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FORMULATION AND EVALUATION OF NIOSOMES OF MIRTAZAPINE FOR NASAL DELIVERY

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ABSTRACT

Mirtazapine is a tetracyclic anti-depressant drug approved by USFDA to treat depression. It is a BCS class II drug with a low oral bioavailability of 50% due to first pass metabolism. Thus, the purpose of this research work was to enhance the bioavailability of the drug by enhancing its solubility by incorporating it in to niosomal vesicles loaded in to an in-situ gel to enhance its permeability across the biological membrane. Niosomes were prepared by thin film hydration method and optimized by using 3^2 full factorial design. Mirtazapine loaded niosomes were further incorporated in to an Poloxamer 407 and Carbopol 934 in-situ gel base. The vesicle size of all niosomal suspension batches ranged between 202-245 nm. The vesicle size of the optimized batch F5 was found to be 211.7 nm with PDI of 0.166. The zeta potential value of F5 was found to be 0.6 mV. The % EE of all niosomal batches was found to be in a range of 69.3% – 83.7% and the cumulative % release was found to be in a range of 75.2% – 84%. DSC, XRD studies were performed for pure drug and niosomal batch F5. All the gel formulations ranged between 17.3±0.03 sec to 27.3±0.03 sec. Gelling temperature was found to be in a range of 44^{o}C±0.00 to 53^{o}C±0.0^{o}C and mucoadhesive results were found to be in a range of 10.3 ±0.023 to 14.5±0.060g. In-vitro drug release was found to be in a range of 68.3%-74.6%.

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INTRODUCTION

The aim of this research work was to enhance the bioavailability of the drug by enhancing its solubility by incorporating it in to niosomal vesicles loaded in to an in-situ gel to enhance its permeability across the biological membrane. Depression (according to WHO) is a common mental disorder, characterized by sadness, loss of interest or pleasure, feelings of guilt or low self-worth, disturbed sleep or appetite, feelings of tiredness and poor concentration. It can be long lasting or recurrent, substantially impairing a person's ability to function at work or school, or cope with daily life. At its most severe, depression can lead to suicide. When mild, depression can be treated without medicines but, when moderate or severe, people may need medication and professional talking treatments. Non-specialists can reliably diagnose and treat depression as part of primary health care. Specialist care is needed for a small proportion of people with complicated depression or those who do not respond to first-line treatments. Depression often starts at a young age. It affects women more often than men, and unemployed people are also at high risk.[1] Depression has been linked to problems or imbalances in the brain with regard to the neurotransmitter's serotonin, norepinephrine, and dopamine. The evidence says it is very difficult to actually measure the level of neurotransmitter in a person's brain.[2]

In October 2016, the National Institute of Mental Health and Neurosciences (NIMHANS) in Bengaluru released a mental health survey that said that the incidence of depression is roughly one in every 20 Indians or 5% of the population.[3]

Neurotransmitters are chemical messengers in the brain that are the means by which nerve cells communicate with each other. The illustration in figure 1.1, depicts the junction between two nerve cells. Packets of neurotransmitter molecules are released from the end of the presynaptic cell (the axon) into the space between the two nerve cells (the synapse). These molecules may then be taken up by receptors (such as serotonin receptors) of the postsynaptic nerve cell (the dendrite) and thus pass along their chemical message. Excess molecules are taken back up by the presynaptic cell and reprocessed. There are three neurotransmitters, known chemically as monoamines they are, serotonin, dopamine and norepinephrine. Decreased levels of these neurotransmitters contribute to depression. Several things might potentially go wrong with this process and lead to neurotransmitter deficiency. Some of these possibilities include:

- Not enough of the neurotransmitter (for example, serotonin) is produced
- Not enough receptor sites to receive the neurotransmitter.
- The neurotransmitter is being taken back up too quickly (into the presynaptic) before it can reach receptor sites.
- Chemical precursors (molecules from which neurotransmitters are built) may be in short supply.
- Molecules that facilitate the production of neurotransmitters, such as specific enzymes, may be in short supply.[4]

Depression is a common mental disorder, characterized by sadness, loss of interest or pleasure, feelings of guilt or low self-worth, disturbed sleep or appetite, feelings of tiredness and poor concentration which is caused due to imbalance in the release of neurotransmitters responsible for transmittance of chemical messages. Mirtazapine was selected, as it possess anti-depressant properties and as it is the only tetracyclic antidepressant approved by USFDA to treat depression. As Mirtazapine has low solubility and low oral bioavailability of 50% due to first pass metabolism, studies were carried out to formulate Mirtazapine in to niosomes to enhance solubility and niosomes were incorporated in to an In-situ gel base to enhance permeability across biological membrane, and deliver the drug directly to the brain by avoiding first pass metabolism through nasal route.[5]

In the present investigation, Mirtazapine was loaded in to niosomes which was further incorporated in to an in-situ gel base that shall gel at nasal temperature. The aim of the study was to enhance the bioavailability of the drug bypassing first pass metabolism by delivering via nasal mucosa directly to the brain.

MATERIALS AND METHODS

Materials

Mirtazapine was purchased from Micro labs, Bangalore, India. Cholesterol (Hi-media Ltd., Mumbai), Span 60 (SD Fine Chemicals Ltd, Mumbai), Carbopol 934 (SD Fine Chemicals Ltd, Mumbai), Poloxamer 407 (SD Fine Chemicals Ltd., Mumbai). All other chemicals were obtained from SD Fine Chemicals Ltd., Mumbai.

Methods

Preparation of Niosomes by thin film hydration method

Mirtazapine loaded Niosomes were prepared by thin film hydration method. Based on the statistical approach which generated 9 different formulae for different concentrations of surfactant (span 60) and cholesterol. These concentrations of span 60 and cholesterol were dissolved in 10 mL of Di-ethyl ether in a rotary flask. This mixture was subjected to formation of thin film on the rotary flask at 60^{o}C. Traces of organic solvent were removed by subjecting the rotary flask to vacuum at 50^{o}C for 15minutes. The thin film formed was then rehydrated with phosphate buffer pH- 6.4 loaded with 10mg of Mirtazapine dissolved in 3 mL of ethanol for 15 minutes. Resultant solution was Ultra-sonicated for 10minutes to form Niosomes.

EXPERIMENTAL DESIGN

Optimization studies for the given procedure was performed by using 3^2 full factorial design (Design Expert Software Trial version 11.0), 9 runs were generated. Concentration of span 60 (X_1) and cholesterol (X_2) was selected as independent variables. Particle size (Y_1), % Entrapment Efficiency (Y_2), and PDI (Y_3) were selected as dependent variables. It signifies how the responses change when the two factors are changed concurrently.

Generation of polynomial equations

Various response surface methodology (RSM) computations for the current optimization study were performed employing Design Expert software. Polynomial models including interaction were generated for all the response variables using multiple linear regression analysis (MLRA) approach.

Statistical analysis of data

The effect of independent variables up on the responses was checked by using statistical tools, that is ANOVA by using Design expert software (version 11.0). A value of $p < 0.05$ was considered statistically significant.

Generation of 3D response surface plots

For the measured responses, three-dimensional plots were generated to determine the change of the response surface. These types of plots are beneficial in the study of the effect of two factors on the response at one time. It signifies the effect of independent variables on the responses by graphical presentation.

CHARACTERIZATION OF DRUG LOADED NIOSOMAL SUSPENSION

Determination of particle size and PDI.

Particle size and PDI for all the proposed formulations was analyzed by Malvern particle size analyzer.

Entrapment Efficiency

Percent Entrapment Efficiency (% EE) of the niosomal suspension was determined by using centrifugation method. 5 mL of freshly prepared niosomal suspension was taken in centrifuge tube and centrifuged (REMI Instruments) at 9000 rpm for 45 minutes. 1 mL of supernatant containing unentrapped drug was withdrawn and diluted with phosphate buffer pH 6.4 and analyzed at 290 nm using UV Spectrophotometer (Shimadzu) against phosphate buffer pH 6.4 as blank. Entrapment Efficiency was calculated by the formula:

$$\% \text{ EE} = \frac{\text{Total amount of drug added} - \text{Drug in supernatant}}{\text{Total amount of drug added}} \times 100$$

% Drug content of Niosomes

Niosomal suspension of Mirtazapine with an equivalent of 10 mg of drug was taken and Ethanol was added to extract the drug by causing lysis of Niosomes. The solution was filtered using Whatman filter paper. 0.1 mL of the solution obtained was taken in 100 mL volumetric flask and volume was made up with phosphate buffer pH 6.4 and the amount of drug present was analyzed by UV Spectrophotometer at 290 nm.

Scanning Electron Microscopy

Scanning Electron Microcopy was carried out for the best formulation F5 to determine the surface morphology of Niosomes.

Zeta Potential Analysis

Charge on Mirtazapine loaded niosomal vesicles was determined using Zeta Potential Analyzer (Horiba SZ 100). Analysis time was kept 60s and charge on Niosomes preparation was determined at 25^{o}C.

Differential Scanning Calorimetry (DSC) studies

DSC thermograms were obtained for pure drug and optimized formulation to investigate any drastic changes with thermal behavior of either drug or excipients. The interaction studies were carried out using differential scanning calorimetry, DSC curves (known as thermo grams) represented as heat flow v/s temperature or time. A typical DSC curve is characterized by the 'baseline' (part of the curve obtained during steady state conditions when no reaction or transition occurred), the peak caused from transitions or reactions, interpolated baseline, initial peak temperature (Ti), extrapolated peak onset temperature (Te), peak maximum temperature (Tp), extrapolated peak offset temperature (Tc), and final peak temperature (Tf). The thermograms of Mirtazapine pure drug and optimized niosomal formulation were obtained. Sample was weighed (5.00-8.00 + 0.5 mg) and placed in sealed aluminum pans, before heating under nitrogen flow at a scanning rate of 10° C/min conducted over a temperature range below 200°C, respectively.

X-Ray Diffractometry (XRD).

X-ray diffractometry is one of crystallography characterization tool. It is used as an analytical tool in identification of the constituents of crystalline phases. It is used to identify any changes in the pure drug, excipient, or methods that might alter the drug efficacy. Mirtazapine pure drug and its niosomal suspension were subjected to X-ray crystallographic studies. The powder X-ray diffraction pattern was recorded using an X-ray diffracto meter (D8 Advance, BRUKER) with 2.2 KW copper as an anode material and X-ray tube as a source. The sample was analyzed using lynux eye detector and filtered using Ni filter.

FTIR Analysis

FTIR was performed for Optimized Niosomal Suspension (batch F5), Optimized gel formulation G5 and for all the physical mixtures, to determine for any Drug-Excipient In-compatibility. The graph obtained for this formulation was evaluated for the peaks of pure drug Mirtazapine.

In-Vitro Drug Release of Niosomes.

Activation of Dialysis Membrane

Activation of Dialysis membrane having a pore size of 2.4 nm and weight cut off of 12,000-14,000 Dalton was performed by soaking the membrane for half an hour in 0.1 Mm EDTA (Ethylenediaminetetraacetic acid) solution, followed by soaking the membrane in 2% sodium bicarbonate for half an hour and 2 hours in distilled water.

In-Vitro drug release study

In- Vitro drug release of Mirtazapine loaded Niosomes was performed by diffusion technique. Franz diffusion cells was employed to perform these studies. Different volumes of Mirtazapine niosomal suspension containing fixed weight of 10mg was taken in the donor compartment. The receptor compartment was filled with phosphate buffer pH 7.4 as the receptor medium. The donor and receptor compartments were separated by semi-permeable cellulose dialyzing membrane. The medium in the receptor compartment was stirred on a magnetic bar at 50 rpm for 8 hours maintained at $37 \pm 0.5^{o}C$. 5 mL of samples were withdrawn and replaced with fresh buffer solution to maintain sink conditions. Obtained sample was diluted and analyzed for the drug release by using UV- Spectrophotometer.

PREPARATION OF NIOSOMAL IN SITU GEL FORMULATION

Screening of optimized poloxamer 407 concentration

Thermoreversible In-situ gel was prepared by cold method that is by dissolving 17% of Poloxamer 407 concentration in cold water ($4^{o}C$). A hazy solution was formed which was kept in refrigerator ($2\text{-}4^{o}C$) overnight for complete dissolution resulting in a clear solution. Niosomes with drug equivalent to 10mg was added to Poloxamer 407 solution. Then, 0.5% of Carbopol 934 was added to the poloxamer 407 containing Niosomes with continuous stirring at $4^{o}C$. The formulated gel was stored at $4^{o}C$ for further evaluation. Concentration of Poloxamer 407 and Carbopol 934 was selected based on preliminary trials.[6]

CHARACTERIZATION OF NIOSOMAL IN-SITU GEL FORMULATION

Determination of gel strength

An accurately weighed quantity of 30g of gel was placed in a 50mL graduated measuring cylinder and was allowed to form a gel in a water bath at $37^{o}C$. By applying 50g of weight in to the gel with the help of a cylinder, the time taken by the weight to go down through the gel was measured as gel strength.[7]

Determination of pH and Clarity.

The apparent pH of the gel product was determined by using digital pH meter. Clarity of various formulations were determined by visual inspection under black and white background and it was graded as turbid (+), clear (+ +), very clear (+++).

Spreadability and Gelling Time

It is evident from the theory of mucoadhesion, a mucoadhesive formulation that has high spreadability and high surface tension adheres strongly to the mucus membrane. For accessing spreadability a glass slide was used. Provision was made so as to keep the slide in hot water, the sheep nasal mucosa was pasted from the serosal side on the slide and hot water was circulated for 15-20min for equilibrating a temperature of mucosa at $34 \pm 2^{o}C$. One drop of formulation was placed on the mucosa at an angle of 120^{o} and the distance travelled by the drop before it gets converted in to gel was recorded and the time taken by it to convert it in to gel was recorded. [8]

Viscosity Measurement

Viscosity was measured by using Brookfield Viscometer coupled with spindle no 21 at 50 rpm before gelation and after gelation of the In-situ gel.

% Drug content

Drug (Niosomes) must be uniformly distributed throughout the sample. Samples from different sites in the container were analyzed for drug content. Ethanol was added to lyse the vesicles. 0.1 mL of formulation was diluted in 100 mL of phosphate buffer pH 6.4 and absorbance measured against the blank at 290 nm.

Gelling Temperature

4mL aliquot of the gel was transferred to test tubes, immersed in a water bath at $25^{o}C$ and sealed with aluminium foil. The temperature of water bath was increased by increments of $0.5^{o}C$. The samples were then examined for gelation which occurs when the meniscus take longer to move.[8]

Determination of Mucoadhesive strength

Mucoadhesive property was determined using a modified physical balance. Sheep nasal mucosa was used as biological membrane, which was fixed under one pan of the balance with the help of cyanoacrylate glue and was hydrated with 100 µL of phosphate buffer pH 6.4 maintained at 34±2°C. Accurately weighed amount of 1 g of gel was stuck to the inverted beaker (250 mL) using glue and the height of the balance was adjusted to accommodate a glass container below the pan where membrane was glued. A preload of 10 g was applied in order to allow the formation of mucoadhesive joints. After a 3 min rest period, the preload was removed and gradually the weight was added to the other pan until the gel was detached from the mucosal surface. The total weight required for the complete detachment of the gel was recorded.[9]

In-Vitro Drug Release Study

In- Vitro drug release of Niosome loaded In-situ gel was performed by diffusion technique. Franz diffusion cells was employed to perform these studies. Different volumes of gel containing niosomal suspension in a fixed weight of 10mg was taken in the donor compartment. The receptor compartment was filled with phosphate buffer pH- 7.4 as the receptor medium. The donor and receptor compartments were separated by semi-permeable cellulose dialyzing membrane. The medium in the receptor compartment was stirred on a magnetic bar at 50rpm for 8 hours maintained at 37 ± 0.5^{o} C. 5 mL of samples were withdrawn and replaced with fresh buffer solution to maintain sink conditions. Obtained sample were diluted and analyzed for the drug release by using UV-Spectrophotometer.

Comparative Release Behaviour Studies and Compartitive Permeation Studies

Pure drug Mirtazapine, optimized Niosomal suspension of Mirtazapine, optimized drug loaded Niosomes incorporated in to an In-situ gel were compared for their release behaviour and permeability studies.

In-Vitro release studies

In-vitro release study for the prepared optimized batches that is F5 optimized niosomal formulation, G5 optimized In-situ gel formulation and pure Mirtazapine solution were determined for their release behaviour. Results were obtained and compared.

Ex-Vivo Permeation Studies

Ex vivo permeation study for the prepared batches that is pure mirtazapine solution, optimized niosomal suspension F5 and optimized In-situ gel formulation G5 was performed by using Franz diffusion cell across sheep nasal mucosal membrane obtained from the local slaughter house which was stored in phosphate buffer pH 6.4 to stabilize. The sheep nasal mucosal membrane was mounted in between the donor and the receptor compartment of the diffusion cell. The position of the donor compartment was adjusted so that the mucosa just touches the permeation medium. Formulation equivalent to 10mg of drug was placed in the donor compartment which was in contact with the mucosal surface of the membrane, while the receptor compartment was filled with phosphate buffer pH 7.4 and its temperature was maintained at $37°\pm0.5^{o}$C. At predetermined time point 1mL of sample was withdrawn from the receptor compartment and replaced with the same volume of fresh medium. The samples were analyzed for percent of drug permeated from the formulations by UV spectrophotometer at 290 nm.

Histopathological studies

Freshly excised sheep nasal mucosa was collected from a local slaughter house and was kept in phosphate buffer pH 6.4 for 15 min. Sheep nasal mucosa pieces having uniform thickness of 0.2 mm were taken. Mucosa treated with phosphate buffer pH 6.4 was taken as a negative control and the mucosa treated with isopropyl alcohol was taken as a positive control. Other two mucosae were treated with optimized Niosomal formulation and optimized In-situ gel for 1 h separately. After 1 hour, both the mucosae were rinsed with phosphate buffer pH 6.4 and all of them were carried to the pathological laboratory in 10% formalin for the preparation of pathological slides. The sectioned tissue was then stained with haematoxylin and eosin. The prepared pathological slides were studied under an optical microscope at x10 and x40 resolution for any sign of inflammation like, sloughing of epithelial cells, necrosis or haemorrhage.[10]

FTIR Analysis of Optimized In-Situ Gel G5.

FTIR was performed for Optimized In-Situ Gel Formulation (batch G5) to determine for any Drug-Excipient In-compatibility. The graph obtained for this formulation was evaluated for the peaks of pure drug Mirtazapine.

Short Term Stability Studies

Stability studies were assessed by keeping Niosomal suspension and Niosomal In-situ gel in sealed glass vials and storing them room temperature and 4 ± 2^{o}C for a period of 30 days. The samples were withdrawn at a period of one month and evaluated for different parameters.

RESULTS AND DISCUSSION

DATA OPTIMIZATION

Generation of Polynomial equations

Statistical model generated interactive polynomial terms for each response, equations is as follows: -

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 AB + \beta_4 A^2 + \beta_5 B^2$$

Where, Y is the independent variable, β_0 is the arithmetic mean response of the 9 runs and β_1 is the estimated co-efficient for the factor A. the main effects of the amount of A and B signifies the average result, when the factors were changed one at a time from their lower to higher values. The interaction terms (AB) show how the response changes when two factors are concurrently changed. The data obtained from DOE strongly signifies that particle size, PDI and %EE are dependent on the selected independent variables. Conclusions can be drawn from the following polynomial equations depending on the mathematical sign it carries that is positive and negative sign, indicating synergistic and antagonistic effect.

Y_1 – (PS) = 209.67 + 14.00 A + 5.17 B + 4.25 AB + 9.00 A^2 + 0.5000 B^2

Y_2– (%EE) = 76.62 + 3.03 A + 3.68 B – 0.2925 AB – 4.50 A^2 +2.88 B^2

Y_3 – (PDI) = 0.1434 + 0.0545 A + 0.0068 B – 0.0070 AB + 0.0238 A^2 + 0.0058 B^2

Where, A = Concentration of Span 60

B = Concentration of Cholesterol

Statistical analysis of data

Analysis of variance (ANOVA) was applied to recognize insignificant factors. Data was evaluated using Design-Expert Software (version 11.0). From the data obtained it was evident that p-value was found to be less than 0.05 ($p<0.05$) for all the dependent variables. Model F value for PS, %EE and PDI was found to be 25.42, 11.13, 23.70 respectively which implies the model is significant. R-squared is a statistical measure of how close the data are to the fitted regression line. It is also known as the coefficient of determination, or the coefficient of multiple determination for multiple regression. R-squared is a goodness-of-fit measure for linear regression models. This statistic indicates the percentage of the variance in the dependent variable that the independent variables explain collectively.

ANOVA for quadratic models of Particle Size (Y_1).

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1570.92	5	314.18	25.42	0.0116	significant
A-Surfactants	1176.00	1	1176.00	95.14	0.0023	
B-cholesterol	160.17	1	160.17	12.96	0.0368	
AB	72.25	1	72.25	5.84	0.0944	
A^2	162.00	1	162.00	13.11	0.0362	
B^2	0.5000	1	0.5000	0.0404	0.8535	
Residual	37.08	3	12.36			
Cor Total	1608.00	8				

Table 2: ANOVA for quadratic models indicating Model-F value of Particle Size.

Std. Dev.	3.52		**R^2**	0.9769
Mean	216.00		**Adjusted R^2**	0.9385
C.V. %	1.63		**Predicted R^2**	0.7196
			Adeq Precision	13.3534

Table 3: ANOVA for quadratic models indicating R-Squared value of Particle Size.

ANOVA for quadratic models of Entrapment Efficiency (Y_2).

	Source	Sum of Squares	df	Mean Square	F-value	p-value	
	Model	193.73	5	38.75	11.13	0.0375	significant
	A-Surfactants	55.21	1	55.21	15.86	0.0283	
	B-cholesterol	81.03	1	81.03	23.28	0.0170	
	AB	0.3422	1	0.3422	0.0983	0.7744	
	A^2	40.50	1	40.50	11.63	0.0421	
	B^2	16.65	1	16.65	4.78	0.1166	
	Residual	10.44	3	3.48			
	Cor Total	204.17	8				

Table 4: ANOVA for quadratic models indicating Model-F value of %EE.

	Std. Dev.	1.87		**R^2**	0.9489
	Mean	75.54		**Adjusted R^2**	0.8636
	C.V. %	2.47		**Predicted R^2**	0.4609
				Adeq Precision	9.9619

Table 5: ANOVA for quadratic models indicating R-Squared value of %EE.

ANOVA for quadratic models of PDI (Y_3).

	Source	Sum of Squares	df	Mean Square	F-value	p-value	
	Model	0.0195	5	0.0039	23.70	0.0129	significant
	A-Surfactants	0.0178	1	0.0178	108.28	0.0019	
	B-cholesterol	0.0003	1	0.0003	1.70	0.2831	
	AB	0.0002	1	0.0002	1.19	0.3550	
	A^2	0.0011	1	0.0011	6.90	0.0785	
	B^2	0.0001	1	0.0001	0.4135	0.5660	
	Residual	0.0005	3	0.0002			
	Cor Total	0.0200	8				

Table 6: ANOVA for quadratic models indicating Model-F value of PDI.

Std. Dev.	0.0128		**R^2**	0.9753
Mean	0.1632		**Adjusted R^2**	0.9341
C.V. %	7.86		**Predicted R^2**	0.7002
			Adeq Precision	11.7421

Generation of 3D Response Surface Plots.

For the measured responses, three-dimensional plots were generated to determine the change in the response surface. These plots generated were found to be beneficial in the study of the effect of two factors on the response at one time.

Surface plot exhibiting the effect of Cholesterol and Surfactant Concentration on Particle Size (nm).

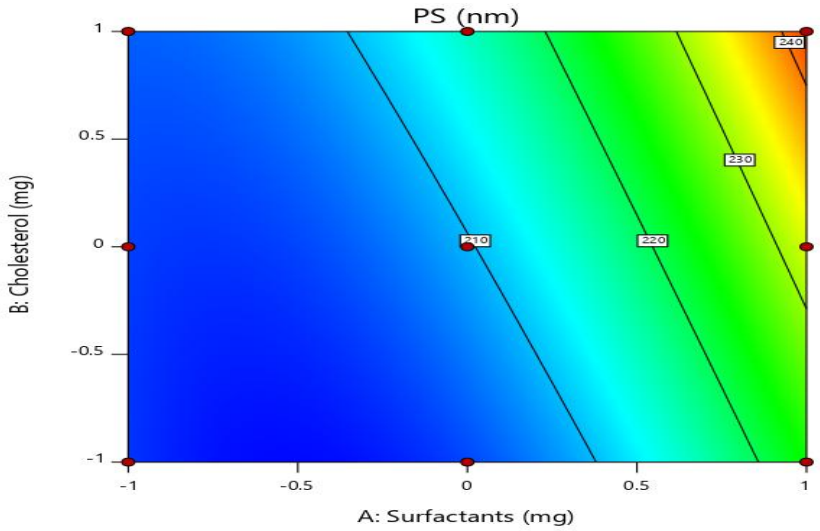


Fig 5.1: Contour Plots for particle size signifying the effects of two factors on response.

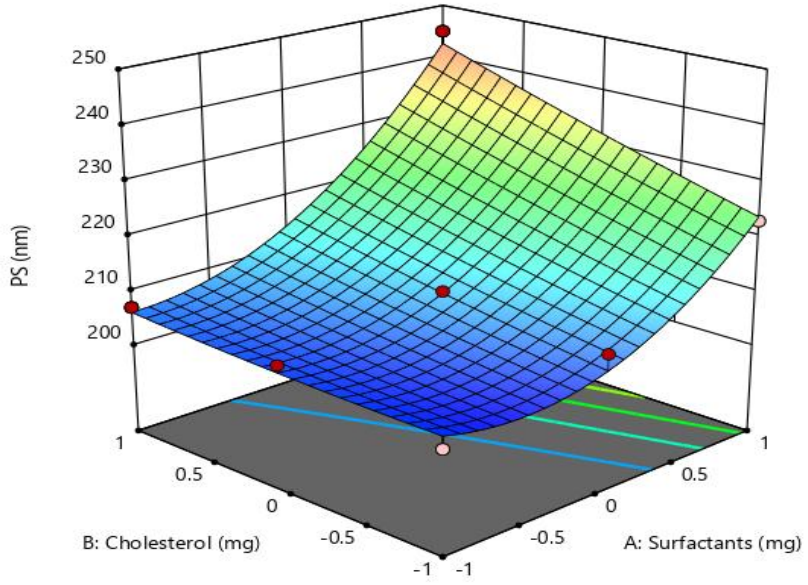


Fig 5.2: 3D Response Surface Plots for particle size.

Effect of Surfactant and Cholesterol Concentration on Particle Size.

Surfactant is an important component in the formation of niosomal vesicles and variation in the concentration may affect the particle size. From the observation gained from the response surface plots, it was found that increase in concentration of surfactant increases the particle size. Similarly, an increase in the concentration of cholesterol increases particle size.

Surface plot exhibiting the effect of Cholesterol and Surfactant Concentration on %EE.

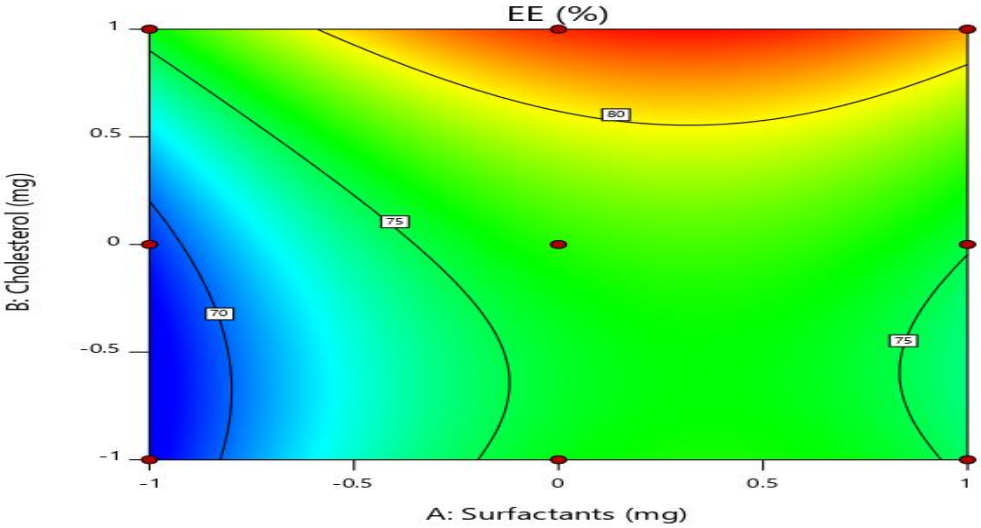


Fig 5.18: Contour Plots for Entrapment Efficiency Signifying the effects of two factors on response.

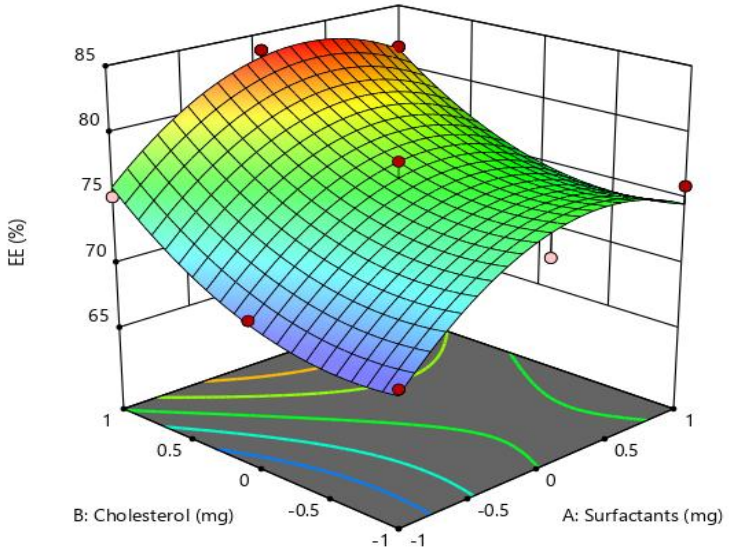


Fig 4: 3D Response Surface Plots for Entrapment Efficiency.

Effect of Surfactant and Cholesterol Concentration on Entrapment Efficiency.

The results obtained indicated that, on initially increase in the concentration of span 60, increased the %EE. But further increase in the concentration of span 60 resulted in decreased %EE. The number of niosomes formed increases with initial increase in the concentration of surfactant, and the niosomes had increased %EE. A further increase in the concentration of surfactant showed decrease in %EE. This could be due to formation of mixed micelles along with the niosomal vesicles with high concentration of surfactant, which may lead to lowering of entrapment efficiency. The concentration of cholesterol was found to have an varying effect on % EE. Increase in the concentration of cholesterol showed increase in % EE. This could be due to the function of cholesterol which at high concentration, prevents the gel-state transformation in to liquid ordered phase which results in an increase in rigidity of the resulting bilayers, thus increasing the stability of niosomes and %EE. But the function of cholesterol was found to be unstable and hence, variations in %EE was observed this could be due to the fact that, cholesterol competes with drug for the space within the bilayer, resulting in disruption of the vesicular membrane and removal of drug from the bilayer.[11]

Surface plot exhibiting the effect of Cholesterol and Surfactant Concentration on PDI.

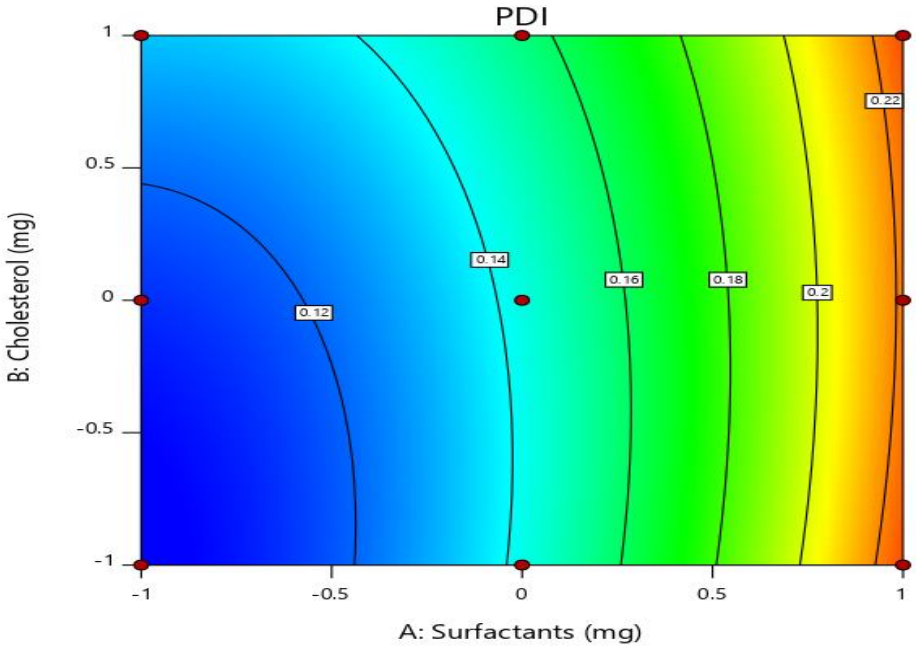


Fig 5: Contour Plots for PDI Signifying the effects of two factors on response.

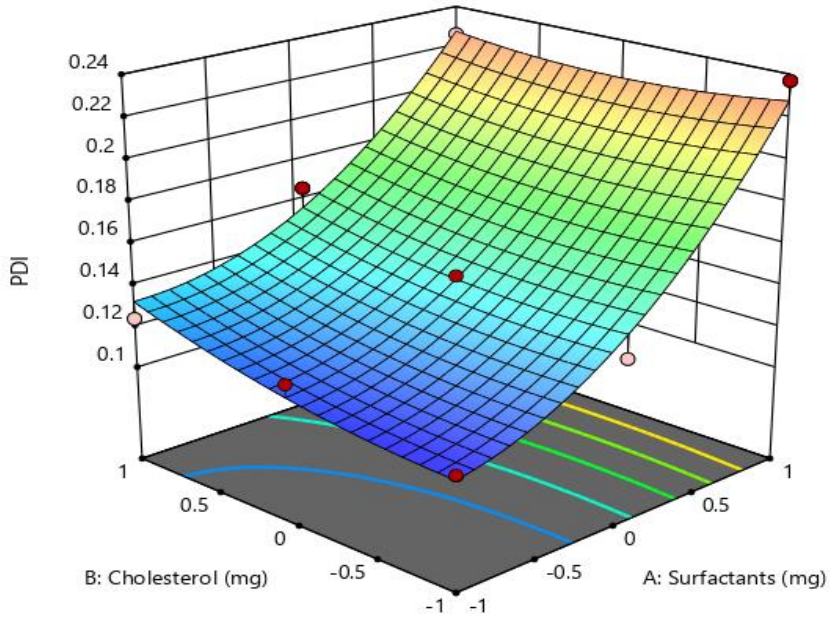


Fig 6: 3D Response Surface Plots for PDI.

Effect of Surfactant and Cholesterol Concentration on PDI.

From the results obtained, it was evident that, with an increase in the concentration of Cholesterol and Span 60, there was a significant increase in PDI of the formulation.

Table 1. Composition of Mirtazapine loaded niosome by 3^2 factorial design.

Code	Factor A Surfactant (mg)	Factor B Cholesterol (mg)	PS (nm)	%EE	PDI	% Drug Content
F1	1	0	231	73.6	0.214	93.2 ±0.033
F2	0	0	210	77.89	0.145	89.30±0.063
F3	-1	1	207	75.02	0.124	88.2±0.06
F4	1	-1	223	76.03	0.237	87.16±0.006
F5	0	1	211.7	83.74	0.166	92.51±0.06
F6	0	-1	208	74	0.131	86.8±0.001
F7	1	1	245	81.6	0.226	87.8±0.001
F8	-1	0	206	69.37	0.119	87.80±0.009
F9	-1	-1	202	68.46	0.107	86.83±0.053

PARTCLE SIZE AND PDI

The vesicle size of all niosomal suspension batches ranged between 202-245 nm. The vesicle size of F5 batch is 211.7 nm.

ENTRAPMENT EFFICIENCY

Entrapment Efficiency of all niosome formulations ranged between 69.3% – 83.7%. The %EE of F5 batch was found to be 83.7%.

% DRUG CONTENT OF NIOSOMES

% Drug Content of all 9 optimized niosomal batches ranged from 86.6±0.001% to 93.2 ±0.003%. % drug content of the optimized F5 batch was found to be 92.51±0.06 %.

SCANNING ELECTRON MICROSCOPY

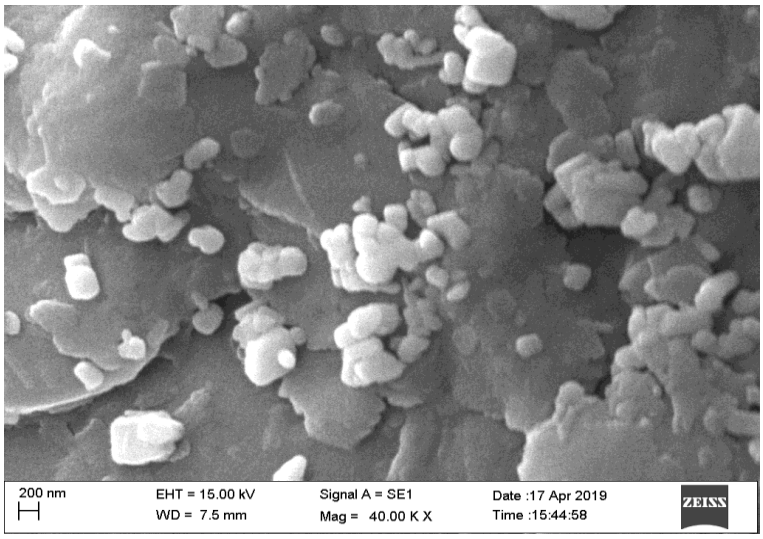


Fig.7: SEM of F5 formulation 40.00 K X magnification.

The prepared niosomal formulation (F5) was characterized for morphology. The SEM reveals that the niosomal particles are spherical, with a smooth surface and a particle size that lies in nanosized range and having an average diameter of 200nm.

ZETA POTENTIAL ANALYSIS

The value of zeta potential was found to be 0.6mV. It specified that prepared niosomes have sufficient charge to prevent aggregation of vesicles.

Calculation Results

Peak No.	Zeta Potential	Electrophoretic Mobility
1	0.6 mV	0.000005 cm2/Vs
2	--- mV	--- cm2/Vs
3	--- mV	--- cm2/Vs

Zeta Potential (Mean) : 0.6 mV
Electrophoretic Mobility Mean : 0.000005 cm^2/Vs

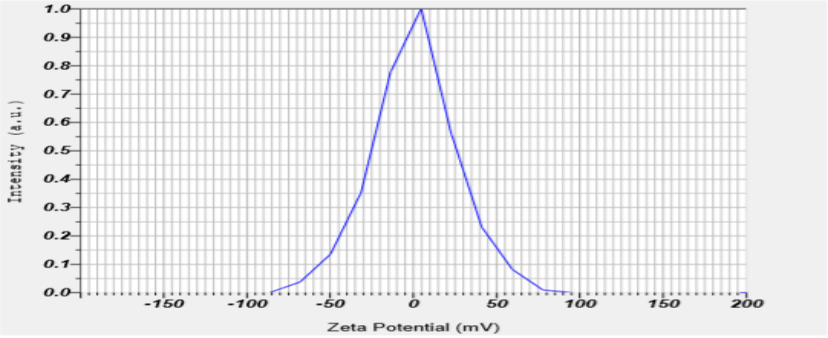


Fig.8: Zeta Potential of of F5 formulation.

Differential Scanning Calorimetry

DSC studies was carried out for pure Mirtazapine and niosomal suspension of Mirtazapine. The thermogram generated for pure Mirtazapine, it revealed occurrence of one sharp endothermic peak at 130.9°C. representing a typical crystalline nature of the drug. When incorporated in to niosomes the characteristic endotherm of Mirtazapine disappeared, exhibited a wide endothermic peak at 108.9°C indicating that Mirtazapine entrapped in niosomes was partially converted to amorphous form.

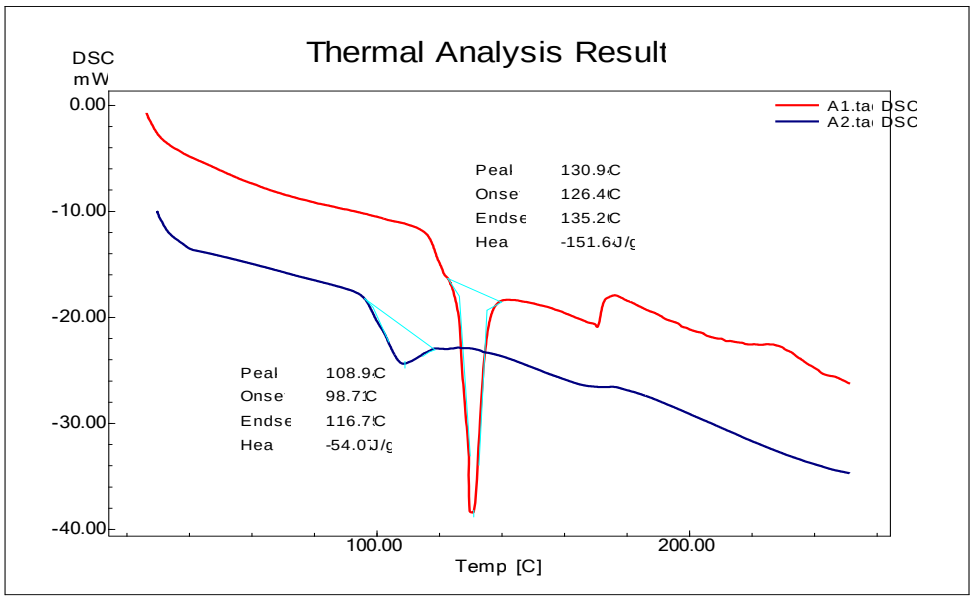


Fig. 9: DSC Thermograms of Pure Drug and Optimized Formulation F5.

X-RAY DIFFRACTOMETRY (XRD)

XRD studies was carried out for pure drug and optimized formulation from 5–60 degrees at 2θ with a scan speed of 0.5 degree/min. The results obtained from the study was in agreement with DSC studies. Mirtazapine is a creamy white crystalline powder which exhibited numerous distinctive peaks at a diffraction angle at various relative intensity. Mirtazapine exhibited a strong and characteristic XRD pattern consistent with a crystalline powder. Whereas, formulation F5 showed loss of peaks and more diffusive peaks indicative of amorphous form of Mirtazapine.

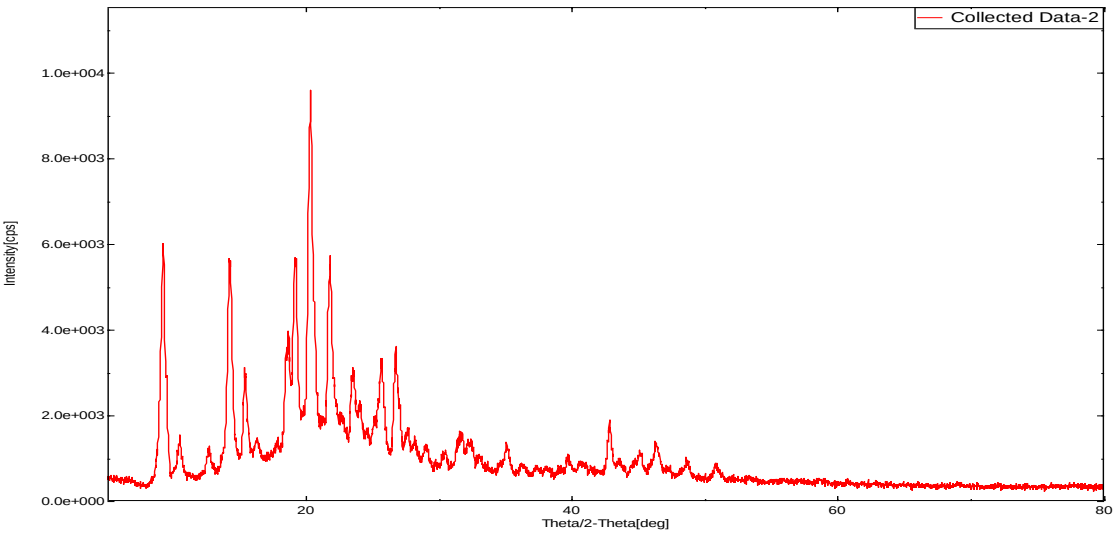


Fig. 10: XRD of pure Mirtazapine.

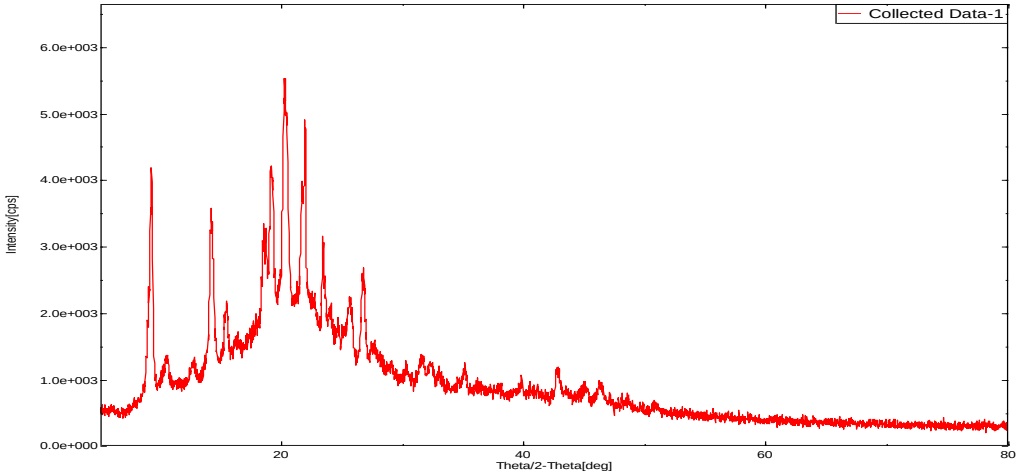


Fig. 11: XRD of Optimized Niosomal Suspension Batch F5.

FTIR Analysis (Fourier transform infrared spectroscopy studies)

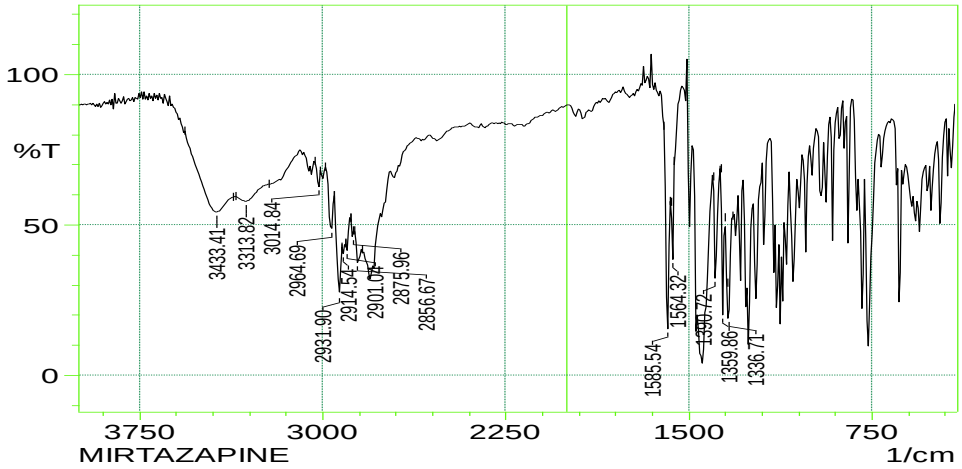


Fig. 12 - FTIR spectra of Pure Mirtazapine.

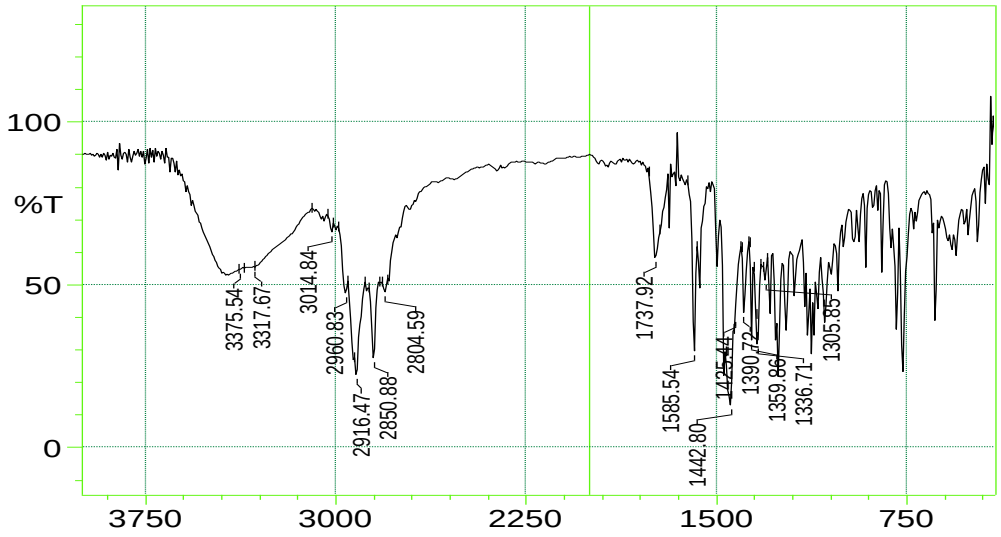


Fig. 13 - I.R spectra of physical mixture of Span 60 and drug.

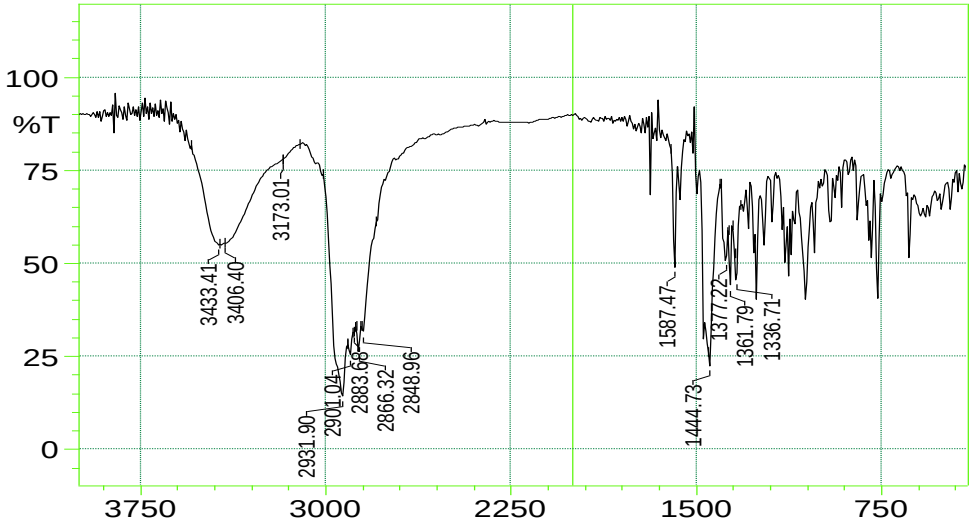


Fig. 14- I.R spectra of physical mixture of Cholesterol and drug.

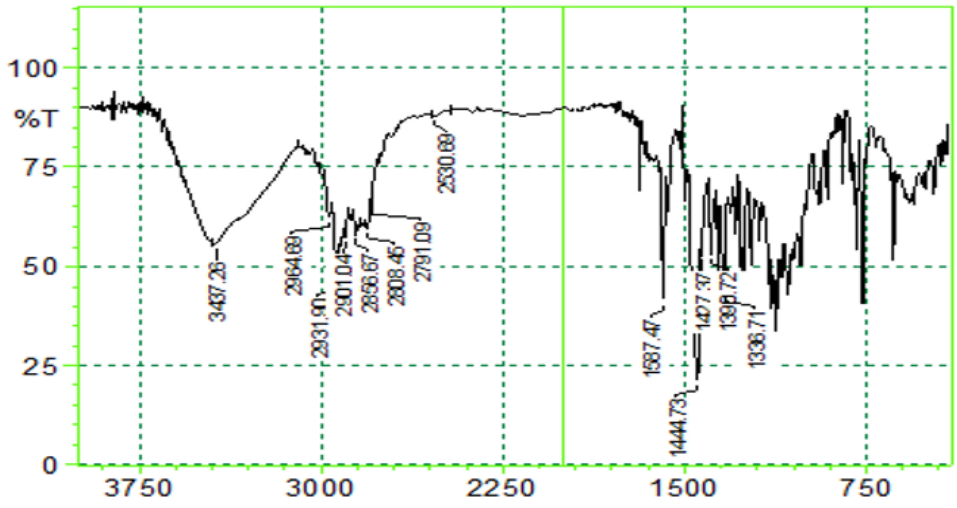


Fig. 15 - I.R spectra of physical mixture of Carbopol 934 and drug.

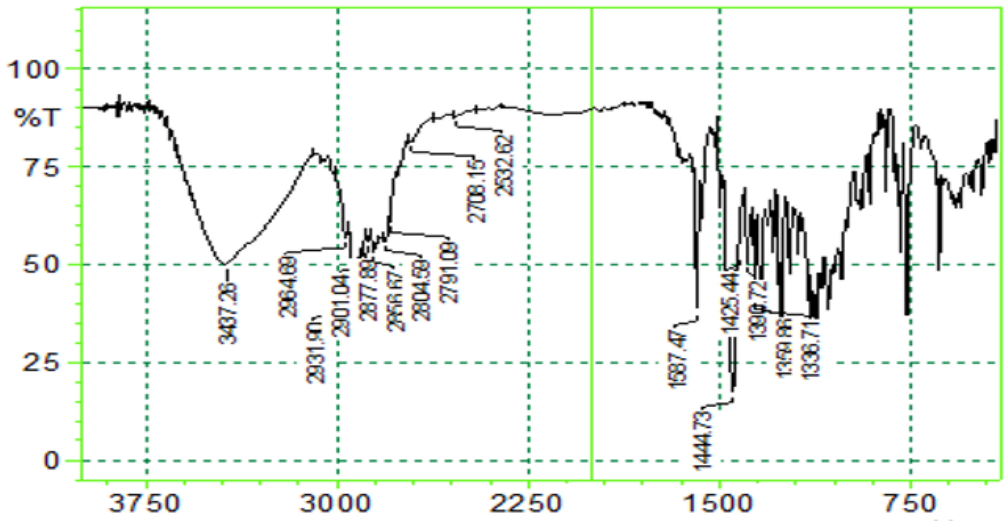


Fig. 16 - I.R spectra of physical mixture of Poloxamer 407 and drug.

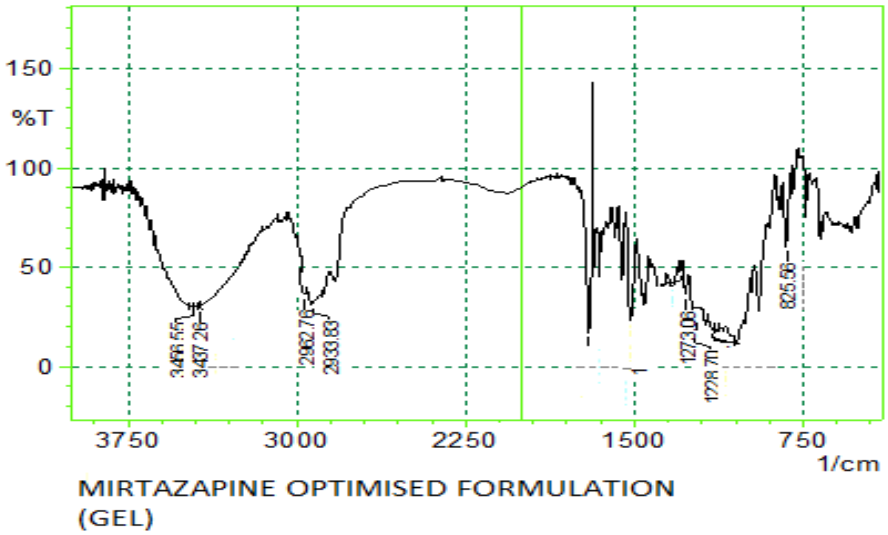


Fig. 17: FTIR of Optimized In-Situ gel Batch G5.

In-Vitro Drug Release Studies.

Table 2: In-Vitro Drug Release of Optimized Formulations.

S. NO	Time (hrs.)	F1 (%)	F2 (%)	F3 (%)	F4 (%)	F5 (%)	F6 (%)	F7 (%)	F8 (%)	F9 (%)
1	0	0	0	0	0	0	0	0	0	0
2	1	8	9.1	7.97	9.43	7.9	9.1	9.2	7.89	9.24
3	2	13.4	10.6	11.5	12.9	14.9	10.4	15.92	11.1	15.8
4	3	20.1	31.7	32.8	33.9	20.1	32.1	20.5	19.7	21.4
5	4	35.4	45.8	46.4	47.9	37.49	41.09	34.59	36.6	35.7
6	5	48.5	52.5	53.1	54	40.9	52.9	38.09	39.8	41.2
7	6	55.9	60.1	60.90	61.7	58.7	60.5	54.8	56.9	61.7
8	7	66.5	72	72.5	72.6	68.50	72.3	62.1	67.1	79.2
9	8	76	76.4	78.8	77.9	79.3	78.9	77.3	78.4	81.4

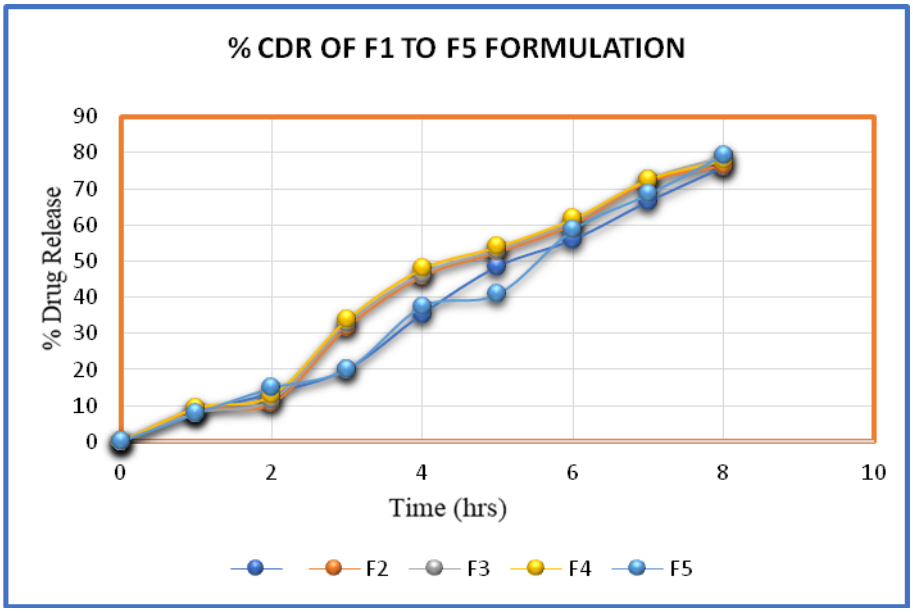


Fig.18 : In-Vitro Drug Release of F1-F5 Formulation.

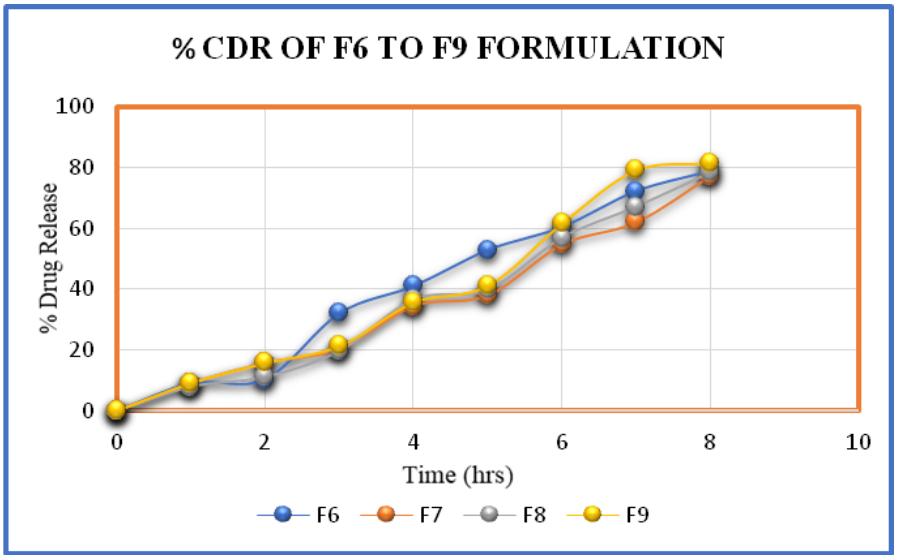


Fig. 19: In-Vitro Drug Release of F6-F9 Formulation.

The In-Vitro drug release was found to be in a range of 76% – 81.4%, the Release % of the optimized formulation F5 was found to be 79.3% at 8th hour.

FORMULATION OF IN-SITU GEL

Table 3: Composition of Drug Loaded (Niosomes) In-Situ Gel.

Formulation code	Drug (% v/v)	Poloxamer 407 (%w/v)	Carbopol 934 (%w/v)
G1	0.5	17	0.1
G2	0.5	17	0.2
G3	0.5	17	0.3
G4	0.5	17	0.4
G5	0.5	17	0.5

EVALUATION OF IN-SITU GEL

Table 4: Goals of Evaluation.

Evaluation parameters	Goal
Gel strength	Maximum
pH	Nasal pH
Clarity	Maximum
Spreadability	Maximum
Gelling Time	Minimum
Viscosity	Sol- optimal for instillation Gel- optimal to prevent mucocillary clearance
% Drug Content	Maximum
Gelling Temperature	Near to Nasal Temperature
Mucoadhesive strength	Optimal to adhere to Nasal Mucosa.

Table 5: Evaluation of the In- Situ Gel Formulations.

Formulation code	Gel Strength (sec)	pH	Clarity	Gelling time (sec)
G1	17.3 ±0.03	6.1 ± 0.03	++	53 ± 0.00
G2	21.6 ± 0.06	6.3 ± 0.026	++	51.6 ± 0.066
G3	23.6 ± 0.006	6.23 ± 0.032	++	46 ± 0.00
G4	24.8 ± 0.002	6.1 ± 0.03	++	46.3 ± 0.032
G5	27.3 ± 0.03	6.26 ± 0.06	++	44 ± 0.00

Determination of Gel Strength

The gel strength of all the gel formulations ranged between 17.3±0.03 to 27.3±0.03 sec, formulation G5 showed highest gel strength value comparatively of 27.3± 0.03 sec.

Determination of pH and Clarity

pH of all the formulation was found to be in a range of 6.1±0.03 to 6.3±0.026. All the formulations were found to be in an acceptable pH range Nasal pH range (5.5 – 6.5). Clarity of all the gel formulations was found to be similar (++), they showed good clarity because both Poloxamer 407 and Carbopol 934 show good aqueous solubility.

Spreadability and Gelling Time

Total surface area of nasal mucosa is reported to be 160cm^2 (12.64cm), and surface area of olfactory mucosa is reported to be 10cm^2 (3.16cm). A glass slide of 7.5cm was taken and a measured 5cm length of nasal mucosal tissue was cut and placed on the slide to determine spreadability. Spreadability of all formulations was found to be in a range of 2.4 ± 0.03cm to 4.4± 0.00cm. Formulation G5 showed spreadability in accordance with the length of olfactory mucosa of 2.4 ± 0.03cm which is near to 3.16cm. Gelling time for all formulations was found to be in a range of 44±0.00sec to 53±0.00sec. Formulation G5 showed low gelling time comparatively of 44±0.00sec.

Table 6: Evaluation of the In- Situ Gel Formulations.

F. Code	Spreadability (cm)	Viscosity (cP)		Drug Content (%)	Gelling temperature (^{o}C)	Mucoadhesive strength (g)
		Initial	After gelation			
G1	4.4 ± 0.00	247	2023	91 ±0.01	40±0.00	10.3 ±0.023
G2	4.6±0.06	368	2520	93±0.03	43.3±0.036	11±0.030
G3	3.8±0.03	452	2650	90±0.09	35.3±0.03	11.8±0.07
G4	3.5 ±0.033	487	3370	93±0.05	42.3±0.33	13.8±0.096
G5	2.8±0.043	579	3596	92.4±0.06	38±0.03	14.5±0.060

Viscosity Measurement.

Viscosity for all the gel formulation was determined as initial viscosity and viscosity after gelation. Initial viscosity of all gel formulations ranged from 257 – 579cP and viscosity after gelation was found to be in a range of 2023-3596 cP.

Drug Content

% Drug Content was determined for all gel formulations and was found to be in a similar range of 90±0.09% to 93±0.05%, indicating the presence of drug in the formulation.

Gelling Temperature

Gelling temperature was carried out for all formulations of gel and the results were found to be in a range of 44°C±0.00 to 53°C±0.00. Formulation G5 showed the lowest gelling temperature of 44°C comparatively.

Determination of Mucoadhesive Strength

Mucoadhesive strength was determined for all gel formulation and the results were found to be in a range of 10.3±0.023g to 14.5±0.060g. Formulation G5 showed high mucoadhesive strength towards the nasal mucosa of about 14.5±0.060g.

In-Vitro Drug Release Study

Table 7. In-Vitro Release of In-Situ Gel Formulations.

S. NO	Time (hrs)	G1 (%)	G2 (%)	G3 (%)	G4 (%)	G5 (%)
1	0	0	0	0	0	0
2	1	8.81	7.01	7.89	7.33	8.436
3	2	15.7	14.7	15.7	17.7	14.76
4	3	24.3	27.5	24.4	22.30	25.26
5	4	30.1	34.11	30.1	27.3	35.03
6	5	39.4	39.6	39.4	34.60	44.86
7	6	46.5	43.20	46.5	48.21	50.43
8	7	55.1	56.4	55.1	53.8	58.74
9	8	61.2	66.9	61.0	63.12	64.85

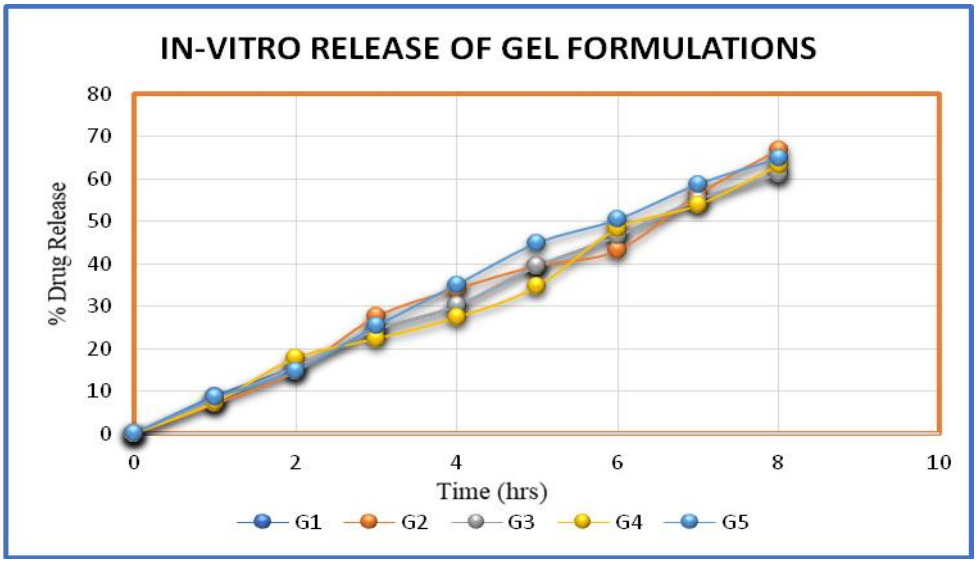


Fig. 20: In-Vitro Drug Release of In-Situ gel Formulation.

Comparative Release Behaviour Studies and Comparative Permeation Studies
In-Vitro drug release studies of pure Mirtazapine, formulation F5 and G5.
Release Behaviour Studies.

Table 8: Comparative In-Vitro Drug Release.

S. NO	Time (hrs.)	Pure Mirtazapine Solution (%)	Niosomal suspension (F5) (%)	In-situ gel (G5) (%)
0	0	0	0	0
1	1	2.08	10.1	9.81
2	2	4.6	17.3	16.6
3	3	8.9	24.6	23.1
4	4	11.02	35.7	32.4
5	5	14.6	50.7	46.9
6	6	17.2	58.2	56.7
7	7	19.2	68.4	64.8
8	8	21.3	76.8	70.2

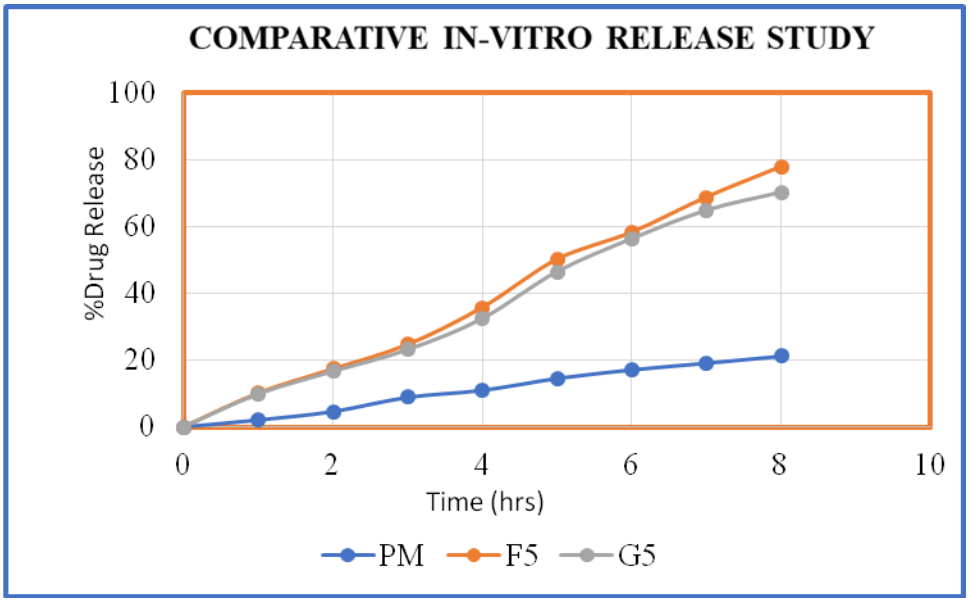


Fig 21: Comparative In-Vitro Release Study.

Ex-Vivo permeability studies of pure Mirtazapine, formulation F5 and G5. Compartitive Permeation Studies.

Table 9: Comparative Permeability Studies.

S. NO	Time (hrs.)	Pure Mirtazapine Solution (%)	Niosomal suspension (F5) (%)	In-situ gel (G5) (%)
0	0	0	0	0
1	1	1.86	9.9	11.1
2	2	3.2	17.3	20.3
3	3	5.6	24.6	28.9
4	4	7.2	30.7	36.8
5	5	10.74	49.7	59.4
6	6	13.44	58.2	63.2
7	7	16.86	63.4	78.5
8	8	18.62	74.8	80.3

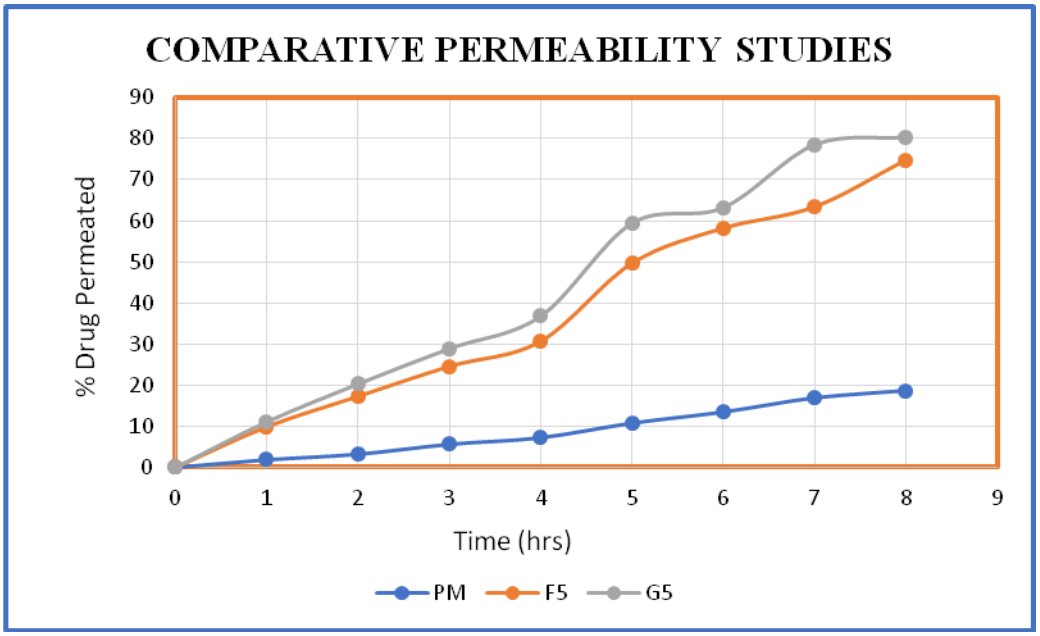
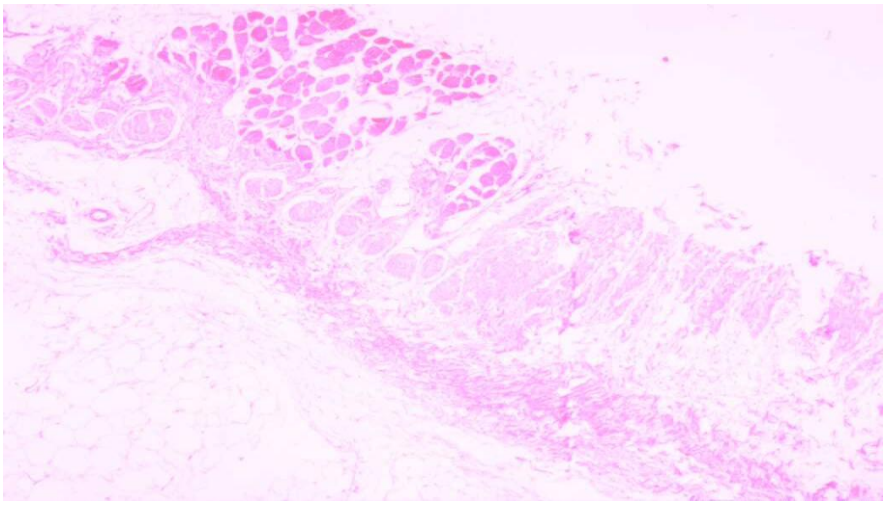


Fig 22: Comparative Permeability Studies.

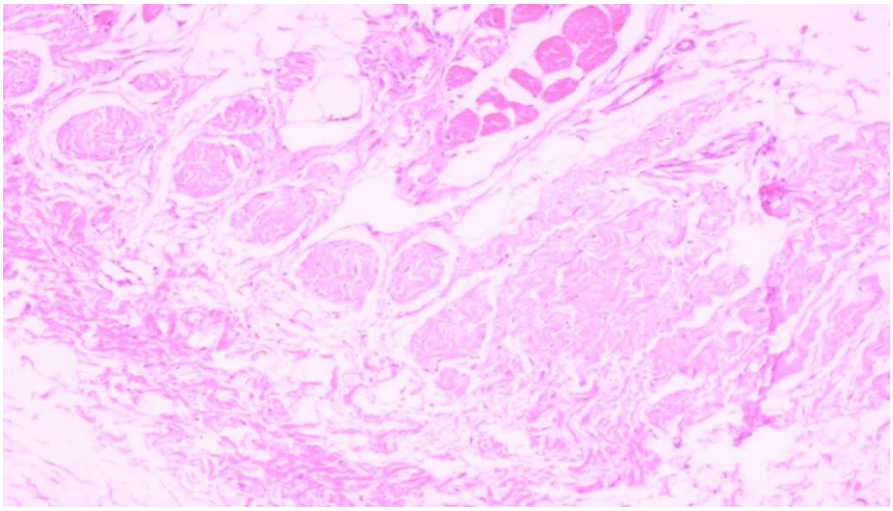


H and E 10x

Fig 23: Histopathology of positive control in 10x.

Results reported that niosomal suspension batch F5 showed high % release as compared to the pure drug solution and (gel) G5 formulation. From the permeation studies it was observed that formulation G5 showed better permeability than pure drug solution and niosomal suspension batch F5.

HISTOPATHOLOGICAL STUDIES
Positive control



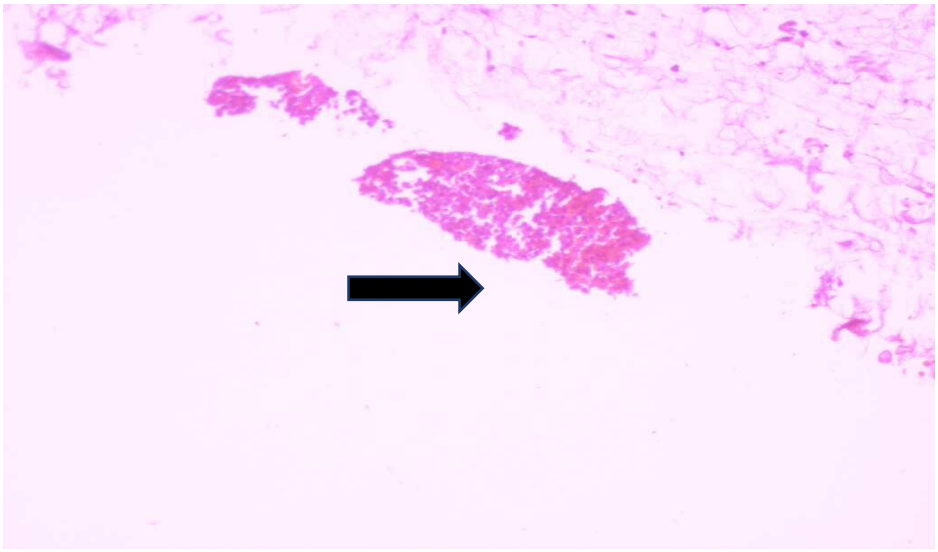
H and E 40x

Fig 24: Histopathology of positive control in 40x.

Microscopy:

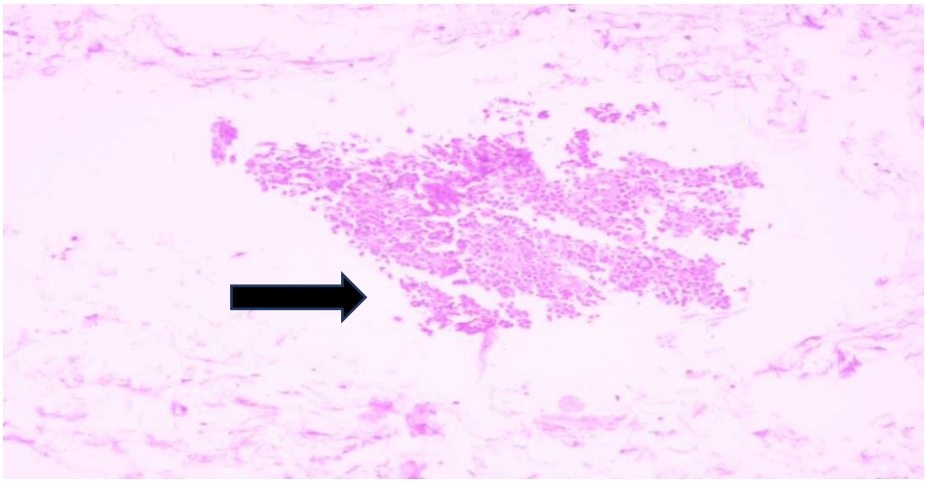
Section studied shows normal mucosa with pseudostratified columnar epithelium. Subepithelium shows normal mucous glands and connective tissue. No inflammation.

Negative Control



Ulcerated epithelium H and E 10x

Fig 25: Histopathology of Negative Control in 10x.

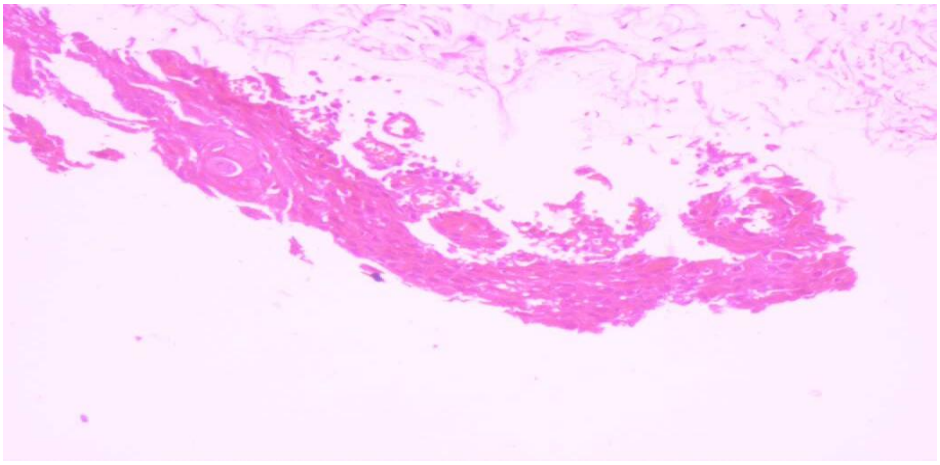


Inflammatory cells. H and E 40x

Fig 26: Histopathology of Negative Control in 40x.

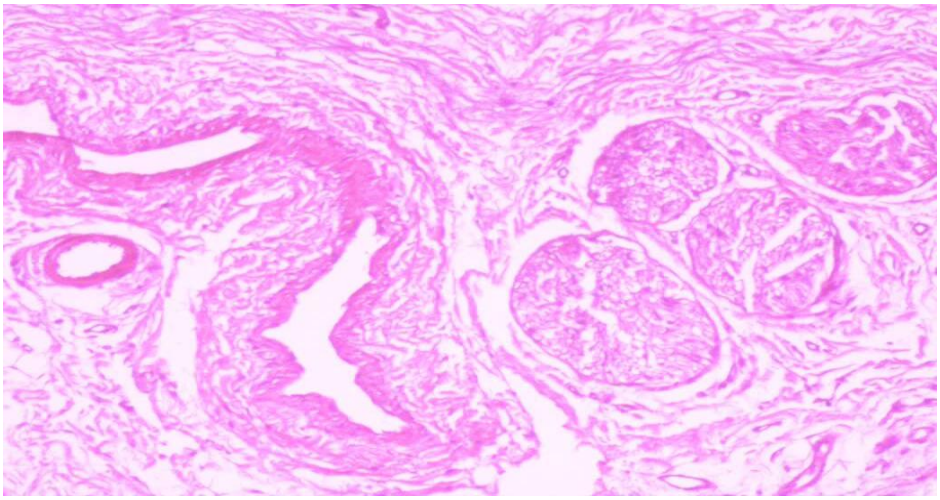
Microscopy: Section studied shows mucosa with pseudostratified columnar epithelium which is ulcerated at many places. Subepithelium shows damaged mucous glands and connective tissue due to sloughing of epithelial cells. Signs of necrosis and mild inflammation seen.

Nasal Mucosa Treated with Optimized Niosomal Formulation (F5).



H and E 10x

Fig 27: Histopathology of Niosomes treated Nasal Mucosa in 10x.

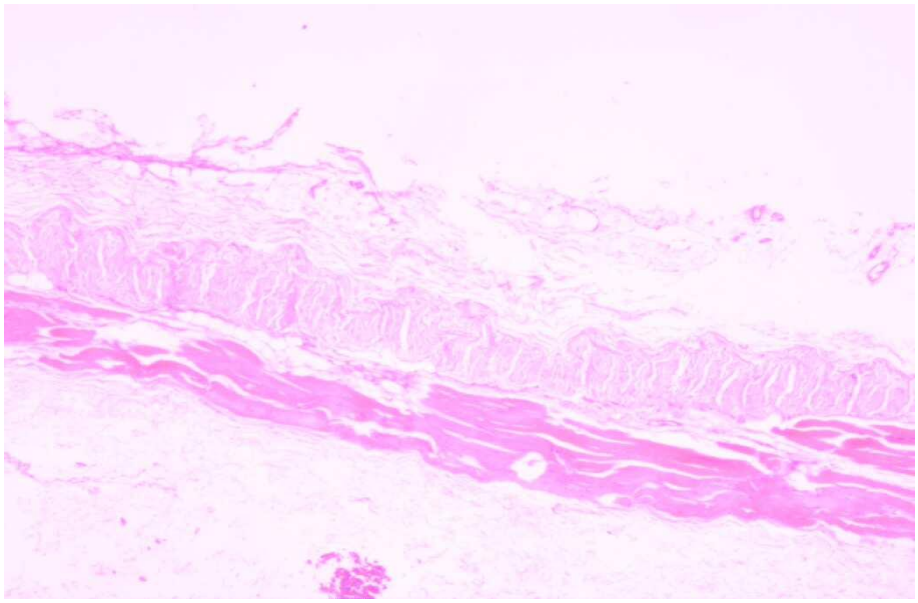


H and E 40x

Fig 28: Histopathology of Niosomes treated Nasal Mucosa in 40x.

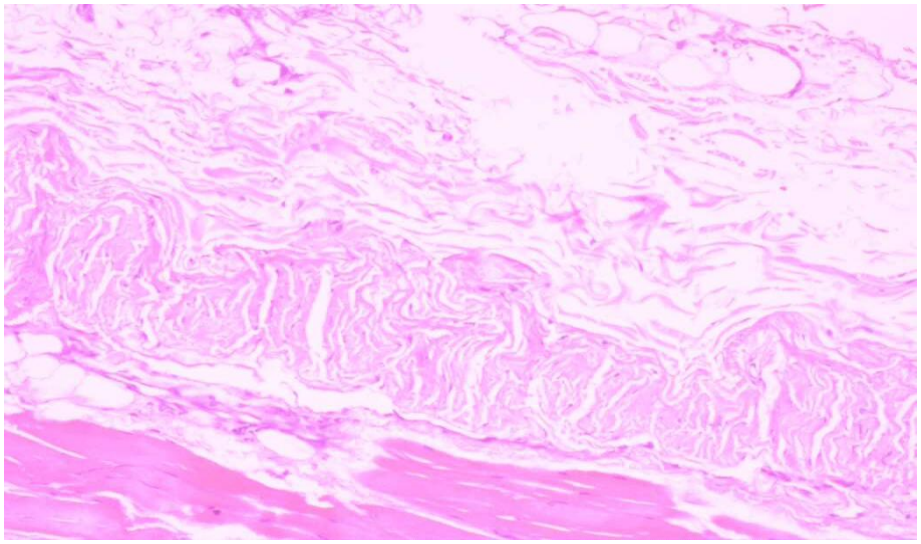
Microscopy: Section studied shows normal mucosa with pseudostratified columnar epithelium. Subepithelium shows normal mucous glands and connective tissue. No signs of necrosis or sloughing of epithelial cells. No inflammation seen.

Nasal Mucosa Treated with Optimized In-Situ Gel Formulation (G5)



H and E 10x

Fig 29: Histopathology of In-Situ Gel treated Nasal Mucosa in 10x.



H and E 40x

Fig 30: Histopathology of In-Situ Gel treated Nasal Mucosa in 40x.

Microscopy: Section studied shows normal mucosa with pseudostratified columnar epithelium. Subepithelium shows normal mucous glands and connective tissue. No signs of necrosis or sloughing of epithelial cells. No inflammation seen.

Short Term Stability Studies.
Niosomes F5 stored at room temperature for 30days.

Table 10: Stability Studies of Niosomes at Room Temperature.

S. NO	Evaluation parameters	Initial day	After 30days
1	Physical appearance	Creamy white	Creamy white
2	%EE	83.74%	79.8%
3	PS (nm)	211.7 nm	216 nm
4	PDI	0.158	0.173

Niosomes F5 stored at 4.0 ± 2.0^{o}Cfor 30days.

Table 11: Stability Studies of Niosomes at 4.0 ± 2.0^{o}C.

S. NO	Evaluation parameters	Initial day	After 30days
1	Physical appearance	Creamy white	Creamy white
2	%EE	83.74%	81.73%
3	PS (nm)	211.7 nm	210 nm
4	PDI	0.158	0.196

In-Situ gel G5 stored at 4±2^{o}Cfor 30days.

Table 12: Stability Studies of In-Situ Gel at 4±2^{o}C.

S.NO	Evaluation parameters	Initial day	After 30days
1	Physical appearance	Transparent gel	Transparent gel
2	pH	6.26	6.43
3	% Drug Content	92.4%	90.06%
4	Viscosity (cP)	Initial 579 cP After Gelation 3576 cP	Initial 1250 cP After Gelation 3800 cP

In-Situ Gel G5 stored at room temperature for 30days.

Table 12: Stability Studies of In-Situ Gel at room temperature.

S. NO	Evaluation parameters	Initial day	After 30days
1	Physical appearance	Transparent gel	Transparent gel
2	pH	6.26	6.51
3	% Drug Content	92.4%	89.63%
4	Viscosity (cP)	Initial 579 cP After gelation 3596 cP	Initial 1250 cP After gelation 3800 cP

Optimized formulations F5 and G5 were stored at two different temperatures that is at room temperature and at 4±2^{o}C for a period of one month and later evaluated for various parameters. Both the formulations were found to be more stable at refrigerated temperature of 4±2^{o}C.

DISCUSSION

The present research work was designed to develop niosomes of Mirtazapine incorporated in to an In-situ gel base. The drug has certain shortcomings like poor oral bioavailability due to low solubility. Hence the present project was carried out to overcome these shortcomings and thereby enhance its therapeutic effectiveness. spectrophotometric estimation of drug by UV method at 290 nm and other studies like partition coefficient and drug- excipient compatibility studies. Partition coefficient value confirmed the lipophilicity of the drug. Compatibility studies showed that there was no interaction between the drug and excipients used in formulation. Mirtazapine showed high solubility in di-ethyl ether, which was used as solvent for formulation of niosomes. Niosomes were prepared by ether injection method, thin film hydration and sonication method of which thin film hydration was selected as ideal method as it gave low particle size and good PDI. Surfactant of different grades was screened based on their particle size, PDI and %EE, of which span 60 gave best results. Studies were carried out for screening of cholesterol: surfactant ratio and optimization of process parameters was performed with an aim to achieve maximum percent drug entrapment and uniform niosome size. 9 formulations were developed, evaluated and optimized through 3^2 full factorial design. Two factors were evaluated each at three levels. Surfactant concentration (X1) and Cholesterol concentration (X2) were selected as independent variables. The dependent variables selected for study were particle size (Y_1), % EE (Y2) and PDI (Y3). Out of 9 formulations F5 was found to be best with a particle size 211.7nm and PDI 0.166, entrapment efficiency of 83.74% and in-vitro release of 82.9%. This formulation, F5 prepared by thin film hydration method was found to be best formulation to be further incorporated into an In-situ gel base of Poloxamer 407 and Carbopol 934. Formulation G5 of In-situ gel was selected as ideal formulation for convenient nasal delivery, which shows a pH of 6.26±0.06, with satisfactory and acceptable, spreadability, gelling temperature, gelling time, gel strength, mucoadhesive strength and viscosity. Histopathological studies performed, showed no signs of inflammation was the result gained. FTIR was performed for F5 niosomal batch and G5 in-situ gel batch and results revealed no in-compatibility. From in-vitro release studies formulation G5 showed a release of 72.73%. Stability studies revealed the stability of both F5 niosomal suspension and G5 gel formulation to be better at 4±2^{o}C as compared to room temperature.

CONCLUSION

These results indicated that niosomal In-situ gel of Mirtazapine can be used to overcome the shortcomings of the pure drug and maintaining therapeutically effective drug concentration for a prolonged period indirectly minimizing drug administration frequency and enhancing the bioavailability of drug by directly delivering the drug to the brain by avoiding first pass effect. Thus, it can be concluded that niosomal In-situ gel represents a promising drug delivery system.

CONFLICT OF INTEREST

The authors declare no conflict of interest

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

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