PREPARATION OF NOVEL VESICULAR CARRIER ETHOSOMES WITH GLIMEPIRIDE AND THEIR INVESTIGATION OF PERMEABILITY

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ABSTRACT

Glimepiride is a third generation sulfonylurea antidiabetic drug. It is practically insoluble in water; this poor aqueous solubility and slow dissolution may lead to irreproducible clinical response or therapeutic failure due to sub therapeutic plasma drug levels. Low oral bioavailability results in wasting of a large portion of an oral dose. To circumvent these drawbacks glimepiride was entrapped in novel vesicular carrier system (Ethosomes) to improve therapeutic efficacy of glimepiride via transdermal route. Therefore current work was aimed to formulate, characterize and evaluate the transdermal potential of ethosomes encapsulating glimepiride. Vesicular shape, surface morphology and entrapment efficiency were determined by optical microscopy, transmission electron microscopy, and minicolumn centrifugation technique respectively. In contrast to liposomes, ethosomes were of more condensed vesicular structure and they were found to be oppositely charged. The ethosomal formulation were found to be more efficient delivery carriers with high entrapment and optimal nanometric size range and low polydispersity index in comparison with plain drug solution and liposomal formulation. The ethosomal formulations exhibited entrapment efficiencies of 42–78%. In vitro percutaneous permeation experiments demonstrated that the permeation of glimepiride through rat skin was significantly increased when ethosomes were used. Kinetics of in-vitro skin permeation showed zero order drug release from formulations. The flux from ethosomes was 3-fold higher than liposomal solution. FT-IR studies revealed that when skin was treated with ethosomal formulation ceramides got loosened leading to breaking of hydrogen bond networks at the head of ceramides due to penetration of ethosomal into the lipid bilayers of SC. Results suggested ethosomes to be the most proficient carrier system for dermal and transdermal delivery of glimepiride.

KEYWORDS: Ethosomes, invitro percutaneous permeation, sustained release, glimepiride

INTRODUCTION

The major advances in vesicle research was the finding that some modified vesicles possessed properties that allowed them to successfully deliver drugs in deeper layers of skin. Transdermal delivery is important because it is a non-invasive procedure for drug delivery. Further, problem of drug degradation by digestive enzymes after oral administration, gastric irritation and discomfort associated with parenteral drug administration can be avoided. Flexible liposomes are common vectors in transdermal drug-delivery systems, with relatively good liquidity and deformability.[1]

In recent years, ethosomes have become new liposome carriers with high deformability; high entrapment efficiency and a good transdermal permeation rate in the drug-delivery system, and are suitable for transdermal administration[2.] Compared with other liposomes, the physical and chemical properties of ethosomes make these more effective for drug delivery through the stratum corneum into the blood circulation, which is very important in the design of a transdermal drug-delivery system.

Glimepiride is a third generation sulfonyl urea drug useful for treatment of diabetes mellitus or type 2 diabetes. It exhibits very low water solubility (< 0.004mg/ml) but highly permeable (class 2) according to the BCS (Jain A., 2008)[3.] The pharmacokinetics and dosage schedule supports once daily sustained release formulations for Glimepiride for better control of blood glucose levels to prevent hypoglycaemia[4], enhance clinical efficacy and patient compliance (Ikegami H, et al., 1986)[5]. Its poor aqueous solubility and slow dissolution may lead to irreproducible clinical response or therapeutic failure due to sub therapeutic plasma drug levels. Low oral bioavailability results in wasting of a large portion of an oral dose (Thummel, 1997; Frick et al., 1998) [6, 7]. To circumvent these drawbacks glimepiride was entrapped in novel vesicular carrier system (Ethosomes) to improve therapeutic efficacy of

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glimepiride via transdermal route. Transdermal deliveries of antidiabetic drugs have been successfully achieved for glipizide, glibenclamide[8].

procured from HIMEDIA, India Ltd. All other reagents used in the study were of analytical grade.

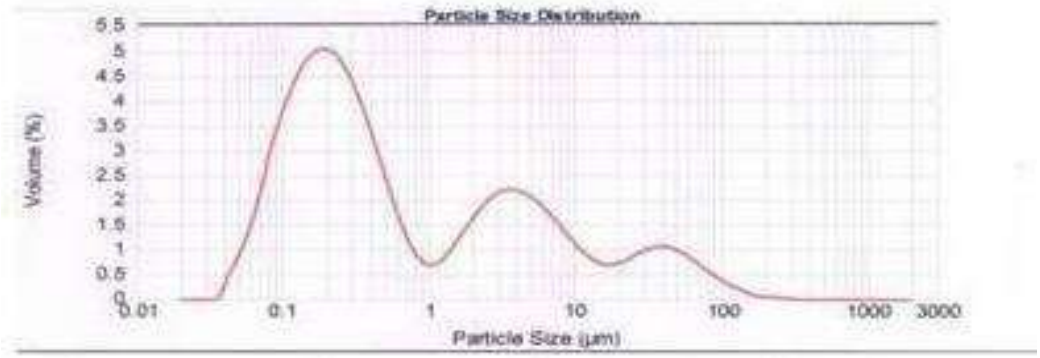

Figure 1 particle size distribution of optimized formulation ETE3

We have successfully achieved sustained delivery for rizatriptan [9,] lovastatin[10,] and 5-FU [11 by] formulating them in to transfersomes. The current study is aimed to formulate and characterize the invitro potential of ethosomes in enhancement of Glimepiride transport across the skin.

MATERIALS AND METHODS

Glimepiride was received as a gift sample from Amit surgical and Pharmaceuticals. High purity soyaphosphatidyl choline (Soya PC) was purchased from Sigma Chemicals. Cellophane membrane (molecular weight cut off (12,000 to 14,000) was

Preparation of ethosomes

The ethosomal formulation was prepared according to the method reported by the Touitou et al (2000)[12] .The ethosomal system prepared here comprised of 0.5-4% phospholipids, 10-50% ethanol, drug, 10% propylene glycol and water to 100% w/w. Phospholipid and drug were dissolved in ethanol-propylene glycol mixture. The mixture was heated to 30ºC in a water bath. The double distilled water heated to 30ºC was added slowly in a fine stream with constant mixing (Mechanical stirrer, Remi Equipment, Mumbai) at 700 rpm in a closed vessel. Mixing was continued for an

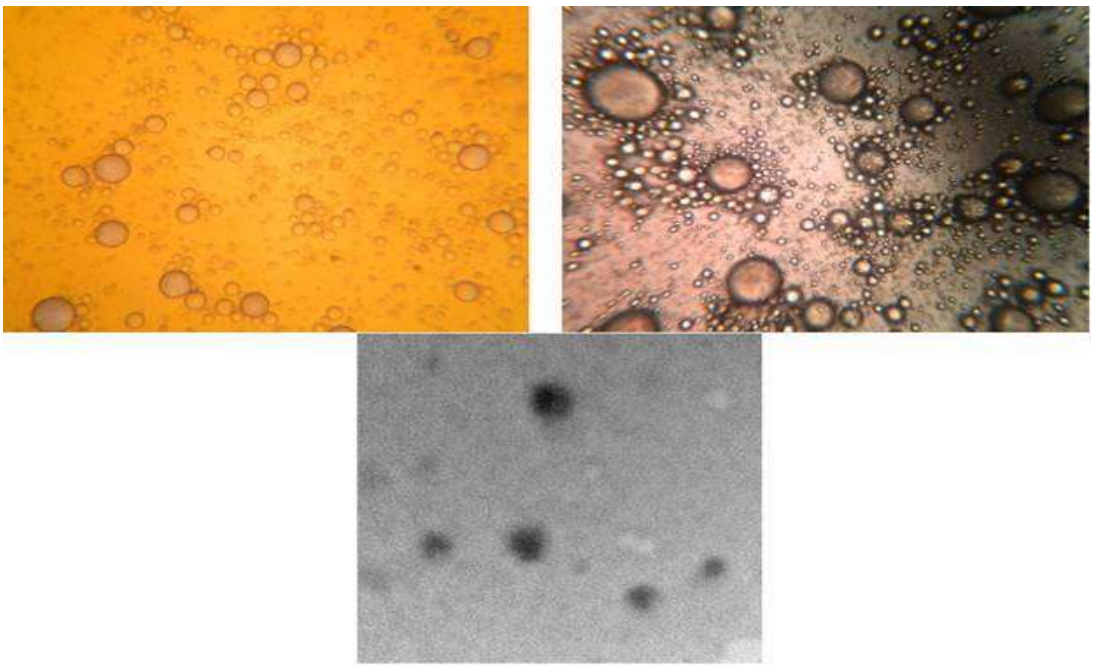

Figure2- A photomicrograph of ETE3 (B) ETE4 (C) TEM photomicrograph of ETE3 Formulation

additional 5 min. The system was kept at 30ºC throughout the preparation. The preparation was sonicated at 4ºC using probe sonicator (at 40 W, Imeco, Ultrasonics, India) in 3 cycles of 5 minutes with 5 minutes rest between the cycles. The final lipid concentration in all ethosomal formulations were 0.5-4% w/w.

Incorporation of glimepiride into vesicle dispersion at saturated concentration

Glimepiride was incorporated into all vesicle formulations at saturating concentration to obtain equal thermodynamic activities. To determine the maximum amount of drug that could be added, increasing amounts drug were added during preparation of ethosomal formulation. It was assumed that the presence of crystals would indicate that the formulation was saturated with drug. Therefore, all drug loaded vesicular formulations were examined over a period of 14 days using phase contrast microscope (Leica, DMLB, Switzerland) for appearance of drug crystals Fig.1C (Honeywell-Nguyen et al., 2003) [13].

Characterization of ethosomal formulations

Vesicles size and size distribution

The vesicles size and size distribution were determined by Dynamic Light Scattering method (DLS), in a multimodal mode using a computerized inspection system (Malvern Zetamaster, ZEM 5002, and UK). For vesicles size measurement, for vesicles size measurement, vesicular suspension was mixed with the appropriate medium (50% v/v ethanol) [14, 15] and measurements were conducted in triplicate.

Vesicles morphology

Ethosomes vesicles were visualized using TEM, with an accelerating voltage of 100 kV. 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% phosphotungstic acid (PTA). A drop of the staining solution was added on to the film and the excess of the solution was drained off with a filter paper. The grid was allowed to air dry thoroughly and samples were viewed in a TEM (Touitou *et al*., 2000)[12].

Elasticity of vesicle membrane

Elasticity of vesicle membrane is a unique parameter of ethosomal formulations because it differentiates ethosomal from other vesicular carriers like liposomes that are unable to cross the stratum corneum intact. The deformability study was done for the ethosomal formulation against the standard liposome preparations. In this study the flux of vesicle suspensions through a large number of pores of known size (a sandwich of polycarbonate filters with pore diameter between 50 and 200 nm depending on the starting vesicle suspension), was driven by an external pressure of 2.5 bars. The amount of vesicle suspension, which was extruded during 5 min, was measured and vesicle size and size distribution were monitored by DLS measurement before and after filtration. The experiment was performed in triplicate and each sample was analyzed twice[16.]

The elasticity of vesicle membrane was calculated by using the following formula as reported by Van den Bergh et al.[17]

$$D = J \times \left({}^{r_x}/_{rp}\right)^2 \text{..... (1)}$$

Where D is the elasticity of vesicle membrane; J is amount of suspension, which was extruded during 5 min; rv = size of vesicles (after passes); and rp= pore size of the barrier.

Turbidity and zeta potential determination

Turbidity of all ethosomal vesicular suspensions was measured by ELICO-CL 52D Nephalometer. In this method, 500 NTU (Nephalometric Turbidity Units) range is set. Then zero reading is set with Millipore water. After this, formulation is transferred to glass cuvettes of capacity 50 ml and placed in the holder inside the instrument. The method is repeated for each formulation and measurement of turbidity is displayed on the screen and expressed as NTU. Zeta potential of the vesicles was determined using Zetasizer (Nano-ZS, Malvern, U.K.). The measurements were made in triplicate.

Entrapment efficiency

The entrapment efficiency of the vesicles was determined by ultracentrifugation method. One ml of the formulation was centrifuged at 4ºC at 14,000 rpm for 1 hr. The supernatant containing the unentrapped drug was decanted. The vesicles lyses was done using Triton-X 100 (0.1%v/v) and after further dilutions it was analyzed for drug content using UV Spectrophotometer at 228nm.[18, 19]

The entrapment efficiency was expressed as percentage of total drug entrapped using the following formula.[20]

Percentage Entrapment = C/T × 100..... (2)

Where, T = theoretical amount of drug that was added, and C = amount of T drug detected after dissolving the vesicles.

In-vitro drug release- through cellophane membrane

Suitable size of membrane (Molecular weight cut of 12,000-14,000, HI Media, Ltd.) was cut and was kept in saline solution for 1 hour before dialysis to ensure complete wetting of the membrane. One ml of the drug-loaded vesicles was placed in the dialysis bag, which was then transferred into 50 ml of phosphate buffer saline (PBS) (pH 6.8). The receiver medium was stirred with a magnetic stirrer which is thermostatically controlled Sample was withdrawn after 0.5-, 1.0-, 1.5-, 2.0-, 3-, 4-, 6-, and 12-hour time intervals and replaced with equal volumes of PBS.[21]

Skin permeation study

The in vitro skin permeation of glimepiride was studied using locally fabricated Franz diffusion cell (Gupta Scientific Labs, Ambala, India) with an effective permeation area and receptor cell volume of 1.0 cm^2 and 20 ml respectively. The temperature was maintained at 32 ± 1ºC. The receptor compartment contained 10 ml PBS (pH 6.8) and was constantly stirred by magnetic stirrer (Expo India Ltd., Mumbai, India) at 100 rpm.

All experiments were conducted in triplicate. The skin was carefully checked through a magnifying glass to ensure that samples were free from any surface irregularity such as tiny holes or crevices in the portion that was used for transdermal permeation studies.[22]

The skin was mounted on a receptor compartment with the stratum corneum side facing upward into the donor compartment. The donor compartment was filled with 1ml of ethosomal formulation i.e. was applied to the epidermal surface of the rat skin. Samples (1ml) were withdrawn through the sampling port of the diffusion cell at predetermined time intervals over 24 h and analyzed. The receptor phase was immediately replenished with equal volume of fresh diffusion buffer. The amount of glimepiride retained in the skin was determined at the end of in vitro permeation experiment (24 h). The skin was washed 10 times with a cotton cloth immersed in methanol. A sample of skin was weighed, cut with scissors, positioned in a glass homogenizer containing 1 ml of methanol, and homogenized. The resulting solution was centrifuged for 10 min at 7000 rpm and then the supernatant was analyzed for drug.[23]

Calculation of permeation parameters

The cumulative amount of drug permeated per unit area was plotted as a function of time, the steady-state permeation rate (Jss) and lag time (LT, h) were calculated from the slope and X-intercept of the linear portion, respectively. The enhancement ratio (ER) was calculated from following equation

$$ER = \frac{\text{Transdermal flux from vesicular formulation}}{\text{Transdermal flux from plain drug}}. \quad \text{..... (3)}$$

Ftir spectral analysis of ethosomal formulation treated and untreated rat skin:

Table 1: COMPOSITION OF ETHOSOMAL FORMULATIONS

Formulation Code	Phospholipid (% w/w)	Ethanol (% w/w)	Isopropyl alcohol (% w/w)	Propylene glycol (% w/w)	Cholesterol (% w/w)
ETE_1	2.0	10	-	10	-
ETE_2	2.0	20	-	10	-
ETE_3	2.0	30	-	10	-
ETE_4	2.0	40	-	10	-
ETE_5	2.0	50	-	10	-
LP^c	2.0	-	-	-	0.15

ETE = Ethosomal formulation containing ethanol

LP^c = Liposomal formulation

At the end of skin permeation study (24hr) SC (stratum corneum) was cut into small circular discs. 7.0% w/v solution of sodium chloride was prepared. 20 ml of 7.0% w/v of sodium chloride solution was placed in 2 conical flasks. Both SC disc were washed, blotted dry, and then air dried for 2 h. Samples were kept under vacuum in desiccators for 15 min to remove any traces of formulation. Fourier transform infra-red (FTIR) spectra was recorded for ethosomal treated and untreated (control) in frequency range of 500 to 4000 cm-1 (Perkin Elmer, Germany).[24]

Stability studies

in comparison to liposomal formulation was probably due to the presence of ethanol in vesicle membrane. Increasing the concentration of ethanol from 15% to 45% increased the entrapment efficiency owing to increase in fluidity of membranes. However, further increase in the ethanol concentration probably made the vesicle membrane leakier, thus leading to decrease in entrapment of glimepiride (Table 2).

The size distribution of ethosomes ranged between tens of nanometers to microns and is known to be influenced by the composition of ethosomes. Table 2 shows that the size of the

Table 2 –CHARACTERISTIC PARAMETERS OF ETHOSOMAL FORMULATION

Formulation Code	Vesicles Size (nm)	PI*	Zeta potential	Turbidity	Elasticity (D)	Entrapment Efficiency
ETE_1	**224 ± 9**	0.042	-1.8 ± 0.2	112 ± 10	15.48 ± 4.0	45.6 ± 4.2
ETE_2	182 ± 8.0	0.035	-6.9 ± 0.8	133 ± 9	27.48 ± 3.9	51.4 ± 5.1
ETE_3	159 ± 6.2	0.037	-4.8 ± 0.5	152 ± 10	35.48 ± 4.1	54.6 ± 5.4
ETE_4	103 ± 6.0	0.056	-6.9 ± 0.8	142 ± 9	12.63 ± 1.4	58.2 ± 3.2
ETE_5	91 ± 5.0	0.054	-9.1 ± 1.1	112 ± 8	11.63 ± 1.3	59.6 ± 4.9
LIPO	117± 3.0	.0089	-1.8 ± 0.2	132 ± 10	4.96 ± 0.8	32 ± 4.1

PI* - Polydispersity index

The purpose of the study is to determine the effect of storage at different temperature conditions on stability of ethosomes. Physical stability studies were conducted by monitoring the change in mean vesicle size and the leakage of encapsulated drug from ethosomal formulations at different time intervals up to 30 days. The dispersions were placed in tightly sealed vials flushed with nitrogen gas and stored at 4 ± 1°C and ambient temperature (28 ± 1°C).[25]

RESULTS AND DISCUSSION

Characterization of ethosomal formulation

The composition of different ethosomal formulations is summarized in Table 1. TEM photomicrograph figure 2 of ethosomes revealed the presence of spherical vesicular structures. The entrapment efficiency for glimepiride in optimized ethosomal and liposomal formulation was 54.6 ± 5.4 and 32± 4.1 respectively. The higher entrapment efficiency of glimepiride in ethosomes

vesicles decreased when the ethanol concentration was increased from 15% to 50%. The largest vesicles of 224 ± 9 nm sizes were present in the preparation containing 10% ethanol, while the smallest vesicles of 91 ± 5.0nm size were present in the preparation containing 50% ethanol. This significant difference in the size of ethosomal formulations is because of the presence of different concentrations of ethanol. Probably, ethanol causes a modification in net charge of the system and confers it some degree of stearic stabilization that may lead to decrease in mean vesicle size. Zeta potential measurement study supported by the above hypothesis as zeta potential was observed to be −4.8±0.5 and −9.1 ± 1.1, respectively, for ethosomal formulation containing 30% and 50% ethanol.

Elasticity measurement

The elasticity of ethosomal vesicle membrane (35.48 ± 4.1) was found to be 7-fold higher than liposomes (4.96 ± 0.8). Higher concentration of

Table 3: SKIN PERMEATION PARAMETERS FOR THE TRANSPORT OF GLIMEPIRIDE THROUGH RAT SKIN

Form. code	J^*_{ss} ($\mu g/cm^2/hr$)	LT^{2*} (h)	P^{3*} (cm/h) $X10^{-3}$	D^{4*} (Cm^2/h)$X10^{-3}$	K^{5*}	ER^{6*}
ETE_1	37.2 ± 0.9	6.6	1.43	4.0	42.0	7.2
ETE_2	57.2 ± 1.6	4.2	3.08	6.2	57.4	15.4
ETE_3	76.5 ± 2.5	3.1	4.86	8.6	66.3	24.3
ETE_4	40.2 ± 0.8	6.9	1.15	3.8	35.5	5.7
ETE_5	18.2 ± 0.7	6.9	1.12	3.8	34.8	5.6
LIPO	26.14 ± 0.9					
PD	3.22 ± 0.2	9.2	0.12	2.8	8.3	

* = Transdermal flux
[2]*= Lag time
[3]* = Permeability coefficient
[4]*= Diffusion coefficient
[5]* = Partition coefficient
[6]* = Enhancement ratio
PD= Plain drug

ethanol present in ethosomes perhaps provided elasticity to vesicle membrane by reducing the interfacial tension of the vesicle membrane.19 In contrast, liposomal formulation contained cholesterol that is known to contribute to rigidity of vesicle membrane.[9] Inclusion of cholesterol in liposomal formulation also significantly (P G .05) decreased the elasticity of vesicle membrane (Table 2). The fluidizing effect of ethanol on ethosomal vesicle membrane has been already confirmed by differential scanning calorimetry and fluorescent anisotropy studies.[26]

In-vitro release studies

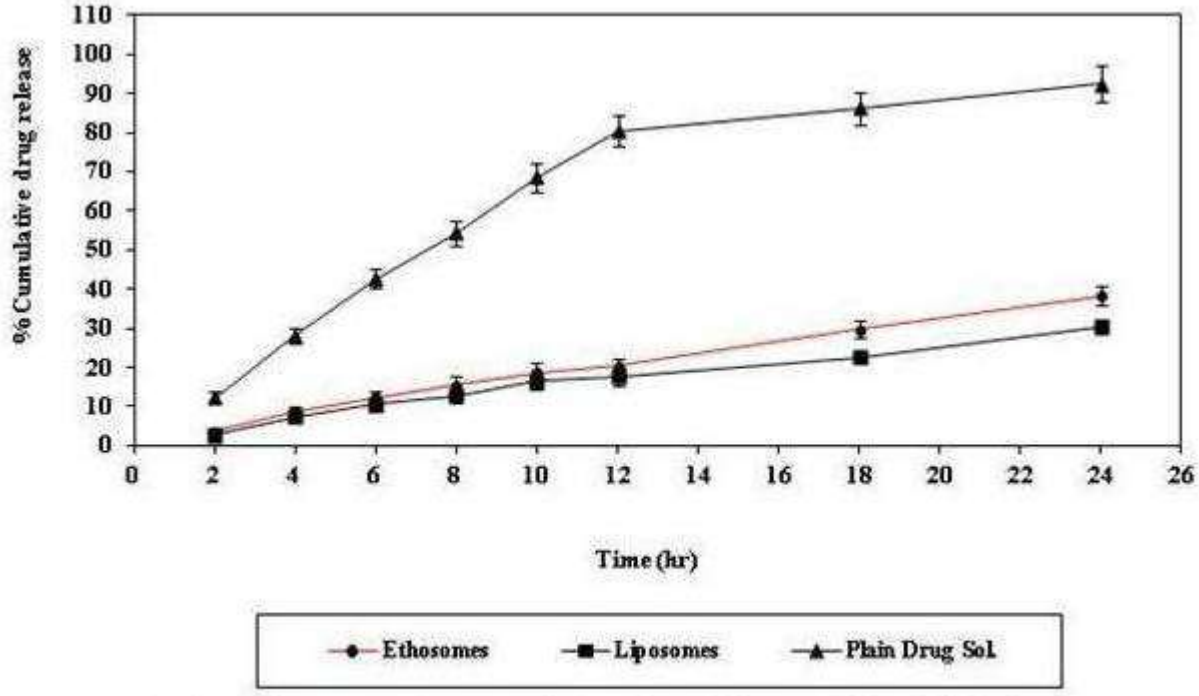

Figure 3 comparative cumulative drug release of glimepiride from ethosomal formulation, liposomal formulation and plain drug solution after 24 hrs

Significant prolongation of glimepiride release across the artificial membrane was achieved with the ethosomal formulation in comparison with the plain drug solution (Figure 3). The cumulative amount of glimepiride released in 24 hr from the ethosomal formulation was 30.4 ±2.3 %, compared 28.3 ±2.3 %, with 99.5 ± 2.2% from the liposomes and control drug solution respectively. Glimepiride release from the ethosomal formulation was steady and slow and decreased as a function of time. This shows the sustained release characteristic of developed vesicular carrier system.

Invitro skin permeation study

Transdermal flux for different ethosomal formulations across excised rat skin ranged between 18.2 ± 0.7 and 76.5 ± 2.5 ug/ cm^2/h (Table 3 & figure 5). The flux from ethosomes was 3-fold higher than that obtained after application of liposomal solution (26.14 ±0.9 ug/ cm^2/h) and 25-fold higher than that of the ethanolic solution

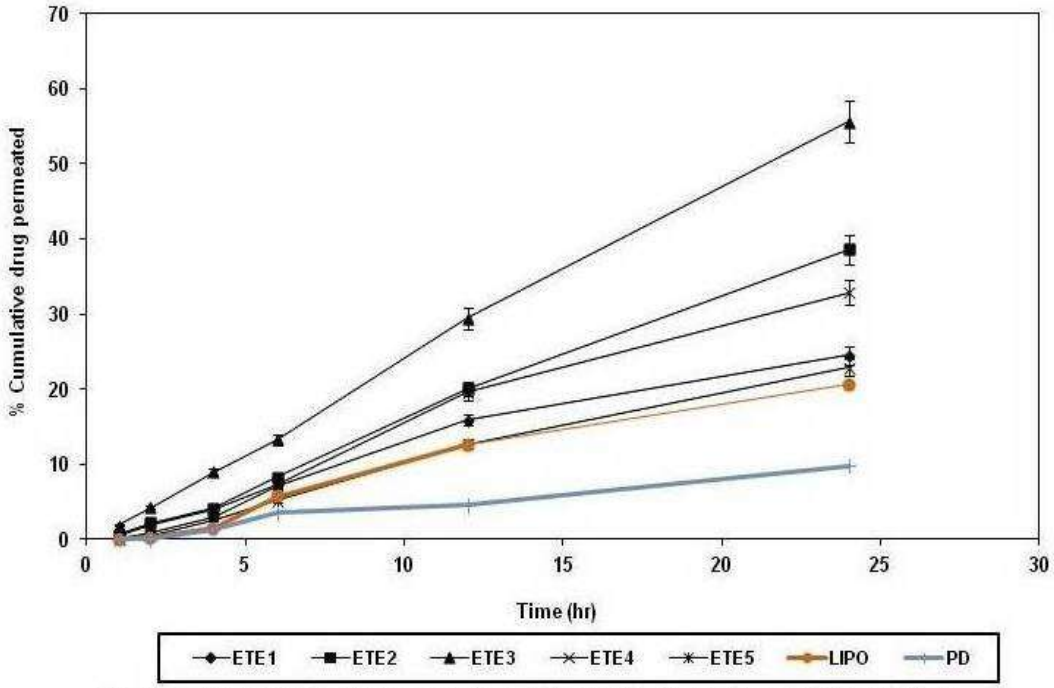

Figure 4. % cumulative drug permeation of glimepiride from ethosomal formulations containing ethanol through rat skin

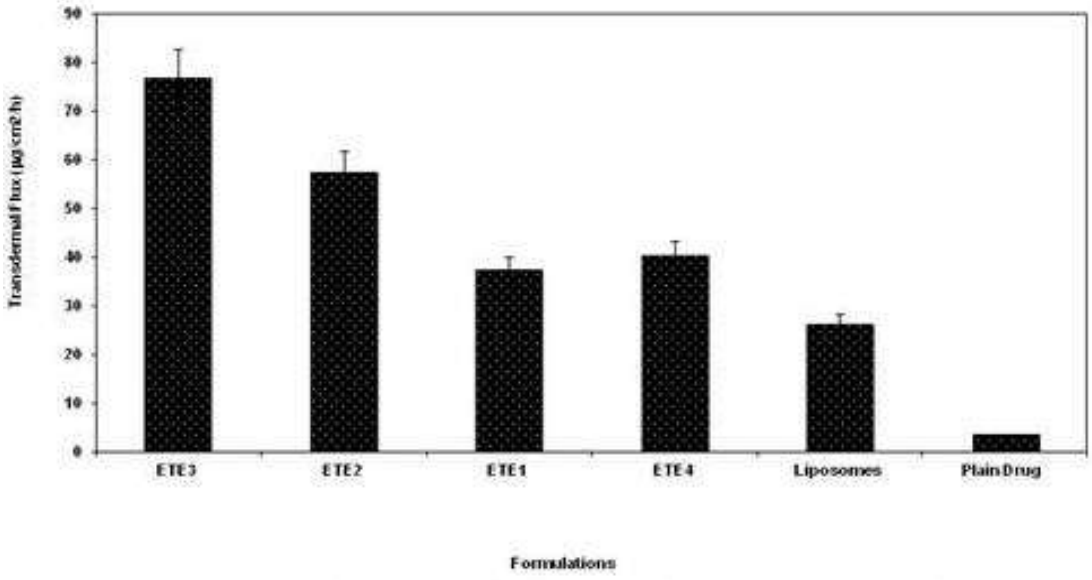

Figure:- 5 Transdermal flux of Glimepiride from ethosomal formulation (ETE3) and control system through the rat skin

of drug (3.22 ± 0.2 ug/cm^2/ h), 4-fold higher than that of the 50 % hydroalcoholic solution of drug (18.2 ±0.7 ug/cm2/h),.The data indicated that the transdermal flux of glimepiride increased with increase in the concentration of ethanol in the formulation. However, further increase (94.5% v/v) in the ethanol concentration resulted in significant reduction in the transdermal flux of glimepiride. This result could be because of the deteriorating effect on the lipid bilayers when ethanol was present in high concentration. The significantly lower vesicle size (159 ± 6.2 nm) and higher membrane elasticity (35.48 ± 4.1) of ETE3 formulation is in consonance with the observed highest transdermal flux of drug. Further, transdermal flux of glimepiride from ethosomal formulation as compared with that from ethanolic solution clearly indicated that the ethosomal system was more effective in delivering glimepiride than ethanol alone, aqueous ethanolic solution or liposomes. Furthermore, better permeation of glimepiride from ethosomes than from ethanol alone suggested some kind of synergistic mechanism between ethanol, vesicles, and skin lipids.

Ethanol has long been known to have permeation enhancement properties. However, the permeation enhancement from ethosomes observed in this work is much greater than would be expected from ethanol alone (Table 3), suggesting some kind of synergistic mechanism between ethanol, vesicles and skin lipids. These data are supported by previous findings that ethanol generally has a fluidizing effect on phospholipid bilayers (Harris et al., 1987) [27]. In comparison to liposomes ethosomes are less rigid. Thus the effect of ethanol, which was considered harmful to classical liposomal formulation, may provide the vesicles with soft flexible characteristic which allow them to move easily and penetrate into deeper layers of the skin.

The effect of ethanol on stratum corneum lipids and on vesicle fluidity as well as a dynamic interaction between ethosomes and the stratum corneum all may contribute to the superior skin penetration ability of ethosomes. The results of the skin vesicle interaction study (figure 6) showed the importance of presence of ethanol in vesicle membrane in establishing disorganization of the skin lipid bilayers and subsequent increase in skin permeability.

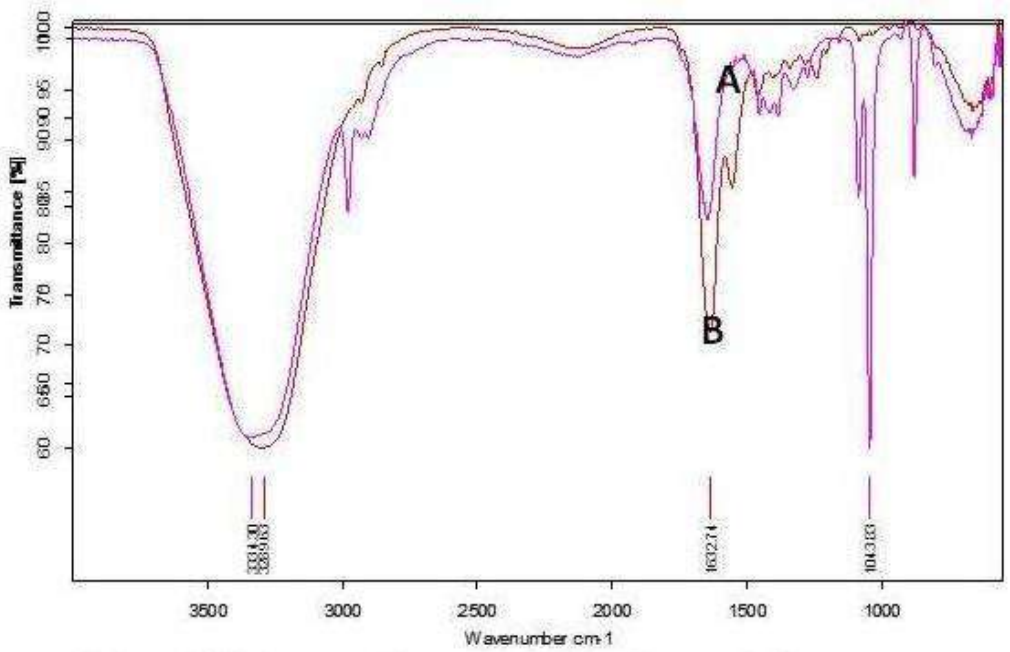

Figure 6 FTIR spectra(A) untreated skin ,(B) treated skin

Ftir spectral analysis of formulation treated and untreated rat skin

FTIR spectrum of untreated Skin showed various peaks due to molecular vibration of proteins and lipids present in the Stratum corneum (Figure 6a). These narrow bands were attributed to the long alkyl chains of fatty acids, ceramides and cholesterol which are the major components of the SC lipids. The two strong bands were due to the amide I and amide II stretching vibrations of SC proteins (Figure 6a). The amide I and amide II bands arisen from C = O stretching vibration and C-N bending vibration respectively. The amide I band consisting of components bands, represented various secondary structure of keratin. There was clear difference in the FTIR spectra of untreated and ethosomal treated skin

with prominent decrease in asymmetric and symmetric CH- stretching of peak height and area (Figure 6b). The rate limiting step for transdermal drug delivery is lipophilic part of SC in which lipids (ceramides) are tightly packed as bilayers due to the high degree of hydrogen bonding. The amide I group of ceramide is hydrogen bonded to amide II group of another ceramide and forming a tight network of hydrogen bonding at the head of ceramides. This hydrogen bonding makes stability and strength to lipid bilayers and thus imparts barrier property to SC [27]. When skin was treated with ethosomal formulation (ET-SP3), ceramides got loosened because of competitive hydrogen bonding leading to breaking of hydrogen bond networks at the head of ceramides due to penetration of ethosomal into the lipid bilayers of SC. Treatment with ethosomal formulation resulted in either double or single peak (Figure 6b) which suggested breaking of hydrogen bonds by ethosomal formulation.[28]

Finally, it can be concluded from the results of our present study that soft vesicle ethosomes improve the transdermal flux, prolongs the release and represent an attractive carrier for sustained transdermal delivery of glimepiride. The efficient skin permeation ability together with the sustained release characteristic make this system a promising candidate for transdermal delivery.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. Madhu Chitkara, Director, Chitkara Institute of Engineering and Technology, Rajpura, Patiala, India and Dr. Ashok Chitkara, Chairman, Chitkara Educational Trust, Chandigarh, India, for support and institutional facilities

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