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FORMULATION & CHARACTERIZATION OF KETOCONAZOLE PRONIOSOMES FOR THE TREATMENT OF CANDIDIASIS

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ABSTRACT:

To overcome drawbacks associated with conventional drug delivery of ketoconazole an attempt is being made to design an alternative drug delivery system in form of Proniosomes. They minimize problems of niosome physical stability such as aggregation, fusion, leaking and provide additional convenience in transporation, distribution, storage and dosing both phospholipids and non ionic surfactants in proniosomes can act as penetration enhancers and help in diffusion of the drug .Proniosomes also avoid problems associated with liposomes like degradation by hydrolysis or oxidation as well as sedimentation, aggregation or fusion during storage.

Therefore, it is envisaged to prepare proniosomes of ketoconazole for the attainment of better therapeutics in candidiasis. The drug is 99% protein bound moreover, it is hepatotoxic hence a cutaneous/topical delivery is more beneficial for the attainment of better therapeutic results. To overcome the side effects associated with conventional delivery and formulation is envisaged for the drug. Prepared proniosome shall penetrate deeper in the skin and led to better and faster therapeutics effect as compared to conventional formulation.

In the present study, the slurry method was used for the preparation and optimization of ketoconazole proniosomes formulation.

KEYWORDS: Ketoconazole, Proniosomes

1.0 INTRODUCTION:

Candidiasis is a fungal infection caused by fungi that belongs to genus candidia. Candidia yeast normally lives on the skin and mucous membranes without causing infection; overgrowth of this disease can cause symptoms to develop. Candidiasis vary depending on the area of the body that is infected. Candidiasis that develops in the mouth or throat is called “thrush” or oropharyngeal candidiasis. Candidiasis is an infection caused by Candida fungi, especially Candida albicans.

Proniosomes :

Proniosomes are dry formulations of surfactant-coated carrier, which can be measured out as needed and rehydrated by brief agitation in hot water. These “proniosomes” minimize problems of niosomes physical stability such as aggregation, fusion and leaking and provided additional convenience in transportation, distribution, storage and dosing.[5]Proniosome derived niosomes are superior to conventional niosomes in convenience of storage, transport and dosing. Size distributions of proniosome derived niosomes are somewhat better that those of conventional niosomes so the release performance in more critical cases turns out to be superior

Proniosomal gel:

Proniosomes are vesicular systems, in which the vesicles are made up of non-ionic based surfactants, cholesterol and other additives. Semisolid liquid crystal gel (proniosomes) prepared by dissolving the surfactant in a minimal amount of an acceptable solvent, namely ethanol and then hydration with least amount of water to form a gel. These structures are liquid crystalline compact niosomes hybrids that can be converted into niosomes immediately upon hydration or used as such in the topical/transdermal applications. Proniosomal gels are generally present in transparent, translucent or white semisolid gel texture, which makes them physically stable during storage and transport. Advantages of Proniosomal gel: Liposomes and niosomes are well known drug/cosmetic delivery systems. But these delivery systems have been reported to have many disadvantages in terms of preparation, storage, sterilization, etc.

Methodology of proniosome preparation :

Preparation of proniosomes- The proniosomes can be prepared by :

1. **Spraying method.**
2. **Slurry method.**
3. **Coacervation phase separation method.**

Application of Proniosomes As A Drug Carrier :

Proniosomes can be used for many purpose in drug delivery. Proniosomes can be used for the topical drug delivery through skin.

Application of Proniosomes[8] :

The application of proniosomal technology is widely varied and can be used to treat a number of diseases. The following are the few uses of proniosomes which are either proven or under research.

1. Drug Targeting
2. Anti-neoplastic Treatment
3. Delivery of Peptide Drugs

4. Uses in Studying Immune Response : Proniosomes as Carriers for Haemoglobin
5. Transdermal Drug Delivery Systems utilizing proniosomes : Localized Drug Action
6. Proniosomes used in Cardiac Disorders
7. Hormonal Therapy
8. NSAID Application
9. Cosmetics/Cosmeceuticals

2.0 PREFORMULATION STUDIES:

Preformulation studies on the obtained sample of drug were performed for identification and compatibility studies.

I. Procurement of drug and chemicals

Ketoconazole was provided as gift sample by Modern Lab, Indore, M.P, India; lecithin was purchased from Himedia Lab., Mumbai and Cholesterol was purchased from Ranbaxy Laboratory, New Delhi. Other solvents were purchased from analytical grade in local firms.

II. Material :

List of Material & Apparatus which are used in preformulation studies is shown in Table 5.1 and Table 5.2.

Table 1. List of material used :

Sr. No	Material	Manufacturer/Supplirers
1	Ketoconazole	Morden Labs, Indore
2	Chloroform	RFCL Limited., Harayana
3	Triethylamine	Samir tech. Chem Pvt Ltd., Vadodara
4	Methanol	HiMedia Laboratories Pvt Ltd., Mumbai
5	Ethanol	Jiangsu Huaxi International Trade Co. Ltd Made in China.,
6	Sorbitol	Nutan Gujarat Industrial Estate., Gujarat
7	D-Mannitol	Qualikems Fine Chem Pvt Ltd.,Vadodara
8	Carbopol 934	High Purity Laboratory Chemicals Pvt Ltd., Mumbai.
9	Conc. Hydrochloric Acid HCl	RFCL Limited.,New Delhi
10	Lecithin	Himedia Laboratories Pvt. Ltd. Mumbai
11	Cholesterol	Ranbaxy Fine Chemical Limited New Delhi
12	Span 60	Ranbaxy Fine Chemical Limited New Delhi

Table 2 List of Apparatus :

Sr. No	Instrument	Manufactures
1	Weight Balance	Wensar
2	Melting Point	Khera Industries, Delhi
3	U.V.(Double Beam)	Systronic double beam spectrophotometer 2203
4	FTIR	Bruker
5.	Electronic Balance	Wensar PG B600
6.	Magnetic Stirrer	Remi Magnetic Stirrers, Mumbai
7.	Ultra Sonicator	Telsonic ,Mumbai
8.	Scanning Electron Microscope	JEOL-6390A
9.	Zeta Meter	Zetatrac

III. Physical Appearance :

The drug (ketoconazole) powder was examined for its organoleptic properties like colour and odour.

IV. Solubility Estimation :[30]

The sample was qualitatively tested for its solubility in various solvents. It was determined by taking 10 mg of drug sample in 10 ml of solvent excess drug in test tubes and well solubilized by shaking, according to IP. Various solubility terms is shown in Table 5.3.

Table 3 Various Solubility Terms :

Descriptive term	Parts of solvent required for parts of solute
Very soluble	Less than 1
Freely soluble	From 1 to 10
Soluble	From 10 to 30
Sparingly soluble	From 30 to 100
Slightly soluble	From 100 to 1000
Very slightly soluble	From 1000 to 10000
Practically insoluble or insoluble	10,000 more

Table 4 Solubility Profile of ketoconazole

Sr. No.	Medium	Solubility Profile	Sign used
1	Water	Insoluble	—
2	Methanol	Soluble	++
3	Ethanol	Sparingly Soluble	+
4	0.1 N hydrochloric Acid	Freely Soluble	++++
5	0.1 N Sodium Hydroxide	Insoluble	—
6	Ether	Insoluble	—
7	Dichloromethane	Freely Soluble	++++
8	Chloroform	Soluble	++

Where, Freely Soluble : ++++, Soluble : ++, Sparingly Soluble : +, Insoluble : —

V. Melting Point Determination :[30]

The Melting point was determined by the capillary method using Digital Melting point apparatus.

Table 5. Melting Point of Ketoconazole

Sr. No.	Melting Point (°C)	Average
1.	143	141 ±2°C
2.	141	
3.	142	

Values are expressed as Mean ± S.D.; n=141 ± 2°C.

Table 6 Preformulation of various Tests For Identification of Ketoconazole :

Parameter	Standard Value given in I.P.	Values found in case of ketoconazole gift sample
Physical Appearance	Color :A White to off-White, Crystalline Powder Odour : Odourless	Color : A White to off-White, Crystalline Powder Odour : Odourless
Solubility	Freely soluble in 0.1N HCl, Dichloromethane and Soluble in methanol, chloroform.	Freely soluble in 0.1N HCl, Dichloromethane, Soluble in methanol, chloroform, Sparingly soluble in Ethanol and Insoluble in Water and Ether
Melting Point	143˚C	141˚C ± 2˚C

VI. Determination of Wavelength of Maximum Absorbance (λ max)[30]:

10 mg of drug (ketoconazole) was weighed accurately and transferred to 10 ml of volumetric flask. Then add 0.1 N HCl was added to dissolve the drug completely. The volume was made up to 10 ml with solvent. The prepared sample was 1000 µg/ml. 1ml of above solution was then transferred to another 10 ml volumetric flask and diluted it up to the 10 ml of solvent. The prepared sample was 100 µg/ml. Another 1ml of this solution was taken and diluted upto 10 ml this gave solution of concentration 10 µg/ml. Now scan sample between 200 – 400 nm in U.V. Spectrophotometer.

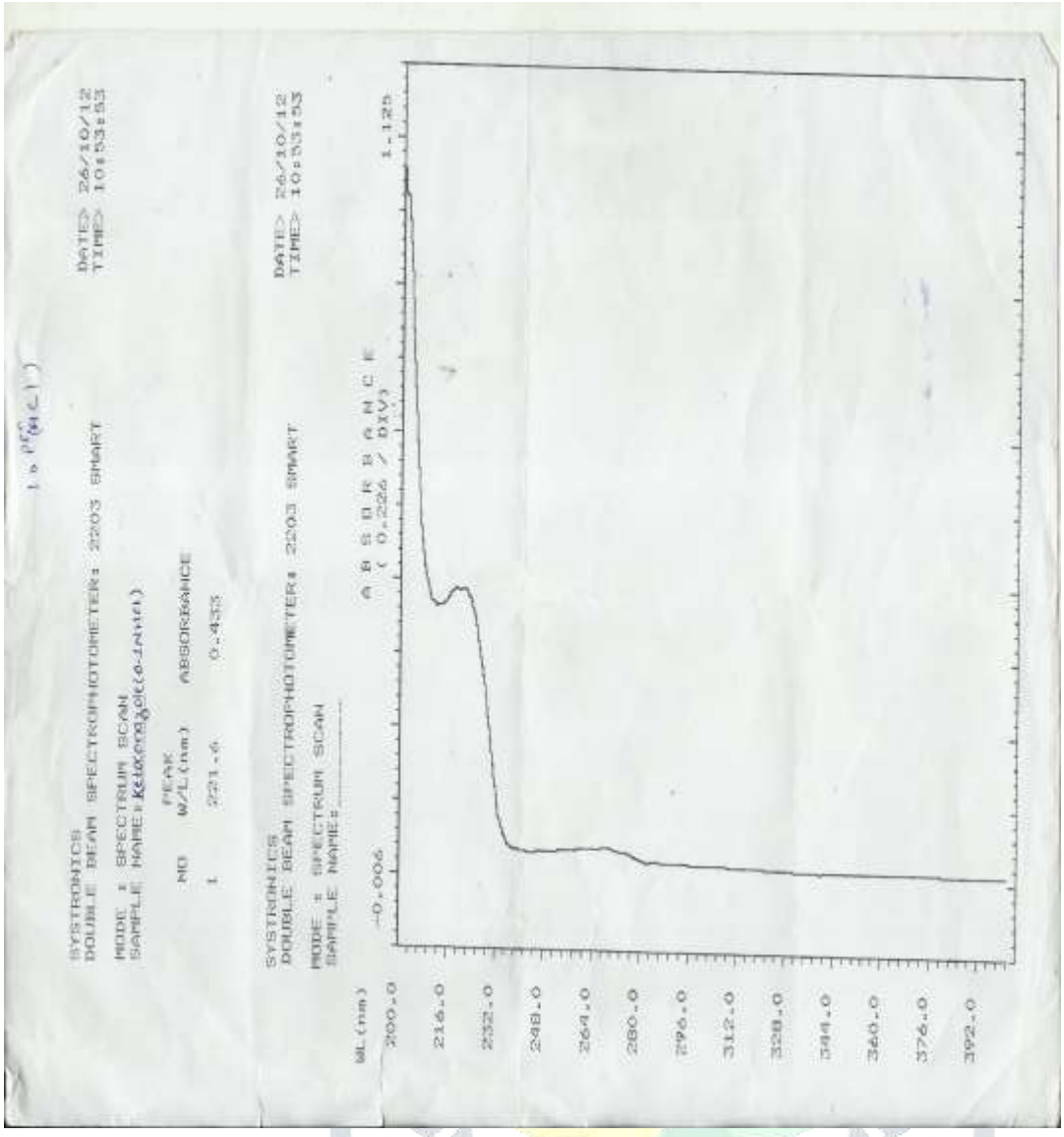


Figure 1 Determination of λmax of ketoconazole in 0.1N HCl

VI.a Preparation of Calibration Curve of Ketoconazole :

The calibration curve was plotted between the concentration and absorbance. The calibration curve of 5-25 µg/ml was carried out in 0.1 N HCl.

Table 7 Calibration Curve of Ketoconazole in Medium 0.1N HCl at λ_{max}221.6 nm

Sr.No.	Concentration(µg/ml)	Absorbance
1.	5	0.200
2.	10	0.390
3.	15	0.575
4.	20	0.755
5.	25	0.940

VI.b Standard Curve of 0.1N HCl :

The standard curves of ketoconazole were prepared in 0.1N HCl solution, at λ_{max} 221.6 nm. The data were regressed to obtain the straight line.

Figure 2 Standard curve in 0.1N HCl of ketoconazole

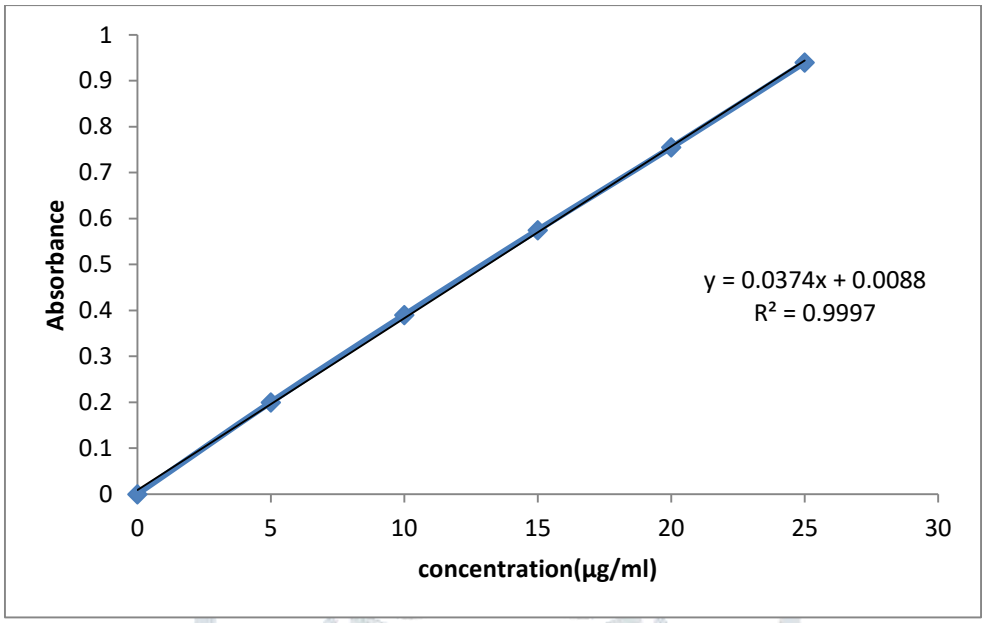


Table 8. Statistical Parameter Related to Standard Curve of ketoconazole at 221.6 λ_{max}nm

S. No.	Absorption Data	Parameter	Values
1	Standard Curve in 0.1 N Hydrochloric Acid	Beer's Law Range	5-25 µg/ ml
		Regression Coefficient	$R^2 = 0.999$
		Regressed Line Eqaution (y = mx +c)	y =0.037 x + 0.008

VII. Determination of Partition Coefficient :

25mg of drug in three separating funnels containing organic and aqueous phase (25 ml each) were shaken for 2 hrs in a wrist action shaker for equilibration. Two phases (n-octanol : water) were separated and the amount of the drug in aqueous phase was analysed spectrophotometrically the partition coefficient of the drug in phases was calculated by using formula:

$$\text{Partition Coefficien t, K} = \frac{\text{Amount of drug in organic layer}}{\text{Amount of drug in aqueous layer}}$$

Table 9 Partition Coefficient Values of Drug

Sr. No.	Medium	Partition Coefficient (Log P)
1	n – octanol : Water	3.026

VIII. Drug – Excipient Compatibility Study[30] :

Drug – Excipient compatibility study done by FT-IR spectroscopy. The presence of characteristic peaks associated with specific structural characteristics of the drug molecule was noted.

a. Fourier-Transform Infra Red spectroscopy (FTIR) Study:

In FTIR study used KBr pellets of drug and excipient are used in solid sample it was detected .The IR spectrum of drug substance was authenticated using IR spectroscopy. The presence of characteristic peaks associated with specific structural characteristics of the drug molecule was noted.

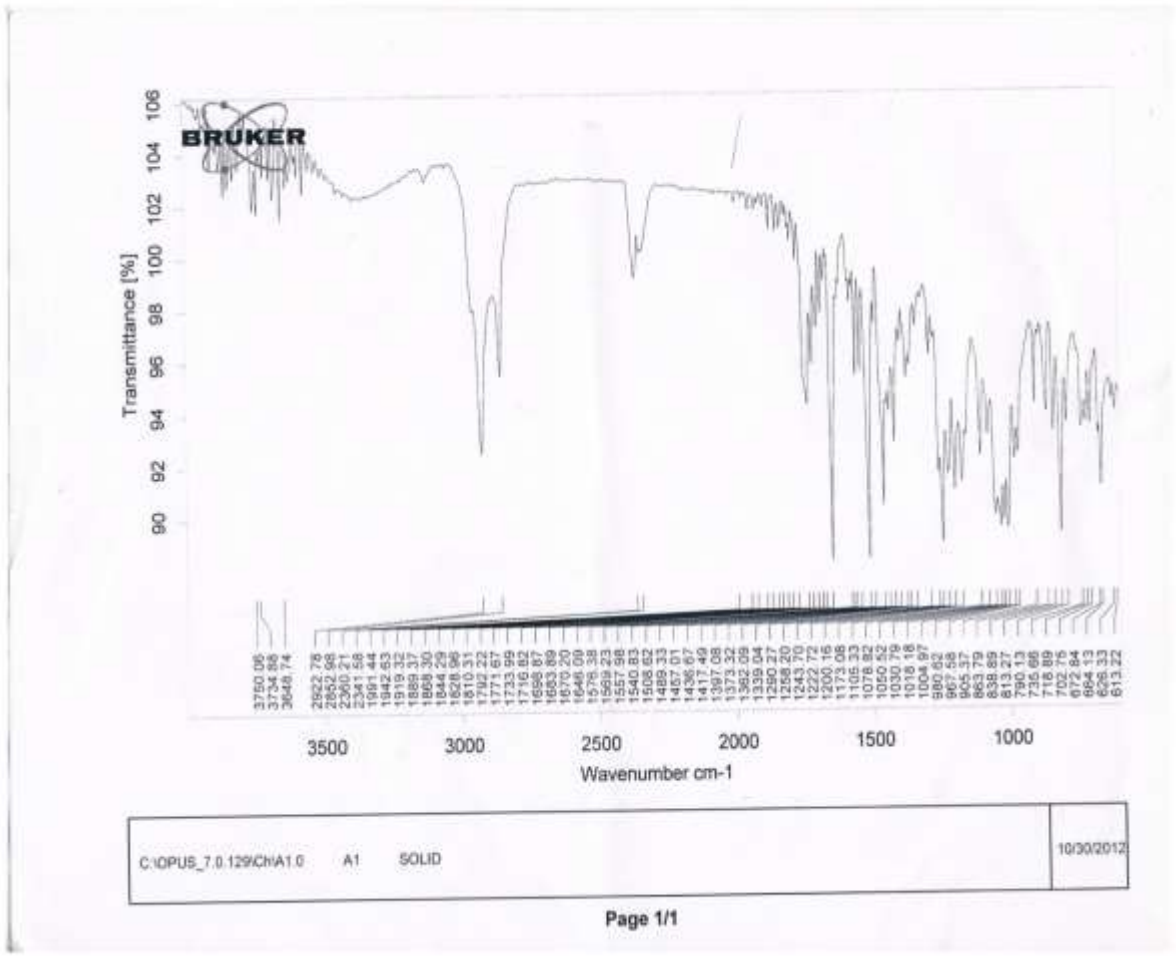


Figure 3 FTIR of Ketoconazole

Table 10: Important band frequencies in IR spectrum of ketoconazole

S. No.	Named Group	Reported Band frequency (cm^{-1})	Band frequency obtained (cm^{-1})
1.	C=O Stretching (amide)	1750	1698.87
2.	C-N Vibration	1400	1397.08
3	CH_3 Bend (Alkane)	1375	1373.32
4.	C=C Stretch (Aromatic)	1475	1457.01
5.	C-Cl Aikylhalides	785	790.13

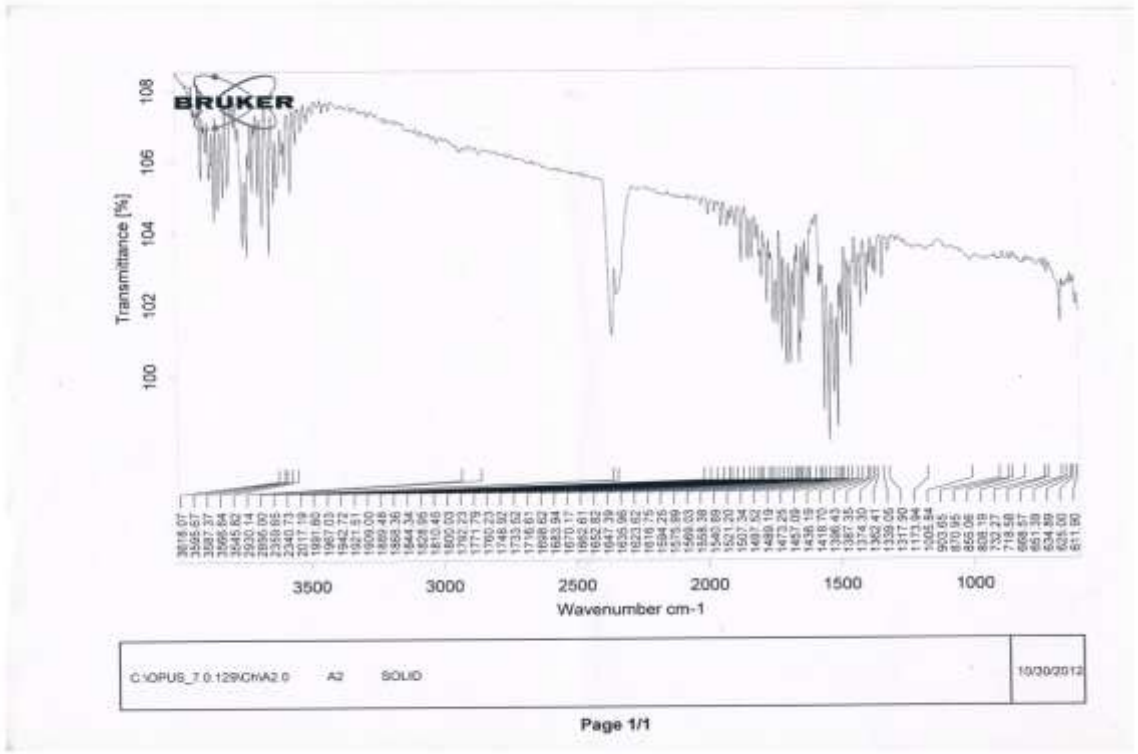


Figure 4. FTIR of Ketoconazole, Lecithin, Cholesterol, Span

IX. Results and Discussion :

The drug (ketoconazole) powder was examined for its organoleptic properties like colour is a white to off-white, crystalline powder and odour is odourless.

The Solubility of pure drug (ketoconazole) was found freely soluble in 0.1N HCl, Dichloromethane, soluble in methanol, chloroform, Insoluble in water, ether and sparingly soluble in ethanol.

Melting point of ketoconazole was found to be 141±2˚C.

Ketoconazole solution was scanned in the U.V. range of 200-400 nm using Systronic UV Visible spectrophotometer. The spectrophotometric method of analysis of ketoconazole at λ_{max} 221.6 nm. The slope and intercept of the calibration curve were 0.037 and 0.008 respectively. The correlation coefficient 'r^2' values were calculated as 0.999.

The partition coefficient of ketoconazole was found to be 3.026.

The FTIR spectra of drug with Lecithin, Cholesterol and Span blends were compared with the FTIR spectrum of the pure drug. It indicates no interaction between ketoconazole, lecithin, cholesterol and Span.

3.0 PREPARATION AND CHARACTERIZATION :

The proniosomes were prepared by slurry method as reported by Chandra and Sharma, 2008: with slight modification in concentration of span 60 and cholesterol.[31]

- 500mg of Mannitol was placed in a 100ml of round bottom flask attached to a rotary evaporator.
- 180mg Lecithin, 160mg Span 60, 40mg Cholesterol and 20mg Ketoconazole mixture in 12ml Chloroform and 3ml Isopropylalcohol mixture (4:1) was added slowly onto Mannitol powder bed.
- Care was taken not to overweight the powder paste.
- The rotary evaporator was maintain at a temperature of 65˚C using water bath and the flask was rotated at 60 rpm under vacuum.
- The dried material was finally removed and kept under vacuum overnight.

I. Percentage Entrapment Efficiency[32] :

Percent entrapment efficiency was determined by centrifugation method. In this the proniosomes were hydrated centrifuged at 18000 rpm for 40 minutes at 5˚C in order to separate unentrapped drug. The supernatant was discarded. The pellet was digested with triton × 100 (1% w/v) and the entrapped drug was extend with PBS (pH 7.4). The solution was filtered and absorbance noted after suitable dilution. The drug concentration in the resulting solutions was assayed spectrophotometrically at 221.6 nm.

Entrapment Efficiency is expressed as the % of drug entrapped

$$\% \text{ entrapment efficiency} = \frac{\text{Drug in proniosomes}}{\text{Total drug}} \times 100$$

II. Microscopy and Vesicle Size[33] :

A drop of proniosomal gel was spread on a glass slide and examined for the vesicle structure and presence of insoluble drug crystals under the optical microscope with varied magnification power. Photomicrographs were taken for proniosome using a digital camera with 100X optical 200m.

III. Optimization of Formulations :

These include ratio of constitutive Lipid (Lecithin and Cholesterol), Surfactant:Lipid and Drug:Excipients. The optimization was carried out by varying one of the parameter and keeping other parameters constant.

1. Optimization of Ratio of Constitutive Lipids (Lecithin and Cholesterol) :

The ratio of constitutive lipids (Lecithin and Cholesterol) were optimized with respect to % entrapment efficiency and vesicle size. Proniosomes bearing Ketoconazole were prepared by varying the molar ratio of the Lecithin and Cholesterol 8:1 to 5:4 and optimized to get the maximum entrapment efficiency. The proniosomes were prepared by slurry method to optimize Lecithin:Cholesterol ratio. The results are given in table 6.1. .

Table 11 : Optimization of Lecithin:Cholesterol ratio for the preparation of proniosomes

Formulation Code	Lecithin :Cholesterol	% Entrappment Efficiency	Vesicle Size(µm)
L_1SD	8:1	80.4 ±2.3	7.8 ± 0.35
L_2SD	7:2	76.3 ±2.5	6.2 ±0.24
L_3SD	4:1	81.6 ±1.8	7.9 ±0.05
L_4SD	2:7	65.9 ±1.5	6.1 ±0.28
L_5SD	6:3	77.4 ±2.0	5.9 ±0.25
L_6SD	5:4	70.9 ±1.9	5.8 ±0.24

Values are expressed as Mean ± S.D.; n = 3

2. Optimization of Surfactant and Lipid in ratio :

The concentration of Span 60 and cholesterol (4:1) was optimized using Slurry method on the basis of % entrapment efficiency and vesicle size. The proniosomes were prepared by changing the molar concentration of surfactant and lipid and the proniosomes were characterized for % entrapment efficiency and vesicle size. The results are given in table 6.2.

Table 12 Optimization of Surfactant and Lipid :

Formulation Code	Surfactant : Lipid	% entrapment efficiency	Vesicle Size(µm)
L_3S_1D	4:1	95.4 ±2.0	8.5 ±0.40
L_3S_2D	7:3	85.6 ±2.8	7.3 ±0.32
L_3S_3D	3:2	83.5 ±2.2	7.3 ±0.31
L_3S_4D	2:3	88.6 ±2.1	7.3 ±0.30
L_3S_5D	3:7	75.3 ±1.8	6.6 ±0.25
L_3S_6D	1:4	60.5 ±1.5	6.1 ±0.27

Values are expressed as Mean ± S.D.; n = 3

3. Optimization of Drug : Excipients (span 60, cholesterol, lecithin) :

In optimization the excipients were kept constant and only drug ratio was changed. In this Study the % entrapment efficiency and vesicle size. The % entrapment efficiency and vesicle size were determined. Observation are recorded in table 6.3.

Table 13 Optimization of Drug: Excipients (Span 60 and Cholesterol) :

Formulation Code	Drug:Span60:Cholesterol:Lecithin	% entrapment efficiency	vesicle Size (µm)
$L_3S_1D_1$	1:50	96.5±2.3	8.6 ±0.40
$L_3S_1D_2$	2:100	85.4 ±1.8	8.4 ±0.38
$L_3S_1D_3$	3:150	81.2 ±1.6	8.2 ±0.35
$L_3S_1D_4$	4:200	79.5 ±1.2	7.4 ±0.31
$L_3S_1D_5$	5:250	75.2 ± 1.2	7.0 ±0.30
$L_3S_1D_6$	6:300	70.3 ±1.5	6.2 ±0.28

Values are expressed as Mean ± S.D.; n = 3

4. Optimized Formulation :

$L_3S_1D_1$: Compositon of proniosomes of Ketoconazole :

- **Drug : 20 mg**
- **Span 60 : 160 mg**
- **Cholesterol : 40 mg**
- **Lecithin : 180 mg**
- **Mannitol : 500 mg**
- **Chlroform & IPA : 12 & 3 m**

The proniosomes prepared by slurry method. Then, used the evaluation of $L_3S_1D_1$. The % entrapment efficiency and vesicle size.

Table 14. $L_3S_1D_1$ of % entrapment efficiency and vesicle size :

S. No.	% entrapment efficiency	vesicle size(µm)
1.	96.5± 4.8	8.6 ± 0.40

IV. Preparation of Ketoconazole Proniosomes Gel :

Gel was prepared with the formula optimized by Chandra and Sharma, 2008.

- Proniosomes powder was weighed into screw cap vials to which was added water at 80˚C.
- The vials were vortexed mixed for completed and uniform hydration.
- The Proniosomal preparation were then converted into gel by approximately diluting the proniosomes and adding Carbopol 934 (1%w/v) for ease of handling.
- The final Ketoconazole concentration achieved was 2%w/w.

V. FORMULATION ASPECT OF PRONIOSOMES :

Proniosomes gel is comprised of ingredients like lecithin, cholesterol, non-ionic surfactants (span60), carbopol gel, alcohol and aqueous phase.

(a) Lecithin : It acts as penetration enhancer.

(b) Cholesterol : In proniosomal gel, cholestrol plays roles likes prevents leakage of the drug from vesicles.

(c) Surfactants : Used span 60 high value, hence large size vesicles are formed. In proniosomal gel, surfactants plays roles likes increase drug flux rate approach the skin.

(d) Solvent : Alcohol has great influences on vesicles size. For providing the softness of vesicles membrane.

(e) Mannitol : It is used in proniosomes formation.

(f) Carbopol Gel : Carbopol gels have been used as dermal base for proniosome and niosomes. This was because of hydrophilic nature, bioadhesive properties, compatibility with vesicular structures and desirable viscosity. In addition, Carbopol 934 was used because it was effective in thick formulations, possess good clarity in water or hydro-alcoholic topical gels and forms clear gel with hydro-alcoholic systems.

VI. Microscopy[33]

A drop of proniosomal gel was spread on a glass slide and examined for the vesicle structure and presence of insoluble drug crystals under the optical microscope with varied magnification power. Photomicrographs were taken for proniosome using a digital camera with 100X optical 200m.

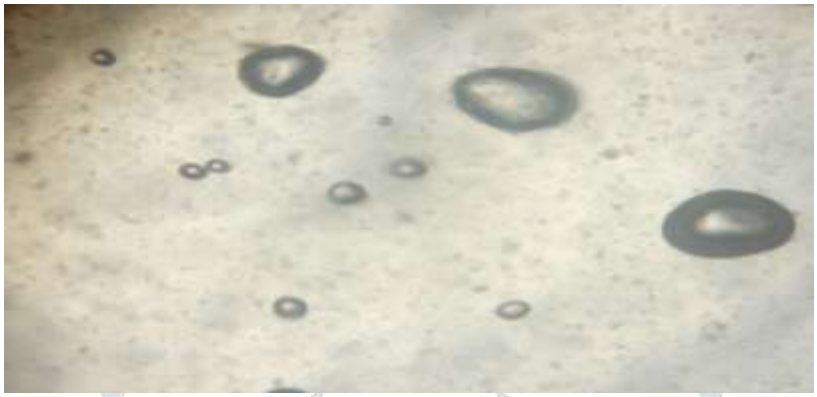


Figure 5. Optical Microscopy of Proniosome gel (ketoconazole) ($L_3S_1D_1$)

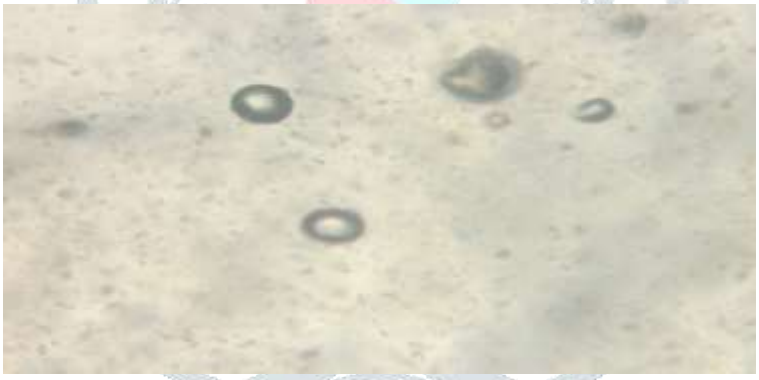


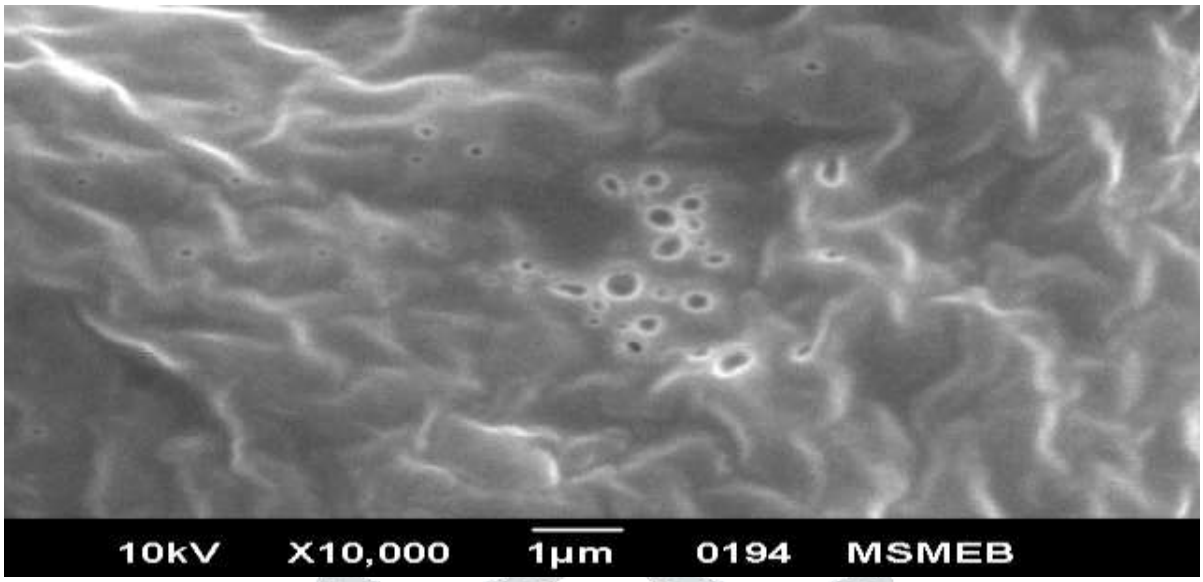
Figure 6. Optical Microscopy of Proniosome gel (Ketoconazole) $L_3S_1D_6$

VII. Vesicle Morphology[34] :

The proniosome gel were mounted on metal stubs and the stud was then coated conductive gold with sputter coater attached to the instrument.

The photographs were taken using a JEOL-6390A scanning electron microscope.

Figure 7. SEM of Proniosomal Gel of Ketoconazole ($L_3S_1D_1$) :



VIII. Zeta Potential and Zeta Sizer[35] :

The Brownian motion power spectrum is analyzed with the Nanotrac controlled reference technique of particle sizing to determine the **M**odulated **P**ower **S**pectrum, a component of the power spectrum resulting from the oscillating particles. Zeta Potential is calculated from the **MPS** signal. Also determined are the particle mobility (velocity per electric field), particle charge and particle size.

Zeta Potential Report

Sample Details

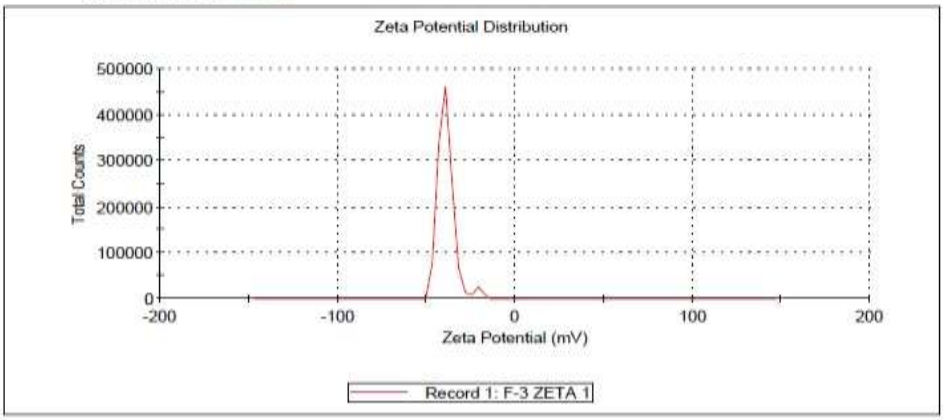
Sample Name:	F-3 ZETA 1		
SOP Name:	mansettings.dat		
General Notes:			
File Name:	F-3 ZETA.dts	**Dispersant Name:**	Water
Record Number:	1	**Dispersant RI:**	1.330
Date and Time:	Thusday, February 21 2013, 5:1	**Viscosity (cP):**	0.8872
		Dispersant Dielectric Constant:	78.5

System

Temperature (°C):	25.0	**Zeta Runs:**	13
Count Rate (kcps):	94.9	**Measurement Position (mm):**	2.00
Cell Description:	Clear disposable zeta cell	**Attenuator:**	7

Results

			Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV):	-38.5	**Peak 1:**	-39.1	96.5	4.04
Zeta Deviation (mV):	5.16	**Peak 2:**	-20.4	3.5	2.37
Conductivity (mS/cm):	0.138	**Peak 3:**	0.00	0.0	0.00
Result quality	Good				



Malvern Instruments Ltd
www.malvern.com

DTS Ver. 5.03
Serial Number : MAL1023461

File name: F-3 ZETA.dts
Record Number: 1
21 Feb 2013 5:16:24 PM

Figure 8 Zeta Potential

Size Distribution Report by Intensity

Sample Details

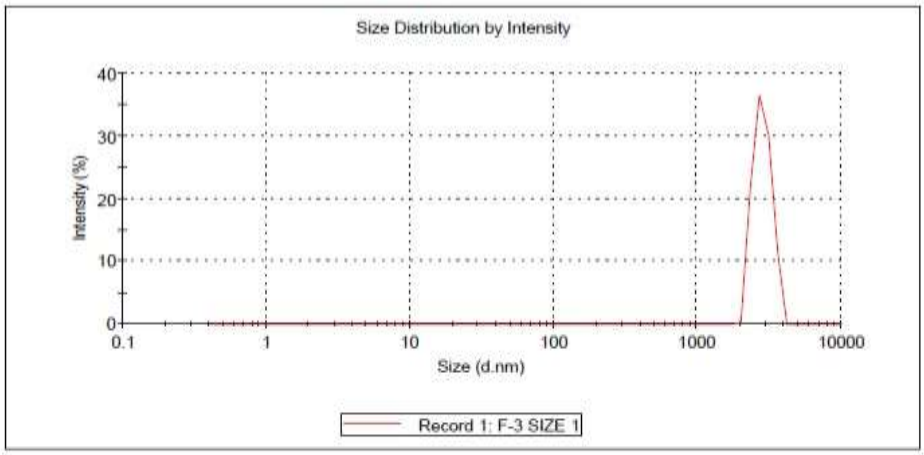
Sample Name:	F-3 SIZE 1		
SOP Name:	mansettings.dat		
General Notes:			
File Name:	F-3 SIZE.dts	**Dispersant Name:**	Water
Record Number:	1	**Dispersant RI:**	1.330
Material RI:	1.59	**Viscosity (cP):**	0.8872
Material Absorbtion:	0.01	**Measurement Date and Time:**	Thusday , February 21 2013...

System

Temperature (°C):	25.0	**Duration Used (s):**	70
Count Rate (kcps):	181.4	**Measurement Position (mm):**	4.65
Cell Description:	Disposable sizing cuvette	**Attenuator:**	6

Results

			Diam. (nm)	% Intensity	Width (nm)
Z-Average (d.nm):	3111	**Peak 1:**	2819	100.0	395.7
PdI:	0.657	**Peak 2:**	0.000	0.0	0.000
Intercept:	0.886	**Peak 3:**	0.000	0.0	0.000
Result quality	Refer to quality report				



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DTS Ver. 5.03
Serial Number : MAL1023461

File name: F-3 SIZE.dts
Record Number: 1
21 Feb 2013 5:11:18 PM

Figure 9. Zeta Sizer

IX. Physical Parameter of Proniosomal Gel :

Proniosomes gel formulations were characterization for spreadability and homogeneity.

IX.a. Spreadability :

It was determined by wooden block and glass slide apparatus. Weights of about 10g were added to the pan and the time was noted for upper slide (movable) to separate completely from the fixed slide.

Spreadability was then calculated by using the formulation.

$$S = M . L / T$$

Where,

S = Spreadability
M = Weight tied to upper slide
L = Length of glass slide
T = Time taken to separate the slide completely from each other

IX.b. Homogeneity :

All developed gels were tested for homogeneity by visual inspection after the gels have been set in the container.

Table 15. $L_3S_1D_1$ of % homogeneity :

S.No.	Homogeneity	Observation
1.	Proniosomes gel shows good homogeneity with absence of lumps.	Proniosomes gel were smooth and turbid.

X. Result and Discussion :

The entrapment efficiency is maximum in Lecithin:Cholestrol ratio of optimized preparation L_3SD i.e. 81.6±1.8. The entrapment efficiency is maximum in Surfactant:Lipid ratio of optimized preparation L_3S_1D i.e. 95.4±2.0. The entrapment efficiency is maximum in Drug:Excipient ratio optimized formulation $L_3S_1D_1$ i.e. 96±2.3.

Vesicle size is large in Lecithin:Cholestrol ratio of optimized preparation L_3SD i.e. 7.9±0.05. Vesicle size in Surfactant:Lipid ratio of optimized preparation L_3S_1D i.e. 8.5±0.4. Vesicle size in Drug:Excipient ratio optimized formulation $L_3S_1D_1$ i.e. 8.6±0.4.

In optical microscopy the photograph shows that proniosomes are unilamellar vesicles having spherical shape and no aggregation or agglomeration was observed optimized formulation $L_3S_1D_1$. In this formulation the vesicle size is large. In optical microscopy the photograph shows that proniosomes are unilamellar vesicles having spherical shape and no aggregation or agglomeration is observed. But in this (L_3S_1D6) formulation the vesicle size is small.

SEM image of proniosomes showed that most of vesicles are well identified, spherical and discrete with sharp boundaries having large internal aqueous space.

Zeta potential and zeta sizer are used for determining the colloidal properties of the prepared. The $L_3S_1D_1$ formulation of zeta potential is -38.5 and zeta deviation is -5.16. Then the zeta potential result is good. The $L_3S_1D_1$ formulation of zeta Sizer is z-averge is 3111 and diameter of peak1 is 2819. Zeta Potential and Zeta Sizer of $L_3S_1D_1$ formulation which was found to have a better physical stability.

The value of spreadibility of all proniosomal gel formulations ranged from 13.4 to 14.3 (g.cm/sec).

All developed gel showed good homogeneity with absence of lumps. All developed preparations were clear and transparent.

4.0 IN-VITRO DIFFUSION STUDY[36] :

Invitro diffusion studies of proniosomal gel was carried out using a dialysis bag as a donar compartment. Proniosomal gel equivalent 200 mg of drug was taken in a dialysis membrane and placed in a beaker containing PBS (pH 7.4) of 100ml, which acted as the receptor compartment. Previously, the dialysis membrane was soaked in warm water for 10min and both ends were sealed with closure clips after adding the proniosomal preparation. The beaker was placed over a magnetic stirrer (100rpm) and maintained at 37±1℃. At predetermined time intervals during 24 hrs, aliquots (1ml) were withdrawn and replace with fresh PBS (pH 7.4). The sink condition were maintained throughout the experiment. Samples withdrawn were suitably diluted and analyzed spectrophotometrically at 221.6 nm for Ketoconazole.

Release Kinetics :

The results obtained from in-vitro drug release studies were shown in table adopting three different mathematical models of data treatment as follows :

- % Cum. Drug release vs Time (Zero order rate kinetics)
- % Cum. Drug retained vs. Time (First order rate kinetics)
- % Cum. Drug release was plotted against √T (root time) (Higuchi model)

Table 16 : In-vitro Drug release of Proniosomal Gel formulation ($L_3S_1D_1$) :

S. No.	Time (min)	Cumulative drug release	% Cumulative drug release	Drug remaining	Sq. root time
0	0	0	0	0	0
1	30	8.396±0.6	8.396±0.6	91.61±2.5	5.477
2	60	25.387±1.2	25.387±1.2	74.62±2.8	7.745
3	90	35.217±1.7	35.217±1.7	64.79±1.2	9.486
4	120	55.325±2.7	55.325±2.7	44.68±2.2	10.954

5	**150**	**65.231±1.2**	**65.231±1.2**	**34.77±1.7**	**12.247**
6	**180**	**75.321±2.7**	**75.321±2.7**	**24.68±1.2**	**13.416**
7	**210**	**80.231±2.0**	**80.231±2.0**	**19.77±0.9**	**14.491**
8	**240**	**86.298±2.5**	**86.298±2.5**	**13.71±0.6**	**15.491**

Values are expressed Mean±S.D., n=3

Table 17 : Drug Release Mechanism ($L_3S_1D_1$) and for their constant values $R^2$and K :

S. No.	Formulation Code	Zero Order		First Order		Higuchi	
		R^2	K	R^2	K	R^2	K
1.	$L_3S_1D_1$	0.995	0.272	0.14	-0.120	0.814	4.738

5.0 STABILITY STUDY[37]:

The optimized formulation was tested for stability studies. Formulation was divided into 3 sample sets and stored at :

- 5˚C ± 2˚C in refrigerator.
- 25˚C ± 2˚C
- 40˚C ± 2˚C

The samples were withdrawn after 15, 30, 45 days tested for drug retained.

Table 18. Data showing Stability Studies of proniosomal gel ($L_3S_1D_1$) at 5˚C ± 3˚C,

Time (days)	Vesicle Size(µm)	(%) residual drug content
0	5.6 ±0.28	100±1.5
15	5.5±0.27	100 ±0.02
30	5.5±0.27	99.99 ±1.54
45	5.4±0.21	99.99 ±2.0

Values expressed are Mean S.D., n = 2

Table 19. Data showing Stability Studies of proniosomal gel ($L_3S_1D_1$) at 25˚C ± 2˚C, 60 ± 5% RH.

Time (days)	Vesicle Size(µm)	(%) residual drug content
0	5.6 ±0.28	100 ±1.5
15	5.6±0.28	99.9±1.0
30	5.7±0.29	99.9±1.5
45	5.7±0.29	99.8±2.0

Values expressed are Mean S.D., n=2

Table 20. Data showing Stability Studies of proniosomal gel ($L_3S_1D_1$) at 40˚C ± 2˚C, 75 ± 5% RH.

Time (days)	Vesicle Size(µm)	(%) residual drug content
0	5.6 ±0.28	100.00±1.4
15	5.7±0.29	99.8±1.8
30	5.7±0.29	99.7±1.1
45	5.8±0.30	99.3±2.0

Values expressed are Mean±S.D., n=2

6.0 SKIN IRRITANCY TEST[38] :

Briefly, six healthy rabbit of either sex having average weight of 3.5 kg were selected for the study. The right back part of rabbits (4 cm^2 area) was shaved carefully. Adequate amount of ketoconazole proniosomal gel was applied to shaved skin area (Group I). Same way, Standard Irritant **(**Formalin) was applied to the shaved skin (Group II), which served as control. The selected formulation equivalent to 200mg drug was applied on shaved rat skin for the determination of irritation characteristics. The applied area was covered by cotton and bandage. The visual observations were carried out at regular intervals of 12, 24, 48 hours for symptoms such as lesions and erythema. The symptoms, lesions and erythema were graded as 3=severe, 2=moderate, 1=mild and 0=absent.

Table 21. Skin Irritation score of standard irritant (formalin) and ketoconazole proniosomal gel :

Hours	Score							
	Standard Irritant (Formalin)			mean	ketoconazole proniosomal gel			mean
12	3	2	1	2	0	0	0	0
24	2	2	1	1.67	0	0	0	0
48	2	1	1	1.33	0	0	0	0

Figure 10. : Skin Irritation test of optimized formulation($L_3S_1D_1$) on rabbits

I. Results and Discussion :

In-vitro release : The release study was conducted for $L_3S_1D_1$ formulation. The formulation was found to provide 86.25% release with a period of 4 hours. The Higuchi describes the release from the system where solid drug is dispersed in insoluble matrix. Drug release is good in $L_3S_1D_1$ formulations.

Stability Studies were carried out after storing the selected formulation at 3 different temperature 5˚C ± 3˚C, 25˚C ± 2˚C and 40˚C ± 2˚C for 45 days. The vesicle size and % residual drug content was monitored every 15 days.

Results showed that there were no significant changes observed in the vesicles size, drug retained of formulation at 5˚C ± 3˚C. It confirms that formulation $L_3S_1D_1$ was stable at the end of 45 days.

On the other hand nominal change in formulation was observed in the vesicle size, % residual drug content diffusion after 45 days at 25˚C ± 2˚Cand 40˚C ± 2˚C temperature.

The results of drug retain studies showed higher drug leakage at higher temperature. Acceleration in drug leakage at higher temperature as observed in storage stability suggested keeping the proniosomal gel in the refrigeration condition.

Skin Irritancy test : No erythema was found when optimized formulation $L_3S_1D_1$ was applied on rabbit and compared with standared irritant.

7.0 SUMMARY AND CONCLUSION :

Oral administration, ketoconazole is poorly absorbed and has a very low half-life of 2 hour. To overcome these inherent drawbacks associated with conventional drug delivery of ketoconazole, an attempt is made to design an alternative drug delivery system in forms of proniosomal gel.

The drug (ketoconazole) powder was examined for its organoleptic properties like colour, it was a white to off-white, crystalline powder and odour is odourless.

The Solubility of pure drug (ketoconazole) was found freely soluble in 0.1N HCl, Dichloromethane, soluble in methanol, chloroform, Insoluble in water, ether and sparingly soluble in ethanol.

Melting point of ketoconazole was found to be 141±2˚C.

Ketoconazole solution was scanned in the U.V. range of 200-400 nm using Systronic UV Visible spectrophotometer. The spectrophotometric method of analysis of ketoconazole was used for drug estimation with λ_{max} found to be 221.6 nm. The slope and intercept of the calibration curve were 0.037 and 0.008 respectively. The correlation coefficient 'r^2' values were calculated as 0.999.

The partition coefficient of ketoconazole was found to be 3.026.

The FTIR spectra of drug with Lecithin, Cholesterol and Span blends were compared with the FTIR spectrum of the pure drug. It indicates no interaction between ketoconazole, lecithin, cholesterol and Span.

To enhance retention time of drug at the affected area, proniosomal gel of ketoconazole were prepared by slurry method using span 60 and cholesterol in different concentration.

The formulations were prepared to study the effect of surfactants and cholesterol concentration on various parameters such as % entrapment efficiency, vesicle size, shape and surface morphology, physical parameters of proniosomal gel.

The entrapment efficiency is maximum in Lecithin:Cholestrol ratio of optimized preparation L_3SD i.e. 81.6±1.8. The entrapment efficiency is maximum in Surfactant:Lipid ratio of optimized preparation L_3S_1D i.e. 95.4±2.0. The entrapment efficiency is maximum in Drug:Excipient ratio optimized formulation $L_3S_1D_1$ i.e. 96.5±2.3.

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The value of spreadibility of all proniosomal gel formulations ranged from 13.4 to 14.3 (g.cm/sec). The values of spreadability indicate that the gel is easily spreadable with minimal of shear. Spreadibility of proniosomal gel formula is good it is easily spreadable.

All developed gel showed good homogeneity with absence of lumps. All developed preparations were clear and transparent.

In-vitro studies : Proniosome gel of ketocoazole are formulated by different ratio of span 60 and cholesterol. The release study was conducted for $L_3S_1D_1$ formulation. The formulation was found to provide 86.25% release with a period of 4 hours. The Higuchi describes the release from the system where solid drug is dispersed in insoluble matrix. Drug release is good in $L_3S_1D_1$ formulations.

Stability Studies were carried out after storing the selected formulation at 3 different temperature 5˚C ± 3˚C, 25˚C ± 2˚C and 40˚C ± 2˚C for 45 days. The vesicle size and % residual drug content was monitored every 15 days. Results showed that there were no significant changes
formulation $L_3S_1D_1$ was stable at the end of 45 days.

On the other hand nominal change in formulation was observed in the vesicle size, % residual drug content diffusion after 45 days at 25˚C ± 2˚Cand 40˚C ± 2˚C temperature.

The results of drug retain studies showed higher drug leakage at higher temperature. This may be due to the higher fluidity of lipid bilayers at higher temperature resulting in higher drug leakage. Loss of drug from the vesicles stored at elevated temperature may be attributed to the effect of temperature on the gel to liquid transition of lipid bilayers together with possible chemical degradation of the phospholipids, leading to defects in membrane packing. Acceleration in drug leakage at higher temperature as observed in storage stability suggested keeping the proniosomal gel in the refrigeration condition.

Skin Irritancy test : No erythema was found when optimized formulation $L_3S_1D_1$ was applied on rabbit and compared with standared irritant.

8.0 CONCLUSION :

A successful attempt was made to develop proniosomal gel for topical delivery of ketoconazole using different ratio of span and cholesterol concentration and evaluated for different in-vitro and skin irritancy test.

Proniosomes are dry formulations of surfactant-coated carrier, which can be measured out as needed and rehydrated by brief agitation in hot water. These “proniosomes” minimize problems of niosomes physical stability such as aggregation, fusion and leaking while providing additional convenience in transportation, distribution, storage and dosing. Proniosome derived niosomes are superior to conventional niosomes in convenience of storage, transport and dosing. Stability of dry proniosomes is expected to be more stable than enhanced as compared to pre-manufactured niosomal formulation.

Proniosomes gel of ketoconazole for the attainment of better therapeutics in candidiasis. The drug is 99% protein bound moreover, it is hepatotoxic hence a cutaneous/topical delivery is more beneficial for the attainment of better therapeutic results.

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