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Enhanced Skin Permeation of Verapamil Hydrochloride ThroughEthanolic Liposomes

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ABSTRACT

The purpose of this work was to formulate and characterize a novel vesicular carrier system ethosomes containing verapamil hydrochloride. Verapamil hydrochloride seems to be a suitable candidate for transdermal drug delivery because of its short biological half life, high hepatic clearance and low bioavailability (approx 20%).Verapamil hydrochloride loaded ethosomes were prepared from 2% soya phosphatidylcholine, 30% ethanol, 10% propylene glycol and water and characterized for various parameters as vesicle shape, vesicle size and size distribution, entrapment efficiency, stability studies and *in vitro* skin permeation studies through excised rat skin (Sprague Dawley) using a locally fabricated Franz diffusion cell.The entrapment efficiency of ethosomeswas found to be 64.2±3.4%. *In vitro* skin permeation of verapamil hydrochloride through excised rat skin (Sprague Dawley) revealed that ethosomesled to an enhanced transdermal flux (54.4±1.41µg/cm^2/h) of verapamil hydrochloride as compared to liposomes (11.6±2.12µg/cm^2/h). Decreased lag time (0.7 h) was observed in case ofethosomes. Our results suggested ethosomes to be the most efficient carrier system for transdermal delivery ofverapamil hydrochloride.

KEY WORDS: Transdermal delivery,Ethosomes, Verapamil hydrochloride.

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INTRODUCTION

Ethosomes are innovative and specially designed vesicular systems developed for transdermal and dermal delivery of bioactives. These are ethanol containing soft and malleable lipid vesicles tailored for enhanced skin permeation of drugs. Ethosomes can be applied topically for local as well as systemic drug administration.Ethosomes contain relatively high concentration of ethanol that is responsible for their better skin permeation ability. Because of their unique structure ethosomes are able to encapsulate and deliver highly lipophillic molecules such as minoxidil and testosterone as well as cationic drugs such as trihexyphenidyl and propranolol. Ethosomes have ability to deliver compounds to cells in culture. Plasmids and insulin have been delivered using ethosomal carrier.The important feature of these vesicles is their soft structure which carries the incorporated active agent into the skin lipid bilayers and promotes its delivery[1-5].

The impermeable barrier nature of the skin always challenges and attracts scientists. The outer most layer of the skin, the stratum corneum provides a protective barrier that prevents the loss of physiologically essential substances and provides greatest resistance to penetration andis the rate limiting step of percutaneous absorption.Stratum corneum behaves as a hydrophobic membrane and the limiting factor is the slow diffusion through the dead horny layer of skin[6].Various strategies as iontophoresis, microporation, electrophoresis, sonophoresis, pressure wave, magnetophoresis, chemical approaches as chemical permeation enhancers and prodrug approach have been utilized to modify the skin barrier[7]. Colloidal and vesicular carrierswith special formulation design are more successful in recent years because of their enhanced skin permeation potential.Different nanosized carriers as liposomes, niosomes, nanoemulsion, elastic liposomes, ethosomes and solid lipid nanoparticles are able to change or modify the barrier nature of skin through different mechanisms[8-10].

Ethosomes are gaining much more attention of researchers for dermal/transdermal delivery of therapeutics because they are able to cross the barrier nature of skin.These are phospholipid nanovesicles with ability to penetrate intact through the skin due to its high ethanol content. They are capable of delivering high concentrations of active agents to deep skin layer and/or the systemic circulation, regulated by its composition as well as physical characteristics.The basic difference between ethosomes and liposomes is their composition. Ethosomes contain relatively high concentration of ethanol that extracts skin lipids and provides flexibility to vesicle membrane. High ethanol content is responsible for better skin permeation ability of ethosomes. Liposomes contain cholesterol that gives rigidity to vesicle membrane. Hence, liposomes are able to deliver drugs to

outer layers of skin only. Soft vesicular ethosomes improve the transdermal flux, prolong the release and represent an attractive carrier for sustained transdermal delivery of drugs[11-12].

Verapamil 2-(3,4-dimethoxyphenyl)-5-{[2-(3,4-dimethoxyphenyl)ethyl](methyl)amino}-2-(1-methylethyl) pentanenitrile hydrochloride is commonly used calcium channel blocker for management of hypertension, supraventricular tachycardia, hypertrophic cardiomyopathy, angina pectoris, mitral stenosis, migraine prophylaxis. It is also used to treat hypertension associated with renal failure[13].Verapamil hydrochloride either alone or in combination withtrifluoperazine and magnesium sulfatemay be utilized inpeyronie's disease topically and transdermally[14-15].Low oral bioavailability of verapamil hydrochloride (approx 20%) due to hepatic first pass metabolism and short biological half-life (4.8 hr) restricts its oral administration. Its short biological half life, high hepatic clearance and low bioavailability support its strong candidature for transdermal drug delivery. For effective, sustained delivery of verapamil hydrochloride noninvasive delivery through transdermal route utilizing vesicular approach i.e. ethosomes may be a suitable option.Some researchers previously established feasibility oftransdermal delivery of verapamil hydrochloride through excised rat skin and porcine ear skin.Gungor et al formulated verapamil hydrochloride transdermal patches (matrix type) and studied various terpenes as penetration enhancer to enhance skin permeation. Transdermal drug delivery of verapamil hydrochloride and amlodipine besylate through microneedles and microneedle rollers showed improved transdermal flux through porcine ear skin[16-21].

The aim of present investigation was to formulate and characterizeethosomes as a delivery system for transdermal delivery of verapamil hydrochloride, a drug having low oral bioavailability (approx 20%), short biological half-life and extensive first pass metabolism.Ethosomes are innovative vesicular systems with ability to penetrate intact, through the human skin due to the high deformability.Physicochemical characteristics of ethosomes allow this vesicular carrier to transport active substances more efficaciously through the stratum corneum into the deeper layers of skin.The important feature of these vesicles is their soft structure which carries the incorporated active agent into the skin lipid bilayers and promotes its delivery.Prepared ethosomal vesicles were characterized for various physio-chemical attributes i.e. vesicle shape, size, surface morphology, entrapment efficiency, stability studies and *in vitro* skin permeation through excised rat skin(Sprague Dawley).

EXPERIMENTAL SECTION

Materials and methods

Soya phosphatidylcholine (99%), Sephadex G-50, Triton X-100 were purchased from Sigma (St. Louis, MO, USA). Verapamil hydrochloride was received as a gift sample from Samarth Life

Sciences Pvt. Ltd. (Solan, India). All other chemicals and solvents were of analytical grade and freshly prepared distilled water was used wherever required.

Preparation of vesicular systems

The ethosomal colloidal suspension was prepared by cold method[22]. The ethosomal systems prepared here comprised of 2-4% w/w of soya phosphatidylcholine, 20-40% of ethanol and 10% propylene glycol. Soya phosphatidylcholine was dissolved in mixture of ethanol and propylene glycol. Freshly prepareddistilled water containing drug was added slowly in a fine stream with constant mixing at 700 rpm on a mechanical stirrer (Remi Equipment, Mumbai, India) in a house built closed container. Mixing was continued for an additional 5 min. The system was kept at 30±1ºC throughout the preparation. The final suspension of ethosomes was left to cool at room temperature for 30 min.Conventional liposomal formulations were prepared by the Cast film method[23].Soya phosphatidylcholine (2%) and cholesterol (0.88%) were dissolved in minimum quantity of chloroform: methanol mixture (3:1) in a round bottom flask. The organic solvent was removed in rotary evaporator (Rotary Evaporator, Superfit, Ambala, India) under reduced pressure to form a thin film on the wall of the flask. Final traces of solvent were removed under vaccum overnight. The deposited lipid film was hydrated with PBS (pH 6.5) of drug at 60 rpm for 1 hr at 55ºC. Both the vesicular systems were then passed through sandwich of 200 and 400 nm polycarbonate filter (Milipore, USA).

Table No. 1 Composition of different ethosomal formulations

S. No.	Formulation Code	Soya PC (% w/w)	Ethanol (%w/w)	Propylene glycol (% w/w)	Drug (% w/w)
1.	ET2A	2	20	10	0.2
2.	EL2B	2	30	10	0.2
3.	EL2C	2	40	10	0.2
4.	EL3A	3	20	10	0.2
5.	EL3B	3	30	10	0.2
6.	EL3C	3	40	10	0.2
7.	EL4A	4	20	10	0.2
8.	EL4B	4	30	10	0.2
9.	EL4C	4	40	10	0.2

Incorporation of verapamil hydrochloride in ethosomes

Verapamil hydrochloride was incorporated into vesicular formulations at saturating concentration. To determine the maximum amount of drug incorporated, increasing amounts of verapamil hydrochloride were added during preparation of ethosomes. Vesicular formulations were examined over a period of 14 days using Light Microscopy (Leica, DMLB, Switzerland).

Characterisation of ethosomes

Vesicle Shape and Surface Morphology

Ethosome vesicles were visualized using a Philips transmission electron microscope (CM12 Electron Microscope, Eindhoven, Netherlands) with an accelerating voltage of 100 kV. Samples were negatively stained with a 1% aqueous solution of phosphotungstic acid. Ethosomal suspension was dried on a microscope carbon-coated grid for staining. The excess solution was removed by blotting. After drying, the specimen was viewed under the microscope at a 10-100k-fold enlargement.

Surface morphology of ethosomal vesicles was investigated by Scanning Electron Microscope (SEM). One drop of ethosomal system was mounted on clear glass stub, air dried and coated with Polaron E 5100 Sputter coater (Polaron, UK) and visualized under Scanning Electron Microscope (Leo-435 VP, Cambridge UK).

Vesicle Size Analysis

The vesicle size and size distribution were determined by Dynamic Light Scattering (DLS) method, in a multimodal mode using a computerized inspection system (Malvern Zetamaster, ZEM 5002, Malvern,UK). For vesicle size measurement, vesicular suspension was mixed with the appropriate medium PBS (pH 6.5) and the measurements were conducted in triplicate.

Nephalometric Measurements

Turbidity of different ethosomal and liposomal formulations were determined using a Nephalometer (Superfit, Mumbai, India) taking PBS (pH 6.5) as blank.

Entrapment Efficiency

Entrapment efficiency of ethosomal system was determined by Ultracentrifugation (Ultracentrifuge, Hitachi, Singapore). Ethosomal preparation that were kept overnight at 4ºC were spun in ultracentrifuge at 3000 rpm for 1hr.

In-Vitro Skin Permeation Studies

The in-vitro skin permeation studies with drug loaded ethosomal formulations were carried out using a locally fabricated diffusion cell and percentage cumulative drug release and transdermal flux were evaluated.Abdominal skin of albino rats (4-5 weeks old) was mounted between the donor and receptor compartments. The effective permeation area and receptor cell volume of the diffusion

cell were 1cm2 and 10 ml respectively. The temperature was maintained at 32±1ºC. The receptor compartment contained 10 ml PBS (pH 6.5) and was stirred constantly with a magnetic stirrer (Expo India Ltd, Mumbai, India) at 100 rpm. The 1 ml of each (a) ethosomal formulation, (b) liposomal formulation, (c) hydroethanolic solution, (d) aqueous drug solution, was applied to the epidermal surface of the rat skin using micropipette. Samples were withdrawn through the sampling port of the diffusion cell at predetermined time intervals over 24 hr and analyzed for drug content. Receptor phase was immediately replenished with an equal volume of fresh buffer. In-vitro drug release study from ethosomes was repeated with cellophane membrane using the same method as described above.

The amount of verapamilhydrochloride remained in the skin was determined at the end of the in-vitro permeation experiment (24 hr). The skin was washed 10 times using acotton cloth immersed in methanol. A sample of skin was weighed, cut with scissors, positioned in a glass homogenizer containing 1ml of methanol and homogenized for 5 min with an electric stirrer. The resulting solution was centrifuged for 10 min at 700 rpm. The supernatant was analyzed for drug.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 (TL, hr) were calculated from the slope and X-intercept of the linear portion, respectively.

Physical Stability of Ethosomes

The ability of vesicles to retain the drug was assessed by keeping the ethosomal vesicles in sealed vials (10 ml capacity) after flushing with nitrogen at different time periods (1, 15, 30, 60, 90 and 120 days). Samples were withdrawn periodically and analyzed for the drug content. The ethosomes was also assessed quantitatively by measuring size and morphology of the vesicles over time using DLS and TEM.

RESULTS AND DISCUSSION

Ethanol is known as an efficient penetration enhancer and believed to act by affecting the intercellular region of the stratum corneum, thus enhancing penetration. Though earlier it was thought that due to the interdigitation effect of ethanol on lipid bilayers, high concentration of ethanol were detrimental to liposomal formulations. Recently embodying high concentration of ethanol in liposomes has been reported to provide the greater fluidity and malleability leading to the formation of soft vesicles known as ethosomes. Various formulation variables which may affect the formulation i.e. phospholipid and ethanol concentration were studied in order to produce optimized vesicles. The cold method reported by Touitou et al., 2000 was followed for the preparation of

ethosomal vesicles using soya phosphatidylcholine, ethanol and propylene glycol. Ethosomes prepared from 2% soya phosphatidylcholine, 30% ethanol, 10% propylene glycol and water was found to be optimized formulation.

For optimization of drug amount, the ethosomes wereentrapment efficiency. The maximum concentration of verapamil hydrochloride that could be incorporated into ethosomes was found to be 20mg with percentage entrapment efficiency of 61.2±2.8%. On further increasing the amount of drug, precipitated drug crystals were observed, this precipitation of drug crystals may be due to the saturation of the vesicles. Further, a lower entrapment of verapamil hydrochloride in ethosomes may be due to the hydrophilic nature of the drug.prepared using varying concentrations of drug, followed by morphological characterization and determination of

Table No. 2 Drug loading in ethosomal formulations

S. No.	Amount of drug (mg)	Microscopic observation	Entrapment efficiency (%)
1.	5	NO	48.3±3.4
2.	10	NO	52.7±5.1
3.	15	NO	57.1±1.6%.
4.	20	NO	61.2±2.8%
5.	25	O	58.4±2.2%

NO- Not observed, O- Observed

Visualization by TEM showed that ethosomes had a unilamellar spherical structure and this confirmed the existence of vesicular structure at higher concentration of ethanol. Surface morphology and three dimentional nature of ethosomes was confirmed further by analysis of preparation by SEM. Vesicle size and size distribution was determined using Dynamic Light Scattering (DLS) method.

Conventional liposomes prepared by cast film method had an average vesicle size of 434±3.7 nm. The polydispersity index for optimized ethosomal formulations were found in the range of <0.1 suggesting that the formulations were homogeneous. Polydispersity index increased with ethanol concentration for all ethosomal formulations. This may be due to the formation of different structure at higher ethanol concentration.

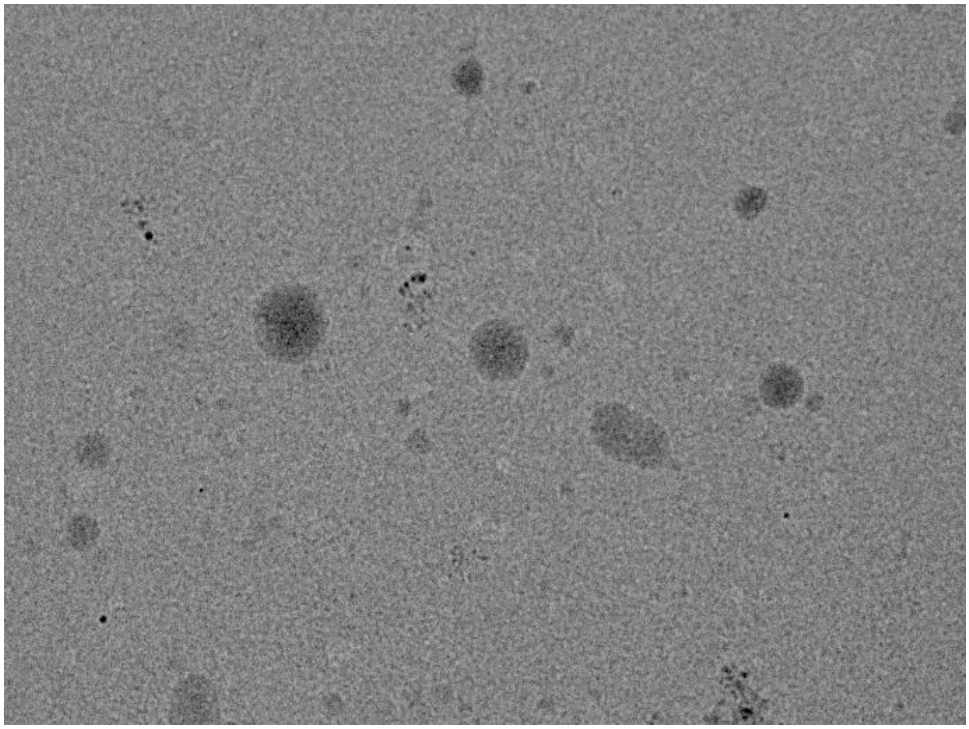

Fig.1 TEM (Transmission Electron Microscopy) Photograph of Ethosome (X 30,000)

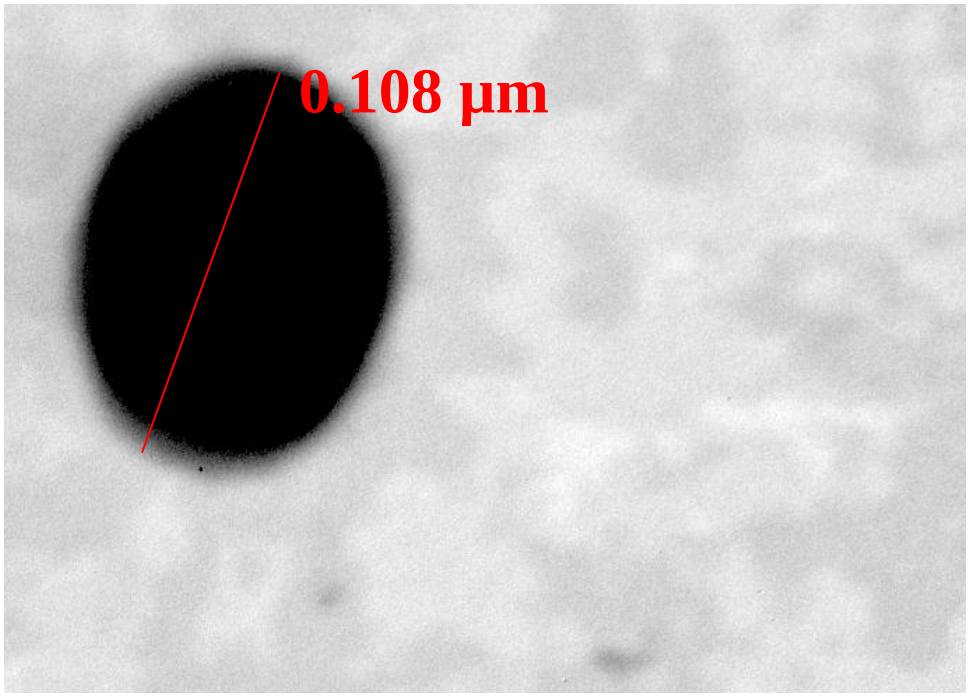

Fig. 2 TEM (Transmission Electron Microscopy) Photograph of Ethosome (X 80,000)

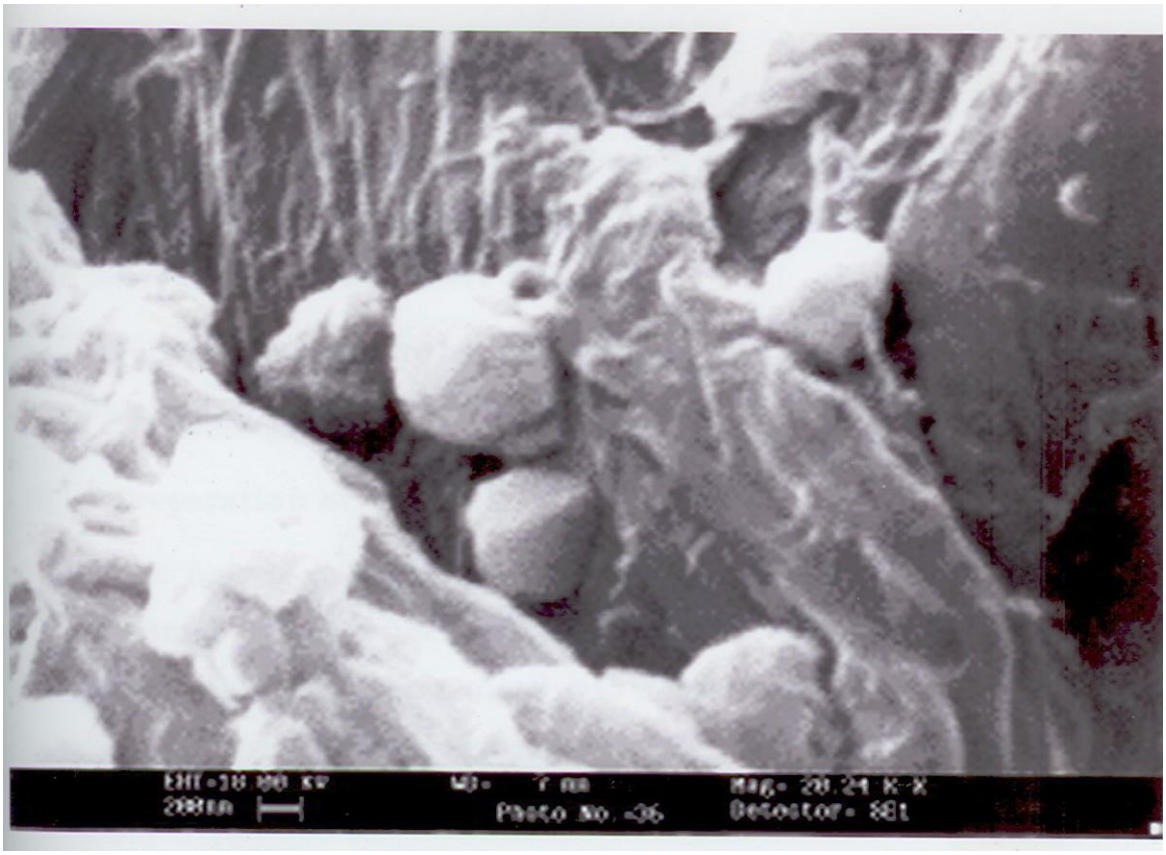

Fig. 3 SEM (Scanning Electron Microscopy) Micrographs of Ethosomes

Turbidity of different ethosomal formulation was measured via Nephalometer. Turbidity was increased with increasing ethanol concentration upto 30%, with further increase in ethanol concentration rapid decrease in turbidity of vesicle dispersion was observed. This may be due to the increased fluidity of lipid bilayers at 30% ethanol concentration resulting in increased turbidity of vesicle dispersion. Maximum turbidity at 30% ethanol concentration suggested the fairer number of vesicles and better effect of ethanol on vesicle stability. At higher ethanol concentration decreased turbidity of vesicular system may be due to the solubilization of lipid bilayer.

Entrapment efficiency is the percentage fraction of the total drug incorporated into the formulation. The effect of ethosomal components i.e. ethanol and phospholipid on the entrapment efficiency of drug was studied. The entrapment efficiency of verapamil hydrochloride in ethosomes (30% ethanol) and liposomes was found to be 61.2±2.8% and 39.3±1.8, respectively. The entrapment efficiency first increased upto 30% ethanol concentration and with further increase in ethanol concentration decrease in entrapment efficiency was observed. Ethosomal formulation containing 30% ethanol showed maximum drug entrapment. This is due to the increased membrane permeability of vesicles at higher ethanol concentration that leads to decrease in the entrapment efficiency of ethosomal formulation. The effect of phospholipid concentration enhancement on entrapment efficiency was not as significant.

Table No. 3Entrapment efficiency and turbidity of different elastic liposomes

S. No.	Formulation Code	Turbidity (N.T.U.	Entrapment efficie
1.	ET2A	26±3.2	44.3±1.9%
2.	EL2B	33±1.9	61.2±2.8%
3.	EL2C	24±2.4	50.8±3.1%
4.	EL3A	27±4.6	46.5±1.6%
5.	EL3B	31±4.5	61.5±2.9%
6.	EL3C	25±1.6	51.4±3.4%
7.	EL4A	27±5.4	46.6±2.3%
8.	EL4B	29±3.7	62.4±1.7%
9.	EL4C	24±4.3	52.4±3.2%
10.	Liposomes	19±3.2	39.3±1.8

The physical stability of ethosomal vesicles was investigated by keeping these formulations for a period of 120 days and vesicle size and shape was monitored by DLS & TEM. Vesicular size measurements of ethosomes stored at room temperature for various time periods showed no significant difference. Electron microscopic visualization of ethosomes stored at room temperature for 120 days showed no significant difference in shape and lamellarity suggesting stabilizing effect of ethanol in the formulation. Probably, ethanol confers a negative net charge on the surface of the system thus avoiding or delaying the formation of vesicle aggregates, due to the electrostatic repulsion. Liposomes showed greater aggregation and increased vesicle size in 120 days.

Table No. 4Stability of ethosomes: vesicle size over time

Time After Preparation	Vesicle Size	
	ET2B	Liposomes
1	108±2.8	454±7.0
15	119±3.1	475±10.0
30	124±2.4	496±14.0
60	132±4.2	509±11.0
90	138±1.6	541±13.0
120	144±3.4	578±17.0

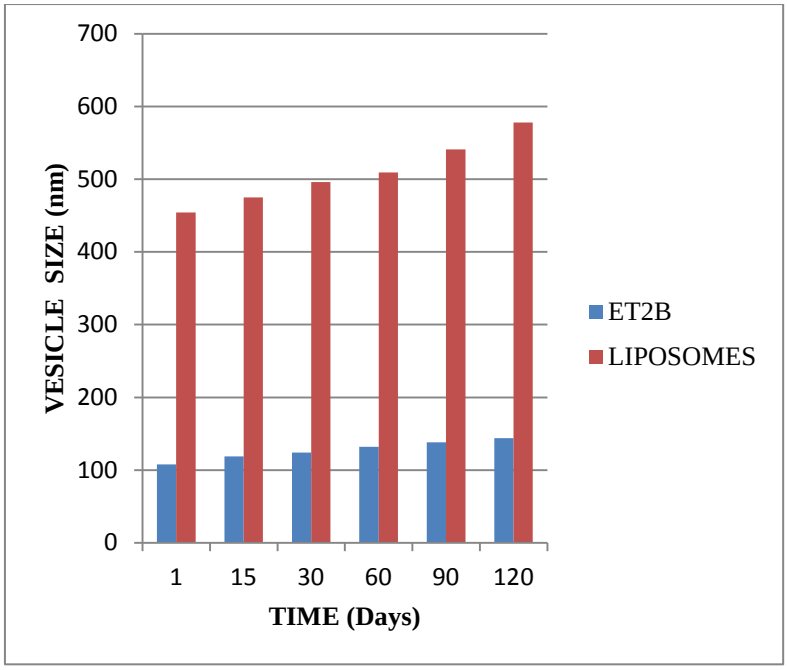

Fig. 4 Effect on vesicle size of ethosomes and liposomes after storage

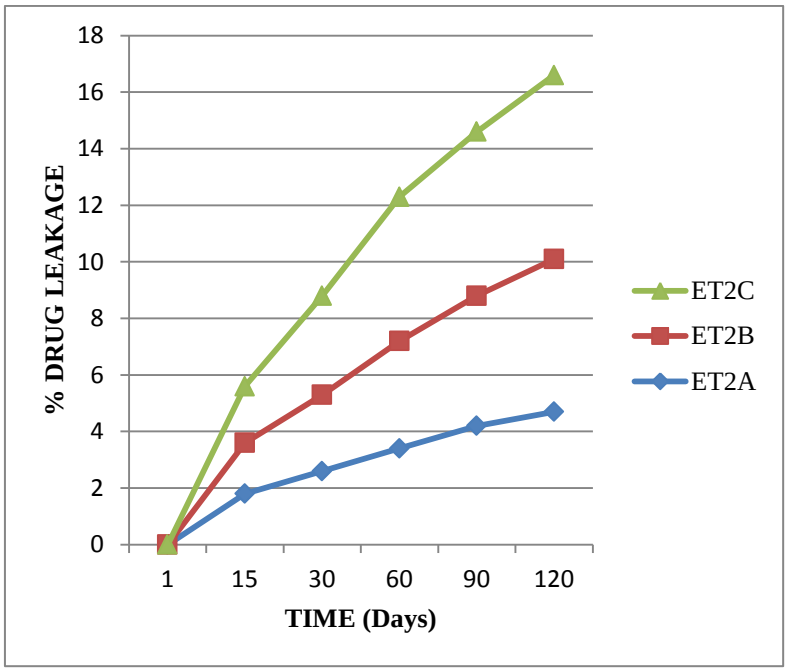

Fig. 5 Extent of drug leakage from ethosomes at different time (days)

The ability of ethosomal vesicles to deliver verapamil hydrochloride was investigated by determining the release rate through cellophane membrane, transdermal flux across excised rat skin and skin deposition of the drug. 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 slope of the linear portion. The steady state transdermal flux for different ethosomal formulations was observed between 51.4±2.12 μg/h/cm2 and 17.6±2.44 μg/h/cm2 across the rat skin. The ET2B formulation

provided the maximum transdermal flux 51.4±2.12µg/h/cm2 with minimum lag time (0.7 h). Conventional liposomes and plain drug solution provided significantly lower flux values and longer lag time of 11.4±1.23 µg/cm2/h, 2.3 h and 3.14±0.88µg/cm2/h, 2.9 h; respectively as compared to ethosomes.

The ET2B formulation showed the highest percent drug skin deposition of 6.74±1.3 as compared to other formulation containing different concentration of phospholipid and ethanol. The value of transdermal flux depends on the ethanol concentration. As the ethanol concentration increases, transdermal flux increases upto 30% and further increase in ethanol concentration, transdermal flux decreases.

Ethosomal vesicles showed higher skin deposition suggesting sustained release effect of these vesicular carriers. These vesicles provide lower lag time, hence rapid distribution of drug in the depths of the skin. A lower transdermal flux was observed from ethosomal formulations ET3B & ET4B containing higher phospholipid concentration as compared to ET2B. Higher phospholipid concentration decreased fluidity of ethosomal bilayers, hence resulting in decreased transdermal flux. The permeation enhancement from ethosomal vesicles may be due to the complex interactions between the system components (ethanol, phospholipid and water) and the skin. Thus the effects of ethanol, which were considered harmful to classic liposomes, may provide the vesicles with soft flexible characteristic, which allow them to more easily penetrate into deeper layers of the skin.

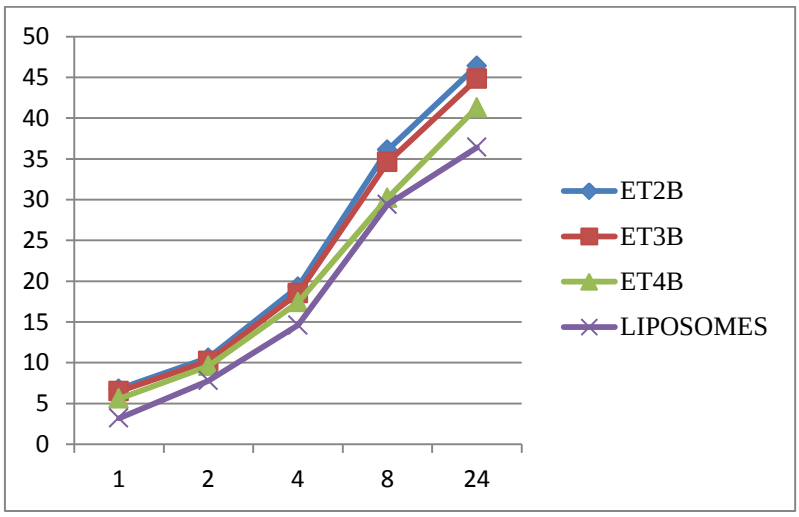

Fig. 6 % Cumulative Drug Release from ethosomes for 24 hours

Table No. 5 Composition and characterization of verapamil hydrochloride loaded ethosomes

S.No.	Parameters	Ethosomes ET2B	Liposomes	Plain drug solution
1	Vesicular shape	Spherical, unilamellar	Spherical, multiilamellar	–
2	Vesicular size (nm)	108±5	434±7.0	–
3	Polydispersity index	0.23	0.058	–
4	% Entrapmemt efficiency	61.2±2.8%	39.3±1.8	–
5	Transdermal Flux across rat skin (μg/cm^2/h)	51.4±2.12	11.4±1.23	3.14±0.88
6	Lag time (h)	0.7	2.3	2.9
7	Percent skin drug deposition	6.74±0.4	1.78±2.5	1.19±1.6

Ethosomal vesicles, containing 2% soya phosphotidylcholine and 30% ethanol was found to be best formulation with maximum entrapment efficiency (61.2±2.8%), maximum transdermal flux (51.4±2.12μg/h/cm^2) and minimum lag time (0.7 h).

CONCLUSION

The aim of the current investigation was to evaluate transdermal potential of novel vesicular carrier,ethosomes containing verapamil hydrochloride. Enhanced transdermal flux and decreased lag time of drug loaded ethosomes suggested the efficacy of ethosomal system as a carrier for transdermal delivery of verapamil hydrochloride and problems associated with verapamil hydrochloride could be sorted out. Commercialization of these vesicular systems could open new possibilities and challenges towards better therapies.

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