Journal of Current Pharma Research
(An Official Publication of Human Journals)
An International Peer Reviewed Journal For Pharmacy, Medical & Biological Science
DOI: 10.25166 CODEN: JCPRD6 NLM ID: 101744065
ISSN: Print: 2230-7834 Online: 2230-7842
SJIF Impact Factor: 6.913

Human Journals
Review Article
June 2023 Vol.:18, Issue:1
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A Review on Development and Characterization of Ethosomes - A Novel Transdermal Drug Delivery System

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Submitted: 02 June 2023
Accepted: 22 June 2023
Published: 25 June 2023

Keywords: Skin, Transdermal drug delivery, ethosomes, preparative methods, characterization.

ABSTRACT

Skin is the largest organ of the human body that restricts the movement of drugs into the systemic circulation. A topical drug delivery system is a system where the drug reaches the systemic circulation. The major obstacle in this route is the low diffusion rate of drugs across the stratum corneum. Exosome are a novel vesicular carrier showing enhanced delivery of drugs to the deeper layers of skin. The autosomal system is composed of phospholipid, ethanol, and water. Ethosomes are “ethanolic liposomes”. Ethanol has long been known to have permeation-enhancing properties. The size of ethosomes may vary from nanometers to microns and they permeate through the skin layers more rapidly and possess significantly higher transdermal flux. In this review, we have focused on the advantages, disadvantages, preparative methods, and characterization of ethosomal formulation.

INTRODUCTION

Ethosomal systems are novel permeation-enhancing lipid carriers embodying ethanol-containing lipid vesicles with interdigitated fluid bilayers. In contrast to liposomes and deformable liposomes, ethosomes have been shown to exhibit high encapsulation efficiency, skin deposition ability, and depth of skin penetration for a wide range of molecules including lipophilic drugs, and are effective at delivering molecules to and through the skin**. [1]** Whether for pharmaceutical purposes, gene therapy, vaccination, or cellular transformations in biomedical research the delivery of molecules through the biological membrane has become a major focus of research in recent years. Results from the intracellular delivery of the labeled phospholipids indicate that the autosomal components themselves may penetrate the fibroblasts. The probe is found throughout the cell structure, in the membrane, and the cytoplasm. **[2]**

Advantages of ethosomes (perspective)

1**.** With this delivery technology, proteins, and peptides can be easily supplied.

2. Ethosomal system can be easily commercialized and is passive and non – invasive.

3. raw materials used are really harmless and easily penetrable.

4. Patient adherence to this drug delivery system is high.

5. Ethosomal formulation is administered in gel or cream in semisolid form for high patient compliance.

6. Also been used in other industries such as veterinary medicine and cosmetics.

7. Simple method for drug delivery comparing iontophoresis and sonophoresis.

Disadvantages of ethosomes [3].

1. Molecular size of the drug should be reasonable.

2. May not be economical.

3. poor yield.

4. Skin irritation and dermatitis due to excipients and permeation enhancers.

5. loss of product during transferring from organic to water media.

Mechanism of enhanced drug penetration

Ehosomes is a novel vesicular carrier showing enhanced delivery of drugs to the deeper layers of skin. The mechanism of enhanced penetration also demonstrated by the same author in 2000. The autosomal system is composed of phospholipid, ethanol, and water. Ethanol has long been known to have permeation enhancing properties. However, the permeation enhancement from ethosomes observed in this work was much greater than would be expected from ethanol alone, suggesting some kind of synergistic mechanism between ethanol, vesicles and skin lipids**[4]**. In comparison to liposomes, ethosomes are less rigid, confirmed by relatively low fluorescence anisotropy of the phosphatidylcholine probe AVPC (entry- vinlyphosphatidyl choline) as well as low T_m in differential scanning calorimetric studies. Thus the effects of ethanol which were considered harmful to classic liposomal formulations may provide the vesicles with soft flexible characteristics which allow them to more easily penetrate deeper layers of the skin. Another contribution to the high skin penetration could be made by the interaction of ethanol and phospholipid vesicles with stratum corneum. It has also been suggested that mixing phospholipids with the subcutaneous lipids of the intercellular layers enhances the permeability of the skin. **[5]**

The following figure also given by **Touitou *et al*** illustrates a hypothetical model of how ethosomes may enhance permeation of drugs through the stratum corneum lipids. First ethanol disturbs the organization of the stratum conium lipid bilayer and enhances its lipid fluidity. The flexible ethosomal vesicles can then penetrate the disturbed subcutaneous bilayers and even forge a pathway through the skin by their particulate nature. The release of drug and absorption by deep layers could be the result of the fusion of ethosomes with skin lipids and drug release at various points along the penetration pathway. When ethosomal carriers which contain ethanol and soft small vesicles are applied to the skin, it follows stratum corneum and pilosebaceous pathways.

Ethosomes

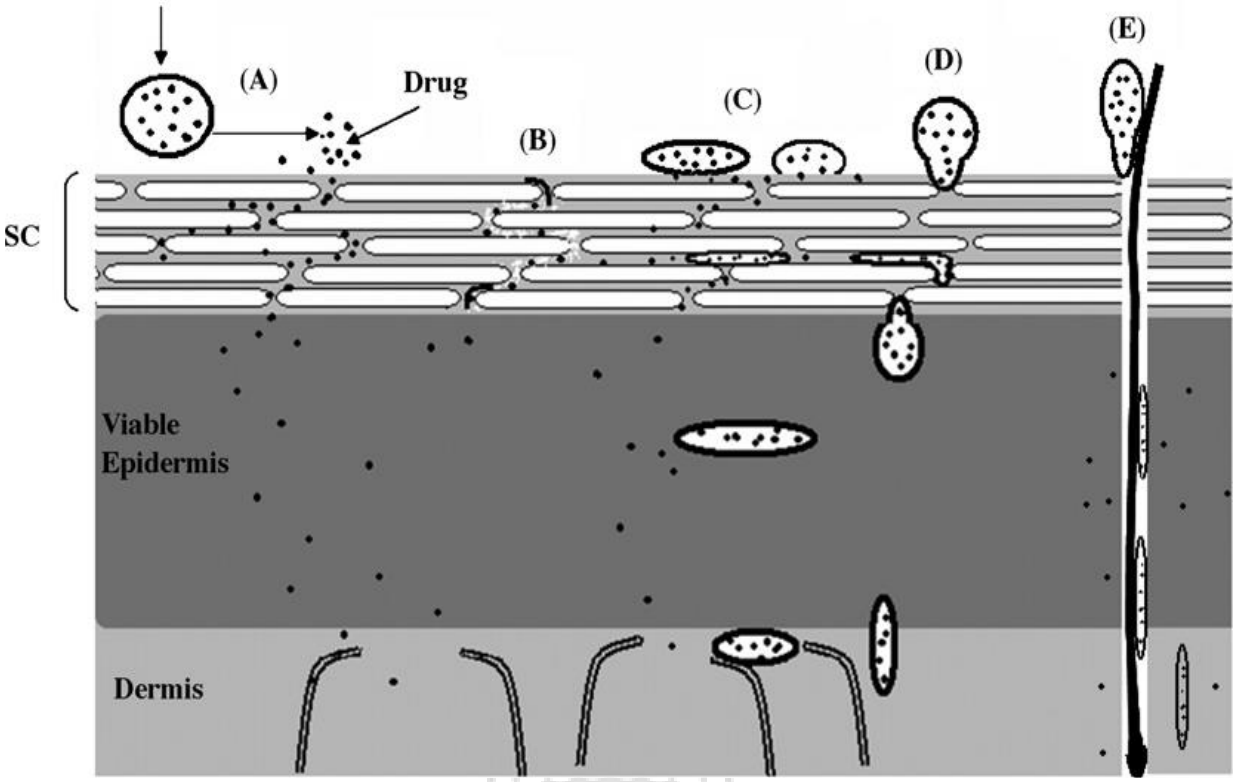

FIGURE-1: POSSIBLE MECHANISMS OF ACTION OF ETHOSOMES AS SKIN DRUG DELIVERY SYSTEMS. (A) is the free drug mechanism, (B) is the penetration-enhancing process of those components, (C) indicates vesicle adsorption to and/or fusion with the stratum conium (SC), and (D) illustrates intact vesicle penetration into or into through the intact skin (E) illustrates penetration of vesicles through hair follicles modified from. [5]

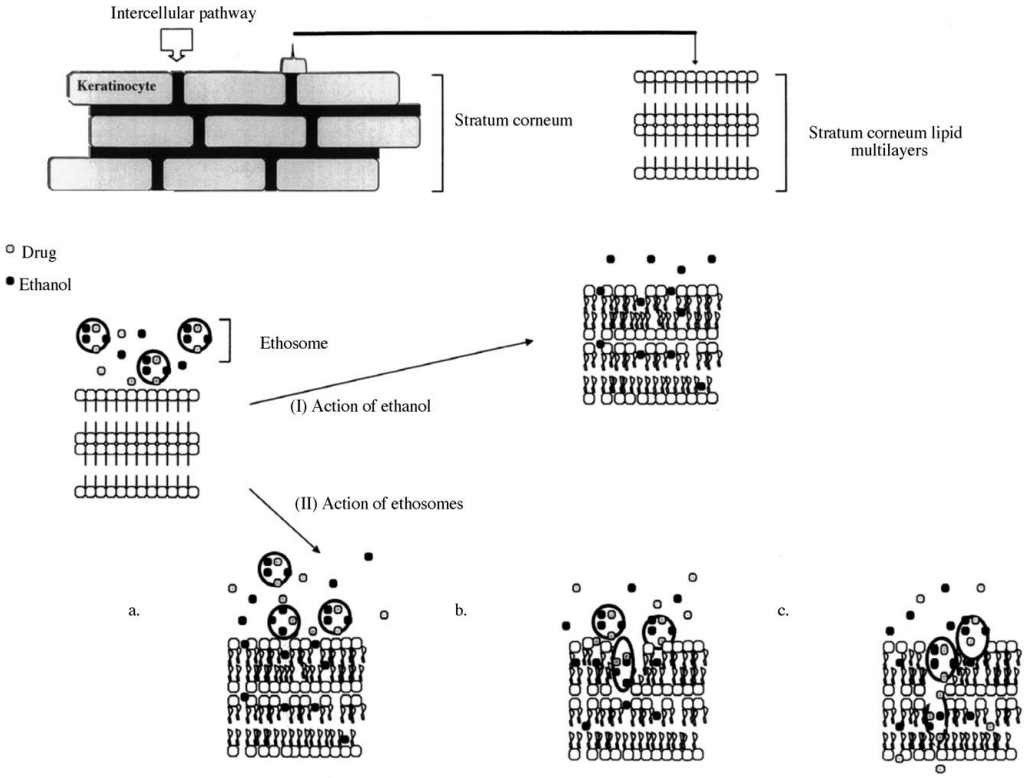

FIGURE-2: PROPOSED MODEL FOR SKIN DELIVERY OF DRUGS FROM THE ETHOSOMAL SYSTEM.

The mechanism of autosomal drug delivery is reviewed by **[6].**In his study, drug solution in 30% ethanol showed a slight, non-significant cumulative drug permeated relative to the aqueous control. This could suggest that the penetration-enhancing effect of ethanol is not an operating mechanism in the observed enhancement of skin delivery of drugs by ethosomes. The proposal that ethanol may increase the flexibility of the vesicles, allowing them to more easily penetrate deeper layers of the skin, could be supported. This may be followed by structural changes in deep layers of the stratum cornium, with subsequent enhancement of drug penetration. **[7]**

Preparation methods:

1. Injection to ethanolic solution method

From the currently available references, this method is first reported by E.Touitou *et al*. and Jain *et al*. In this method, the drug and phosphatidyl choline is dissolved in ethanol and taken

in a hermetically closed glass bottle. Double or triple distilled water heated to 30±1ºC is added slowly in a fine stream to the lipid solution with constant stirring at 700rpm with a mechanical or magnetic stirrer. Mixing is continued for additional 5 minutes. The system is maintained at 30±1ºC during the preparation and then left to cool to room temperature for 30 minutes. The resulting vesicle suspension is homogenized by passing through polycarbonate membrane with extruder or sonication by a probe sonicator.

2. Ethanol injection - sonication method

This method is followed by Zhao *et al*. In this method the phospholipid is dissolved in ethanol in a glass bottle. The drug is dissolved in double distilled water and stirred by a magnetic stirrer. The glass bottle is connected to a syringe hermetically, which permits the supplement of ethanol, but avoided evaporation. After dissolution of the drug, the phospholipid – ethanol solution is added into the drug solution at a flow rate of 200µl/min, and then the mixture is finely homogenized at 50ºC by sonication for 5 minutes (300 watt) by a sonifier probe type ultrasonic instrument. The obtained colloidal solution is filtered through 0.22 µm disposable filters and then the ethosomes formulation containing ethanol and water is obtained in the filtrate. All the processes are carried out under the protection of nitrogen at room temperature.

3. Classic mechanical dispersion method

This method is reported by Dubey *et al*., Fang *et al*. and Curic *et al*. In this method phosphatidyl choline is dissolved in chloroform: methanol 3:1 mixture in a clean round bottom flask followed by removal of the organic solvents using a rotary flash evaporator above the lipid transition temperature to form a thin lipid film under vacuum overnight followed by hydration with different concentration of hydro ethanolic mixture containing drug with 60rpm for 1 hour at the corresponding temperature. The preparation vortexed followed by sonication at 4ºC using a probe sonicator.

The same method is followed by Tadros *et al*. with emulsification by sonication followed by solvent evaporation in rotary flash evaporator. In this procedure the lipid dissolved in a a small volume of Di ethyl ether: Chloroform 1:1 mixture in a round bottom flask. An aqueous phase containing water soluble drug is added to the organic phase such that organic to aqueous phase ratio was 5:1. The mixture is then sonicated for 10 minutes. A stable white emulsion produced from which the water and organic solvent mixture slowly evaporated at

55ºC using rotary flash evaporator until a thin film formed on the wall of the flask. The resulting film kept vacuum to eliminate the traces of organic solvent. This film was hydrated with different concentration of hydro ethanolic mixture for 1 hour with rotation. The resulted formulation left at room temperature for 1 hour and then sonicated for 20 minutes at 4ºC.

Vesicular characterization [8]

1. Vesicular size and zeta potential

This can be determined by Dynamic Light Scattering (DLS) technique using a computerized inspection system. The water-ethanol solution of the same proportion of the formulation or saline or pH: 6.8 phosphate buffer saline (PBS) is used as sample diluents. The vesicular suspension is mixed with the appropriate medium and the measurements are taken. **[9]**

2. Visualization for vesicular shape and surface morphology

Transmission Electron Microscope (TEM) is used for the visualization of ethosomal vesicles. The vesicular suspension is placed on carbon coated grid or copper grid and the material excess is removed with a filter paper. This is stained with aqueous solution of phosphotungstic acid or 2% uranyl acetate solution. Finally, the grid is semi-dried andexamined under Transmission electron microscope.

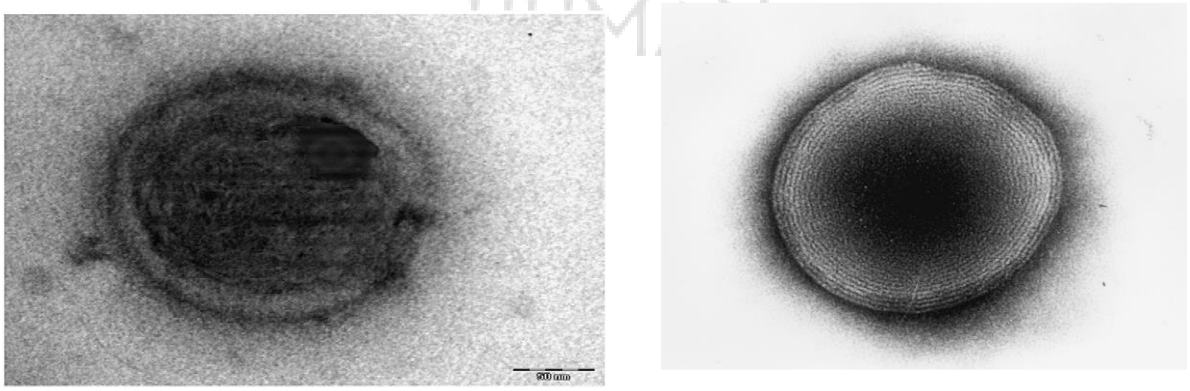

FIGURE- 3: VISUALIZATION OF ETHOSOMAL VESICLES TEM (A) MAGNIFICATION 315 000 (B) MAGNIFICATION 1,10,000 [10]

For vesicle characterization, freeze fracture transmission electron microscopy also used. After centrifugation the samples at 30,000g for 30 min at room temperature, are examined by means of the freeze fracture microscopy technique: samples are impregnated with 30% glycerol and then frozen in partially solidified Freon 22, freeze fractured in a freeze fracture device (-105ºC, 10–6 mm Hg) and replicated by evaporation from a platinum/carbon gun.

The replicas are extensively washed with distilled water, picked up onto Formvar-coated grids and examined with a transmission electron microscope (TEM).

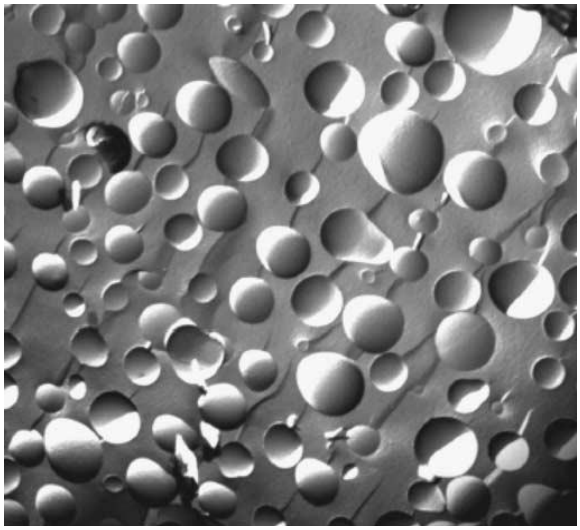

FIGURE-4: FREEZE FRACTURE ELECTRON MICROGRAPHS OF ETHOSOME FORMULATION CONTAINING AMMONIUM GLYCYRRHIZINATE

Scanning Electron Microscope (SEM) is also used to characterize the surface morphology of the ethosomes. One drop of ethosomal suspension is mounted on a clear glass stub, it is air dried and coated with gold and visualized under Scanning electron microscope. **[11]**

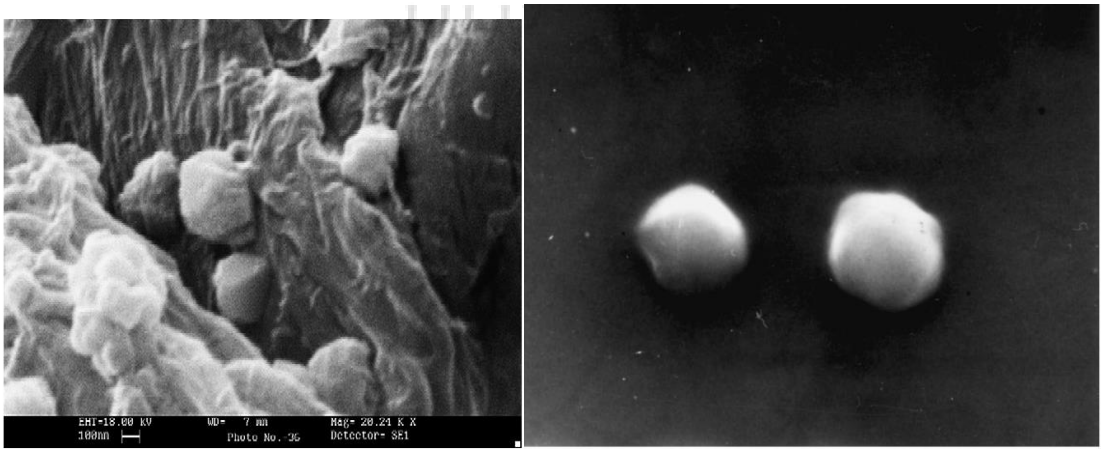

FIGURE-5: VISUALIZATION OF ETHOSOMAL VESICLES BY SEM. (A) TESTOSTERONE ETHOSOMES (B) METHOTREXATE ETHOSOMES

3. Drug entrapment efficiency

Drug entrapment efficiency can be determined by 3 methods,

(a) Ultra centrifugation method

(b) Sephadex G-50 minicolumn centrifugation method

(c) Dialysis method

(a) Ultra centrifugation method

After preparation, the ethosomal formulation is centrifuged at 4°C at a speed ranging from 15,000 rpm to 56,000 rpm for a time ranging from 30 minutes to 4 hours by ultra centrifuge. The required rotation speed of the centrifuge depends upon the individual formulation characteristics. Usually, higher revolution speed will cause the separation of the supernatant in less time duration. The amount of drug in supernatant and ethosomal pellet is determined. The amount of drug in the ethosomal sediment is determined after lysing the vesicles by triton X-100 0.5% w/w. From this entrapment efficiency is determined by

$$E.E = D_E / D_E - D_S \quad \text{or} \quad E.E = D_T - D_S / D_T$$

Where,

D_E ─ Amount of drug in the etosomal sediment

D_S ─ Amount of drug in the supernatant

D_T ─ Theoretical amount of drug used to prepare the formulation.

(b) Sephadex G-50 minicolumn centrifugation method

The prepared ethosomal vesicles separated from the free (unentrapped) drug by a sephadex G-50 minicolumn centrifuge. The collected vesicles were lysed by Triton X-100 0.5% W/V and entrapped efficiency is determined by the formula explained in ultra centrifugation method (Touitou *et al.*,2000).

(c) Dialysis method

Cellulose acetate dialysis membrane is kept in saline for 1 hour before dialysis to ensure complete wetting of the membrane. The formulation is placed in the dialysis bag, which is

then transferred into the receiver medium stirred by magnetic stirrer. Samples are withdrawn at regular time interval with the replacement of receptor volume. After 4 hours when free drug is separated, the vesicles are lysed using Triton X-100 and analyzed for drug content. **[13]**

4. Depth of skin penetration

Depth and mechanism of skin penetration of fluorescent probe loaded ethosomes is investigated using Confocal Lasser Scanning Microscopy(CLSM). The probe loaded vesicles are first passed through the sephadex G-50 minicolumn to separate the unentrapped probe and formulation is applied non-occlusively for 8 hours to the dorsal skin of nude albino rat. The rat is then sacrificed by heart puncture; dorsal skin was gently teared off any adhering fat and/or subcutaneous tissue. The skin is sectioned into pieces of 1mm^{2}size.The full skin thickness is optically scanned at different increments (usually10-20µm) through the z axis of a CLS Microscope. Optical excitation is carried out with a 488 nm for Rhodamine red (RR) at 527 nm for D-289 (4-(4-di ethyl amino) stearyl-N- methyl pyridinium iodide) and 488 nm for calcein. This entire study can also be performed in invitro by fixing the rat skin in the Franz diffusion cell and applying the probe-loaded ethosomes. After the contact period the skin is washed and analyzed by laser scanning.

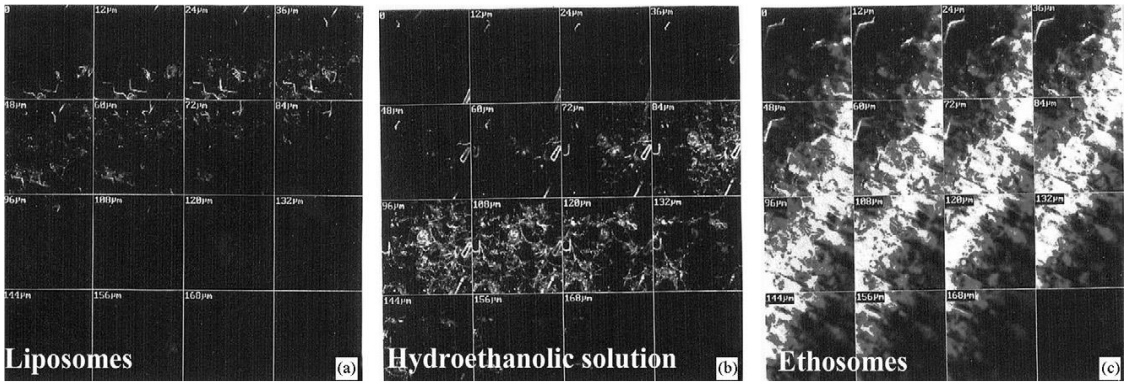

FIGURE-6: CONFOCAL LASER SCANNING MICROSCOPY MICROGRAPHS OF MOUSE SKIN, AFTER APPLICATION OF THE FLUORESCENT PROBE D-289 IN TRADITIONAL LIPOSOMES, HYDROETHANOLIC SOLUTION AND ETHOSOMES

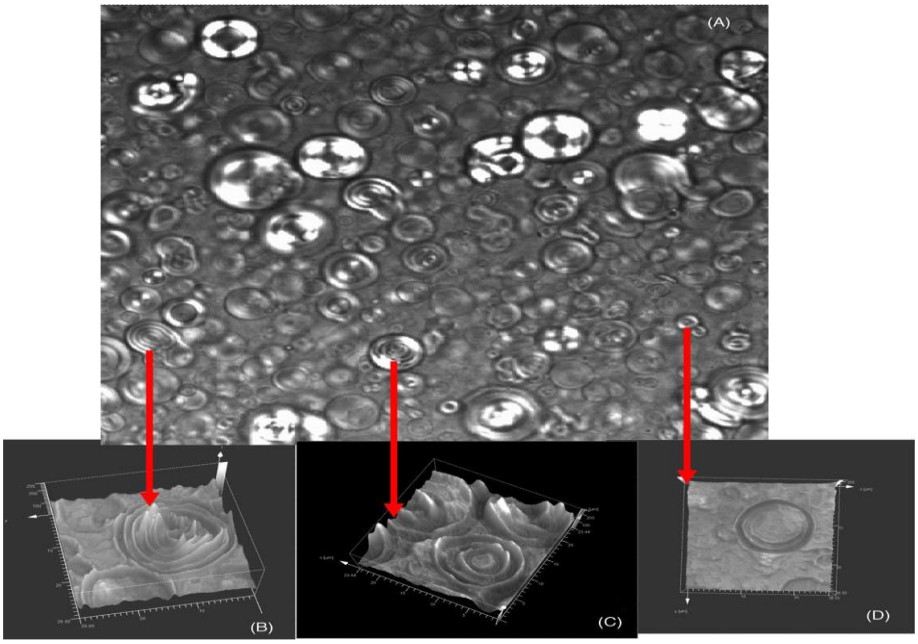

FIGURE-7: MICROPHOTOGRAPHS CORRESPONDING TO DPPC MULTILAMELLAR LIPOSOMES BY CLSM USING TRANSMITTED CHANNEL(A) THE WHOLE FIELD; (B) SIX LAMELLAE; (C) THREE LAMELLAE. (D) UNILAMELLAR VESICLE

(5) *Invitro* drug release studies (skin permeation studies)

Franz diffusion cell is mostly used for invitro skin permeation studies. Valia-Chien cell was used for permeation study of Minoxidil and Trihexyphenidyl Hclethosomes by Touitou *et al.* Skin of new born mice, abdominal and dorsal skin of male nude mouse, rat skin, male albino rabbit pinna skin taken from inner ear, dermatomed human cadaver skin from abdominal areas and human abdominal skin obtained by plastic surgery were used for the permeation studies. Synthetic semi permeable membranes made of cellulose material also used for the invitro drug release studies.

To get the skin from animals, the animal is sacrificed by chloroform inhalation. The hair of test animals was carefully trimmed short (<2mm) with scissors and the skin is separated from the underlying connective tissue with a scalpel. The excised skin was placed on the skin is gently teased off for any adhering fat and/or subcutaneous tissue. The obtained skin is mounted between the donor and receptor compartment with the stratum conium side facing upward into the donor compartment. The prepared Franz diffusion cell is either used immediately after preparation or after stored in the refrigerator (4^{o}C) in order to hydrate the skin overnight. Caution is taken to remove all bubbles between the underside of the skin and

the acceptor solution.

Isotonic phosphate buffer solution like phosphate buffered saline P^H: 6.5, P^H: 7.4 and citrate phosphate buffer P^H: 7.4 and hydroethanolic solution were used as receptor solution. In some studies 0.11% w/v of formaldehyde containing medium also used. During this studies 32±1^{o}C (skin surface temperature) is maintained. Some authors maintained human body temperature (37±1^{o}C) as itself. Stirring is maintained by a magnetic stirrer throughout the experiment. The ethosomal formulation is applied on the skin in donor compartment, which is then covered with a parafilm to avoid any evaporation process. Samples are withdrawn through the sampling port of the diffusion cell at predetermined time intervals over 12 hours to 24 hours and analyzed for drug content by respective analytical techniques. The receptor phase is immediately replenished with equal volume of fresh medium maintained at the same temperature as that of receptor compartment. Skin conditions are maintained throughout the experiments. **[14]**

(6) Skin retention study

The amount of drug retained in the skin is determined at the end of the invitro permeation experiment. The skin is washed using a cotton cloth immersed in methanol. The drug retention is determined by two methods. **[15**]

(a) Skin destruction method: The sample of skin is weighed, cut with scissors, positioned in a glass homogenizer containing 1ml methanol and homogenized for 5minutes with an electric stirrer. The resulting solution is centrifuged for 10 minutes at 7000rpm. The supernatant is analyzed for drug content by suitable analytical technique.

(b) Skin extraction method: After completion of the invitro permeation study the receptor content is completely removed and replaced by 50%v/v ethanol in distilled water and kept for a further 12 hours, then the drug content is determined. This receptor solution diffused through the skin, disrupting any liposome structure and extracting deposited drug from the skin, thus giving a measure of skin deposition.

(7) Skin irritancy studies[16]

Irritancy of different formulation is determined in male albino rabbits. The animals divided into groups as required. One group receives ethosomal formulation and one group receives hydroethanolic solution of drug having an ethanol concentration used in ethosomal

formulation. The score of erythema is determined. This study was reported by V.Dubey *et al* for melatonin ethosomes and demonstrated that ehanol present in the ethosomal formulation is not able to act as a skin erythema inducing agent, even though present in high concentration.

(8) Differential scanning calorimetry (DSC) measurements

The transition temperature (T_m) of vesicular lipids is measured using modulated differential scanning colorimetry with programmed heating rate of 10^{o}C/min under constant nitrogen stream within a range of -50^o to +50^{o}C. The amount of sample usually carried out this experiment is 20±5mg. Calorimetric studies demonstrate low T_m values for ethosomal system as compared to liposomes suggesting a fluidizing effect of ethanol on phospholipid bilayers. Thus ethosomes considered as a soft liquid state vesicles with fluid bilayers. Further T_m of the drug-loaded ethosomal system is similar to that of ethosomal systems, suggesting presence of drug in ethosomal core. If this is not similar indicates presence of drug in bilayer.**[17]**

(9) Storage and physical stability of ethosomes

The vesicular suspensions are kept in sealed vials after flushing with nitrogen and stored at different temperatures 4±2^{o}C (actual storage temperature), 25±2^{o}C (room temperature). The stability of ethosomes was assessed quantitatively by monitoring size and morphology of the vesicles overtime using dynamic light scattering technique and TEM. For assessing the skin permeability of stored ethosomal system, confocal laser microscopic studies were performed. **[19]**

ACKNOWLEDGEMENT:

The authors are thankful to the management and Principal for their co-operation and constant support.

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