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Formulation Development and *In-vitro/ Ex-vivo* Assessment of Mucoadhesive Microemulsion for Nasal Delivery of Centrally Acting Drug Modafinil

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ABSTRACT

The aim of this study was to formulate the microemulsion (ME) for nasal delivery of BCS Class-II drug Modafinil, used for the treatment of narcolepsy and attention deficit hyperactivity disorder (ADHD) optimized by 'D-Optimal Mixture design' and evaluation of its *in-vitro* and *ex-vivo* potential. Modafinil loaded mucoadhesive microemulsion (MME) was prepared with chitosan and concentration of chitosan was optimized by evaluating its stability after dilution, particle size and percentage transmittance. D-optimal mixture experimental design was applied to optimize microemulsion. The optimized batch revealed smaller droplet size (< 100nm), maximum % transmittance (>95% for 100 times diluted formulation) and was studied for stability for 3 months. The potential of chitosan loaded ME was compared to drug loaded ME system with a view to Permeation Coefficient and Diffusion Coefficient. The rate and extent of % drug diffusion was studied by using dialysis sac method, shows drug suspension had less release (39.658 ± 0.964 %) as compared to drug loaded ME (53.6413 ± 1.06 %) and MME (69.618 ± 1.035 %) formulations in 4 hr. Ex-vivo study was performed using isolated tissue of the sheep nasal mucosa, approximately 50% drug released within 4 hrs, 2 hrs and 1.5 hrs from drug suspension, drug loaded ME and MME respectively. The significant difference ($p<0.05$) was observed for *in vitro* and *ex vivo* drug release between ME,MME and drug suspension. Therefore MME can be used as a possible alternative dosage form of Modafinil to improve its therapeutic potential.

INTRODUCTION

Nasal drug delivery system has gained importance as an additive approach to the oral and parenteral routes for systemic and targeted drug delivery of Central Nervous System (CNS) acting drug[1].There has been increasing interest in using the nasal pathway as a promising approach to deliver the CNS acting moiety. The interest arising for the delivery to the nasal route is due to presence of vascularized epithelium with larger surface area for drug absorption, lower enzymatic activity compared to GIT, circumvent of first pass metabolism, direct transport of absorbed drug into the systemic circulation and it is non invasive route having ease of application and self administration is possible. Pharmacological treatment of CNS disorders requires that drugs must attain its efficacious concentration in the brain [1,2].

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This therapeutic objective requires that drugs are able to cross the brain barriers successfully (i.e., BBB, BCSF barrier) and in brain parenchyma cellular compartments such as astrocytes, microglia, oligodendrocytes, neurons [3].The nasal delivery seems to be a favorable way to circumvent the obstacles for blood brain barrier (BBB) and blood cerebrospinal fluid (BCSF) barrier, thus allowing the direct drug delivery to the central nervous system (CNS).It is the only site in the human body where the nervous system is in direct contact with the surrounding environment. The nasal route, therefore offers a potential for drugs targeting to the brain and provide more opportunities for the entry of drug in the CNS [4,5].

The design and development of new drug delivery system with an objective of enhancing the efficacy of existing drug is an ongoing process in pharmaceutical research. Since there are many types of drug delivery systems that have been developed, one in particular the colloidal drugs delivery system has great potential for achieving

the goal in drug targeting [6,7].Several claims have been made in favors of developing nasal formulations containing liposomes, microspheres, nanoparticles and micro-emulsions for intranasal drug delivery [8]. These systems can include, besides the drug, enzymatic inhibitors, nasal absorption enhancers or/and mucoadhesive polymers in order to improve the stability, membrane penetration and retention time in nasal cavity. Those efficacious treatment strategies can be implemented with the concept of microemulsion system as it is isotropic and thermodynamically stable system consist of oil, surfactant and co-surfactant. Microemulsion is a colloidal like preparation in which globule size ranging from 10-200nm [7,8].

Nasal drug delivery offers a number of attractions, including the potential of mucosal drug delivery and the avoidance of syringes and needles for dosing. Drugs belong to BCS Class-II and CNS acting shows added advantages for brain targeting via nasal delivery [9]. Drugs administered intranasal respond better than any other route and response improve when co-administration with an adjuvant, chitosan. Chitosan is a copolymer of glucosamine and N-acetyl glucosamine (cationic biopolymer), typically derived from crustaceans and fungi, and is available in high purity grades suitable for pharmaceutical applications. Chitosan has been demonstrated to enhance the transmucosal uptake, and thus increase bioavailability, of a wide range of small molecules, peptide and protein drug compounds. The polymer is mucoadhesive, provides efficient contact time and reduces the chances of nasal clearance of drug; able to modulate epithelial tight junctions, and has immune stimulant properties. The safety of chitosan and chitosan-based delivery has also shown potential in numerous preclinical and clinical investigations [8].

Modafinil, a BCS class-II drug, marked as a suitable and safe candidate for the Narcolepsy. Narcolepsy disorder is the second-leading cause of excessive daytime sleepiness (after obstructive sleep apnea) [10]. It is a lifelong sleep disorder that makes patient feel overwhelmingly tired, and in severe cases, have sudden uncontrollable sleep attacks. About one in 2,000 people have some form of narcolepsy [11]. The cause of narcolepsy is still unknown, but recent research suggests that many people with narcolepsy with cataplexy have low levels of the neurotransmitter hypocretine, a chemical that regulates arousal, wakefulness and appetite [11,12]. Sleep disorders are more common in children with attention deficit hyperactivity disorder (ADHD) and psychiatric disorder. In children and young people whose ADHD is unresponsive to methylphenidate, atomoxetine and dexamfetamine, further treatment may include the use of medication (after referral to tertiary services) unlicensed for the treatment of ADHD (such as bupropion, clonidine, modafinil and imipramine). (National institute for health and care excellence (NICE) published a guideline for unlicensed or off-label medicine) [13]. Although ADHD can't be cured, it can be successfully managed and some symptoms may improve as the child ages. Despite being the most commonly studied and diagnosed psychiatric disorder in children and adolescents, the cause in the majority of cases is unknown, research efforts continue. The World Health Organization estimates that it affected about 39 million people as of 2013.

The objectives of this study were to develop the best possible formulation of microemulsion and mucoadhesive microemulsion containing Modafinil, BCS class-II drug using D-Optimal Mixture design, to perform its characterization and evaluating its potential for *in-vitro* release profiles and *ex-vivo* permeation study.

MATERIAL AND METHODS

Materials

Modafinil was provided as a gift sample by Alembic Pharmaceutical, Baroda. Capmul MCM C8 was gifted by Abitec Corporation, USA. Polysorbate-80 and PEG-400 were purchased from Spectrochem, Vadodara, India. Chitosan-652 was used for Nose to Brain Microemulsion (NTB ME) which was purchased from SiberHegner, Mumbai, India. HPLC grade water was obtained by filtering double distilled water through nylon filter paper 0.45µm pore size and 47mm diameter, purchase from Pall Life sciences, Mumbai, India. All other chemicals and reagents used were of pharmaceutical grades.

METHODS

RP-HPLC analysis of Modafinil

Chromatography was performed on Shimadzu (Shimadzu Corporation, Kyoto, Japan) chromatographic system equipped with Shimadzu LC-20AT pump and Shimadzu SPD-20AV absorbance detector. Samples were injected through a Rheodyne 7725 injector valve with fixed loop at 20 µl. The chromatographic separation was performed using a Supelco C8

(250 mm × 4.6 mm i.d., 5 µm particle size) column. Separation was achieved using a mobile phase consisting of Methanol: Water: acetic acid in the ratio 500:500:1(v/v) pumped at a flow rate of 1 ml/min. The eluent was monitored using UV detector at a wavelength of 220 nm. The column was maintained at 40°C and an injection volume of 20 µl was used. Water used in the mobile phase was doubled distilled passed through the vacuum filtration through 0.45 µm nylon membrane filter and degassed in an ultrasonic bath prior to use. Data acquisition and integration was performed using LC Solution software [16,27].

Solubility studies

The solubility study for Modafinil in different types of oils, surfactants, and co-surfactants was conducted and maximum solubility of Modafinil was determined by adding an excess amount of drug in each vehicle; initially 20 mg of drug was taken in 2 ml of oil, surfactant or co-surfactant in a glass vial, which was followed by gentle heating at 40 °C in water bath for solubilization. Then mixture was vortexed for 10 minutes using cyclomixer for proper mixing and kept in isothermal shaker for 48 hours at 37 °C to attain equilibrium[14]. The equilibrated samples were then centrifuged at 5000 rpm for 15 min. If sample remained clear without settling of drug, then same process was repeated by adding excess of drug until it ascertain its maximum solubility. When added quantity of drug was unable to dissolve, clear supernatant was isolated and diluted with suitable solvent methanol/acetonitrile followed by filtration through a 0.45 µm membrane filter or sometimes centrifugation was done prior to filtration [14,15].The concentration of Modafinil was determined in oils, surfactants or co-surfactants using RP-HPLC. All studies were repeated thrice, with similar observations being made between repeats.

Screening of surfactant: co-surfactant (Smix) ratio based on pseudo ternary phase diagram Based on drug solubility, ternary phase diagram was developed for selected oil, surfactant and co-surfactant. The physical state of the microemulsion was marked on a pseudo-ternary phase diagram with one axis representing aqueous phase, the second axis representing oil and the third representing a mixture of surfactant and co-surfactant (Smix) at fixed weight ratio [14]. Ternary phase diagram was developed using aqueous titration method. The weight ratio of surfactant to co-surfactant was varied as 3:1, 2:1 and 1:1. For each pseudoternary phase diagram at a specific Smix weight ratio, oil was added to the Smix at 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9, respectively with constant stirring(14-18). To the resultant mixtures, water was added drop wise till the first sign of turbidity and visually analyzed in order to identify the end point. The amount of water was noted down in order to complete the pseudoternary phase diagrams and then an appropriate weight ratio were selected based on these results. In order to prepare stable microemulsion system, selection of microemulsion region from phase diagram was based on the fact that microemulsion remains clear even on infinite dilution.

Formulation of microemulsion and mucoadhesive microemulsion

A fixed quantity of drug was dissolved in oil using vortex mixer (Remi Motors Ltd., India). Required amount of Smix was added to the mixtures with continuous stirring followed by sonication until clear drug dispersion was obtained [19]. Then, aqueous phases was added dropwise with continuous stirring. It was warmed at 40 °C using a water bath for 30 min with intermittent shaking to ensure complete mixing. Then, it was stored at room temperature for 24 hrs to attain its equilibrium [20].

Optimization of Drug loaded Microemulsion Formulation Using D-Optimal Mixture Design

This experimental design was used as a statistical tool to quantify relation between critical formulation variables and measured response for optimization. Here, concentration of oil (Capmul MCM C8) as X1, concentration of Smix (Tween 80 and PEG 400)as X2 and concentration of aqueous phase(Sodium acetate buffer pH 5.0) as X3 were selected. From the preliminary trials and pseudoternary phase diagram their minimum as well as maximum concentrations were determined (Table 1) from which design was created by the software and it was characterized for specified parameters. Total 16 formulations with 5 centre points were prepared on the basis of D-Optimal Mixture experimental design. They were assessed for droplet size (Y1) and % transmittance (Y2) after 100 times dilution with aqueous media (Table 2).

Data analysis and model evaluation

Since the variables in the mixture models are correlated, so partial least squares regression

was used to fit quadratic models. The component variables were scaled to unit variance and centered before analysis. The resulting models were assessed for the goodness of fit (R^2) and ANOVA analyses. Equation generated by the model was evaluated for the predicted batches. Once the validity of the models was established, the coefficients of the model terms and response surface contour plots were used for the analyses of the effect of mixture composition on the particles size and % Transmittance.

Maximum drug loading in optimized batch of microemulsion

Different batches of the microemulsion were formulated by adding specified quantity of drug in the increasing order and mixing properly. Each preparation was analysed for particle size, % transmittance and stability on dilution at initially and after 4 hr.

Optimization of Chitosan concentration for finalized microemulsion system

Many excipients like Carbopol, Polycarbophil, and Chitosan etc. were applicable for the preparation of the mucoadhesive microemulsion (MME) but chitosan provides added advantages like good solubility at acidic nasal pH which gave controlled gelling, less prone to precipitation and enhance drug transport across the nasal mucosa [14, 21-23]. Optimized microemulsion system was studied for the determination of optimum concentration of the chitosan. Different concentrations of chitosan solution (0.20, 0.25, 0.30, 0.35, 0.40 % W/W) were evaluated for the visual inspection on dilution, viscosity, globule size and % transmittance.

Characterization

Thermodynamic stability testing

To evaluate the thermodynamic stability, Modafinil loaded ME and MME were subjected to heating cooling cycle (4˚C and 45˚C), centrifugation test and freeze thaw cycle (-21˚C and +25˚C) [24]. Physical stability was continuously monitored throughout the experiment to study effect of formulation parameters on the drug stability in the drug loaded microemulsion and mucoadhesive microemulsion. Various aspects like phase separation, turbidity, particle size, zeta potential etc. at room temperature were observed [25,26].

Dilution stability

Stability of diluted drug loaded ME and MME were evaluated by dilution which was performed by diluting specified quantity of formulation with different dilution medium like water and sodium acetate buffer pH 5.0 at 100 times [26]. The diluted microemulsions were stored for 12 hours at room temperature and observed for any signs of phase separation, reported as stable or unstable.

Transmittance

Drug loaded ME and MME formulation (10, 50, 100 times) were diluted with distilled water and the % transmittance (% Ts) was measured at 650nm using UV spectrophotometer (UV 1700, Shimadzu, Japan) keeping distilled water as a blank [15, 19].

Globule size and zeta potential

Drug loaded ME and MME formulations were diluted 100 times with aqueous phase and evaluated for the droplet size, polydispersity index (PDI) and zeta potential of the dispersion was determined using clear disposable zeta cell using Malvern Zeta Sizer Nano ZS 90 (Malvern Instruments, Malvern, UK) [15,18,20].

Assay

Optimized formulation of drug loaded ME and MME systems were analyzed for the content of modafinil [15,18]. Here, 500 µl of drug loaded microemulsion and mucoadhesive microemulsion was diluted using methanol up to 2 ml, further 1 ml of this solution was diluted 10 times, again 1 ml of above solution was diluted upto 10 times with methanol and as per analytical method and amount of drug was determined by validated RP- HPLC method. Area was measured at absorption maxima of 220 nm in RP-HPLC [16, 27]. Test was performed in triplicate.

pH

The pH of drug loaded ME and MME systems were measured using pH meter (Electro Lab), both the systems were previously diluted with double distilled water. All the measurements were performed in triplicate and the results are expressed as mean ± SD.

Conductance

The conductivity measurements (Conductivity meter, Elco CM 180) help in determining whether diluted drug loaded ME and MME systems have oil-continuous or water-continuous phase. The solubilization of water phase in the selected oily mixture was monitored quantitatively by measuring the electrical conductivity (σ) using an electro-conductometer [18].

Table 1: Finalization of concentration range for Drug loaded Microemulsion formulation

Sr. No.	Class	Name of Excipients	Concentration Range (% W/W)	
			Low	High
1	Drug	Modafinil	25 mg (for the batch of 5 gm)	
2	Oil	Capmul MCM C8	5	8
3	Smix	Tween-80: PEG-400	47	58
4	Aqueous Phase	Sodium Acetate Buffer pH 5	37	45

Viscosity

The differentiation of low-viscous newtonian from non-newtonian flow is particularly important for undiluted drug loaded ME and MME. Filling of very low-viscous formulations into suitable container can lead to loss of filling mass due to splashing around the dosing nozzle of the machine. This typically increases the rate of leaking as well as the weight variability of the units. Viscosity was determined using Brookfield digital Viscometer, DV-1, Prime.

Cloud point

Modafinil loaded ME and MME were diluted with water in the ratio of 1:100, and the sample was placed in a water bath with the temperature increasing gradually, spectro-photometric analysis was carried out to measure % transmittance of the sample at every gradual increment of the temperature up to 85 °C.

Histopathology study

The nasal-cavity mucosa of a sheep was obtained from the local slaughter house. Within 15 min of the sacrifice of the animal, the nasal cavity was fully exposed by a longitudinal incision through the lateral wall of the nose while avoiding the damage of the septum. Following, the mucosa was carefully removed and immediately immersed in 900 ml of ice-cold Ringer's solution for 15 to 30 min. Six sheep nasal mucosa with uniform thickness were mounted on the Franz diffusion cell [22-24].

One mucosa was treated with 0.5 ml PBS 6.4, another one was treated with isopropyl alcohol and remaining with microemulsion and mucoadhesive microemulsion for 1 hr. After 1 hr the mucosa were rinsed with PBS pH 6.4 and carried to the pathological laboratory in 10% formalin for the preparation of slides. The sheep nasal mucosa treated with PBS pH 6.4 and isopropyl alcohol taken as negative and positive control respectively. The prepared pathological slides were studied under optical microscope for any sign of toxicity of the cells and finally it was evaluated by comparing the results of drug loaded products with positive and negative group.

Transmission Electron Microscopy

The morphology of MME systems were investigated using Transmission electron microscopy (TEM) [18,19]. TEM analysis was performed using a Transmission electron microscope (Jeol, JEM - 1011). Briefly, it was carried out by operating at acceleration voltage of 100kv. The formulation was diluted up to 50 times with distilled water for the analysis. Approximately 2 min after sample deposition (1-2 µl), the carbon-coated copper grid (300 mesh, 3mm) was tapped with filter paper to remove surface water and air dried. The image was taken with Transmission Electron Microscope.

***In-Vitro* Drug release study by diffusion through dialysis bag/ sac**

Previously activated dialysis sac was thoroughly washed with water, then it was filled with 1% SLS (sodium lauryl sulphate) and examined for leaks and again washed (28).The *in vitro* release for drug suspension, drug loaded ME and MME was estimated. In-vitro drug release studies were performed using a Franz diffusion cell at 37.0 ± 0.5 °C for 4hr. Dialysis membrane was presoaked in simulated nasal fluid for 15 min [29]. Presoaked membrane was mounted between the donor and receiver compartment of the diffusion cell and clamped into position [30].Formulation for analysis (0.5ml containing 4mg drug) was uniformly spread over the membrane from the donor compartment side and additionally 1.5 ml sodium acetate buffer pH 5 was added. The reservoir fluid 20ml (simulated nasal fluid) was maintained in the receiver compartment at 37.0 ± 0.5°C. Samples (1ml) were withdrawn from the receiver compartment after every time point (15, 30, 45, 60, 90, 120, 180, 240 min) and replaced by an equivalent amount of temperature (37.0 ± 0.5°C) equilibrated fresh media. The samples

were analyzed, after adequate dilutions, by RP-HPLC method at 220nm.

***Ex-vivo* Drug permeability study by using isolated sheep nasal mucosa**

The freshly excised sheep nasal mucosa was collected from the slaughter house in PBS PH 6.4. The membrane was identified and separated from the nasal mucosa [18]. The excised nasal membrane was stabilized on Franz diffusion cell with 12 mm diameter in a way that the nasal membrane surface just flushes the diffusion fluid.Each0.5 ml of drug loaded ME, MME and drug suspension were placed in the donor compartment of Franz diffusion cell along with 1.5 ml of sodium acetate buffer pH 5 added additionally [19]. Receptor compartment containing stimulated nasal fluid was stirred with teflon coated magnetic stirrer at 37 ± 1^0C. Samples from the receptor compartment were withdrawn at predetermined time intervals of 15, 30, 45, 60, 90, 120, 180, 240 min and replaced by an equal volume of acetate buffer PH 5. The samples were analyzed by RP-HPLC method at 220nm as mentioned earlier. The experiment was performed in triplicate. The mean cumulative values for % drug release and the diffusion and permeability coefficient for the different formulations were calculated.

Determination of diffusion coefficient (D)

$$D = Kp * h / K$$

Where, D is the diffusion coefficient,
K is the octanol / PBS partition coefficient
h is the thickness of the biological membrane

Permeability coefficient (Kp) is the determined by,

$$Kp = Jss / Cd$$

Where, Kprepresents permeability coefficient,
Jss is the steady-state flux = Slope of the graph of Cumulative permeation (μg/h.cm^2) vs Time (hr)
Cd is the initial concentration of drug in donor compartment = 4000 μg /cm

Stability Study

Both the formulations were filled into amber colored glass containers and subjected to stability studies at different conditions like 25°C ± 2°C, 60%± 5% RH and 40°C ± 2°C,75%± 5 % RH. Samples were withdrawn at interval of every month for analysis over a period of 3 months [31,32]. Drug content was analyzed using a previously developed and validated stability-indicating RP-HPLC method and subjected for different characterization parameters pH, Assay, Zeta potential, Globule Size and % Transmittance.

RESULT AND DISCUSSION

Nasal delivery of microemulsion is the one of the most promising approach for the effective delivery of CNS acting drugs. Keeping these criteria in mind, the study was designed in such a way that the results can ensure the predetermined behavior of the drug delivery system in vivo. Here, non-ionic surfactants used with chitosan act as a permeation enhancer and improve the contact time with nasal mucosa which were used in the study since they are known to be less affected by pH and changes in ionic strength.

Solubility Study

Results from solubility studies are reported in Figure 1. As seen in the figure, Clove oil, Capmul MCM C8, Tween-80, Tween-20 and PEG-400 showed the highest solubilization capacity for Modafinil. Here, clove oil leads to nasal irritation and/or nasal toxicity, therefore Capmul MCM C8 was selected as oil. Tween-80 and Tween-20 doesn't show any significant differences but Tween-80 shows opening of tight junction in the nasal mucosa which will improve the drug diffusion profile ofthe preparation. The rest of the excipient was PEG-400 selected as Co-surfactant. The components used in the system should have high solubilization capacity for the drug as well as they must ensure the solubilization of the drug in the dispersion.

On the basis of the solubility study of Modafinil, selected excipients were usedto construct pseudo ternary phase diagram. Capmul MCM C8, Tween-80 and PEG-400 were selected as oil, surfactant and co-surfactant respectively, while sodium acetate buffer pH 5.0 was selected as an aqueous phase. The diagram (Figure 2) showshigh region (black colour) which suggest formation of stable microemulsion. Higher monophasic region in phase diagram was found for the Smix/Km ratio 3:1 and 2:1 than 1:1, shows stable microemulsion formulation. However, Km 3:1 was with higher concentration of surfactant leads to nasal irritation and other side effects. Here Km 3:1 shows lesser concentration of co-surfactant while drug has maximum solubility in the co-surfactant. So, to impart more drugs loading Km ratio 2:1 was selected for further analysis.

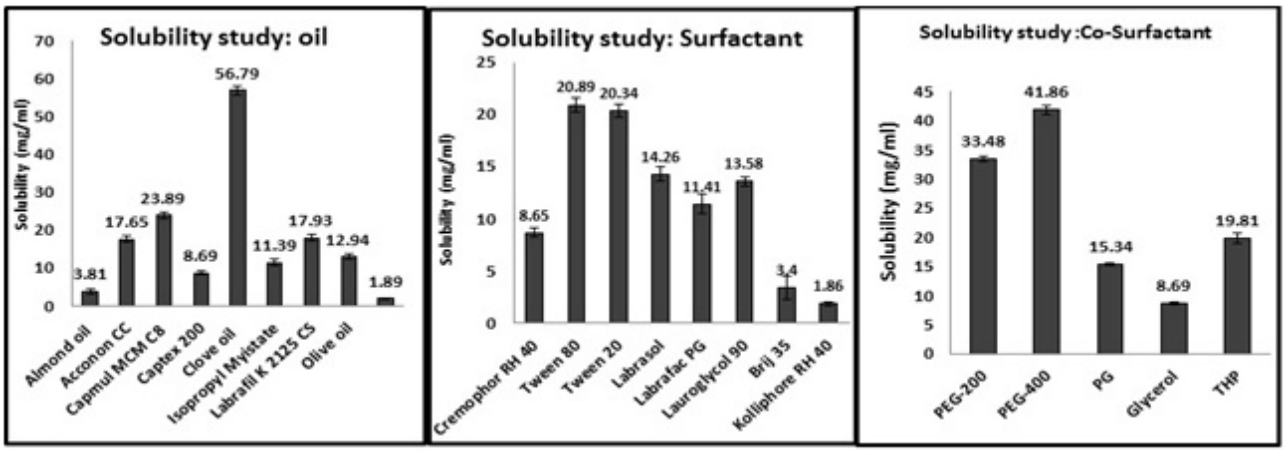

Figure 1: Solubility Study of Modafinil in different excipients (oil, surfactant and co-surfactant)

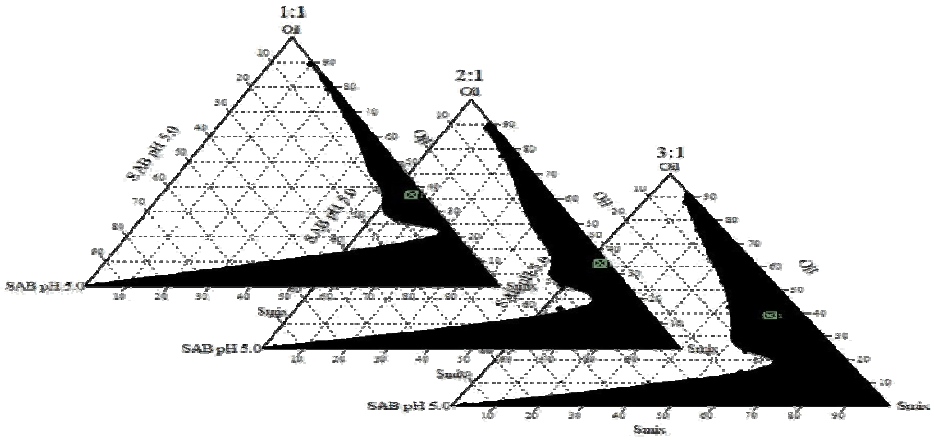

Figure 2: Pseudo Ternary Phase Diagram at different Km value

Table 2: D-optimal mixture experimental design for the optimization of microemulsion

Exp. Run	X1(%)	X2 (%)	X3(%)	Observed Value		Predicted Value		Residual Value	
				Y1 (nm)	Y2 (%)	Y1(nm)	Y2 (%)	Y1 (nm)	Y2 (%)
1	5.00	57.997	37.002	48.16	99.34	48.05	99.22	0.11	0.12
2	5.063	49.936	45.00	52.61	98.49	53.04	98.23	-0.43	0.26
3	7.359	54.157	38.483	63.2	96.93	65.63	96.88	-2.43	0.05
4	5.00	55.111	39.888	50.08	99.11	50.39	99.33	-0.31	-0.22
5	7.645	49.486	42.867	66.63	96.31	67.07	95.71	-0.44	0.6
6	7.988	50.664	41.346	68.28	96.32	68.64	95.97	-0.36	0.35
7	6.749	56.250	37.00	57.33	97.38	57.62	97.39	-0.29	-0.01
8	5.864	50.888	43.24	56.89	97.72	56.61	97.59	0.28	0.13
9	8.00	52.064	39.935	68.63	96.32	67.95	96.23	0.68	0.09
10	5.0631	49.936	45.00	53.42	98.06	53.04	98.24	0.38	-0.18
11	7.9979	47.002	45.00	70.12	94.32	70.02	94.51	0.1	-0.19
12	8.00	52.064	39.935	67.61	95.74	67.95	96.23	-0.34	-0.49
13	6.249	52.182	41.567	58.21	97.18	28.81	95.19	29.4	1.99
14	7.9973	47.002	45.00	70.12	94.32	70.02	94.51	0.1	-0.19
15	6.749	56.250	37	57.33	97.38	57.62	97.39	-0.29	-0.01
16	5.00	57.997	37.002	48.16	99.34	48.05	99.22	0.11	0.12

Formulation Optimization of Drug Loaded Microemulsion System

From the results of phase diagrams, Capmul MCM C8, Tween-80, PEG-400 and Sodium acetate buffer pH 5.0 were finalized as oil, surfactant, co-surfactant and aqueous phase respectively. MEwas prepared incorporating 25 mg drug with the use of selected formulation ingredients. As shown in Table 2, sixteen batches with 5 center points were prepared by using design expert software and evaluated.

Optimization of Drug Loaded Microemulsion

Experimental designs were used as a systematic approach to simultaneously identify excipients and the ratio of the selected excipients. Furthermore, concentration of the excipients was selected based on (pseudo) ternary mixture designs which were demonstrated in Table 1 and batch design from Design Expert V 7.0 were shown in Table 2. Here, statistical design was used to study the combined effect of them on the mean globule size (nm) and % Transmittance of the formulation.

The mean globule size was ranged between 48.16 to 70.12 nm, which indicates that the response was sensitive towards the studied factor. To identify the significant parameters and their interactions, analysis of variance was performed for each parameter. The values of the coefficients of X1, X2 and X3 are related to the effect of these variables on the response. The equation for droplet size was as follows;

Y1= 33.01661*X1 - 0.68518*X2 + 1.0141*X3 - 0.8343*X1*X2 -0.3136*X1*X3+0.04513*X2*X3

From the Table 3, P value less than 0.05 of ANOVA, it can be inferred that the interaction term AB (X1.X2) and BC (X2.X3) have significant effect on the mean droplet size. From the 3D surface plot in figure 3A; amount of Capmul MCM C8 was found to have directly proportional relationship with the mean droplet size while its combination effect with surfactant and co-surfactant shows positive impact. Combination effect of X2.X3 shows antagonistic effect on the particle size means as their concentration increases particle size decreases.

Transmittance was ranged between 94.32-99.34%, which indicates that the response was sensitive towards the studied factor. To identify the significant parameters and their interactions, analysis of variance was performed for each parameter. The values of the coefficients of X1, X2 and X3 are related to the effect of these variables on the response. The equation for % Transmittance was as follows;

Y2= 11.9831*X1 + 0.5447*X2 + 0.13236*X3 - 0.12173*X1*X2 -0.13912*X1*X3 0.02976*X2*X3

Table 3: p-Value for the ANOVA analysis of different parameters

Source	p-value	
	Globule Size	% Transmittance
Model	< 0.0001	< 0.0001
Linear Mixture	< 0.0001	< 0.0001
AB	0.0477	0.2753
AC	0.1046	0.2741
BC	0.0285	0.0504
Lack of Fit	0.3758	0.1030

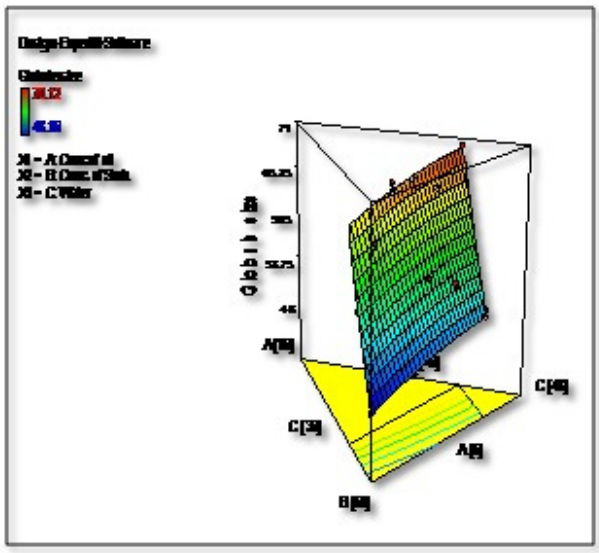

Figure 3(A): 3D plot for particle size

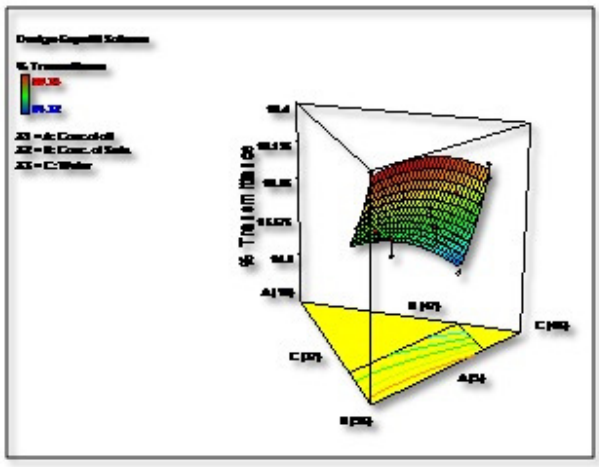

Figure 3(B): 3D plot for % transmittance

Table 4: Predicted batch of analysis for the microemulsion within design space

Sr. No.	Parameters	Predicted	Observed	Residual difference
1	**Globule size(nm)**	66.343	66.47 ± 0.02	-0.127
	%Transmittance	95.5161	95.37 ± 0.09	0.1461
2	**Globule size(nm)**	54.5609	54.51 ± 0.04	-0.0509
	%Transmittance	97.7918	97.63 ± 0.09	0.1618
3	**Globule size(nm)**	49.2809	49.14 ± 0.03	0.1409
	%Transmittance	99.0549	99.09± 0.12	-0.0351

Table 5: Variables for desirability plot and goals for response

Variables	Goal	Limit (%W/W)	Limit (%W/W)
A: Oil	In range	5.0	10.0
B: Smix	In range	47.00	57.99
C: Sodium Acetate Buffer pH 5.0	In range	37	45.0
Globule size	Minimize	48.16	60
%Transmittance	Maximize	98	99.34

Table 6: Desirability Plot for Optimization of the microemulsion

Exp. Run	X1	X2	X3	Particle size (nm)	% T	Desirability
1	5.00	57.584	37.416	48.040	99.2809	0.991

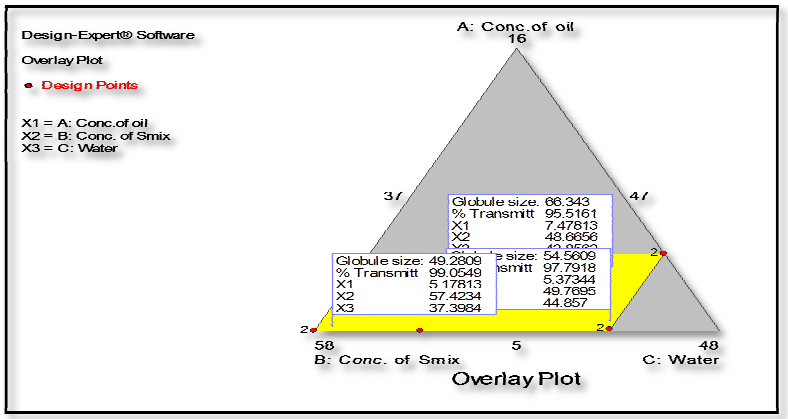

Figure 4: Overlay plot of experimental design

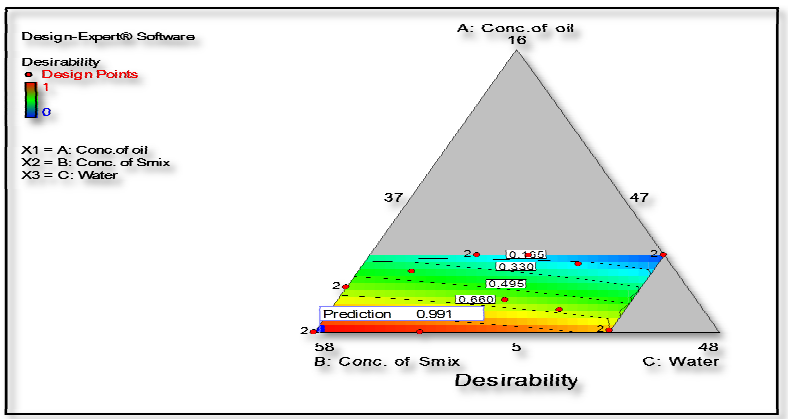

Figure 5: Desirability plot for optimization of microemulsion

It was found that variables (X1, X2 and X3) and their interactions (X2.X3) had significant effect on response Y2. For interaction term P value less than 0.05 of ANOVA suggest that X2.X3 has significant effect on % Transmittance. From the 3D surface plot in figure 3B; X1, amount of Capmul MCM C8 was found to have negative effect, suggesting an indirect proportional relationship with % Transmittance. Its combination effect like oil with Smix and water shows negative. It means that high concentration of oil provides microemulsion system with less % Transmittance in correlation. Surfactant and co-surfactant shows positive and direct proportional relationship with % Transmittance means it increases % T.

Overlay plot for check point analysis

Experimental design was used for multiple responses Y1: Globule Size and Y2: % Transmittance, it is necessary to obtain a region that provides optimum values of factors. Overlay plot (Fig. 4) can be obtained by superimposing contour plots of responses Y1 and Y2 which displays the area of feasible response values in the factor space. The region highlighted in yellow color is the area in which a slight variation in the critical variables won't affect the final response and the response will be in desired range. Regions that do not fit the optimization criteria are shaded gray while design space which is accepted colored yellow. Flags can be placed in overlay plot that shows predicted values of desired response with optimized values of variables. Table 4 shows there is no significant difference between predicted and experimental value of each of particle size and % transmittance of the same formulation.

Desirability Study for the Optimization of Modafinil Loaded Microemulsion

Further optimization of microemulsion was based on the desirability which may range from 0 to1 based on the more to less particle size or one can consider less to more %Transmittance. Based on the maximum desirability, one formulation (desirability = 1.0) was chosen for confirmation and further optimization of Modafinil microemulsion as shown in Fig 5. Parameters for the desirability batch were shown in Table 5 and desirable batch is shown in table 6. Evaluation of desirability batch shows in Table 7.

Microemulsion was formulated 3 times repeatedly with above stated factor. Residual error was calculated which is significantly lower for both the response which indicate ruggedness of mathematical model and high prognostic ability of the experimental design. The formula for optimized batch is shown in Table 8.

Table 7: Evaluation of desirability batch

Response	Experiment value	Predicted value	Residual Difference
Y1	47.49 ± 0.31	48.04	-0.55
Y2	99.20 ± 0.07	99.2809	0.08

Table 8: Optimized batch of drug loaded microemulsion

Sr. No.	Component	Optimized concentration
1	Drug: Modafinil	25 mg
2	Oil: Capmul MCM C8	5 % W/W
3	Smix (2:1): Tween-80 and PEG-400	57.584 % W/W
4	Aqueous Phase: Sodium Acetate Buffer pH 5.0	37.416 % W/W

Maximum drug loading in optimized batch of microemulsion

Different batches of microemulsion were formulated and specified quantity of drug in the increasing order was added in each batch. Particle size of drug loaded microemulsion was analysed initially while % transmittance and stability on dilution (100times) were determined initially and after 4 hr.

From Table 9 it can be understood that drug loading doesn't affect particle size as a main responsive factor. Effect of drug loading evaluated for dilution stability illustrate up to addition of 40 mg of drug (batch F4) dilution of microemulsion remained stable but on the addition of 45 mg of Modafinil in microemulsion, it was unstable on dilution within the time duration of 4 hr. So, F4 batch with drug loading efficiency of 40 mg was selected for further optimization of addition of mucoadhesive agent.

Optimization of Chitosan Concentration for MME: Batch F4 with drug loading 40mg and aqueous phase sodium acetate buffer pH 5.0 was further optimized for amount of mucoadhesive agent by addition of different concentration of chitosan. Chitosan has dual advantage that it can act as a mucoadhesive agent as well as permeation enhancer too. Different batches were prepared to optimize its concentration by analyzing particle size, % transmittance and dilution stability.

Table 9: Effect of Drug Loading on Stability of the Microemulsion System

Batch. No.	Drug Loading (mg)/ 5 gm	Particle Size (nm)	% Transmittance		Stability on Dilution (100 times)	
			Initially	After 4 hr	Initially	After 4 hr
F1	25	47.49±0.31	99.20±0.07	99.14±0.09	Stable	Stable
F2	30	47.63±0.28	99.18±0.09	99.18±0.11	Stable	Stable
F3	35	47.39±0.32	99.29±0.04	99.17±0.02	Stable	Stable
F4	**40**	**47.75±0.26**	**99.06±0.07**	**99.11±0.03**	**Stable**	**Stable**
F5	45	48.09±0.42	99.21±0.03	91.37±1.27	Stable	Precipitation

Table 10: Optimization of chitosan concentration in F4 batch

Sr. No.	% of Chitosan	Particle Size (nm)	% Transmittance	Stability on Dilution (100 times)	
			Initially	**Initially**	**After 4 hr**
1.	0.0	47.75±0.26	99.20±0.07	Stable	Stable
2.	0.2	53.15±0.24	99.14±0.09	Stable	Stable
3.	0.25	57.94±0.31	99.09±0.07	Stable	Stable
4.	**0.3**	**59.81±0.24**	**98.89±0.03**	**Stable**	**Stable**
5.	0.35	65.94±0.26	96.16±0.19	Stable	Haziness
6.	0.40	69.37±0.19	94.21±0.11	Haziness	----

Table 11: Optimized formula for Drug loaded Microemulsion and Mucoadhesive Microemulsion

Sr. No.	Ingredient	Drug loaded ME	Drug loaded MME
1	Drug	40 mg	40 mg
2	Capmul MCM C8	5 % W/W	5 % W/W
3	Smix (Tween-80 and PEG-400) 2:1	57.58 (38.386 : 19.193) % W/W	57.58 (38.386 : 19.193) % W/W
4	Sodium acetate buffer pH 5.0	37.416 % W/W	37.416 % W/W
5	Chitosan	-----	0.3 % W/W

Table 12: Thermodynamic Stability Testing for Drug Loaded Microemulsion and Mucoadhesive Microemulsion

Sr. No.	Test	Observation		Inference
		Drug loaded ME	**Drug loaded MME**	
1	Heating Cooling Cycle	Remain Clear without any sign of turbidity	Remain Clear without any sign of turbidity	Both were Stable
2	Centrifugation Test	No phase separation	No phase separation	Both were Stable
3	Freeze Thaw Stress Testing	Formulation doesn't produce any precipitation or color change	Formulation doesn't produce any precipitation or color change	Both were Stable

Table 13: Effect of different dilution medium (Dilution Factor 100) on ME systems

Sr. No.	Solvent	Drug loaded ME			MME		
		% T	**Globule Size (nm)**	**PDI**	**% T**	**Globule Size (nm)**	**PDI**
1	Distilled water	99.16 ± 0.12	47.59 ± 0.19	0.165 ± 0.07	98.27 ± 0.22	57.09 ± 0.24	0.176 ± 0.13
2	Sodium Acetate pH 5.0	99.20 ± 0.14	47.75 ± 0.26	0.234 ± 0.05	98.89 ± 0.03	59.81 ± 0.24	0.219 ± 0.18

Table 14: % Transmittance for 100 times diluted microemulsion systems

Sr. No.	Dilution Factor (with sodium acetate buffer pH 5)	% Transmittance	
		Drug loaded ME	MME
1	10 times	97.24 ± 0.17	96.67 ± 0.13
2	50 times	98.12 ± 0.19	98.02 ± 0.14
3	100 times	99.20 ± 0.14	98.89 ± 0.03

Table 15: Viscosity of the ME and MME before and after dilution

Sr. No.	Content	Viscosity (cP)	
		ME	MME
1	Initially	968.21 ± 2.36 cP	1190.21 ± 3.67 cP
2	After 10 times dilution	8.74 ± 0.75 cP	21.86 ± 0.42 cP
3	After 100 times dilution	0.77 ± 0.27 cP	2.34 ± 0.19 cP

Table 16: Diffusion Co-Efficient

Sr. No.	Formulation	Permeation Co-Efficient (µg/hr.cm^2)	Diffusion Co-Efficient (µg/hr.cm)
1.	Drug Suspension	0.26008	0.04601
2.	Drug loaded Microemulsion	0.3613135	0.06508
3.	Drug loaded MucoadhesiveMicroemulsion	0.50851	0.09002

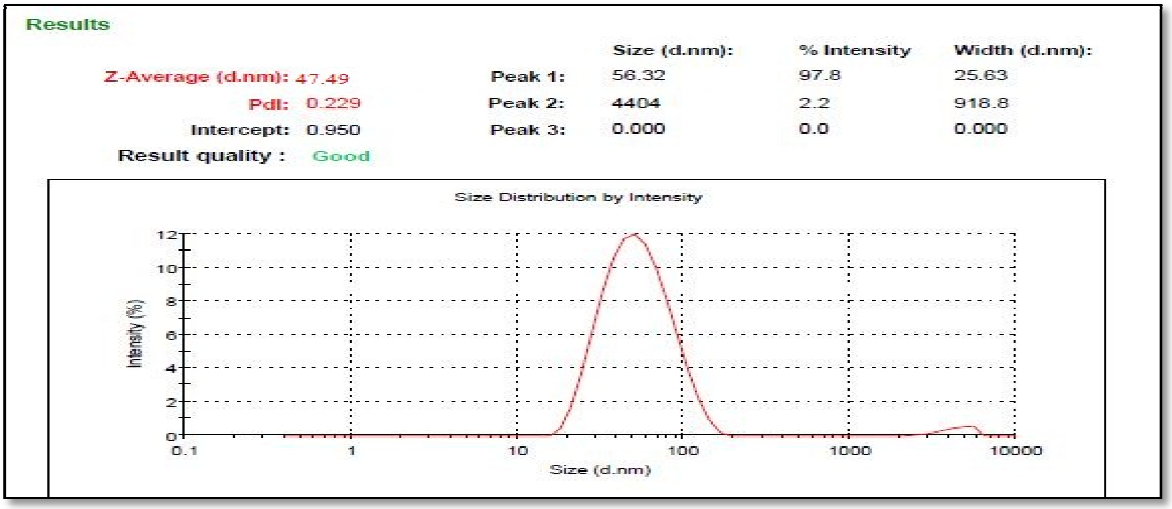

Figure 6(A): Particle size of Modafinil Loaded Microemulsion (ME)

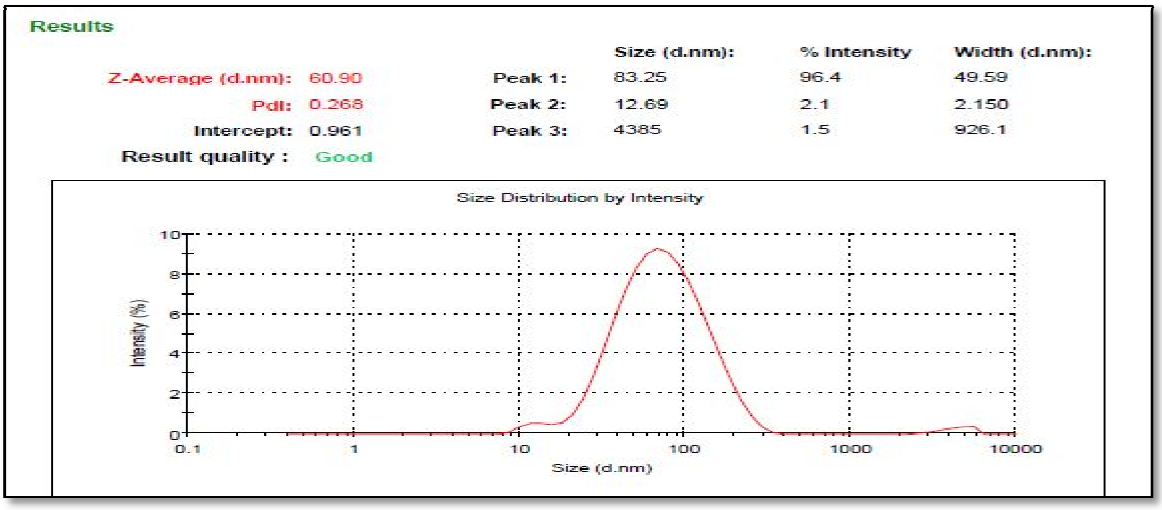

Figure 6(B): Particle size of Modafinil Loaded Mucoadhesive Microemulsion (MME)

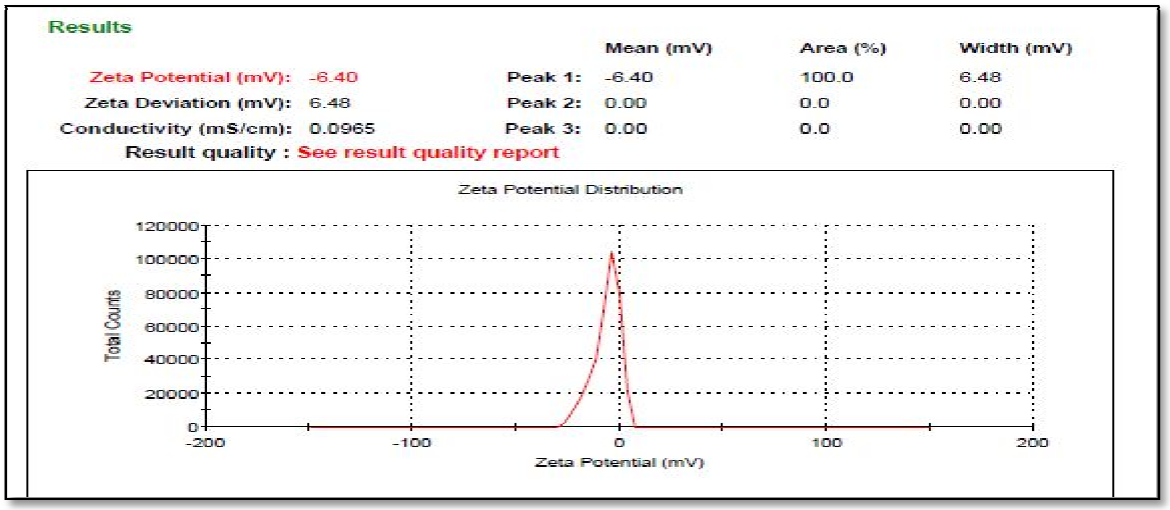

Figure 7(A): Zeta Potential of Modafinil Loaded Microemulsion (ME)

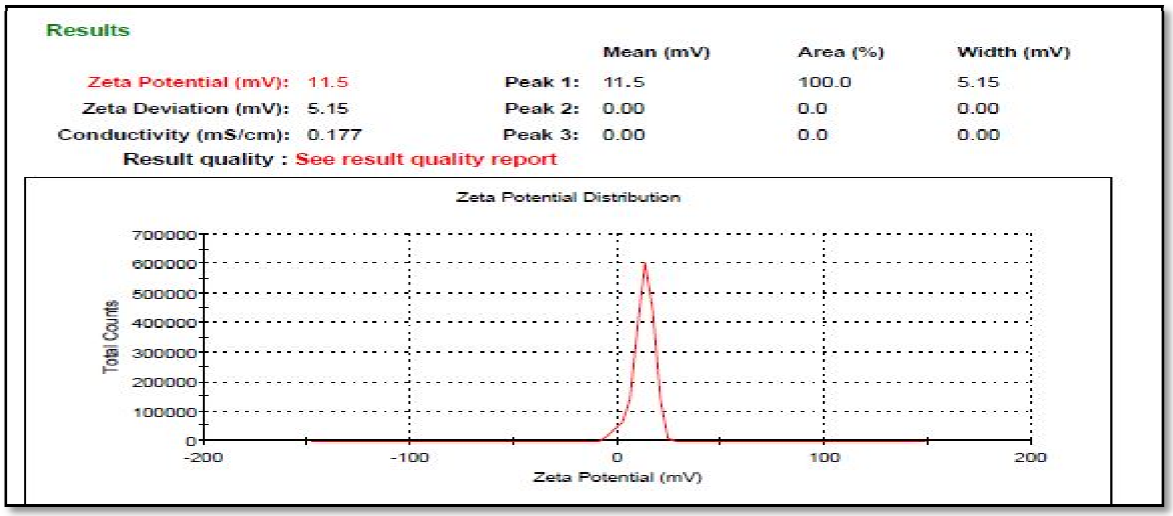

Figure 7(B): Zeta Potential of Modafinil Loaded MucoadhesiveMicroemulsion (MME)

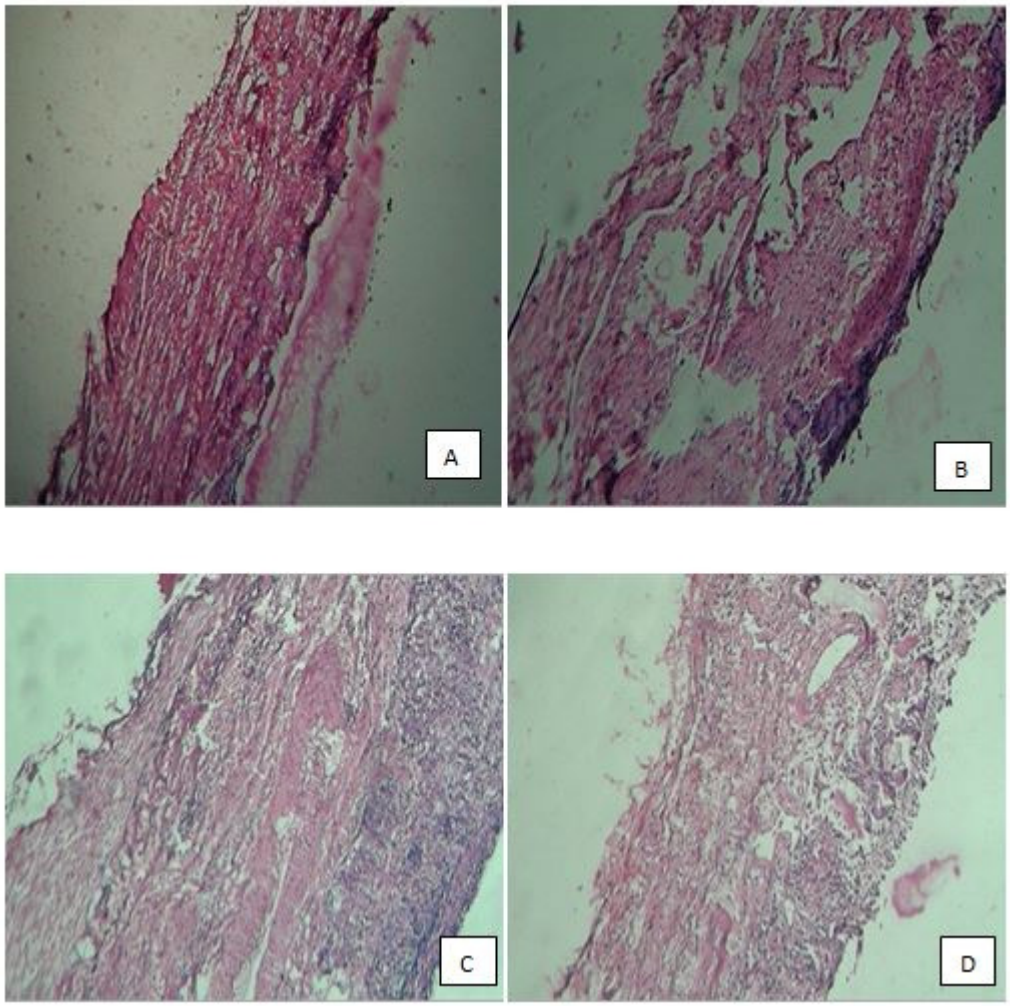

Figure 8: Histopathology study for evaluation of Nasal Toxicity
(Negative control-A, Positive control-B, Drug loaded microemulsion-C, Mucoadhesive microemulsion-D)

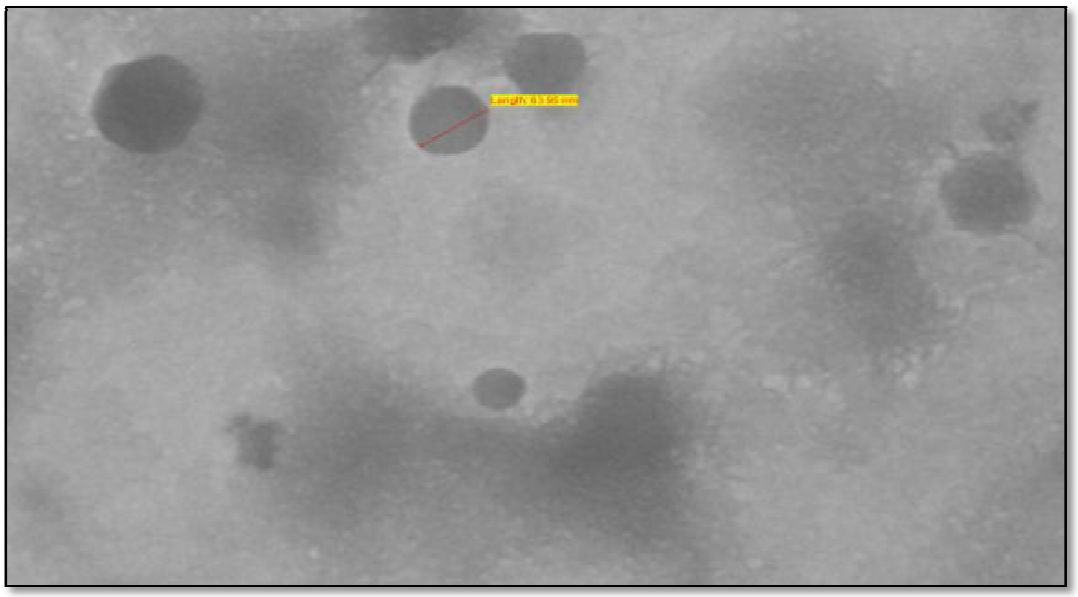

Figure 9: TEM image of the Mucoadhesive Microemulsion of Modafinil

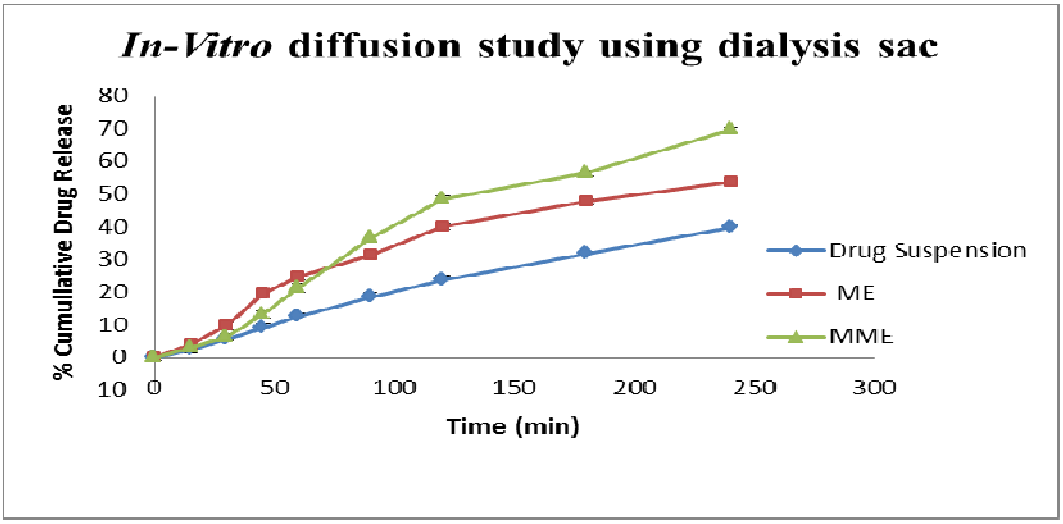

Figure 10: *In-vitro* diffusion profiles of drug suspension, drug loaded ME and MME

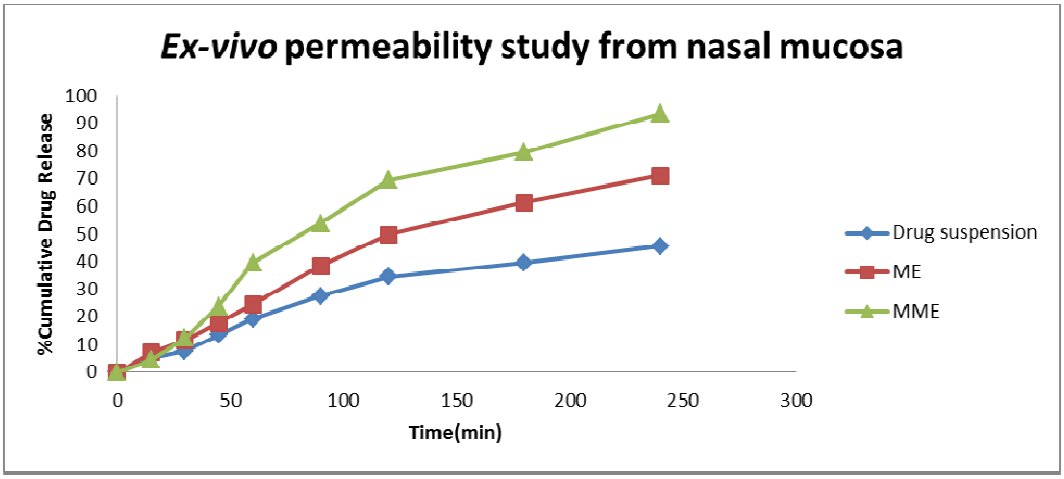

Figure 11: *Ex-vivo* drug permeation profiles for drug suspension, Drug loaded ME and MME using sheep nasal mucosa

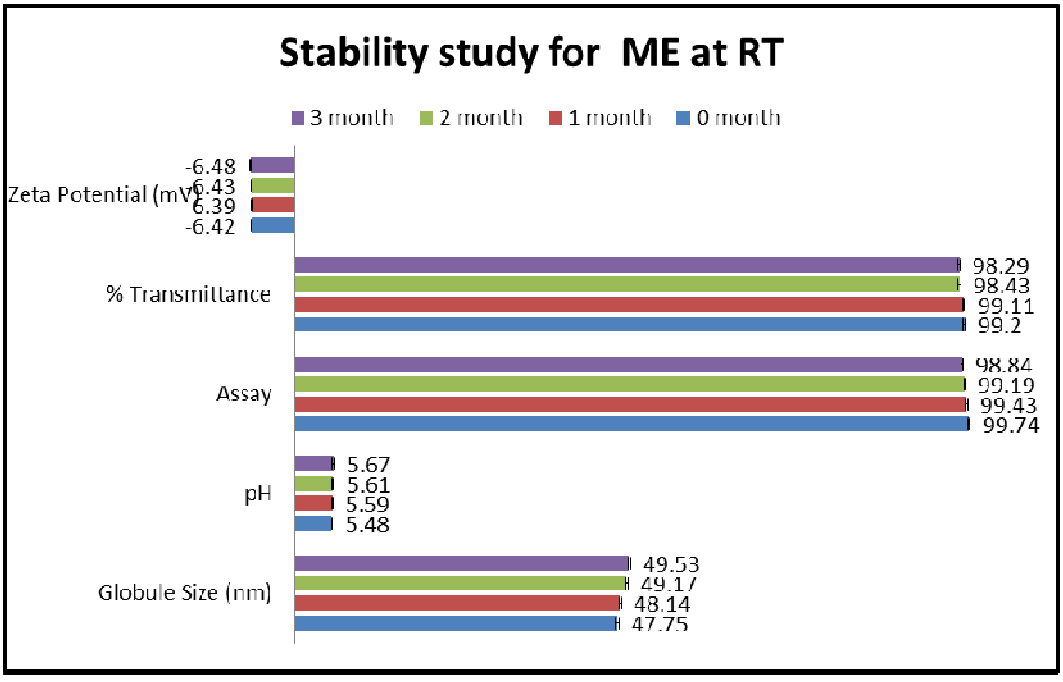

Figure 12(A): Stability study of Drug loaded ME at Room Temperature

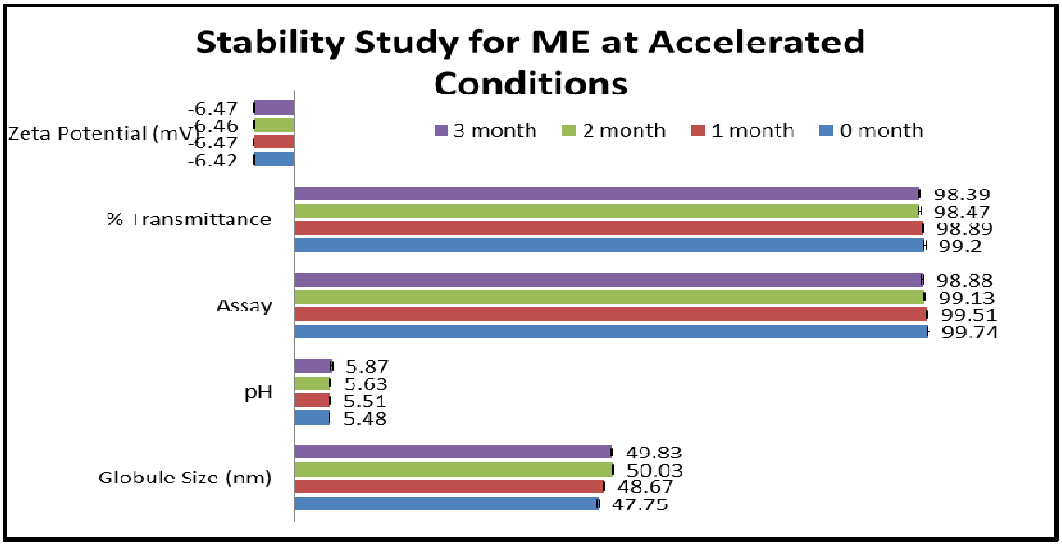

Figure 12(B): Stability study of Drug loaded ME at Accelerated Conditions

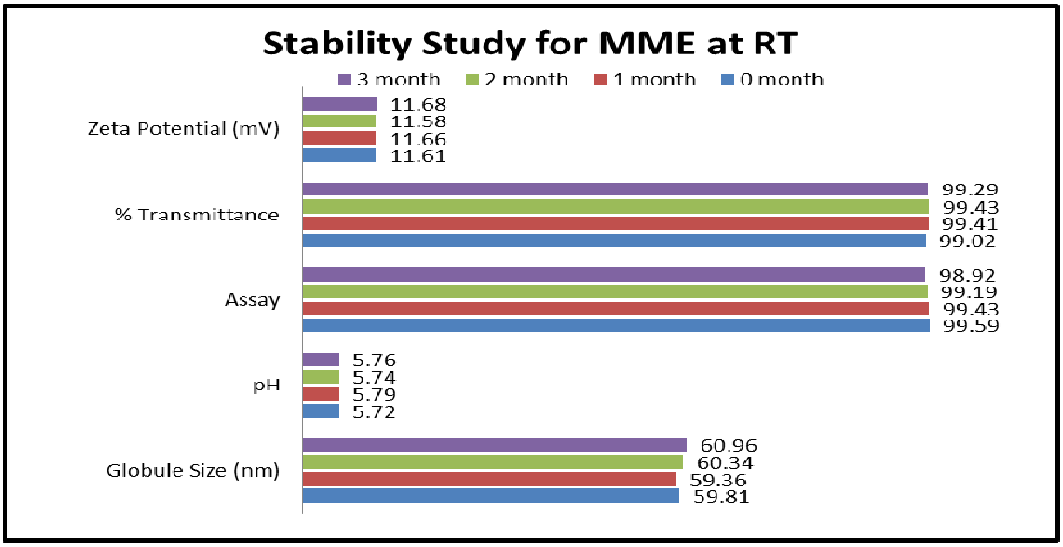

Figure 13(A): Stability study of MME at Room Temperature

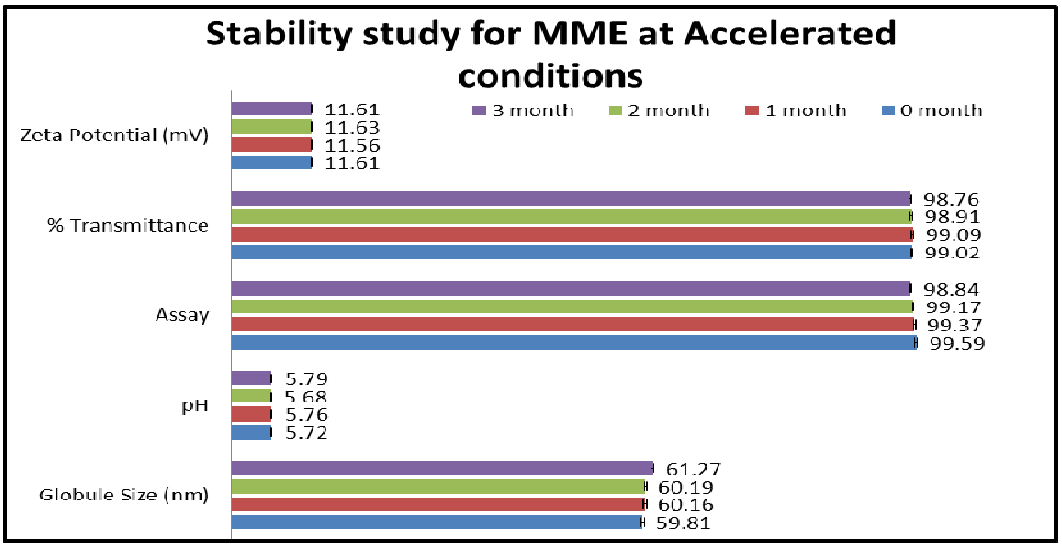

Figure 13(B): Stability study of MME at Accelerated Conditions

From Table 10 it can be concluded that on dilution of mucoadhesive formulation having concentration of chitosan more than 0.3%doesn't formulate stable microemulsion system. As the concentration of chitosan increases particle size also increased, % transmission lowered and showed instability (haziness) on 100 times dilution. From the result obtained, batch F4 with chitosan concentration of 0.3% was selected as a finalized batch for the further characterization of the formulation.

Optimized Formula for Drug Loaded Microemulsion and Mucoadhesive Microemulsion System:

In Table11 optimized formula for drug loaded microemulsion and mucoadhesive microemulsion are shown with excipients/ingredients.

Characterization

Thermodynamic Stability Testing

It was observed that formulation passed the heating cooling cycle test therefore it was further exposed to centrifugation which does not show any phase separation hence, it was taken for freeze thaw stress test. After freeze thaw stress test, it was found that both the formulations showed good stability with no phase separation, creaming/cracking or any precipitation of the particles. During thermodynamic study, there was not any phase separation or precipitation of drug occurred. It indicated good stability of optimized formulations of drug loaded ME and MME at different temperature condition as shown in Table 12.

Small globule size of the formulation enhances the permeation through the mucus membrane and provides larger surface area by the same for drug absorption. The Z-average size of the resultant 100 times diluted drug loaded ME and MME were 47.75±0.26 nm and 59.81±0.24 nm with PDI value of 0.234±0.05 and 0.272±0.04 respectively in Figure 6(A) and 6(B), indicating that the system had narrow size distribution with very good uniformity of the particle size.

The Zeta potential of the 100 times diluted drug loaded ME and MME systems were found to be -6.42 ± 0.02 (Figure 7(A)) and 11.61 ± 0.04 mV (Figure 7(B)) respectively. The Zeta potential indicates the degree of repulsion between adjacent and similarly charged particles in dispersion.. The zeta potential values were found to be indicating good stability of formulation.

Robustness to dilution

Drug loaded ME and MME formulations of modafinil were evaluated by diluting specified quantity of formulation at dilution factor 100 times with distilled water an sodium acetate buffer pH 5.0;it was found to be robust to dilution and doesn't show any sign of precipitation or turbidity. No precipitation of the drug or phase separation was observed even on keeping the sample for 12 hours at room temperature. This revels that optimized formulation was stable on dilution for 12 hrs shown in Table 13 for 100 times diluted drug loaded ME and MME.

pH:

The excipients used in the formulation decide the pH of the final preparation. Change in pH may change zeta potential of formulation which may affect the stability of preparation. Optimized drug loaded microemulsion ME and MME formulation shows pH 5.48±0.07 and 5.72±0.09. Here pH is an important parameter for nasal administration. Formulations with neutral pH may enhance chances of microbial infection of nasal cavity because at less acidic pH, lysozymes of nasal cavity became inactive. So pH adjustment was done with sodium acetate buffer pH 5.

% Transmittance Measurement

The value of % Transmittance of 10, 50, 100 times diluted (Sodium acetate buffer pH 5.0) drug loaded ME and MME was found to be nearer to 100 %, indicates that the system is optically clear which a primary requirement for microemulsion as shown in Table 14.

Conductance

Conductivity measurements provide a means of determining whether the diluted drug loaded ME and MME shows Acetate Buffer pH 5.0 as a continuous phase. Conductivity value of optimized ME and MME formulation was found to be 96.21 ± 0.37 µS/cm and 177.63 ± 0.68 µS/cm respectively.

Viscosity Measurement

Viscosity of the Modafinil ME and MME was measured by using Brookfield viscometer at 25 °C temperature. Spindle 64 was selected and rotated at 100 rpm for measurement of viscosity before and after dilution with aqueous phase (10 and 100 times dilution). However, the viscosity of the system gradually decreased with an increase in the aqueous phase (Table 15).

Cloud point measurement

The cloud point is the temperature above which the formulation clarity turns into cloudiness. It is an essential factor in the ME and MME consisting of nonionic surfactants, and responsible for the successful formation of a stable *in-vivo* microemulsion. When the temperature is higher than the cloud point, an irreversible phase separation will occur and it will lead to decreases drug adsorption, because of the dehydration of the polyethylene oxide moiety. Hence, the cloud point for drug loaded ME and MME must be above 37°C, which will avoid these problems. The cloud point of optimized drug loaded ME formulation was found to be 72 ± 2 °C at which % Transmittance was 78.37 ± 0.87 % and for MME formulation it was found to be 68 ± 3 °C at which % Transmittance was 74.29 ± 0.87 %. Therefore, it would suggest a stable microemulsion formed on *in-vivo* administration for the both the microemulsion system.

Assay

Assay of the drug loaded ME and MME gives the amount of drug present in the formulation. Ideally the amount of the drug content in the optimized formulation should be in the range of 95 ± 105 %. Thus, from the result it can be concluded that the optimized formulation of drug loaded ME and MME, which contain drug content 99.74 ± 0.13 % and 99.59 ± 0.07 % respectively were in the limit as per required.

Histopathology Study

The prepared formulations (drug loaded ME and MME) were subjected to nasal toxicity study to evaluate the safety of the ingredients used in the formulation. The optical microscopy images of nasal mucosa treated with formulations are shown in Figure 8.

The nasal mucosa treated with PBS pH 6.4 showed intact epithelial layer without any damage while mucosa treated with isopropyl alcohol (mucociliary toxic agent-positive control) showed destruction of the epithelial layer and other nasal tissues. The prepared formulations were subjected to nasal toxicity to study the safety profile of the ingredients used in the formulation. The nasal mucosa treated with test preparation shows no damage to the mucosal layer. So, it can be predicted that prepared formulation seems to be safe for nasal administration.

Trannsmission electron microscopy

The morphology for 100 times diluted MME, was studied using TEM. The TEM image is shown in Figure9. The microemulsion seems to have uniformly distributed spherical drug loaded globules. No aggregation of the globules indicates the stability of the system. The globule size seemed to be in agreement with the result obtained from globule size analysis using Zetasizer. These images show that it has a uniform size and possess smooth surface morphology.

***In vitro* drug release study**

The % release of drug was calculated against time and plotted the graph and whole experiment was performed in the triplicate. *In-vitro* diffusion profile was studied by diluting 0.5 ml of formulation up to 2 ml using sodium acetate buffer pH 5.0 and 2 ml of this diluted preparation (20mg/2ml) was added on the diffusion sac and bath temperature was maintained at 37±0.5°C.

In vitro drug diffusion studies for drug suspension, drug loaded ME and MME are shown in Figure 10; provides predictive approaches for release pattern of Modafinil from drug suspension, drug loaded ME and MME; reveal that release of Modafinil from drug suspension was very less (39.658 ± 0.964 %) as compared to drug loaded ME (53.6413 ± 1.06) and MME (69.618 ± 1.035) formulations in 4 hr. MME shows somewhat steady drug release pattern than ME.

***Ex-vivo* Drug permeability study and determination of diffusion co-efficient**

The % cumulative drug release was calculated against time and graph was plotted, whole experiment was performed in the triplicate. *Ex-vivo* diffusion profile was studied by freshly excised and treated sheep nasal mucosa. Bath temperature was maintained at 37±0.5°C throughout the experiment.

Ex-vivo drug permeation studies from freshly excised sheep nasal mucosa for drug suspension, drug loaded ME and MME of modafinil is shown in Figure11. Approximately 50 % drug released from drug suspension, drug loaded ME and MME; within 4 hrs, 2 hrs and 1.5 hrs respectively. Diffusion profile was improved for drug loaded ME and MME due to more solubilization of drug. MME shows better release profile compared to other formulations due to presence of permeation enhancer.

It shows that within 4 hr of the time period more than 45%, 80% and 90% of the drug diffused from the drug suspension, drug loaded ME and MME respectively. Thus, it can be inferred that diffusion of the drug from the nasal mucosa can be enhanced with MME which can lead to enhancement of bioavailability.

The diffusion coefficient of all three formulations had been shown in Table 16. The results were reveal that the formulation successfully diffused through nasal mucosa and among all three formulations the mucoadhesive microemulsion showed highest diffusion coefficient and better drug release profile.

Stability Study

Both the formulations do not show any drastic change in physical appearance during real time and accelerated stability studies (40°C ± 2°C / 75%RH ± 5%) concluded from Fig. 12(A) to 12(B). They shows good stability, remain clear at all the storage conditions, stable on dilution with no signs of drug precipitation or cloudiness. This indicates that the drug remained in the solubilized form at accelerated stability conditions (40±2 °C/75 ±5 % RH). There is no significant decrease in modafinil concentration. % transmittance, pH, particle size and zeta potential were observed at different time laps (0, 1, 2, 3 months) for drug loaded ME as well as MME formulation as shown in figure 13(A) and 13(B) which doesn't produce any considerable changes indicating that formulation remains stable. Thus, drug loaded ME and MME for modafinil were found to be stable under room temperature as well as at the accelerated condition (40 ± 2 °C/75 ± 5 % RH) for 3 months.

CONCLUSION

The poor aqueous solubility and first pass metabolism of Modafinil leads to variable low oral bioavailability. So, in the present investigation attempt was made to improve the permeability of Modafinil and to achieve uniform drug release by developing appropriate formulation for intra nasal application. Drug loaded ME and MME formulations were optimized by using D-Optimal Mixture design. Both of them were characterized for various parameters and found to be Transparent (nearby 99% Transmittance) with Z-average size 47.75 ± 0.26 nm with PDI value of 0.234 ± 0.05, zeta potential -6.42 ± 0.02mV for Drug loaded ME and 59.81±0.24 nm with PDI 0.272±0.04 and zeta potential 11.61 ± 0.04 mV for MME. The zeta potential values were found to be indicating good stability of Formulation. Histopathology study indicates that both the formulations were non toxic. The *in-vitro* and *ex-vivo* drug release suggested that there was uniform and improved drug diffusion in MME as compared plain drug suspension and drug loaded ME. TEM image also revealed that the MME formulation has uniform globules and size of droplet was comparable to the result obtained from the Zeta sizer. The Stability study for the 3 months confirmed the stability of Drug loaded ME and MME formulations. Finally based on the above results it can be concluded that the MME is suitable for improving the diffusion as well as bioavailability of poorly water soluble drug Modafinil. So, it can be concluded that MME can be used to increase solubility and permeation of poorly water soluble drug Modafinil by nasal route.

Acknowledgement

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