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Enhanced transdermal permeation of indinavir sulfate through Stratum Corneum via. Novel permeation enhancers: Ethosome

Sheo Datta Maurya*

[1] *Department of Pharmacy, IEC- CET, Knowledge Park-I, Greater Noida, INDIA*

ABSTRACT

The aim of the present study was to investigate the potential of ethosomal formulations for transdermal delivery of indinavir sulphate from ethosomes. All the system were characterized for vesicle morphology, particle size and entrapment efficiency by Scanning Electron Microscopy , Transmission Electron Microscopy, Differential light scattering, centrifugation, elasticity and turbidity respectively. The effect of different formulation variable on skin permeation of Indinavir Sulphate was studied via synthetic semipermeable membrane or skin of new born mice by using diffusion cell. The selected system were incorporated into Carbopol934P gel and evaluated for both drug permeation and mice skin deposition. The optimized ethosomal formulation showed transdermal flux 25.01±0.34 μg/cm^2/hr across rat skin as compared to 2.98±0.21 μg/cm^2/hr for plane drug solution, 4.28±0.54 μg/cm^2/hr for hydroethanolic solution and 9.7±0/21 μg/cm^2/hr for classical liposomes. The obtained flux was nearly 7.5 and 12.04 times higher than conventional liposomal formulation bearing indinavir sulphate and plain drug solution Finally it was concluded from the study that, ethosomes can increase the transdermal flux, prolong the release and present an attractive route for sustained delivery of Indinavir Sulphate.

Key words: Ethosome, Transdermal Flux, Indinavir Sulphate, Transmission Electron Microscopy

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INTRODUCTION

In the past decades, topical delivery of drug by liposomal formulation have evoked considerable interest, it has been evident that traditional; liposomes are of little or no value as carrier for transdermal delivery of drug, because they do not deeply penetrate skin but remains confined to upper layer of the stratum corneum. To overcome problem of poor skin permeability Cave et al. [1] and Touitou et al. [2] recently introduce two new vesicular system

transfersomes and ethosomes incorporated edge activator (surfactant) and penetration enhancer (alcohols and polyols), respectively, to influence the properties of vesicles and stratum corneum [3].

Ethosomes are soft malleable vesicles composed mainly of phospholipid, ethanol (relatively high concentration) and water. These soft vesicles represents novel vesicular carrier for enhanced delivery to/through skin. The size of ethosome vesicles can be modulated from tens of microns to nanometres. This carrier presents interesting features correlated with its ability to permeate intact through the human skin due to its high deformability [4]. The high concentration of ethanol makes the ethosome unique, as ethanol is known for, its disturbance of lipid bilayer organization; therefore when integrated into a vesicle membrane, it gives that vesicle the ability to penetrate the stratum corneum. Also because of their high ethanol concentration, lipid membrane is packed less tightly than conventional vesicles, but has equivalent stability allowing a more malleable structure and improves drug distribution ability in stratum corneum lipid.

Indinavir sulphate an approved by the FDA (Food and Drugs Administration) on March 13, 1996, for use in Combination (a dose of 800 mg orally every 8 hr) with other protease inhibitors, nucleoside analogues or reverse transcriptase inhibitors and indicated for the treatment of HIV-1 infection in adults and paediatric patients. Indinavir is prescribed as a sulphate salt due to superior gastrointestinal solubility and absorption when compared with the free base. Clinical experience with indinavir has demonstrated that it has a relatively narrow therapeutic window and is frequently associated with nephrotoxicity, which may manifest as a syndrome of renal colic, tubulointerstitial nephritis or even acute renal failure. Prolonged use of indinavir is associated with chronic elevations in serum creatinine [5-10].

The transdermal delivery system offers many advantage over their corresponding oral, injectables and inhaler systems, including (1)improving the systemic bioavailability of drug by avoiding first pass metabolism by liver (2) achieving a controlled constant drug delivery profile. Achieving controlled transdermal drug delivery system of Indinavir Sulphate is challenging, as it is a hydrophilic drug with a logP value less than 0.2. Therefore the study is focused to develop an efficient ethosomal system that could be incorporated into a suitable gel to be easily applied by patients for constant and effective therapeutic benefits [11- 12].

MATERIALS AND METHODS: [13-22]

Indinavir Sulphate was gift sample from Ranbaxy Laboratories, New Delhi, India. Soya lecithin was purchased from Hi Media Ltd. Mumbai. Ethanol Methanol, Chloroform Fluroscein sodium, Propylene glycol, was purchased from Central Drug House, New Delhi. Ethanol was procured from Qualigens fine chemicals, Mumbai. All other chemicals were of analytical grade and double distilled water used throughout the experiment.

Method of preparation of vesicular formulation:

The ethosomal formulation was prepared according to the method reported by Touitou et al. the ethosomal system prepared were composed of 2-6% phospholipid, 10-40% ethanol, drug , 10% propylene glycol and water to 100% w/w. phospholipid, drug and probe (Flurocein sodium) were dissolved in ethanol/propylene glycol mixture, the mixture was heated to 30^0C in a water bath. The double distilled water heated to 30^0C was added slowly in a fine stream, with constant mixing (mechanical stirrer) at 700 rpm in a closed vessel, .mixing was continued for additional 5 min. The system was kept at 30 ºC throughout preparation. The final milky solution of

ethosomes was left to cool at room temperature. The preparation was homogenised by using sonicator for 15 min. Liposomes were prepared by thin film method by dissolving the phospholipids and cholesterol in minimum quantity of chloroform: methanol mixture (3:1 v/v) in a round bottom flask. The organic solvent was removed in a rotary evaporator under reduced pressure to form a thin film on the wall of flask. Final trace of solvent was removed under vacuum, overnight. The deposited lipid film was hydrated with aqueous solution of drug at 60 rpm for one hour at room temperature. The preparation was sonicated using sonicator for 15 min.

Table- 1: Batch Specification of Different Formulation.

For. code	PL (%w/w)	Ethanol (%w/v)	PPG (%w/v)	CHOL (%w/w)	FNa (%w/w)	Drug (%w/w)
E-1	2.0	10	10	-	0.20	0.5
E-2	2.0	20	10	-	0.20	0.5
E-3	2.0	30	10	-	0.20	0.5
E-4	2.0	40	10	-	0.20	0.5
E-5	4.0	30	10	-	0.20	0.5
E-6	4.0	30	10	-	0.20	0.5
E-7	6.0	40	10	-	0.20	0.5
E-8	6.0	40	10	-	0.20	0.5
Liposome	6.0	-	10	0.20	0.20	0.5
HD Solution	-	30	10	-	0.20	0.5
PD Solution	-	-	10	-	0.20	0.5

*****HD solution- hydroethanolic solution ***** PD solution- plane drug solution *CHOL Cholestrrol
*PL Phospholipid (Soyalecithin) *PPG Propylene Glycol * FNa Fluroscein sodium *LP Liposome
*****ZP zetapotential *PI Poly dispersity index *EE Entrapment efficiency

Vesicular characterization:

Ethosomes vesicles were visualized by using electron microscope and transmission electron microscope (AIIMS New Delhi), with an accelerating voltage of 80 kV. A drop of ethosomal sample was placed on to a carbon coated 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 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 dried thoroughly and sample was viewed in a transmission electron microscope, which confirms the unilamellar and multilameller three dimensional natures or structure of phospholipids vesicles.

Vesicle Size, Zeta Potential and Zeta Sizes Distribution Analysis:
The effect of phospholipids and ethanol concentration on the size and distribution of ethosome vesicles was investigated by using Malvern Zetasizer (Centre for Pharmaceutical Nanotechnology, Pharmaceutics Dept. NIPER).

Drug Entrapment Efficiency:
Indinavir Sulphate entrapped within the ethosomes was estimated after removing the unentrapped drug. The unentrapped drug was separated from the ethosomes by subjecting the dispersion to centrifugation in a cooling centrifuge (Remi Equipments, Mumbai) at 22000 rpm at a temperature of 4°C for 30 minutes, where upon the pellets of ethosomes and the supernatant containing free drug were obtained. The ethosome pellets were washed again with phosphate buffer to remove any unentrapped drug by centrifugation. The combined supernatant was analyzed for the drug content after suitable dilution with phosphate buffer solution by measuring absorbance at 265.5 nm using shimadzu 1700 UV spectrophotometer.

$$\textbf{Encapsulation efficiency} = (A1 - A2)*100/A1$$

A1- Amount of Indinavir Sulphate added initially, **A2**- Amount of Indinavir Sulphate determined in the filtrate by spectrophometry, **A1-A2** - Represents the amount of Indinavir Sulphate entrapped in the formulation.

Vesicle Elasticity Measurement:
The elasticity of ethosomes vesicles were measured by extrusion method. The ethosomal formulation were extruded through filter membrane (pore size diameter- 100 nm), using a stainless steel filter holder having 50 mm diameter, by applying a pressure of 2.5 bar. The quantity of vesicle suspension, extruded in 5 minutes was measured.

$$E = J^* (r_v / r_p)^2$$

Where, **E**- Elasticity of vesicle, **J**- Amount of suspension extruded in 5 minutes, r_v and r_p are vesicle size after extrusion and pore diameter of filter membrane.

***In-vitro* drug permeation and skin drug deposition study:**
In-vitro release of Indinavir Sulphate from ethosomal formulation was studied using locally fabricated diffusion cell. The effective permeation area of the diffusion cell and receptor cell volume was 1cm^2 and 20 ml, respectively. The temperature was maintained at 37±1º C. The receptor compartment contained 20 ml of phosphate buffer solution (pH-7.4) and was constantly stirred by magnetic stirrer at 100 rpm. The synthetic semi-permeable membrane or the skin of the new born mice was mounted between the donor and receptor compartments. The ethosomal formulation was applied to the membrane. Sample were withdrawn through sample port of the diffusion cell at predetermined time interval over 24 hours and analyzed by UV spectrophotometer. The receptor phase was immediately replenished with equal volume of phosphate buffer solution of pH 7.4. Sink condition was maintained throughout the experiment. The second stage of *in-vitro* release was employed to determine the amount of drug deposited on the skin. The receptor content was completely removed and replaced by 50% (v/v) ethanol in distilled water and kept for further 12 hours for same condition as for *in-vitro* release study, and then the absorbance of resulting solution was measured spectrophotometrically for the amount of drug deposited in the skin.

Effect of Temperature on the Release of Drug from Ethosomes:
The temperature of the release medium may alter the viscosity of the ethosomal suspension. The release studies were conducted for 2 hrs to find the behavior of ethosome, at different temperature (same as *in-vitro* drug release study).

Transdermal Flux:
The cumulative amount of drug permeated per unit area was plotted as a function of time and steady state transdermal flux were calculated from the linear portion of the curve.

Physical Stability of ethosomes
The physical stability of the Indinavir Sulphate incorporated ethosome was determined for their drug content at different storage temperature for 3 months. Sample of Indinavir Sulphate ethosome (ET-8) was centrifuged at 22,000 rpm for 45 minutes and pallet obtain was mixed with 10 ml of phosphate buffer solution of pH-7.4. These ethosome suspensions were stored at different temperature i.e. refrigerated temperature (2-8^{0}C), room temperature (28±2^{0}C), body temperature (37^{0}C) and 45^{0}C. Same day 1.0 ml of each sample from their different storage temperature was withdrawn and assayed spectrophotometrically at 265.5 nm. The same quantity of aliquot (0.5 ml of fresh phosphate buffer of pH 7.4) was added to all samples. The same study was performed for 3 months.

Statistical Analysis
The mean and standard deviation was calculated by Graph Pad Instat3.0. The statistical analysis was carried out employing analysis of variance (ANOVA) by using the software PRISM (Graph Pad) 5.0. Differences were considered statistically significant at $p < 0.05$.

RESULT AND DISCUSSION

Visualization by transmission electron microscope and scanning electron microscope showed that ethosomes has a lamellar vesicular structure, and this confirms the existence of vesicular structure at higher concentration of ethanol (Figure 1-4).

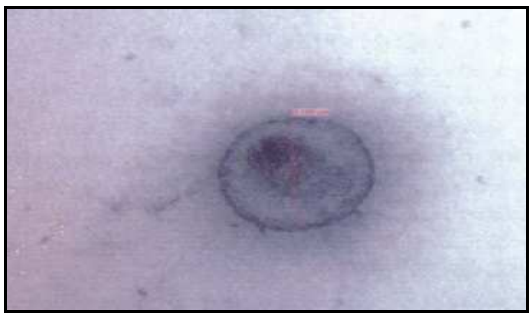

Figure-1: Optical Microscopic photograph ethosome loaded with Indinavir Sulphate

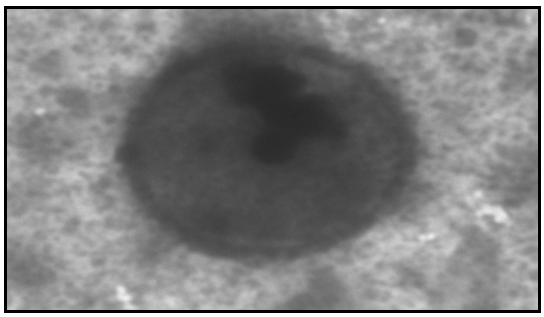

Figure-2: Transmission Electron Microscopic of photograph of ethosomes loaded with Indinavir Sulphate

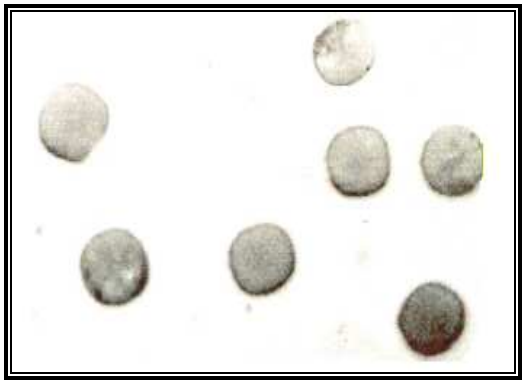

Figure-3: Transmission Electron Microscopic photograph of ethosomes loaded with Indinavir Sulphate

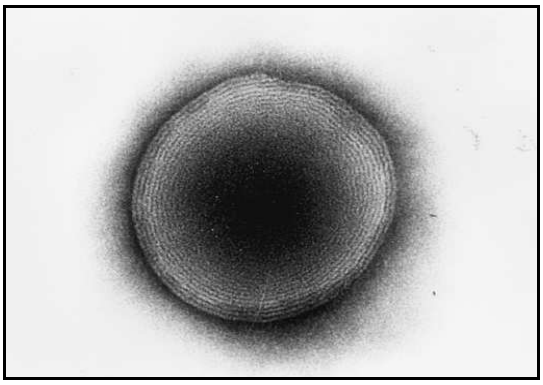

Figure-4: Transmission Electron Microscopic photograph of ethosomes loaded with Indinavir Sulphate

The effect of phospholipids and ethanol concentration on the size distribution of ethosomes vesicles was investigated, using Malvern Zetasizer. The ethosomal formulation prepared with 40% alcohol and 1% PL showed an average vesicle size 538.32 nm (Figure 5-8). In the ethanol concentration range of 10% to 40% the size of vesicle decreases with increase in ethanol concentration. This indicates that at higher ethanol concentration the membrane thickness reduced considerably, probably due to formation of a phase with interpenetrating hydrocarbon chain that will lead to decrease in size of ethosome vesicle on increasing concentration of ethanol. PI was determined as a measure of homogenesity of formulation. Small value of Polydipersity index (PI<0.3) indicate a homogenous population of ethosome vesicles, while PI value>0.3 indicate high hetrogenesity. Zeta potential measurement study supported by the above hypothesis, as zeta potential tends to be more negative as the concentration of alcohol increases (Table 2).

Table-2:, Vesicle Size, Polydispersity Index, Zeta Potential, Vesicle Elasticity and Entrapment Efficiency of different formulation

S. No.	Formulation code	Vesicle size(nm)	Polydispersity index	Zeta potential	Vesicle elasticity (E)	%Entrapment efficiency
1.	E-1	674.89	0.112	-14.8	25.6±2.1	40.02±2.9
2.	E-2	609.36	0.179	-19.8	29.4±2.3	42.11±2.8
3.	E-3	599.13	0.326	-21.3	39.8±1.9	43.39±2.5
4.	E-4	538.32	0.649	-22.0	38.6±2.2	43.06±2.9
5.	E-5	614.33	0.324	-13.2	26.8±2.8	45.96±3.2
6.	E-6	602.72	0.204	-16.4	22.4±2.5	45.79±2.4
7.	E-7	714.41	0.623	-13.8	25.3±3.1	47.21±3.1
8.	E-8	790.98	0.486	-14.7	20.8±2.6	50.26±2.7
9.	Liposome	820.45	0.345	-10.5	6.94±1.2	40.25±3.8

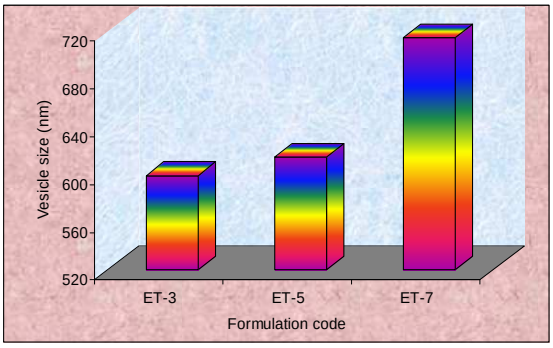

Figure-5: Effect of phospholipid concentration on entrapment efficiency

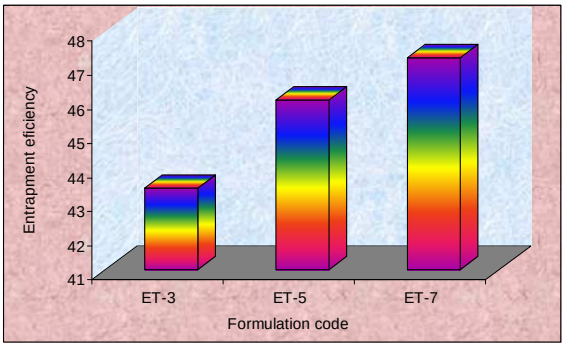

Figure-6: Effect of phospholipid concentration. on the size of vesicles

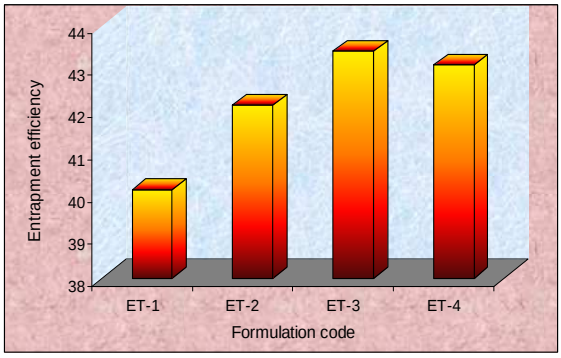

Figure-7: Effect of ethanol concentration on the entrapment efficiency

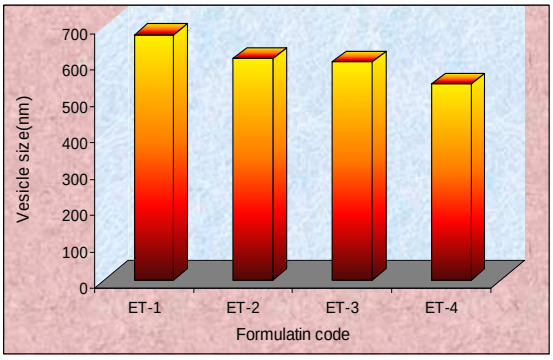

Figure- 8: Effect of ethanol concentration on the size of vesicles

The entrapment efficiency of different vesicular formulation and in traditional liposomes was calculated as percent total drug entrapped. The greatest entrapment of Indinavir Sulphate in ethosomes (50.26±2.7) as compared to conventional liposomes (40.25±3.8) could be attributed to the greater retentivity of Indinavir Sulphate in ethanol present in ethosomal core (Table 2). The data indicate that entrapment efficiency depends on ethanol concentration, as the concentration increases up to 30%, results in increase in entrapment efficiency of ethosomal formulation. With further increase in ethanol concentration entrapment efficiency decreases, owing to increase fluidity of membrane and vesicles become more permeable that leads to decrease in entrapment efficiency of ethosomal formulation. The elasticity of ethosomal vesicle membrane (38.6±2.5) was found to be - 5.5 fold higher than liposome (6.94±2.1) (Table 2). Higher concentration of ethanol present in ethosomes perhaps provided elasticity to vesicle membrane by reducing the interfacial tension of the vesicle membrane (Table-2).

The *in-vitro* release study suggested that the penetration enhancing effect might be of greater importance in enhance skin delivery of Indinavir Sulphate by ethosomal vesicles under non occlusive condition, than intact vesicle permeation into the stratum corneum (SC). Possible interaction of vesicles with layers of SC, promoting impaired barrier function of these layers to the drug with less well packed intracellular structure forms, and with subsequent increased in skin partitioning of drug play a major role in increased skin delivery of drug. For hydrophilic drug penetration enhancing effect seems to play a more important role in enhanced skin delivery than in case of lipophilic drug, since permeation of hydrophilic molecule tends to be more enhanceble (Table-3).

Table-3: Skin permeation profile of different formulation of Indinavir Sulphate calculated from *in-vitro* drug release study through rat skin (after 24 hr).

Formulation code	% Indinavir Sulphate permeated (µg%)	Transdermal flux (µg/cm^2/hrs)	Regression coefficient (R^2)	Enhancement ratio (ER)
E-1	22.26±0.67	13.54±0.45	o.985	4.5
E-2	24.96±0.46	14.61±0.67	0.991	4.9
E-3	28.69±0.97	17.34±0.78	0.983	5.8
E-4	32.00±0.46	17.54±0.78	0.980	5.9
E-5	28.40±0.46	17.69±0.42	0.991	6.0
E-6	34.68±0.67	22.54±0.54	0.961	7.6
E-7	29.49±0.89	18.95±0.31	0.972	6.7
E-8	36.00±0.67	25.01±0.34	0.971	8.4
Liposome	17.50±0.89	9.700±0/21	0.968	3.2
HD Solution	8.950±0.48	4.280±0.54	0.978	1.4
PD Solution	5.800±0.69	2.980±0.21	0.966	-

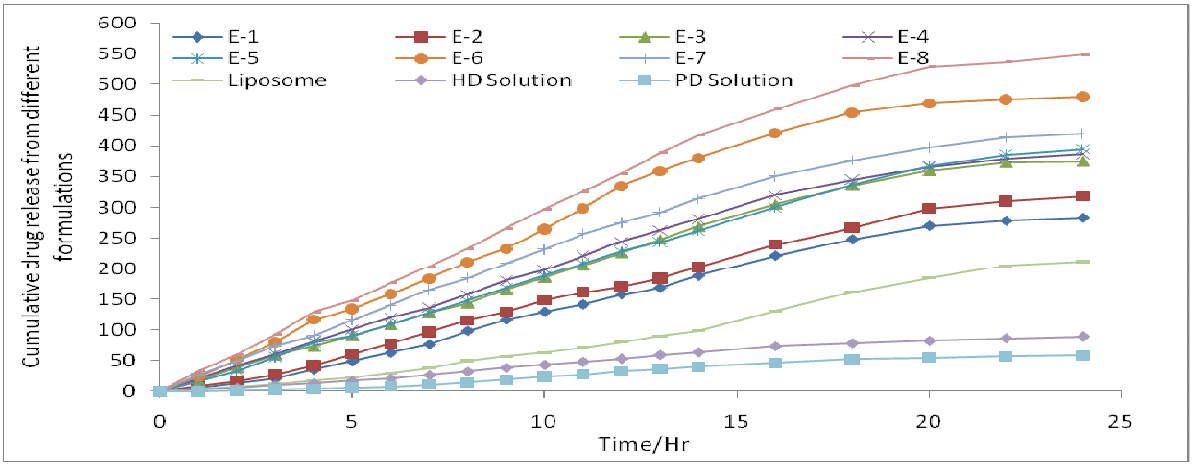

Figure-9: Comparative cumulative release of drug (µg/cm^2) different from formulations

Ethanol used in the preparation of ethosome is a well known penetration enhancer and increase penetration of Indinavir Sulphate through skin was suggested of a synergistic mechanism between ethanol vesicles and skin lipid. Ethosomal formulations contain ethanol in their composition that interacts with lipid molecules in the polar head group regions, resulting in an increased fluidity of the SC lipids. The high alcohol content is also expected to partial extract the SC lipids. These processes are responsible for increasing inter and intracellular permeability of ethosomes.

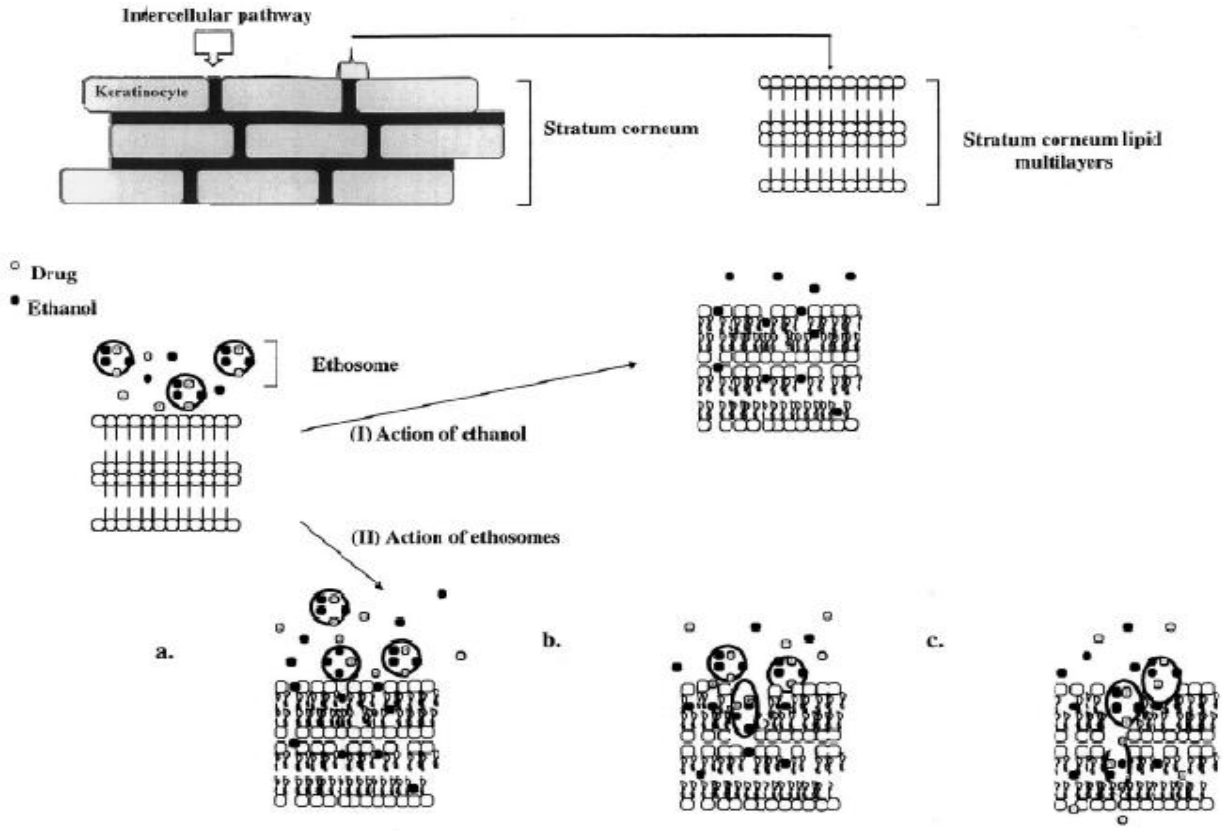

Figure-10: Proposed mechanism for penetration of molecule from ethosomal system across the lipid domain of stratum corneum (*Courtesy:* Touitou *et al.).*

Propylene glycol used in formulation widely used as a penetration enhancer in topical formulation, either alone or in combination with other fatty acids. It will enhance solubility and partitioning of drug in SC, and increase the flux of PG and permeant across SC.

The release profile of ethosomes and liposomes are shown in figure 9 and it will clearly show that the release rate of ethosomes was higher than the liposomes. The higher drug releases of ethosomes as compared to liposomes are because of ethanol presents in ethosomes vesicles. Ethanol imparts elasticity to vesicle membrane and enhance rate of permeation of drug across SC barrier and increase rate of permeation of Indinavir Sulphate from ethosomes. The percent of Indinavir Sulphate deposited in skin from liposomal formulation and from ethosomal formulation was higher than the percent deposited from both hydroethanolic solution and plane drug solution (Figure 11). Results showed that as the concentration of ethanol increases the amount of percent drug deposited decreases. This may be explained with respect to the role of ethanol in ethosome formulation, which favours or enhance the permeation of the hydrophilic drug Indinavir Sulphate through stratum corneum and demonstrate that to permeate skin, the drug must be released first. So the % Indinavir Sulphate deposited in skin was higher with formulation E-1and E-2 containing 10% and 20% ethanol indicating that it need more time to permeate through the skin layers; the fact that ethosomal formulation E-1 have lowest and E-4 have the highest permeation rate among the ethosomal formulation also clarify this (Table-3).
The data suggested that the value of transdermal flux depends on the ethanol concentration (Figure 12). As the concentration of ethanol increases, transdermal flux of Indinavir Sulphate increased. The flux of ethosome was 8.4 folds higher than that obtain from plane drug solution, and 2.6 fold higher than liposomal formulation (Table-3).

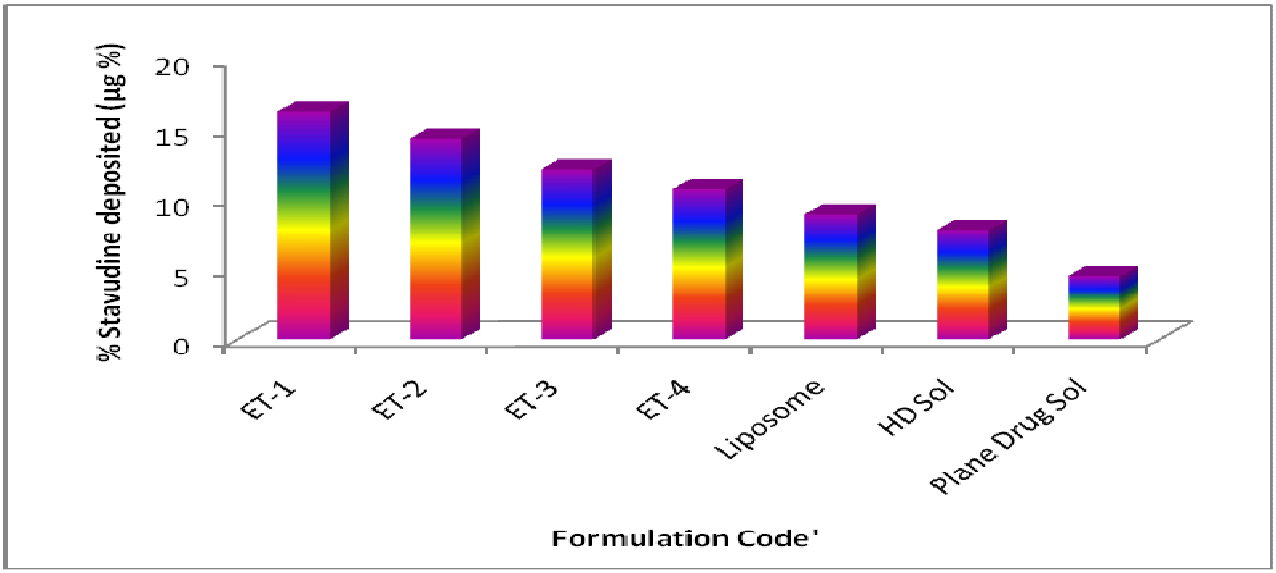

Figure-11: Percent Indinavir Sulphate deposited on mice skin in-vitro after 12 hours of extraction with 50% hydroalcoholic solution at 37^0C±0.5^0C.

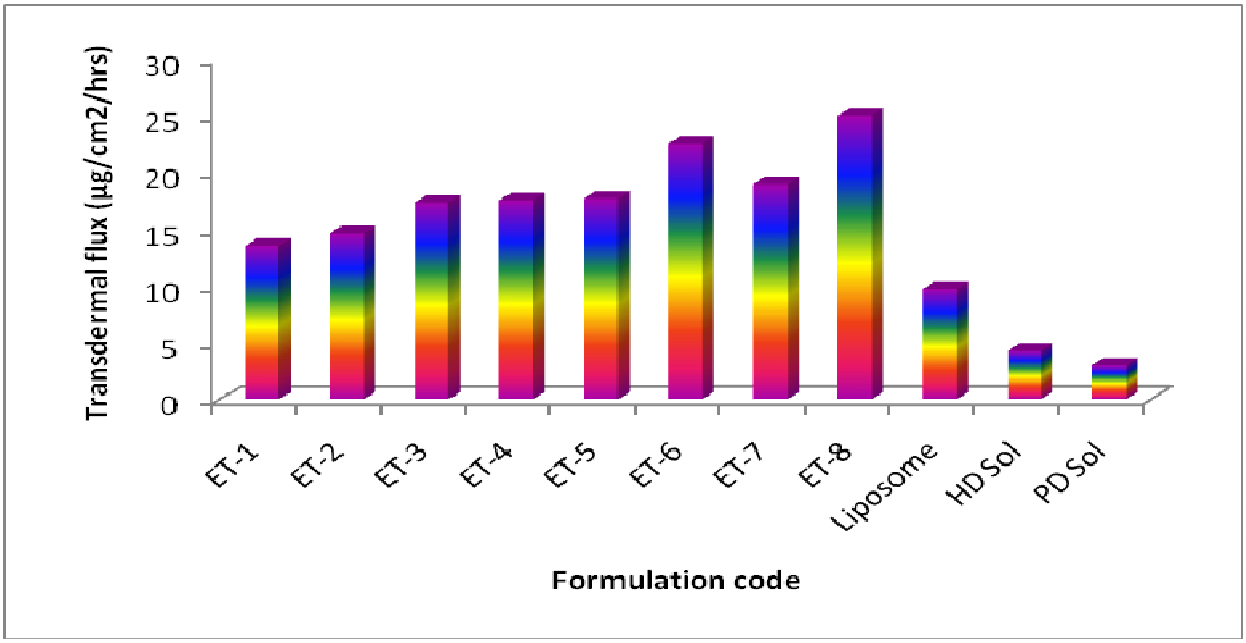

Figure-12: Transdermal Flux of different formulation

The temperature of the release medium may alter the viscosity of the ethosomal suspension. Generally the human body temperature is 37°C. However in the clinical cases, such as fever, the variation of temperature can change the *in-vitro* drug release behavior when ethosomes are sensitive to the temperature (Table-4). The temperature of the medium has no significant effects on Indinavir Sulphate release from ethosomes in the range 37°C - 39°C. This means that drug release from the ethosomes in non-temperature sensitive *in-vitro* at body temperature. But with increased temperature, the Indinavir Sulphate release rate increased. At elevated temperature greater drug release from the system was observed, that might be ascribed to the effect of on the

gel to sol transition of lipid bilayer together with the possible chemical degradation of the phospholipids, leading to defect in the membrane packing which leads to increases the rate of diffusion of drug from ethosomal system, which will attribute to increased release rate of drug from ethosomes at elevated temperature.

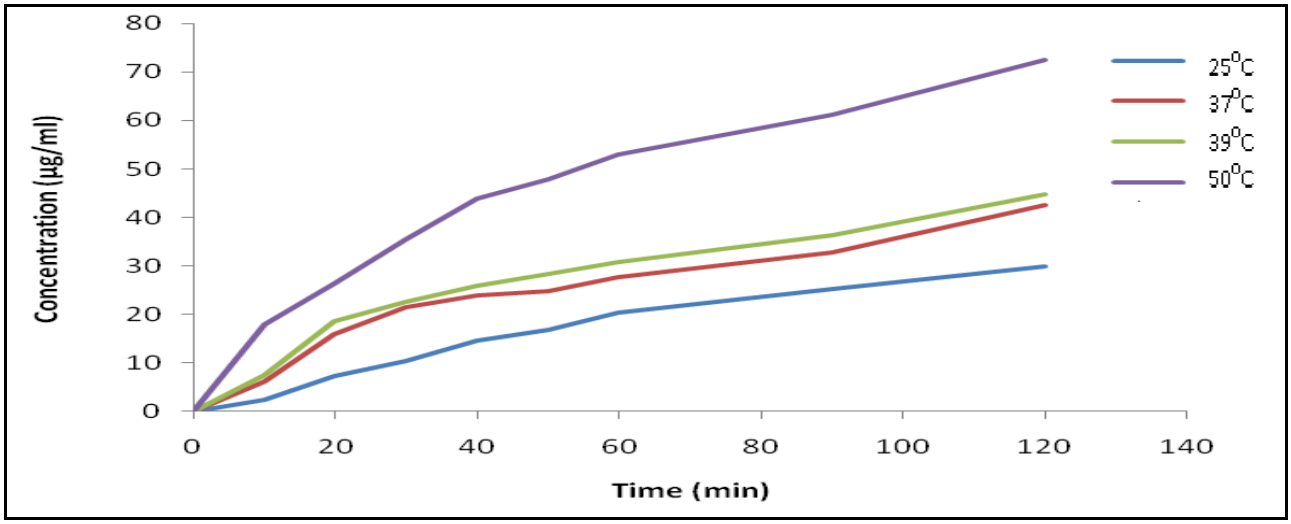

Figure-17: In-vitro release of Indinavir Sulphate from ethosome (ET-5) at 25°C, 37°C, 39°C, 50°C.

The stability studies was carried out by storing ethosomal preparation at different temperature i.e. refrigeration temperature (2-8^{0}C), room temperature (28^{0}C ± 2^{0}C), body temperature (37^{0}C ± 1^{0}C) and 45^{0}C ± 1^{0}C.The drug remains entrapped after 3 months at various storage temperature i.e. refrigeration temperature (2-8^{0}C), room temperature (28^{0}C ± 2^{0}C), body temperature (37^{0}C ± 1^{0}C) and 45^{0}C± 1^{0}C was found to be 91.72%, 67.75%, 8.84% and 6.81% respectively. The data suggested that the refrigeration temperature was found to be most suitable for storage of Indinavir Sulphate containing ethosomes. As seen from result, leakage rate was slow and only 8.18% of total entrapped content was released after storage for 90 days. The result of stability studies at room temperature indicate that ethosomes are stable for first 30 days and shows only 7.68% drug loss from total drug entrapped, after that rapid loss of drug start. Where as ethosomes stored at 37^{0}C ± 1^{0}C and 45^{0}C ± 1^{0}C will show rapid loss of drug and after 90 days only 8.84% and 6.81% of total drug remained entrapped in formulation (Table 4). The stability profile of Indinavir Sulphate loaded ethosomal formulation evaluated for substantial loss of drug at various temperature suggested the storage of the ethosomal formulation at refrigerated temperature (2-8 ºC), as at the elevated temperature greater drug loss from the system was observed, that might be ascribed to the effect of on the gel to sol transition of lipid bilayer together with the possible chemical degradation of the phospholipids (increase in bond deformity that cause breakage of bilayer structure of phospholipid) leading to defect in the membrane packing. Still a small amount of drug leaked out of the ethosomal system at refrigerated temperature also (8.18 %), because of hydrosoluble nature of Indinavir Sulphate.

Table-4: Comparative Stability of ethosome containing Indinavir Sulphate at different temperature after three months.

S. No	Time in months	% Drug retained at 2-8^{0}C	% Drug retained at room temp.	% Drug retained at 37^{0}C	% Drug retained at 45^{0}C
1.	0	100.0	100.0	100.0	100.0
2.	1	98.00	92.32	78.50	68.45
3.	2	95.68	78.45	49.48	39.75
4.	3	91.72	69.75	8.840	6.810

CONCLUSION

Novel penetration enhancer, ethosomes were prepared successfully by mechanical-dispersion method for prolonged as well as controlled release of Indinavir Sulphate across SC. The prepared formulation were characterized for various evolutionary parameters like vesicle size, entrapment efficiencies, vesicles elasticity, *in-vitro* drug deposition study and rate of transdermal flux across stratum corneum and prepared formulation were also characterized for *in-vitro* release studies by using cellophane membrane (semipermeable membrane), skin of new born mice for *in-vitro* study.

From *in-vitro* drug release studies, it is concluded that by changing the ratio of PL and ethanol, Indinavir Sulphate release can be controlled for a prolonged period of time by reducing possible side effects occurred during conventional therapy. Ethosomes of different vesicle size and drug content could be obtained by varying the ratio of PL and ethanol.

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