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Formulation Development and In-vitro Evaluation of Minoxidil Bearing Glycerosomes

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Abstract Present study was undertaken to assess the potential of Glycerosomes as a novel drug delivery system for topical application of Minoxidil. Pre-formulation studies were done for identification of drug as well as for determination of its physiochemical properties. Spectra of various mixtures of drug and excipients do not show any additional peak thus, indicating compatibility with each other. Glycerosomes was prepared by using lipid thin film hydration method. Prepared formulations were evaluated in terms of particle size, surface analysis, zeta potential, entrapment efficiency and *in-vitro* drug release. The formulated Glycerosomes were found to have better surface characteristics and entrapment efficiency. The *in vitro* drug dissolution study was carried out using egg membrane on modified franz diffusion cell and the release mechanisms were explored. The release data was incorporated into various mathematical models and the formulation follows Higuchi as well as Fickian diffusion. Results study proved that Glycerosomes containing Minoxidil can be an excellent therapy for Alopecia.

Keywords: *Glycerosomes, Minoxidil, Lipid thin film hydration, topical drug delivery, novel formulation*

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1. Introduction

Topical route is the most oldest and convenient route for the administration of therapeutic agents because of low cost of therapy and ease of administration which leads to highest level of patient compliance. Conventional formulations are required to be administered in multiple doses and therefore have several disadvantages like poor patient compliance, fluctuations in drug levels. [1,2,3] Topical delivery involve the introduction of a drug to the surface of the body, in a formulation which can be absorbed. [4] Topical drug delivery offer number of potential advantages over conventional drug delivery system such as like avoidance of first pass metabolism, predictable and extended duration of activity, minimizing undesirable side effects, utility of short half-life drugs, improving physiological and pharmacological response, avoiding the fluctuation in drug levels, inter-and intra-patient variations, and most importantly, it provides patient convenience. [5,6]

Recently various strategies have been used to augment the topical delivery of bioactives. Mainly they include iontophoresis, electrophoresis, sonophoresis, chemical permeation enhancers, microneedles and vesicular drug delivery systems. Among these strategies vesicular drug delivery system appear promising. [7] Novel vesicular drug delivery systems aim to deliver the drug at a rate directed by need of body during the period of treatment. [8]The vesicular systems are highly ordered assemblies of one or several concentric lipid bilayers formed, when certain amphiphillic building blocks are confronted with water. [9]

Drug carriers used in topical drug delivery such as liposomes, niosomes, or micro-emulsions have problem that they remains mostly confined to the skin surface and therefore do not transport drugs efficiently through the skin and this led to intensive research in the field, with the introduction and development of new classes of lipid vesicles. Therefore, new, modified formulations have been introduced, such as deformable/elastic liposomes and ethosomes. [10,11]

A new approach to increase liposome properties as dermal and transdermal drug delivery systems by modifying liposomal bilayer fluidity is represented by Glycerosomes, which are composed of phospholipids and water and are characterized by high amount of glycerol (20-50%) like ethosomes these carriers do not contain ethanol as structural component, thus Glycerosomes are harmless and fully accepted compound for topical applications. [12]

2. Material and Methods

2.1. Materials

The excipients and chemicals used for the presents study are detailed as Minoxidil from Balaji Chemicals, Surat; Lecithin from HiMedia Laboratory Pvt.Ltd, Nasik; Cholesterol from LOBA Chemie, Mumbai; Stearylamine from Ottokemi, Mumbai; Glycerol from Nice Laboratory Reagents, Kochi; All the ingredients were AR grade.

2.2. Methods of preparation of Glycerosomes

Lipid thin film hydration method was used in this study for preparation of Glycerosomes, in this method we have to take a 0.5-litre round bottom flask; Minoxidil (2 % of dilution medium) was dissolved in Ethanol. In separate beaker Lecithin (30 mg/ml), Stearylamine (10 % of lecithin) and Cholesterol (2 mg/ml) were co-dissolved in a minimum amount of chloroform. Both organic solutions were mixed and the organic solvent was evaporated until complete dry film was obtained under reduced pressure using a rotary evaporator. Then this dry film was hydrated using glycerol solution in phosphate buffer of pH 7.4 with a concentration ranging from 10 to 50% w/v (Table 1).

Table 1. Composition of different formulations of Glycerosomes

Ingredients mg/10 ml	Formulation code								
	F1	F2	F3	F4	F5	F6	F7	F8	F9
Minoxidil	2	2	2	2	2	2	2	2	2
Lecithin	300	300	300	300	300	300	300	300	300
Cholesterol	20	2o	20	20	20	20	20	20	20
Glycerol (% of 10 ml)	20	20	20	30	30	30	40	40	40
Stearylamine	10	10	10	10	10	10	10	10	10

3. Preformulation Studies

3.1. Identification of Drug

The drug sample was identified by various analytical techniques such as UV/VIS spectroscopy, FTIR spectroscopy, and Melting point.

3.1.1. UV Spectrum

i) Absorbance spectra of Minoxidil in 0.1 M HCl and NaOH [13]

Dissolve 20.0 mg in 0.1 M HCl and dilute to 100.0 ml with the same solvent (solution a). Dilute 2.0 ml of solution (a) to 100.0 ml with 0.1 M HCl (solution b) and dilute 2.0 ml of solution (a) to 100.0 ml with 0.1 M NaOH (solution c). Examine solutions (b) and (c) spectrophotometrically using UV double beam spectrophotometer (Systronics PC based double beam spectrophotometer 2202) between 200 nm and 400 nm.

ii) Absorbance spectra of Minoxidil in Phosphate buffer of pH 7.4

Minoxidil stock solution (100 µg/ml) was prepared in Phosphate buffer of pH 7.4 and scanned spectrophotometrically using UV double beam spectrophotometer (Systronics PC based double beam spectrophotometer 2202) in a range from 200 nm to 400 nm.

3.1.2. FT-IR Spectrum

Fourier Transform Infrared Spectrophotometer (Bruker) was used for structural analysis of drug. A small amount of Minoxidil was taken and scanned in the range 4000 to 400cm^{-1}.

3.1.3. Melting Point

A capillary melting point apparatus was used to determine melting point of Minoxidil. USP has described specification for capillaries for melting point determination i.e. 10 cm length, 0.8-1.2 mm internal diameter and 0.2-0.3 mm wall thickness and sufficient amount of the dry powder should be packed down to form a 2.5-3.5 mm high column in the bottom of the tube.

Minoxidil was placed in a vaccum desicator at room temperature and dried for about 24 hours. Then dried Minoxidil was tightly filled in the capillary tube up to 3 mm height by tapping the closed end of capillary on a solid surface. Liquid paraffin, which is a heat transfer agent, was filled in the Thiele tube. Thiele tube was clamped on the stand and thermometer (with tied capillary) was dipped into the liquid paraffin and was tied on the stand. Thiele tube was heated from the below to raise the temperature and the temperature at which Minoxidil melt, was recorded. These were performed in triplicate and average value was recorded.

3.2. Partition Coefficient

Accurately weighed (200mg) drug was transferred in conical flask containing hydrophilic solvent i.e. water and the hydrophobic solvent i.e. n-Octanol (20 ml each) as this solvent has the ability to mimic the aqueous membrane interface, due to its chemical characteristics. Conical flask was shaken till equilibrium was attained with drug and transfers the mixture to separating funnel. Allow the two layers to separate and analyze spectrophotometrically. [14]

Partition coefficient was calculated by using following equation:

$$P_{o/w} = \frac{\textit{concentration of drug in n-octanol}}{\textit{concentration of drug in water}}.$$

3.3. Drug Excipient Compatibility Studies [15]

3.3.1. Physical Compatibility Studies

The samples i.e. drug alone, excipients alone and homogeneous mixer of drug and each excipients in specified ratios were kept at accelerated conditions i.e. 60°C in sealed glass vials and 40°C/75% RH in open glass vials (punctured to enable exposure to RH conditions). These samples were then examined against a control samples kept at 4°C after 30 days.

3.3.2. Chemical Compatibility Studies

Minoxidil and each of excipients were mixed in 1:1 and placed in glass vials. The glass vials were sealed and placed in stability chamber at 40°C and 75% RH for one month. The FT-IR spectra was taken and analyzed for any shift in major peaks of the Minoxidil.

3.3.3. Analytical Methodology for the Drug

In order to evaluate the Minoxidil quantitatively, analytical methodology using spectrophotometric method was carried out.

4. Characterization of Glycerosomes

4.1. Particle Size Analysis by Beckman Coulter

The particle size of the prepared Glycerosomes was measured by dynamic light scattering particle size analyzer (Beckman Coulter). The prepared formulations were diluted with deionised water and sonicated for 1 min with an ultra-sound probe before measurement.

4.2. Surface and Shape Analysis by Transmission Electron Microscopy:

The shape and surface characteristics of Glycerosomes were analyzed by Transmission Electron Microscope (H-7500 Hitachi, Japan). The sample was diluted with HPLC water and stain of Phosphotungstic acid (PTA 1%) was prepared. Then one TEM grid was placed on wax sheet and one drop of diluted sample was put on TEM grid. After some time, this TEM grid was stained by using PTA stain and TEM grid was placed in sample holder to analyze samples under Transmission Electron Microscope. Glycerosomes size is critical not only in determining the efficacy of the therapeutic agent by affecting tissue penetration or even intracellular uptake.

4.3. Zeta Potential Analysis

Surface charges of all the Glycerosomal formulations were determined by zeta sizer. Zeta potential (Mv) of all the formulations was measured by laser doppler electrophoresis at the same concentration as used for particle analysis (Zetasizer, Beckman Coulter).

4.4. Drug Entrapment Efficiency (%)

0.2 ml of Glycerosome suspension (undiluted) was drop wise applied on the top of the gel bed in the center. Columns were centrifuged at 3000 rpm for 3 min to expel and remove void volume containing Glycerosomes in to the centrifuge tube. Elutes was removed and 0.2 ml saline was applied to each column, and centrifuged as previously. Amount of drug entrapped in the Glycerosomes was then determined by lysing the vesicles by Triton X-100 (0.5% v/v) and assayed for drug content by using UV Visible spectrophotometer at 288.3 nm.

The percentage entrapment was calculated by the following equation:

$$Entrapment\ Efficiency = \frac{\textbf{\textit{observed drug content}}}{\textbf{\textit{Initial drug conent}}} \times 100.$$

4.5. *In vitro* Drug Release Studies [16]

Modified franz diffusion cell with a receiver compartment volume of 67 ml and effective diffusion area of 2 cm^2 was used for this study. *In vitro* drug study was performed by using egg membrane in phosphate buffer (pH 7.4). To perform *in vitro* drug release study, egg membrane was mounted horizontally on the receptor compartment of franz diffusion cell. The effective permeation area of donor compartment exposed to receptor compartment was 2 cm and capacity of receptor compartment was 30 ml. The receptor compartment was filled with 18 ml of phosphate buffer (pH 7.4) maintained at 37 ± 0.5°C and stirred by a magnetic bar at 100 rpm. Glycerosomal formulation equivalent to 2 mg drug was placed on the egg membrane and top of the diffusion was covered. At appropriate time interval 5 ml aliquots of the receptor medium was withdrawn and immediately replaced by an equal volume of fresh phosphate buffer (pH 7.4) to maintain sink conditions. The sample was analyzed spectrophotometrically at λ_{max} 288.3 nm.

4.6. Stability Studies

Storage stability of optimized formulation was carried for the short term by storing them in tightly closed container at different temperatures [4 ± 1°C and Room temperature]. Samples were analyzed for residual drug content after a period of 15, 30, 45, 60 and 90 days. Initial drug content was taken as 100% for each formulation.

The log % residual drug content vs. time graph was plotted for the optimized formulation in order to evaluate K (specific rate constant or degradation rate constant).

5. Result and Discussion

5.1. Preformulation Studies

5.1.1. UV Spectrum Analysis

i) Absorbance spectra of Minoxidil in 0.1 M HCl and NaOH: On scanning the UV Visible spectrum of Minoxidil in 0.1 M HCl over the prescribed range (200 to 400 nm), the drug exhibited two Maxima at 229.6, 281.9 nm. On scanning the UV Visible spectrum of Minoxidil in 0.1 M NaOH over the prescribed range (200 to 400 nm), the drug exhibited three maxima at 229.6, 262.4, 288.3 nm.
ii) Absorbance spectrum of Minoxidil in Phosphate buffer of pH 7.4: On scanning the UV Visible spectrum of Minoxidil in Phosphate buffer of pH 7.4 over the prescribed range (200 to 400 nm), the drug exhibited three Maxima at 229.5, 262.8, 288.3 nm. All results are shown in Figure 1.

From these peaks we have selected **288.3 nm** because at this peak linearity was observed in the concentration range of 2-20 µg/ml.

5.1.2. FT-IR Spectral Analysis

Table 2. FT-IR Spectrum band assignments

Sr. No.	IR Bands (cm^{-1})	Assignment
1.	3470, 3445, 3420, 3365	NH(Stretching)
2.	3280, 3040	H-Bonded N-H
3.	2975, 2955, 2844	CH (Stretching Aromatic & Aliphatic)
4.	1644, 1618	C=N (Stretching Aromatic)
5.	1548, 1485, 1475, 1460, 1450	Aromatic C=C (Stretching N-H Bending)
6.	1260, 1248, 1202	N-O (Stretching Aromatic C-N Stretching)

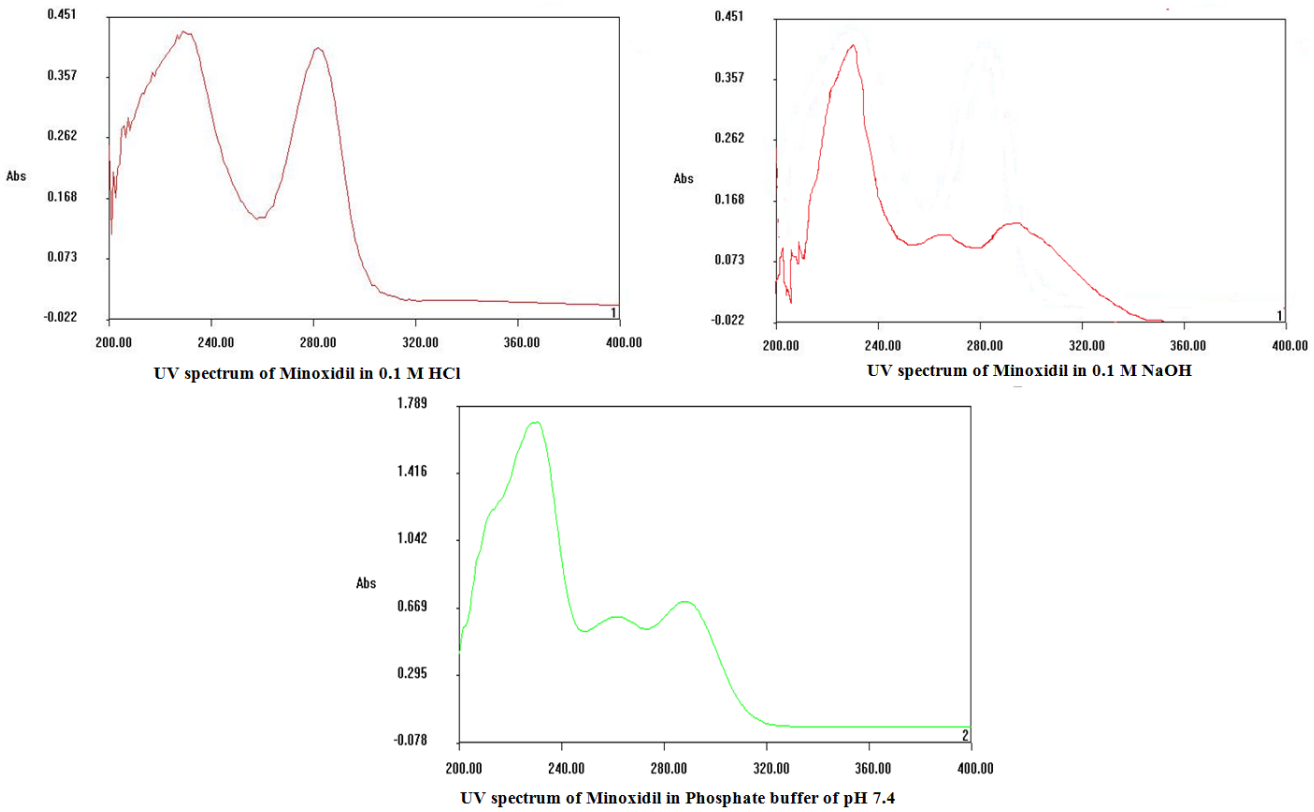


Figure 1. UV spectrum of Minoxidil in different medium

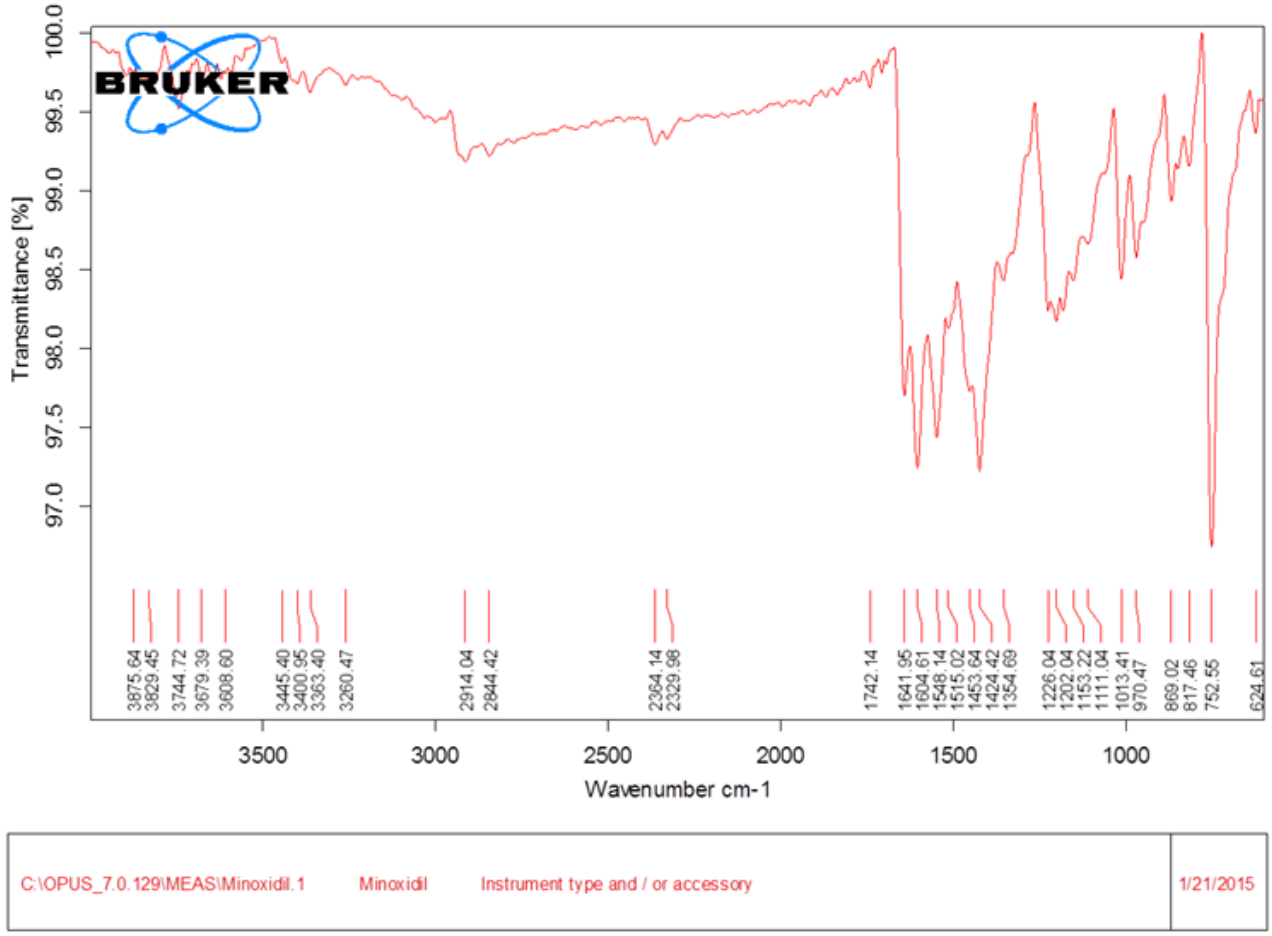


Figure 2. FTIR Spectrum of Minoxidil

The FT-IR spectrum of Minoxidil as recorded in Figure 2 was found in accordance with the FT-IR of standard Minoxidil as shown in Figure 3. Band assignments for the observed spectrum are comparable with standard Minoxidil which indicate identification and summarized in Table 2.

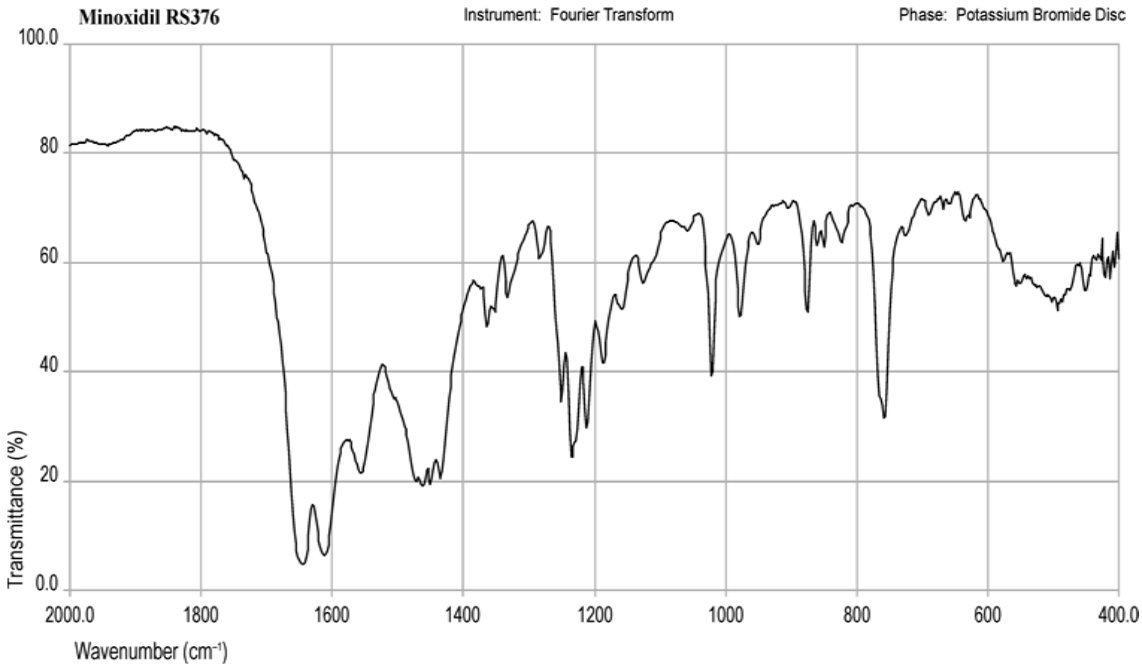


Figure 3. Reference spectrum of Minoxidil from BP

5.1.3. Melting Point

The melting point of drug was found to be 246°C, which is accordance with the standard range of melting point of Minoxidil.

5.2. Partition Coefficient

The standard and observed partition coefficient of Minoxidil is tabulated in Table 3.

Table 3. Standard and observed partition coefficient of Minoxidil

Standard (64)	Observed
Log P-1.24	Log P-1.29

5.3. Drug Excipient Compatibility Study

5.3.1. Physical Compatibility Study

The ratios for physical mixture of drug and the excipients were selected on the basis of probable concentration of the excipients in the formulation. The physical changes were observed for drug and excipients compatibility study. The data obtained is shown in Table 4, indicating model drug and all the excipients are compatible.

Table 4. Compatibility study of the drug with different excipients

Sr. no.	Parameter	Ratio	Initial	Control sample (30 days)	40°C/75% RH – Open (30 days)	40°C/75 % RH – Closed (30 days)	60°C Close (30 days)	60°C Open (30 days)
1	API	Control	✓	✓	✓	✓	✓	✓
2	Glycerol	Control	✓	✓	✓	✓	✓	✓
3	Lecithin	Control	✓	✓	✓	✓	✓	✓
4	Cholesterol	Control	✓	✓	✓	✓	✓	✓
5	API+ Cholesterol	1:1	✓	✓	✓	✓	✓	✓
6	API+ Lecithin	1:1	✓	✓	✓	✓	✓	✓
7	API+ Lecithin+ Cholesterol	1:1:1	✓	✓	✓	✓	✓	✓

Note: ✓ indicates no interaction.

5.3.2. Chemical Compatibility Study

FT-IR studies were performed to detect the possible interactions between Minoxidil, cholesterol and Lecithin. The FT-IR spectrums are being shown in Figure 4, Figure 5, Figure 6, Figure 7, and Figure 8. The characteristic peaks of Minoxidil, cholesterol, Lecithin and physical mixture are presented in Table 5. It was revealed that there were no shifting in the peaks of the absorption bands, hence providing evidence for the absence of interactions in the solid state between Minoxidil and excipients.

Table 5. FT-IR peaks of Minoxidil, Lecithin, Cholesterol and their physical mixture

Sr. No.	Compounds	IR Bands
1.	Minoxidil	3445.40, 3260.47, 1644.95, 1604.61, 1226.04, 1202.04, 752.55
2.	Lecithin	3646.43, 1868.29, 1741.29, 1513.60, 1462.19, 1153.22, 856.56, 691.47
3.	Cholesterol	3285.61, 1135.67, 1052.30, 1018.28, 953.82, 832.53, 802.77
4.	Minoxidil+ Lecithin	3418.67, 3266.63, 3646.43, 1869.07, 1677.04, 1615.69, 1547.90, 1515.91, 1222.97, 851.05, 750.17
5.	Minoxidil+ Cholesterol	3248.69, 1676.15, 1546.92, 1210.97, 1136.97, 1063.52, 987.17, 937.49, 839.98, 740.97

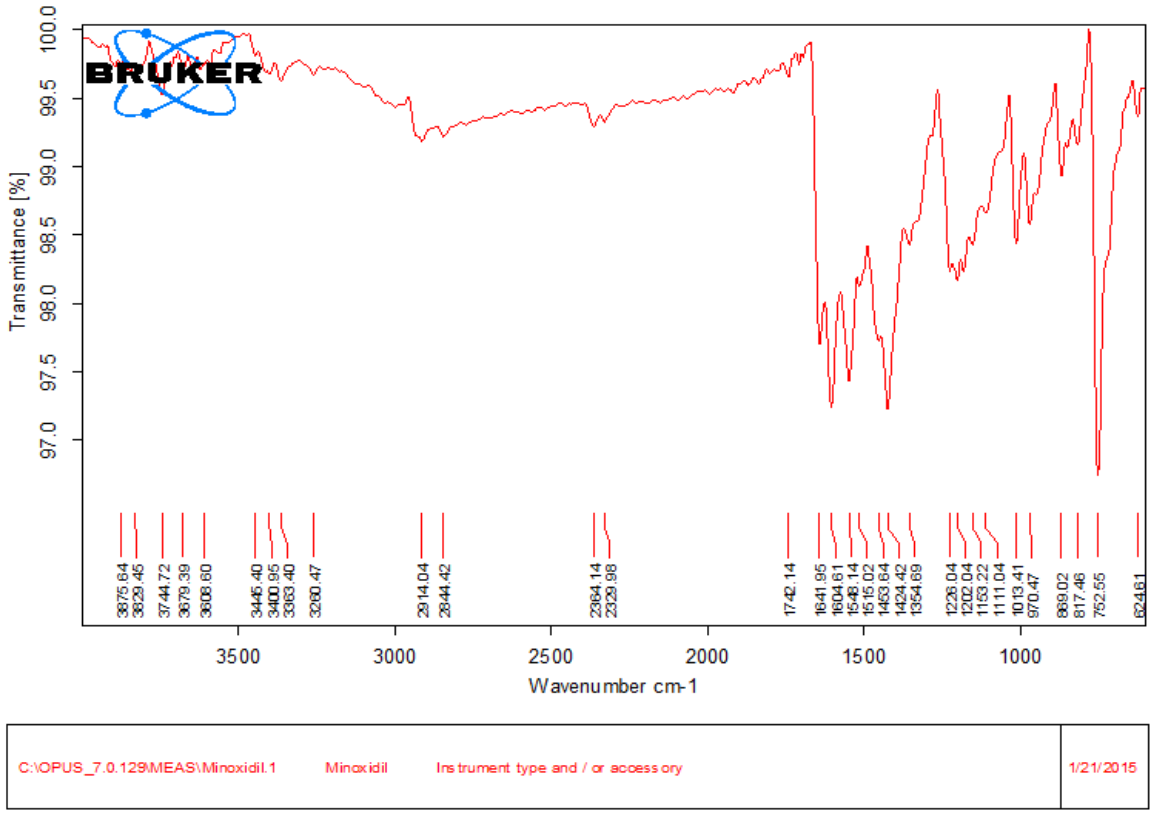


Figure 4. FTIR Spectrum of Minoxidil

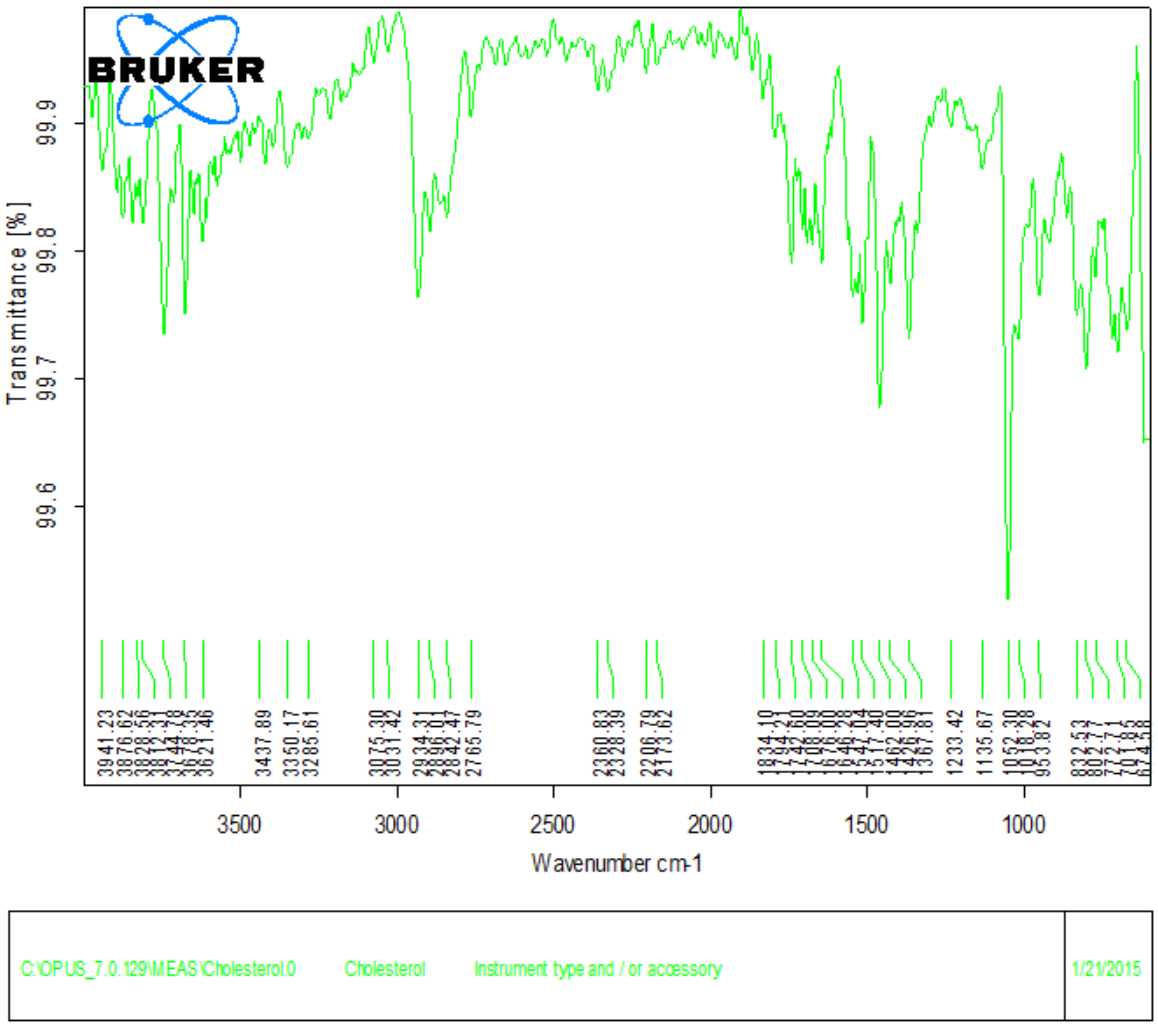


Figure 5. FTIR Spectrum of Cholesterol

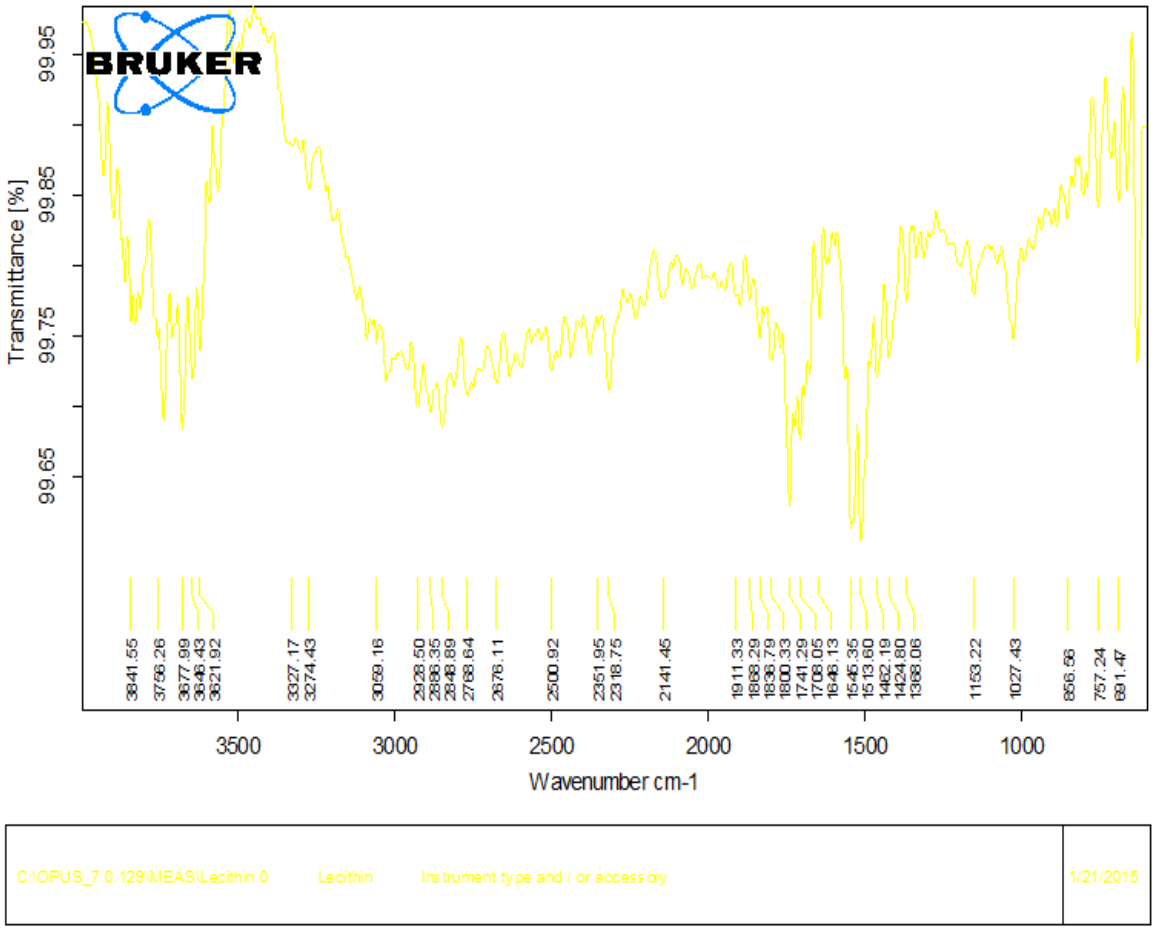


Figure 6. FTIR Spectrum of Lecithin

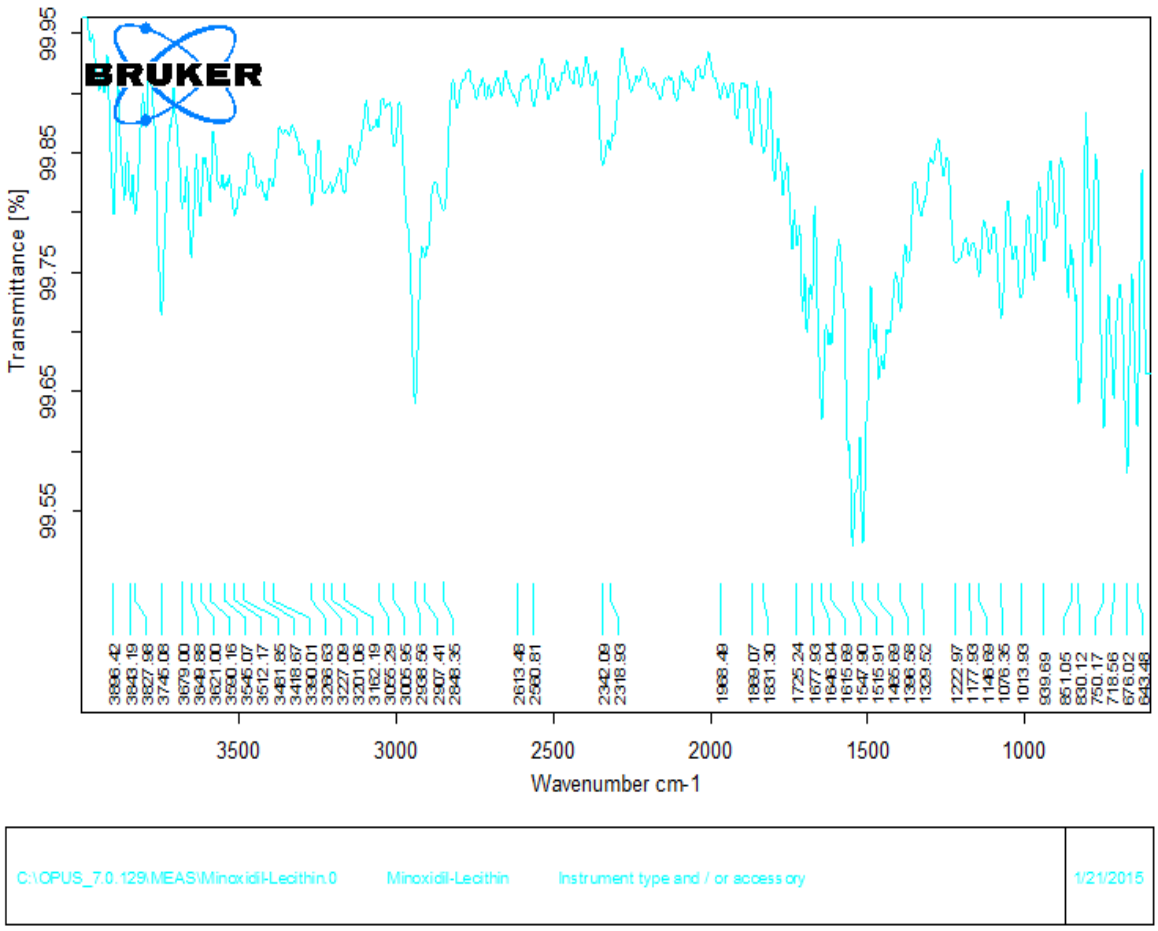


Figure 7. FT-IR spectrum of Minoxidil + Lecithin

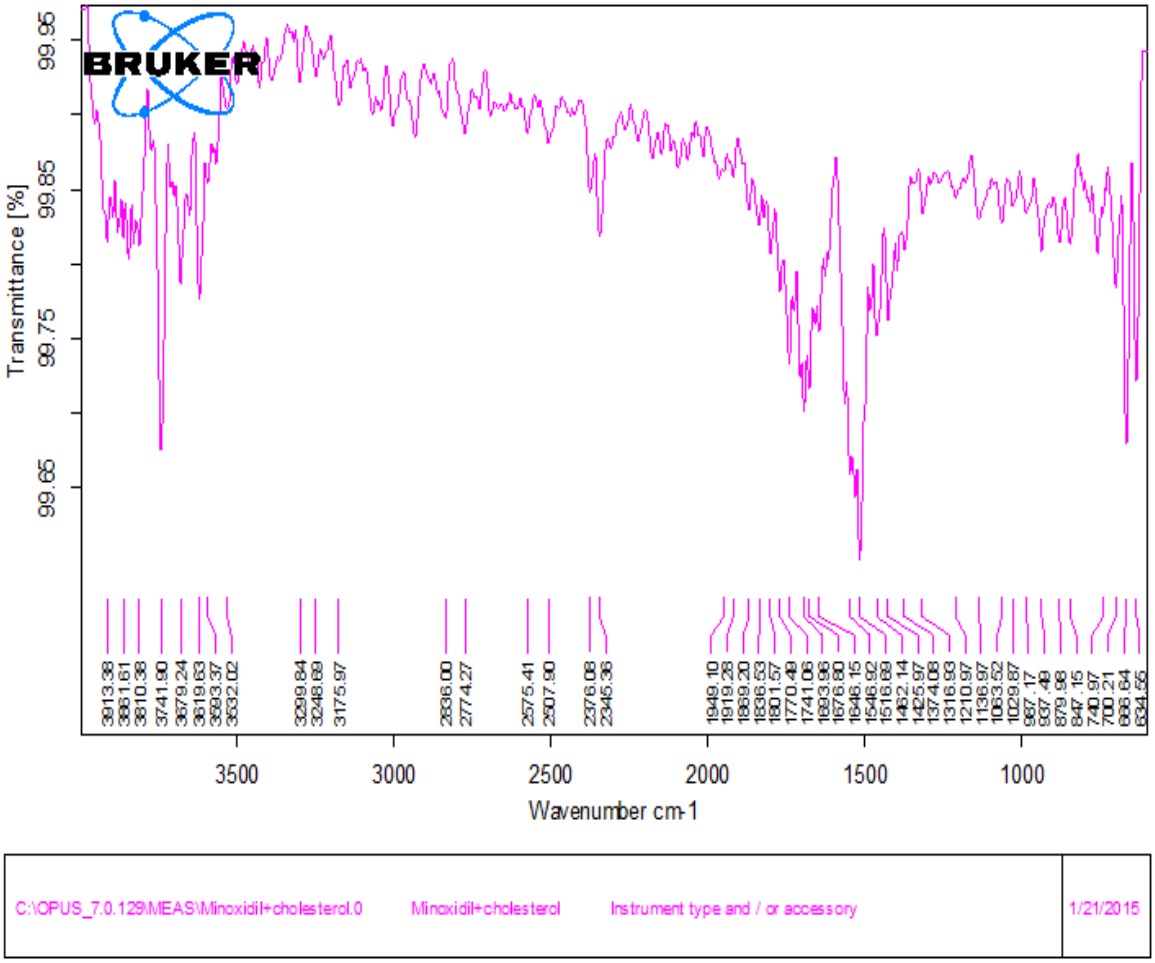


Figure 8. FT-IR spectrum of Minoxidil + Cholesterol

5.4. Standard calibration curve of model drug in Phosphate buffer of pH 7.4

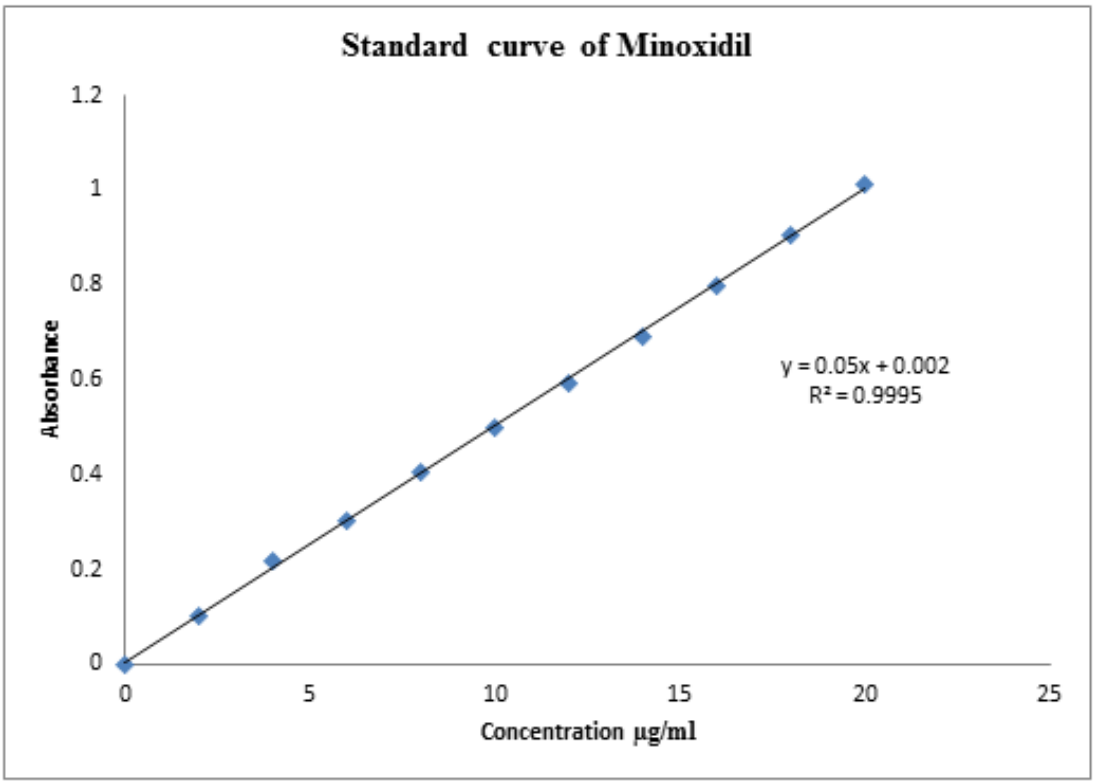


Figure 9. Standard curve of Minoxidil

6. Characterization of Glycerosomes

6.1. Particle size analysis by Beckman Coulter

Particle size analysis is performed by Beckman Coulter and average size of particles is reported as 121.58 nm as shown in Figure 10.

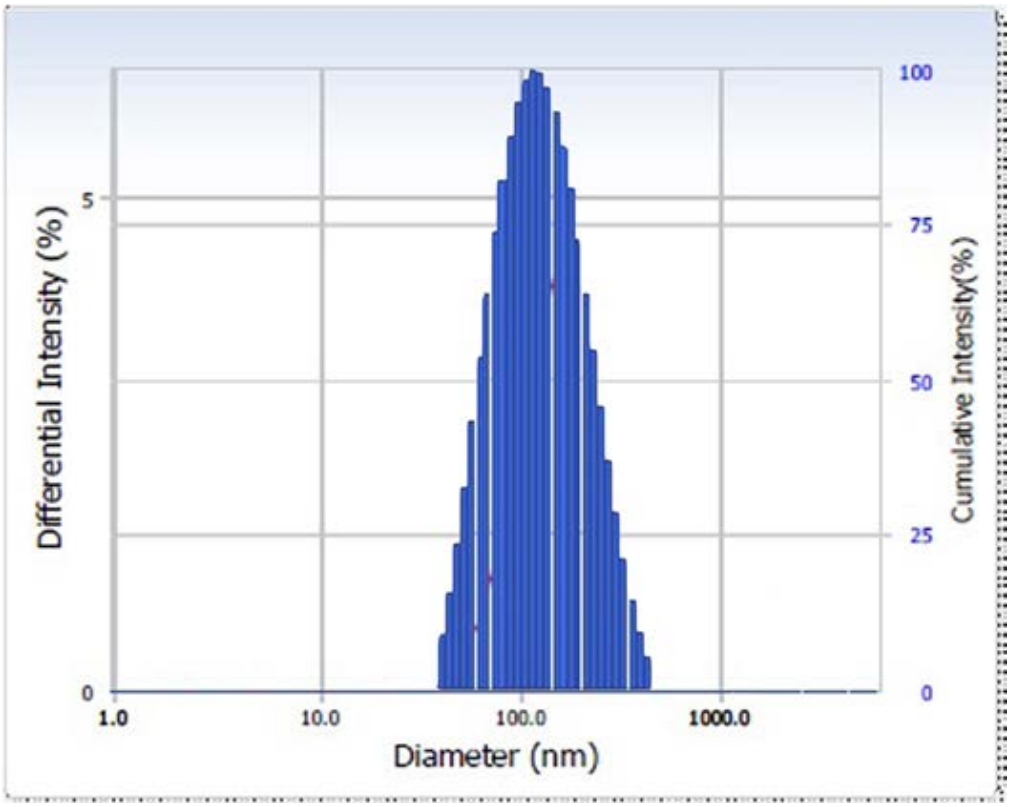


Figure 10. Particle size distribution

6.2. Surface Analysis and Shape by Transmission Electron Microscopy:

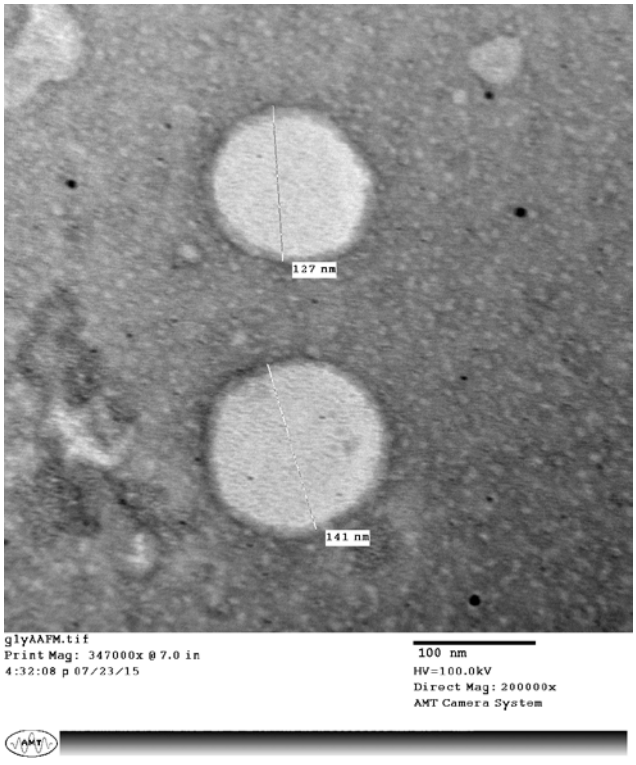


Figure 11. TEM photograph of Minoxidil loaded Glycerosomes

Surface morphology of the Glycerosomes was examined by TEM as shown in Figure 11. Glycerosomes were observed spherical, smooth, vesicular in nature, and morphologically similar without agglomerations.

6.3. Zeta Potential Analysis

Zeta potential analysis was performed by Beckman Coulter, zetasizer and average Zeta potential was reported as -4.5 (mV) as shown in Figure 12.

6.4 Entrapment Efficiency:

Entrapment efficiency of nine formulations (F1-F9) is summarized in Table 6.

Table 6. Entrapment efficiency of Glycerosomes

Batch Code	Entrapment Efficiency (%) ± S.D.
F1	81.45 ± 0.42
F2	79.62 ± 1.24
F3	76.39 ± 1.19
F4	74.61 ± 0.27
F5	72.91 ± 0.86
F6	69.49 ± 1.38
F7	**88.41 ± 0.57**
F8	84.94 ± 1.08
F9	81.42 ± 1.48

BECKMAN COULTER

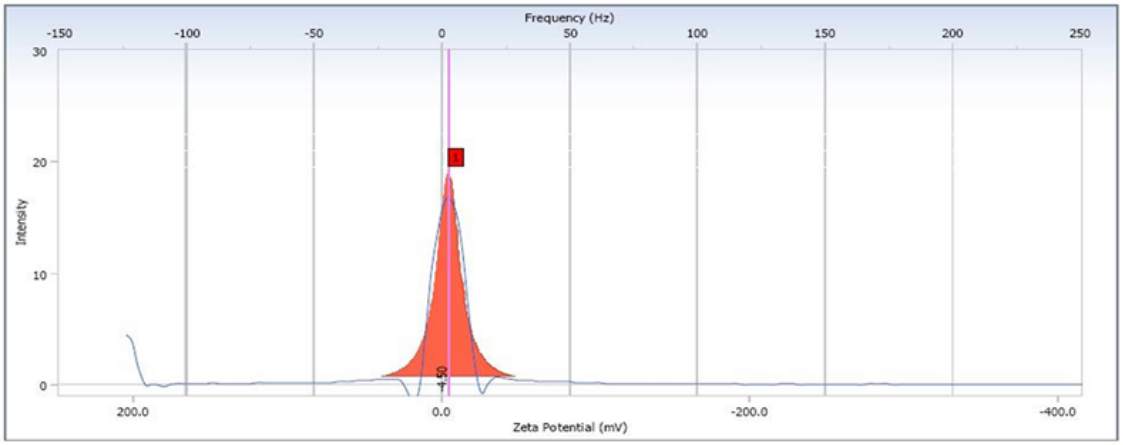
Delsa™ Nano
Common

Distribution Graph S/N : 141612

User	: Common	Group	:	Repetition	: 1/1
Date	: 7/23/2015	File Name	: deepika zeta		
Time	: 13:38:30	Sample Information	:		
SOP Name	: ZETA			Security	: No Security

Version 3.73 / 2.30

Mobility Distribution



Measurement Results

Zeta Potential	: -4.50	(mV)	Doppler shift	: 2.72	(Hz)
Mobility	: -3.513e-005	(cm²/Vs)	Base Frequency	: 121.0	(Hz)
Conductivity	: 2.0462	(mS/cm)	Conversion Equation	: Smoluchowski	

Zeta Potential of Cell			Diluent Properties		
Upper Surface	: -3.80	(mV)	Diluent Name	: WATER	
Lower Surface	: -6.36	(mV)	Temperature	: 25.0	(°C)
Cell Condition			Refractive Index	: 1.3328	
Cell Type	: Flow Cell		Viscosity	: 0.8878	(cP)
Avg. Electric Field	: -16.03	(V/cm)	Dielectric Constant	: 78.3	
Avg. Current	: -1.64	(mA)			

Peak Data Table of Distribution Graph

Peak	Frequency (Hz)	Intensity	Half Width (Hz)	Zeta Potential (mV)	Mobility (cm²/Vs)
1	2.72	18.94	5.18	-4.50	-3.513e-005
2					
3					
4					
5					

Figure 12. Zeta potential analysis

6.5. *In vitro* Drug Release Study:

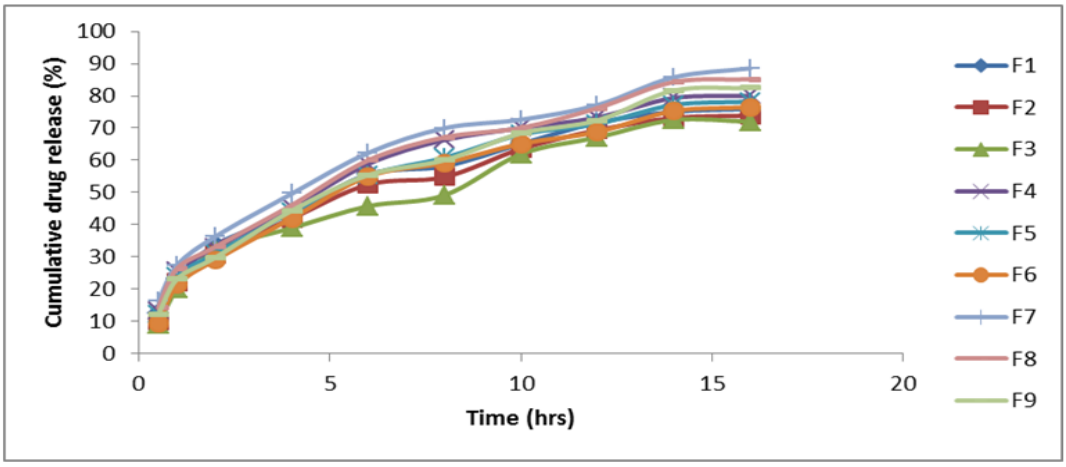


Figure 13. *In-vitro* drug release profiles of Glycerosomes

6.6. Drug Release Kinetics:

Table 7. Regression coefficient (R2) obtained from various kinetics models

Batch code	Zero Order Kinetics	Higuchi Kinetics	Korsmeyer Peppas Kinetics	First Order Kinetics
F1	0.873	0.978	0.975	0.746
F2	0.881	0.981	0.982	0.790
F3	0.892	0.975	0.962	0.714
F4	0.875	0.984	0.975	0.747
F5	0.883	0.986	0.978	0.722
F6	0.881	0.983	0.963	0.687
sF7	**0.887**	**0.99**	**0.988**	**0.784**
F8	0.899	0.991	0.981	0.772

The interpretation of data was based on the values of the resulting regression coefficients the formulation follows Fickian diffusion indicating anomalous transport. So as observed R^2 value of Higuchi model is more as compared to other models. Hence, all Glycerosomes formulations follow Higuchi release kinetics (Table 7).

7. Conclusion

In this study, a new vesicular carrier containing different amount of glycerol, has been developed and characterized, which exhibit many features for dermal application of cosmetic and pharmaceutical products, such as, the controlled release and targeting of drugs, occlusion associated with penetration enhancement, increase of skin hydration and excellent tolerability.

Morphological investigations showed that all vesicles exhibit a spherical shape, with a mean diameter of 121.58 nm. Selection of the appropriate experimental conditions result in the production of Minoxidil loaded Glycerosomes having high entrapment efficiency (88.41 ± 0.57% w/w) and high cumulative percent drug release (88.54 ± 0.23% w/w) at 16^{th} hour.

Over three month of investigation on stability at 4±1°C and room temperature, formulation shows the faster degradation at higher temperature. The results indicate that the ideal storage temperature is a cold place.

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