RESEARCH PAPER

QBD based Voriconazole Nanosuspension for Topical Ophthalmic Application: Design, Optimization and Antifungal Activity Assessment

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ABSTRACT
Voriconazole nanoparticles seem to be an appropriate carrier for ocular medication administration due to their capacity to generate monodispersion with reduced particle sizes, high stability, biocompatibility, and lack of irritating effects on the cornea, iris, and conjunctiva. Voriconazole (VCZ) loaded polymeric nanosuspension was created using Eudragit as a polymer and having the goal of increasing VCZ availability at intraocular level and lowering the frequency of dosage for fungal infections in the eye.
Methods: VCZ-loaded nanosuspension were produced using the solvent evaporation approach with Eudragit RL100. Nanosuspension was physically evaluated using a variety of methods, including particle size, zeta potential, PDI, FTIR, DSC, XRD and TEM. Nanosuspension were studied both in vitro and ex vivo utilizing a modified USP I dissolving device and an all-glass Franz diffusion cell. Antifungal properties of nanosuspension along with commercialized preparation were tested toward Candida albicans as well as Aspergillus flavus.
Results: VCZ-loaded nanosuspension had particle size between 109-122 nm exhibited negative positive potential throughout all batches. VCZ-loaded Nanosuspension's present entrapment efficiency ranged from 91-96%.
Discussion: In-vitro drug release testing revealed the nanosuspension provided most sustained release of VCZ compared to marketed formulation. In-vitro trans corneal permeation studies revealed that optimized formulations outperformed commercial formulations. When comparing to the marketed formulation, improved VCZ nanosuspension had the highest antifungal efficacy against Candida albicans and Aspergillus flavus.
Conclusion: The findings suggested that a mucoadhesive formulation of VCZ loaded nanosuspension may be used as possible delivery strategy for controlling ocular fungal infections.
Keywords: Voriconazole; Nanosuspension; Ocular drug delivery; Eudragit RL100; Trans corneal permeation; Antifungal activity.
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INTRODUCTION

The eye has a complex anatomy and is delicate organ.This structure is composed of both anterior & posterior parts. Efficiency of strong medications which used for managing eye conditions is limited by a number of ocular obstacles. Conventional eye drops are drained by blinking & tear production. Consequently, less than 5% of them are bioavailable [1]. Cornea is composed of endothelium stroma, epithelium which can only be penetrated by small, lipophilic medications [2]. Ocular formulations can be administered intra-ocularly, peri-ocularly, topically. Ophthalmic dose forms may be solid, semi-solid, liquid, or a combination of these. Drops, suspensions, and emulsions are examples of liquid dosing. Eye drops make up over ninety percent of commercialized ophthalmic drugs. [3]. although they have brief residence period, they are employed to provide medicament to frontal area of eye [2]. Although they can administer hydrophobic medications, ocular suspensions and emulsions can cause impaired vision. Semi-solid ocular gels and ointments may greatly extend residence duration. Solid dose forms might be employed for zero order release (insert), water-sensitive drug delivery (powder), or maintaining residence duration (therapeutic contact lens) [3]. Good ocular absorption necessitates both adequate corneal penetration and an ideal medicine concentration with the least amount of the active therapeutic substance & effective pre corneal residence duration. Nano systems are cutting-edge technology designed to promote corneal permeability across biological barriers, protect the medication from the biological environment, prolong drug residence duration, and get past ocular impediments [4].

Effective topical administration has been of interest to scientists for many years. Ensuring appropriate ocular penetration & extending period of pharmaceutical residency is difficult assignment for them. Numerous ocular barriers impede successful medication administration. Ocular administration methods include topical, intra vitreal, intraocular, juxtascleral, sub-conjunctival, intra cameral, & retro bulbar. These days, nanotechnology-based carriers are attractive due to their potential to trap polar & lipid-based medications, increase the duration of residency and enhance medication stability and increase absorption [5]. Nanosuspension, colloidal nanocarriers are made up of lipophilic medications [6]. Improved drug solubility and bioavailability, longer dwell duration, prolonged release of drug are some of their benefits [7]. Eudragit® polymers are the most widely utilized mucoadhesive agents in nanosuspension. The created nanosuspension are made of Tween 80 and Eudragit® RS and RL 100. The resulting formula demonstrated improved permeability, stability, and corneal residence time [8].

Patients may lose their vision as a result of the illness if treatment is delayed. In tropical and subtropical areas of globe, such South India, where it accounts for up to 47% of keratitis cases, fungal keratitis is more prevalent [9]. Voriconazole, a commercially available oral, injectable, and topical treatment, is a modified form of fluconazole with exceptional broad spectrum action. In vitro, voriconazole exhibits broad antifungal efficacy against molds and yeasts. It shows fungicidal action in vitro across most Aspergillus species, molds such as Scedosporium & Fusarium species with significantly lower minimum inhibitory concentration (MIC) than other antifungal drugs. Additionally, it works well against Candida species that are resistant to fluconazole, including Candida krusei, C. glabrata, C. albicans. There are also commercially available forms of Voriconazole, such as VOZOLE, which is powder that has been frozen [10, 11, and 12].

Ophthalmic nanosuspension reportedly uses PLGA, PLDL, Eudragit RL-100 & RS-100, E-100, S-100 & L-100 [13].

They include quaternary ammonium groups in amounts ranging from 8.8% to 12% and 4.5% to 6.8%, respectively. These seem to be a promising polymeric carrier for medication dispersions since they are less soluble at normal pH & can reduce swelling. The quaternary ammonium group gives the polymer a positive charge, which enables it to interact with mucine and anionic drugs. The polymer's positive charge may also boost the anionic cornea's residency on the corneal surface by promoting mucoadhesion [14, 15, 16]. Prior evaluation of the Eudragit polymers for ophthalmic applications revealed that they have high ocular tolerability, improved aqueous humor drug levels, stable drug levels in ophthalmic formulations, and ocular drug availability. To examine the regulation of drug release and permeability properties, Eudragit RL 100 was employed in various ratios [17].

Using Eudragit RL-100 as the polymeric carriers, the current study focuses on creating and characterizing a voriconazole-loaded nanosuspension. Because of its broad range of action, voriconazole, a new imidazole antifungal medication, has significant potential for treating ocular fungal diseases such keratitis. Its low ocular penetration and poor aqueous solubility, however, limit its therapeutic efficacy. In order to stabilize the nanosuspension and improve medication retention on eye, Eudragit RL-100, a biocompatible with controlled release and mucoadhesive qualities, was employed. Formulation attempted to reduce particle size to nanoscale range in order to increase surface area, improve solubility and permit deeper corneal penetration [18–20].

MATERIALS AND METHODS

The pure drug of Voriconazole is received as gift sample from Dr. Reddy's Laboratories, Hydrabad, India.

The Eudragit RL-100 (Evonik industries, Mumbai)& Polyvinyl alcohol (Loba Chemie Pvt. Ltd. Mumbai) respectively.

FORMULATION STUDIES

Preparation of Voriconazole Loaded Nanosuspension by Using Nanoprecipitation Technique

Formulation of preliminary batches of Voriconazole Nanosuspension

Voriconazole-loaded nanosuspension were made using a nanoprecipitation method that was slightly altered from previously described procedure. Medication & ERL100 were dissolved in 10 mm of acetone to create homogeneous mixture for nanosuspension. Next, 26-gauge syringe was used to put prepared medication and polymer solution into 20 milliliters of distilled water with predetermined amount of polyvinyl alcohol at steady speed of 0.5 mm/min. Magnetic stirring was used to homogenize liquid for two hours at steady agitation speed of 700–800 rpm. Final volume of suspension was gathered after excess of acetone was evaporated through air drying. Ultrasonicator (Digital ultra-sonic cleaner, Mumbai, India) was used to further sonicate resultant suspension.

Formulation of final batches of voriconazole loaded nanosuspension using nanoprecipitation technique

Voriconazole nanosuspension were prepared using nanoprecipitation technique. Pure voriconazole &

ERL100 were precisely weighed and dissolved in 10 milliliters of acetone at room temperature. Next, a 26 gauge syringe was used to drop prepared medication & polymer solution into 100 ml of distilled water that included predetermined amount of polyvinyl alcohol at steady speed of 0.5 milliliters per minute. To evaporate solvent, therefore mentioned solution was constantly agitated for three hours. Resulting suspension was further sonicated using an Ultrasonicator (Digital ultra- sonic cleaner, Mumbai, India). Different formulations were prepared using a range of concentrations of polymer & sonication time as per the shown in Table 1.

Table 1. Composition of Preliminary Study of Polymeric Nanosuspension of Voriconazole

Optimization of surfactant	Surfactant	Sr. No.	Voriconazole (mg)	Eudragit RL 100 (mg)	Concentration of surfactant (%)
	i)Poloxamer 407	1	20	100	0.25
		2	20	100	0.5
		3	20	100	0.75
		4	20	100	1
		5	20	100	1.25
	ii)Tween 80	1	20	100	0.12
		2	20	100	0.18
		3	20	100	0.24
		4	20	100	0.3
		5	20	100	0.36
	iii) Polyvinyl alcohol	1	20	100	0.5
		2	20	100	1
		3	20	100	1.5
		4	20	100	2
		5	20	100	2.5
Optimization of concentration of PVA	Polyvinyl alcohol	1	20	100	0.5
		2	20	100	1
		3	20	100	1.5
		4	20	100	2
		5	20	100	2.5
Optimization of drug concentration	Polyvinyl Alcohol	1	10	100	1
		2	20	100	1
		3	30	100	1
Optimization of Eudragit RL 100	Polyvinyl alcohol	1	20	100	1
		2	20	200	1
		3	20	300	1

Fabrication of final batches of voriconazole loaded nanosuspension using nanoprecipitation technique by 3^2 factorial design

Experimental Design

To optimize, we utilized a design tool that represented formulated batches of Full Factorial Design Response Surface Methodology & Design Expert soft-wareTrialversion11.0.0. This approach enabled us to systematically assess the impact of numerous formulation parameters. We had a total of nine trial runs.

In this study, we considered Concentration of Eudragit RL100 (mg) (X_1) & sonication time (sec) (X_2), as our dependent variables, while particle size (nm) (Y_1), Zeta potential (mV) (Y_2) &encapsulation efficiency (%) (Y_3) were selected as our independent variables are given in Table 2. Purpose of this analysis was to understand how these responseschange when we alter values of two selected factors.

Table 2. Design Layout of 3^2 Factorial Designs and Different Factors with Coded Value

Formulation Batches	Factors			
	X_1 Conc. of ERL 100 (mg)		X_2 Sonication Time (Sec)	
VPNS1	+1	0.75	+1	150
VPNS2	0	0.50	+1	100
VPNS3	-1	0.25	+1	50
VPNS4	+1	0.75	0	150
VPNS5	0	0.50	0	100
VPNS6	-1	0.25	0	50
VPNS7	+1	0.75	-1	150
VPNS8	0	0.50	-1	100
VPNS9	-1	0.25	-1	50

Note: +1 is higher level, -1 is lower level and 0 is midlevel for the independent variables

Generation of Polynomial Equations

Response surface methodology employs mathematical model to examine relationship between variables & predict effect to find independent variables on dependent variables. RSM & Design Expert software Trial version 11.0.0 were used to run number of simulations within framework of our present optimization investigation. For every response variable, we created polynomial models with interactions using MLRA method.

Statistical Analysis of Data

It is a scientific procedure that collects & analyses data in order to identify patterns & trends. It is important instrument for study. We employed statistical analysis methods; like ANOVA to investigate impact of independent factors on replies. Statistical significance has been defined as

$p < 0.0001$.

Development of Response Surface Plots in Three Dimensions

To visualize responses we created three-dimensional graphs that display how response surface varies. They are crucial instruments for examining two factors' combined impact on outcome. They provide a graphic representation of how results are influenced by independent variables.

Characterization of Final Batches of Voriconazole Loaded Nanosuspension Using 3^2 factorial Design:

Fourier transforms infrared spectroscopy (FTIR) of drug

Using potassium bromide dispersion method, Fourier Transform Infrared spectrophotometer (IR Affinity1, Shimadzu, Japan) was used to determine the infrared spectrum of VCZ, ERL100, physical mixture, and optimum formulation. Dried potassium bromide was used to finish the base line correction. Spectrum of medicament & kBr mixture has been run, then succeeded by drug with excipients (physical mixture) & %Transmittance has been evaluated at resolution of 4 & 40^{cm-1} scans in spectral range of 4000-400 cm-1 [21].

DSC Study

Voriconazole (VCZ), ERL-100, a physical mixture (VCZ + polymer) in one as to one drug to polymer ratio & freeze-dried VCZ nanosuspension were all subjected to DSC investigations. Sample was placed in aluminium pans & detected in nitrogen environment between 30 & 300°Cat speed of 10°C per min [21].

X-ray diffraction study (XRD)

XRD (BRUKER D8 ADVANCE) is utilized to acquire X-ray diffraction (XRD) profiles for voriconazole. Five milligrams of each material were exposed to monochromatized cu-Ka radiation (1.54184 Å) and analyzed within the 40 to 1000 A° range using a scanning rate of 40^0/min. Voltage as well as current settings are 35 kV as well as 15 mA, correspondingly. [21]

% Entrapment Efficiency

Cooling centrifuge device (Remi, Mumbai, India) was used to centrifuge VCZ nanosuspension (10 ml) for three hours at 10,000 rpm & 10°C. After supernatant was removed, absorbance of free drug content was determined at 256 nm using UV/Visible spectrophotometer (Shimadzu 1800, Japan). By deducting quantity of free drug from original amount of medicament added, EE of has been measured [21] The following formula was used to determine the EE percentage of each batch:

$$\%EE = \frac{\text{Initial Drug } (D_i) - \text{Unentrapped Drug}(D_u)}{\text{Initial Drug } (D_i)} \times 100$$

In-vitro drug release

The modified USP dissolution apparatus 1 (37 ± 0.5 ºC) with two-sided open glass cylinder was used for 12-hour in vitro drug release tests. Dialysis membranes with molecular weight cutoffs of 12000–14000 A° (Himedia, Mumbai) served as diffusion barrier and release barrier. Glass cylinder's terminal section was fitted with pre-soaked dialysis membrane. In each instance, glass cylinder was mounted on stirrer & precisely filled with nanosuspension (2 ml) from open side. stirrer was kept at 37ºC ± 0.5ºC at 100 rpm in 100 cc solution of synthetic tear fluid (pH 7.4). Sample aliquots (5 ml) were taken out with volume replenishment at prearranged intervals. Drug content of removed samples was determined using a UV visible spectrophotometer (Shimadzu 1800, Japan) to measure absorbance at 257 nm. For duration of release, sink conditions were maintained. Data had been graphically evaluated, such as by plotting percentage of medication release vs time [21].

Drug release kinetic data analysis

Fitness of release data within different kinetic model Zero order, First order, Higuchi, Hixson Crowell & Korsemeyer- Peppas release models, had been further investigated using release data acquired from different formulations. Obtained data was estimated using variety of parameters. To understand release process, parameter 'n' as well as time factor 'k', release constant rate 'R', regression co-efficient (R^2), was found using Korsemeyer-Peppas equation. [21]

Antifungal Study

Cup-plate method has been employed to test formulation's antifungal activity. Nutrient agar medium was utilized as antifungal activity medium. 65 grams of nutritious agar were used and powder had been entirely dispersed by boiling it in 1 liter of distilled water with constant stirring & boiling it up to one minute. After that, prepared media was autoclaved for 15 minutes at 1210C. Media were aseptically transferred into glass petri plate with diameter of 93 mm & allowed to solidify. Sterilized cork borer was used to puncture agar plates' surface.

Aspergillus flavus & Candida albicans were streaked onto agar surface. Intial 7 mm wells were filled with an aliquot of commercially available formulation made from 5 µl of appropriately diluted. Since standard sample, aliquot of optimized nanosuspension made from 60 µl of appropriately diluted distilled water was created and put in the second 7 mm wells. Plates were incubated at 250C for 24 hours after being left for 30 minutes. Zone of inhibitions was then measured [21].

TEM Study

TEM has been utilized for assessing nanoparticles' morphology. Transmission electron microscopy (Hitachi H-7500, Tokyo, Japan) was used to observe nanoparticle suspension (5–10 µl) on carbon-coated copper grids. Image capture and analysis were carried out using imaging viewer software [21].

HET- CAM Test (Hen's Egg Test - Chorioallantoic Membrane)

HET-CAM assay was carried out in accordance with ICCVAM guidelines, which were published in Appendix G in November 2006 & modified for our lab setting. 40–50 grams of clean, viable, fresh chicken eggs were from commercial sources. Before being used, these eggs were candled to determine viability and development of embryos; nonviable or faulty eggs were removed. Lastly, study used six eggs per group. This protocol's goal was to outline elements and methods used to assess a test substance's propensity for causing ocular irritation by gauging its capacity to induce significant harmful alterations in chorioallantois membrane of fertilized hen's egg. Effects were measured by:

Coagulation;

Hemorrhage;

Blood Vessel lysis.

These evaluations were taken into account separately before being added together to create a score that was used to categorize the test substance's degree of irritation.

Experimental Design

Six eggs per group (negative& positive controls, test substance)

Control:

To define a baseline for test endpoints and ensure that assay parameters did not inadvertently generate an unpleasant response, each experiment included a 0.9% NaCl (negative control).

Negative Control:

Chorioallantoic membrane was treated with 0.3 ml of 1% SDS & 0.1N NaOH.

Treatment Groups (Formulation):

On the ninth day, 0.3 mL of test formulation was applied to CAM, making sure that at least half of its surface was covered.

Grading: The scores were noted

CAM Preparation:

Choose 50–60 g White Leghorn chicken eggs that are clean, viable, and fresh (no more than 7 days). Candle eggs & dispose of any that were flawed or nonviable. It is not recommended to use eggs that are extremely malformed or those have thin or fractured shells. When preparation, it is best to minimize shaking, needless tilting, knocking.

Put it in an incubator on revolving tray. Eggs should be incubated in still-air incubator at 38.3 ± 0.2°C & 58 ± 2% RH, or in forced-air incubator at 37.8 ± 0.3ºC & 58 ± 2% relative humidity. Up to day eight, hand rotates eggs five times a day.

On eighth day of incubation, candle eggs & destroy those that are faulty or nonviable. They were put back in incubator for one more day with their big end facing up (without being rotated by hand).

On day nine, take eggs out of the incubator so they can be used in test. Any defective or nonviable eggs should be thrown out once they have been candled.

Mark egg's air cell. Using a revolving dentist saw blade, cut area designated as air cell, then remove it.

Use 0.9% NaCl to moisten inner membrane. Solution can be applied using disposable glass pipette. Put egg in incubator for no more than half an hour.

Before using egg in test, take it out of incubator & decant 0.9percent sod. chloride solution. Using forceps, separate inner membrane, being careful not to damage it.

Observations:

For 300 seconds, watch reactions on the CAM. It is important to track & record, in seconds for each of mentioned endpoints to materialize. Following endpoints needed to be monitored:

Haemorrhage (bleeding from vessels)

Vascular lysis (blood vessel disintegration)

Coagulation (intra- and extra-vascular protein denaturation)

Hemorrhage time = observed start (in seconds) of hemorrhage reactions on CAM

Lysis time = observed start (in seconds) of vessel lysis on CAM

Coagulation time = observed start (in seconds) of coagulation formation on CAM.

RESULT AND DISCUSSION:

Formulation Studies

Characterization of preliminary study of polymeric nanosuspension of voriconazole

Optimization of surfactant

Selection of ideal surfactant, surfactant decide were characterize by physical appearance. On the basis of physical appearance, the PVA was selected as non-ionic surfactant for further study as in table 3,.

Table 3. Optimization of surfactant

A) Optimization of surfactant	Surfactant	Sr. No.	Voriconazole (mg)	Concentration of surfactant	Physical appearance
	i) Poloxamer 407	1	20	0.25 %	Agglomeration of particles

		2	20	0.5 %	Agglomeration of particles
		3	20	0.75 %	Agglomeration of particles
		4	20	1 %	Agglomeration of particles
		5	20	1.25 %	Agglomeration of particles
	ii) Tween 80	1	20	0.12 %	Sedimentation of particles
		2	20	0.18 %	Sedimentation of particles
		3	20	0.24 %	Sedimentation of particles
		4	20	0.3 %	Sedimentation of particles
		5	20	0.36 %	Sedimentation of particles
	iii)Polyvinyl alcohol	1	20	0.5 %	Slightly undissolved fibers
		2	20	1 %	Slightly undissolved fibers
		3	20	1.5 %	Bluish opalescence and fibers absent
		4	20	2 %	Bluish opalescence and fibers absent
		5	20	2.5 %	Bluish opalescence and fibers absent

Optimization of PVA concentration

Based on findings from previously mentioned Table 3. PVA was chosen for more formulation research. Several nanosuspension batches were made using varying PVA conc (0.5, 1, 1.5, w/v) with same polymer concentration (100 mg) in order to optimize PVA concentration. After preparation of different batches containing PVA were evaluated by EE (%), amount of permeation & percent permeation & their effect was shown in Table 4. From results, study suggested that, among all ranges of concentrations, the 1.5% (w/v) concentration of PVA produced highest EE with high drug permeation. So, based on results, optimum concentration of PVA was decided for further formulation study.

Optimization of Voriconazole Concentration

After selection of suitable surfactant and its optimized drug concentration in the formulation of final batches, there is requirement of study the effect of Voriconazole (mg) on percent encapsulation efficiency. So, further batches of nanosuspension were designed with different polymer concentration with same concentration of PVA. The composition and result profile of various formulation containing different drug and polymer concentration were represent in Table 4.

Optimization of polymer concentration

After selection of suitable surfactant and its optimized concentration in the formulation of final batches, impact of polymer conc. (ERL100) on zeta potential must be investigated. So, further batches of nanosuspension were designed with different polymer concentration with same concentration of PVA. The composition and result profile of various formulation containing different drug and polymer concentration were represent in Table 4.

From Table 4, results were demonstrated that optimum concentration of drug & polymer with same concentration of PVA provides higher EE (%) with maximum percent drug permeation from nanosuspension. Results of 100mg polymer concentration were able produced higher % encapsulation efficiency i.e. 92.36 % & minimum zeta potential 21.2 mV. So, on basis final results

which are obtained from Table 4, final design was decided in form of factorial design i.e. 3^2 factorial designs.

Table 4. Optimization of PVA, voriconazole, Polymer Concentration

Optimization of PVA, voriconazole, polymer, concentration	PVA (% w/v)	Voriconazole (mg)	Eudragit RL 100 (mg)	Particle Size (nm)	Zeta Potential (mV)	EE (%)
PVA	0.5	20	100	122.5	23.9	91.52
	1.0	20	100	115.3	26.0	93.19
	1.5	20	100	109.8	28.4	96.21
Voriconazole	1.5	10	100	121.6	-	90.21
	1.5	20	100	123.2	-	95.25
	1.5	30	100	126.4	-	92.56
Polymer	1	-	100	-	21.2	92.36
	1	-	200	-	24.6	91.24
	1	-	300	-	27.3	90.43

Formulation of voriconazole loaded nanosuspension

Particularly for weakly water soluble medications like voriconazole, nanosuspension technology seems to be compelling formulation strategy. Most popular technique for creating nanosuspensions at laboratory scale is nanoprecipitation. It is thought to be more cost-effective technique of preparing nanoparticles because it requires less equipment & chemicals. Procedure calls for synthesis of two phases: solvent phase, where medication is soluble & anti solvent phase, where drug is insoluble. Rapid gradient-driven diffusion of acetone from organic droplets into the aqueous phase occurs when organic phase is gradually added to aqueous phase. Drug & polymer spontaneously precipitate and form anosphere matrix because they are both insoluble in aqueous phase.

To improve precipitation, a supersaturated drug & polymer solution in acetone was made. The organic solvent was evaporated under magnetic agitation to accomplish final solidification into nanosphere. For nanosuspension to be physically stable & to maintain required viscosity, PVA, highly aqueous soluble surfactant, was required. According to ICH Q3C (R4) recommendations, acetone, an ICH class 3 solvent, was chosen for organic phase preparation since it is less harmful than methanol and chlorinated solvents. Additionally, Eudragit RL-100 and ICZ demonstrated good solubility in it. Prepared nanosuspensions had a translucent, milky white appearance & showed no indications of particle debris (fig. 1). It is clear that batches with high drug/polymer ratio had a milky white look.

Fig. 1. Image of prepare final batches of voriconazole loaded polymeric nanosuspension.

CHARACTERIZATION OF FINAL BATCHES OF VORICONAZOLE NANOSUSPENSION USING 3^2 FACTORIAL DESIGN:

Data Optimization

Quadratic Equations:

$$\mathbf{Y = \beta 0 + \beta 1\ A + \beta 2\ B + \beta 3\ AB + \beta 4\ A^2 + \beta 5\ B^2}$$

Where, Y= Response Variable,

β0, β1 ...β5 = Coefficient,

A= Conc. of ERL 100,

B= Sonication time.

When each factor is changed independently between more or less values ranges, average outcome is displayed by major influences of factors A and B. Y represents independent variable in above equation, 0 represents the average response over nine runs & 1 represents calculated coefficient for factor A. Conversely, interaction terms (AB) indicate how the result changes when both variables are altered simultaneously. Particle size, zeta potential & percent encapsulation efficiency are significantly impacted by a few independent characteristics, according to an analysis of DOE data. Above polynomial equations are helpful for drawing conclusions from mathematical signs they include. While a negative sign denotes an antagonistic effect where factors oppose one another, a positive sign suggests a synergistic influence where elements work together.

Y1–(PS)= +110.59 +1.98 A -9.33 B -0.025 AB +0.32 A^2 -2.03 B^2,

Y2–(ZP)= +19.17 +2.33 A +6.88 B +0.30 AB -0.20 A^2 +1.55 B^2,

Y3–(%EE)= +89.18 + 1.91 A -5.72 B +0.077 AB +0.025 A^2 -1.78 B^2.
Where, A= Conc. of ERL 100, B = Sonication time

Statistical Analysis

To find unimportant components, ANOVA was used. The results demonstrated that all dependent variables had p-values less than p<0.0001. Additionally, Tables 9–14 show that the model F values for PS, ZP, and %EE were 0.9995, 0.9979, and 0.9989, respectively, indicating their relevance. R-squared, a statistical metric, measures the proximity of data points to fitted regression line. It is also called the coefficient of determination or in multiple regressions, coefficient of multiple determinations.

A surface plot depicts show varying concentrations of ERL100 & sonication time impact percentage of encapsulation efficiency. Initially, increasing concentration of ERL 100 caused a increase in % encapsulation efficiency .However, as ERL 100 concentration continued to rise, % entrapment efficiency showed an upward trend. This shift could be attributed to higher production of nanosuspension with increasing polymer concentration, consequently enhancing % encapsulation efficiency.

Similarly, increasing sonication time resulted in a decrease in % encapsulation efficiency. Boosting nanosuspension stability and resulting in a higher % entrapment efficiency, particularly at high concentrations (Fig.2. C).

A surface plot shows effect of polymer and sonication time on particle size (nm). The concentration of ERL 100 plays an important role in nanosuspension production, influencing particle size. The response surface plots clearly indicate that increasing concentration of ERL 100 result in larger particle size. Additionally, an increase in sonication time is associated with smaller particle sizes (Fig.2. A).

Another surface plot shows the effect of ERL 100 and Sonication time on zeta potential. According, to the studies, raising the quantities of ERL 100 results to decrease the zeta potential and sonication time causes larger zeta potential of nanosuspension. (Fig .2. B).

Table 5. ANOVA for Quadratic models of Particle Size (Y1)

Source	Sum of Square	Df	Mean Square	*F*-value	*p*-value Prob > F	significant
Model	554.74	5	110.95	1180.53	<0.0001	significant
A- Conc of ERL100	23.60	1	23.60	251.13	0.0005	-
B-Sonication time	522.67	1	522.67	5561.38	<0.0001	-
AB	2.500E-003	1	2.500E-003	0.027	0.8808	-
A^2	0.20	1	0.20	2.13	0.2402	-
B^2	8.27	1	8.27	87.98	0.0026	-
Residual	0.28	3	0.094	-	-	-
Cor Total	555.02	8	-	-	-	-

Abbreviations: Df-Degree of freedom, F-value – Ratio of two variation,*P*- value Probability value.

Table 6. ANOVA for Quadratic Models Indicating Model F-value of Particle Size (Y_1)

Std. Dev.	0.31	R^2	0.9995
Mean	109.44	Adjusted R^2	0.9986
C. V. %	0.28	Predicted R^2	0.9947
PRESS	2.96	Adeq. Precision	90.422

Abbreviations: Std. Dev.- Standard Deviation, C.V – Coefficient of variance, R^2 – Coefficient of determination, Adeq. Precision – Adequate precision.

Table 7. ANOVA for Quadratic models of Zeta Potential (Y_2)

Source	Sum of Square	Df	Mean Square	*F*-value	*p*-value Prob > F	significant
Model	322.19	5	64.44	289.97	0.0003	significant
A-Conc of Polymer	32.67	1	32.67	147.00	0.0012	-
B-Sonication time	284.28	1	284.28	1279.27	<0.0001	-
AB	0.36	1	0.36	1.62	0.2928	-
A^2	0.080	1	0.080	0.36	0.5908	-
B^2	4.81	1	4.81	21.62	0.0188	-
Residual	0.67	3	0.22	-	-	-
Cor Total	322.86	8	-	-	-	-

Abbreviations: Df-Degree of freedom, F-value – Ratio of two variation, *P*-value – Probability value.

Table 8. ANOVA for quadratic models indicating model F-value of Zeta Potential

Std. Dev.	0.47	R^2	0.9979
Mean	20.07	Adjusted R^2	0.9945
C. V. %	2.35	Predicted R^2	0.9750
PRESS	8.08	Adeq. Precision	47.891

Abbreviations: Std. Dev. - Standard Deviation, C.V – Coefficient of variance, R^2 – Coefficient of

determination, Adeq. Precision – Adequate precision.

Table 9. ANOVA for Quadratic models of % Encapsulation Efficiency (Y_3)

Source	Sum of Square	Df	Mean Square	F-value	p-value Prob > F	significant
Model	224.37	5	44.87	543.18	0.0001	significant
A-Conc of Polymer	21.85	1	21.85	264.49	0.0005	-
B-Sonication time	196.20	1	196.20	2374.86	<0.0001	-
AB	0.024	1	0.024	0.29	0.6272	-
A^2	1.250E-003	1	1.250E-003	0.015	0.9099	-
B^2	6.30	1	6.30	76.27	0.0032	-
Residual	0.25	3	0.083	-	-	-
Cor Total	224.62	8	-	-	-	-

Abbreviations: Df-Degree of freedom, F-value – Ratio of two variation, *P*-value – Probability value.

Table 10. ANOVA for quadratic models indicating model F-value of % Encapsulation Efficiency

Std. Dev.	0.29	R^2	0.9989
Mean	88.01	Adjusted R^2	0.9971
C. V. %	0.33	Predicted R^2	0.9881
PRESS	2.69	Adeq Precision	64.996

Abbreviations: Std. Dev. - Standard Deviation, C.V – Coefficient of variance, R^2 – Coefficient of determination Adeq. Precision – Adequate precision.

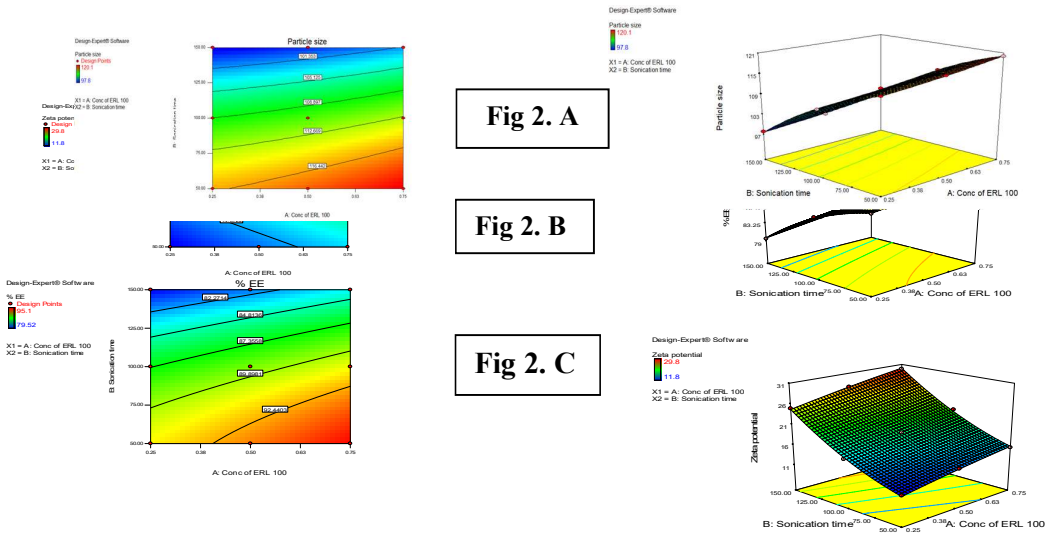


Fig. (2). Indicates Surface plots and 3D responses of **A)** Particle Size, **B**) Zeta Potential, **C)** %Encapsulation Efficiency.

Particle Size

Particle size in the prepared batches varied from 97.8 nm to 120.1nm. The VPNS 7 and VPNS 4 detectors measured the smallest and largest particle sizes respectively. The correlation between particle size and processing parameters, such as polymer and sonication time content was discovered. The VPNS 4 batch have a median size of less than 290 nm, making them suitable for prospective ocular applications. There was a notable correlation between quantity of ERL 100 & average size of VPNS formulations. Higher concentrations of ERL 100 led to increase in particle size as shown in table 11.

Zeta Potential

In current evaluation, all batches demonstrated zeta potential of more than11.8mV.VPNS-2 had greater zeta potential of 29.8mV. Interestingly, as ERL 100 content increased, zeta potential increased for all nanosuspension formulations Table 11. Data suggest that concentration of polymer ERL100 increased, zeta potential decreases. It is worth noted that polymer concentration enhances zeta potential while also influencing polymer levels in all VPNS formulations, resulting in observed trends.

% Encapsulation Efficiency

%EE calculates the percentage of total mass that is encapsulated in polymeric carrier. Every

formulation showed an encapsulation efficiency of higher than 79.52% in current valuation. VPNS-4 had a greater encapsulation effectiveness of 95.10%. Interestingly, as ERL 100 content increased, percentage encapsulation efficiency increased for all nanosuspension formulations Table 11. Data suggest that concentration of polymer ERL100 increased, drug encapsulation efficiency increases. It is worth noted that polymer concentration enhances encapsulation efficiency while also influencing polymer levels in all VPNS formulations, resulting in observed trends.

Table 11. Physicochemical characterization of Voriconazole Polymeric nanosuspension

Sr. No.	Formulation batches	Particle size (nm)	Zeta Potential (mV)	Encapsulation Efficiency (%)
1.	VPNS1	112.9	21.8	91
2.	VPNS2	101.6	29.8	83.7
3.	VPNS3	110.8	19.1	89
4.	VPNS4	120.1	15.4	95.10
5.	VPNS5	116.2	11.8	91.23
6.	VPNS6	118	13.9	93.1
7.	VPNS7	97.8	25	79.52
8.	VPNS8	108.7	16.2	87.6
9.	VPNS9	98.9	27.6	81.9

CHARACTERIZATION OF VORICONAZOLE LOADED NANOSUSPENSION:

FTIR Spectroscopy

To assess potential drug–excipient interactions, the FTIR spectra of pure voriconazole (A), Eudragit RL-100 (B), polyvinyl alcohol (PVA) (C), physical mixture (D), and the optimized nanosuspension (E) were compared. Pure voriconazole displayed its distinctive absorption bands due to O–H stretching (~3200–3500 cm^{-1}), C–H stretching (~3000 cm^{-1}), and strong peaks in the fingerprint region (1500–1000 cm^{-1}) associated with C–F, C–N, and aromatic vibrations. Eudragit RL-100 and PVA exhibited their typical polymeric peaks, including ester carbonyl stretching about ~1730 cm^{-1} and wide hydroxyl stretching near ~3300 cm^{-1}, respectively.

There was no chemical incompatibility during simple mixing, as evidenced by the retention of all main distinctive peaks of voriconazole and excipients in the physical combination without any notable shifting. Due to drug dispersion at the nanoscale and potential hydrogen bonding with stabilizers, the improved nanosuspension also maintained the key drug peaks with only slight broadening and decreased intensity. Voriconazole's chemical stability within the nanosuspension was confirmed by the lack of new peaks or significant peak disappearance, indicating satisfactory drug-excipient compatibility.

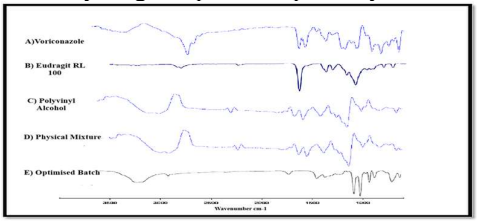


Fig. (3). The Overlay of FTIR of Voriconazole loaded Nanosuspension **A)** Voriconazole, **B)** ERL-100, **C)** PVA, **D)** Physical Mixture and **E)** Optimized batch.

DSC Thermogram

DSC is a method for examining how a substance melts and recrystallizes. Figure 4 displays graph of voriconazole, eudragit RL-100 & physical mixture of voriconazole and eudragit RL-100, and lyophilized voriconazole loaded nanosuspension (VPNS4) samples. At 129°C, voriconazole thermogram displayed a sharp endothermic peak indicative of crystalline substance. Eudragit RL-100's thermal curve features a strong melting endotherm at 67.12°C. Eudragit RL-100 has a glass transition temperature (T g) of 150.28°C. The voriconazole and eudragit RL-100 physical mixture displayed distinctive peaks at 73.98°Cand 162.33°C. Because voriconazole was present, the lyophilized nanosuspension's DSC curve had an endotherm at 174.32°C. These findings imply that there hasn't been any drug-related interaction.

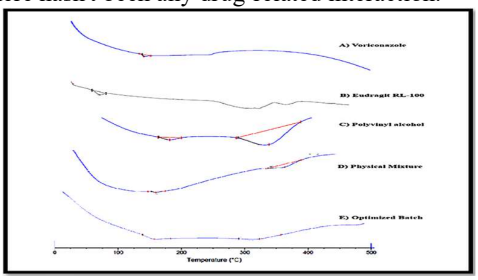


Fig. (4). The Overlay of DSC Thermogram of Voriconazole loaded Nanosuspension A) Voriconazole, B) ERL-100, C) PVA, D) Physical Mixture and E) Optimized Batch.

XRD Spectra

Powder X-ray Diffraction technique was employed to examine physical features of medication that was encapsulated. XRD of pure voriconazole are shown below, it was showed that pattern of the voriconazole exhibited sharp crystalline peaks at 2θ

values, which proved that voriconazole was crystalline form. Amorphous nature of Eudragit RL-100 was demonstrated by its broad and scattered highest peak at two-fold levels. Voriconazole peaks in the XRD spectrum in of a physical blend (VCZ + ERL-100) indicated that medication's crystallinity pattern remained unchanged following physical combination of drug and polymers. In contrast, voriconazole-loaded formulation (VPNS4) pattern displayed sharp, powerful peaks, indicating that drug's crystal structure persisted in nanosuspension (Fig. 5).This finding showed that, following integration in nanosuspension form, physical mixing had no discernible impact on drug diffraction (i.e., a somewhat zigzag pattern).

Fig.(5) The Overlay of XRD for Voriconazole loaded Nanosuspension A) Voriconazole, B) ERL-100, C) PVA, D) Physical Mixture and E) Optimized batch

Encapsulation Efficiency

EE is ratio of the actual mass of drug trapped in a polymeric vehicle to initial amount of drug delivered. All formulations within current trial had encapsulation efficiencies more than 75.10%. VPNS4 has greater encapsulation effectiveness of 95.10 with a 2:0.5 drug polymer ratio & 1.5% w/v PVA. Since particle size grew within batches, related percent encapsulation effectiveness of all nanosuspension formulations as polymer conc. increased. (Fig. 6.) Findings indicated that drug encapsulation efficiency rose dramatically when medicament to polymer ratio increased because of its increasing viscosity. Thickness of droplet exiting the syringe grows as viscosity of organic phase rises.

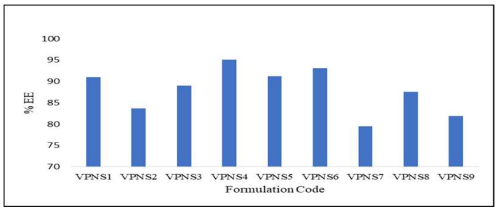
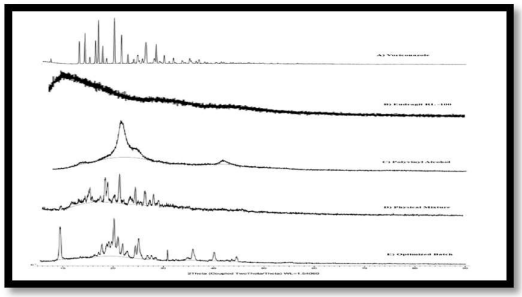


Fig 6. Graphical representation of Encapsulation Efficiency of Voriconazole Loaded Nanosuspension.

***In -Vitro* Dissolution Study**

In contrast to what is usually expected for poorly soluble medications, the in vitro dissolution profiles showed a considerable improvement in the release behaviour of voriconazole from nanosuspension formulations. A quick initial release was followed by a prolonged release phase in all formulations (VPNS1–VPNS9). With a cumulative drug release of roughly 90–98% in 10–12 hours, VPNS1 and VPNS2 demonstrated the fastest dissolution of all the tested batches, demonstrating efficient particle size reduction and enhanced wettability. Additionally, VPNS4 and VPNS5 demonstrated excellent release performance, with almost full drug release (~100%) by 14–16 hours. VPNS7–VPNS9, on the other hand, showed comparatively slower release patterns, which could indicate variations in stabilizer concentration or particle agglomeration. Voriconazole's better saturation solubility, larger surface area, and smaller particle size are all responsible for the modified nanosuspension



formulations' faster rate of dissolution. Overall, the study demonstrates that the dissolving properties of weakly water-soluble voriconazole are greatly improved by nanosuspension technology, with VPNS4 (or optimized batch) emerging as the best-performing formulation.

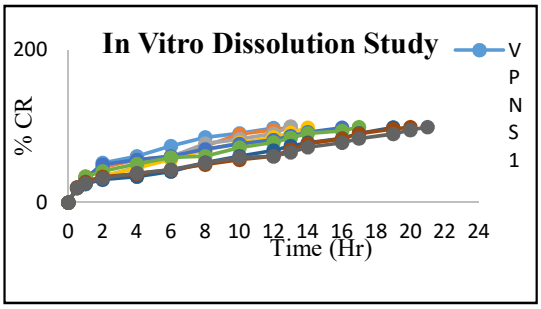


Fig. 7. *In Vitro* Dissolution Study of Voriconazole Loaded Nanosuspension.

Release Kinetic Study

To clarify medicament release behaviour, release kinetic data of voriconazole-loaded nanosuspension formulations (VPNS1–VPNS9) were fitted to several mathematical models. With high R2 values ranging from 0.9359 to 0.9892, indicating diffusion controlled drug release from nanosuspension matrix, Higuchi model was found to best characterize the release kinetics for the majority of formulations. The release exponent (n) values for all formulations were below 0.5 (0.2902–0.523), and the Korsmeyer–Peppas model demonstrated strong linearity (R^2 = 0.9035–0.996), suggesting that the drug release mostly

followed Fickian diffusion. This demonstrates that diffusion, not erosion or swelling-controlled processes, was primary mechanism by which the drug molecules were released.

In Higuchi & Korsmeyer–Peppas models, VPNS4 showed best goodness fit (R^2 = 0.9899) among the batches, suggesting a more reliable and consistent release pattern. The Hixson–Crowell model displayed significantly lower R2 values, indicating that particle erosion and surface area changes had less of an impact on drug release.

Overall, the kinetic study confirms efficacy of formulation technique for prolonged drug release by showing that voriconazole release from produced nanosuspensions follows a diffusion-controlled, non-swelling matrix mechanism.

Table 12. *In Vitro* Release Kinetics of Voriconazole Loaded Nanosuspension

Formulation Code	R^2				Korsemeyer Peppas		Drug Transport	Release Mechanism
	Zero Order	First Order	Higuchi Kinetics	Hixson Crowell Model	R^2	n (slope)		
VPNS1	0.91777	0.93112	0.98044	0.98355	0.90449	0.5233	Fickian Diffusion	Non swellable matrix diffusion
VPNS2	0.96331	0.91883	0.97911	0.94577	0.97762	0.31667	Fickian Diffusion	Non swellable matrix diffusion
VPNS3	0.98444	0.97786	0.98892	0.89377	0.9966	0.498	Fickian Diffusion	Non swallable matrix diffusion
VPNS4	0.99223	0.83404	0.98887	0.94333	0.98999	0.396	Fickian Diffusion	Non swallable matrix diffusion
VPNS5	0.93552	0.84955	0.97622	0.94456	0.90035	0.3443	Fickian Diffusion	Non swallable matrix diffusion
VPNS6	0.96886	0.80838	0.98044	0.93320	0.99935	0.2902	Fickian Diffusion	Non swallable matrix diffusion
VPNS7	0.99962	0.73777	0.95833	0.89990	0.92667	0.3768	Fickian Diffusion	Non swallable matrix diffusion
VPNS8	0.98447	0.80775	0.93599	0.88233	0.96220	0.3059	Fickian Diffusion	Non swallable matrix diffusion
VPNS9	0.99928	0.74760	0.96166	0.89444	0.98122	0.3536	Fickian Diffusion	Non swallable matrix diffusion

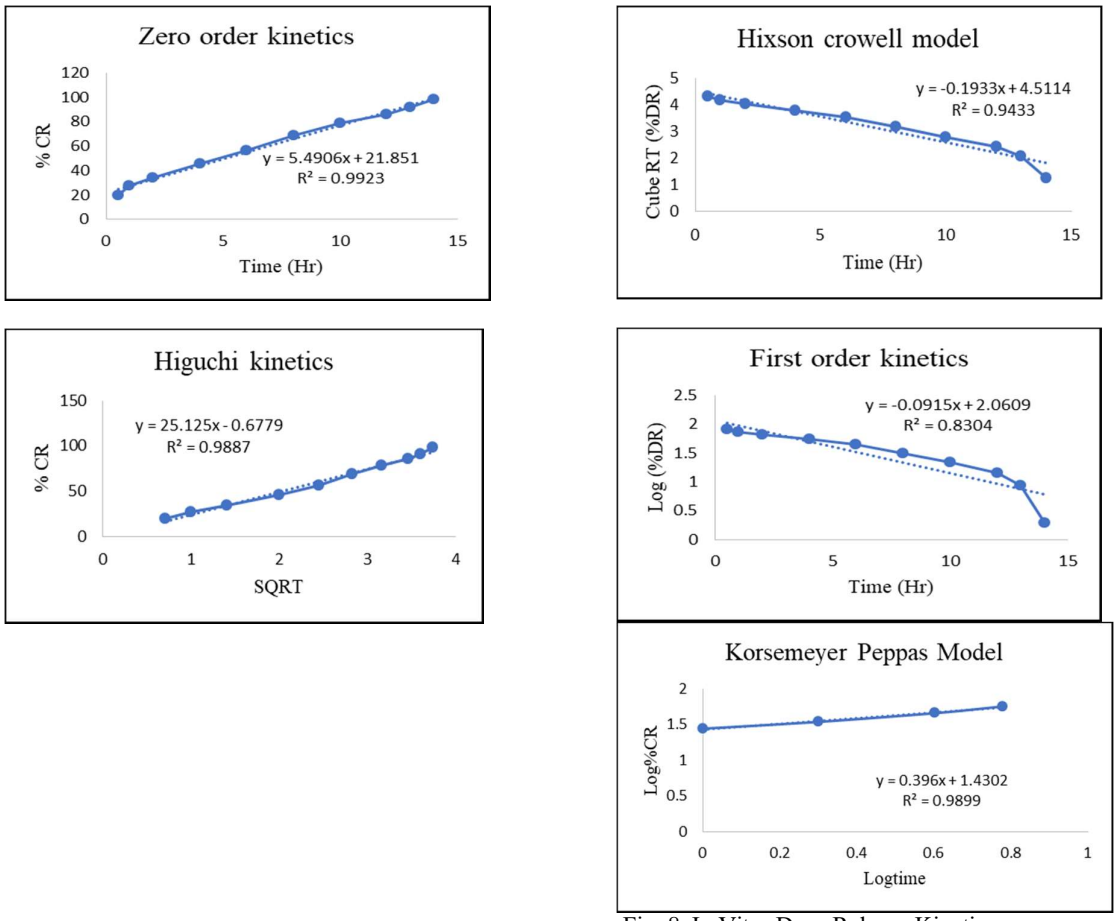


Fig. 8. In Vitro Drug Release Kinetics of Voriconazole Loaded Nanosuspension of Optimized Formulation (VPNS4).

Antifungal Study

Agar diffusion method was used for microbiological assay investigations. Compared to VOZOLE (1% w/v) & voriconazole aqueous solution, results show that voriconazole nanosuspension considerably inhibits development of Candida albicans and Aspergillus flavus. Table 17 and Fig. 9 illustrate the mean diameter of ZOI with Candida albicans & Aspergillus flavus. Zone of inhibition is examined at fifty micro gram per ml conc. in order to optimize MICs of VPNS4 & marketed product. For both formulation and procedures for remainder antifungal investigation, this concentration was regarded as minimum inhibitory concentration (MIC). Findings of antifungal study shown that, in comparison to other formulations, optimized formulation, VPNS4, produced maximum zone of inhibition (17.20 & 14.40 mm) for Candida albicans & Aspergillus flavus. In contrast, the zone of inhibition of placebo exhibits lowest values (2.00 & 2.20 mm) against Aspergillus flavus and Candida albicans. ZOI for Candida albicans and Aspergillus flavus in commercially available Voriconazole eye drop (VOZOLE) was 10.80 & 9.40 mm, respectively. These values are marginally higher than those of placebo formulation but lower than those of aqueous solution voriconazole with both Candida albicans & Aspergillus flavus.

The ZOI values for Candida albicans & Aspergillus flavus were 12.50 & 11.20 mm, resp. Voriconazole aqueous solution was able to inhibit fungal growth somewhat less than optimal formulation.

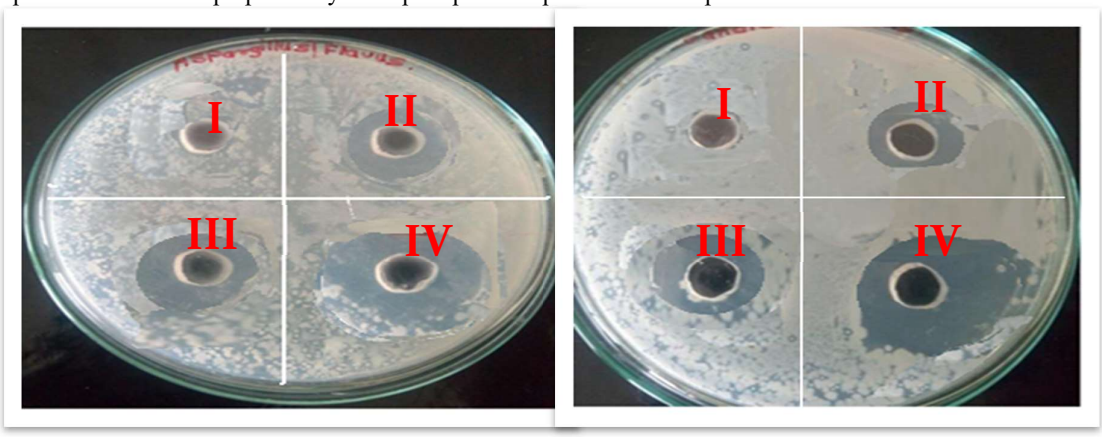
Table13. A comparative study of antifungal activity of voriconazole loaded Nanosuspensions

Sr no.	Solution	Zone of inhibition (mm)	
		Candida albicans	*Aspergillus flavus*
I	Placebo (Acetone +Methanol +Eudragit RL-100).	2.00	2.20
II	Marketed Formulation. (VOZOLE) (50µg/ml)	10.80	9.40
III	Voriconazole Ophthalmic Solution. (50µg/ml)	12.50	11.20
IV	Voriconazole loaded nanosuspension (VPNS4) (50µg/ml)	17.20	14.40

Fig.9. Antifungal study using I. Placebo (Eudragit RL-100+ Methanol +Acetone) II. Marketed eye drop (VOZOLE) III. Voriconazole aqueous solution IV. Voriconazole nanosuspension (VPNS4) against *Candida albicans & Aspergillus flavus* by agar diffusion method after 24 hour

TEM Study

TEM has been utilize for study structure of voriconazole nanosuspension. Figure 10 displays images of optimized VPNS-4 prepared by nanoprecipitation process. TEM pictures show a circular form with flat



nanosuspension interface, indicating that nanoparticles may have stabilized. Nanosuspension may not irritate the ocular surface. Nanoparticles showed a monodispersion distribution and a limited range of particle sizes.

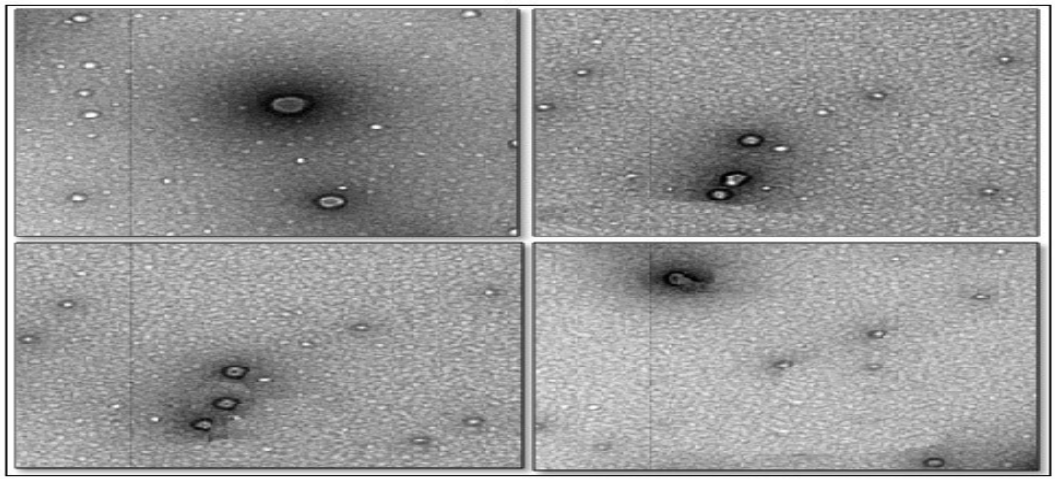


Fig. 10. Transmission electron microscopy images of VPNS4 nanosuspension.

HET- CAM Test (Hen's Egg Test - Chorioallantoic Membrane)

All the formulations tested was Non-Irritant when compared to negative control are depicted in Fig.11. and in Table 14

Table14. Comparative HET-CAM Irritation Assessment of Voriconazole Nanosuspension and Control Samples

Formulations	**Normal Saline as Control**			**SDS and NaOH as Negative Control**			**SDS and NaOH as Negative Control Placebo Nanosuspension**			**Voriconazole Loaded Nanosuspension**		
	Scores (Times in Min)											
	0.5	1	2	0.5	1	2	0.5	1	2	0.5	1	2
Egg1	0	0	0	5	5	5	0	0	0	0	0	0
Egg2	0	0	0	7	5	5	0	0	0	0	0	0
Egg3	0	0	0	7	5	5	0	0	0	0	0	0
Egg4	0	0	0	9	5	5	0	0	0	0	0	0
Egg5	0	0	0	5	5	5	0	0	0	0	0	0
Egg6	0	0	0	5	5	5	0	0	0	0	0	0
Mean	0	0	0	6.3	5	5	0	0	0	0	0	0

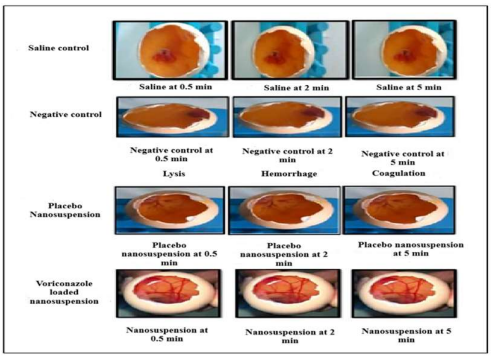


Fig. 11. Comparative antifungal study of voriconazole loaded nanosuspension (VPNS4) against *candida albicans* and *aspergillus flavus*

Conclusion

The current study shows that a promising method for ocular medication administration is voriconazole-loaded polymeric nanosuspension made with Eudragit RL100. The optimized formulation demonstrated the necessary physicochemical stability, high entrapment efficiency, and nanoscale particle size for successful ocular delivery. Studies conducted both in vitro and in vivo demonstrated improved ocular bioavailability by confirming prolonged drug release and increased transcorneal penetration in comparison to the commercial formulation. Additionally, the nanosuspension demonstrated better antifungal activity against Aspergillus flavus and Candida albicans. All things considered, this mucoadhesive nanosuspension technology is a good substitute for traditional ocular formulations since it may lower dose frequency and improve therapeutic efficacy in the treatment of ocular fungal infections.

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