EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 8
Issue: 11
247-258
Year: 2021

FORMULATION, DEVELOPMENT AND EVALUATION OF POSACONAZOLE NANOEMULSION FOR OCULAR DELIVERY

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Article Received on 09/09/2021 **Article Revised on 30/09/2021** **Article Accepted on 20/10/2021**

ABSTRACT
The aim of the present study was to formulate and evaluate the nanoemulsions of antifungal drug Posaconazole for its sustained and prolong effect. Pseudoternary phase diagrams were constructed, and various nanoemulsion formulations were prepared using Isopropyl myristate (IPM) as oil, Tween-80 as a surfactant and Transcutol P as a co-surfactant.A total of seven formulations was selected from the nanoemulsion region and all formulations were evaluated for the attribute such as viscosity, droplet size, pH, refractive index, surface tension, poly-dispersity index, zeta potential, osmolarity, drug content and in vitro drug release. The optimized nanoemulsion was subjected to TEM, ex vivo permeation, ocular irritancy, antifungal activity, sterility, in vivo studies and stability. The formulated nanoemulsions NE-1 to NE-7 showed acceptable physicochemical properties. NE-2 nanoemulsion was clear, transparent and elegant in appearance when compared to the other formulations. From primary evaluation, the NE-2 nanoemulsion was selected as optimized formulation because of higher zeta potential and drug content value and lower particle size as well as the poly-dispersity index. Posaconazole was effectively formulated in nanoemulsion form and can be a good candidate for ocular delivery as it holds high penetration through the cornea and good therapeutic efficacy for the treatment of fungal keratitis together with sustained effect.

KEYWORDS: Ocular nanoemulsion, Microemulsion, Nanotechnology, Novel drug delivery system, Fungal keratitis, Keratomycosis.

INTRODUCTION
Various ocular diseases require frequent administration of the drug and poor ocular bioavailability of drugs (< 1%) from the conventional ophthalmic formulations is a serious concern.[1] Conventional ophthalmic delivery systems possess low ocular bioavailability because of rapid tearing and transient residence time,[2] reduced therapeutic response due to lacrimal secretion and nasolacrimal drainage[3] and precorneal drug loss in few minutes.[4]

To overcome the drawbacks of conventional ophthalmic dosage forms, many improvements have been done to improve the corneal drug absorption and minimize pre-corneal drug loss. Nanotechnology-based systems with an appropriate particle size can be designed to ensure low irritation, adequate bioavailability, increased residence time and ocular tissue compatibility. Several nanocarriers, such as nanoparticles, nanoemulsions, nanosuspensions, etc. have been developed and have shown promising results for improving ocular bioavailability.[5]

Nanoemulsions promote the solubilization of both hydrophilic and hydrophobic drug candidates. The nano-size of the droplets that are adsorbed in the cornea assists in the avoidance of nasolacrimal drainage.Additionally, nanoemulsions can achieve sustained drug release along with high permeation and thereby improved ocular bioavailability.[6]

Fungal keratitis is a very serious and potentially sight-threatening corneal infection that most commonly develops in patients after trauma or in those with a compromised corneal surface.[7]

Contact lens wear and contact lens solution have also been identified as a risk factor for fungal keratitis.The most commonly implicated species organisms include Aspergillus, Fusarium, Scedosporium apiospermum,

phaeohyphmycetes, Candida albicans and other Candida species.[8]

Fungal keratitis infection is difficult to treat and it can lead to severe visual defectsor even blindness. It is worldwide in distribution but is more common within the tropical and subtropical climate zones.[9]

Because of relatively reduced systemic toxicity and better corneal penetration, several antifungal azole compounds such as Econazole, Fluconazole, Voriconazole, Posaconazole, etc. can be used for fungal keratitis.[10] Posaconazole act by inhibiting the synthesis of an essential component of the fungal cell membrane (ergosterol) by binding and inhibiting the enzyme lanosterol 14α-demethylase.[11]

The objective of the present study was to formulate and characterize nanoemulsion of Posaconazole to investigate the practicability of nanoemulsion for ocular administration to offer high penetration of the drug through the cornea and sustained-release effect.

MATERIALS AND METHODS

Materials

Posaconazole, Isopropyl myristate, Tween-80, Transcutol P, Dialysis membrane, n-Octanol, Ethanol, Methanol, etc.All chemicals used were of analytical grade.

Methods

Determination of solubility of Posaconazole in oils, surfactants and co-surfactants

- **Assessment of oil**

Posaconazole belongs to the triazole family of medication and is highly lipophilic in nature. Screening of different oils was carried out to find out the suitable oil which can be used as the oil phase in nanoemulsion. The solubility of Posaconazole in various oils like oleic acid, Isopropyl myristate, olive oil, triacetin and castor oil was measured.

- **Assessment of surfactants**

Surfactants are the compounds that are used at different concentrations as emulsifiers. For the preparation of nanoemulsion, the use of nonionic surfactants was chosen due to its high effectiveness, efficiency, biodegradability and low toxicity concerns. The solubility of Posaconazole in various surfactants like Tween-20, Tween-80, Cremophore EL, Labrasol and Span 20 were determined.

- **Assessment of co-surfactants**

Co-surfactants enhance the effectiveness of a surfactant. The solubility of Posaconazole in various co-surfactants like Transcutol P, PEG 200, PEG 400, and propylene glycol was also determined. (12)

Construction of pseudoternary phase diagrams

Construction of phase diagram is a valuable approach to demonstrate various components and series of interactions that can occur when different components are mixed. The simple water titration method at 25 °C was employed to construct the pseudo-ternary phase diagrams.The pseudoternary phase diagrams of oil, surfactant and cosurfactant was plotted using TernPlot-Excel Plotting program to determine the regions of nanoemulsion formation.[13]

Preparation of Nanoemulsions

O/w nanoemulsions of Posaconazole were prepared by using an appropriate amount of deionized water (DW) as aqueous phase, IPM as oil phase, Tween 80 and Transcutol-P were used as a surfactant and co-surfactant phases, respectively.

An appropriate amount of Posaconazole (0.3% w/w) was dissolved in the oil phase. This oil phase was then added to the mixture of surfactant and co-surfactant phases, and finally, the aqueous phase was added to the mixture and vigorously stirred and vortexed to get nanoemulsion. A total of seven formulations were selected from the nanoemulsion region for further study. Benzalkonium chloride was added as a preservative in all prepared nanoemulsions in a concentration of 0.005% w/w.[14]

Thermodynamic stability screening

To assess the thermodynamic stability of drug-loaded nanoemulsions, clarity, phase separation, droplet size, and drug content were evaluated.

- ***Centrifugation***

Nanoemulsion formulations were centrifuged at 3500 rpm for about half an hour. Those nanoemulsions which failed to exhibit any phase separation were taken for the heating-cooling cycle.

- ***Heating-cooling cycle***

About six cycles were performed at two different temperatures i.e., refrigerated temperature (4°C) and the higher temperature (45 °C), with storage at each temperature for not less than 48 hours. Nanoemulsion formulations, which were stable at above-stated temperatures, were subjected to freeze-thaw testing.

- ***Freeze-thaw cycle(accelerated aging)***

Three freeze-thaw cycles between temperature -21 °C and +25 °C with storage for not less than 48 hours was done for the nanoemulsions formulations.[15]

Characterization of Nanoemulsions

Viscosity measurement

The viscosity of the prepared nanoemulsions was determined by using cone and plate viscometer (Brookfield Engineering Laboratories, USA), spindle 40.

pH measurement

pH of the nanoemulsion formulations was measured by using Digital pH meter (Systronics, Ahmedabad).The pH values were recorded immediately after preparations of the nanoemulsions.[16]

Droplet size measurement

The droplet size distribution of the nanoemulsion can be determined by photon correlation spectroscopy which analyzes fluctuations in light scattering due to the Brownian motion of the particles, using a Zeta seizer 1000 HS (Malvern Instruments, UK).[17]

Refractive index measurement

The refractive index of nanoemulsions was determined using an Abbe's type refractometer (Nirmal International, New Delhi, India) at 25 ± 0.5 °C.[18]

Surface tension measurements

Surface tension measurements were carried out at 20°C using a thermostatically controlled processor tensiometer K100 (Kruss GmbH, Germany) provided with a Du Nouy ring (ring radius 9.545 mm, wire diameter 0.37 mm).[19]

Poly-dispersity measurement

The poly-dispersity index of nanoemulsions was measured by Photon Correlation Spectroscopy. The measurements should be performed at temperature of 25 °C.[20]

Zeta potential measurement

Samples of prepared nanoemulsion were placed in a plastic cuvette and positioned in the path of the laser. The light scattered was collected at 14.8˚ and detected using a photomultiplier tube. Each sample was analyzed three times and the zeta potential of the sample was determined from the average of the runs.[21]

Osmolarity determination

Osmolarity was measured using Micro Osmometer (model 3300, Advanced Instruments Inc., USA). Osmolarity is an important parameter by which one can predict the irritability of the formulation caused to the eyes. It was calculated by using the following equation[22]

$$Milli\ osmoles\ per\ liter = \frac{Mass\ in\ grams}{Molecular\ Weight\ (\frac{g}{ml})}\ Particle\ number\ x\ 1000$$

Drug content determination

Drug concentration in nanoemulsion preparation was measured by spectrophotometer. Posaconazole content in the solution was measured by adding nanoemulsion with a known quantity of solvent (Methanol) and it gets miscible. Absorbance was measured after suitable dilutions in UV-VIS spectrophotometer and % drug content was calculated.[23]

Transmission Electron Microscopy

Morphology and structure of the nanoemulsions were studied using transmission electron microscopy operating at 200 kV and capable of a 0.18 nm point to point resolution.[24]

In vitrorelease studies of Posaconazole

In vitrodrug release studies of the developed nanoemulsions were performed in triplicate in a USP dissolution tester apparatus type II at 34 ± 0.5 ºC to simulate the ocular surface temperature. The receptor phase was composed of 100 ml Phosphate Buffer SalinepH 7.4 and it was constantly stirred at 50 rpm throughout the experiment. Drug release was executed through a dialysis membrane of 12,000 Da. At definite time intervals, about 1 ml of the receptor phase was withdrawn and replaced by the fresh buffer to maintain a constant volume within the apparatus. Drug concentration was determined spectrophotometrically at wavelength 263 nm. The amount of drug released in percent was plotted versus time. The mean dissolution rate was calculated for each nanoemulsion formulation.

The drug release data of formulation NE-2 were fitted to various mathematical models such as zero order as cumulative % of drug released vs. time, first order as log cumulative % of drug remaining vs. time and Higuchi's model as cumulative % drug released vs. square root of time. To determine the mechanism of drug release from formulations, the data were fitted into Korsmeyer Peppas equation as log cumulative % of drug released vs. log time.[25]

Ex Vivo Permeation Studies

Preparation of Corneas

Goat eyeballs were procured from a local slaughterhouse within 30 minutes after sacrifice and were transported immediately to the laboratory in normal saline maintained at 4 ºC temperature. The eyeballs were dissected and the corneas were excised in such a way that some part of the sclera was attached. It was washed with cold saline and then, placed in PBS pH 7.4.

Permeation studies

Permeation studies of the nanoemulsions were carried out by using Franz-diffusion cells of 20 mm internal diameter and 15 ml receiver capacity. The phosphate buffer was used as a diffusion medium. 200 µl of the samples were withdrawn at different time intervals and replaced with an equal volume of phosphate buffer (pH 7.4) to maintain equilibrium at regular intervals for about 24 hours and samples were analyzed by HPLC. The amount of drug permeated per unit area through the excised cornea (µg/cm^2) versus time (h) graph was plotted and the equations used for calculating flux (J, µg/cm^2/h) and corneal permeability coefficient (Papp, cm/s) were as follows.

Flux

J = dQ/(dt A)

Where; J = Flux, M = Cumulative amount of drug permeated, A = Membrane surface area

Permeability coefficient
Papp = J/Cd

Where; J = Flux, Cd = Initial drug concentration in the donor phase

Corneal Hydration
After completion of the permeation studies, each cornea was weighed and noted as W1. Then it was soaked in methanol, allowed to dry overnight, reweighed and noted as W2. Corneal hydration was calculated from the differences in corneal weights W1 and W2.
% CH= (W1-W2) / (W1 x W2) x 100[26]

Ocular irritation studies
The optimized nanoemulsion formulation was used for eye irritancy study. The rabbit was observed periodically for any sort of irritation in cornea, iris and conjunctiva.

Table 1: Test conditions for ocular irritancy test.

Method for test	**Draize test**
Strain of rabbit	New Zealand White Albino
Weight of rabbit	2-3 Kg
Volume of formulation instilled	50µL
Left eye of rabbit	Sterile water for injection
Right eye of rabbit	Optimized formulation

Table 2: Score rating for eye irritancy study.

S. No.	**Score**	**Irritancy Rating**
1.	0	None
2.	1	Slight
3.	2	Mild
4.	3	Moderate
5.	4	Severe

Total Score = Sum of all scores obtained for the cornea, iris, and conjunctivae.[27]

Antifungal activitystudy
The disc diffusion technique was selected for the study of antifungal activity. These tests were carried out using the cultures of Candida albicans in Sabouraud-dextrose agar media. An amount of 15 ml of the media with a 24-hour subculture of C. albicans was distributed in each petri-dish of 10 cm diameter and allowed to solidify. On solidification, the microbial suspension was spread with the help of sterilized cotton swab on the surface of the media.

The filter paper disc was prepared from Whatmann's filter paper, which was then impregnated with drugs having Posaconazole in nanoemulsion (Test), Posaconazole in homogenized IPM mixture (Positive Control) and Nanoemulsion without active ingredient ie., Posaconazole (Negative Control) onto the surface of agar plates, over which a culture of the microorganisms has been streaked. The zone of inhibition diameter (mm) were then recorded and compared.[28]

Test for Sterility
The sterility test was carried out as per the IP (2014) method.

Table 3: Test Conditions for sterility testing.

Method of sterility test	Direct Inoculation
Medium	Sterile Fluid Thioglycolate, Soyabean casein digest
Volume of individual test solution	5ml
Positive control	• For aerobic bacteria: Sterilized media inoculated with *Bacillus subtilis* • For aerobic bacteria: Sterilized media inoculated with *Bacteriodes vulgatus* • For fungi: Sterilized media inoculated with Candida albicans
Negative control	Sterile media
Incubation time	14 days for detection of bacterial and fungal contamination.
Incubation Temperature	• FTGM at 30-35ºC for anaerobic bacteria • SCDM at 20-25 °C for aerobic bacteria & fungi
Method of detection	Visual inspection of turbidity

The 1 ml sterile optimized nanoemulsion formulation was taken and this formulation was diluted with 100 ml sterile water for injection, form this about 5 ml test solution was added in each medium.[29, 30]

In vivo drug study
In vivo pharmacokinetic studies of Posaconazole nanoemulsion were done on New Zealand white rabbits weighing 2.0 to 3 Kgs, free of clinically observable ocular surface diseases. The rabbits were fed a balanced diet and maintained in a temperature-controlled room, (20 °C to 24 °C) and humidity (55% to 65%) before the experiment. The pharmacokinetics parameters like maximum drug concentration (Cmax) and the time required to achieve maximum concentration (Tmax) were obtained by the plotting graph of concentration

versus time and area under the curve (AUC) was calculated.

Drug kinetics in Plasma

18 rabbits were divided into 3 groups of six rabbits each. Each group was further divided into 2 groups of three rabbits each. In every group, Group 1 received control (Normal saline) and Group 2 received test (optimized nanoemulsion formulation) samples of 30 µl each. 200 µl of blood samples were collected into heparinized tubes at different time intervals up to 24 hours' time-points through the marginal vein. After collection, samples were immediately stored at temperature (-20 °C) until the HPLC analysis was performed.[31]

Drug kinetics in Aqueous Humor

24 rabbits were used corresponding to 8 sampling points at different time intervals up to 24 hours. Each rabbit was placed in the separate restrainer, 30 µl of test sample and control (Normal saline) were topically applied on to the right eye and left eye respectively. 100 µl of aqueous humor was collected from three rabbits at each time point by inserting 22 G needle of an insulin syringe into the anterior segment of the eye through the cornea without causing any injury or damage to iris and lens. Before collecting the samples, rabbits were anesthetized by injecting intramuscularly the combination of 35 mg/kg ketamine hydrochloride and 5 mg/kg xylazine. The eye was locally anesthetized by a 4% xylocaine solution. After collection, samples were immediately stored at temperature (-20 °C) until the HPLC analysis was performed.[32]

Accelerated stability studies

The principal objective of stability testing was to determine the influence of environmental factors like temperature and humidity on the degradation of Posaconazole nanoemulsion. These studies help in establishing the storage conditions for prepared drug formulations.

As per ICH guidelines (Q1), the optimized nanoemulsion formulations were subjected to accelerated stability studies at different temperatures for three months at temperature 40 ± 2 °C. The optimized formulation was tested for globule size, pH, drug content, viscosity, release and permeation studies after 3 months.[33]

Statistical Analysis

All values presented in this study are the average of triplicate experiments for the same time points. All the results were expressed as mean ± standard deviation (SD). Analysis of variance (ANOVA) was performed to determine the level of significance between the means. A *p-value* of <0.05 was considered to be statistically significant. Statistical analysis was performed using GraphPad Prism for Windows.

RESULTS AND DISCUSSION

Determination of solubility of Posaconazole in oils, surfactants and co-surfactants

Solubility of Posaconazole in different oils, surfactants and co-surfactants was carried out to find out the components of the nanoemulsions which shows maximum solubility and can be used for the preparation of the formulation.

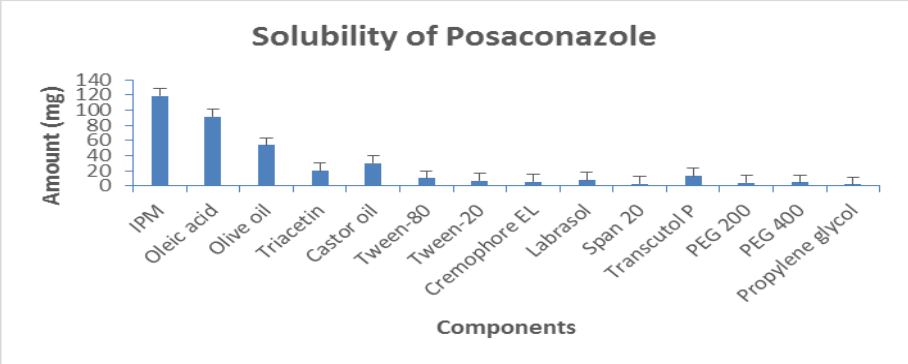


Figure 1: Solubility of Posaconazole in oils, surfactants and co-surfactants.

Posaconazole shows maximum solubility in Isopropyl myristate (oil), Tween-80 (Surfactants) and Transcutol P (Co-surfactants). Hence, they are selected as major components in Posaconazole nanoemulsion.

Construction of pseudoternary phase diagrams

These pseudo-ternary phase diagrams (consisting of oil, Smix and water) demonstrated an extensive region of nanoemulsion formation. Four phase diagrams were constructed with a surfactant to co-surfactant mixtures (Smix) in ratios of 1:1, 1:2, 2:1 and 3:1 and combinations of different weight ratios of oil and Smix mixtures were prepared.

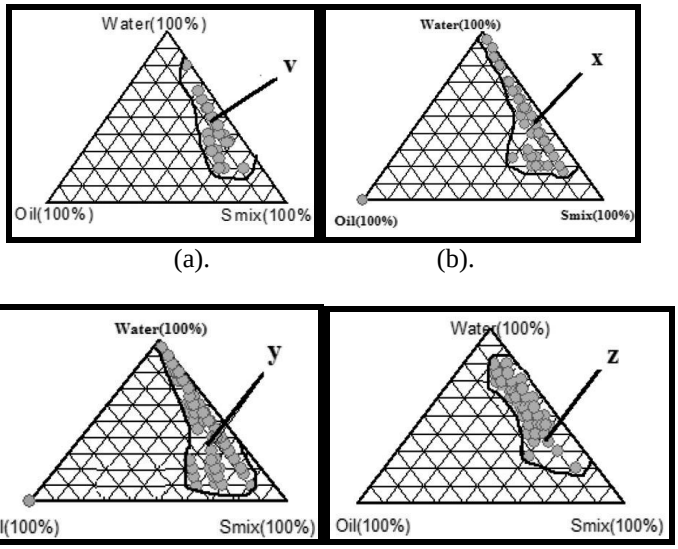


Figure 2: Pseudo-ternary phase diagram of oil (IPM), water and Smix; Tween-80: Transcutol-P (a): (1:1) presenting nanoemulsion region (v), (b): (1:2) presenting nanoemulsion region (x), (c): (2:1) presenting nanoemulsion zones and (d): (3:1) presenting nanoemulsion zones.

Preparation of Nanoemulsions

A total of seven formulations were selected from the nanoemulsion region and the final composition of the same is depicted in the table below.

Table 4: Composition of Posaconazole Loaded Nanoemulsions.

Formulations	S : CoS ratio	Oil (%) IPM	Smix (%)		Deionized water (%)	Total
			Tween-80	Transcutol-P		
NE-1	1:0	10.0	20.0	-	70	100
NE-2	1:1	10.0	10.0	10.0	70	100
NE-3		15.0	10.0	10.0	65.0	100
NE-4	2:1	10.0	15.0	8.0	67.0	100
NE-5		15.0	13.0	7.0	65.0	100
NE-6	3:1	10.0	20.0	6.0	64.0	100
NE-7		15.0	19.0	5.0	61.0	100

All the contents were expressed in terms of w/w.

Thermodynamic stability screening

- ***Centrifugation:***

It shows the effect of centrifugation and revealed promising physical stability; no signs of creaming, phase separation or cracking were observed. Cloudiness was observed when the nanoemulsions were frozen at a temperature of −21 °C.

- ***Heating-cooling cycle***

The nanoemulsions passed the alternate heating–cooling cycles and remained clear.

- ***Freeze-thaw cycle***

Clarity of the cloudy nanoemulsions obtained in the centrifugation stage upon freezing at −21 °C was recovered upon thawing.

Characterization of Nanoemulsions

Viscosity measurement

Posaconazole nanoemulsions viscosity ranged between 4.18 ± 0.26 to 7.31 ± 0.80. The viscosity values of all Posaconazole nanoemulsions were less than 10.0 mPas. All Posaconazole nanoemulsions exhibited a Newtonian behavior as expected from nanoemulsions.[Table 5]

pH measurement

The pH of prepared formulations found near to the biological pH with values ranged from 6.1 ± 0.5 to 6.9 ± 0.7. The pH of tear solution is near to 7.4 and the acceptable range for ophthalmic formulations is 5 to 8 as the human eye can tolerate pH changes within this range. Hence, the prepared nanoemulsion formulations can be considered suitable for ophthalmic use.[Table 5]

Droplet size measurement

The droplet size of nanoemulsions ranged from 49.24 ± 2.74 to 72.76 ± 3.84 nm confirming the formation of

nanoemulsions.Due to the small droplet size of nanoemulsion NE-2, its surface areas were assumed to be high. Hence, it provided a high concentration gradient and improved Posaconazole permeation from the formulation.[Table 5]

Refractive index measurement

Posaconazole nanoemulsions had refractive index values ranging from 1.340 ± 0.001 to 1.359 ± 0.004. It is recommended that eye drops for ophthalmic use should have refractive index values not higher than 1.476. Hence, refractive indices are found within the recommended values.[Table 5]

Surface tension measurements

The surface tension of the prepared Posaconazole nanoemulsions found between 41.6 ± 4.5 and 50.1 ± 1.9 mN/mwhich is more or less similar to that of the lachrymal fluid (i.e., 40 to 50 mN/m). The low surface tension of nanoemulsions guarantees an excellent spreading effect on the cornea and mixing with the precorneal film components, thus probably enhancing the contact between the drug and the corneal epithelium.[Table 5]

Poly-dispersity measurement

The polydispersity index values found <1.00 and ranged from 0.143 ± 0.015 to 0.517 ± 0.021 confirming the homogeneity of nanoemulsions due to the narrow size distribution of droplets. [Table 5]

Zeta potential measurement

The zeta potential values of nanoemulsions ranged from -15.68 ± 3.12 to -24.37 ± 4.23 mV. [Table 5]

Osmolarity determination

Osmotic pressure for prepared nanoemulsions ranged from 312.8 ± 12.4 to 355 ± 10.3 mOsm/kg. It is in an acceptable range of osmotic pressure for ophthalmic formulations and is non-irritant to the eyes. [Table 5]

Drug content determination

Drug content of nanoemulsions ranged from 93.53 ± 1.61 to 98.94 ± 1.56 %. No significant difference in drug content was observed in all the formulated nanoemulsions. All the formulation showed uniform drug content. [Table 5]

Table 5: Physico-chemical evaluation of Posacoanzole nanoemulsions.

S. No.	Formulation Code	Viscosity (mPa s)	pH	Droplet size (nm)	Refractive index	Surface tension (mN/m)	Poly dispersity index	Zeta potential (mV)	Osmolarity (mOsm Kg^{-1})	Drug content (%)
1.	NE-1	4.65 ± 0.33	6.2 ± 0.7	58.55 ± 3.84	1.346 ± 0.007	45.3 ± 3.4	0.209 ± 0.110	-15.68 ± 3.12	329.2 ± 16.6	97.82 ± 0.07
2.	NE-2	4.18 ± 0.26	6.7 ± 0.2	49.24 ± 2.74	1.340 ± 0.001	41.6 ± 4.5	0.143 ± 0.015	-24.37 ± 4.23	316.4 ± 15.2	98.94 ± 1.56
3.	NE-3	4.56 ± 0.27	6.6 ± 0.4	61.81 ± 4.55	1.358 ± 0.002	46.8 ± 3.7	0.254 ± 0.017	-17.26 ± 2.04	312.8 ± 12.4	94.53 ± 1.15
4.	NE-4	4.21 ± 0.25	6.9 ± 0.7	72.76 ± 3.84	1.348 ± 0.005	50.1 ± 1.9	0.236 ± 0.015	-23.45 ± 4.60	326.4 ± 16.1	96.54 ± 1.59
5.	NE-5	5.17 ± 0.52	6.1 ± 0.5	63.54 ± 5.12	1.344 ± 0.001	49.5 ± 1.8	0.312 ± 0.003	-20.20 ± 1.97	349.7 ± 11.9	95.91 ± 2.35
6.	NE-6	7.31 ± 0.80	6.8 ± 0.6	70.53 ± 6.04	1.359 ± 0.004	48.1 ± 4.0	0.278 ± 0.044	-21.81 ± 2.44	355.2 ± 10.3	93.53 ± 1.61
7.	NE-7	5.53 ± 0.24	6.6 ± 0.3	61.29 ± 5.38	1.347 ± 0.002	47.9 ± 2.5	0.517 ± 0.021	-18.48 ± 2.08	332.6 ± 16.5	94.61 ± 1.93

From primary evaluation the NE-2 batch was selected because we got higher zeta potential value and lower particle size as well as a lower poly-dispersity index. Drug content was also found maximum for NE-2 formulation. As nanoemulsions NE-2 formulation was also found clear, transparent and elegant when compared to the other formulations. So, it is considered as the optimized formulation for further studies.

Transmission Electron Microscopy

TEM image of prepared nanoemulsion system of posaconazole optimized formulation NE-2 is shown in the figure below. It may be confirmed from the figure that nanoemulsion droplets in the developed system were circular and uniform in shape. Posaconazole drug particles integrated into oil droplet which was surrounded by a water molecule and it validates the formation of oil in water (o/w) nanoemulsion system.

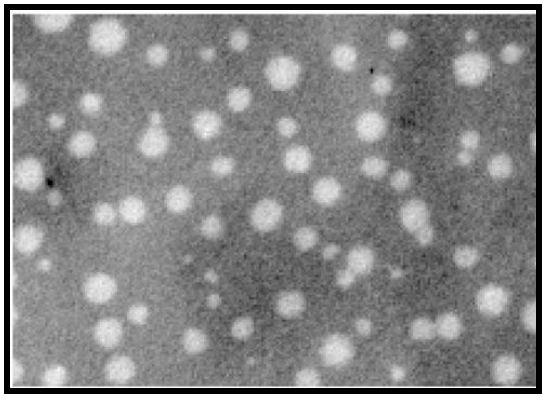


Figure 3: TEM image of optimized Posaconazole nanoemulsion NE-2.

In vitrorelease studies of Posaconazole

The cumulative amount of drug release from nanoemulsion formulations NE-1, NE-2, NE-3, NE-4, NE-5, NE-6 and NE-7 were found to be 97.5, 99.8, 96.2, 95.1, 97.4, 91.6 and 95.7 respectively in 24 hours.

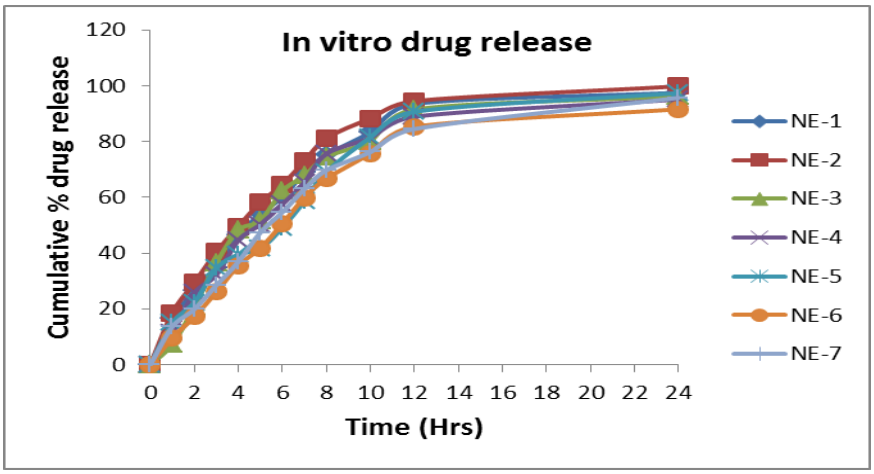


Figure 4: In vitro drug release of Posaconazole from nanoemulsions.

The formulation NE-2 showed a better in vitro drug release profile across the membrane when compared to the other formulations.

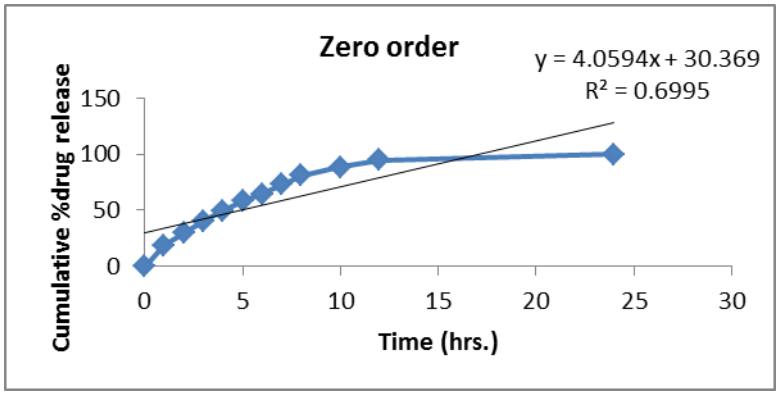
Release kinetics of Posaconazole nanoemulsions

In vitro release data was fitted with various release equations and kinetic models like the Zero-order, First-order, Higuchi and Korsemeyer Peppas.

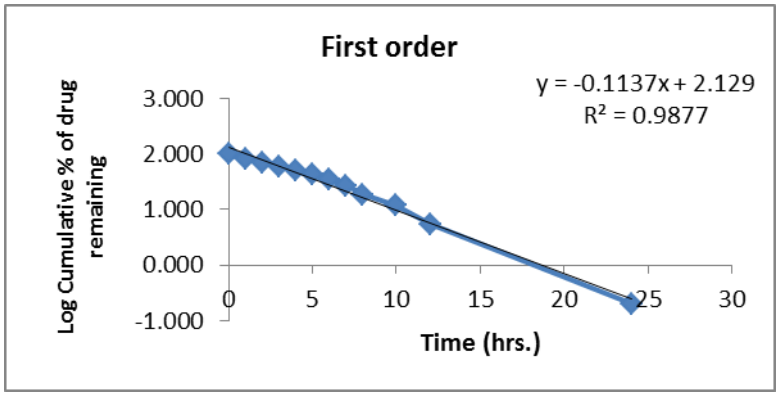
Table 6: Release kinetics data of all Posaconazole nanoemulsions.

S. No.	Formulation Code	Zero order Regression value (R^2)	First order Regression value (R^2)	Higuchi model Regression value (R^2)	Korsmeyer Peppas model Regression value (R^2)	Korsmeyer Peppas model (Slope)
1.	NE-1	0.726	0.950	0.924	0.658	1.072
2.	NE-2	0.699	0.987	0.917	0.631	1.057
3.	NE-3	0.709	0.945	0.907	0.747	1.207
4.	NE-4	0.727	0.945	0.919	0.703	1.122
5.	NE-5	0.785	0.965	0.931	0.686	1.081
6.	NE-6	0.768	0.930	0.920	0.760	1.164
7.	NE-7	0.778	0.988	0.937	0.711	1.109

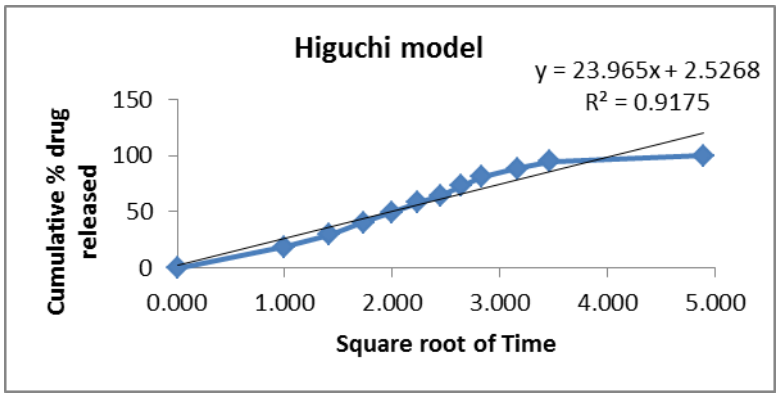
The Regression value (R^2) of all the 4 mathematical models was compared for the prepared nanoemulsion formulations to determine the mechanism of drug release. R^2 was found maximum for First-order release kinetics which indicated the release of drugs from all the tested formulations followed first-order release rate kinetics. Hence, it delivered the drug in a sustained manner for a prolonged period of time.



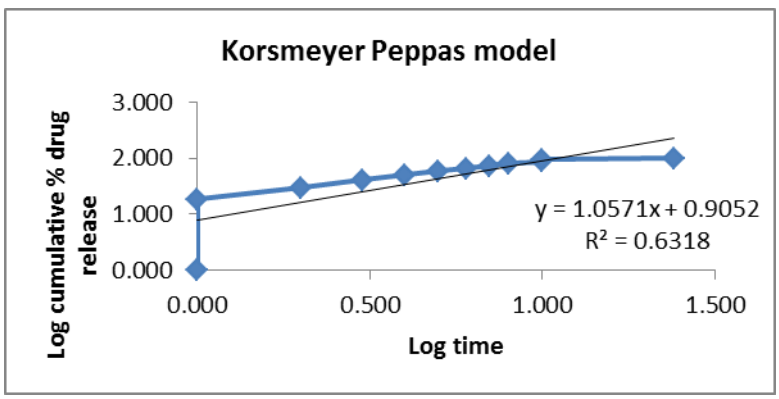
(a)



(b)



(c)



(d)

Figure 5: Release kinetics of the optimized nanoemulsion NE-2; (a): Zero order release kinetics, (b): First order release kinetics, (c): Higuchi model release kinetics and (d): Korsmeyer Peppas model release kinetics.

Ex Vivo Permeation Studies

The amount of drug permeated per unit area through the excised cornea (µg/cm^2) versus time (h) graph was plotted.

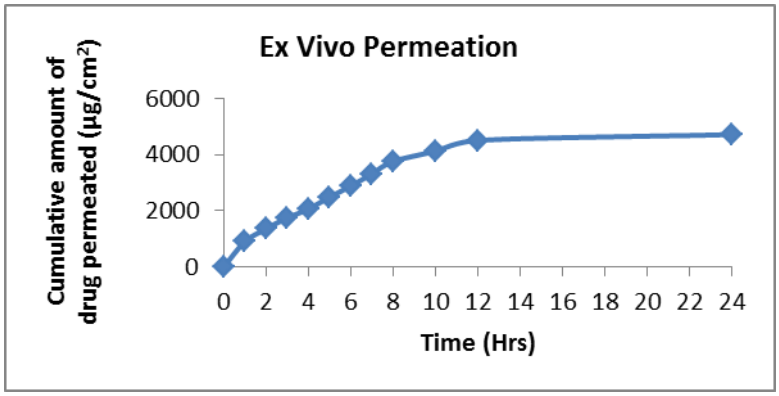


Figure 6: Posaconazole permeation studies through goat corneas from nanoemulsion NE-2.

The obtained results of the drug (Posaconazole) permeated through the excised goat cornea confirmed that the formulation is well suitable for the human eye.

Table 7: Ex Vivo Permeation data of optimized nanoemulsion NE-2.

S. No.	Attributes	Result
1.	Flux	596.4 ± 6.09 µg / hr / cm^2
2.	Corneal permeability coefficient (Papp)	1.28×10^{-5} cm/s
3.	Corneal hydration	78.52 ± 0.28 %

Ocular irritation studies

No irritancy was observed in the cornea, iris and conjunctiva (total score = 0) upon periodic application of the nanoemulsion for a week, which suggests the non-irritant action of the formulation. Hence, the optimized nanoemulsion was considered safe for ocular delivery.

This action is attributed to the use of oils, nano size of particles, narrow polydispersity index and the acceptable pH range of the formulation prevents the occurrence of any irritation into the eye.

Antifungal activity study

In the antifungal activity study of the test nanoemulsion NE-2, the inhibition zone values of the test nanoemulsion were compared to control and it was found that the test nanoemulsion was significantly different from control, P<0.005. From the obtained result and by comparing the inhibition zone values of the test with the control, it was observed that the optimized formulation NE-2 had a large zone of inhibition. So, it may be therefore concluded that the optimized nanoemulsion NE-2 exhibited better antifungal activity.

Table 8: Antifungal activity of Posaconazole nanoemulsion NE-2.

Diameter of Zone of inhibition (mm)					
Negative (-ve) Control	Positive (+ve) Control	Nanoemulsion Formulations NE-2 (Test)			
		C1 50 µg/ml	C2 100 µg/ml	C3 150 µg/ml	C4 200 µg/ml
-	6.82 ± 0.85	8.65 ± 0.57	12.46 ± 1.25	19.62 ± 0.46	28.79 ± 1.18

Test for Sterility

In order to access the sterility of the prepared nanoemulsion two different media were taken and formulation NE-2 was incubated for 14 days at 30 ºC to 35 ºC in FTGM and 20 ºC to 25 ºC in SCDM. There was no appearance of turbidity and no evidence of microbial growth in the optimized nanoemulsion hence, the formulation NE-2 examined, passes the sterility test.

In vivo drug study

The efficacy of Posaconazole nanoemulsion was studied in the rabbit eye model of Aspergillus flavus induced keratitis. The various pharmacokinetic parameters like Cmax, Tmax, and area under the curve of Posaconazole after ocular administration into the rabbit eye were determined for plasma and aqueous humor. The distribution of Posaconazole after administration of nanoemulsion in the aqueous humor was found significantly higher in comparison to the plasma. This confirmed the improved penetration properties of the

Posaconazole through nanoemulsion across the cornea and high precorneal residence of nanoemulsion lowering nasolacrimal drainage.

Table 9: Pharmacokinetics parameters in rabbits of optimized nanoemulsion formulation NE-2.

Parameter	Plasma	Aqueous humor
Cmax (µg/ml)	43.64 ± 8.32	92.28 ± 27.85
Tmax (h)	1.5 ± 0.1	2 ± 0.0
AUC (µg.h/ml)	456 ± 143.11	2564.45 ± 208.24

Accelerated stability studies

The stability study of optimized formulation (NE-2) was conducted according to ICH guidelines; the formulation was stored at 40 °C and 75 % relative humidity for 3 months.

Table 10: Stability data of optimized nanoemulsion NE-2.

S. No.	Parameters	Before Stability testing	After Stability testing		
			1 month	2 months	3 months
1.	Physical appearance	No change	No change	No change	No change
2.	Globule size (nm)	49.52 ± 2.35	50.41 ± 0.22	50.99 ± 0.32	52.22 ± 0.04
3.	pH	6.6 ± 0.3	6.5 ± 0.6	6.3 ± 0.1	6.3 ± 0.3
4.	Drug content (%)	98.96 ± 1.22	98.13 ± 0.48	97.68 ± 0.74	97.15 ± 1.06

The stability studies results signified that there was no significant change in physical appearance, globule size, pH and drug content after 3 months. The formulated nanoemulsion was found chemically stable and possess adequate shelf life until 90 days.

CONCLUSION

From the results obtained and discussion generated therefrom, encouraged conclusions were drawn and written. The drug Posaconazole holds good therapeutic efficacy for administration via the ophthalmic route for the treatment of fungal keratitis. The possibility to formulate it as a nanoemulsion and the various parameters that were evaluated helps to understand the usefulness of the drug. Using an appropriate combination of oil, surfactants and co-surfactants one can achieve sustained release up to 24 hours. The drug remained intact and stable in the nanoemulsion during storage, with no significant changes in appearance and drug content. Thus, the study demonstrates that the nanoemulsion formulation can be employed to improve the solubility of the poorly water-soluble drug-like Posaconazole.

CONFLICT OF INTEREST

None to declare.

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