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Formulation and Characterization of Long Circulating Liposomes of Anti Fungal Drug

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Abstract: Posaconazole *is an effective antifungal drug, used as first line treatment for Invasive Aspergillosis. The present work focusses on formulation of PEGylated liposomes to achieve longer circulation in the blood, prevent the liposomes from opsonisation by mononuclear phagocytic system of RES. The higher plasma concentration of Posaconazole leads to visual disturbance and dermatological side effect, which are minimized by PEGylation. Compatibility studies like FTIR (Fourier Transform IR) and DSC (Differential Scanning Calorimetry) showed that lipids or drug do not interact and are highly compatible. Liposomes were prepared by thin lipid film hydration method, applying 32 full factorial design with various molar ratio of phospholipids like DPPC /Cholesterol /DSPE mPEG2000. Vesicular size and zeta potential was found within desired range of 100-300 nm to effectively pass through intra venous circulation. Long circulating liposomes were found to have entrapment efficiency of 75-85%. TEM (Transmission Electron Microscopy) showed distinctive spherical shaped vesicles. Long circulatory effect was confirmed from biodistribution study. Stability studies of long circulating liposomes were carried out and Pegylated liposomes successfully showed long circulatory action inside the body and minimized side effect of drug with appreciable antifungal activity determined by microbiological assay (zone of inhibition).*

Keywords: Posaconazole, Invasive aspergillosis, Long Circulating Liposomes, Thin film hydration method

I. INTRODUCTION

Overview of illness

The fungus Aspergillus species is the cause of the disease aspergillosis. Allergies, lung infections, and infections in other organs such as the kidney, brain, etc. are among the range of illnesses. Aspergillosis causes include: extended use of antibiotic medications during treatment.

People with compromised immune systems, such as HIV/AIDS patients Individuals receiving treatment with chemotherapy or steroids (weakened immune system as a result of these drugs'toxicity) the incurable aspergillosis. The illness kills patients whose immune systems are compromised. The earlier the diagnosis is made, the better off they are, but sadly, there isn't a reliable single diagnostic test. Treatment must frequently begin as soon as a condition is suspected. Typically, this illness is identified clinically in a inserted via the nose) is frequently used to aid in the confirmation of the diagnosis. Blood tests, particularly those that look for antigens, and cultures are typically required to confirm the disease. In individuals with weakened immune systems, the fungus can spread from the lung through the bloodstream to the brain or other organs, such as the skin, heart, kidneys, or eyes. Generally speaking, this is a sign that the illness is more severe and that the patient is at a higher risk of dying.

Amphotericin B (needed in higher doses, also associated with significant kidney and organ damage), Posaconazole (prophylactic), caspofungin and micafungin (partially effective), itraconazole (reports of resistance observed), and Posaconazole (first line treatment; oral/IV administration).

Introduction to Long Circulating Liposomes Long-circulating liposomes are longer in the circulation than conventional liposomes, which have the drawback of being eliminated from circulation. A stealth liposome [23] is a spherical vesicle

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with a membrane made of phospholipids bilayer that is used to deliver drugs or genetic material into cells. Coating liposomes with PEG decreases the rate of uptake by macrophages (stealth effects), evades immune system detection, and results in a prolonged presence of liposomes in circulation, giving them plenty of time to escape via leaky endothelium.

II. MATERIALS AND METHODS

Materials

Adventus Laboratories (India) Pvt. Ltd. (Baroda) provided the Posaconazole. Lipids: DSPE-MPEG-2000, (DPPC) 1.2-Dipalmitoyl-sn-glycero-3-phosphocholine The phosphoethanolamine methyl-1, 2Distearoyl-sn-glycero-3 polyethyleneglycolconjugate-2000 Na^+ salt was obtained from Lipoid GmbH, Ludwigshafen (Germany) and Cholesterol from Loba chemie Pvt. Ltd. (Mumbai) All the other chemicals were of analytical grade.

Liposome formulation

Film Hydration Method was used to create vesicles at lower pressure. In a solvent mixture including methanol and chloroform (9:1), Posaconazole was dissolved in various ratios of lipid and cholesterol. The solution's round-bottom flask was connected to the Equitron Roto Evaporator (EQUITRON ROTA EVAPORATOR) in an inert nitrogen environment at a reduced pressure (500–600 mm Hg), temperature, and RPM of 50°C and 30 rpm, respectively. Rotate the flask until the solvent evaporates and a thin, transparent coating forms on the flask walls. It was kept under regulated pressure and vacuum for the entire night. Next, 15 millilitres of phosphate buffer saline pH 7.4 were added to hydrate the film. To prevent deterioration, the produced vesicles were sonicated in an ice bath for a predetermined amount of time (per the design arrangement). SUVs were sterilized in an aseptic setting using membrane filtration. Vials were used to store the gels. Water was used to reconstituted the lyophilized SUVs for injection

Screening of processing parameters

Screening of process parameter was done on basis of formulation process by trial and error method which affects on particle size and PDI of liposomes. Formulation parameters are following Speed of rotary evaporator (30, 50, 60, 90 rpm) Temperature of rotary evaporator (500C, 600C) Sonication cycles [pulses on/off (15/5), (30/30)

III. OPTIMIZATION OF THE FORMULATION

Experimental Design

A 32-fold factorial design was used in the study project.The molar ratio of DPPC: Cholesterol and DPPC: Cholesterol: DSPE-mPEG 2000 were the elements taken into consideration in the experiment. Sonation time was an additional component, and the responses that were recorded were proportion of entrapment efficiency and vesicle size. The codes for the three levels of the Long Circulating Liposomes 2 factor were -1, 0 and +1, in that order. Thin film hydration was also utilized to generate conventional liposomes, which were then used to compare liposome release studies conducted in vitro.

For Conventional Liposomes: three batches with molar ratio of DPPC: Cholesterol was 6:1, 7:2, 8:3 and the corresponding sonication time was 5, 10 and 15 minutes respectively.

For Long Circulating Liposomes:

Levels in Coded Value: -1, 0, +1.

Factor A (Molar ratio DPPC: Cholesterol: DSPE- mPEG 2000): (6:1:0.2), (7:2:0.8), (8:3:1.4)

Factor B (sonication time): 5, 10, 15 minutes respectively.

The above factors with their levels and response of each of the experiments were fed in the design expert software 7.0 and the further analysis of the data was done using the software

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IV. METHOD OF ESTIMATION OF POSACONAZOLE

UV-Visible Spectroscopy method

Posaconazole was estimated by UV-Visible spectrophotometry using phosphate buffer saline (PBS) pH 7.4. To determine absorption maxima 10µg/ml solution was prepared and scanned between 200-4000 nm using spectrophotometer (Shimadzu 1800, UV-Visible double beam Spectrophotometer). For preparation of calibration curve, the aliquots of 0.5, 1, 1.5, 2, 2.5, 3, 3.5 ml of the stock (0.1 mg/ml) were serially diluted with 10 ml of PBS to get 5, 10, 15, 20, 25, 30 µg/ml concentration. Absorbance of each solution was measured at 255 nm against PBS as a blank. The data were subjected to linear regression analysis.

HPLC method for estimation of Posaconazole

A rapid and sensitive RP–HPLC method with UV detection (256 nm) for routine analysis of Posaconazole was developed. Chromatography was performed with mobile phase acetonitrile: water (50:50 v/v) with flow rate of 1.0 ml min^{-1}. The calibration curve was plotted with five concentrations 5–25 µg/ml solutions of Posaconazole, prepared in acetonitrile: water (50:50 v/v) mixture **Drug-Excipient Compatibility study FTIR study for drug-Lipid interaction**
The samples of pure drug Posaconazole, DPPC, DSPE-mPEG2000 and Cholesterol individually and drug-lipid combination (mixed with IR grade KBr) were prepared. All the samples were placed between IR transmitting windows in FTIR Spectrophotometer (Bruker, ALPHA- T) and scanned over a range of 4000 to 400 cm^{-1}.

DSC study for drug-Lipid interaction

The DSC study was performed of pure drug, DSPE- mPEG2000 and mixture (Drug: Cholesterol: DPPC: DSPE-mPEG2000 in ratio of 1:1:1:1). Samples were taken in standard aluminium pan and heated for 30 minutes at a rate of 10°C/min till 300°C under constant nitrogen stream (20 ml/min).

EVALUATION OF FORMULATION

Transmission Electron Microscopy

Negative electron microscopy (Tecnai 20, Philips, Holland) technique was used to study liposomal shape and lamellarity. Size and size distribution of vesicles was determined by photon correlation spectroscopy (PCS) using Zetasizer Nanoseries (Malvern Instruments, Malvern and Worcestershire, UK). Each sample was taken in the disposable cuvette and analysis was performed at 250C with an angle of detection of 900.

Zeta-Potential

Zeta potential (ZP) of vesicular dispersion was determined using Zetasizer Nanoseries (Malvern Instruments, Malvern, Worcestershire, UK). Charge on vesicles and their mean ZP values with standard deviation (±S.D.) were obtained directly from the instrument. Particles having high zeta potential value show higher stability, as the particles of the same formulation having same charge on it will repel each other, and will avoid aggregation.

Entrapment Efficiency

The extruded liposomal samples were centrifuged at 3,000 rpm in refrigerated centrifuge at 4°C to pelletize the unencapsulated drug. The supernatant was centrifuged at 10,000 rpm to pelletize the drug loaded liposomes. Absolute alcohol (1ml) was used to disrupt the liposomes. The pellets were resuspended and diluted with PBS pH 7.4. The concentration of the encapsulated drug was measured as absorbance at 255 nm using UV-visible spectrophotometer (Shimadzu 1800, UV-Visible double beam Spectrophotometer). The absorbance was converted into drug concentration using a standard curve. The encapsulation efficiency was calculated as

$$\%\ Encapsulation\ Efficiency = \frac{Encapsulated\ drug}{Total\ drug} * 100$$

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Diffusion Medium: pH 7.4 phosphate buffer saline Membrane for permeation: Dialysis membrane 50 Temperature: 37°C Sampling Interval: 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 16, 20, 22 and 24 hrs Volume of Diffusion Medium: 150 ml
In- Vitro drug release study was performed in Dialysis membrane 50 with solution in it and tied at both the end with a thread. The dialysis bag was put into a beaker containing 150 ml of phosphate buffer saline pH 7.4. The whole system was kept at 37°C and stirred continuously by magnetic stirrer. At a sampling interval time 5 ml of the aliquot was withdrawn and the absorbance of the aliquot was measured in UV Spectrophotometer. 5 ml of the Phosphate buffer saline 7.4 was replaced to the system to maintain sink condition.

Antifungal activity

The antimicrobial studies of the optimized formulations and of the marketed formulation against *Aspergillus niger* were performed. For the development of inoculum, Lyophilized strip of strain of *A. niger* (ATCC 16888) was subcultured in nutrient broth and incubated at 25 ^{0}C for 48 hrs. Potato dextrose agar media seeded with the strain of *A. niger* (ATCC 16888) was allowed to solidify in the sterilized petri plate Posaconazole drug solution was placed into the bore created in potato dextrose agar at suitable distance and plates were incubated at 25^{0}C for 48 hrs.The diameters of the zones of inhibition were measured

Biodistribution study

Bioanalytical Assay of Posaconazole in rat plasma

Bio analytical assay of Posaconazole in rat plasma was carried out by RP-HPLC method. The calibration curve of samples were prepared by spiking 100µL of Wistar rat plasma and mixing with various concentration of the appropriate standard working solution prepared to obtain VRC final concentrations of 50, 100, 250, 500, 1000 and 2500 ng/ml.
Standard stock solution of Ketoconazole (Internal Standard) was prepared at 500µg/mL similarly. This solution was successively diluted to result in a final concentration of 4µg/mL of Ketoconazole. Prior to the chromatographic analysis, all samples of Posaconazole and IS were deproteinized by addition of ice-cold methanol and vortexed for 30 sec and centrifuged at 12,000 rpm, 4 ◦C for 15 min. Supernant solution was collected and diluted with proper mobile phase and chromatographic analysis was carried out
In vivo animal study
Animal experiments were carried out according to IAEC (Institutional Animal Ethical Committee) containing Protocol No: PIPH 7/14. The rats were housed under standard conditions and had ad libitum access to water and standard laboratory diet. Posaconazole solution of marketed preparation and optimized long circulating liposomal formulation of Posaconazole was administered intravenously by tail vein in Wistar rats. Blood sample was collected from retro orbital plexus of lightly anaesthetised rats at 1hr, 6hr, and 24 hr. Animals were euthanized and tissues (lungs and kidney) were isolated and homogenized by
homogenizer (Silent Crusher MHeidolph). The supernatant (plasma) was taken and mixed with methanol and centrifuged in refrigerated centrifuge to isolate plasma. The resulting supernatant was subjected to HPLC (LC-10ADVP - Shimadzu) for analysis

Stability studies

Stability study of long circulating liposomes was done at refrigerated storage condition 5 ± 2^{0}C and 25 ± 2^{0}C at 65% RH ± 5% RH under accelerated stability study in Humidity controlled oven (Remi Instruments Ltd). The particle size, size distribution and drug encapsulation efficiency were measured periodically

V. RESULT AND DISCUSSION

Method of estimation of Posaconazole (UV and HPLC method)

UV Spectroscopy of drug Posaconazole in Phosphate Buffer Saline in pH 7.4 λ_{max} of Posaconazole was found at 255 nm. This (λ values) was selected for rest of the analysis. From the calibration curve, regression analysis gave equation as y =0.025x- 0.035 (R^2=0.998). A calibration curve obtained from HPLC method followed by regression analysis gave y= 15.99x + 27.07 (R^2 = 0.995)

Drug-Excipient Compatibility study:

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FTIR study for drug-Lipid interaction: [80] [Figure 2] The spectra of individual components as well as the combination of drug lipid were obtained. From the spectra the following peaks were observed:

Alcohol (-OH) **3415.15 cm^{-1}**
Alkane (-CH_3) **2985.86 cm^{-1}**
Imines (-C=N-) **1586.07 cm^{-1}**
Fluoride (Ar-F) **1013.59 cm^{-1}**
Ester (C=O) **1735.64 cm^{-1}**
Phosphate ester (P=O) **1163.08 cm^{-1}**

From the spectra, peak of drug is distinctly obtained in the physical mixture. There is no interaction between the drug and lipids indicating drugs and lipids are compatible

Conventional Liposomes with Vesicle size, Zeta potential, PDI and % Entrapment Efficiency.

Molar ratio of DPPC: Cholesterol (6:1, 7:2, 8:3)

Sonication time (min) (5, 10, 15). Molar ratio and sonication time affects vesicle size and %E.E. As with very low sonication time, vesicle size was increased and % entrapment efficiency is less. And with high sonication time, vesicle size was decreased and % Entrapment efficiency is more.

Long Circulating Liposomes with Vesicle size, Zeta potential, PDI and % Entrapment Efficiency.

Factor A: Molar ratio of DPPC: DSPE-mPEG 2000: Cholesterol (6:1:0.2, 7:2:0.8, 8:3:1)

Factor B: Sonication time (min) (5, 10, and 15)

Table: Result obtained for Long Circulating Liposomes

Optimization of long circulating liposomes: Optimization of long circulating liposomes was done by 3^2 full factorial designs. For long circulating liposomes two factors were molar ratio of DPPC: CHOL: DSPE-mPEG-2000 and Sonication time. The responses measured were Vesicle size and % E.E. after reconstitution of lyophilized product with water for injection. ANOVA was applied and the following results were obtained. [Table 1]

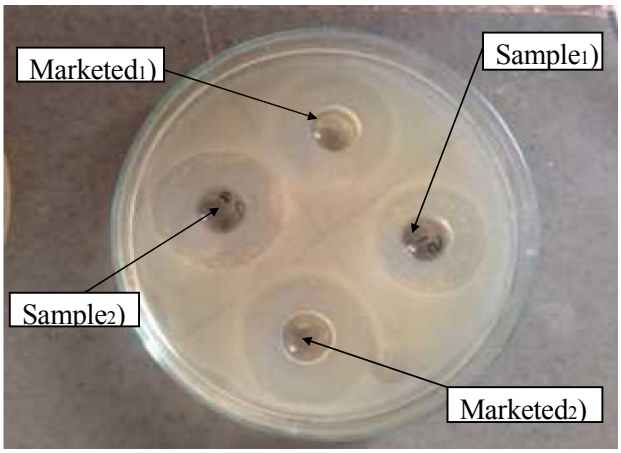

Figure 5: Zone of Inhibition of Posaconazole

% Entrapment Efficiency:

ANOVA for % E.E

% E.E. = + 70.12 + 9.6 * A +2.93 * B -0.90 * A * B
+ 1.85 * A^2 + 0.42 * B^2 **(Full model ANOVA)**

% E.E. = 71.394 + 8.466 * A + 1.608 * B **(Reduced model ANOVA)**

PREPARATION OF THE CHECK POINT BATCH

Using the design expert, the design space was prepared which gave size range from 120 - 140 nm and % E.E. from 75 - 85% and from the resulting design space, randomly one flag point was selected and the batch was prepared and evaluated for size and % E.E. The resulting parameters were compared with the standards provided by the software.

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When we had narrowed down the size selection region from 125-140 nm and % E. E range selected was 75-85% the design expert has shown the following design space that will give the desired range of result.

Results of check point batch: By selecting any point from the design space a batch was prepared. The vesicle size and % EE was found to be 126.7±0.8 nm (predicted: 129.902 nm) and 80.55±0.88%
(82.96 %) respectively.

Transmission Electron Microscopy

The average vesicular size and zeta potential of the optimized batch obtained from Zeta sizer instrument was 126.7±0.8 nm and -17.3 respectively.

Invitro Release Study

Release study of the liposomal formulation and marketed formulation was performed using dialysis membrane, the following table shows the % CDR with time. In case of free Posaconazole more than 92.95 % drug was released within 8 hr. and Conventional liposomal formulations produced slower Posaconazole release was more than 87.365 % within 12 hr. whereas long circulating liposomal formulations produced slower Posaconazole release was more than 89.344 % within 24 hr. [Table 2]

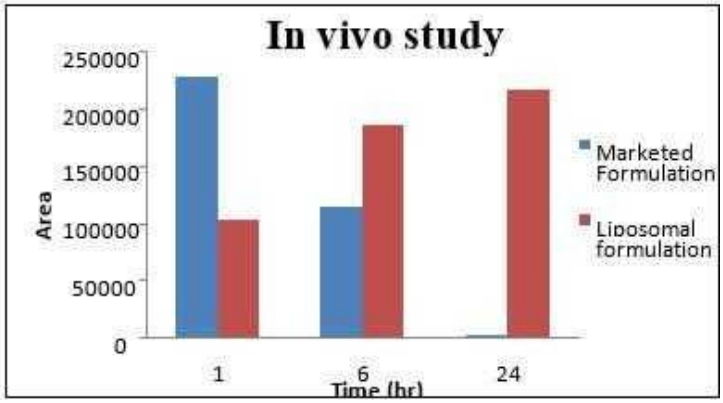
Antifungal activity

Marketed Formulation showed average diameter of 18.3 mm and liposomal formulation showed average diameter of 18.4 mm inhibition which is similar to the zone of inhibition of marketed formulation of liposomal formulation.

Biodistribution study

After 1hr, 6hr and 24hr, drug was extracted out from blood plasma and analysed using HPLC. In case of long circulating liposomal injection plasma peaks obtained at 1hr, 6hr and 24 hr with increase in peak area. Hence, it is concluded that the pegylated liposomes exhibits long circulation in the body. This was also confirmed by peak area of drug in HPLC chromatogram.

Time (hr)	Peak Area (mV.s)	
	Marketed Formulation	Pegylated Liposomes
1	227028	102935
6	114131	185672
24	2339	215689



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Stability study

Duration	Storage Condition	Vesicle Size (nm)	% Entrapment Efficiency
0th day	5 ± 2° C; 60% ± 5% RH	127.23±1.5	80.55±0.28
15th day	5 ± 2° C; 60% ± 5% RH	132.5±1.8	78.85± 0.42
30th day	5 ± 2° C; 60% ± 5% RH	148.8±2.3	72.65± 0.39

Stability study was carried for a month for long circulating Liposomes. Formulation was stable at temperature of 5 ± 2° C and Relative Humidity of 60% ± 5%. There was no significant change in the Entrapment Efficiency of the drug or the size of the liposomes. However, when formulation of liposomes were subjected to 25° ± 2° C and 60% ± 5%. There was loss of liposomal structure and entrapment efficiency.

VI. CONCLUSION

The objective of present work was to prepare and evaluate long circulating liposomes of antifungal drug. Long Circulating Liposomes containing Posaconazole were prepared by Thin Film Hydration technique. In present study, 32 full factorial design was applied in which molar ratio and sonication time were taken as independent variables and Vesicle size and % Entrapment Efficiency as dependent variables. The optimized formulation showed the particle size around 126.7±0.8 nm with entrapment efficiency 80.55±0.88 %. In-vitro release study shows that liposomes seem to be a sustained dosage form of Posaconazole vital for treatment of Invasive Aspergillosis. Zone of Inhibition optimized formulation showed that Posaconazole exhibited anti-fungal activity which was found almost similar to marketed formulation. Long circulatory effect was successfully found out from bio distribution study. Optimized formulation exhibited good stability during 1 month storage at 5° C ± 2° C at 65% ± 5% RH. Thus, it was concluded that Posaconazole loaded long circulating liposomes were suitable and desired dosage form for the treatment of Invasive Aspergillosis

REFERENCES

[1]. Vidyasagar M, Srinivas B, Parameshwar P, Ganesh A, Kumar RR. Darivatization and Pharmacological Evaluation of New 4 Substituted 5-Phenyl-3-Mercapto-1, 2, 4–Triazoles. Asian Journal of Research in Chemistry. 2013;6 (8):731-4.

[2]. Indian Pharmacopoeia, Volume-II. Government of India. Ministry of health and Family welfare New Delhi, The controller of publication 2007; p.1278-1279.

[3]. Beckett AH, Stenlake JB. Practical Pharmaceutical Chemistry. 4th ed. part II, New Delhi: CBS Publisher and Distributor; 1997. P. P. 158-164.277.

[4]. U.S.P. Asian Edition, Rockville: United Pharmacopoeial Convention Inc; 2005. p. 2386-2389.

[5]. ICH, Q2R1 Validation of Analytical Procedures: Text and Methodology; International Conference on Harmonization, Geneva; 1996.

[6]. ICH. Q1A (R2), Stability testing of new drug substances and products, International conference on Harmonization. Geneva, 2003.

[7]. Parameshwar Pabba NR, Ramesh E, Arjun N. HYPOGLYCEMIC AND ANTI-LIPIDEMIC EFFECTS OF HYDRO-ETHANOLIC EXTRACT OF HELCTERES ISORA LINN. Inventi Impact: Ethnopharmacology. 2010 Oct 21