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Formulation Development and Evaluation of Ethosome of Stavudine

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ABSTRACT

The main objective of the present work was to develop transdermal delivery of stavudine, a hydrophobic drug used for the treatment of AIDS, 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 and centrifugation respectively. The effect of different formulation variable on skin permeation of stavudine was studied via synthetic semipermeable membrane or skin of new born mice by using diffusion cell. The selected system were incorporated into HPMC 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±0.21μg/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 liposome. 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 stavudine.

Key words: *Ethosome, Transdermal Flux, Stavudine, Transmission Electron Microscopy*

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 transferosomes 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. As compared to classical liposomes that delivered drug to outer layers of skin, ethosomes were shown to enhance permeation through the stratum corneum barrier.[5] Horwtiz et al.[6] in a two armed, double blind, randomized clinical study, compared the efficacy of 5% acyclovir in a novel liposomal carrier (ethosomes) was compared to that of a commercial 5% acyclovir cream (Zovirax Cream), and reported that a 5% acyclovir ethosomal preparation compared to the 5% acyclovir cream showed significant improvement in treatment of herpetic infection. Dayan et al.[7] investigated the delivery of trihexyphenidyl HCl (THP) from ethosomes versus classic liposomes, and concluded that in comparison to

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standard liposomes, ethosomes had higher entrapment efficacy and a greater ability to deliver entrapped fluorescent probe to the deeper layer of skin. The flux of THP through nude mouse skin from THP ethosomes was higher than liposomes. Similarly Lodzki et al.[8] designed a transdermal delivery system for cannabidiol by using ethosomal carrier, concluded that transdermal application of ethosomal cannabidiol prevented the inflammation and edema induced by sub plantar injection of carrageenan in the same animal model. Thus ethosomes enabled cannabidiol skin permeation and its accumulation in a depot at level that demonstrated. Jain et al.[9] prepared ethosomes of zidovudine and characterized them for in-vitro and in-vivo and studied effect of different formulation variables on skin permeation of zidovudine by using keshry-chien type of diffusion cell. Results indicated that, optimized ethosomes formulation showed better transdermal flux across rat skin and concluded that ethosomes can increase transdermal flux and prolongs the release time of drug. Similarly Touitou et al.[10] prepared ethosomal drug delivery system of testosterone and minoxidil and studied the depth of skin permeation and demonstrated that the ethosomes enhanced the delivery of drug to skin in terms of both depth and quantity of skin permeation of testosterone and minoxidil.

The aim of present work was to study the possibility of transdermal controlled delivery of stavudine. The transdermal delivery system offers many advantage overthier corresponding oral, injectable 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 stavudine is challenging, as it is a hydrophilic drug with a logP value 0.145. 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 [13-16]

Stavudine 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:

The ethosomal formulation was prepared according to the method reported by Touitou et al. the ethosomal system prepared were composed of 1-3% 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 vertex shaker 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 vortexted using vertex shaker for 15 min.

Vesicular characterization:

Ethosomes vesicles were visualized by using transmission electron microscope, 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% phosphotungustic 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 multilamellar 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.

Drug Entrapment Efficiency:

Stavudine 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 45 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.

Encapsulation efficiency = (A1 – A2)*100/A1

A1- Amount of stavudine added initially, **A2**- Amount of stavudine determined in the filtrate by spectrophometry, **A1-A2** - Represents the amount of stavudine 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 stavudine 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±1° 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.

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.

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 1-2).

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. 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 1).

The entrapment efficiency of different vesicular formulation and in traditional liposomes was calculated as percent total drug entrapped. The greatest entrapment of stavudine in ethosomes (50.26±2.7) in compared to conventional liposomes (40.25±3.8) could be attributed to the greater retentivity of stavudine in ethanol present in ethosomal core. 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). Higher concentration of ethanol present in ethosomes perhaps provided elasticity to vesicle membrane by reducing the interfacial tension of the vesicle membrane (Figure-3).

The *in-vitro* release study suggested that the penetration enhancing effect might be of greater importance in enhance skin delivery of stavudine 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 (Figure 4).

Ethanol used in the preparation of ethosome is a well known penetration enhancer and increase penetration of stavudine 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.

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 percent of stavudine deposited in skin from liposomal formulation and from ethosomal formulation was higher than the percent deposited from both hydroethanolic solution and plane drug solution. 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 stavudine through SC and demonstrate that to permeate skin, the drug must be released first. So the % stavudine deposited in skin was higher with formulation ET-1and ET-2 containing 10% and 20% ethanol indicating that it need more time to permeate through the skin layers; the fact that ethosomal formulation ET-1 have lowest and ET-4 have the highest permeation rate among the ethosomal formulation clarify this (Figure 6).

The data suggested that the value of transdermal flux depends on the ethanol concentration. As the concentration of ethanol increases, transdermal flux of stavudine 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 (Figure 7).

CONCLUSION:

Novel penetration enhancer, ethosomes were prepared successfully by mechanical-dispersion method for prolonged as well as controlled release of stavudine 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, stavudine 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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Table- 1: Batch Specification of Different Formulation

For. Code	PL (%w/w)	Ethanol (%w/w)	PPG (%w/w)	Chol (%w/w)	Drug (%w/w)	Vesicle size(nm)	PI	ZP	EE
ET-1	1.0	10	10	-	0.3	674.89	0.112	-14.8	40.02±2.9
ET-2	1.0	20	10	-	0.3	609.36	0.179	-19.8	42.11±2.8
ET-3	1.0	30	10	-	0.3	599.13	0.326	-21.3	43.39±2.5
ET-4	1.0	40	10	-	0.3	538.32	0.649	-22.0	43.06±2.9
LP	1.0	-	-	0.10	0.3	820.45	0.345	-10.5	40.25±3.8
HD sol	-	30	10	-	0.3	-	-	-	-
PD sol	-	-	10	-	0.3	-	-	-	-

*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

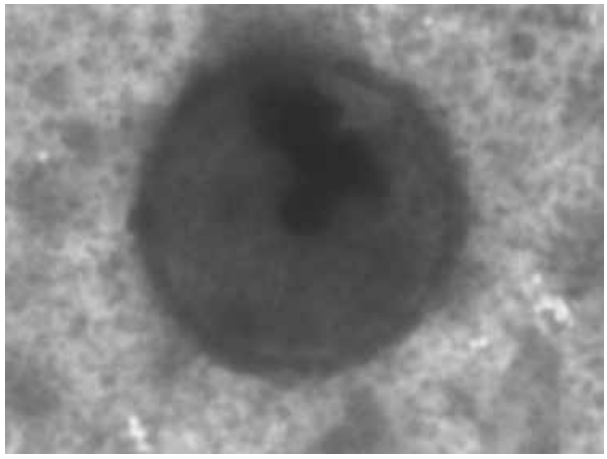

Figure-1: Transmission Electron Microscopic photograph of ethosomes loaded with stavudine

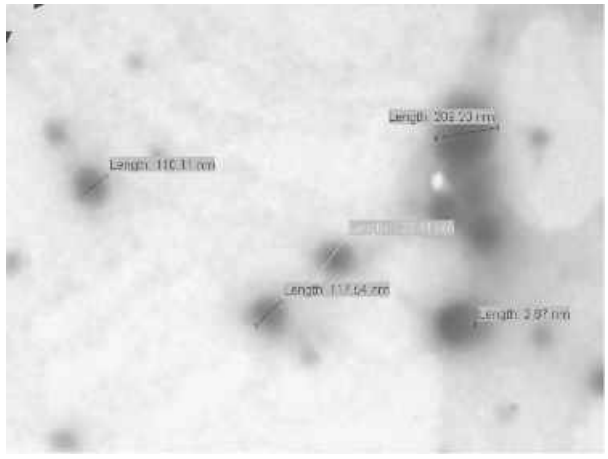

Figure-2: Transmission Electron Microscopic photograph of ethosomes loaded with stavudine

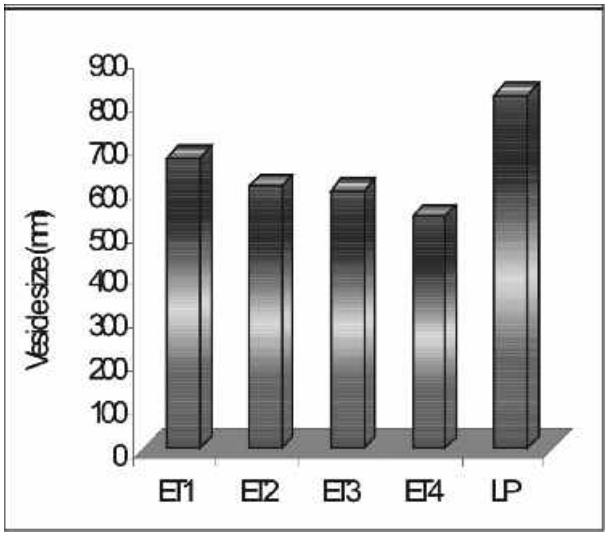

Figure-3: Vesicle size of different formulation

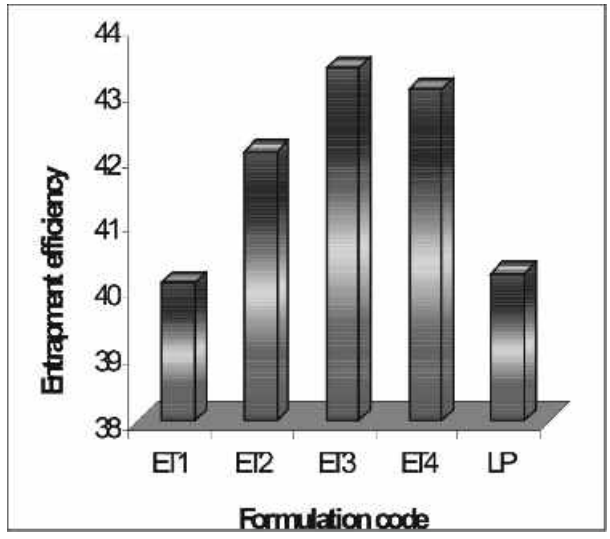

Figure-4: Entrapment efficiency of different fomulation

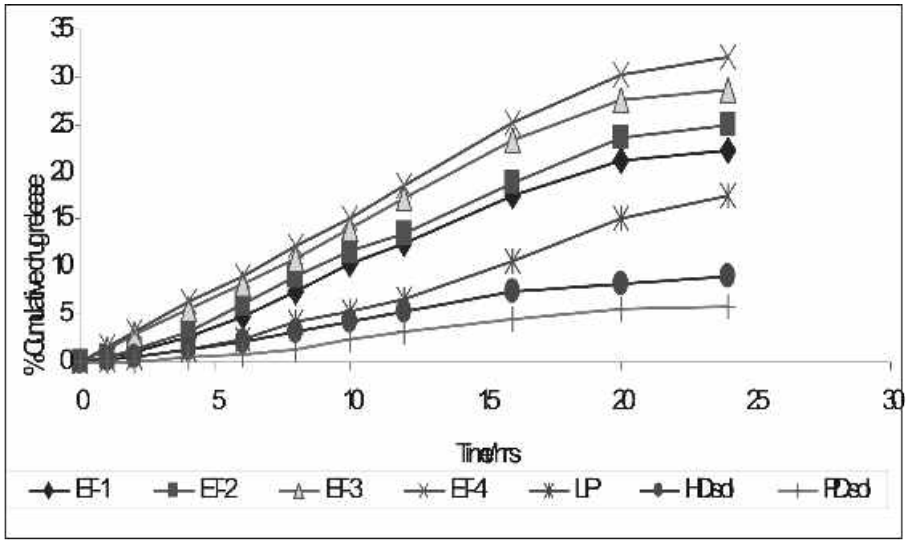

Figure-5: Percent comparative cumulative release of drug (µg/cm^2) different from formulation

Table-2: Skin permeation profile of different formulation of stavudine calculated from in-vitro drug release study through rat skin (after 24 hr).

Formulation code	% Stavudine permeated	% Stavudine deposited	Transdermal flux (µg/cm^2/hr)	Regression coefficient (R^2)	Enhancement ratio
ET-1	22.26±0.67	16.29±0.45	13.54±0.45	0.985	4.5
ET-2	24.96±0.46	14.30±0.78	14.61±0.67	0.991	4.9
ET-3	28.69±0.97	12.15±0.89	17.34±0.78	0.983	5.8
ET-4	32.00±0.46	10.68±0.56	17.54±0.78	0.980	5.9
Liposome	17.5±0.89	8.910±0.67	9.7±0/21	0.968	3.2
HD Solution	8.95±0.48	7.800±0.78	4.28±0.54	0.978	1.4
PD Solution	5.8±0.69	4.500±0.23	2.98±0.21	0.966	-

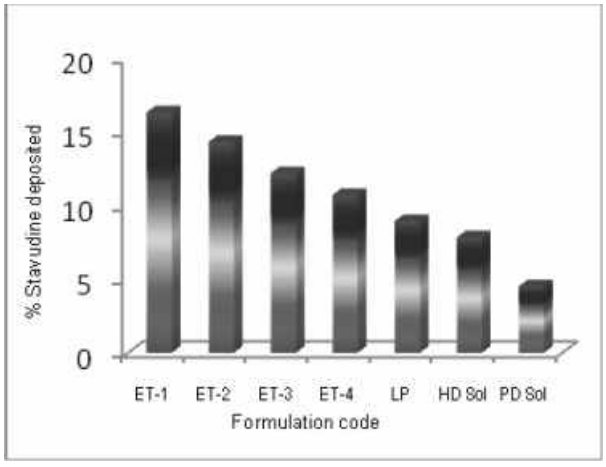

Figure-6: Percent stavudine deposited on mice skin in-vitro after 12 hours of extraction with 50% hydroalcoholic solution at 37^{0}C±0.5^{0}C.

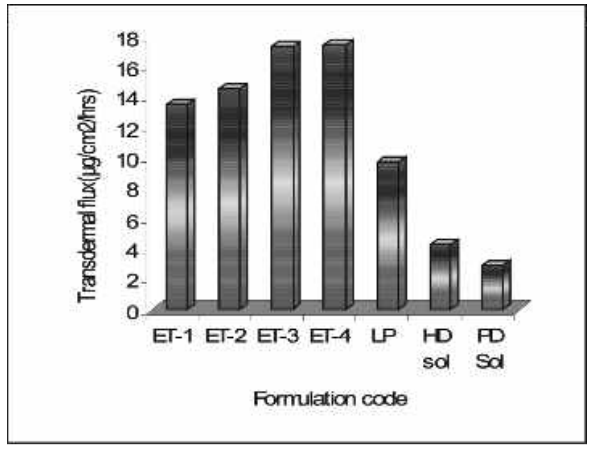

Figure-7: Transdermal Flux of different formulation

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