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PREPARATION AND EVALUATION OF SIMVASTATIN ETHOSOMES

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Abstract:

The purpose of the present investigation is aimed to prepare and evaluate simvastatin ethosomes containing different concentration of ethanol and phospholipids by sonication for size reduction of vesicles. Designed ethosomes are characterized for Size and shape, Entrapment efficiency, Release study and Stability study, The effect of sonication also studied on the characteristics of simvastatin ethosomes. To formulate ethosomal gel, characterize the prepared formulation using cold method and carryout various evaluation parameters which includes vesicular shape and surface morphology, vesicular size, size distribution, drug content, storage-physical stability of ethosomes, entrapment efficiency and in vitro drug diffusion study of ethosomal gel.

Key words: ethosomes, phospholipids, sonication, gel and cold method.

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INTRODUCTION:

Optimization of drug delivery through human skin is important in modern therapy. Recently, the transdermal route vied with oral treatment as the most successful innovative research area in drug delivery [1-3].

Transdermal delivery is an important delivery route that delivers precise amount of drug through the skin for systemic action. Improved methods of drug delivery for biopharmaceuticals are important for two reasons; these drugs represent rapidly growing portion of new therapeutics, and are most often given by injection. Discovery of new medicinal agents and related innovation in drug delivery system have not been only enabled the successful implementation of novel pharmaceutical, but also permitted the development of new medical treatment with existing drugs. Throughout the past two decades, the transdermal patches has become a proven technology holding the promise that new compound could be delivered in a safe and convenient way through the skin. Since the first transdermal patch was approved in 1981 to prevent nausea and vomiting associated with motion sickness, the FDA has approved through the past 22 years more than 35 transdermal patch products spanning 13 molecules [4,5,6].

The vesicles have been well known for their importance in cellular communication and particle transportation for many years. Researchers have understood the properties of vesicle structures for use in better drug delivery within their cavities, that would allow to tag the vesicle for cell specificity. Vesicles would also allow to control the release rate of drug over an extended time, keeping the drug shielded from immune response or other removal systems and would be able to release just the right amount of drug and keep that concentration constant for longer periods of time. One of the major advances in vesicle research was the finding a vesicle derivative, known as an ethosomes [7,8,9].

Ethosomal carriers are systems containing soft vesicles and are composed mainly of phospholipid (Phosphotidyl choline; PC), ethanol at relatively high concentration and water. It was found that ethosomes penetrate the skin and allow enhanced delivery of various compound to the deep strata of the skin or to the systemic circulation [10-15].

Simvastatin is a lipid-lowering agent that is derived synthetically from the fermentation of Aspergillus terreus. It is a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase (hydroxymethylglutaryl COA reductases), which is the rate-limiting enzyme in cholesterol biosynthesis. It may also interfere with steroid hormone production. Due to the induction of hepatic LDL receptors, it increases breakdown of LDL cholesterol.

Present work to formulate ethosomal gel. Characterize the prepared formulation using cold method. To carryout various evaluation parameters which includes vesicular shape and surface morphology, vesicular size, size distribution, drug content, storage-physical stability of ethosomes, entrapment efficiency. To carry out *in vitro* drug diffusion study of ethosomal gel.

MATERIALS AND METHOD:

Simvastatin from Chandra Labs Hyderabad, India. Soya Lecithin from BrightLaboratories. Ethanol and Chloroform from S.D.Fine Chemicals, Mumbai. Carbopol-940 from Research lab fine chem. Industries,Mumbai.

Preparation of Calibration Curve

An accurately weighed 100 mg of Simvastatin was dissolved in methanol and volume was made upto pH 6.8 phosphate buffer as per I.P and make up the volume up to 100 ml in a volumetric flask, (Stock Solution: I, 1000 µg/ml) . From this 5 ml of solution were pipette out and make up the volume up to 100 ml (Stock Solution: II, 50 µg/ml). Then the aliquots were prepared, whose concentration ranging from 0 to 30 µg/ml and the absorbance were measured at 239 nm[53] by using UV Spectrophotometer Labomed, (Model No: 2602) against the reagent blank.

Compatibility Studies:

IR spectroscopy can be used to investigate and predict any physicochemical interactions between different components in a formulation and therefore it can be applied to the selection of suitable chemically compatible excipients.

Preparation of Ethosomes Containing Simvastatin (By Cold Method)

Preparation of Simvastatin ethosomes was followed by method suggested by Touitou et al., with little modification.[7.]

The ethosomal system of Simvastatin comprised of 2-5 % phospholipids, 20-50 % ethanol, 10 % of propylene glycol, 0.005g of cholesterol and aqueous phase to 100 % w./w. Simvastatin (20mg) was dissolved in ethanol in a covered vessel at room temperature by vigorous stirring. Propylene glycol was added during stirring. This mixture was heated to 30^0 in a separate vessel and was added to the mixture drop wise in the center of the vessel, which was stirred for 5min at 700rpm in a covered vessel. the vesicle size of ethosomal formulation can be decreased to desire extend using sonication[29] or extrusion[30] method. Finally, the formulation is stored under refrigeration[31].Ethosomes were formed spontaneously by the process.

Table 1: Composition of Different Ethosomal Formulations

Ethosomal formulation	Lecithin (Soya lecithin%)	Ethanol (%)	Propylene glycol (%)	Drug (g)	Cholesterol(g)	Water
SF_1	2	20	10	0.025	0.005	q.s
SF_2	3	20	10	0.025	0.005	q.s
SF_3	4	20	10	0.025	0.005	q.s
SF_4	5	20	10	0.025	0.005	q.s
SF_5	2	30	10	0.025	0.005	q.s
SF_6	2	40	10	0.025	0.005	q.s
SF_7	2	50	10	0.025	0.005	q.s

Table 2: Composition of Different Ethosomal Gel Formulation

Gel formulation	Simvastatin ethosomal suspension(ml)	Carbopol (%)	Triethanolamine (ml)	Phosphate buffer (pH 6.8)
G-1	20	1	0.5	q.s
G-2	**20**	**1.5**	**0.5**	**q.s**
G-3	20	2	0.5	q.s

Preparation of Simvastatin ethosomal gel

The best achieved ethosomal vesicles suspension, formula SF-2 was incorporated into carbopol gel (1%, 1.5%, 2% w/w).the specified amount of carbopol 940 powder was slowly added to ultrapure water and kept at 100^0c for 20min. tri ethanolamine was added to it dropwise. Appropriate amount of formula SF-2 containing Simvastatin (1% w/w) was then incorporated into gel-base. Water q.s was added with other formulation ingredients with continuous stirring until homogenous formulation were achieved (G-1,G-2 and G-3).

Characterization of Ethosomes

Size and Shape Analysis

Microscopic analysis was performed to determine the average size of ethosomes . A sample of ethosomes was suitably diluted with distilled water in order to observe individual vesicle and a drop of diluted suspension was put on a glass slide covered with cover slip and examined under microscope (magnification 15 × 45 X). The diameters of 150 vesicles were determined randomly using calibrated eyepiece micrometer with stage micrometer. The average diameter was calculated using the formula.

Average diameter = nd / n

n = number of vesicles

d = diameter of vesicles

Sonication reduced the vesicular size. Since vesicular size of these vesicles could not be analysed using microscopic method at magnification 15 × 45 X. Hence analysis of sonicated vesicles was done under a special microscope which is connected with a software and photomicrographs were taken under 400 and 800 magnification. Further selected photomicrographs were analysed for size analysis by using special software "particle size analysis" developed by BIOVIS. This special software works on images of photomicrographs with standard dimension.

Scanning Electron Microscopy:

Determination of surface morphology (roundness, smoothness and formation of aggregates) of ethosomal gel with polymer was carried out by scanning electron microscopy (SEM).

Entrapment Efficency

The entrapment efficiency of ethosomal vesicle was determined by ultracentrifugation 10 ml of (ethosomal suspension) each sample was vortexed for 2 cycles of 5 min with 2 minutes rest between the cycles. 1.5ml of each vortexed sample and fresh untreated ethosomal formulations were taken into different centrifugal tubes. These samples were centrifuged at 20,000 rpm for 3 hours. The supernatant layer was separated, diluted with water suitably and drug concentration was determined at 239 nm in both vortexed and unvortexed samples. The entrapment efficiency was calculated as follows

$$\text{Entrapment Efficiency} = \frac{T - C}{T} \times 100$$

'T' is total amount of drug that detected from supernatant of vortexed
'C' is the amount of drug unentraped and detected from supernatant of unvortexed

Characterization of Gel

Surface morphology: The surface morphology of the ethosomal was determined by using scanning electron microscope using gold sputter technique. The system was vacuum dried, coated with gold palladium, and observed microscopically.

Organoleptic Characteristics: The formulations were tested for its psycho rheological properties like color, odor, texture, phase separation and feel upon application(grittiness, greasiness)

Washability: A small quantity of gel was applied on the skin. After washing with water, checked for whether the gel was completely washable or not.

pH: Solution of 1gm of gel dissolved in 30ml of distilled water(pH 7) was prepared. The pH of the ethosomal gel was determined by using digital pH meter, measured by bringing the probe of the pH meter in contact with the samples.

Drug Content and Content Uniformity: 1g of gel was dissolved in a 100ml of phosphate buffer pH 6.8 for 48 hrs with constant stirring using magnetic stirrer.solution was filtered and observed with u.v spectrophotometer at λmax 239 nm.the measurements were made in triplicate.

In-Vitro Release Studies

Drug Release Study from Dialysis Membrane

The skin permeation of Simvastatin from ethosomal formulation was studied using open ended diffusion cell specially designed in our laboratory according to the literates. The effective permeation area of the diffusion cell and receptor cell volume was 2.4 cm and 200 ml respectively. The temperature was maintained at 37 ± 0.5°C.[2]

The receptor compartment contained 200 ml of pH 6.8 buffer and was constantly stirred by magnetic stirrer at 100 rpm. Prepared dialysis was mounted between the donor and the receptor compartments. Ethosomal formulation was applied to the dialysis membrane and the content of diffusion cell was kept under constant stirring then 5 ml of samples were withdrawn from receptor compartment of diffusion cell at predetermined time intervals and analysed by spectrometric method at 239 nm after suitable dilution. The receptor phase was immediately replenished with equal volume of fresh pH 6.8 buffer. Triplicate experiments were conducted for drug release studies

In-vitro Release Kinetics:

To analyze the in vitro release data various kinetic models were use to describe the release kinetics. The zero order rate Eq. (2) describes the systems where the drug release rate is in-dependent of its concentration. The first order Eq.(3) describes the release from system where release rate is concentration dependent. Higuchi (1963) described the release of drugs from insoluble matrix as a square root of time dependent process based on Fickian diffusion.

The results of in vitro release profile obtained for all the formulations were plotted in modes of data treatment as follows:

Zero order kinetics:

Zero order release would be predicted by the following equation:

$$\mathbf{At = A_0 – K_{0t}}$$

Where,

At = Drug release at time't'
A_0 = Initial drug concentration.
K_0 = Zero- order rate constant (hr-1)

When the data is plotted as cumulative percent drug release versus time, if the plot is linear then the data obeys Zero – order kinetics and its slope is equal to Zero order release constant K_0**.**

First Order Kinetics:

First - order release could be predicted by the following equation:

$$\mathbf{Log\ C = log\ C_0 – K_t / 2.303}$$

Where,

C = Amount of drug remained at time't'
C_0 = Initial amount of drug.
K = First - order rate constant (hr^{-1}).

When the data plotted as log cumulative percent drug remaining versus time, yields a straight line, indicating that the release follow first order kinetics. The constant 'K_1' can be obtained by multiplying 2.303 with the slope value.

Higuchi's Model:

Drug release from the matrix devices by diffusion has been described by following Higuchi's classical diffusion equation:

$$\mathbf{Q = [D\epsilon / \tau(2A – \epsilon Cs)\ Cst]1/2}$$

Where

Q = Amount of drug release at time't'
D = Diffusion coefficient of the drug in the matrix.
A = Total amount of drug in unit volume of matrix.
Cs = Solubility of drug in the matrix. ϵ = Porosity of the matrix.
τ = Tortuosity.

t = Time (hrs at which q amount of drug is released).

Above equation can be simplified as if we assume that 'D', 'Cs' and 'A' are constant. Then equation becomes:

$$Q = Kt_{1/2}$$

When the data is spited according to equation i.e. cumulative drug release versus square root of time yields a straight line, indicating that the drug was released by diffusion mechanism. The slope is equal to 'K' (Higuchi's 1963).

Korsmeyer Equation / Peppa's Model:

To study the mechanism of drug release from the liposomal solution, the release data was also fitted to

Test	Description
Colour	white powder

the well-known exponential equation (Korsmeyer equation/ Peppa's law equation), which is often used to describe the drug release behavior from polymeric systems.

$M_t / M_\alpha = Kt^n$

Where

M_t / M_α = The fraction of drug released at time't'.

K = Constant incorporating the structural and geometrical characteristics of the drug / polymer system.

n = Diffusion exponent related to the mechanism of the release.

Above equation can be simplified as follows by applying log on both sides,

$$\text{Log } M_t / M_\alpha = \text{Log } K + n \text{ Log } t$$

Mechanism of Drug Release

Table 3: Diffusion Exponent and Solute Release Mechanism for Cylindrical Shape

S.No	Diffusion	Exponent (n) Overall solute
1.	0.45	Fickian diffusion
2.	0.45<n<0.89	Anomalous (non-Fickian) diffusion
3.	0.89	Case-II transport
4.	n>0.89	Super case-II transport

Stability Studies

Stability study was carried out for Simvastatin ethosomal preparation at two different temperature i.e. refrigeration temperature (4 ± 2° C) at room temperature (27 ± 2° C) for 8 weeks (as per ICH guidelines). The formulation was subjected to stability study and stored in borosilicate container to avoid any sort of interaction between the ethosomal preparation and glass of container, which may affect the observations.

***In-vitro* Stability Release Study**

Stability of drug and stability of vesicles are the major determinant for the stability of formulation, studies were carried to evaluate total drug content at room temperature (27±2° C) and refrigeration temperature (4±2° C).samples was collected for every 2 weeks and absorbance was seen at 239nm in U.V spectrometer.

RESULTS AND DISSCUSSION:

Pre-Formulation Studies

Description

These tests were performed and the results were illustrated in the following table:

Table no 9: Table showing the description of Simvastatin (API)

Result

The results were found as per specifications**.**

Solubility

These tests were performed and the results are illustrated in the table

Table 4: Table showing the Solubility of Simvastatin (API) in various solvents.

Solvents	Solubility
Water	Insoluble
Methanol	Soluble
Ethanol	Soluble

Melting Point

This test is performed and the result was illustrated in the following table.

Table 5: Table showing the melting point of API's

Material	Melting Point
Simvastatin	139^0c

Result: The Result was found to be within limit.

Construction Of Calibration Curve

Table 6: Calibration Curve Data of Simvastatin

CONCENTRATION IN µg/ml	ABSORBANCE AT 239 nm
0	0
4	0.171
6	0.253
8	0.337
10	0.421
12	0.514
14	0.602

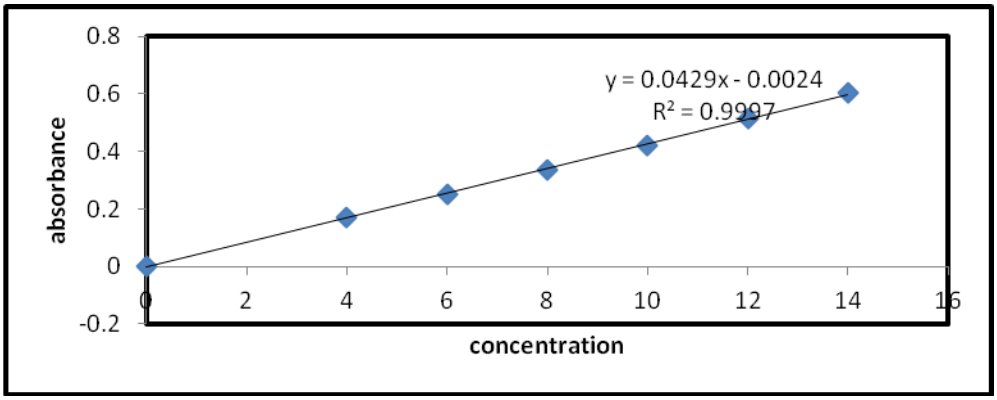

Fig 1: Calibration Curve Data of Simvastatin

Drug-Excipient Compatibility Studies

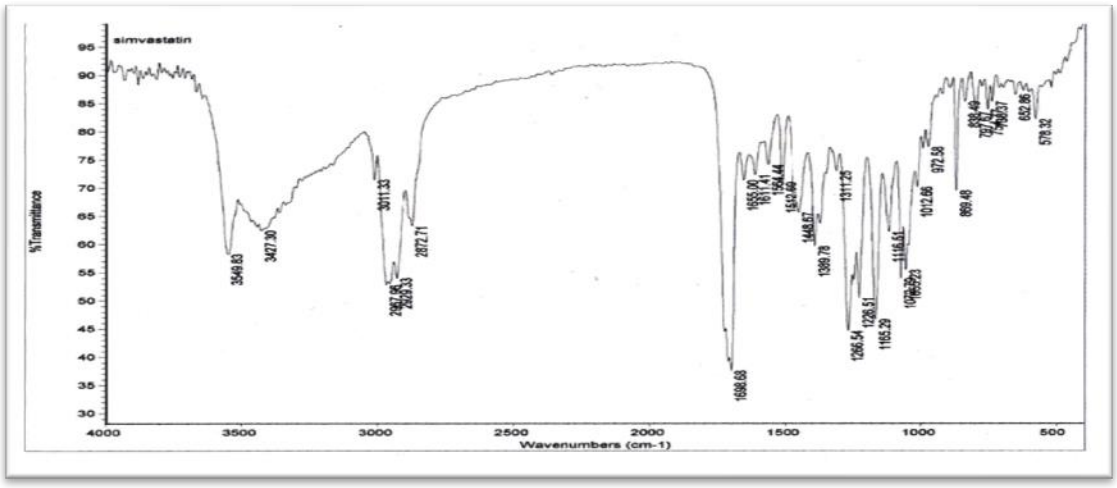

Fig 2: FTIR Spectra of Simvastatin Pure Drug

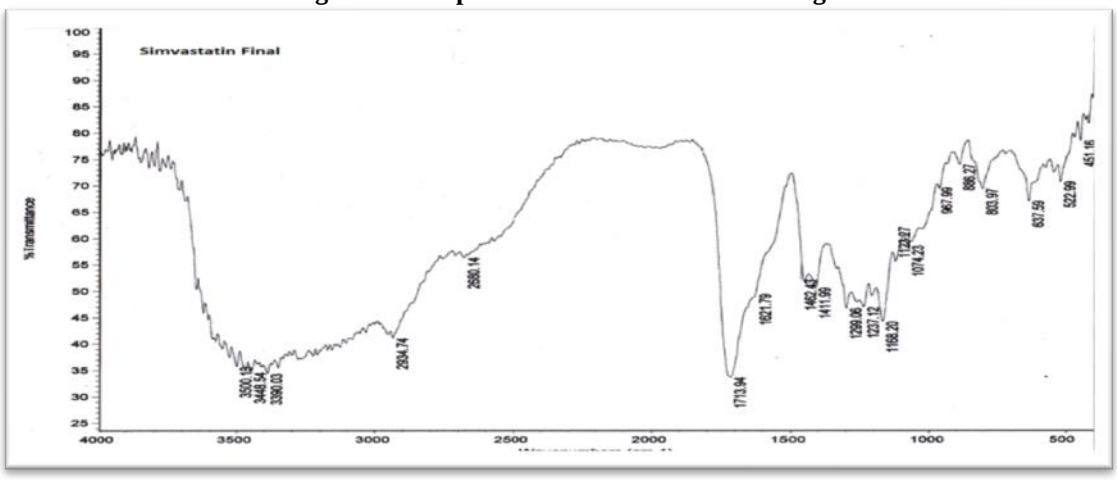

Fig 3: FTIR Spectra of Simvastatin Optimized Formulation

Evaluation of Ethosomal Gel

Table 7: Organoleptic Characteristics of Ethosomal Gel

Organoleptic Characteristics:	Color: golden yellow Greasiness: Non greasy Grittiness: Free from grittiness Ease of application: Easily/smoothly applied Skin irritation: No skin irritation
Washability:	Easily washable without leaving any residue on the surface of the skin.

Size and Shape Analysis

Microscopic analysis was performed under different magnification to visualize the vesicular structure, lamellarity and to determine the size of ethosomal preparations.

Scanning Electron Microscope (SEM)

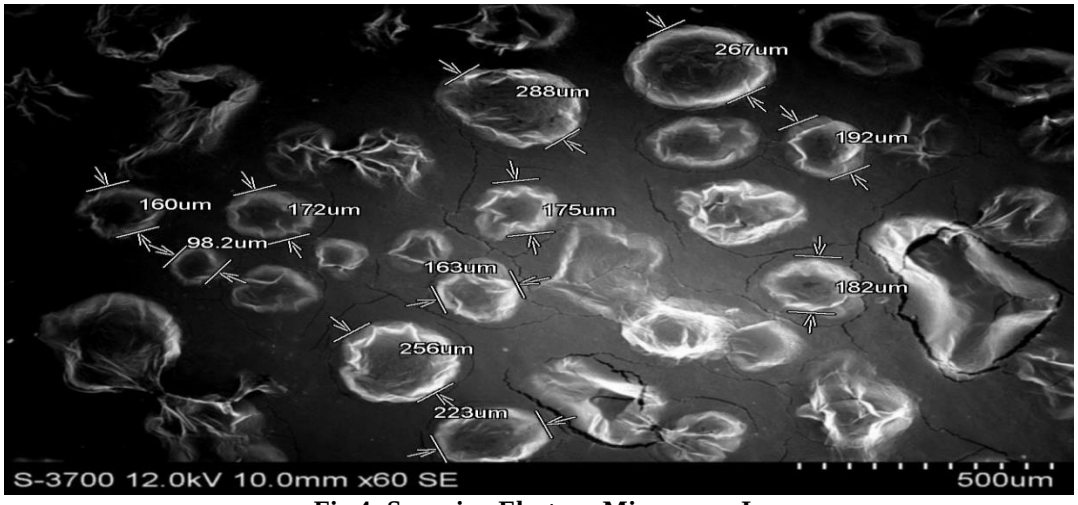

Fig 4: Scanning Electron Microscope Image

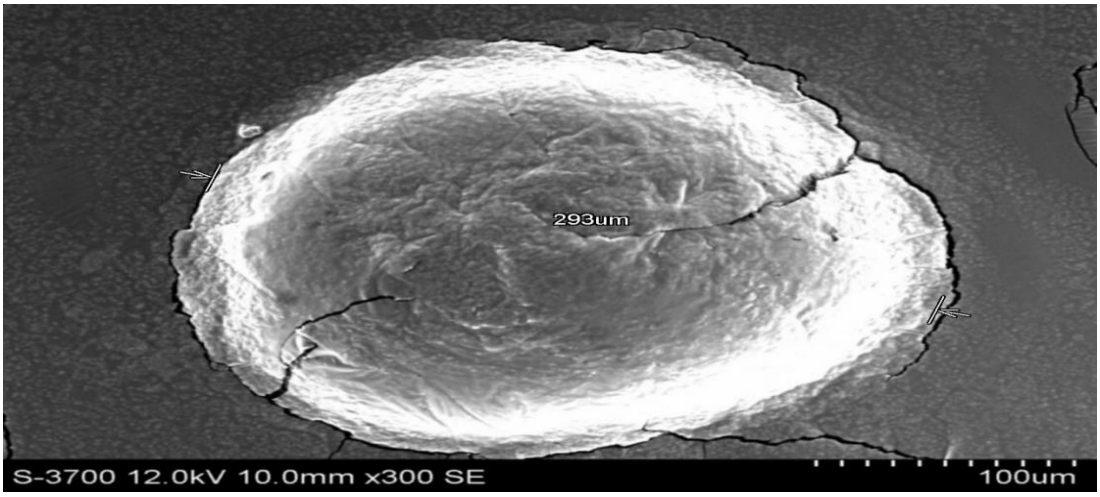

Fig 5: Scanning Electron Microscope Image

Entrapment Efficiency

Once the presence of bilayer vesicles was confirmed in the ethosomal system, the ability of vesicles for entrapment of drug was investigated by ultra centrifugation. Ultra-centrifugation was the method used to separate the ethosomal vesicles containing drug and un-entrapped or free drug, to find out the entrapment efficiency.

Table 8: Drug Entrapment Efficiency of Simvastatin Ethosomal Gel

Formulation code	Entrapment efficiency(%)
SF1	81.2
SF2	85.6
SF3	77.5
SF4	75.2
SF5	85.1
SF6	72.5
SF7	70.6

The maximum entrapment efficiency of ethosomal vesicles as determined by ultracentrifugation was 85.6% for ethosomal formulation containing 20% ethanol (SF2). As the ethanol concentration increased from 20% to 50% w/w, there was increase in the entrapment efficiency and with further increase in the ethanol concentration (>30% w/w) the vesicle membrane becomes more permeable that lead to decrease in the entrapment efficiency. Results of entrapment efficiency also suggest that 3% phospholipid is optimal concentration for entrapment efficiency and hence increased or decreased in concentration of phospholipid reduces the entrapment efficiency of vesicles.

Entrapment efficiency of ethosomal formulations are significantly different and are reported in Table.

Increase in entrapment efficiency may be due to the possible reduction in vesicle size. The detrimental effect on the vesicle during ultra-centrifugation which are larger in size. Sonication gives the more uniform lamellae, smaller vesicle and uniform size and hence it may be the reason for higher vesicular stability and lesser vesicular disruption during ultra centrifugation.

P^H measurements

The pH of organogels was measured by using electrode based digital pH meter.

The pH values for all formulations were in the range of 6.6 to 6.8

Table 9: Drug Content

Formulation code	Drug content(%)
SF1	98.6
SF2	99.3
SF3	98.6
SF4	98.3
SF5	95.3
SF6	95.6
SF7	99.7

***In-Vitro* Release Studies**

Table 10: *In-Vitro* Cumulative % Drug Release Profile for Ethosomal Gel

Time (min)	SF1	SF2	SF3	SF4	SF5	SF6	SF7
5	5.42	6.26	4.57	3.6	2.31	1.46	0.75
10	13.5	15.95	11.8	10.08	9.42	8.4	6.26
15	27.15	30	26.04	25.2	24.4	22.97	19.68
30	35.68	41.68	33.02	32.04	31.24	30.01	28.48
60	41.68	53.3	39.6	38.7	37.02	35.68	33.02
120	55.5	57.7	53.3	52.4	49.7	47.1	42.6
240	62.22	64.4	60.44	58.6	55.5	53.5	49.7
360	68.5	70.6	67.1	65.7	64.4	62.22	57.77
720	78.2	82.6	77.3	75.5	73.3	70.6	67.1
1440	88.4	93.7	89.3	87.11	78.2	75.5	72.44

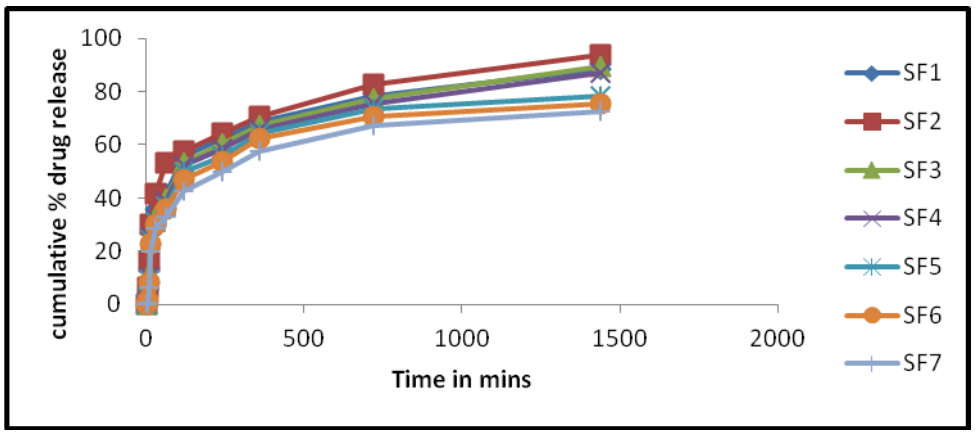

Fig6: Cumulative Drug Release of *In-Vitro* Studies

Kinetic Studies:

Table 11: Release Kinetics for Optimized Formulation

	Zero	First	Higuchi	Peppas
	% CDR Vs T	Log % Remain Vs T	%CDR Vs √T	Log C Vs Log T
Slope	0.053783679	-0.00123944	2.384253094	0.553650277
Intercept	32.25808743	1.889245875	18.16474588	0.501982141
Correlation	0.771969509	-0.97816641	0.909688584	0.903260348
R 2	0.595936923	0.956809534	0.827533319	0.815879257

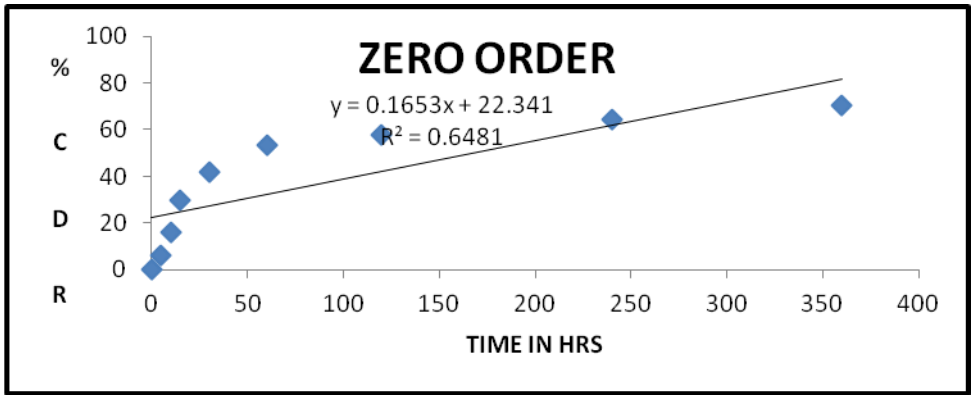

Fig 7: Zero Order Plot for Optimized Formulation

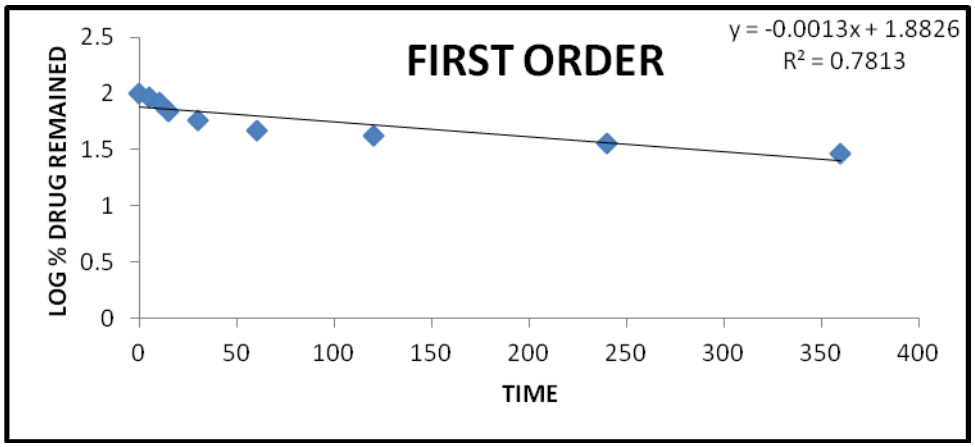

Fig 8: First Order Plot for Optimized Formulation

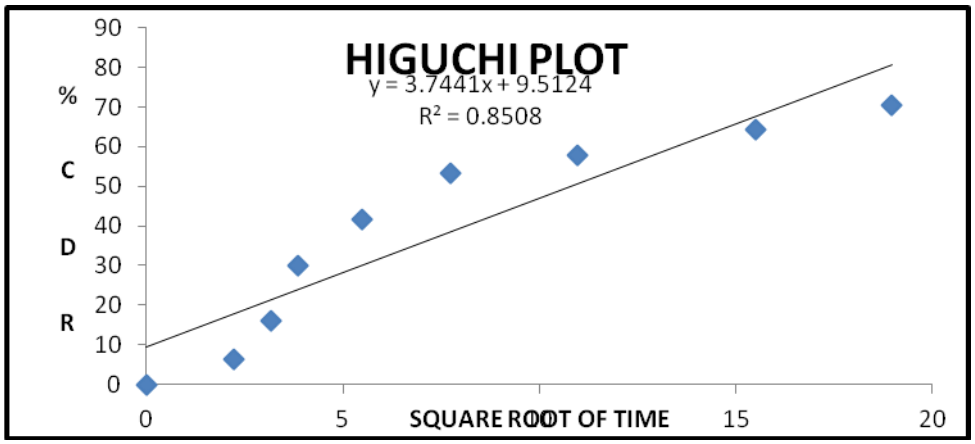

Fig 9: Higuchi Plot for Optimized Formulation

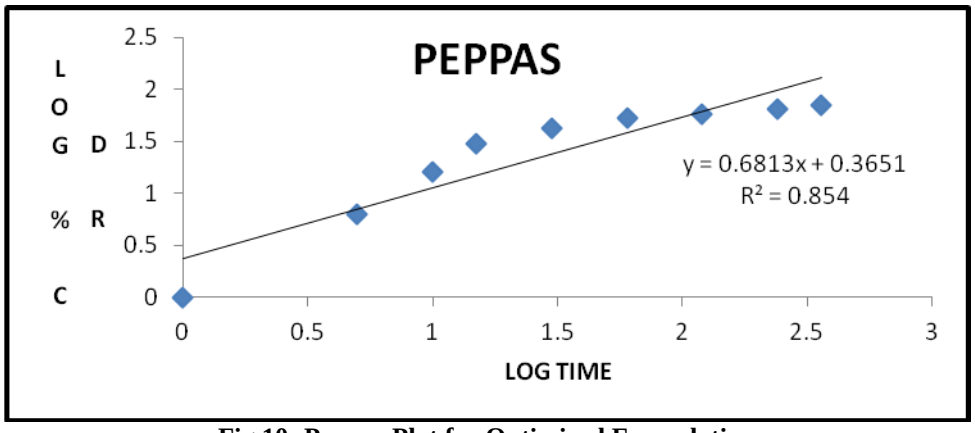

Fig 10: Peppas Plot for Optimized Formulation

Stability Study

Ethosomal formulations were observed for any change in appearance or color for a period of 8 weeks. There was no change in appearance in Ethosomal formulations throughout the period of study.

In-vitro Stability Release Study

Since the stability of drug and stability of vesicles are the major determinant for the stability of formulation, studies were carried to evaluate total drug content at room temperature (27±2° C) and refrigeration temperature (4±2° C). Stability study could not be carried out at higher temperature (>room temperature) because phospholipid was used as the component for ethosomes and gets deteriorated at higher temperature.

Loss in percentage of drug was not more than 4 percentage.Highest drug loss was observed at room temperature after 8 weeks as compared to refrigeration temperature. Results also showed that there was no significant change.

Loss in percentage of drug release during stability studies of the optimized batch (4±2 ° C & 27±2 ° C)

Table 12: Loss in percentage drug during stability studies

Formulation code (SF2)		Percentage of drug release
Initial	4±2 ° C	93.7
	27±2 ° C	93.7
After 2 weeks	4±2 ° C	93.7
	27±2 ° C	92.8
After 4 Weeks	4±2 ° C	92.9
	27±2 ° C	91.5
After 6weeks	4±2 ° C	92.2
	27±2 ° C	90.4
After 8 weeks	4±2 ° C	91.5
	27±2 ° C	90.2

The results of characterization are significant and encouraging, and these were discussed in light current concept. Summary of this work and some of important conclusions are discussed in the next chapters “Summary” and “Conclusion”.

CONCLUSION:

Ethosomes prepared by sonication method were more uniform and small in size which is essential for skin penetration. While comparing the entrapment efficiency, ethosomes containing 20% w/w ethanol and prepared by soncation showed highest value respect to all other formulation; so it is concluded ethosomal prepared by sonication and containing 20 % w/w ethanol as the best formulation considering all other aspects.

Stability studies carried out for a period of 8 weeks showed no changes in the characteristics of ethosomes and further the loss of drug is not more than 4 %. When effect of sonication was compared on ethosomal formulation, sonicated formulation possessed better or suitable characteristics (smaller size, uniform size, distribution, highest entrapment efficiency and higher transdermal flux).

From the above observations it can be concluded sonicatation is an essential tool for the preparation of ethosomes.

Thus, the specific objectives listed in the introduction chapter of this thesis were achieved namely design, characterization and release studies of Simvastatin ethosomes. Certainly these finding can be applied for transdermal drug delivery of Simvastatin. Further these findings may help the industry for development and scaling up a new formulation.

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