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NEOMYCIN SULPHATE ADMINISTRATION USING AN ULTRADEFORMABLE GEL FOR DEEPER SKIN INFECTIONS

[1]Ankit Dixit, [1]Anup Kumar Sirbaiya*, [2]Stuti Verma*

[1]Maharana Pratap School of Pharmacy, Gaura, Mohanlal Ganj Lucknow-226301.

[2]Sagar College of Pharmacy, NH-28, Lucknow Faizabad By Pass Road, near Rasauli, Barabanki Uttar Pradesh 225001

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Corresponding Author:
Anup Kumar Sirbaiya
Stuti Verma

Abstract

In the present study we have shown that elastic liposome of neomycin sulphate was highly efficient in eradicating S. aureus-induced intradermal infections. The treatment with elastic liposomes neomycin sulphate applied on the skin was much more efficient than the conventional hydro-gel. The clinical implications of the data presented here suggest the possibility of substituting conventional antibiotic treatment with this novel vesicular treatment. This would result in decreased drug exposure and the associated side-effects, thereby potentially increasing patient compliance. In addition to these possible therapeutic benefits, a rapid bacterial kill and short therapy courses with elastic liposomes of neomycin sulphate could ultimately result in reducing treatment costs and minimizing bacterial resistance. These findings may open new avenues for the treatment of deep dermal infections by local application of tailored antibiotic elastic liposomes. Elastic liposomes have been studied as a possible vehicle for deep dermal delivery of neomycin sulphate, an antibacterial agent. The study confirmed that elastic liposomes are a very promising carrier for the deep dermal delivery of neomycin sulphate as revealed from an enhanced antibacterial potency, skin penetration, transdermal flux, skin deposition, and higher entrapment efficiency.

Introductions

Elastic liposomes (Transfersomes)

The name means “carrying body”, and is derived from the Latin word 'transferre', meaning ‘to carry across’, and the Greek word ‘soma’, for a ‘body’. A transferosome carrier is an artificial vesicle designed to be like a cell vesicle or a cell engaged in exocytosis, and thus suitable for controlled and, potentially targeted, drug delivery. Term transferosome and the underlying concept were introduced in 1991 by Gregor Cevc. Numerous groups have since been working with similar carriers, frequently under different names (elastic vesicle, flexible vesicle, Ethosome, etc.) to describe them. In broadest sense, a Transfersome is a highly adaptable and stress-responsive, complex aggregate, Transfersomes penetration through the pores in stratum corneum, the outermost layer of the skin as shown in fig.1. Its preferred form is an ultradeformable vesicle possessing an aqueous core surrounded by the complex lipid bilayer. Interdependency of local composition and shape of the bilayer makes the vesicle both self-regulating and self- optimizing. This enables the Transfersome to cross various transport barriers efficiently, and then act as a Drug carrier for non-invasive targeted drug delivery and sustained release of therapeutic agents.

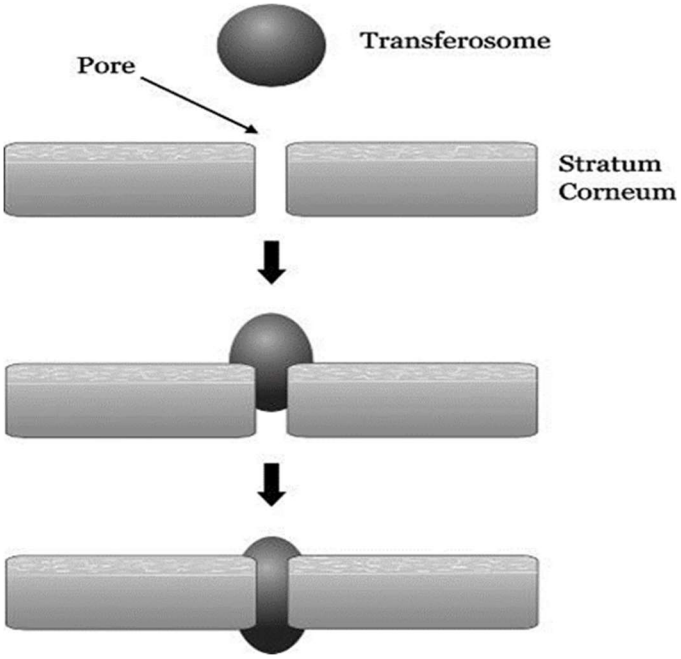

Fig 1. Transfersomes penetration through the pores in stratum corneum, the outermost layer of the skin.

Composition and mechanism of action elastic liposomes

The carrier aggregate is composed of at least one amphiphat (such as phosphatidylcholine), which in aqueous solvents self-assembles into lipid bilayer that closes into a simple lipid vesicle. By addition of at least one bilayer softening component (such as a biocompatible surfactant or an amphiphile drug) lipid bilayer flexibility and permeability are greatly increased1.

The resulting, flexibility and permeability optimized, Transfersome vesicle can therefore adapt its shape to ambient easily and rapidly, by adjusting local concentration of each bilayer component to the local stress experienced by the bilayer. In its basic organization broadly similar to a liposome, the Transfersome thus differs from such more conventional vesicle primarily by its "softer", more deformable, and better adjustable artificial membrane. For example, a Transfersome vesicle applied on an open biological surface, such as non-occluded skin, tends to penetrate its barrier and migrate into the water-rich deeper strata to secure its adequate hydration. Barrier penetration involves reversible bilayer deformation, but must not compromise unacceptably either the vesicle integrity or the barrier properties for the underlying hydration affinity and gradient to remain in place.

Being too large to diffuse through the skin, the Transfersome needs to find and enforce its own route through the organ. The Transfersome vesicles usage in drug delivery consequently relies on the carrier's ability to widen and overcome the hydrophilic pores in the skin or some other (e.g. plant cuticle) barrier. The subsequent, gradual agent release from the drug carrier allows the drug molecules to diffuse and finally bind to their target. Drug transport to an intra-cellular action site may also involve the carrier's lipid bilayer fusion with the cell membrane, unless the vesicle is taken-up actively by the cell in the process called endocytosis2.

Preparation and Optimization of elastic liposomes

Formulation ingredients for elastic liposomes: Elastic liposomes vesicles are prepared in a similar manner as liposomes, except that no separation of the vesicle-associated and free drug is required. All the methods of preparation of elastic liposomes are comprised of two steps. Table 1. shows Ingredients for elastic liposomes. First, thin film is prepared, hydrated and then brought to the desired size by sonication; and secondly, sonicated vesicles are homogenized by extrusion through a poly carbonate membrane83.

Table 1. Ingredients for elastic liposomes.

S. No.	Ingredients	Uses
1.	Soya phosphatidylcholine **(PHOSPHOLIPON® 90 G)**	Vesicles forming component
2.	Tween-80 Span-80	Provides flexibility
3.	Chloroform: Methanol (3:1)	As an organic solvent
4.	Phosphate buffer saline (pH 7.4)	As a hydrating medium

Preparation of elastic liposomes

Preparation of drug free elastic liposomes: Formulations of elastic liposomes were prepared by conventional rotary evaporation method. Appropriate amounts of PHOSPHOLIPON®90 G (375-475 mg) were dissolved in a minimum amount of chloroform/methanol (3:1). The organic solvent was evaporated in rota evaporator (Rotavapour Popular lab India) and solvent traces were removed

by maintaining the lipid film under vacuum overnight. The films were hydrated with PBS pH 7.4 (10 ml) by vortexing for 1 h. For elasticity determinations, the hydrated vesicles were sonicated for 30 min. using bath sonicator (Ultrasonics Mumbai). The elastic liposomes were homogenized by extrusion 5 times through a Sandwich of 0.20µm polycarbonate membranes yields the homogenized suspension of elastic liposomes.

Preparation of drug loaded elastic liposomes: Formulations of elastic liposomes were prepared by conventional rotary evaporation method. Appropriate amounts of PHOSPHOLIPON®90 G (375-475 mg) were dissolved in a minimum amount of chloroform/methanol (3:1)as shown in table.2. The organic solvent was evaporated in rota evaporator (Rotavapour Popular lab India) and solvent traces were removed by maintaining the lipid film under vacuum overnight. The films were hydrated with PBS pH 7.4 (10 ml) containing 1% w/v neomycin sulphate by vortexing for 1 h. For elasticity determinations, the hydrated vesicles were sonicated for 30 min. using bath sonicator (Ultrasonics Mumbai). The elastic liposomes were homogenized by extrusion 5 times through a Sandwich of 0.20µm polycarbonate membranes yields the homogenized suspension of elastic liposomes of neomycin sulphate3.

Composition of different Elastic Liposomes (EL) formulations

Table. 2 Formulation codes of EL formulations.

S. No.	Ingredients(5% w/v)	Formulations									
		NT 1	**NT2**	**NT 3**	**NT 4**	**NT 5**	**NT 6**	**NT 7**	**NT 8**	**NT 9**	**NT10**
1.	Phospholipon® 90 G: Tween-80	95:05	90:10	85:15	80:20	75:25	_	_	_	_	_
	Phospholipon® 90 G: Span-80	_	_	_	_	_	95:05	90:10	85:15	80:20	75:25
2.	Chloroform: Methanol (2:1)	q.s. (As minimum quantity to dissolve Phospholipids: surfactant mixture).									
3.	PBS (pH 7.4) [Drug 1%]	q.s. (To make up volume up to 10 ml).									

Optimization of elastic liposomes

Optimization of vesicle system was done on the basis of vesicle size and entrapment efficiency by varying the type of surfactant and concentration of surfactant.

Vesicular size analysis: The vesicle sizes of EL were measured in one set of triplicates in a multimodal mode in Malvern Zetasizer 2000 (Malvern, UK). Prior to the measurement, vesicles were diluted with PBS pH 7.4 and the measurements were taken in triplicate.

Entrapment efficiency: The neomycin sulphate entrapment capacity by EL was determined by centrifugation method with cooling microcentrifuge (Remi instruments ltd Mumbai) at 40C at 12000 rpm for 120 min. The supernatant (free drug in PBS pH7.4) and sediment (vesicle

entrapping the neomycin sulphate) were collected separately. Supernatant and sediment (lysis of vesicles by Triton X-100 0.5% v/v) was removed and analyzed for drug quantity. The concentration of neomycin sulphate following the 100-1000-fold dilution was assessed by furfural assay method as shown in fig.2. The entrapment efficiency was calculated using the following equation.

$$EE\ \% = [(T-S)/\ T] \times 100$$

Where,
T is the total amount of NS detected both in supernatant and sediment. S is the of NS detected only in the supernatant amount.

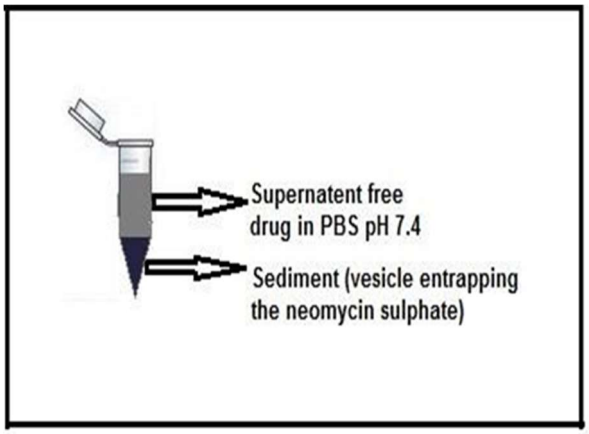

Fig 2. Separation of sediment and supernatant in ependroff tube.

Characterization of elastic liposomes

Vesicle shape and surface morphology: Transmission Electron Microscope (TEM), Hitachi (H-7500), was used as a visualizing aid for elastic liposomal vesicles. A drop of the sample was placed on to a carbon-coated copper grid to leave a thin film. Before the film dried on the grid, it was negatively stained with 1% aqueous solution of phosphotungstic acid (PTA). A drop of the staining solution was added on to film and the excess of the solution was drained off with filter paper. The grid was allowed to air dry. After drying the specimen was viewed under the microscope at 100–100000 k-fold enlargements at an accelerating voltage of 100 kV and the measurements were taken in triplicate4.

Polydispersity index (PI) and Zeta potential detremination: The vesicle size and size distribution of EL were measured by Malvern Zetasizer 2000 (Malvern, UK). Vesicular suspensions were mixed with the appropriate medium (PBS, pH 7.4) and the measurements were taken in triplicate in a multimodal mode. All measurements were performed at 25 ◦C after 5min of thermal equilibration. The Malvern Zetasizer 2000 (Malvern, UK) detects backscattering at an angle of 173o for an improved sizing of especially larger particles at higher concentrations. A spherical form of particles, a viscosity of the medium of 0.89 mPas and refractory index of 1.33 were assumed.

Extent of vesicle skin penetration by CLSM: Depth of skin penetration of Rhodamine Red loaded elastic liposomes was investigated using confocal laser scanning microscopy (CLSM). The probe-loaded vesicles (Rhodamine Red) were separated from the unentrapped probe by centrifugation method. Thereafter formulation was applied non-occlusively for 8 h to the dorsal skin of 5- to 6-week-old rat (Albino wistar strain). Treated animal was kept in a separate cage and maintained under laboratory conditions. Food and water were allowed ad labitum. All investigations were performed as per the protocol approved by the Institutional Animals Ethical Committee of Collage of pharmacy IPS Academy, Indore, India. The rat was sacrificed by heart puncture; dorsal skin was excised and washed with distilled water. The excised skin was then placed on aluminium foil and the dermal side of the skin was gently teased off any adhering fat and/or subcutaneous tissue. The skin was sectioned into the pieces of 1 mm2 size and evaluated for depth of probe penetration. The full skin thickness was optically scanned at different increments through the z-axis of a CLS microscope (LSM 510 Meta with an attached universal Zeiss epifluoroscence microscope). Optical excitations were carried out with a 543 nm Argon laser beam and fluorescence emission was detected above 623 nm for RR.

Preparation of elastic liposomal-gel

Preparation of carbopol-gel: The concentration of carbopol in the hydrogels was 1, 2, 3, and 4% (w/w). The hydrogels were prepared by the following procedure: Carbopol was dispersed in distilled water in which propylene glycol (15 % w/w), and the preservatives (mixture of methyl paraben 0.2 % (w/w) and propyl paraben 0.2 % w/w) were previously added and left to stay for 24 hours. The mixture was stirred the next day (500 rpm/min, for 15 minutes) until the carbopol resin was homogeneously dispersed and then neutralized by the drop wise addition of Triethanolamine (with gentle stirring to avoid inclusion of air), until a transparent gel was obtained as shown in table.3. The pH of the gel base thus obtained was 7.0 (neutral pH).

Table. 3 Formulation ingredients for Carbopol gel.

S. No.	Ingredients	Quantity (% w/w)	Quantity (10g)
1.	Carbopol resin	1 – 4	100 - 400 mg
2.	Propylene glycol	15	1500 mg
3.	Methyl paraben	0.2	20 mg
4.	Propyl paraben	0.2	20 mg
5.	Triethanolamine	To adjust pH	To adjust pH
6.	DM water	q.s.	q.s.

Preparation of elastic liposomal-gel: Elastic liposomal suspension was incorporated into carbopol hydrogels in the ratio of 1:1 (w/w) by mixing them into the gel with mixer at 200 rpm/min for 5 minutes. The concentration of neomycin sulphate was 0.5 % (w/w), and the concentration of carbopol was 0.5, 1.0, 1.5, and 2.0 % (w/w), depending on the carbopol hydrogel used for

incorporation as shown in table.4. The composition of the final liposomal hydrogels is represented in table 3. A combination of preservatives was used, that is, the mixture of parabens (methylparaben, and propylparaben,) in order to enhance their preservative activities, since their synergistic effects were reported.

In addition, the preservative efficacy of parabens is also improved by the addition (2–15%) of propylene glycol, which was contained in liposomal gels. However, the reported synergistic effects enable the use of lower preservative concentrations, being very important since these gels are intended to be used on the skin of patients suffering from different bacterial skin diseases, and higher preservative concentrations (especially of parabens) could lead to additional irritancy or contact dermatitis. This adverse effect may also be increased due to the use of elastic liposomal formulations, since elastic liposomes could enhance the preservatives' penetration, despite the fact that they are not encapsulated. Therefore, lower concentrations of the parabens' mixture were used. Plain drug gel was prepared similarly by adding 100 mg of drug, instead of the liposome5.

Table .4 Formulation codes of Elastic liposomal-gel formulations.

S. No.	Formulation code	Type of Carbopol	Concentration of carbopol in gel (10 g)	Concentration of carbopol in EL-gel (20 g)
1	NTG 1	934	1 % (100 mg)	0.5 % (50 mg)
2	NTG 2	934	2 % (200 mg)	1 % (100 mg)
3	NTG 3	934	3 % (300 mg)	1.5 % (150 mg)
4	NTG 4	934	4 % (400 mg)	2 % (200 mg)
5	NTG 5	940	1 % (100 mg)	0.5 % (50 mg)
6	NTG 6	940	2 % (200 mg)	1 % (100 mg)
7	NTG 7	940	3 % (300 mg)	1.5 % (150 mg)
8	NTG 8	940	4 % (400 mg)	2 % (200 mg)

Optimization of Elastic Liposomal-gel

Optimization of elastic liposomal-gel was done on the basis of viscosity, spreadabilty and extrudability by varying the type of carbopol and concentration of carbopol.

Viscosity: Brookfield Rotational digital viscometer (DV I+) was used to measure the viscosity (in cps) of the prepared elastic liposomal-gel formulations. The spindle (TF S-96) was rotated at 10 rpm. Samples of gels were allowed to settle over 15 min. at the room temperature before the measurements were taken.

Spreadability: The spreadabilty was determined by modified wooden block and glass apparatus. The apparatus consists of a wooden block, with fixed glass slide and a pulley. A pan was attached to another glass slide (movable) with the help of a string. For the determination of spreadabilty,

measured amounts of elastic liposomal-gels was placed in the fixed glass slides, the movable glass slide with a pan attached to it, was placed over the fixed glass slide, such that the elastic liposomal-gels was sandwiched between the two slides for 5 min. the weight was continuously removed, now about 20 g of weight was added to the pan. Time taken for slides to separate was noted.
Spreadability was determined using the following formula:

S=M.L/T

Where,
S is spreadabilty in g cm/sec, M is mass in grams,
L is the length of glass,
T is time in seconds.

Extrudability: For evaluation of gel formulations extrudability was determined. It was based upon the quantity in % of gel extruded from lacquered aluminium collapsible tube containing elastic liposomal-gel which was pressed and firmly crimped at the end. When the cap was removed, elastic liposomal-gel extruded until pressure dissipated. Weight in grams extruded by 0.5 cm ribbon of elastic liposomal-gel in10 seconds was determined6.

$$E = (We / Wt) X 100$$

Where,
E = Extrudability (%)
We = Amount of formulation extruded (g)
Wt = Total amount of formulation in tube (g)

Measurement of pH: The pH of elastic liposomal-gel was checked by using a digital pH meter at constant temperature. Prior to this, the pH meter was calibrated and then the electrode was directly dipped into elastic liposomal-gel formulation and constant reading was noted.

Drug content: The drug content of optimized EL-gel was determined briefly, 1g of EL-gel equivalent to 5mg of neomycin sulphate was diluted with 10 ml distilled water, mixed until a slightly opaque dispersion was obtained and afterwards the dispersion was centrifuged at 3000 rpm for 30 min. The supernatant was collected separately while the sediment was discarded. The supernatant was filtered through the polycarbonate membrane filter (0.20 µm) and was diluted with 10 ml of distilled water and analysed for drug quantity. The concentration of neomycin sulphate was determined by measuring the absorbance at 277nm using spectrophotometer after treating them by furfural assay method.

Comparative evaluation of aqueous solution of drug, plain hydro- gel, EL-suspension and

EL-gel.

Vesicle skin interaction studies: This study was performed to determine the vesicles to be compatible with the skin. The vesicles were applied on the skin of rats (Male Albino Wistar rat, 5–6 week old, 80–100 g) in order to observe the ultrastructural changes in the skin upon exposure to various formulations. Elastic liposomes was applied topically to the skin for 6 h, animal was sacrificed, skin was excised and stored in formalin solution (10%) in PBS (pH 7.4) followed by dehydration with alcohol. It was then treated with anti-media and embedded in paraffin for fixing. Similar procedure was followed to prepare control (without application of any formulation) and elastic liposomal-gel skin section. Sections of 5 µm thicknesses were cut from each piece and stained with haematoxylin and eosin and histological changes in stratum corneum, epidermis and dermis were examined under optical microscope (BX40; Olympus, Tokyo, Japan).

In-vitro drug release and Drug deposition study: The in-vitro skin permeation of neomycin sulphate loaded elastic liposomal formulations was studied using locally fabricated Franz diffusion cell with an effective permeation area and receptor cell volume of 2.54 cm2 and 16 ml, respectively. The temperature was maintained at 32 ± 10C. The receptor compartment contained 16 ml PBS (pH 6.4) and was constantly stirred by magnetic stirrer at 100 rpm. All investigations were performed as per the protocol approved by the Institutional Animals Ethical Committee of Collage of pharmacy IPS Academy, Indore, India. The rat was sacrificed by heart puncture; dorsal skin was excised and washed with distilled water. The excised skin was then placed on aluminium foil and the dermal side of the skin was gently teased off any adhering fat and/or subcutaneous tissue. The rat skin was mounted on a receptor compartment with the stratum corneum side facing upward into the donor compartment. The elastic liposomal formulation (200 µl) was applied on the skin in donor compartment. Samples (1 ml) were withdrawn through the sampling port of the diffusion cell at predetermined time intervals over 24 h and analyzed for drug content by furfural assay. The receptor phase was immediately replenished with equal volume of fresh diffusion buffer. Triplicate experiments were conducted for each study. Similar experiments were performed with aqueous NS solution, plain hydro-gel and elastic liposomal-gel7.

The amount of neomycin sulphate deposited in the skin was determined at the end of the in vitro permeation experiment (24 h). The skin was washed 10 times using a cotton cloth immersed in methanol. The skin samples were washed with 5 ml of distilled water and the neomycin sulphate content of the skin was determined by homogenizing it with distilled water (10 ml) and assaying the filtrate (0.22 um filter), the amount of neomycin sulphate was determined by measuring the absorbance at 277nm using spectrophotometer after treating them by furfural assay method. The cumulative amount of drug permeated, Transdermal flux (J) µg/cm2/h, and skin deposition were calculated.

Antibacterial activity: Comparative evaluation of developed system with simple gel, aqueous solution of drug and drug loaded suspension of elastic liposomes for antibacterial potency of formulation by microbiological assay was determined.

Preparation of inoculums

- The nutrient agar (1.4 gm) was dissolved in 50 ml of distilled water and heated until it was completely dissolved,
- The media was sterilized for 15 min. at 15 lbs. in autoclave.
- The sterilized media (5ml) was transferred aseptically into the each test tube and kept in slant position for solidification.
- The loop of culture was taken from the recently grown slant and inoculated on the surface of another slant in a zigzag manner.
- The inoculated slants were kept for the incubation at 370C ±0.50C for 18 hours.
- After incubation, the stock suspension was prepared by collecting the surface growth in 3 ml of sterile saline.

Preparation of plates: Weighed quantity of antibiotics media (nutrient agar) reconstitute with the distilled water in flat-bottomed flask. The media was sterilized by autoclave at 15 lbs for 15 min then the media was cooled at about 400C after cooling microorganism suspension was added to the media. 25 ml of media was transferred into the each petri plates. The plates were allowed to stand at room temperature for one hour. After solidification bore was done with the aid of cork borer (8 mm diameter).

Sampling procedure

- The gel (0.5 gm) was taken and poured at their respective bore.
- Petri plates were kept for the diffusion of sample for 2 h.
- After 2 hrs Petri plates were incubated at 370C for 24 h.
- Zone of inhibitions were calculated after the incubation period.

In-vivo study: In-vivo study of the EL formulations was performed on Rat model of deep dermal staphylococcal infection. Briefly, according to the animal protocol the experimental set of 60 rats was randomly divided into five groups: rats topically treated with EL-gel, rats topically treated with plain hydrogel, rats topically treated with elastic liposomal suspension, rats topically treated with aqueous solution of neomycin sulphate and controls (untreated rat). The animals were challenged with 108 cfu/rat S. aureus ATCC 29213. At least three rats from each treatment group were sacrificed at each time interval. 5 groups of rat with 12 rats in each group were inoculated intracutaneously with bacteria. The intracutaneous injection (0.1 ml) containing live bacteria were applied to the back of each animal previously shaved. Treatment was conducted by application of the above-mentioned formulations. A daily dose of 0.5 % w/w of neomycin sulphate was administered to the skin from each system in two divided doses. The treatment was started 72 h following inoculation of the bacteria and lasted for 2 weeks. The progress of intradermal wounds was clinically monitored for local inflammatory reaction and photographed once daily. The skin area corresponding to the infection site and underlying tissues from the animals sacrificed on days 0, 3, 7 and 10 after the challenge were removed and processed for histopathological evaluation8.

Stability study: The optimized EL suspension batch NT3 and its optimized batch of EL-gel NTG2 were kept in sealed vials (30 ml capacity) and stored at 2-8 0C (refrigerated temperature), 25 ± 2 0C (room temperature, RT) for 3 months. The sample from either batch at each temperature was taken at definite time intervals and observed visually under microscope for change in consistency (crystalline structure) and the ability of vesicles to retain the drug (i.e., drug- retentive behaviour) was assessed. For determination of the drug retention the sample were centrifuged at 12000 rpm for 120 min, and then the supernatant was analyzed for free drug concentration. All measurements were performed in triplicate (n=3).

Method

Preparation of Neomysin Sulphate loaded elastic liposomes Elastic liposomes were prepared by rotator evaporation method with slight modification in which drug was dissolved in methanol to give a concentration of 1.0% w/v of drug solution. The accurately weighed amounts (10% w/v) of phospholipids and surfactant (7:3 ratio) were taken in a clean, dry, round-bottom flask and this lipid mixture was dissolved in minimum quantity of methanol and chloroform mixture in ratio of 2:1. The round bottom flask was rotated at 45º angle using rotator evaporator at 40°C in order to make uniform lipid layer. The organic solvent was removed by rotary evaporation under reduced pressure at the same temperature (40°C). Final traces of solvents were removed under vacuum overnight. The prepared lipid film in the inner wall of round bottom was hydrated with 10% w/v of drug solution in water followed by rotating the flask containing mixture of drug by rotation at speed of 60 rev/min for 1 hr. After complete hydration of film, the prepared formulation of elastic liposomes was subjected to sonication at 40 C in 3 cycles of 10 minutes with 5 sec rest between the cycles. The prepared formulation was stored at 4°C in closed container till further use for analysis9.

Preparation of Gel Base

Carbopol 934 (1-3%w/v) was accurately weighed and dispersed into double distilled water (80ml) in a beaker. This solution was stirred continuously at 800 rpm for 1 hour and then 10ml of propylene glycol was added to this solution. The obtained slightly acidic solution was neutralized by drop wise addition of 0.05 N sodium hydroxide solutions, and again mixing was continued until gel becomes transparent. Volume of gel was adjusted to 100 ml and then sonicated for 10 min on bath sonicator to remove air bubbles. Final pH of the gel base was adjusted to 6.5. Gel was also prepared with plain drug by adding 10 mg of drug and dispersed properly by following same procedure given above. The same procedure was used to formulate noisome containing gel in which previously prepared liposomes cake was added in place of plain drug. Elastic liposomes preparation corresponding to 6.0 % w/w (6.0 mg of drug in 100 mg of gel) of drug was incorporated into the gel base to get the desired concentration of drug in gel base.

Optimization of elastic liposomes

Optimization of lipid: surfactant ratio

In the elastic liposomal formulation, the lipid: surfactant ratio was optimized by taking their

different ratio such as 8:2, 7:3, 6:4 and 5:5 ratio and all other parameters were kept remain constant. The prepared formulations were optimized on the basis of average particle size, polydispersity index and % entrapment efficiency (table. 5)

Table 5: Optimization of lipid: surfactant concentration

Formulation code	Soya PC: Span 80 (% w/v)	Drug (% w/v)	Average particle size (nm)	% Entrapment efficiency	PDI
ELSL-1	8:2	1.0	356.02±4.23	76.5±4.78	2.56
ELSL-2*	**7:3**	**1.0**	**251.05±3.12**	**72.03±3.39**	**1.09**
ELSL-3	6:4	1.0	230.87±1.90	41.48±3.23	0.98
ELSL-4	5:5	1.0	287.43±1.90	28.73±2.14	0.91

Optimization of drug concentration

Drug concentration was optimized by taking different concentration of drug and prepared their formulation and all other parameter such as Soya PC: Span 80, sonication time kept remain constant. The formulation optimized on the basis of entrapment efficiency, average vesicle size and PDI (table.6).

Table 6: Optimization of drug concentration

Formulation code	Soya PC: Span80 (% w/v)	Drug (% w/v)	Average particle size(nm)	% Entrapment efficiency	PDI
ELSL-5	7:3	1.0	251.02±1.09	72.03±2.39	1.09
ELSL-6*	**7:3**	**1.5**	**262.25±3.37**	**76.03±2.49**	**0.96**
ELSL-7	7:3	2.0	230.87±2.29	76.48±3.23	0.97

Optimization of sonication time

Sonication time was optimized by sonicating the formulation for different time i.e 30, 60 and 90 sec at 40 C in 3 cycles of 10 minutes with 5 sec rest between the cycles, optimization was done on the basis of average particle size, % Entrapment efficiency and PDI (table 7 and table 8).

Table 7: Optimization of sonication time

Formulation code ELSL-6	
Phospholipid: Surfactant (10 % w/v)	7:3
Drug (% w/v)	1.5
Sonication time (sec)	60

Table 8: Optimized formulation elastic liposomes

Formulation code ELSL-6	
Phospholipid: Surfactant (10 % w/v)	7:3
Drug (% w/v)	1.5
Sonication time (sec)	60

CHARACTERIZATION OF ELASTIC LIPOSOMES

Vesicle size

Microscopic analysis was performed to determine the average size of prepared elastic Liposomes. Formulation was diluted with distilled water and one drop was taken on a glass slide and covered with cover slip. The prepared slide were examined under trinocular microscopic at 400 X. The diameters of more than 150 vesicles were randomly measured using calibrated ocular and stage micrometer10.

Surface charge and vesicle size

The vesicles size and size distribution and surface charge were determined by Dynamic Light Scattering method (DLS) (Malvern Zetamaster, ZEM 5002, Malvern, UK). Zeta potential measurement of the elastic liposomes was based on the zeta potential that was calculated according to Helmholtz–Smoluchowsky from their electrophoretic mobility. For measurement of zeta potential, a zetasizer was used with field strength of 20 V/cm on a large bore measures cell. Samples were diluted with 0.9 % NaCl adjusted to a conductivity of 50 lS/cm.

Table 9: Characterization of Optimized formulation of elastic liposomes

Characterization .	Average vesiclesize (nm)	% Entrapment efficiency	PDI	Zeta Potential(mV)
ELSL-6	178.37±5.07	71.03±2.49	0.189	-17.3±2.4

Entrapment efficiency

Entrapment efficiency of Neomysin Sulphate in enthosomal formulation was determined using the

Sephadex G-50 column. The weighed amount of Sephadex G-50 was properly mixed with sufficient amount of distilled water in a beaker and kept for 24 h for complete swelling. After complete swelling, Sephadex dispersion was placed in a 1 mL capacity of PVC syringe (Dispovan) packed with glass wool and a small piece of Whatman filter paper at the bottom end to provide stability for the Sephadex column at 3,000 rpm. The entrapment efficiency of Neomysin Sulphate in elastic liposomes vesicle was determined by ultracentrifugation, 10mL of elastic liposomes formulation were passed from the column. The amount of drug not entrapped in the elastic liposomes was determined by passing the formulation from the Sephadex column, centrifuging at 3,000 rpm, and collecting the elution using the equation given below. After removing the un-entrapped drugs, the elastic liposomes were collected and lysed using 1 % Triton X100; and then centrifuged. The supernatant layer was separated, diluted with water suitably and drug concentration was determined at 239 nm using UV spectrophotometer as shown in table.9.

$$\%\ Entrapment\ Efficiency = \frac{\text{Theoretical } drug\ content - Practical\ drug\ content}{\text{Theoretical drug content}} \times 100$$

pH measurements

pH of selected optimized formulations was determined with the help of digital pH meter. Before each measurement of pH, pH meter should be calibrated with the help of buffer solution of pH 4, pH 7 and pH 9. After calibration, the electrode was dipped into the vesicles as long as covered by the vesicles. Then pH of selected formulation was measured and readings shown on display were noted11.

Characterization of Elastic Liposomes Containing Gel

Measurement of viscosity

Viscosity measurements of prepared topical Liposomes based gel were measured by Brookfield viscometer using spindle no. 63 with the optimum speed of 10rpm; viscosity was found to be 3776cps.

Drug content

Accurately weighed equivalent to 100 mg of topical Liposomal gel was taken in beaker and added 20 ml of 0.01N HCl. This solution was mixed thoroughly and filtered using Whatman filter paper no.1. Then 1.0 mL of filtered solution was taken in 10 mL capacity of volumetric flask and volume was made upto 10 mL with 0.01 N HCL. This solution was analyzed using UV-Spectroscope at λmax 225 nm. Drug content of topical Liposomal based gel is shown in table no 6.

Extrudability study

Extrudability was based upon the quantity of the gel extruded from collapsible tube on application of certain load. More the quantity of gel extruded shows better extrudability. It was determined by applying the weight on gel filled collapsible tube and recorded the weight on which gel was

extruded from tube. Extrudability of gel required 170 grams of weight to extrude a 0.6cm ribbon of gel in 6 seconds.

Spreadibility

Spreadibility of formulation is necessary to provide sufficient dose available to absorb from skin to get good therapeutic response. An apparatus in which a slide fixed on wooded block and upper slide has movable and one end of movable slide tied with weight pan. To determine spreadibility, placing 2-5 g of gel between two slide and gradually weight was increased by adding it on the weight pan and time required by the top plate to cover a distance of 10 cm upon adding 80g of weight was noted. Good spreadibility show lesser time to spread as shown in table.11.

In Vitro drug diffusion study

The in-vitro diffusion study is carried by using Franz Diffusion Cell. Egg membrane is taken as semi permeable membrane for diffusion. The Franz diffusion cell has receptor compartment with an effective volume approximately 60 mL and effective surface area of permeation 3.14sq.cms. The egg membrane is mounted between the donor and the receptor compartment. A two cm2 size patch taken and weighed then placed on one side of membrane facing donor compartment. The receptor medium is phosphate buffer pH 7.4. The receptor compartment is surrounded by water jacket so as to maintain the temperature at 32 ± 0.5°C. Heat is provided using a thermostatic hot plate with a magnetic stirrer. The receptor fluid is stirred by Teflon coated magnetic bead which is placed in the diffusion cell.

During each sampling interval, samples are withdrawn and replaced by equal volumes of fresh receptor fluid on each sampling. The samples withdrawn are analyzed spectrophotometrically at wavelength of drug as shown in table 10.

Table 10: In vitro drug release study of prepared gel formulation

S. No.	Time (hr)	% Cumulative Drug Release
1	0.12	6.93±0.5
2	0.5	12.45±0.9
3	1	16.59±1.2
4	2	19.26±1.7
5	4	22.5±1.3
6	6	25.19±2.5
7	8	26.76±2.4
8	12	32.69±2.9
9	24	41.36±1.8
10	48	52.06±2.6
12	72	61.6±3.2

Table 11: Characterization of gel-based formulation of elastic liposomes

Characterization	Viscosity (cps)	%Entrapment efficiency	Release after 72hr	Extrudability (g)	Spreadibility (g.cm/sec)

GELSL-6	178.37±5.07	71.03±2.49	61.6±3.2	170	5.16

Stability Studies

Stability study was carried out for drug loaded elastic liposomes at two different temperatures i.e. refrigeration temperature (4.0 ±0. 2°C) and at room temperature (25- 28±2°C) for 3 weeks. The formulation subjected for stability study was stored in borosilicate container to avoid any interaction between the formulation and glass of container. The formulations were analyzed for any physical changes and drug content (table 7.8).

Table 12: Characterization of Optimized formulation of elastic liposomes

Characteristic	Time (Month)					
	1 Month		2 Month		3 Month	
Temperature	4.0 ±0. 2°C	25-28±2°C	4.0 ±0. 2°C	25-28±2°C	4.0 ±0. 2°C	25-28±2°C
Average particle size (nm)	178.37±5.07	192.56±3.43	179.32±2.49	238.54±4.87	186.84±5.84	583.54±6.99
% EE	67.37±2.52	48.62±1.39	62.37±2.52	35.29±1.08	52.37±2.52	23.83±2.11
Physical Appearance	Normal	High turbid	Normal	High turbid and agglomeration	Normal	High turbid and agglomeration

Result and Discussion

Average Vesicle Size and Zeta Potential

Prepared formulations of elastic liposomes were optimized on basis of vesicle size, shape, surface charge and entrapment efficiency as shown in table 12. Vesicle size of elastic liposomes were examined under trinocular microscopic (magnification 400X) and also determined by light scattering method (Malvern Zetasizer, ZEM 5002, and UK) and found that average vesicle size of optimized formulation ELSL-6 was 178.37±5.07 nm. The PDI was found 0.189 and Zeta potential was -17.3±2.4. It was observed that the vesicles size of elastic liposomes was increase with increasing the concentration of phosphotidylcoline and similarly vesicle size was decease with increasing the concentration of span 80 due to its surfactant action. There was no significant difference in average vesicle size was observed with increasing the drug concentration. But in increasing the sonication time the size vesicle was decrease from 218.42±6.09 to 145.29±7.80 after 90 sec of sonication12.

Polydispersity Index (PDI)

The low PDI value represents the uniformity of formulation in which there is no major difference in size of vesicles. The PDI of optimized formulation (ELSL-6) was found 0.189. PDI of formulation was varied with increasing or decreasing the concentration ratio of lipid and surfactant and sonication time. It was observed that when lipid ratio in formulations was decreased and surfactant concentration was increased then the PDI was found decrease. Lipid and surfactant ratio with 7:3 ratio was the optimum ratio and it was given low PDI value of 1.09 in formulation ELSL-

2.

When sonication time increase from 30 to 90 second then the PDI value was 0.309, 0.189 and 0.487 respectively for ELSL-5, ELSL-6 and ELSL-7. It was observed that on 90 second of sonication time the PDI was increased and it was due to the high mechanical forced of sonication waves which was resultant in heat generation which leads to agglomerates or denaturing the lipid molecules after breaking the vesicles.

% Entrapment efficiency

% Entrapment efficiency of optimized elastic liposomes formulation (ELSL-6) was found 71.03±2.49%. It was observed that the percent drug entrapment was decrease with increasing the concentration of surfactant and on increasing the time of sonication. It is due to the leaching out the drug from vesicles on increasing the mechanical force by sonication and size reduction of size liposomes on increasing the concentration of surfactant due to their surfactant action. It was clearly shown in table 3 and figure…. that when formulation was sonicate for 30 sec then the % EE was 72.03±2.39 (ELSL-5) and when it sonicate for 60 and 90 sec then the %EE was found 71.03±2.49 (ELSL-6) and 45.48±3.23 (ELSL-7) respectively. The 60 sec is selected as optimized time for sonication because it provided the required size of vesicle 178.37±5.07nm and good %EE i.e 71.03±2.49 with low PDI value of 0.189. The ELSL-6 formulation was selected as optimized formulation13.

Stability Study

Stability study data was revealed that the optimized formulation (ELSL-6) stable after 3 months of storage at 4ºC while at 25-28±2°C, the formulation was found unstable. Stability of formulation was observed on the basis of % drug remain, average vesicles size and physical appearance. The average vesicle size of liposomes was found 178.37±5.07, 179.32±2.49 and 186.84±5.84 nm after 1, 2 and 3 month of storage at 4.0 ±0. 2°C while at 25-28±2°C the average vesicle size was found 192.56±3.43, 238.54±4.87 and 583.54±6.99nm after 1,2 and 3 month of storage. Drug remaining in liposomal formulation was 48.62±1.39, 35.29±1.08 and 23.83±2.11 % after 1, 2 and 3 month of storage at 25-28±2°C while there was no significant changes in % drug remain and physical appearance in liposomal formulation was observed after 3 month of storage at 4ºC as shown in table 1214.

Characterization of gel-based formulation of prepared gel containing Neomycin Sulphate loaded Liposomes

Prepared gel was prepared and evaluate for viscosity, % entrapment, extrudability, spreadability and drug release study. It was found that viscosity of prepared gel was 178.37±5.07 cps, % Entrapment efficiency was 71.03±2.49%, Extrudability was 170 g and Spreadibility (g.cm/sec) was found that 5.16 (g.cm/sec) respectively (table 7). In vitro Drug Release: In vitro drug release from elastic liposomes was carried out using Frenze diffusion cell method and found 61.6±3.2% in 72 hr. In first 30 min it was 12.45±0.9% drug release which slightly high. It was due to the

release of free drug present in bag after leaching from liposomes. Drug release from elastic liposomes formulation was found in very sustained and controlled manner and follow Higuchi and Korsemeyer Peppas relase kinetic. Drug release in 24 hr was 41.36±1.8 and 52.06±2.6 in 48 hr.

Conclusion

The objective of the present work was to investigate the dermal delivery of neomycin sulphate (NS), a model aminoglycoside antibiotic, from elastic liposomes (EL). The phospholipid used to prepare the EL dispersion was soya phosphatidylcholine (Phospholipon®90 G) and tween 80 (T) or span 80 (S) as surfactant and also formulation of the EL-gel by carbopol resins and the comparative study of both formulations. Elastic liposomes were characterized for vesicular shape, vesicular size, zeta potential, entrapment efficiency, stability, in vitro skin penetration and in vivo animal model to determine its wound healing efficacy.

Transmission Electron Microscopy (TEM) and Zeta Seizer (ZS) defined EL as spherical, unilamellar structures, low polydispersity (<0.5) and nanometric size range (186 ± 5.85 nm). Entrapment efficiency of NS in EL carrier was found to be (34.6 ±0.15 %). Stability profile of prepared system was found that they are stable for 14 days store at 40C were as the EL-gel were stable for 2 months. Confocal laser scanning microscopy (CLSM) experiments revealed that EL facilitated the co-penetration of antibiotic and phospholipid to the deeper layers of the rat skin up to180 μm. NS loaded EL shows an enhanced transdermal flux of 17.71μg/cm2/h, amount of drug released as 14.16 % and skin deposition of 10.34 %.

The in vivo experiments demonstrated a very efficient healing of S. aureus- induced deep dermal infections when the rats were treated with Elastic liposomal systems of neomycin sulphate. Histological evaluation of the skin treated with elastic liposomal antibiotic revealed no bacterial growth and normal skin structure. The in vivo results suggested that when NS applied to the infected skin area from EL resulted in efficient inhibition of the infection, stopping its development. In the design of these experiments, we chose the aqueous solution of NS to serve as a control, in order to exclude the possibility of any artifacts due to the action of phospholipon and tween 80, components of the EL systems. In EL systems, phospholipon fluidizing both the vesicle and the SC lipids and tween 80 form the vesicles are more flexible, so that the skin is more penetrable. The suggested mechanism for penetration enhancement involves a synergistic interaction between phospholipid, vesicles and skin lipids.

When EL carriers are applied to the skin, several simultaneous processes may occur, involving the stratum corneum and pilosebaceous pathways. Phospholipid disturbs the organization of the intracellular lipid bilayers in SC and enhances their fluidity. The soft elastic liposomes can then enter the disturbed SC bilayers and create a pathway through the skin lipids. Thus, the drug is released from elastic liposomes in the deep skin strata as a result of fusion of vesicles with skin lipids along the penetration pathway.

Efficient delivery of antibiotics to deep skin strata from elastic liposomal applications could be highly beneficial, reducing possible side effects and other drawbacks associated with systemic treatment.

Use of lipid vesicles in delivery system for skin treatment has increasing attention in recent years. However, it is generally agreed that classic liposomes are of little or no value as carrier for transdermal drug delivery because they do not penetrate the skin, but remain conformed to the upper layer of the stratum corneum. Elastic liposomes are specially optimized particles or vesicles, which can respond to an external stress by rapid and energetically inexpensive, shape transformations. Such highly deformable particles can thus be used to bring drugs across the biological permeability barriers, such as skin. Drug loaded elastic liposomes can carry drug across the skin.

With all these benefits of the elastic liposomes over the conventional dosage form there is a drawback they have very less stability. From our result we concluded that the stability of the elastic liposomes are of just 14 days, but there stability and ease of application had been enhanced by formulating elastic liposomal-gel (i.e. entrapment of the vesicular system in the gel).

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