Section: Research Paper

Development and characterization of lipidic nano-cochleate for topical delivery of Itraconazole

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Abstract

Clinical investigation showed that itraconazole exhibit various adverse effect such as nausea, vomiting, and other gastric disturbances. To minimize these side effects, itraconazolenano-cochleatesformulation was developed and delivered to topical site which can minimize the frequency and intensity of adverse effect. The need of these study due to the drug has low bioavailability (55%) because of low aqueous solubility and first pass effect. Nano-cochleates are prepared by trapping method by using the phosphatidyl serine as a lipid and calcium chloride as a metal cation. In the current study design expert base risk assessment was exploited for the preparation of itraconazole loaded nano-cochleae. The Box-Behnken design was utilize for risk assessment and optimization for various formulation and process parameter. Five central point were utilized in this design for the accomplishment of the goal.The optimized formula yielded 247nm particle size, 0.364 PDI, -30.8mV zeta potential, and 93% entrapment efficiency. An optimized batch in-vitro drug release study was carried out. The selected formulation was loaded into HPMC K 5M gel for in-vitro testing. The antifungal study on Trichophyton rubrum (T. rubrum) culture revealed an 18.5±0.5mm zone of inhibition with the nano-cochleate formulation. A design expert assist in understanding the interaction between process and formulation parameters. In the future, the prepared nano-cochleate gel could be a potential alternative for topical fungal infection.

Keywords: Itraconazole, nano-cochleates, topical drug delivery, design of expert, antifungal formulation

Introduction

Superficial fungal infections of the hair, skin and nails are a major cause of morbidity in the world[1]. Currently, limited number of antifungals are available for treating the fungal infections because of increasing the multi resistance and the adverse effect are the major

obstacles for fungal infection therapy[2]. Mycoses range extent from superficial infection involveouter layer of stratum cornea of the skin to infection involved in rain, heat, lungs, liver and kidney[3]. Fungal infection is a 4th most common skin disease affecting 984 million people[4]. Approximately 1.6 million people die each year because of fungal infection[5]. In men dermatophytosis is most cause of fungal infection[6]. Dermatophytosis caused by the following fungi such as Trichophyton, Microsporum and Epidermophyton, while the trichophyton rubrum is the most common agent[7]. Tinea is term used as dermatophytosis. This infection is higher in tropical and subtropical zones, in region with hot and humid climates[8&9].

Patient suffering from immunocompromised disorder (HIV and AIDS), undergo anticancer therapy, or undergoing transplantation or medical device implantation or some serious health related disorders or some weaker in health system are more prone to infected by fungi[10]. Currently antifungal are treated for polyenes, azoles and echinocandins. Azoles are most common is used for treatment of mycoses[11]. In some case antifungal resistance is one of the immersing issues.

There are wide range of azoles are available for treatment of mycoses e.g. Itraconazole, fluconazole, ketoconazole[12]. Synthetic antifungal agent of the imidazole class itraconazole, it works by slow the growth rate of fungi. Mainly its use to treat or kill the fungal infection. The drug is specifically targeted to fungal membrane and disturb their function[13]. Azoles groupinterfere with the inhibition of fungal cytochrome P-450 dependent enzyme lanosterol 14-α-demethylase. When this enzyme inhibited it blocks the conversion of lanosterol to ergosterol which disturb fungal cell membrane synthesis[12]. Clinically treatment of cutaneous mycoses with itraconazole involves oral administration. Topical drug delivery is a conventional treatment for cutaneous mycoses. For topical use gel formulation is suitable, the gel formulation is easy removal from the skin and suitable for delivery of drug[14]. The topical treatment is effective for dermatological disease[15]. In past decades nanostructured drug carrier is the alternated approach in formulation of topical administration[16].

Cochleate delivery show a new technology for the clinical important drug[17]. Cochleate is a lipid base drug delivery system. In this solid particle made up of large lipid bilayer sheet in this sheet no internal aqueous space. Cochleate are well organized in encapsulation on a hydrophobic or hydrophilic drug molecule and positively or negatively charged moiety. They are formed by the self-assembled negatively charged lipid or cations, for example phosphatidylserine and calcium. The cochleate technology applicable for topical, oral as well as parenteral route. Cochleate formulation enhance the bioavailability, safety of drug and efficacy by decreasing the side effects[18,19].

Materials and Methods

Materials

The drug Itraconazole was purchased from Dr. Hetero Lab, pvt. Solvent Chloroform was purchased from Vajra chemicals (Mumbai, India). Ethanol was purchased from Merk chemicals (Mumbai). Soya Phosphatidyl Serine lipid was purchased from Vitaegen Life science (Nagpur). Then Calcium Chloride was purchased from Merk chemicals (Mumbai). HPMC K 5M was purchased from the Research-lab fine chemicals industries, (Mumbai). Sabouraud Dextrose Agar was purchased from HiMedia (Mumbai). Dimethyl Sulfoxide was

purchased from Research-lab fine chemical industry (Mumbai). Culture of Trichophyton Rubrum was Purchased form a National Centre for Microbial Resource (NCMR) Pune.

Analytical method development for itraconazole

Solubility of itraconazole is determine by using various organic solvents such as chloroform, DMSO, methanol etc. Chloroform solvent was selected for itraconazole drug for analytical method development. Then the standard 10 ug/ml solution of itraconazole in chloroform was prepared and then scanned from a 200 to 400 nm in UV (Ultra-Violet) spectrophotometer (UV 1700, Shimadzu, Japan.) for detection of lambda max. when the lambda max was detected then the preparation of sample in concentration of 2-10 ug/ml and their absorbance was measured. Then the linearity curve was plotted in between 2-10 ug/ml to obtained a linearity equation. Phosphate buffer saline (pH 5.8) was used drug release study[20].

Method of preparation of Itraconazole Nano-cochleate

First selection of appropriate lipid for formulation of a stable nano-cochleate formulation. In this study nano-cochleate were prepared byTrapping film method. In this preparation lipid and drug was taken in a 1:5 ratios. First aqueous phase was prepared, for formation of aqueous phase phosphatidyl serine was dissolve in water and stir for 10 min, formation f liposomes take place. Then another beaker drug dissolve in a solvent we selected. Then mix the aqueous phase in organic phase. Formation of drug dissolving liposomes take place this formation stir for10 min. Then addition of a (metal ion) calcium chloride rapidly in drug loaded solution and this formation stir for 2 hrs at constant RPM. Last cochleate formation take place[21&22].

Box-Behnken Design

The box-Behnken design (BBD) is a 3-level design consisting of a cube having a central point. The design start with the independent variable,that affect the product or a process. Box-Behnken design was suitable for three independent variables[23&24].

A three-levels, four-factor Box-Behnken Design was employed to statistically optimize the formulation variables.

A. **Independent Variables:** F1 (Sonication Time), F2 (Stirring Speed), F3 (Concentration of Calcium chloride).
B. **Dependent Variables:** R1 (Particle Size), R2 (Zeta Potential), R3 (PDI), R4 (Entrapment Efficiency).

Table 1. Levels of Independent Variables

Independent Variables	Low Level (-1)	Medium Level (0)	High Level (+1)	Dependent Variables
Sonication Time F1	10	15	20	Particle Size, Zeta Potential, PDI,
Stirring SpeedF2	450	500	550	

Concentration of Calcium Chloride (µl) F3	230	250	270	Entrapment Efficiency

Experiments are often run at distinct factor values, called levels. In every run of the experiment, combination of levels of the factors are being investigated and involved. The Box-Behnken design using Response Surface Methodology (RSM) was chosen because it allows determination of influences of the factors on nano-cochleates properties with a minimum number of experiments. Design of Expert (DoE) technique used to study the effect of various variables on the experiments.

With the help of DoE obtained optimized formulation in minimum numbers of experiments runs and lower the chance of product failure. By the help of Box-Behnken design Quadratic response surface and polynomial model appropriately obtained. It is appropriate approach for understanding the effect of formulation variables (independent factors) and their related effect on the response variables (dependent factors). The main effects (F1, F2, F3) signify average result of alternating one factor at a time from its lowest to highest values. The interaction terms (F1F2, F1F3, and F2F3) promotes changes in responses. 17 formulations are obtained in this formulation there are 5 central points are detected. This formulation assessed for response variables i.e. Particle size (R1), Zeta potential (R2), PDI (R3), Entrapment efficiency (R4). Each response fitted to a quadratic equation; significance of model was assessed by ANOVA and lack of fit test with the help of Design Expert Software Ver. 13. (Stat-Ease Inc., Minneapolis, USA)[25-27].

Table 2: Box-Behnken Design

	Factor 1	**Factor 2**	**Factor 3**
Run	**A: sonication time**	**B: stirring speed**	**C: concentration of calcium chloride**
	Min	Rpm	µl
F1	10	450	250
F2	15	450	230
F3	20	500	270
F4	15	500	250
F5	15	500	250
F6	10	550	250
F7	15	500	250
F8	10	500	230
F9	20	500	230
F10	15	500	250
F11	10	500	270
F12	15	550	270
F13	20	550	250
F14	15	500	250

F15	15	450	270
F16	20	450	250
F17	15	550	230

Optimization of formula

The basic intension of BBD was to optimized the sonication time, stirring speed and concentration of calcium chloride. 17 runs are generated from design in this 17runs there are 5 central points obtained, formulation consisting of optimized results were selected. The selected formulation was further examined for particle size, zeta potential, PDI & entrapment efficiency.

Preparation of optimized batch of Nano-cochleate

First accurately weigh amount of phosphatidyl serine (5%), dissolve in a aqueous phase. Then another beaker accurately weigh amount of Itraconazole (1%), dissolve in a solvent chloroform. Then addition of a aqueous phase in a organic phase take place, stir for 10 min. Then addition of a metal ion calcium chloride rapidly 270ul and stir for 2 hrs. the stirring speed is a 550rpmthen the mixture was sonicated for 20 min. cochleate formation rapidly formed and further analysis was done.

Characterization of Nano-cochleates

Particle size, Zeta potential, PDI

The particle size, Zeta potential and PDI (Polydispersity Index) determined by using particle size analyser (Horiba nano analyser & SZ-100).0.1ml of the nano-cochleate formulation was dissolved in 100 mlof water and stir gently stirring in a glass beaker then 1 ml of the aliquot was withdrawn and placed in a polystyrene cuvette this cuvette placed in a thermostatic sample chamber maintained at 25^0c and 3 runs for 30 sec was performed. Zeta potential determined by dynamic light scattering using zeta sizer (Horiba nano analyser & SZ-100). In zeta potential high negative charged shows present in fatty acids in formulation. PDI reflects the uniformity of the particle diameter and measures the particle homogeneity[27].

Entrapment Efficiency (EE%)

The entrapment efficiency (EE%) of nano-cochleate was determined indirectly by calculating the difference between the total amount of itraconazole added to the formulation and remaining is the aqueous medium after separation the nano-cochleates. This separated dispersion was centrifuged at 10,000 rpm for 1 hr using Micro centrifuge. Then the supernatant was removed and amount of corporated drug was measured by taking the absorbance of appropriate dilutes supernatant at 267nm using UV spectrophotometer[27].

$$\textbf{Entrapment Efficiency (\%)} = \frac{W\ (\text{Initial drug}) - w\ (\text{free drug})}{W\ (\text{initial drug})} \times 100$$

Differential Scanning Calorimetry (DSC)

DSC analysis of the drug, lipid and formulation was done. Instrument was calibrated with the indium. The small amount of sample was taken in a aluminium cuvette and sealed it. The DSC instrument was started 2 hrs before the analysis for preheating purpose. The gas flow rate was 30 ml/min. The analysis was performed in between 25^0c to 600^0c[28].

Fourier Transform Infrared Spectroscopy (FTIR)

In FTIR (Fourier Transform Infrared Spectroscopy) use the interaction between of drug and lipid. The FTIR study was performed using the potassium bromide (KBr) as blank. KBr mixed with drug as a ratio of 300:1 ratio. Sample was filled into the cuvettes and sample was detected in a (Jasco FTIR-4100). The sample was scanned from 400 to 4000 cm^{-1}[29].

In-vitro Drug Release

The diffusion studies of the prepared nano-cochleate are performed by using dialysis cellophane membrane bag (surface area 2.7cm²). Nano-cochleate sample (5ml) is taken in cellophane membrane and the diffusion studies are carried out at 37 ± 1 °C using 50 ml of phosphate buffer (pH 5.8) as the dissolution medium. 0.5 ml of each sample was withdrawn periodically at 0, 30, 60, 90, 120, 150, 180, 210, and 240 min and each sample replaced with equal volume of fresh dissolution medium in order to maintain sink condition. Samples are analyzed by UV- spectrophotometer at 267 nm for drug content[30].

Anti-fungal Activity

Fungal culture of Trichophyton Interdigitale (MCC1157) was used in this study. Sabouraud dextrose agar (SDA) medium was used for maintenance of fungal culture and stored at 4^0c before being used in experiment. For fungal assessment SDA medium was employed for development of the fungus.

The fungal strain was maintained on SDA agar plate and store at 40^0c. about 20 ul of drug formulation were placed on a petri plate. Drug solution used as a positive control on SDA. All the plates are incubated with 28^0c for one week. Then the zone of inhibition of measured[31&32].

Microscopic study

Microscopic study was done under the Motic microscope. The image which gives the overview idea of the formulation. The sample of drop placed into a glass slide. Then the focused using the 4x, 10x magnification.

Preparation of itraconazolenano-cochleate gel

A gel was formed using a HPMC K 5M (1%w/v) as the gel matrix.The distilled water heated up to 80-90ºC and HPMC was added with continuous stirring and it allow to cool. Then formulation was dispersed in the gel with continuous stirring for 10 to 15 min. The pH of the formulation was adjusted to 5-6 by using the triethanolamine and then methyl paraben and propyl paraben preservatives was added in to gel formulation. Then gel formulation was homogenized until desired consistency[33].

Measurement of pH

The topical formulation pH range is 5-7measure by using the pH meter. For pH testing, 1gm of gel was dissolve in a 10 ml of water.

Viscosity

The viscosity of gel was determined by using Brookfield viscometer (DV-II+ Pro) with T-shaped spindle (S-94) at 5 rpm[35].

Spreadability

For testing spreadability,1 gm of the gel was weighed on to the center of the glass slide-1. Glass slide-2 was placed on the gel. 50 grams of weight was placed on the glass slides. The weight was removed after 2 minutes and the diameter of the gel spreaded onglass slide was measured. The test was performed in triplicates for the gel[34].

Spreadability (S) =M*L / T

Where, M= Weight placed on the glass slide, L= Length of glass slide, T= Time taken to cover distance by upper slide.

In vitro diffusion study using cellophane membrane and release kinetics:

The in vitro diffusion study was carried out through cellophane membrane using Franz diffusion cell. The diffusion medium consisted of phosphate buffer pH 5.8. The formulation equivalent to 100mg of drug was applied to 3.46 cm^2 area of cellophane membrane mounted at the lower end of doner compartment. The donor and receptor compartments were held together using a rubber band. Receptor compartment containing 13ml of diffusion medium maintained at 37± 0.5^0c and stir with magnetic stirrer. Sample was collected at predermined time intervals (0, 30, 60, 90, 120, 150, 180, 210 and 240 min) and replaced with fresh buffer. The concentration of drug determined using a UV spectrophotometer at 267 nm[36&37].

Result and Discussion

Preformulation study of itraconazole drug

The prepared formulations were studied for preformulationparametes as shown in Table no. 3

Table no. 3: Preformulation Study of Drug

Parameter	**Observation**
Physical Description	Solid
Structure	Crystalline
Colour	White
Odour	Odour of cherry
Taste	Unpleasant taste
pH	7.3 pH
Solubility	Soluble in organic solvent
Melting point	166.2^0c

Analytical method development for itraconazole

The lambda max of itraconazole was found to be 267 nm. The linear equation and regression coefficient obtained from the linearity curve were y = 0.0393x + 0.0062 and R^2 = 0.9953 respectively.

Formulation by using Experimental design

Response data for all experimental runs of Box-Behnken design are presented in Table 4. The responses were fitted into Quadratic models. The obtained models were validated using an

ANOVA. p values lower than 0.05 indicated that the regression equations were statistically significant. According to the results, polynomial models representing particle size, PDI, zeta potential, Entrapment Efficiency were generated which are shown further in terms of coded factors.

Table 4: Observed response for randomized runs in the Box-Behnken Design

Run	Sonication time (Min) F1	Stirring speed (rpm) F2	Amount of Calcium chloride (µl) F3	Particle size (nm) R1	Zeta potential (mV) R2	PDI R3	Entrapment Efficiency (%) R4
F1	10	450	250	398	-12.4	0.39	81
F2	15	450	230	265	-17.4	0.47	86
F3	20	500	270	335	-32.5	0.36	79
F4	15	500	250	281	-28.8	0.4	91
F5	15	500	250	315	-26.3	0.38	94
F6	10	550	250	300	-22.2	0.48	96
F7	15	500	250	278	-16	0.42	91.5
F8	10	500	230	227	-19.7	0.41	85
F9	20	500	230	375	-25.4	0.5	89
F10	15	500	250	320	-23	0.39	92.6
F11	10	500	270	410	-18.1	0.45	84.4
F12	15	550	270	375	-24.1	0.25	87.7
F13	20	550	250	380	-30.5	0.56	92.8
F14	15	500	250	325	-27.4	0.43	93
F15	15	450	270	221	-28.5	0.33	91.6
F16	20	450	250	400	-19	0.37	89.6
F17	15	550	250	310	-23	0.47	90.5

Determination of particle size

Quadratic equation for particle size =+303.80 – 13.87 F_1 + 0.1250 F_2 – 40.25 F_3 + 7.00 F_1F_2 – 55.75 F_1F_3 + 9.75 F_2F_3 + 52.35 F_1^2 + 25.85 F_2^2 - 19.40 F_3^2

The quadratic equation negative sign of F1 (Sonication time), positive sign for F2 (Stirring speed), and negative sign for F3 (calcium chloride). Suggested that the decrease in the sonication time and decrease in the amount of calcium and increase in the stirring speed, the particle size increases.

Determination of zeta potential

Quadratic equation for Zeta potential = - 19.66 + 0.8625 F_1 + 1.49 F_2 + 3.63 F_3 - 4.73 F_1F_2 – 2.05 F_1F_3 + 0.8500 F_2F_3 + 1.17 F_1^2 - 7.23 F_2^2 - 2.91 F_3^2

The above quadratic equation represents the quantitative effect of predictor variables (independent variables) on the measured response (zeta potential). The equation positive sign for F1, F2, F3 suggested that increase in all three independent variables, the zeta potential also increases.

Determination of PDI

Quadratic equation for PDI = + 0.3980 + 0.0075 F_1 + 0.0287 F_2 - 0.0462 F_3 +0.0250 F_1F_2 – 0.0450 F_1F_3 + 0.0623 F_2F_3 + 0.0623 F_1^2 - 0.0102 F_2^2 - 0.0303 F_3^2

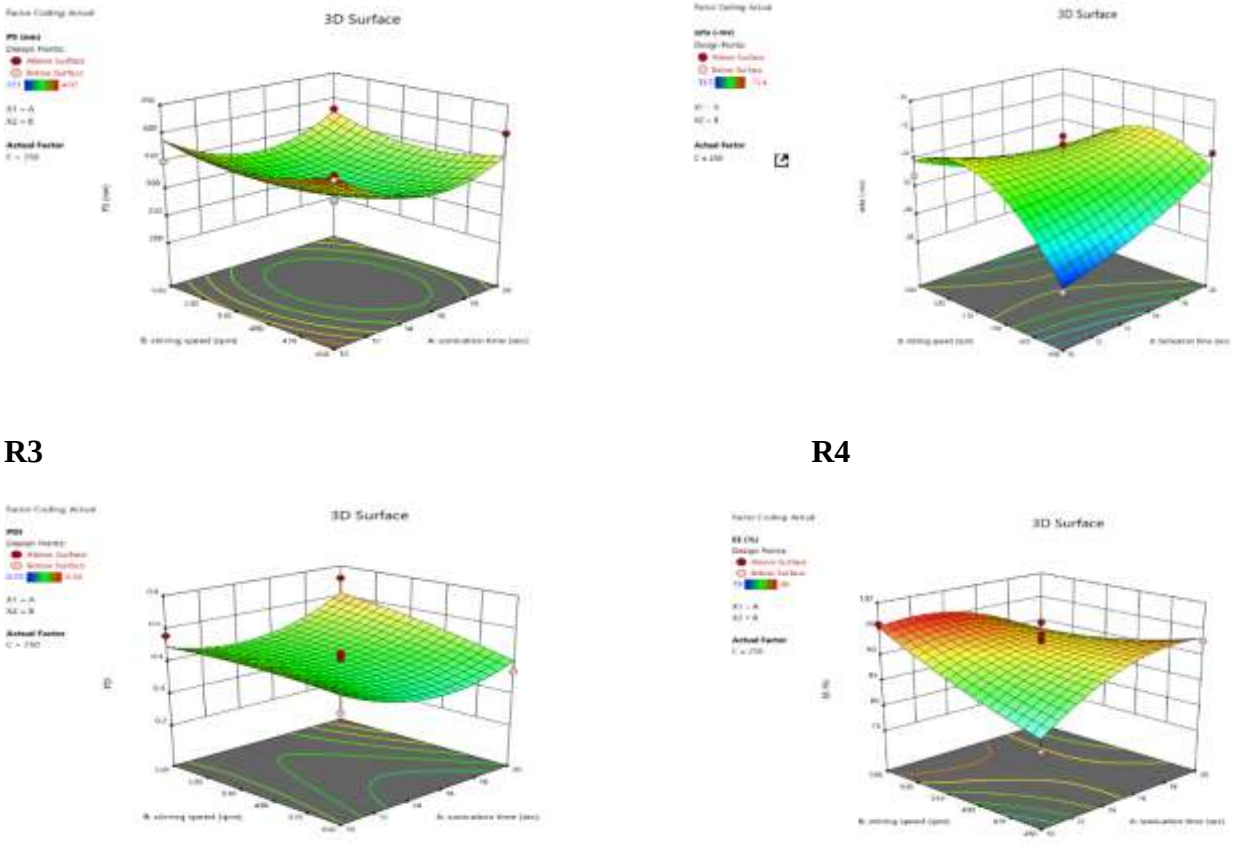
The above equation showed to the positive sign of F1 and F2 and negative sign of F3 indicates that the increase in sonication time and stirring speed and decrease in conc. calcium chloride, the PDI increases.

Determination of Entrapment Efficiency

Quadratic equation for Entrapment Efficiency = + 92.42 + 0.4875 F_1 + 1.54 F_2 - 0.9750 F_3 - 4.58 F_1F_2 – 2.35 F_1F_3 - 2.10 F_2F_3 - 3.60 F_1^2 + 1.00 F_2^2 - 4.47 F_3^2

Entrapment efficiency shows that increase in entrapment efficiency with increase in sonication time and stirring speed and decrease with conc. of calcium chloride.

R1 **R2**



R3 **R4**

Fig 1: 3D responses surface plot of all the dependent variables

Optimized batch evaluation

The F13 batch was selected as optimized formulation and evaluated for further parameters (Table 5).

Table 5: Validation of Design of Expert Software

Sr. no.	Parameter	Predicted Value	Experimental Value
1	Particle size (nm)	282.07 nm	247.9 nm
2	Zeta potential (mV)	-31.6 mV	-30.8 mV
3	PDI	0.35	0.364
4	Entrapment Efficiency (%)	89.46 %	92 %

The optimized batch was selected for further characterization. It shows maximum entrapment efficiency. The mean particle size was found to be 247.9 nm i.e. the range is specified for nano-cochleates. PDI of the formulation was found to be 0.364 thus the formulation was stable. Zeta potential is used for predicting dispersion stability. ±30 zeta potential is sufficient for physical stability. The formulation showed the -30.8mV zeta potential and entrapment efficiency find to be 92%.

Differential Scanning Calorimetry (DSC)

DSC is an effective thermal analysis technique which gives information on the melting point, solid phase transition and chemical degradation. It is useful in drug-excipient interaction in formulation. The DSC showed there is slightly difference between the lipid and PM sample.

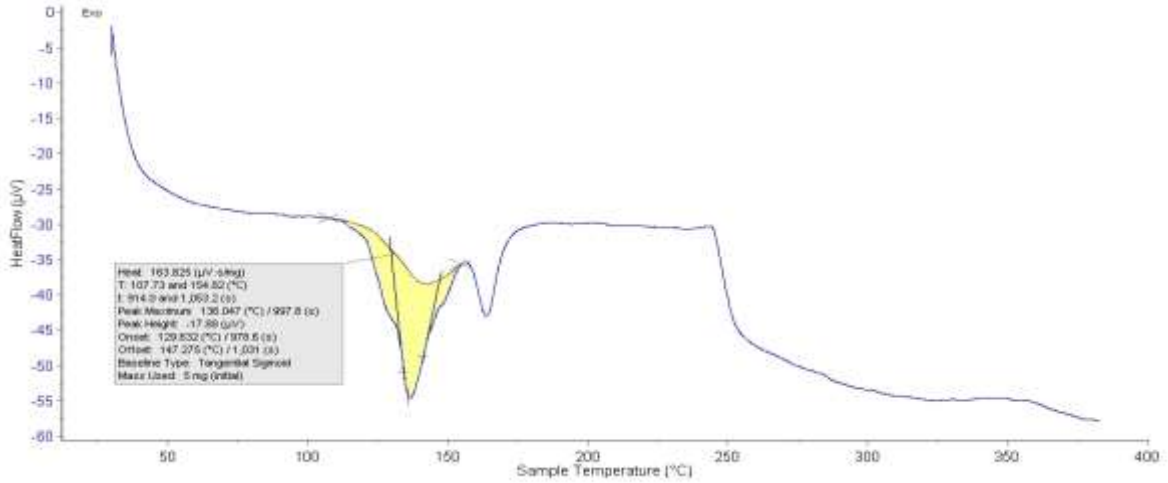


Fig 2: DSC result of the formulation of Nano-cochleates

Fourier Transfer Infrared Spectroscopy (FTIR)

The spectra of itraconazole shows a C-N stretching at a 1230cm^{-1}, N=N=N stretching at 1509cm^{-1}, and last C=O stretching at 1698cm^{-1}. The spectra of phosphatidyl serine shows many peaks such as followed C=C bending at 678cm^{-1}, C-H bending at 829cm^{-1}, C-O stretching at 1078cm^{-1}, CH deformation at 1464cm^{-1}, C=C stretching at 1646cm^{-1}, C=O stretching at 1737cm^{-1}, N-H stretching 2922cm^{-1}, and last O-H stretching at 3648-3750cm^{-1}. The spectra of physical mixture showed no major shifts in the functional peaks between the spectrum of drug and lipid used.

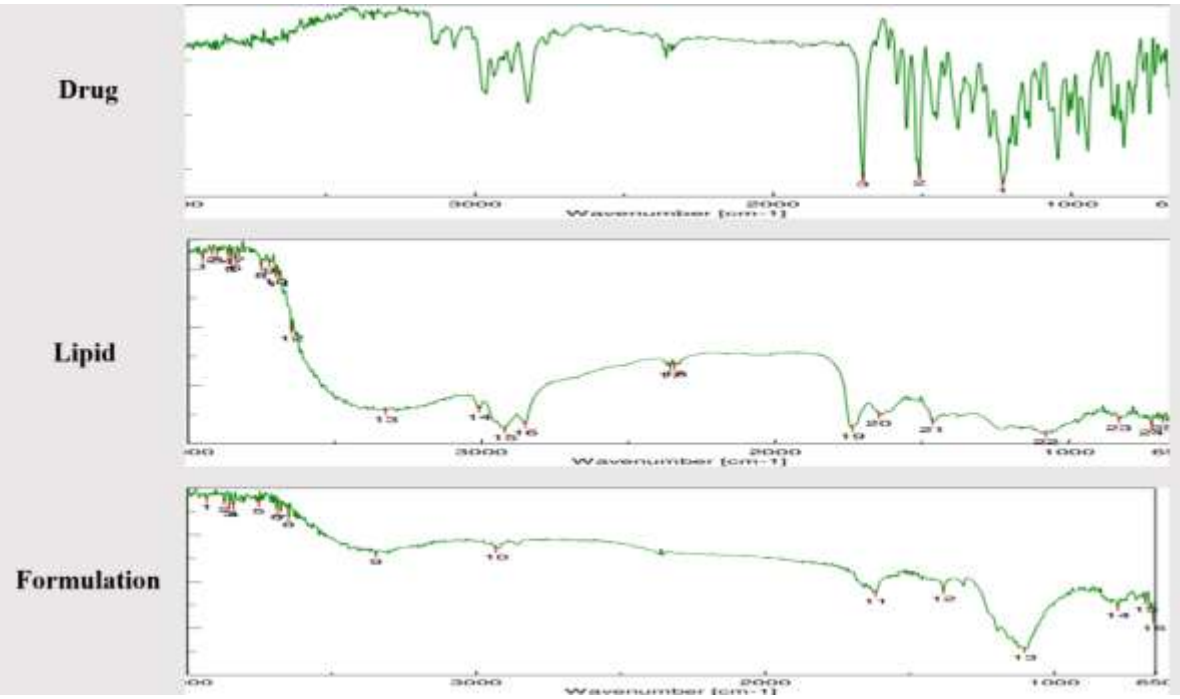


Fig 3: FTIR spectra of drug, lipid and he formulation

Antifungal Activity

The study was performed in duplicate the zone of inhibition was calculated with help of a ruler. The zone of inhibition of cochleate formulation exhibit 18.5 ±0.5mm.

Fig 4: Zone of inhibition (ZoI) of nano-cochleate formulation

Microscopic Study

Microscopic study was done under the Motic microscope. The image which gives the overview idea of the formulation**.**

Fig 5: Microscopic Study of nano-cochleate formulation

Preparation of itraconazolenano-Cochleate Gel

Prepared of nano-cochleate gel formulation was formulated using HPMC K 5 M 1.0% (w/v) as the gel matrix. Then distilled water heated up to 80-90^{0}c. with continuous stirring and it allow to cool. Then formulation was dispersed in the gel with continuous stirring for 10 to 15 min. The pH o the formulation was adjusted to 5-6 using the triethanolamine and gel formulation was homogenized until desired consistency.

Table 6: Nano-cochleate gel formulation

Ingredients	**Concentration**
Phosphatidyl Serine	4.64%
Itraconazole	0.928%
Chloroform	10ml
Calcium Chloride	270ul
HPMC K 5M	1%
Triethanolamine	q.s. to pH 5.8
Methyl paraben	0.2%
Propyl paraben	0..1%
Water	Q.S. to 25 ml

Characterization of ItraconazoleCochleate Gel

pH, Viscosity and Spreadability

The apparent pH of itraconazolecochleate gel was found to be 6.5. The pH is slightly acidic and is physiologically compatible with the skin. The viscosity of gel was found to be 49,153 cP at 5 rpm by using T-shaped spindle. Spreadability of gel was determined by pressing 1 gm of gel between two glass plates. Initial diameter of gel was noted. A constant weigh of 50 gm was kept on the plate assembly for 2 min and the increase in diameter is noted S= 4.57 cm.

In-vitro Drug Release

The nano-cochleate (NC) is excellence tool for enhancing the solubility of hydrophobic drugs, the bioavailability of drug enhanced with small globule size. Achieve the better drug release and therapeutic effect drug was distributed into the nano-cochleate globules. At the end of 4hr, the drug was released from topical itraconazolenano-cochleate was (71.42%).Maximum release of drug was found within 4hr was 68.89 % in itraconazolenano-cochleate formulation and marketed itraconazole gel drug release was 70.5%.As shown in Fig 6.

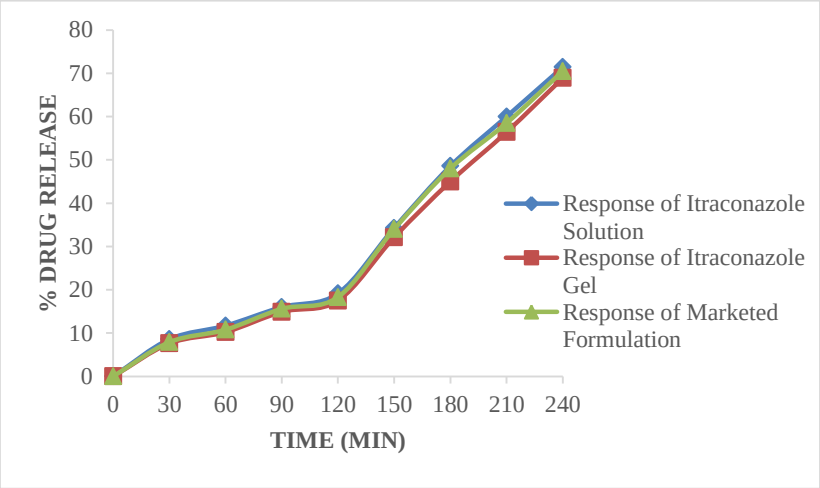


Fig 6: % Drug Release study of itraconazole

Conclusion

The present study is evident that the tested itraconazole drug is utilized in the treatment of mycoses infection. This study successfully demonstrated the use of the BBD for the optimization of different responses of formulation.The preparation of cochleate using trapping method were studied for particle size, effect of rpm, effect of sonication time, addition of concentration of calcium chloride and entrapment efficiency.The optimum concentration of calcium chloride was found to be 270 ul and sonication time 15 min for better formation of cochleate.Optimize this system and found the phosphatidyl serine lipid combination effect high entrapment efficiency and improve therapeutic effect.Itraconazole drug is used for the formation of antifungal cochleates they show good antifungal activity.The optimization of antifungal cochleates using Box-Behnken experimental design was performed. The optimized formula of cochleate was phosphatidyl serine 1.25 gm, itraconazole 250mg, chloroform 10ml, sonication time is 19 min, stirring speed is 547 rpm and conc of calcium chloride was 270ul.The formulation has a high entrapment efficiency 91.6% and low particle size 247nm with PI 0.364, stable zeta potential -30.8 mV.

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