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Original Article

Formulation Development with Box-behnken design Study of Ondansetron HCl Ethosome for chemotherapy induced nausea vomitting

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ABSTRACT

Aim of this work was evaluation of Ethosome by box-behnken design study of Ondansetron HCl Ethosome for physicochemical characterization was carried out by scanning electron microscopy, zeta sizer, particle size analyser and freeze fracture performed.Ethosomes shown a higher % EE greater ability to deliver entrapped drug because of ethonol effect**.** The optimized batch of Ethosome has zeta potential of -24 mv, vesicle size was found to 242.3nm (PDI = 0.245).*In-vivo* relese study performed using Guinea pig skin through Franz's cells for (24 hr). Ethosome shows higher flux by Hyton and chains analysis. Gel prepared by using Carbopol 940 shows pesudoplastic flow. Ondansetron HCl undergoes first-pass metabolism,so its bioavailability may be improved when delivered through transdermal route. The 3-D response plots were constructed from linear model obtained from the regression analysis through Design Expert®.. ANOVA on the basis of p-value was found to be less than 0.05 at 95% Confidence limit by student-t test.

Keywords: vesicle, box-behnken design, ondansetron HCl, Ethosomes , permeation enhancer.

INTRODUCTION

Cancer Treatment suffer from side effects of nausea and vomiting ,To prevent or minimize these side effects of anticancer treatments with 5 HT-3 antagonist such as tropisetron, ondansetron, granisetron and dolasetron, known as serotonin receptor antagonists have been widely administered either parenteral or orally on a daily basis. The transdermal delivery of antiemetic drug is an interesting concept, and seems to be beneficial in a great many patients with chemotherapy induced nausea vomitting[1].

Ondansetron HCl is a potent and selective 5-hydroxytryptamine (5HT-3) receptor antagonist with antiemetic activity indicated for the prevention or treatment of nausea and vomiting associated with cytotoxic chemotherapy radiotherapy and postoperative nausea and vomiting. Ondansetron HCl is rapidly absorbed from the gastrointestinal tract and reaches maximum concentration in serum after approximately 1.6 hr. Ondansetron HCl undergoes first-pass metabolism so its bioavailability may be improved when delivered through transdermal route. So there is a need to develop a transdermal formulation of Ondansetron HCl which increases patient compliance and non-invasive .The word vesicle' having a biological origin actually means a bubble of liquid within a cell. Technically a vesicle is a small, intracellular, membrane-enclosed sac that stores or transports substances within a living cell[2] .Touitou (1998) discovered and investigated lipid vesicular systems embodying ethanol in relatively high concentration and named them Ethosomes. The basic difference between Liposomes and Ethosomes lies in their composition.The high concentration of ethanol (20-50%) in ethosomal formulation could disturb the skin lipid bilayer organization. Touitou (19980) discovered and investigated lipid vesicular systems embodying ethanol in relatively high concentration and named them Ethosomes. Ethanol acts as a penetration enhancer through the skin. The mechanism of its penetration enhancing effect is well-known. Ethanol penetrates into intercellular lipids and increases the fluidity of cell membrane lipids and decrease the density of lipid multilayer of cell membrane. Increased cell membrane lipid fluidity caused by the ethanol of Ethosomes results increased skin permeability. So the Ethosomes permeates very easily inside the deep skin layers, where it got fused with skin lipids and releases the

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drugs into deep layer of skin. Liposome have problem of drug leakage which is overcome by using Ethosome. Stability of vesicle is enhanced by use of the ethanol. Ondansetron HCl BCS -3 drug, so permeation of the vesicle containing Ondansetron HCl enhanced virtue of ethanol effect of ethanol and ethosomal effect of Ethosomes. Literature revels that no work is reported on ondansetron in gel based vesicular delivery of antiemetic drug is an interesting concept but its clinical use has found limited application due to remarkable barrier properties of the outermost layer of the skin.

Literature also suggests that Ethosomes has better stability than Liposomes, niosomes and more permeation than Liposomes and liquid drug solutions. First time attempt to develop liposomal and ethosomal TDDS for antiemetic drugs for the treatment of chemotherapy induced nausea and vomiting (CINV).Development of sustain release ethosomal and liposomal transdermal gel of ondansetron HCl. Attempt to increase permeation of ondansetron HCl through liposomal and ethosomal based system . Prolonged drug release Reduce dose frequency Improve patient compliance [3]. The animal experiment was approved by Institutional Animal Ethics Committee (IAEC) of Government College of Pharmacy, Aurangabad India (Ref. No. GCPA/IAEC/2012/555- Date: 4/6/2012) and carried out as per the guidelines of the committee.

MATERIAL AND METHODS

Ondansetron HCl from (Cipla Mumbai), Was a Kind Gift Soya Lecithin from **(**ResearchLab), Cholesterol from (Dipa Laboratory Chemicals), Carbopol 940 From (Noveon), Ethanol extra pure from (Loba Chemie) All Other Chemicals Was of Analytical grade. Animal guinea pig from Wockhardt .The animal experiments was approved by Institutional Animal Ethics Committee (IAEC) of Government College of Pharmacy, Aurangabad, India (Ref. No. GCPA/IAEC/2012/555- Date: 4/6/2012).

Preparation of Ethosome: Preliminary batches of the Ethosomes was prepared by hot methode using soya lecithin (10-40mg), ethanol (20-50%) and cholesterol (10 -40 mg) as independent variable. The optimized batch (E5) was selected by Box-Behnken optimization (Table1) and In this method phospholipid soya lecithin is dispersed in water by heating in a water bath at 40°C until a colloidal solution is obtained. In a separate vessel ethanol, propylene glycol 0.5ml and cholesterol are mixed and heated to 40°C. Once both mixtures reach 40°C the organic phase is added to the aqueous once. The drug initially dissolved in ethanol. The vesicle size of ethosomal formulation can be decreased to the desire extent using Sonication for 15 min. Then formulation is stored under refrigeration at 4°C for 24 hr[4].

Preparation of Ethosomal Gel: Ethosomal suspension containing Ondansetron HCl was incorporated into Carbopol 940 (20%w/w) as a gelling agent with constant stirring using a Teflon-coated magnetic bead, and the resulting mixture was then refrigerated at 4^0C for 24 hr to obtained a completely hydrated, homogeneous and clear solution[5]. After solution as removed from refrigerator placed at room temperature, until it forms a completely hydrated, homogeneous, and clear gel. (Table2)

Characterization of Vesicles

In-vivo Permeation Studies: The guinea pig skin was mounted on modified franz diffusion cell. In-vivo evaluation of permeation rates of preliminary and optimized ethosomal (E5) was performed respectively and drug solution containing same concentration of Ondansetron HCl through guinea pig skin was studied (As control)[20,21]. At the predetermined sampling intervals, aliquots of 1 ml was withdrawn periodically and replaced with the same volume of fresh receptor fluid (20%PEG).Skin Permeation was studied for 24 hr. Experiments for preliminary batches was performed in triplicate. Drug concentrations was measured by UV-Spectrophotometric method. After the experiment the skin was cut into pieces and kept in 20%v/v (PEG-400) in water for 24hr to determined skin drug content. Then solutionwas filtered and analyzed by RP-HPLC method[6]. From this amount of drug remain in skin was determined By Hyton and Chien Equation (table 1, 2 and Fig :11)

$$C_n^1 = Cn\ (Vt/Vt - Vs)(C_n^1 - 1/Cn - 1$$

Entrapment Efficiency Determination: Ethosomal Suspension prepared by hot methd Method was further optimized for the entrapment efficiency[7,8,9]. The prepared Ethosomes was kept overnight at 4^0C and ultra centrifuged (Megafuge 1.0 R, Heraeus, Hanau, Germany) for 5 hr at 14000 rpm. The free (unentrapped) Ondansetron HCl concentration was determined in the supernatant spectrophotometrically (Shimadzu UV–1601 PC Double Beam, Kyoto, Japan) at λmax 310.5nm. The Ondansetron HCl entrapment percentage was calculated from the following formula:

EE = [(Qt – Qs)/ Qt] * 100

Skin Irritation Studies: The skin irritation potential of the optimized batch(E5) of Ethosomes was investigated in guinea pigs[10]. Skin irritation following single application (single insult challenge) was assessed by a visual erythema scoring method (Fig1).

Vesicle Size and Size Distribution: Vesicle size of optimized (E5) batch of Ethosomes and was determined using zeta sizer (Beckmann coulter counter) (Fig2).

Microscopic Examination: MicroscopyOlympus® Cx31 Equipped With Magnus Pro.V.3.0. Software of the Optimized E5 Formulations of Ethosomes Revealed the Presence of Ethosomal vesicle.

Zeta Potential Analysis: Zeta potential of optimized (E5) batch of Ethosomes was determined using zeta sizer (Beckmann coulter counter) (Fig4). which was initially calibrated according to Beckmann instrument specification[11,12].

Surface Morphology Evaluation: Scanning electron microscopy (Jsm-760 0f Philips) Photomicrographs of optimized (E5) batch of Ethosomes vesicle was taken using a scanning electron microscope to study the surface morphology of Vesicle (Fig5).

Evaluation of Ethosomal Gel Formulations: The gel formulation containing Ondansetron HCl was evaluated for pH, viscosity, consistency and clarity, drug content uniformity, histopathology (Table3) (Fig8).

Freeze Fracture Analysis: Vesicle characterization, the samples, after centrifugation for 30 min at room temperature (Microcentrifuge Ole Dich. NCL Pune),was examined by means of the freeze fracture microscopy technique: samples were impregnated with 30% glycerol and then frozen in partially solidified Freon 22, freeze fractured in a freeze fracture device (_105 8C, 10–6 mm Hg) and replicated by evaporation from a platinum/carbon gun[13]. The replicas were extensively washed with distilled water, picked up onto Formvar-coated grids and examined with a Philips CM 10 transmission electron microscope.(Fig9) .

Statistical Analysis Optimization: E5 batch showing max entrapment 74%, flux 27.52(μg/cm^2/hr), permeability coefficient 2.4(cm/hr) was selected for further studies by box-behnken optimization cube plot. ANOVA on the basis of p-value was found to be less than 0.05 at 95% Confidence limit by student-t test. (Fig 5-10.)Data analysis of factorial batches with statistical software has been very popular, especially for a small number of factors. For t = 3 factors, the Box-Behnken (BB) design requires only 12 runs, plus a recommended n = 3 center point. Box-Behnken (BB) was used for the study and 3 factors were evaluated variables of study were formula percentage of Ethanol, soya lecithin and Cholesterol. The dependent parameter was drug entrapment, flux and permeation. Experimental design can be defined as the strategy for setting up experiments in such a manner that the information required is obtained as efficiently and precisely as possible. The factorial experimental designs are suitable over traditional optimization in terms of minimum number of experiments and ease in evaluation of statistical significance of independent factors on dependent variables. The factorial design requires lesser efforts than that of traditional optimization methods[14,15].

The factorial design can serve as an essential tool to understand the complexity of mechanisms of pharmaceutical formulations. The polynomial equations are used to evaluate the statistical significance of the obtained responses. An asymmetrical general factorial experimental design was used for study. The ethanol, soya lecithin and cholesterol were independent variables and entrapment (%) flux (ug/cm2/hr) and permeability coefficient ug/hr were responses of the study.

Y=b0+b1 X1+b2 X2+b12 X1X2+b11X12+b22X22

Where, Y is the dependent variable, isb_0s the arithmetic mean response of the twelve runs and bi(b1,b2,,b12,b11 and b22)is the estimated coefficient for corresponding factor Xi (X1,X2,X12,X11,and X22), which represents the average results of changing one factor at a time from its low to high value. The interaction term (X1X2) depicts the changes in the response when two factors are simultaneously changed. The polynomial terms (X12 and X22) are included to investigate nonlinearity[19,20]

The final equations in terms of coded values of factors and actual values of factor obtained from ,
Final Equations for % entrapment in Terms of Coded Factors:
Entrapment =+42.38+0.45 * A[1]+1.44 * A[2]+6.15 * A[3]-6.32 * B-2.86 *C+6.35 * A[1]B-4.50 * A[2]B-1.02* A[3]B-1.54* A[1]C-3.41 * A[2]C+4.08* A[3]C-3.32 * BC
(r2 =0.0277015)

Final Equations for flux in Terms of Coded Factors:
Flux =+14.54-9.05 * A[1]-4.51 * A[2]+17.23 * A[3]-4.76 * B+2.41* C+5.90* A[1]B

+8.15 * A[2]B-18.08* A[3]B-1.05 * A[1]C-0.84* A[2]C+2.92 * A[3]C+0.23* BC
(r2 =0.024947)

The percent entrapment and flux of factorial batches is shown in the Tables 14. The summary of response as reported earlier reveals dependence of flux on the concentration ethanol and soya lecithin while cholesterol, another independent variable was found to have overall no significant effect on the entrapment and flux. The effects of variables can be studied by the above equations. The regression coefficient values are used to validate the model fitting [22-23]. The regression coefficient was high indicating the adequate fitting of the quadratic model for response entrapment and flux. The polynomial equations can also be used to draw conclusions considering the magnitude of co-efficient and mathematical sign it carries; i.e. positive or negative. If the terms in the equation are positive it contribute positively to the response similarly if the terms is negative it contribute negatively to the response. In the present study positive coefficient of independent factor ethanol showed that it contributes positively to entrapment and flux and leads to enhancement of the drug release at all response points and it is a significant variable in the drug release. However, the negative coefficient of soya lecithin of equations of flux and entrapment indicates significance of independent variable. The analysis of variance study of the data also showed same results revealing the ethanol as significant variable (P value <0.05) at flux, permeability coefficient. As response point and insignificant at entrapment response, while the cholesterol, soya lecithin was insignificant variable at all response point. It again indicates the significance of ethanol in the drug release from the developed formulation of ethosomes.

The 3-D response plots were constructed from linear model obtained from the regression analysis through Design Expert® in which the responses were represented by bars as a function of independent variables as shown in the Figures9 to 12. The relationship between the response and independent variables can be directly visualized from the response plots. The response and interaction plots used to observe the response's dependence on the input variables to predict this response over the whole of the domain, and possibly also at its periphery.[16,17,18]

RESULTS

Size of optimized E5 batch of Ethosomes was found in nano range (242.3 nm). It was observed that increase in concentration of ethanol reduces vesicle size which leads to enhancement in permeation and flux. Polydispersity index of 0.245 indicates that vesicles are monodispersed with distribution of vesicles from 242.3 nm to 349.20 nm range. Vesicle size of optimized Microscopic examination of the optimized Ethosomesbatch formulations revealed the presence of vesicles. Zeta potential of optimized Ethosomes E5batch % entrapment efficiency of optimized was found to be 74%. Flux of optimized Ethosomes E5batch was found to be 27.52 (µg/cm^2/hr). Permeability coefficient of optimized Ethosomes E5 batch and flux of was found to be 2.4 (cm/hr) respectively. Surface morphology analysis performed by using scanning electron microscopy.skin sensitivity test shows no erythema for optimized E5 so suitable for transdermal application.ethosome as better drug delivery for Ondansetron HCl.

DISCUSSION

In the present study an attempt was made to formulate, optimise and develop ethosomal system of Ondansetron HCl with the aim to have rapid onset of action which will last for prolonged period of time. In preliminary trial batches for preliminary studies two levels of ethanol, two levels of cholesterol and two level of soya lecithin was selected based on entrapment efficiency. Box-behnken design was used for further optimization containing twelve batches. Three independent variables ethanol, soya lecithin and cholesterol was used against entrapment efficiency and flux as dependent variables sows significant effect on formulation. E5 batch showing max entrapment 74%, flux 27.52 (µg/cm^2/hr), permeability coefficient 2.4(cm/hr) was selected for further studies. Ethosomal gel containing ethosomal suspension (E5) and Carbopol 940 as gelling agent (20 % w/w) was prepared and compared with plain gel containing same drug concentration for in vitro drug release. Comparative *In-vitro* drug release study of plain drug solution, drug in ethosomal suspension form, gel with plain drug and gel with ethosomal suspension was carried out for 24 hours. It was found that cumulative release and flux of ethosomal suspension was more than drug solution containing same drug concentration (less Lag time) and ethosomal gel showed enhanced permeation as compared to plain gel. prepared gels was evaluated for pH, viscosity, consistency and uniformity of content.

CONCLUSION

Entrapment increases with increase in concentration of ethanol ethosomal gel shows increased in permeation than plain gel containing same drug concentration. Finally, it can be concluded that Ondansetron HCl can be successfully formulated in gel based ethosomal TDDS which can be used to achieve faster onset of action and the formulation can still prolong the drug delivery for more than 24 hours. However it requires further study on human cadaver skin, in vivo study and establishment if *In-vitro* relations

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Table 1: Ethosomal Box-behnken design

Batch Code	ONDA HCl (mg)	Ethanol(ml)	Soya lecithin (mg)	Propylene glycol (ml)	Cholesterol (mg)	Entrapment (%)	Flux (ug/cm2/hr)	Permeability coefficient($x10^{-3}$) (cm/h)
E1	8	3.50	10.00	0.5	10.00	49	1.5	0.014
E2	8	4.00	10.00	0.5	15.00	51	3.82	0.33
E3	8	3.00	10.00	0.5	15.00	41	0.6608	0.0539
E4	8	4.00	15.00	0.5	20.00	44	6.36	0.0564
E5	8	4.00	15.00	0.5	10.00	74	27.52	2.4
E6	8	3.50	20.00	0.5	20.00	47	3.3	0.0294
E7	8	3.50	20.00	0.5	10.00	38	26.93	2.3
E8	8	3.00	15.00	0.5	20.00	42	8.264	0.0733
E9	8	4.00	20.00	0.5	15.00	56	9.1	0.015
E10	8	3.00	20.00	0.5	15.00	50	10.72	0.957
E11	8	3.00	15.00	0.5	10.00	43	12.31	1.093
E12	8	3.50	10.00	0.5	20.00	61	10.71	0.951

*Box-behnken design

Table2: Ethosomal gel composition

Composition	Ingredients	Quantity
Plain Ondansetron HCl	ONDA HCl	8 (w/w %)
	Carbopol 940	20 (w/w %)
	Water	80 (V/V%)
Ethosomal Gel	Ethosomal suspension (E5)	80 (V/V%)
	Carbopol 940	20 (w/w %)

Table 3: Ethosomal gel evaluation

Formulation code (E5)	pH	Viscosity (cp)	Clarity	Content Uniformity
Ethosomal gel	5.20	15± 0.26	++	98.01%

*****+ = Poor ++ = Good +++ = Excellent

Table 4: Ethosome parameter evaluation

Sr.no	Parameter	Ethosomes
1	% EE (%)	74
2	Particle size (nm)	242.3
3	Flux(µg/cm^2/hr)	27.52
4	Permeability cefficient(cm/hr)	2.4
4	P.D	0.245
5	Zeta potential	-14.12

Table 5: Factorial Batches for Ethosome by Box-behnken design

Formulation code	***ONDA HCl (mg)***	***Ethanol(ml)***	***Soya lecithin (mg)***	***Propylene glycol (ml)***	***Cholesterol (mg)***
E1	*8*	*3.50*	*10.00*	*0.5*	*10.00*
E2	*8*	*4.00*	*10.00*	*0.5*	*15.00*
E3	*8*	*3.00*	*10.00*	*0.5*	*15.00*
E4	*8*	*4.00*	*15.00*	*0.5*	*20.00*
E5	*8*	*4.00*	*15.00*	*0.5*	*10.00*
E6	*8*	*3.50*	*20.00*	*0.5*	*20.00*
E7	*8*	*3.50*	*20.00*	*0.5*	*10.00*
E8	*8*	*3.00*	*15.00*	*0.5*	*20.00*
E9	*8*	*4.00*	*20.00*	*0.5*	*15.00*
E10	*8*	*3.00*	*20.00*	*0.5*	*15.00*
E11	*8*	*3.00*	*15.00*	*0.5*	*10.00*
E12	*8*	*3.50*	*10.00*	*0.5*	*20.00*

Table6:Summary of Statistical Design

Factor	***Names***	***Units***	***Type***	***Subtype***	***Min***	***Max***	***Cod***	***Values***	***Std.***
A	***SOYA LECITHIN***	***mg***	***Numeri***	***Contin***	***10.00***	***20.00***	***1.000=10.00***	***1.000=20.00***	***4.08***
B	***CHOLEST EROL***	***mg***	***Numeri***	***Contin***	***10.00***	***20.00***	***1.000=10.00***	***1.000=20.00***	***4.08***
C	***ETHANOL***	***ml***	***Numeri***	***Contin***	***3.00***	***4.00***	***1.000=3.00***	***1.000=4.00***	***0.41***

Table7:Summary of Responses

Response	***Description***	***Units***	***Obs.***	***Analysis***	***Min***	***Max***	***Mean***
Y1	***Entrapment***	***%***	***12***	***Polynomial***	***Min***	***Max***	***Mean***
Y2	***Flux***	***(ug/cm^2/hr)***	***12***	***Polynomial***	***38***	***74***	***49.6667***
Y3	***Permeation coefficient***	***cm/hr***	***12***	***Polynomial***	***0.6608***	***27.52***	***10.0996***

Table 8 : % EE ANOVA for Response Surface Linear Model

Sum of Source	***Square***	***Mean Df***	***F Square***	***P-Value***	***Prob> F***	
Model	*327.75*	*3*	*109.25*	*1.11*	*0.234*	***Significant***
A-SOYA LECITHIN	*15.13*	*1*	*15.13*	*0.15*	*0.188*	
B-CHOLESTEROL	*12.50*	*1*	*12.50*	*0.13*	*0.232*	
C-ETHANOL	*300.13*	*1*	*300.13*	*3.04*	*0.199*	

Table9:Flux ANOVA for Response Surface Linear Model

Sum of Source	***Squares***	***Mean Df***	***F Square***	***P-Value***	***Prob> F***	
Model	*711.48*	*6*	*118.58*	*4.01*	*0.0746*	***significant***
A-SOYA LECITHIN	*139.10*	*1*	*139.10*	*4.70*	*0.0823*	
B-CHOLESTEROL	*196.28*	*1*	*196.28*	*6.63*	*0.0497*	
C-ETHANOL	*27.55*	*1*	*27.55*	*0.93*	*0.0789*	

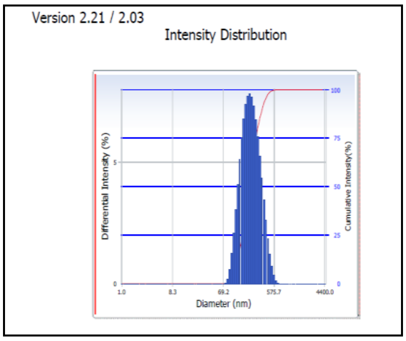
Table 10: Permeation Coefficient ANOVA for Response Surface Linear Model

Sum of Source	***Squares***	***Mean df***	***F Square***	***P-Value***	***Prob> F***	
Model	*6.66*	*6*	*1.11*	*3.05*	*0.01206*	***significant***
A-SOYA LECITHIN	*0.48*	*1*	*0.48*	*1.31*	*0.03042*	
B-CHOLESTEROL	*2.76*	*1*	*2.76*	*7.58*	*0.0402*	
C-ETHANOL	*0.049*	*1*	*0.049*	*0.13*	*0.01294*	

Fig 1: Skin Irritation Study

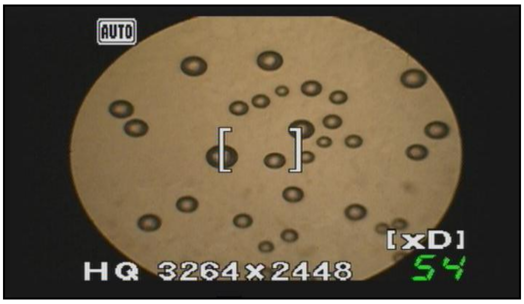
Visual observation	Erythema score	Conclusion	Suitability
 For Ethosome	0	No erythema	Suitable

Fig 2: Vesicle Size of Ethosome E5 batch



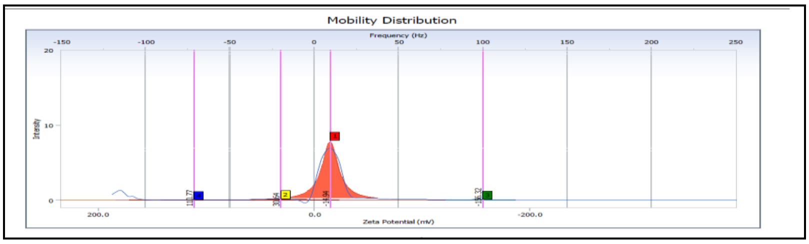
* E5 batch

Fig 3: Microscopic Examination of Ethosome E5



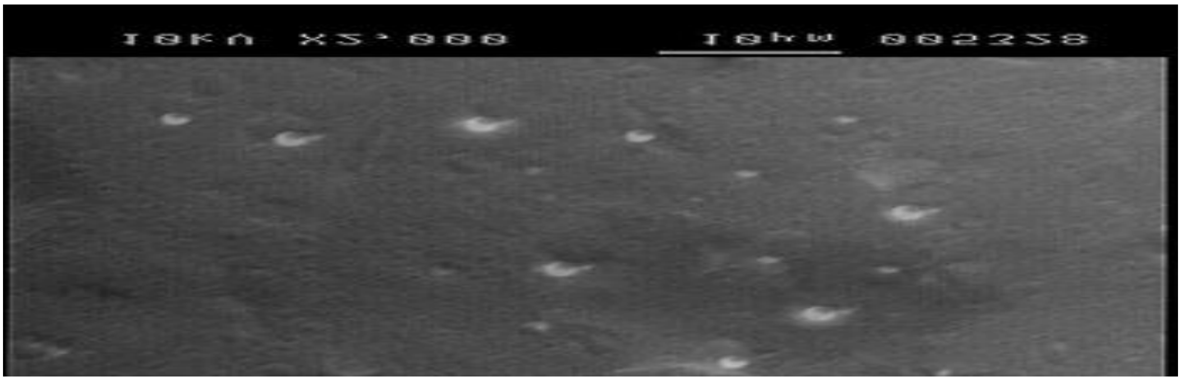
* Ethosome E5

Fig 4: Zeta Potential Analysis of Ethosome E5



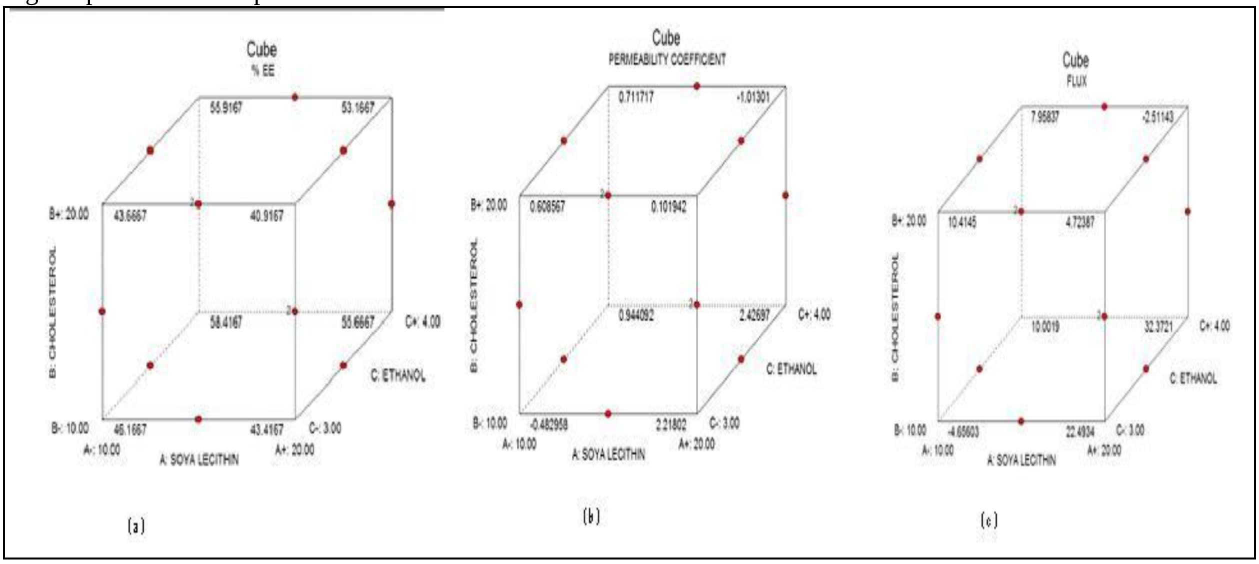
* Ethosome E5

Fig 5: Surface Morphology Study of Ethosome E5

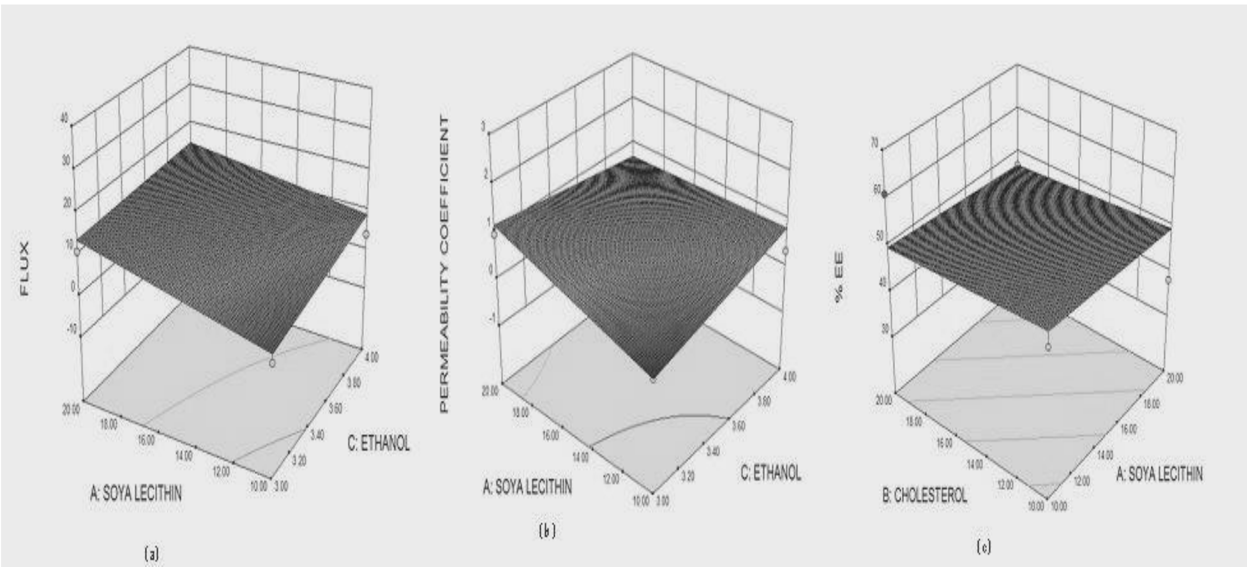


* Ethosome E5

Fig 6: optimization Graphs for Ethosomes E5

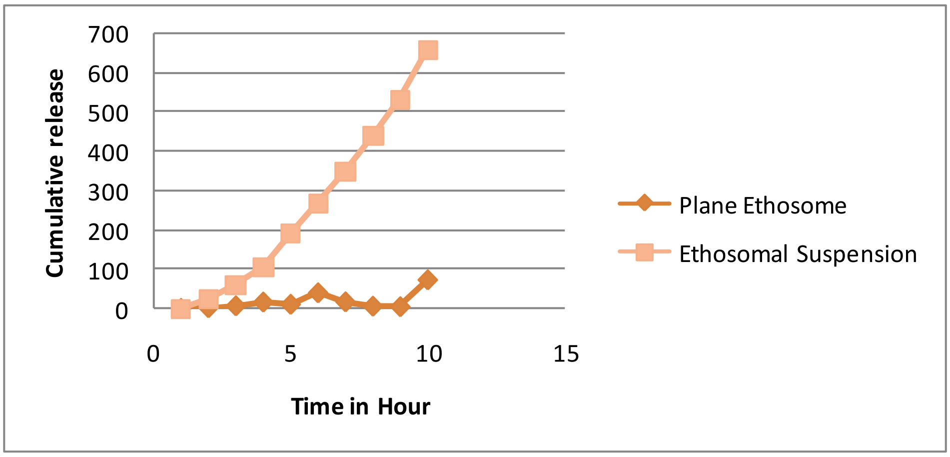


*cube plot Ethosomes E5



* response surface plot for Ethosomes E5

Fig 7:cumulative release for Ethosome E5



*   Ethosome E5

Fig8: Histopathology of Guinea pig skin with E5 Ethosome

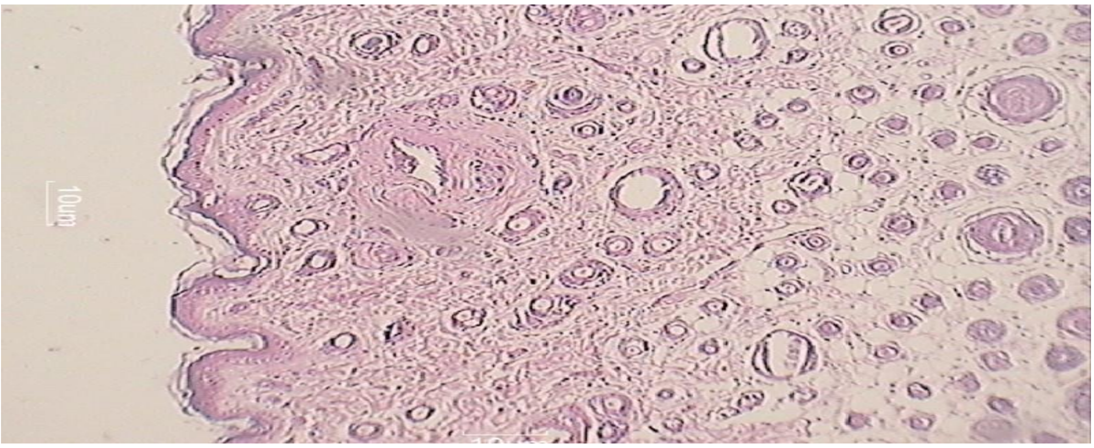


Fig9: Freeze Fracture of   E5 Ethosome

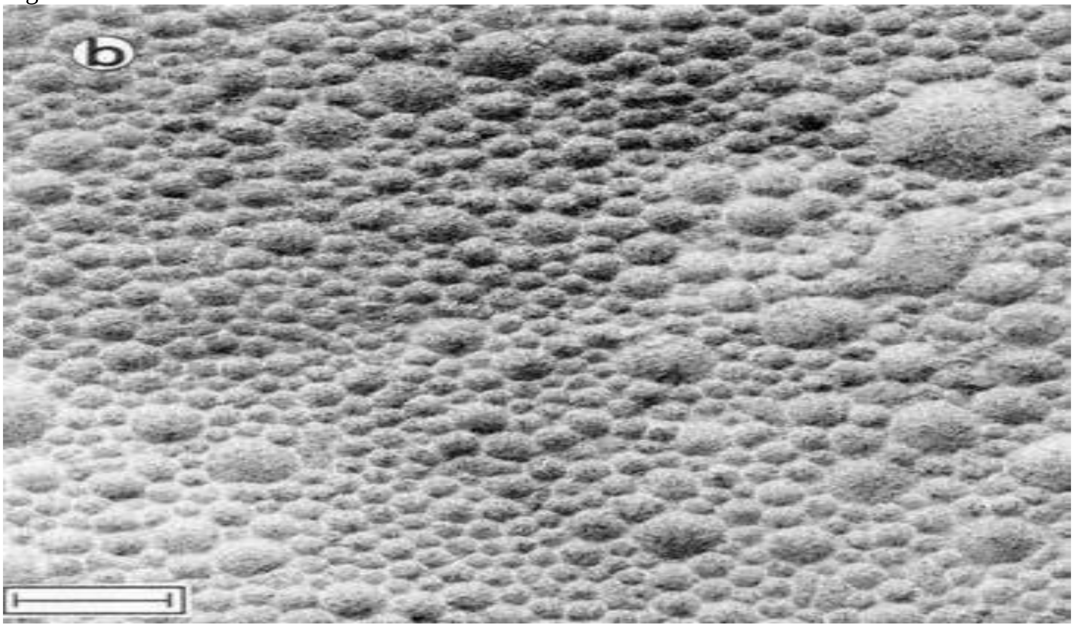
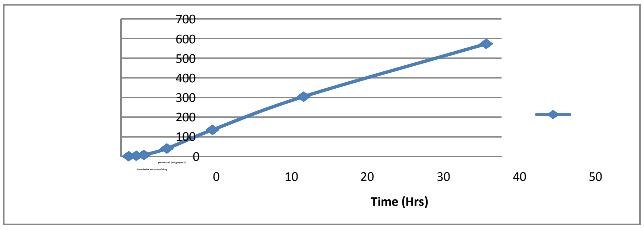
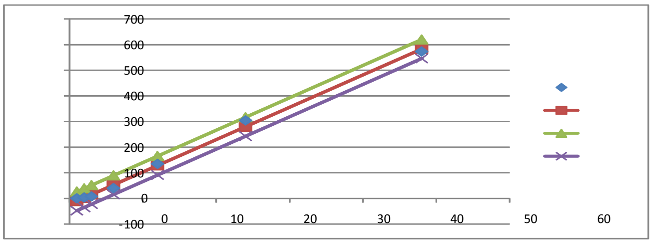


Fig:10 Spreadsheet template for quantification and non-linear regression analysis of data generated from *in vitro* skin permeation study.

	A		B	C	D	E	F	G
1			**In-vitro permeability study**					
2	Sample name		ONDA in NMP 20 mg/ml			Date	07-07-213	
3	Diffusion cell no.			4				
4	Diffusion cell volume (ml)	*Vr*		14				
5	Surface area exposed (cm^2)	*A*		6.15				
6	donor cell drug conc	*Cd*		20000				
7	Sampling volume	*Vs*		1				
8	**Sampling hr**		**Measured conc in receptor medium**	**Actual drug conc in receptor cell**	**Drug loss in sampling**	**Sum total of previous measured samples**	**Cumulaitve amt of drug permeated**	**Cumulaitve amt of drug permeated per unit area**
9	***t***		***Cn***	***Vr.Cn***	***Vs.Cn***	***Vs .∑ Cm***	***Q***	***Q/cm^2***
10	1		0.2762	3.8668	0.2762	0.2762	3.8668	0.6287
11	2		1.3694	19.1716	1.3694	1.6456	20.8172	3.3849
12	3		3.0482	42.6748	3.0482	4.6938	47.3686	7.7022
13	6		16.211	226.9540	16.2110	20.9048	247.8588	40.3022
14	12		54.0327	756.4578	54.0327	74.9375	831.3953	135.1862
15	24		119.7569	1676.5966	119.7569	194.6944	1871.2910	304.2750
16	48		222.2222	3111.1108	222.2222	416.9166	3528.0274	573.6630
17–30								
31	Slope (Permeation flux) (micgm/cm2/hr)	**J**	12.56075	Diffusion parameter **D'**	0.0278			
32	Intercept (Lag time)	**L *t***	6.00000	Partition Parameter **K'**	0.0226			
33	Permeability coefficient	**P**	0.00063					
34								
35			**NONLINEAR REGRESSION ANALYSIS**					
36	**Time**		**Amt permeated/ unit area**	**Model equation**	**UpperCI**	**Lower CI**	**D'**	0.0918
37	1		0.6287	-10.3054	26.9268	-47.5376	**K'**	0.0011
38	2		3.3849	2.3156	39.5478	-34.9166	**Mean_of_y**	152.1632
39	3		7.7022	14.9366	52.1688	-22.2956	**df**	5.0000
40	6		40.3022	52.7996	90.0318	15.5674	**SE of y**	14.4840
41	12		135.1862	128.5256	165.7579	91.2934	**R^2**	**0.9962**
42	24		304.2750	279.9776	317.2099	242.7454	**Critical t**	2.5706
43	48		573.6630	582.8817	620.1139	545.6494	**CI**	37.2322
44–57								






```
cell C10 formula =   $C$4*B10                                      Copy to cell C16
cell D10 formula =   $C$7*B10                                      Copy to cell D16
cell E10 formula =   D10
cell E11 formula =   D11+E10                                       Copy to cell E16
cell F10 formula =   C10
cell F11 formula =   C11+E11                                       Copy to cell F16
cell G10 formula =   F10/$C$5                                      Copy to cell G16
cell B31 formula =   SLOPE(Linear portion of graph)
cell B32 formula =   INTERCEPT(Linear portion of graph)
cell D31 formula =   (1/(6*$B$32))
cell B33 formula =   ($B$31/$C$6)
cell D32 formula =   ($B$33/$D$31)
cell C37 formula =   ($C$5*$G$37*$C$6)*(($G$36*A37)-(1/6))         Copy to cell C43
cell G38 formula =   AVERAGE(B37:B43)
cell G39 formula =   COUNT(B37:B43)-COUNT(G36:G37)
cell G40 formula =   SQRT(SUM((B37:B43-C37:C43)^2/$G$39))           Press Ctrl+Shift+Enter
cell G41 formula =   1-SUM((B37:B43-C37:C43)^2)/SUM((B37:B43-$G$38)^2   Press Ctrl+Shift+Enter
cell G42 formula =   TINV(0.05,$G$39)
cell G43 formula =   G42*G40
cell D37 formula =   C37+$G$43                                     Copy to cell D43
cell E37 formula =   C37-$G$43                                     Copy to cell E43
```

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