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FORMULATION AND CHARACTERIZATION OF 5-FU LOADED ETHOSOMES FOR THE TREATMENT OF ACTINIC KERATOSIS

Pharmacy

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ABSTRACT

The present investigation aims to evaluate the transdermal potential of elastic liposomes (Ethosomes) loaded with 5 Fluorouracil (5 FU), an effective antineoplastic agent with poor skin permeation. 5 FU ethosomes were prepared, optimized and characterized for vesicular shape and surface morphology, vesicular size and size distribution, stability, entrapment efficiency, in vitro skin permeation test, skin irritancy test. Transmission electron microscopy, Dynamic light scattering shows spherical vesicles and nanometric size range (140.6 nm). Percent entrapment efficiency of the optimized formulation (Et63/20/40) was found to be 76.6 + 0.52%.Fourier Transformed-Infra Red (FT-IR) data generated to assessed the fluidity of skin after the application of formulation. Further, a better skin tolerability suggested that ethosomes may offer a suitable approach for transdermal drug delivery of 5 FU. These results can be considered as a step forward for the transdermal drug delivery system of certain problematic molecules like 5 FU for various types of skin cancers like Actinic Keratosis (AK), Squamous Cell Carcinoma (SCC), and Basal Cell Carcinoma (BCC) etc.

KEYWORDS

Ethosomes; Transdermal; Ethanol; Phospholipid; 5 Fluorouracil

INTRODUCTION

Fluorouracil is a drug that is a pyrimidine analog which is used in the treatment of cancer. It works through noncompetitive inhibition of thymidylate synthase. Due to its noncompetitive nature and effects on thymidine synthesis, 5-FU is frequently referred to as the "suicide inactivator". It belongs to the family of drugs called antimetabolites. Like many anti-cancer drugs, 5-FU's effects are felt system wide but fall most heavily upon rapidly dividing cells that make heavy use of their nucleotide synthesis machinery, such as cancer cells. 5 FU can be used topically for treating actinic (solar) keratoses and some types of basal cell carcinomas of the skin [1].

As a pyrimidine analogue, it is transformed inside the cell into different cytotoxic metabolites which are then incorporated into DNA and RNA, finally inducing cell cycle arrest and apoptosis by inhibiting the cell's ability to synthesize DNA. It is an S-phase specific drug and only active during certain cell cycles [2,3]. In addition to being incorporated in DNA and RNA, the drug has been shown to inhibit the activity of the exosome complex, an exoribonuclease complex of which the activity is essential for cell survival.

Touitou discovered lipid vesicular systems ethosomes embodying ethanol in relatively high concentration. Ethosomes contain phospholipids, alcohol (ethanol and isopropyl alcohol) in relatively high concentration and water. Unlike classical liposomes, ethosomes were shown to permeate through the stratum corneum barrier and were reported to possess significantly higher transdermal flux in comparison to liposomes. The exact mechanism for better permeation into deeper skin layers from ethosomes is still not clear. However, synergistic effects of combination of phospholipids and high concentration of ethanol in vesicular formulations have been suggested to be responsible for deeper distribution and penetration in the skin lipid bilayer [4].

The use of vesicular system for the treatment of skin and related diseases has attracted increasing attention in recent years. Topical delivery of drug by liposomal formulations has also evoked a considerable interest. Despite intensive research, results of the interaction of liposomes with skin are contradictory. Recent approaches in delivering drug through skin have resulted in new vesicular carrier system, ethosomes [5]. Ethosomes are interesting and innovative vesicular systems that have appeared in the fields of pharmaceutical technology and drug delivery. It has a capability to permeate the skin in intact form due to its high deformability. Ethosomes are soft, malleable vesicles tailored for enhanced delivery of active agents. It allows the active substances to transport more efficaciously through stratum corneum and to the deeper layers of the skin [6].

Thus this carrier system is suitable for both topical and systemic drug administration. Ethosomes are phospholipid vesicular system containing relatively high concentration of alcohol is very much effective in dermal or transdermal delivery of lipophilic, hydrophilic or amphiphilic molecules including low and high molecular weight compounds.

Ethosomes are mainly used for the delivery of drugs through transdermal route. The transdermal delivery is one of the most important routes of drug administration. The main factor which limits the application of transdermal route for drug delivery is the permeation of drugs through the skin [7]. Human skin has selective permeability for drugs. Lipophilic drugs can pass through the skin but the drugs which are hydrophilic in nature can't pass through. Water soluble drugs either show very less or no permeation. To improve the permeation of drugs through the skin various mechanisms have been investigated, including use of chemical or physical enhancers, such as iontophoresis, sonophoresis, etc. Liposomes, niosomes, transferosomes and ethosomes also have been reported to enhance permeability of drug through the stratum corneum barrier. Permeation enhancers increase the permeability of the skin, so that the drugs can cross through the skin easily [8-12].

In this research an attempt has been made to formulate the 5 FU loaded ethosomes for the treatment of actinic keratosis. The Ethosomal encapsulation of 5 FU was found to increase the skin residence tie, reduction of side effects and duration of therapy. Ethosomes are prepared using phospholipid, cholesterol, ethanol and water by hand shaking method with required modifications after optimizing the process and formulation variables. We also carried out some experiments to evaluate the 5 FU loaded ethosomes and studied prolonged release properties of this carrier.

EXPERIMENTAL

Materials

The 5-FU was received as a gift sample from Biochem Pharmaceutical Industries Mumbai, (India). Phospholipid (Phospholipon 85G) was obtained as a gift sample from Lipoid GmbH Ludwigshafen, Germany. Ethanol was purchased from Changshu Yangyuan Chemical Co., Ltd., Jiangsu, China (Mainland). Cholesterol was purchased from HiMedia Laboratory Pvt. Ltd. Mumbai, (India). All other ingredients used were of analytical grade.

Fluorouracil Solubility

The solubility of the 5 FU was determined in different polar and non-polar solvents. 5 FU was dissolved in different solvents separately and kept for 24 hours at 25 ^{0}C. The concentration of drug in respective above solutions were determined by UV spectrophotometer (Shimadzu 1700, Japan).

Preparation of 5 FU loaded ethosomes

Ethosomes were prepared following classic mechanical-dispersion method [13]; 63 mg Phospholipon 85 G and 20 mg Cholesterol were dissolved in small amount of methanol: chloroform (1:3). This solution was completely dried by hand shaking method in a clockwise or anticlockwise direction at 45 degree angle to form a lipid film on the wall of round-bottom flask. This lipid film was then hydrated using water-ethanol mixed solution (40% v/v ethanol) containing 5 FU previously dissolved. The preparation was mixed well and all samples were sonicated for 10 minutes, then immediately sealed and stored in darkness at a temperature of 4°C. Various formulation variables e.g. phospholipid concentration, cholesterol concentration and ethanol concentration which would affect the preparation and properties of ethosomes were identified and studied. The results were shown in Table I.

Determination of entrapment efficiency

The drug entrapment percentage was determined by ultracentrifugation method and by using dialysis bags [14]. Vesicle preparation was kept overnight at 4°C and centrifuged for 2 hrs at 40,000 rpm. Free 5 FU was assayed by UV spectrophotometer (Shimadzu 1700, Japan) in the supernatant. The 5 FU entrapment percentages was calculated from the relationship:EE = Qt – Qs/ Qt x 100

where EE is the entrapment efficiency, Qt is the theoretical amount of % FU that was added, and Qs is the amount of 5 FU detected only in the supernatant.

FT-IR analysis of human cadaver skin

The prepared human skin as mentioned above was treated with 200µl of Ethosomal formulation, 40% hydroethanolic solution for 6 h. The treated skin was then washed with distilled water and dried. The FT-IR spectrum of cadaver skin was recorded in range of 3000-1000 cm-1 using FT-IR spectrophotometer (Shimadzu 8400S, Japan). The FT-IR spectrum of control human cadaver skin was also recorded [15].

Transmission electron microscopy (TEM)

Ethosomal preparation was examined by TEM. A drop of sample solution was placed in a carbon coated grid and excess solution was removed by blotting and negatively stained with aqueous solution of phototungstic acid. The excess of staining solution was removed with a filter paper or by blotting in 60s. Finally after drying the grid was examined under JEM-1230 transmission electron microscope (JEOL, Japan), at 10-100 K fold magnification [16].

Optical Microscopy

The ethosomal dispersion was spread on the glass slide using a glass rod [17]. Formation of multilamellar vesicles was confirmed by examining the ethosomal suspension under an optical microscope (Dolphin International Ltd, India) with magnification of 10x. Photograph of vesicles was taken using SONY DSC W310.

Fluoroscence Microscopy

The ethosomal dispersion was mixed with fluorescence dye or crescent dye and the dispersion was spread in the glass slide with a glass rod. Vesicles were observed using VanGuard 1200ECM Fluorescence Trinocular, Newyork [18].

Differential Scanning Calorimetry

Differential scanning calorimetry was performed by using (Pyris 6 DSC, Jade DSC V1.12, USA). The samples were placed in aluminium pans and were crimped, followed by heating under nitrogen flow (20 ml/min) at a scanning rate of 10°C/min from 30°C to 350°C. Aluminium pan containing same quantity of indium was used as reference. The sample weight was about 4.3 mg. The heat flow as a function of temperature was measured for both the drug and drug-excipient mixture [19].

Vesicular size, size distribution and zeta potential

The vesicular size, size distribution and zeta potential of vesicles were determined by dynamic light scattering (DLS) using a computerized inspection system (Malvern Zetasizer DTS Ver. 5.03, MAL1023461 Malvern UK). For size measurements, vesicular suspension was mixed with the appropriate medium (usually water). The measurements were conducted in triplicate, in a multimodal mode of 60s each at a medium stable count rate. The electric potential of the ethosomes, including its Stern layer (zeta potential) was determined as described, by injecting the diluted system in to a zeta potential measurement cell [19].

In vitro drug release studies (Skin permeation studies)

The in vitro skin permeation of 5FU loaded ethosomes was studied using Franz diffusion cell with effective permeation area and receptor cell volume of 1.0 cm^2 and 10 ml, respectively. The temperature during the whole study was maintained to 32±1 °C. The receptor compartment contained 10 ml PBS (pH 6.5) and was constantly stirred by magnetic stirrer at 100 rpm. Human cadaver skin from upper limb were obtained from Netaji Subhash Chandra Bose Medical College, Jabalpur, India and stored in freshly prepared formalin solution. The skin was washed and then checked through magnifying glass to ensure that surface is free from any surface irregularities such as tiny holes or crevices in the portion that was used for transdermal permeation studies. After a proper check skin was mounted on the receptor compartment with the stratum corneum side facing upwards into the donor compartment. The Ethosomal formulation (200 µl) was applied on the skin in donor compartment, which was then covered with parafilm to avoid any evaporation process. Samples were withdrawn through sampling port of diffusion cell at pre determined time intervals over 24 h and analyzed for drug content by UV spectrophotometer. Sink condition was immediately be maintained after every sampling with equal volume of PBS (pH 6.5). Similar experiment was repeated with hydroethanolic 5 FU solution (1% drug dispersed in40% hydroethanolic solution).

The amount of 5 FU retained in the skin was determined at the end of the *in vitro* permeation experiment (24 h). The skin was washed with methanol and homogenized for 5 min with electric stirrer. The resulting solution was centrifuged for 10 min at 7000 rpm. The supernatant was analyzed for drug content by UV spectrophotometer [20-22].

Skin irritancy test

The skin irritancy (erythema) of ethosomes and hydroethanolic solution (40% v/v ethanol) was reported elsewhere [23]. Briefly, irritancy of formulation was determined in male albino rabbits (180-200 gms). The animals were housed in an air-conditioned room (20°C) and hair at the back was trimmed short, 24 h before the beginning of assay. The animals were divided in three groups of six each. First and second group received Ethosomal and hydroethanolic solution respectively. Three squares were drawn on each side of the back of each rabbit, and 200 µl formulations were applied on each square. After exposure of 32 h, the test substance was removed and exposed skin was scored depending on the degree of erythema, as follows: no erythema- 0, very slight erythema (barely perceptible- light pink) – 1, well defined erythema (dark pink) – 2, moderate to severe erythema (light red) – 3.

pH Measurements

The pH measurements of formulation were carried out using a electronic pH meter (EQUIP-TRONICS Model EQ 610) by dipping the glass electrode completely into the ethosomal dispersion so as to cover the electrode.

Stability Studies

The accelerated stability studies were carried out in accordance with the ICH guidelines. The ability of vesicles to retain the drug was assessed by keeping the ethosomal suspension at different temperature. Optimized ethosome formulation was selected for stability studies of vesicles. The vesicular suspension was kept in sealed vials (10ml) at 4 ± 2ºC and at room temperature for 45 days. Percent entrapment and vesicles size was determined at different time intervals [24].

RESULTS AND DISCUSSION

The method used to quantify the 5 FU in to the systems was ultracentrifugation method. It was observed that maximum entrapment percentage was reached at 40% (v/v) ethanol, 20 mg of CHOL, and 63 mg of phospholipid. The maximum 5 FU entrapment in ethosomes estimated using ultracentrifugation method was found to be 76.6 ± 0.52% as shown in Table I. The greater entrapment of 5 FU in ethosomes could be attributed to greater solubility of 5 FU in ethanol present in ethanol core.

Table I: Physicochemical characteristics of 5 FU loaded ethosomes

Sample EtPH/CHOL/ ETH	Mean diameter	Zeta Potential	Polydispersity Index	Encapsulation Efficiency
Et61/20/40	132.5	-19.8 mV	0.215	64.23 ± 0.96
Et62/20/40	137.2	-20.5 mV	0.316	68.06 ± 0.20
Et63/20/40	140.6	-15 mV	0.374	76.6 ± 0.52
Et64/20/40	141.4	-14.8 mV	0.382	58.1 ± 0.26
Et65/20/40	142.3	-14.6 mV	0.391	55.4 ± 1.10
Et63/10/40	124.8	-19.2 mV	0.218	71.3 ± 0.28
Et63/15/40	136.2	-18.7 mV	0.284	74.6 ± 0.28
Et63/20/40	140.6	-15 mV	0.374	76.6 ± 0.52
Et63/25/40	162.4	-14.5 mV	0.276	72.6 ± 0.76
Et63/30/40	184.2	-14.2 m V	0.384	70.8 ± 0.57
Et63/20/10	215.8	-13.9 mV	0.519	67.3 ± 1.00
Et63/20/20	196.2	-14.2 mV	0.468	70.6 ± 1.25
Et63/20/30	164.5	-14.6 mV	0.412	73.3 ± 1.04
Et63/20/40	140.6	-15.0 mV	0.374	76.6 ± 0.52
Et63/20/50	134.8	-19.4 mV	0.306	72.0 ± 0.50

FT-IR spectral profile of stratum corneum provides a measure of the fluidity of stratum corneum lipids. On comparison of spectral profile of untreated SC, treatment of SC with hydroethanolic solution and ethosomes resulted in a shift to a higher frequency and an absorbance broadening for both the C-H symmetric stretching absorbance frequency peak near 2850.34 cm-1 and C-H asymmetric stretching absorbance near 2924.14 cm-1 (Table II and Fig. 1). These results suggests that in case of hydroethanolic solution the ethanol could probably increase the rotational freedom of acyl chains leading to increased fluidity of skin lipids. Also a synergistic penetration enhancing effect is observed in case of ethosomes, as visualized from our FT-IR data (higher the frequency shift and absorbance broadening as compared to hydroethanolic solution), suggesting greater mobility of SC lipids on application of ethosomes penetrate the skin via disrupted stratum corneum's lipid bilayer organization.

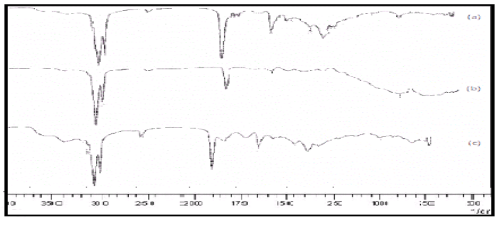


Fig. 1: (a) FTIR of Untreated Skin (b) FTIR of skin treated with hydroethanolic solution (c) FTIR of skin treated with 5FU loaded ethosomes

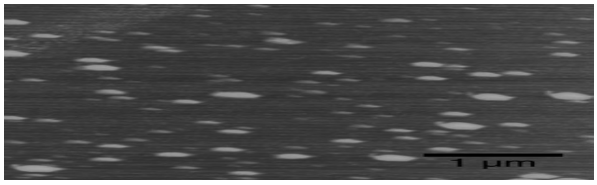
Table II: Effect of various formulations on C-H symmetric and C-H asymmetric stretching absorbance shifts on the acyl chains of stratum corneum lipids

Treatments	C-H symmetric stretching	C-H asymmetric stretching
No treatment	2850.34	2924.14
40% Hydroethanolic solution	2852.07	2925.20
Ethosomes	2854.74	2926.11

5 FU loaded ethosomes ($Et_{63/20/40}$) were negatively stained and observed by TEM. These appeared as spherical structure, confirming the vesicular characteristics (Fig. 2).

Fig. 2: Visualization of 5 FU loaded ethosomes by TEM

The ethosomal dispersion was spread on the glass slide using a glass rod. Formation of multilamellar vesicles was confirmed by examining the ethosomal suspension under an optical microscope with magnification of 10x (Fig. 3). Photograph of vesicles was taken using SONY DSC W310.



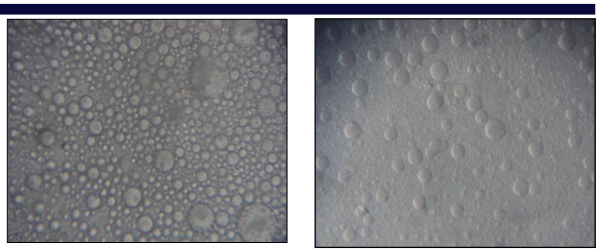


Fig. 3: Photographs from optical microscope

The ethosomal dispersion was mixed with fluorescence dye or crescent dye and the dispersion was spread in the glass slide with a glass rod. Vesicles were observed using Fluoroscence microscope as shown in

(Fig. 4).

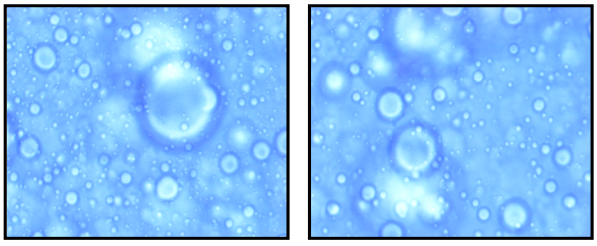


Fig. 4: Photographs from Fluoroscence Microscope

The possible interaction between the drug and the excipients were studied by DSC. The results of DSC studies are given in (Fig. 5). Pure 5 Fluorouracil showed a sharp endotherm at 283.07°C corresponding to its melting point/transition temperature. There was no appreciable change in the melting endotherms of the physical mixture (5 FU + Polymer) compared to pure drug. indicated the absence of any interactions between drug and additives used in the preparation. However there was slight decrease in the melting point of the drug. It was also observed that there was a noticeable reduction in the enthalpy of the formulation with compare to 5 FU formulation (Et63/20/40) showed -4.6 j/mg. The lowest melting point was 202.88°C.

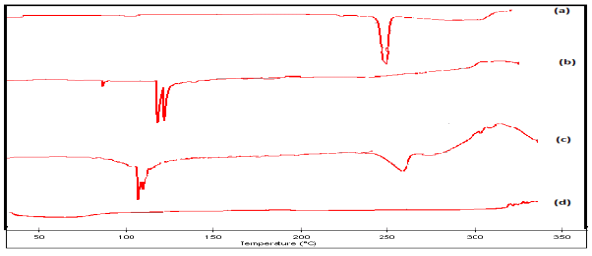


Fig. 5: Thermogram of Pure Drug, Pure Phospholipid and Physical mixture (a) Thermogram 5 FU (b)Thermogram Phospholipon G and (c) Thermogram Physical Mixture (Phospholipon G + 5 FU) (d) Thermogram of Loaded Ethosomes

The effect of phospholipid and ethanol was investigated using DLS. The average particle size of ethosomes ranges between 124.8 nm and 215.8 nm. For ethosomes prepared with 40% ethanol and 63 mg of phospholipid, the data showed a narrow particle size distribution with an average size of 140.6 nm (Fig. 6). It has been observed that on increasing the concentration of ethanol the mean particle size decreases with the smallest particles in concentration 50% (134.8 nm). We observed that there is initial decrease in Poly Dispersity Index (PI) of ethosomes with increasing amount of ethanol upto 40% ethanol but on further increase in the amount of alcohol (50%) leads to decrease in PI. This suggests that there exists an optimal amount of ethanol which can be incorporated in the formulation composed of small vesicle. The PI The charge of the ethosomal vesicles is an important parameter that can influence both vesicular properties such as stability, as well as skin vesicle interaction. The PI of the optimized preparation was found to be 0.374. The presence of ethanol in the system induces negative charge over vesicles. The charge of the ethosomes containing 40% ethanol was found to be -15 mV.

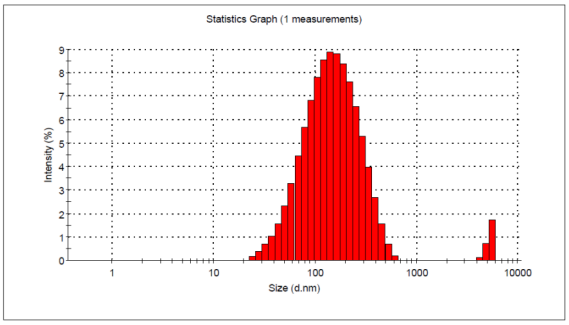


Fig. 6: Showing size distribution of an Optimized preparation

Invitro permeation studies give us valuable information about the product behaviour in vivo. The drug permeated dictates the amount of drug available for absorbtion. In vitro locally fabricated franz diffusion cell measurements were used to investigate the transport enhancement potential of ethosomal vesicles. This method is commonly used in skin research and normally gives good correlation with the invivo data.

The ability of ethanolic vesicles to deliver 5FU was investigated by determining the flux of 5FU across dermatomed human skin. The cumulative amount of 5FU permeated per unit area across excised human cadaver skin via various formulations was plotted as a function of time (Fig. 7).

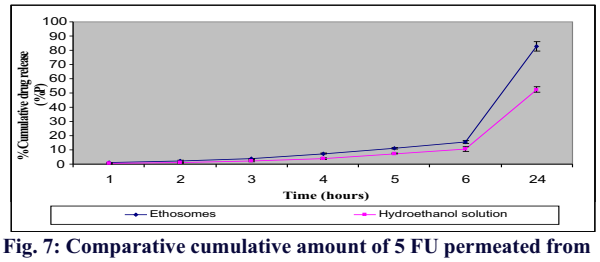


Fig. 7: Comparative cumulative amount of 5 FU permeated from ethosomal formulation and hydroethanolic solution in a 24 h study via human cadaver skin

The enhanced transdermal efficacy obtained from ethsomes is mainly due to two reasons i.e. ethanol effect and ethosomal effect. In ethanol effect, interaction of ethanol with intercellular lipid occurs which increase the fluidity and decrease the density skin lipid. This effect is followed by ethosomal effect which includes interlipid penetration and permeation by opening new pathway due to malleability and fusion and thus the drug is released in deep dermal tissue.

A very important characterstic to be evaluated before the proposal of a carrier as a potential transdermal drug delivery system is its in vivo skin tolerability/irritancy. Measurement of erythema scores upon exposure of hairless rat to various formulation including saline solution (0.9% w/w NaCl solution, control), ethosomal formulation, hydroethanolic solution and blank formulation revealed that saline, ethosomes and blank formulation showed no significant erythema, while hydroethanolic solution showed a remarkable erythema. This demonstrates that ethanol present in ethosomal formulation is not able to act as a skin erythema inducing agent even if it is present in high concentration.

The pH measurements of formulation were carried out using a pH meter by dipping the glass electrode completely into the ethosomal dispersion so as to cover the electrode. The pH of the final optimized preparation was found to be 5.8.

The ability of vesicles to retain the drug was assessed by keeping the ethosomal suspension at different temperature. Optimized ethosome formulation (Etho 5) was selected for stability studies of vesicles. The vesicular suspension was kept in sealed vials (10ml) at 4 ± 2ºC and at room temperature for 45 days. Percent entrapment and ethosomes size was determined at different time intervals. The size of the ethosomes increased more in case of room temperature as compared to 4 ± 2ºC. It was observed that the ethosomal system was more stable at 4 ± 2°C showed in (Table III).

Table III: Stability studies at different time interval

Time (Days)	Percent Entrapment (4 ± 2°C)	Percent Entrapment (Room Temperature)	Vesicle Size (4 ± 2°C)	Vesicle Size (Room Temperature)
1	76.6 + 0.52	76.6 + 0.52	140.6	140.6
15	76.3 + 0.48	75.2 + 0.28	143.2	144.3
30	75.8 + 0.60	74.8 + 0.42	145.8	148.4
45	74.1 + 0.58	68.4 + 0.80	146.4	152.8

CONCLUSION

The current investigation reveals that 5 Fluorouracil loaded ethosomes provided enhanced transdermal drug permeability, higher entrapment efficiency and low skin irritancy potential, thus leading to the generic conclusion that this approach for transdermal delivery of 5 Fluorouracil.

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