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Formulation and Evaluation of Elastic Liposomes of Clotrimazole

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Abstract

The objective of present study is to develop and evaluate the Elastic liposomes of Clotrimazole so as to provide the sustained release and to enhance the anti-fungal activity of the drug. Elastic liposomes were prepared by rotary evaporation method using Span 80 as an surfactant. The prepared Elastic liposomes were evaluated for entrapment efficiency, vesicle size, vesicle size distribution, no. of vesicles, stability study, *in-vitro* drug release, *ex-vivo* skin permeation study and microbiological study. The entrapment efficiency of resultant optimized Elastic liposomes was 73.5%±1.61, vesicle size (100nm), vesicle size distribution (in the normal size range of 1-10µm), models of *in-vitro* drug release confirmed sustained release and non-fickian diffusion, *ex-vivo* studies confirmed higher skin permeation and morphological studies confirmed enhanced anti-fungal activity of optimized Elastic liposomes.

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Key words:
Elastic liposomes, Span 80, Sustained release, Anti-fungal activity

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INTRODUCTION

Transdermal drug delivery is a non-invasive, user-friendly delivery method for therapeutics. However, its clinical use has found limited application due to the remarkable barrier properties of the outermost layer of skin, the stratum corneum (SC) [1,2,3]. Physical and chemical methods have been developed to overcome this barrier and enhance the transdermal delivery of drugs. One of such techniques was the use of microneedles to temporarily compromise the skin barrier layer. This method combines the advantages of conventional injection needles and transdermal patches while minimizing their disadvantages. As

compared to hypodermic needle injection, microneedles can provide a minimally invasive means of painless delivery of therapeutic molecules through the skin barrier with precision and convenience. The application of transdermal delivery to a wider range of drugs is limited due to the significant barrier to penetration across the skin which is allied primarily with the outermost stratum corneum layer of the epidermis. The barrier nature of skin inhibits the penetration of most drugs. The use of lipid vesicles in a delivery system for skin treatment has attracted increasing attention in recent years, but it remains controversial. Most relevant reports cite the localization effect of liposomes with transport processes reported in a few cases, depending on the formulation. In recent years, there has been an increasing interest in the development of novel vesicular approaches for effective transcutaneous immunization. Vesicular carriers i.e. Elastic liposomes, conventional liposomes, niosomes etc., elicit immune response by different mechanisms. [4,5]. Some lipids directly lower the skin permeability barrier, which resides primarily in the stratum corneum. The most recent development in vesicle design for transcutaneous bioactive delivery is the use of Elastic liposomes, which differ from conventional liposomes and niosomes by their characteristic fluid membrane with high Elasticity. In Elastic liposomes, Elasticity is stress controlled, owing to the composition dependence of the membrane bending energy [6]. Elastic liposomes passage through the normally confining pores is then governed by the basic principles of elastomechanics.

Elastic lipsomes have been defined as specially designed vesicular particles, consisting of at least one inner aqueous compartment surrounded by a lipid bilayer with appropriately tailored properties. Accordingly, Elastic liposomes resemble lipid vesicles, liposomes, in morphology but functionally, Elastic liposomes are sufficiently deformable to penetrate pores much smaller than their own size. They are metastable, which makes the vesicle membrane ultraflexible and thus the vesicles are highly deformable. Thus the present study were to refine the formulation of Elastic liposomes of Clotrimazole that will release the drug over a prolonged period of time i.e. sustained release, enhanced anti-fungal activity [7,8.9].

2. MATERIAL AND METHOD:

2.1 Material

Clotrimazole was received as a gift sample from Ind-swif Pharmaceuticals Pvt. Ltd, Soya phosphatidylcholine Hi media Ltd, Mumbai), Span 80, Cholesterol, Choloroform, Ethanol (S.D fine chemical Ltd.), Methanol (E.Merck Ltd.)All the reagents and solvents used were of analytical grade satisfying pharmacopoeial standards.

2.2 Preparation of Elastic liposomal formulations

The Elastic liposomes were prepared by rotary evaporation method. Different batches of Elastic liposomes were prepared using different proportions of surfactant, phospholipids and drug. The accurately weighed amounts of phospholipids and surfactant were taken in a clean, dry, round-bottom flask and this lipid mixture was dissolved in small quantity of chloroform-methanol mixture (2:1). The organic solvent was removed by rotary evaporation under reduced pressure at 40°C. Final traces of solvents were removed under vacuum overnight. The deposited lipid film was hydrated with 7 % v/v ethanol i.e. solution of drug by rotation at 60 rev/min for 1 hr.

The resulting vesicles were swollen for 2 h at room temperature, these were bath sonicated for 10 min. Compositions of different batches of Elastic liposomes as shown in Table 1.

Table 1: Compositions of different Elastic liposomal formulations.

S. No.	Formulation code	*Soya PC (mg)	Span 80 (mg)	Solvent (ml)	
				Chloroform	Methanol
1	EL-SP1	95	5	10	5
2	EL-SP2	90	10	10	5
3	EL-SP3	85	15	10	5
4	EL-SP4	80	20	10	5
5	EL-SP5	75	25	10	5

Soy PC - Phosphatidylcholine
EL-SP - Elastic liposomal formulation containing different concentrations of Span80

2.3 Drug loading in Elastic liposomal formulation

To determine the maximum amount of drug that can be added in vesicles, increasing amounts of Clotrimazole (4, 6, 8, 10 and 12mg) was added during the preparation of Elastic liposomal formulations. All the drug loaded vesicular formulations were examined for maximum entrapment efficiency and for the appearance of drug crystals over a period of 14 days using optical microscope [10].

2.4 Characterisation of Elastic liposomal formulation

2.4.1 Entrapment Efficiency

For determination of entrapment efficiency the unentrapped drug was separated by the use of the minicolumn centrifugation method. The amount of drug entrapped (Total amt. of drug- unentrapped drug) in the vesicles was then determined by disrupting the vesicles using methanol followed by filtration and amount of drug was quantified spectrophotometrically.

$$\text{Percentage entrapment} = \frac{\text{Entrapped drug } (\mu g) \times 100}{\text{Total drug added } (\mu g)}$$

2.4.2 Vesicles Size

Elastic liposomal vesicles were visualized by using Moragagni 268D TEM with an accelerating voltage of 100 kV. A drop of the sample was placed onto a carbon-coated copper grid to leave a thin film on the grid. The grid was allowed to air dry thoroughly and the samples were viewed on a transmission electron microscope [11].

2.4.3 Vesicle size distribution

The vesicle size before sonication was determined by optical microscopy using stage eyepiece micrometer calibrated using micrometer scale. A total of 100 particles per batch were counted for their size [12] . After sonication the vesicle size was determined by dynamic light scattering method using particle size analyzer.

2.4.5 Number of vesicles per cubic mm

Elastic liposomal formulation (without sonication) was diluted five times with 0.9 % NaCl solution and number of Elastic liposomes per cubic mm was counted by optical microscopy using haemocytometer. The Elastic liposomes in 64 squares were counted and number of vesicles per cubic was calculated.

2.4.6 *In vitro* drug release studies through cellophane membrane

In-vitro drug release studies were carried out in modified Diffusion cell using Dialysis membrane (Himedia laboratories Pvt Ltd: dry, unwashed, open ended; flat width: 28.46mm; inflated diameter: 17.5mm; Length: 1m). The membrane was soaked in Phosphate buffer pH 6.8 for 9-12 hours and it was clamped carefully to one end of the hollow glass tube of dialysis cell (2.3 cm diameter; 4-16 cm^2 area). Then Elastic liposomal gel was spread uniformly on the dialysis membrane. 50 ml of Phosphate buffer pH 6.8 was taken in a beaker, which was used as receptor compartment. The donor compartment was kept in contact with the receptor compartment. This whole assembly was kept on a magnetic stirrer and the solution on the receptor side was stirred continuously using a magnetic bead. The temperature of the cell was maintained at 37°C. Sample (5 ml) was withdrawn at 1 hour time intervals up to 24 hours and replaced with equal amounts of fresh dissolution media.

Samples were analysed spectrophotometrically at 26

0 nm and % Cummulative drug release was calculated.

2.4.7 Skin permeation studies

Ex-vivo drug permeation study was carried out in a modified Diffusion cell, using rat skin. A section of skin was cut and clamped carefully to one end of the hollow glass tube of dialysis cell (2.3 cm diameter; 4-16 cm^2 area) keeping the dorsal side upward. Then Elastic liposomal gel was spread uniformly on the skin. Phosphate buffer pH 6.8 was used as dissolution media. The donor compartment was kept in contact with receptor compartment. This whole assembly was kept on a magnetic stirrer and the solution on the receptor side was stirred continuously using a magnetic bead and temperature of the cell was maintained at 37°C. Sample (5 ml) was withdrawn at 1 hour time intervals up to 24 hours and replaced with equal amounts of fresh dissolution media. Samples were analyzed spectrophotometrically at 260 nm and % drug permeated was calculated.

2.4.8 Stability studies

Stability is technically defined as the capacity of particular formulation in a specific container/closure system, to remain within its physical, chemical and therapeutic specification. The optimized Elastic liposomal formulations were stored in glass vials at room temperature and in refrigerator (4±2°C) for 60 days [16].

2.4.9 Microbiological assay

The microbiological assay was done to determine the activity of prepared formulation with the strain *Candida albicans* and the Sabouraud agar medium Ditch plate technique was used evaluation of bacteriostatic or fungistatic activity of a compound. It is mainly applied for semisolid formulations. Previously prepared Sabouraud's agar dried plates were used one gram of Elastic lipoosomal gel were placed in a ditch cut in the plate. After incubation for 18 to 24 hours at 37 °C . Then the % inhibition was measured as follows [17, 18, 19].

% Inhibiton=

$$\frac{L2}{L1} \times 100$$

L1- Total length of the streaked culture.
L2- Length of inhibiton.

2.5 RESULTS:

Different batches of Elastic liposomes were prepared with Span 80 and Phosphatidylcholine using rotary evaporation method. Span 80 was selected as edge activator surfactant because it is biocompatible and pharmaceutically acceptable. Phosphatidylcholine was used as bilayer forming agent. In the present study, 7% v/v ethanol was used as hydrating agent because ethanol is known to extract stratum corneum lipids and alter the barrier property of intracellular lipoidal route, thereby allowing higher drug permeation.

Drug loading was carried (EL-SP3 arbitrary selected) out to check how much maximum drug can be loaded to the Elastic liposomal formulation. Result mentioned in the Table 2 and Fig-1-2 indicated that no crystal appeared in the formulation up to 10 mg and crystals appeared in 12 mg formulation. Results indicated that 10 mg drug can be maximum loaded to the Elastic liposomal formulation.

Table 2: Drug Loading in Elastic Liposomal Formulations

Sr. No.	Amount of drug (mg)	Observations
1.	4	CNA
2	6	CNA
3	8	CNA
4	10	CNA
5.	12	CA

CNA- crystals not appeared
CA- crystals appeared

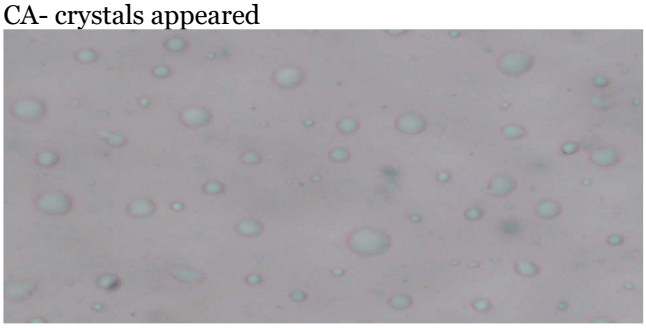

Fig-1: Photomicrograph of Elastic liposomes(EL-SP3)

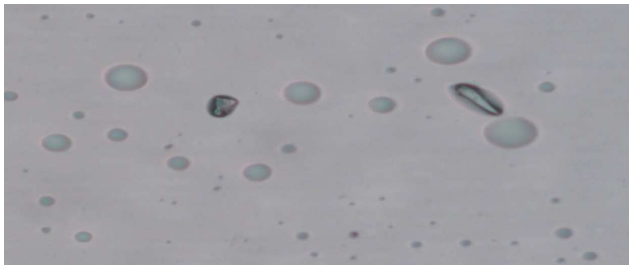

Fig-2: Photomicrograph of EL-SP3(12mg) with crystals

Elastic liposomes were evaluated by various parameters like Vesicle shape, Vesicle size, Entrapment Efficiency, No. of vesicles, *In-vitro* drug release, *Ex-vivo* skin permeation study, Stability study and microbiological assay.

Entrapment Efficiency of different Elastic liposomal formulations was investigated using minicoloum centrifugation method. Results are shown in (Table 3). Formulation EL-SP (85:15) has maximum entrapment efficiency, so it was selected for further evaluation parameters and activity. The effect of surfactant concentration on the entrapment efficiency of Clotrimazole has been shown diagrammatically in Fig-3.

Table 3: Entrapment efficiency of different formulations

Formulation Code	Surfactant concentration (% w/w)	Entrapment efficiency (%)
EL-SP1	5	56.6±1.54
EL-SP2	10	63.6±2.10
EL-SP3	15	73.5±1.61
EL-SP4	20	48.8±2.68
EL-SP5	25	45.9±2.87

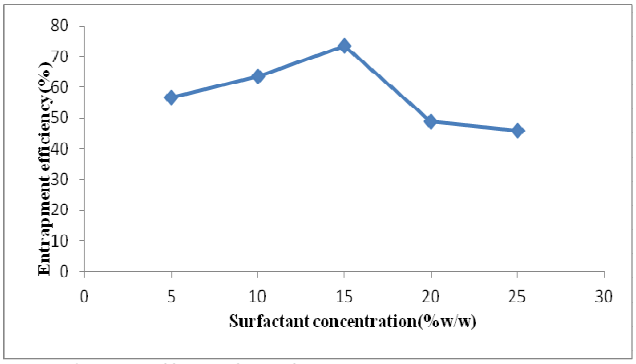

Fig-3: Effect of Surfactant Concentration on Entrapment Efficiency of Different Formulation

Vesicle size of optimized EL-SP3 formulation was found to be 100nm (as shown in Fig-4) i.e. in the normal size range of 1 to 10µm (1-10,000 nm) that was previously determined using TEM. For better skin permeation the vesicle size must be of 100-400 nm.

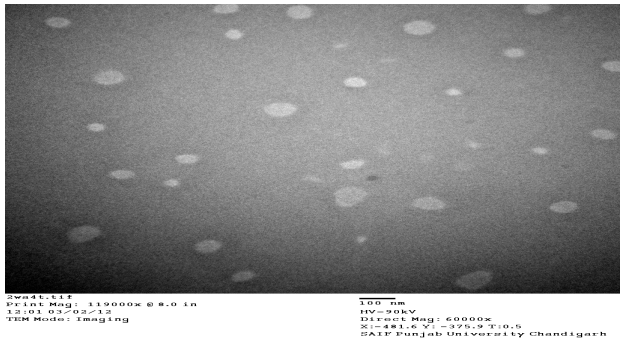

Fig-4: TEM image of EL-SP3

Vesicle size distribution is required to check how much % intensity of particles of Elastic liposomes distributed in the size range. Fig-5 clearly showed that % intensity of particles distributed were in the normal size range (1-10,000 nm).

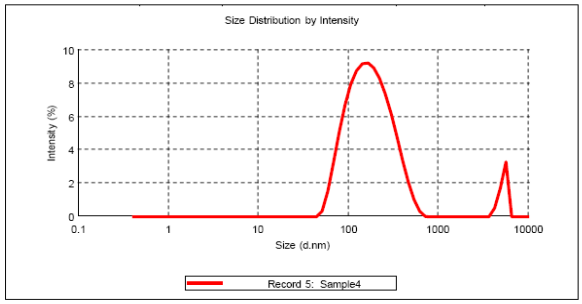

Fig-5: Particle Size distribution of optimized Elastic Liposomal Formulation (EL-SP3)

Number of vesicles per cubic mm is important parameter for optimizing the composition and other process variables. Number of vesicles per cubic mm was determined using haemocytometer. It was observed that with increase in the concentration of surfactant up to optimum level (EL-SP3) there is decrease in the number of vesicles in regular manner, but after the optimum level it started decreasing drastically as shown in Table 4.

Table 4: No. of vesicles of different formulations

Formulation Code	Surfactant concentration (% w/w)	No. of vesicles per cubic mm X 1000
EL-SP1	5	45.3±1.33
EL-SP2	10	42.9±1.67
EL-SP3	15	42.7±1.4
EL-SP4	20	33.4±2.17
EL-SP5	25	24.8±1.85

Formulation EL-SP3 was optimized and found to be suitable for further studies. Gel was prepared (composition as shown in Table 5) for the optimized batch and then Elastic liposomal gel, liposomes gel was subjected to *in vitro* drug release studies using cellophane membrane. After comparing the release rate of Elastic liposomal gel and liposomal gel. In comparison at 6 hr, about 16.47±0.44% and 21.72±0.15% of Clotrimazole was released from the Elastic liposomal gel (EL-SP3) and from liposomal gel respectively. The % release of EL-SP3 gel after 24 hrs was found to be 62.11%±0.10 which was less as comparison liposomal gel. The results indicated that EL-SP3 gel showed sustained release after 24 hrs (i.e. less % age drug release after 24hrs) as graphically shown in Fig-6.

Table 5: Composition of Elastic liposomal gel

Elastic liposomes gel ingredients	
Elastic liposomes	Eqv. to 10% of drug
Carbopol 934	1%
Distilled water	q.s

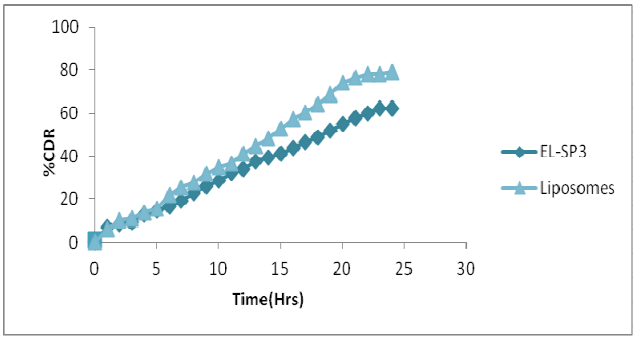

Fig-6: *Invitro* drug release of EL-SP3, Liposomes across cellophane membrane

The *ex-vivo* skin permeation of Clotrimazole from different formulations was studied using locally modified diffusion cell. In comparison at 6 hr, about 15.6%±0.26 and 15.2±0.40% of Clotrimazole was permeated from the Elastic liposomal gel (EL-SP3) and from liposomal gel respectively. The results indicated that after 24 hrs % drug permeation for EL-SP3 gel was 79.51±0.37 i.e. more as comparison to liposomal gel (63.18±0.64).

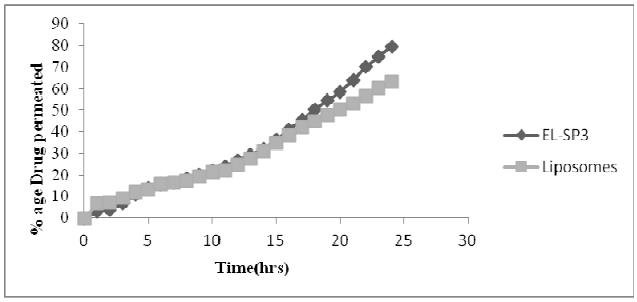

Fig-6: Ex-vivo skin permeation of EL-SP3 gel, Liposomes gel across rat skin

The dissolution models were applied to determine the kinetics of optimized El-SP3 formulations and the data obtaines as shown in Table 6. According to data the value of R^2 for zero order model was found to be higher than that of higuchi, first order model and korsermeyer peppas model. So, the EL-SP3 Optimized formulation confirmed to show sustained zero order release. In case of korsermeyer peppas the value of n found to be 0.487 i.e. the drug follows non-fickian diffusion (0.45<n<0.89)

Table 6: Regression analysis (r^2) of release data based on best curve-fitting method for optimized EL-SP3 formulations.

Formulation	Zero order		First order		Higuchi		Korsemeyer peppas	
	n	R^2	n	R^2	n	R^2	n	R^2
EL-SP3	2.142	0.9996	2.018	0.9985	12.86	0.9888	0.4873	0.9903

The optimized Elastic liposomal formulations were stored in glass vials at room temperature and in refrigerator (4±2°C) for 60 days. Formulations were evaluated after every 20 days. No drug crystals were observed after 2 months storage at room temperature

and cold temperature and size remained in the range.

For determining %age minimum inhibitory concentration, microbiological assays were carried out to evaluate the anti-fungal activity of optimized EL-SP3 formulation (F1). Values are summarized in the Table 7 and graphically compared in Fig. 7. Results indicated the %MIC of EL-SP3 optimized formulation was found to be higher than that of marketed gel i.e. our optimized EL-SP3 formulation showed better anti-fungal activity as comparison to marketed gel.

Table 7: Comparison of % MIC

Formulation	MIC ± S.D
F1(Formulation)	52.9 ± 1.33
F2(Marketed gel)	40.1± 1.62

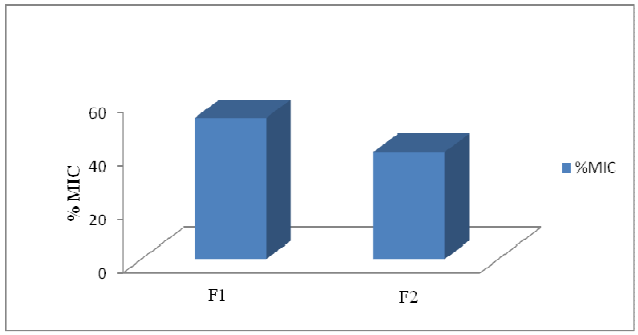

Fig-7 Comparison of %MIC

CONCLUSION:

Elastic liposomes of Clotrimazole were prepared by rotary evaporation method. The optimized Elastic liposomes (EL-SP3) have higher entrapment efficiency, vesicle size (100nm) and vesicle size distribution in the normal size range (1-10µm) , drug release and skin permeation studied up to 24 hours, models indicated optimized Elastic liposomes (EL-SP3) followed zero order sustained release and non-fickian skin diffusion, skin permeation studies indicated higher skin permeation and results indicated optimized Elastic liposomes (EL-SP3) have higher anti-fungal activity.

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