</tbody>
</table>

Typical Properties:

Acidity/alkalinity: pH = 5.0–7.4 for a 2.5% w/v aqueous solution,

HLB : 18–24

Density : 1.06 g/cm³ at 25°C,

Flash point : 260°C,

Melting point : 52–57°C

Moisture content: Poloxamer generally contain less than 0.5% w/w water and hygroscopic at RH > 80%.
**CHAPTER 2**

**MATERIALS AND METHODS**

**Solubility**: Freely soluble in Water, isopropanol and 95% Ethanol.

**Stability and Storage Conditions**

Kolliphor P188 is a stable material. Aqueous solutions are stable in the presence of acids, alkalis, and metal ions. The bulk material ought to be stored in a well-closed container in a cool, dry place.

**Applications in Pharmaceutical Formulation or Technology**

Kolliphor P188 is a stable and the aqueous solutions are stable in the presence of acids, alkalis, and metal ions. The bulk material must be stored in a well-closed container in a cool and dry place.

**Regulatory Status**

Included in the FDA Inactive Ingredients Guide IV ophthalmic preparations; inhalations injections; oral powders, solutions, suspensions, and syrups; topical preparations.
Table 5: List of equipment’s and instruments

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Instrument Name</th>
<th>Model</th>
<th>Make</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Electronic Balance Model</td>
<td>AA-2200</td>
<td>Anamed</td>
</tr>
<tr>
<td>2.</td>
<td>Magnetic Stirrer</td>
<td>2-MLH</td>
<td>Remi</td>
</tr>
<tr>
<td>3.</td>
<td>UV-Visible double beam Spectrophotometer</td>
<td>Sican 2301</td>
<td>Inkarp</td>
</tr>
<tr>
<td>4.</td>
<td>FTIR</td>
<td>IRAffinity-1S</td>
<td>Shimadzu</td>
</tr>
<tr>
<td>5.</td>
<td>Nanotrac</td>
<td>R- 150 Ultra</td>
<td>Microtrac</td>
</tr>
<tr>
<td>6.</td>
<td>Differential Scanning Calorimeter</td>
<td>DSC-60</td>
<td>Shimadzu</td>
</tr>
<tr>
<td>7.</td>
<td>High Performance Liquid Chromatography</td>
<td>SPD-M20A</td>
<td>Shimadzu</td>
</tr>
<tr>
<td>8.</td>
<td>Transmission Electron Microscopy</td>
<td>H-7500</td>
<td>Hitachi</td>
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<tr>
<td>10.</td>
<td>Franz Diffusion Cell</td>
<td>V6B-02</td>
<td>PermeGear</td>
</tr>
<tr>
<td>11.</td>
<td>pH Meter</td>
<td>EQ-610</td>
<td>Equip-Tronics</td>
</tr>
<tr>
<td>12.</td>
<td>Zetasizer</td>
<td>Nano S</td>
<td>Malvern</td>
</tr>
<tr>
<td>13.</td>
<td>Homogenizer</td>
<td>DV-III Ultra</td>
<td>Remi India</td>
</tr>
<tr>
<td>14.</td>
<td>Probe Sonicator</td>
<td>Probe-400</td>
<td>Rivotek Mumbai</td>
</tr>
<tr>
<td>15.</td>
<td>Refrigerated Centrifuge</td>
<td>Fz Power</td>
<td>Plastro crafts Ind. Mumbai</td>
</tr>
<tr>
<td>16.</td>
<td>Trinocular microscope</td>
<td>Metzer</td>
<td>Metzer</td>
</tr>
<tr>
<td>Sr. No.</td>
<td>Chemicals</td>
<td>Suppliers</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------</td>
<td>-----------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>1.</td>
<td>Bimatoprost</td>
<td>Gifted by, Biocon Ltd, Bengaluru.</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Sparfloxacin</td>
<td>Gifted by, Micro Labs, Bengaluru.</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>Water Soluble Chitosan</td>
<td>Pelican Biotech Ltd, Kochin</td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>HPMC E5</td>
<td>Colorcon Ltd, Goa</td>
<td></td>
</tr>
<tr>
<td>5.</td>
<td>Ethanol</td>
<td>Changshu Yangyuan Chemical China</td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td>Kolliphor P188</td>
<td>BASF. Mumbai</td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>Poloxamer 407</td>
<td>Sigma Aldrich. Mumbai</td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td>Potassium Dihydrogen Orthophosphate</td>
<td>Himedia Pvt Ltd. Mumbai</td>
<td></td>
</tr>
<tr>
<td>10.</td>
<td>Sodium chloride</td>
<td>Himedia laboratories pvt ltd, Mumbai</td>
<td></td>
</tr>
<tr>
<td>11.</td>
<td>Benzalkonium chloride</td>
<td>M/s Otto chemika biochemika</td>
<td></td>
</tr>
<tr>
<td>12.</td>
<td>Sodium hydroxide</td>
<td>Himedia laboratories pvt ltd, Mumbai</td>
<td></td>
</tr>
<tr>
<td>13.</td>
<td>Acetone</td>
<td>S D Fine Chemicals Pvt Ltd, Mumbai</td>
<td></td>
</tr>
</tbody>
</table>
2.2 EXPERIMENTAL

2.2.1 PREFORMULATION STUDY:

CHARACTERIZATION OF PURE DRUG:

2.2.1 UV spectroscopy: (Determination of λ.max):
Sparfloxacin was accurately weighed and dissolved in the 0.1 M NaOH : Methanol 60:40 proportion further serial dilutions were carried out by using simulated tear fluid (STF) to obtain solution of 10µg/ml. UV spectrum was run from 200-400nm and λ.max was recorded using UV spectrophotometer (Inkarp – Sican 2301).

2.2.2 FTIR spectroscopy:
The infrared spectrum of the Bimatoprost and Sparfloxacin was recorded using Fourier Transform Infra-Red spectrophotometer (Shimadzu).

2.2.3 Melting Point Determination:
The melting point of Bimatoprost and Sparfloxacin was determined by capillary method. The melting point was also confirmed by DSC (Shimadzu) study.

2.2.4 Solubility Determination:
In 250 ml of conical flask, excess quantity of drug was added to 25ml of different solvents. By using thermostatically controlled mechanical shaker, the flasks were shaken for 48 hrs to achieve solubility equilibrium. The samples were filtered through Whatman filter paper no. 41, diluted suitably using respective solvent and analyzed spectrophotometrically at particular wavelength.
2.2.5 DEVELOPMENT OF UV SPECTROSCOPIC METHOD:

The UV method was developed with some modifications in the procedure of Sharma et al.

CALIBRATION CURVE FOR THE ESTIMATION OF SPARFLOXACIN

i) Preparation of simulated tear fluid: Simulated tear fluid was prepared by dissolving 6.7gm of sodium chloride, 2gm of sodium bicarbonate and 0.08gm of calcium chloride dehydrated in 1000 ml of distilled water. The pH was found to be 7.4 ± 0.1

ii) Preparation of standard stock solution: Standard stock solution of Sparfloxacin was prepared by dissolving 100 mg of drug in 100 ml 0.1M NaOH: Methanol (60:40) to obtain stock solution of 1 mg/ml concentration. This was followed by sonication for 15 minutes. It was finally filtered through Whatman filter paper.

iii) Preparation of sample solutions: Aliquots of stock solutions were subjected to serial dilutions with simulated tear fluid to get solutions with concentration of 2-10 µg/ml.

iv) Determination of λ max: A 10 mcg/ml solution were scanned in the range of 200–400 nm against blank. The absorption maxima were found to be at 289 nm against blank.

v) Preparation of Calibration curve: The absorbance of these dilutions was measured using UV spectrophotometer at 289.0 nm against blank. The absorbance of Sparfloxacin for corresponding concentrations and regression data are given in Table 1. The absorbance were plotted against concentration of Sparfloxacin and shown in Fig 1. The calibration curve will be used for the estimation of Sparfloxacin from the prepared nanosuspension.
2.2.6 DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR ESTIMATION OF BIMATOPROST

The HPLC method was developed with some modifications in the procedure of Suresh Kumar et al.

i) Instrument:

HPLC Model: Shimadzu Prominence

Autosampler: SIL-30 AC

Fixed Capacity Loop: 20 µl

Detector: PDA Detector

Column: Phenomenex, C18 column, 150 mm × 4.6 mm, 5µm

ii) Preparation and Selection of Mobile phase: The preliminary studies on a reverse phase C18 column with different mobile phase combination of Acetonitrile and Triethylamine buffer were studied for estimation of drug. The optimal composition of mobile phase determined to be Acetonitrile: 0.2% Triethylamine pH 7.0 adjusted with orthophosphoric acid (50:50 v/v) and filtered through 0.45µ membrane filter.

iii) Preparation of standard stock solution: Weighed accurately about 10 mg of Bimatoprost and transferred into 100ml volumetric flask. To this added about 70 ml of mobile phase (Triethylamine buffer pH 7.0 and acetonitrile in the ratio of 50:50 v/v) and sonicated for 20min to dissolve it completely. Then the volume was made up to the mark with the mobile phase. Further working standard solution of 6.25, 12.5, 25, 50 and 100µg/ml of Bimatoprost solutions were prepared with mobile phase respectively.
iv) Preparation of sample solution: A quantity equivalent to 100µg/ml was prepared from marketed formulation (label claim 0.1 mg/ml or 0.01% w/v solution) using mobile phase and injected into HPLC system and chromatograms were obtained for sample solutions in triplicate and amount of drug present was calculated using ICH guidelines.

v) Chromatographic Conditions: The mobile phase, Acetonitrile: 0.2% Triethylamine pH 7.0 (50:50 v/v) pumped at a flow rate of 1 ml/min through the column Phenomenex, C18 column, 150 mm × 4.6 mm, 5µm. The mobile phase was degassed prior to use under vacuum by filtration through a 0.45µ membrane filter. Bimatoprost showed good absorbance at 194nm, which was selected as wavelength for further analysis.

vi) Analysis of marketed formulation: 5ml solution was transferred to 10 ml volumetric flask and the volume was made up to the mark using mobile phase. The solution was sonicated for 20 minutes. The solution was filtered through Whatman Filter Paper No.42. The prepared sample solution was chromatographed for 7 minutes run time using mobile phase at 194 nm and a flow rate of 1 ml/min. From the peak area obtained in the chromatogram, the amounts of the drug were calculated by fitting peak area responses into the equation of the straight line representing the calibration curves for Bimatoprost.

3. DRUG-EXCIPIENT COMPATIBILITY STUDY:

The drug with the physical mixture of other excipients like Poloxamer 407, Hydroxypropylmethylcellulose, n- Carboxymethyl chitosan and Kolliphor P188 (in 1:1 ratio) was subjected to storage at room temperature for one month. The mixtures of drug and excipients were then evaluated by IR spectra by using FTIR spectrometer (Shimadzu) and DSC (Shimadzu).
3.1.1 **Differential scanning calorimetric study:** A DSC-60 Differential Scanning Calorimeter (Make - Shimdzu) equipped with an intracooler and a refrigerated cooling system was used to analyze the thermal behavior of drugs and mixture of drug and excipients in hermetically sealed flat aluminium crucibles, with temperature range from 30 to 300ºC according to predetermined melting point of drug. Blank crucible was used to calibrate the DSC temperature. Nitrogen was purged at 30 ml/min through cooling unit. The obtained peaks were analyzed for drug excipient compatibility study.

3.1.2 **FT-IR spectroscopic studies:** Infrared spectra of pure drugs, and with physical mixture of excipients like Poloxamer 407, Hydroxypropylmethylcellulose, n-Carboxymethyl chitosan and Kolliphor P188 were obtained using Fourier-transform infrared (FTIR) spectroscopy (Shimdzu) by diffused cell technique. The spectra were recorded over the wave number 4000 to 400 cm\(^{-1}\). The spectra were analyzed comparatively for drug excipient compatibility study.

4 **PRELIMINARY SCREENING (OPTIMIZATION):**

The preliminary screening was carried out to determine the concentration range of polymer and surfactant to be incorporated during optimization. The concentration of polymer was varied from 0.1% - 0.5 % w/v by keeping the surfactant concentration constant and different formulations was prepared. During the determination of range of surfactants concentration the surfactant concentration was varied from 0.1% - 1 % w/v, keeping the polymer concentration at constant level. The particle size and entrapment efficiency of the obtained particles were observed to select the concentration range of polymer and surfactant for further optimization studies. The various formulations were prepared.
4.1 Formulation of Sparfloxacin Nanosuspension:

Nanosuspension formulations were prepared by using solvent diffusion (H.S.M. Ali et al) method followed by probe sonication (P.P. Ige et al). Weighed quantity of sparfloxacin was dissolved in 2.5ml of organic solvent (acetone) and 2.5ml co solvent (1% Lactic acid). This solution was added drop by drop using syringe fitted with 24-guage needle to 25ml aqueous phase of polymers, water soluble chitosan (3%), HPMC E5 (5%) and surfactants Kolliphor SLS (0.2%) and Poloxamer 407 (0.2%). Then homogenized using high speed homogenizer (REMI Motors Ltd. Mumbai) at 6000 rpm for 20 min and kept it for magnetic stirring for 2 hours to remove residual solvent. This was followed by probe sonication at 30 kHz for 20 min to produce nanosuspensions.

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>Sparfloxacin (% w/v)</th>
<th>Chitosan (% w/v)</th>
<th>HPMC E5 (% w/v)</th>
<th>Poloxamer 407 (% w/v)</th>
<th>Kolliphor P188 (% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNF1</td>
<td>0.3</td>
<td>0.3</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>SNF2</td>
<td>0.3</td>
<td>0.3</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
</tr>
<tr>
<td>SNF3</td>
<td>0.3</td>
<td>0.3</td>
<td>-</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>SNF4</td>
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<td>-</td>
<td>0.5</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>SNF5</td>
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<td>-</td>
<td>0.5</td>
<td>0.2</td>
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<tr>
<td>SNF6</td>
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<td>0.5</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>SNF7</td>
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<td>0.15</td>
<td>2.5</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>SNF8</td>
<td>0.3</td>
<td>0.15</td>
<td>2.5</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>SNF9</td>
<td>0.3</td>
<td>0.15</td>
<td>2.5</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>
4.2 Formulation of Bimatoprost Nanosuspension:

Nanosuspension formulations were prepared by using solvent diffusion (H.S.M. Ali et al) method followed by probe sonication (P.P. Ige et al)\(^3\). Weighed quantity of BIM was dissolved in 5ml of organic solvent (Ethanol). This solution was added drop by drop using syringe fitted with 24-guage needle to 25ml aqueous phase of polymers, water soluble chitosan (1.5%), HPMC E5 (3%) and surfactants Kolliphor P188 (0.2%) and Poloxamer 407 (0.2%). Then homogenized using high speed homogenizer (REMI Motors Ltd. Mumbai) at 6000 rpm for 20 min and kept it for magnetic stirring for 2 hours to remove residual solvent. This was followed by probe sonication at 30 kHz for 15 min to produce nanosuspensions.

<table>
<thead>
<tr>
<th>Formulation Code</th>
<th>Bimatoprost (% w/v)</th>
<th>Chitosan (% w/v)</th>
<th>HPMC E5 (% w/v)</th>
<th>Poloxamer 407 (% w/v)</th>
<th>Kolliphor P188 (% w/v)</th>
</tr>
</thead>
<tbody>
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<td>-</td>
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<td>-</td>
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<tr>
<td>BNF2</td>
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<td>0.2</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
</tr>
<tr>
<td>BNF3</td>
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</tr>
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<td>BNF4</td>
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<td>0.2</td>
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</tr>
<tr>
<td>BNF5</td>
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<td>-</td>
<td>0.3</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>BNF6</td>
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<td>-</td>
<td>0.3</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>BNF7</td>
<td>0.03</td>
<td>0.1</td>
<td>0.2</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>BNF8</td>
<td>0.03</td>
<td>0.1</td>
<td>0.2</td>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>BNF9</td>
<td>0.03</td>
<td>0.1</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>
4.3 Process optimization:

For the nanosuspension formulation preparation the process parameters like homogenization speed, homogenization time, sonication amplitude and sonication time were considered.

4.4 Characterization of Sparfloxacin Nanosuspension

4.4.1 pH

pH is one of the most significant parameter concerned in the ophthalmic formulation. The pH should be such that the formulation will be stable at that pH and at the same time there would be no eye irritation to the patient on ocular instillation of the formulation. The developed nanoformulations were evaluated for pH by using digital pH meter. The instrument was calibrated before each use with standard buffers.

4.4.2 Particle size and charge:

Particle size of the formulated nanosuspension was determined by dynamic light scattering technique using particle size analyzer (Nanotrac R-150 ULTRA, Microtrac Inc.). The Polydispersity Index (PDI) was calculated to check the particle size distribution. The zeta potential of all the formulations was determined by Malvern Zetasizer.

4.4.3 Entrapment efficiency:

The percentage of incorporated Sparfloxacin (entrapment efficiency) was determined spectrophotometrically at 289 nm. After centrifugation of the aqueous suspension, amount of the free drug was detected in the supernatant and the amount of incorporated
drug was determined as the result of the initial drug minus the free drug. The entrapment efficiency (EE %) could be achieved by the following equation:

\[
\text{Entrapment efficiency (\%) = } \frac{W_{\text{initial drug}} - W_{\text{free drug}}}{W_{\text{initial drug}}} \times 100
\]

4.4.4. *In vitro* Drug release study

The *in vitro* drug release profiles of all formulations were determined in 50mL simulated tear fluid, pH 7.4 (STF) using dialysis bags (HI media Laboratories, India) at 37 ± 0.5°C under low speed magnetic stirring. A 3-mL sample was taken at predetermined time intervals and replaced with a same quantity of fresh STF. The sample was then properly diluted and analyzed quantitatively by using a validated UV spectrophotometric method at 289 nm. Each experiment is repeated six times and data was analyzed for release kinetics.

4.4.5. Kinetics of drug release

One of the most important and challenging areas in the drug delivery field is to calculate the release of the active agent as a function of point in time using both simple and complicated mathematical models. The magnitude of such models lies in their effectiveness during both the design phase of a pharmaceutical formulation and the experimental authentication of a release mechanism. In order to recognize an exacting release mechanism, experimental data of statistical significance are compared to a solution of the theoretical model.
To investigate the mechanism for the release and release rate kinetics of the dosage form, the data obtained was fitted into Zero order, First order, Higuchi matrix and Korsmeyer-Peppas model. By comparing the $R^2$ values obtained, the best fit model was selected.

**Zero Order Kinetics**

Dissolution of drug from pharmaceutical dosage forms that do not disintegrate and release the drug slowly assuming that area does not change and no equilibrium conditions are obtained can be represented by the following equation-

$$Wo - Wt = Kt \quad \ldots \ldots \text{Eq. 1}$$

Where, $W_o$ = Initial amount of drug in pharmaceutical dosage form

$W_t$ = Amount of drug in the dosage form at time $t$

$K$ = Proportionality constant.

Dividing this equation by $W_o$ and simplifying

$$f_t = K_o t \quad \ldots \ldots \text{Eq. 2}$$

Where, $f_t$ = 1 - ($W_t/W_o$) which represents the fraction of drug dissolved in time $t$

$K_o$ = Apparent dissolution rate constant or zero order release constant

The pharmaceutical dosage forms following this profile release the same amount of drug by unit time and it is the ideal method of drug release in order to achieve a pharmacological prolonged action. This following relation can express this model.
\[ Q_t = Q_0 + K_0 t \] …… Eq. 3

Where, \( Q_t \) = Amount of drug dissolved in time t,

\( Q_0 \) = Initial amount of drug in the solution and

\( K_0 \) = Zero order release constant.

**First Order Kinetics**

To evaluate the first order release rate kinetics the release rate data were fixed to the following equation.

\[ \log Q_t = \log Q_0 + K_1 t / 2.303 \] …… Eq.4

Where, \( Q_t \) = Amount of drug released in time t,

\( Q_0 \) = Initial amount of drug in the solution and

\( K_1 \) = First order release constant.

The pharmaceutical dosage forms following this dissolution profile, release the drug in such a way that it is proportional to the amount of drug remaining in its interior, so that the amount of drug released by unit of time gets diminished.

**Higuchi Model**

Higuchi developed some theoretical models to learn the release of water soluble and low-soluble drugs incorporated in semisolids and/or solid matrices. Mathematical expressions
were obtained for drug particles dispersed in a homogeneous matrix behaving as the diffusion media.

The Higuchi equation is

\[ f_t = K_H \times t^{1/2} \quad \ldots \text{Eq. 5} \]

Where, \( f_t \) = Amount of drug released in time \( t \) and

\( K_H \) = Higuchi dissolution constant.

**Korsmeyer-Peppas Model**

Korsmeyer, investigated a simple, semi-empirical model describing exponentially the drug release to the elapsed time. To learn this model the release rate data is fitted to the following equation

\[ f_t = \frac{M_t}{M_{\infty}} = K \times t^n \quad \ldots \text{Eq.6} \]

Where, \( \frac{M_t}{M_{\infty}} \) = Fraction of drug release,

\( K \) = Release constant,

\( t \) = Drug release time and

\( n \) = Diffusional exponent for the drug release that is dependent on the shape of the matrix dosage form.

This mathematical model has been used very regularly to depict the drug release from various diverse pharmaceutical modified release dosage forms.
4.4.6. HET CAM Study

HET CAM (Hen’s egg chorioallantoic membrane) study is an substitute to the Draize in vivo rabbit eye test for the recognition of ocular irritations. The hen’s egg chorioallantoic membrane bioassay was accomplished using 10 day fertilized eggs. Prior to use, the eggs were candled to notice the viability of the embryo. Sparfloxacin nanosuspension was comparatively studied with commercial formulation. Experiments were performed in triplicates using sodium chloride as a negative control and sodium hydroxide as a positive control. The CAM was treated with 500 µl of the sample and irritation levels were checked by observing for signs of irritation such as haemorrhage, lysis and coagulation at different time intervals up to 5 min. Potential irritation scores (PIS) were calculated by the formula given below:

\[
PIS = \frac{(301 - h)}{300} \times 5 + \frac{(301 - l)}{300} \times 5 + \frac{(301 - c)}{300} \times 9
\]

Where \(h\) is the time in seconds when haemorrhage appears; \(l\) is the time in seconds when lysis appears; \(c\) is the time in seconds when coagulation appears.

4.4.7. Ocular irritation studies / Draize Test

Eye irritancy potential of a substance was determined on the basis of its ability to cause injury to the cornea, iris and conjunctiva when a substance is applied to the eye. Male Newzeland rabbits weighing 1.5 kg to 2.5 kg were obtained from the animal facility of the Faculty of Pharmacy, KLE University, India. The animals were housed in a temperature-controlled room (22–23° C) with a 12–12 h light dark cycle. The experimental protocols were performed and approved in accordance with institutional
guidelines by the Ethics Committee in Animal Experiment of the KLE University, India with the reference no. (KLECOP/IAEC/RES NO-18). The study was carried out as per National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (protocols n° 251/11 and 211/13). In addition rabbits are fed with green diet only, according to ARVO (Association of Research in Vision and Ophthalmology) declaration for the use of animals in ophthalmic and vision research. The modified Draize technique was designed for the ocular irritation potential of the ophthalmic product. Three groups of 6 male Albino rabbits each were made and were marked as test, positive control and negative control respectively. The negative control group received 0.9% NaCl, the test group received sterile best formulation and positive control group received dioctyl sodium sulfosuccinate. 2-3 drops of test solution was normally placed in the lower cul de sac once a day for a period of 7 days and irritancy was tested at the time interval of 1 hr, 24 hr, 72 hr and 1 week after administration. The rabbits were observed periodically for redness, swelling and watering of the eye.

4.4.8. Cytotoxicity screening

Cytotoxicity screening of Sparfloxacin nanosuspension was performed using resazurin assay. 200 µl of Vero cells with density of $1 \times 10^6$ were cultured in 96 well plate in Dulbecco’s Modified Eagle Medium (DMEM) media containing 5% Foetal Bovine Serum (FBS). The experiment was performed in triplicates. Cells were allowed to incubate for proliferation at $37^0C$ in 5% CO$_2$ incubator for 24 h. The cells were further incubated for 24 h with 50 µl Sparfloxacin nanosuspension. After 24 h exposure of cell lines, 20µl of resazurin (1 mg/ml) was added and the plate was incubated for 4 h in CO$_2$ incubator with 5% CO$_2$ at 37 $^0C$ for 4h. The pink colour resazurin was produced by
reducing the resazurin by mitochondrial dehydrogenase enzyme was analyzed by ELISA plate reader at 573 nm. The % cytotoxicity was calculated by the given formula.

\[
\% \text{ Cytotoxicity} = \frac{AbsU - AbsT}{AbsU}
\]

Where, AbsU is the absorbance of cells treated with control, AbsT is the absorbance of cells treated with 0.3% Sparfloxacin nanosuspension (SNF3).

**4.4.9. Microbiological studies**

The microbiological studies were performed to determine the biological activity of the optimized formulation compared with pure drug solution against a microorganism *Staphylococcus aureus*. A layer of nutrient agar (20 mL) seeded with the test microorganism (0.2 mL) was endorsed to solidify in the Petri plate. Wells were made on the solid agar layer by a sterile borer at 4 mm diameter. Then, a quantity of the formulations (optimized formulation and pure drug solution) containing equivalent amount of drug was individually poured into two cups. Before incubation petri plates kept at room temperature for 4 hours for diffusion of the drug, the plates were incubated at 37°C for 24 hours. The zones of inhibition were found and measured by an antibiotic zone finder. Observations were recorded in triplicate to overcome methodological errors.

**4.4.10. Isotonicity Study**

Isotonicity is a very important part for the parenteral and ophthalmic formulations. Isotonicity has to be maintained to avoid ocular irritation and tissue damage. Ethical approval was taken from the KLE University Ethics Committee for Human Subjects (Ref No. KLEU/ Ethics/2015-16/D-46) subjecting to the human blood sample withdrawal. Optimized formulation SNF3 was subjected to isotonicity testing. In this study the
CHAPTER 2 MATERIALS AND METHODS

formulation was mixed with the few drops of human blood, one drop of anticoagulant was added and observed under microscope at 45X magnification. The observed result is compared with the 0.9% normal saline solution as a standard, 1.5% sodium chloride solution for hypertonic as a positive control and 0.5% sodium chloride solution for hypotonic effect was tested.

4.4.11. Ex vivo corneal permeation studies using goat’s cornea

Goat corneal membrane was used to study the drug permeation. Intact eyeballs of goat were purchased from a slaughter house and shifted to laboratory in cold condition in normal saline maintained at 4°C. The cornea were cautiously removed along with a 5–6 mm of surrounding scleral tissue and washed with cold saline. The corneas were washed and kept in freshly prepared cold solution of artificial tear buffer of pH 7.4. The study was done by using Franz-diffusion cell in such a way that corneum side is constantly remained in a close contact with formulation in the donor compartment. The receptor compartment was full up with STF pH 7.4 at 34 °C ± 0.5 °C. The receptor medium was stirred on a magnetic stirrer. The samples were removed at different time intervals and evaluated for drug content. Receptor phase were replaced with an equal volume of STF (pH 7.4) at each time interval. The percent drug release was calculated and plotted against time to get dissolution rate curves.

4.4.12. In vivo antimicrobial study

Male Newzeland rabbits weighing 1.5 kg to 2.5 kg were obtained from the animal facility of the Faculty of Pharmacy, KLE University, India. The animals were housed in a temperature-controlled room (22–23° C) with a 12–12 h light dark cycle. The
experimental protocols were performed and approved in accordance with institutional guidelines by the Ethics Committee in Animal Experiment of the KLE University, India with the reference no. (KLECOP/IAEC/RES NO-18). The study was carried out as per National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (protocols n° 251/11 and 211/13). In addition rabbits are fed with green diet only, according to ARVO (Association of Research in Vision and Ophthalmology) declaration for the use of animals in ophthalmic and vision research. The 12 rabbits divided into two groups as group A and group B. Right eye of each rabbit was exposed to *Staphylococcus aureus* suspension to develop conjunctivitis and left eye kept as a control. After 24 hours conjunctivitis was induced and treatment was started. The group A was treated 8 times a day with Sparfloxacin suspension and group B was treated with Sparfloxacin nanosuspension (SNF3) three times a day (After every 8 hour) for 7 days. The rabbit eyes were examined every day and photographed.

4.4.13. Morphology

Transmission electronic microscopic (TEM) analysis was done using Hitachi H7500 instrument. The prepared nanosuspension was dropped onto carbon coated copper grid and extra solution was cleared using blotting paper. The grid was subjected to dry for 5min and loaded in the goniometer. TEM micrograph was taken by applying accelerating voltage of 80 kV.

4.4.14. Stability studies of the formulations

The physical stability of the nanosuspension was evaluated according to ICH guidelines on storing the sample for six months. 50 ml of formulation was stored for stability testing in closed amber-colored glass vials at 5 ± 2°C (refrigerator) away from direct light. Ten
ml of the formulation was withdrawn at 1, 3 and 6 month time intervals to determine particle size and drug content, as described earlier

4.5 Characterization of Bimatoprost Nanosuspension

4.5.1. pH

pH is one of the most significant parameter concerned in the ophthalmic formulation. The pH should be such that the formulation will be stable at that pH and at the same time there would be no eye irritation to the patient on ocular instillation of the formulation. The developed nanoformulations were evaluated for pH by using digital pH meter. The instrument was calibrated before each use with standard buffers.

4.5.2. Particle size and charge:

Particle size of the formulated nanosuspension was analyzed by dynamic light scattering technique using particle size analyzer (Nanotrac R-150 ULTRA, Microtrac Inc.). The Polydispersity Index (PDI) was measured to check the particle size distribution. The zeta potential of all the formulations was determined by Malvern Zetasizer.

4.5.3. Entrapment efficiency:

The % entrapment efficiency of Bimatoprost was analyzed by HPLC at the wavelength of 194nm. After centrifugation of the aqueous suspension, amount of the free drug in the supernatant was detected and the amount of drug incorporated was determined as the result of the initial drug minus the free drug. The entrapment efficiency (EE %) can be achieved by the following equation:

\[
\text{Entrapment efficiency (\%) } = \frac{W_{\text{initial drug}} - W_{\text{free drug}}}{W_{\text{initial drug}}} \times 100
\]
4.5.4. *In vitro* Drug release study

The *in vitro* drug release profiles of all formulations were determined in 50mL simulated tear fluid, pH 7.4 (STF) using dialysis bags (HI media Laboratories, India) at 37 ± 0.5°C under low speed magnetic stirring. A 3-mL sample was removed at predetermined time intervals and replaced with a same quantity of fresh STF. The sample was then appropriately diluted and quantitatively analyzed using a validated HPLC method. The mobile phase, acetonitrile:0.2% triethylamine (pH 7.0 adjusted with o-phosphoric acid) (50:50 v/v) pumped at a flow rate of 1 ml/min through the column Agilent, C\textsubscript{18} column (150 mm × 4.6 mm, 5µm)at 194nm. Each experiment is repeated six times and data was analyzed for release kinetics.

4.5.5. Kinetics of drug release

One of the most important and challenging areas in the drug delivery field is to calculate the release of the active agent as a function of point in time using both simple and complicated mathematical models. The magnitude of such models lies in their effectiveness during both the design phase of a pharmaceutical formulation and the experimental authentication of a release mechanism. In order to recognize an exacting release mechanism, experimental data of statistical significance are compared to a solution of the theoretical model.

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4.5.6. HET CAM Study

HET CAM (Hen’s egg chorioallantoic membrane) study is an substitute to the Draize in vivo rabbit eye test for the recognition of ocular irritations. The hen’s egg chorioallantoic membrane bioassay was accomplished using 10 day fertilized eggs. Prior to use, the eggs were candled to notice the viability of the embryo. Bimatoprost nanosuspension was evaluated in comparison with marketed formulation. Experiments were carried out in triplicates using sodium chloride as a negative control and sodium hydroxide as a positive control. The CAM was treated with 500 µl of the sample and irritation levels were checked by observing for signs of irritation such as haemorrhage, lysis and coagulation at different time intervals up to 5 min. Potential irritation scores (PIS) were calculated by the formula given below:

\[ PIS = \frac{(301 - h)}{300} \times 5 + \frac{(301 - l)}{300} \times 5 + \frac{(301 - c)}{300} \times 9 \]

Where \( h \) is the time in seconds when haemorrhage appears; \( l \) is the time in seconds when lysis appears; \( c \) is the time in seconds when coagulation appears.

4.5.7. Ocular irritation studies

Eye irritancy potential of a substance was determined on the basis of its ability to cause injury to the cornea, iris and conjunctiva when a substance is applied to the eye. Male Newzeland rabbits weighing 1.5 kg to 2.5 kg were obtained from the animal facility of the Faculty of Pharmacy, KLE University, India. The animals were housed in a temperature-controlled room (22–23° C) with a 12–12 h light dark cycle. The experimental protocols were performed and approved in accordance with institutional guidelines by the Ethics Committee in Animal Experiment of the KLE University, India.
with the reference no. (KLECOP/IAEC/RES NO-18). The study was carried out as per National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (protocols n° 251/11 and 211/13). In addition rabbits are fed with green diet only, according to ARVO (Association of Research in Vision and Ophthalmology) declaration for the use of animals in ophthalmic and vision research. The modified Draize technique was designed for the ocular irritation potential of the ophthalmic product. Three groups of 6 male Albino rabbits each were made and were marked as test, positive control and negative control respectively. The negative control group received 0.9% NaCl, the test group received sterile best formulation and positive control group received dioctyl sodium sulfosuccinate. 2-3 drops of test solution was normally placed in the lower cul de sac once a day for a period of 7 days and irritancy was tested at the time interval of 1 hr, 24 hr, 72 hr and 1 week after administration. The rabbits were observed periodically for redness, swelling and watering of the eye.

4.5.8. Cytotoxicity screening

Cytotoxicity screening of Bimatoprost nanosuspension was performed using resazurin assay. 200 µl of Vero cells with density of 1 × 10⁶ were cultured in 96 well plate in Dulbecco’s Modified Eagle Medium (DMEM) media containing 5% Foetal Bovine Serum (FBS). The experiment was performed in triplicates. Cells were allowed to incubate for proliferation at 37⁰C in 5% CO² incubator for 24 h. The cells were further incubated for 24 h with 50 µl Bimatoprost nanosuspension. After 24 h exposure of cell lines, 20µl of resazurin (1 mg/ml) was added and the plate was incubated for 4 h in CO² incubator with 5% CO² at 37 ⁰C for 4h. The pink colour resazurin was produced by reducing the resazurin by mitochondrial dehydrogenase enzyme was analyzed by
ELISA plate reader at 573 nm. The % cytotoxicity was calculated by the given formula.

\[
\% \text{ Cytotoxicity} = \frac{AbsU - AbsT}{AbsU}
\]

Where, AbsU is the absorbance of cells treated with control, AbsT is the absorbance of cells treated with 0.03% Bimatoprost nanosuspension (BNF3).

4.5.9. Isotonicity Study

Isotonicity is an important aspect for the ophthalmic and parenteral formulations. Isotonicity has to be maintained to prevent ocular irritation and tissue damage. Ethical approval was obtained from the KLE University Ethics Committee on Human Subjects (Ref No. KLEU/ Ethics/2015-16/D-46) regarding human blood sample withdrawal. Optimized formulation BNF3 was subjected to isotonicity testing. In this study the formulation was mixed with the few drops of human blood, one drop of anticoagulant was added and observed under microscope at 45X magnification. The observed result is compared with the 0.9% normal saline solution as a standard, 0.5% sodium chloride solution for hypotonic and 1.5% sodium chloride solution for hypertonic effect, were tested as a positive control.

4.5.10. Ex vivo corneal permeation studies using goat’s cornea

Goat corneal membrane was used to study the drug permeation. Intact eyeballs of goat were purchased from a slaughter house and shifted to laboratory in cold condition in normal saline maintained at 4°C. The cornea were cautiously removed along with a 5–6 mm of surrounding scleral tissue and washed with cold saline. The corneas were washed and kept in freshly prepared cold solution of artificial tear buffer of pH 7.4. The study
was done by using Franz-diffusion cell in such a way that corneum side is constantly remained in a close contact with formulation in the donor compartment. The receptor compartment was full up with STF pH 7.4 at 34 °C ± 0.5 °C. The receptor medium was stirred on a magnetic stirrer. The samples were removed at different time intervals and evaluated for drug content. Receptor phase were replaced with an equal volume of STF (pH 7.4) at each time interval. The percent drug release was calculated and plotted against time to get dissolution rate curves.

4.5.11. Morphology

Transmission electronic microscopic (TEM) analysis was done using Hitachi H7500 instrument. The prepared nanosuspension was dropped onto carbon coated copper grid and extra solution was removed using blotting paper. The grid was allowed to dry for 5min and loaded in the goniometer. TEM micrograph was taken by applying accelerating voltage of 80kV.

4.5.12. Pharmacokinetic study

Male Newzeland rabbits weighing 1.5 kg to 2.5 kg were obtained from the animal facility of the Faculty of Pharmacy, KLE University, India. The animals were housed in a temperature-controlled room (22–23 °C) with a 12–12 h light dark cycle. The experimental protocols were performed and approved in accordance with institutional guidelines by the Ethics Committee in Animal Experiment of the KLE University, India with the reference no. (KLECOP/IAEC/RES NO-18). The study was carried out as per National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (protocols n° 251/11 and 211/13). In addition rabbits are fed with green diet only, according to ARVO (Association of Research in Vision and Ophthalmology)
declaration for the use of animals in ophthalmic and vision research. The 12 Albino rabbits were randomly divided in two groups of six animals each and treated with the formulation under investigation. Each rabbit received dose of 50μL once into the conjunctival sac of both eyes. The first group was treated with Bimatoprost commercial formulation (Careprost 0.03%), whereas the other group was treated with the prepared Bimatoprost Nanosuspension (BNF3). Aqueous humor (50 μL) was withdrawn at 0.5, 1, 2, 4, 6, 8 and 9 Hr after instillation through the limbus, with a syringe with a 26G needle and stored at -20° C until HPLC analysis. Before paracentesis the rabbits were anesthetized by an intravenous injection of 25 mg/kg of ketamine.

**Sample preparation**

Each aqueous humor sample was mixed with 50μL methanol and 100μL acetonitrile and vortexed for 30 seconds, followed by centrifugation at 14,000 rpm and at 4°C for 10 minutes. The supernatants (20μL) were injected into HPLC and analysed by developed bio analytical method.

**4.5.13. Pharmacodynamic Study (Glaucoma Model)**

Male Newzeland rabbits weighing 1.5 kg to 2.5 kg were obtained from the animal facility of the Faculty of Pharmacy, KLE University, India. The animals were housed in a temperature-controlled room (22–23° C) with a 12–12 h light dark cycle. The experimental protocols were performed and approved in accordance with institutional guidelines by the Ethics Committee in Animal Experiment of the KLE University, India with the reference no. (KLECOP/IAEC/RES NO-18). The study was carried out as per National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals (protocols n° 251/11 and 211/13). In addition rabbits are fed with green diet only, according to ARVO (Association of Research in Vision and Ophthalmology).
declaration for the use of animals in ophthalmic and vision research. The 12 Albino rabbits were randomly divided in two groups of six animals each and treated with the formulation under investigation. Rabbits were sedated by using diazepam (1 mg/kg i.v.) and anaesthetized with ketamine (25 mg/kg i.v.). Newly prepared α-chymotrypsin (50 units) solution made in 0.1 ml of sterile saline was irrigated throughout the cannula into the posterior chamber. The debris of tissue blocked the pathway of aqueous humor outflow in the trabecular meshwork to create the ocular hypertension. Six rabbits with glaucoma were selected and used to know the effect of drugs on IOP. After achieving a steady elevated IOP, Bimatoprost nanosuspension and commercial formulation was administered topically into the left eye whereas, right eye served as control. The IOPs were measured at time zero (just before eye drop instillation) and suitable time intervals.

5) STABILITY STUDIES OF THE FORMULATIONS

The physical stability of the nanosuspension was evaluated according to ICH guidelines on storing the sample for six months. 50 ml of formulation was stored for stability testing in closed amber-colored glass vials at 5 ± 2°C (refrigerator) away from direct light. Ten ml of the formulation was withdrawn at 1, 3 and 6 month time intervals to determine particle size and drug content, as described earlier.

6) STATISTICAL ANALYSIS

Data is expressed as mean ± standard deviation (SD). One way ANOVA followed by Dennett’s test was used for the study (Graph pad prism 5, San Diego, CA, USA). A value of $p < 0.05$ was considered as statistically significant.