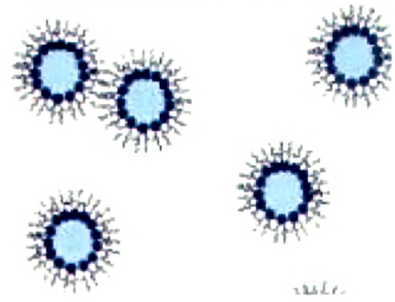
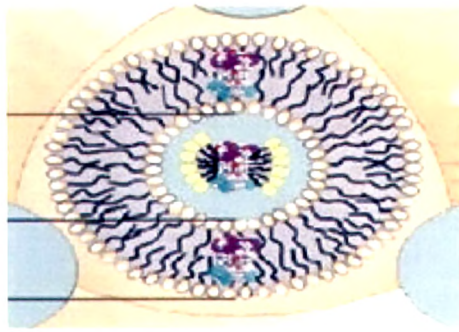


Chapter 5: Preparation, optimization and characterization of microemulsions



5. PREPARATION, OPTIMIZATION AND CHARACTERIZATION OF MICROEMULSIONS

Microemulsions are isotropic systems, which are difficult to formulate than ordinary emulsions because their formulation is a highly specific process involving spontaneous interactions among the constituent molecules. These are thermodynamically stable systems that are broadly categorized into three types: - (1) oil-in-water (o/w) microemulsions, (2) water-in-oil (w/o) microemulsions and (3) bicontinuous microemulsions. Many researchers in various literatures have reported the formulation techniques for microemulsions. These techniques are mainly pseudo ternary diagram construction and titration method (Lawrence et al 2000). Regardless of the type of microemulsion systems, microemulsions can be formulated easily by mixing the oil component with surfactant and cosurfactant components. Aqueous components can be added gradually to the mixture of oil containing surfactant and co surfactant components. Since microemulsions are thermodynamically stable systems, they undergo spontaneous formation facilitated by micelle formation without input of external energy into the system. Ternary phase diagram is a very important tool to study the phase behavior of the microemulsion system. Ternary phase diagram can be represented in a triangular format, in which each coordinate represents one component of microemulsion with 0-100% concentration in the increment of 10%. If four or more components are investigated for microemulsion system, binary mixtures like surfactant/cosurfactant or oil/drug are taken in the ordinates and pseudo ternary phase diagram will be constructed. The advantages associated with titration techniques are that they are rapid, reasonably accurate, precise and economical due to limited number of trial batches. However, the major disadvantage is that though it can provide the true picture of the phase boundary between the polyphasic and monophasic region, the different types o/w, w/o and bicontinuous microemulsion within the monophasic region cannot be identified from the phase diagram which is constructed on the basis of titration method without further characterization.(Lawrence et al 2000).

Table 5.1 Materials and equipments

Material	Source
Nicergoline	Gift samples from Ivax Pharmaceuticals s.r.o, Opava – Komarov, Crech Republic.
Hydergine	Gift samples from Ivax Pharmaceuticals s.r.o, Opava – Komarov, Crech Republic.
Sibutramine Base (SB)	Extracted from Sibutramine hydrochloride monohydrate gifted from Matrix Laboratories Ltd., Secunderabad, India.
Water (distilled)	Prepared in laboratory by distillation
Capmul MCM	Gifted by Abitec Corporation Limited, Janesville, USA.
Labrafac PG, Labrafac Lipophile WL 1349, Labrafil M1944, Transcutol, Labrafac CC, Labrafil M 2125	Gifted by Gattefosse, France
Tween 80, Tween 20, PEG 200, PEG 400, Propylene Glycol	SD Fine chemicals, Mumbai, India
Chitosan 652	Siber Hegner India Pvt. Ltd., Mumbai, India
HPLC grade methanol, glacial acetic acid, sodium acetate	SD Fine chemicals, Mumbai, India
Equipments	**Make**
Calibrated pipettes of 1.0 ml, 5.0 ml and 10.0 ml, volumetric flasks of 10 ml, 25 ml, 50 ml and 100 ml capacity, Funnels (i.d. 5.0 cm), beakers (250 ml) and other requisite glasswares	Schott & Corning (India) Ltd., Mumbai
Analytical balance	AX 120, Shimadzu Corp., Japan
pH meter	Pico^{+} Labindia, Mumbai, India
Viscometer	Brookfeild HADV
Magnetic stirrer	Remi Instrument Ltd., Mumbai, India
Bath sonicator	Ultra Sonic, Trans-O-Sonic, India
Cooling Centrifuge	3K 30, Sigma Laboratory centrifuge, Osterode, GmBH, Germany.
UV-Visible Spectrophotometer	Shimadzu UV-1601, Japan
Conductometer	CM 180 Elico, India
Particle size and zeta potential analyzer	NanoZS, Malvern Instruments, U.K.
Transmission electron microscope	Morgagni, Philips, Netherlands

5.1 METHODS

5.1.1 Preparation of microemulsions and mucoadhesive microemulsions

5.1.1.1 Solubility determination

Solubility of drugs nicergoline (NG), hydergine (HG) and sibutramine base (SB) was determined in different oils, surfactants and cosurfactans. Drugs were added in excess

to different oils, surfactants and cosurfactants and stirred on magnetic stirrer for 24 hours. The samples were centrifuged at 8000 rpm for 10 minutes and the drug content in the supernatant was analysed after proper dilution with methanol as described in analytical sections 3.1.2.2, 3.2.2.2 and 3.3.2.2 for NG, SB and HG respectively. The drug solubilities were calculated and tabulated in Table 5.2 for NG, SB and HG.

5.1.1.2 Construction of phase diagram

Pseudo-ternary phase diagram is constructed to obtain the appropriate components and their concentration ranges that can result in large existence area of microemulsion. Once the appropriate microemulsion components are selected, ternary pseudo phase diagram is constructed to define the extent and nature of the microemulsion regions. To produce such diagrams, a large number of samples of different composition are prepared. Based on the solubility study the pseudo ternary phase diagrams of oil (capmul MCM), surfactant (tween-80), cosurfactant (transcutol) and distilled water were developed for the drugs NG and HG. While, for SB pseudo ternary phase diagrams of oil (capmul MCM), surfactant (tween-80), cosurfactant (ethanol) and distilled water were developed.

The pseudo ternary phase diagrams were developed by the water titration method. Aliquots of each surfactant and cosurfactant mixture (S_{mix}) were mixed with the oil at ambient temperature. For each phase diagram, the ratio of oil to the S_{mix} was varied as 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 (v/v). Water was added drop wise to each oil-S_{mix} mixture under vigorous stirring. After equilibrium, the samples were visually checked and determined as being clear microemulsions. No heating was done during the preparation. Phase diagrams were constructed using Chemix software and are shown in figure 5.1 and 5.2 for the two systems respectively. The area of the monophasic region was used as a tool for the selection of suitable surfactant to co-surfactant ratio for respective drugs.

5.1.1.3 Preparation of microemulsions

Based on the phase diagram, the optimum S_{mix} ratio was selected and the drug loaded microemulsions were prepared by dissolving the drug in the oil-S_{mix} mixture then

titrated with the continuous phase. The external phase was added in a drop wise manner under vortex mixing. The process was optimized for the speed and time of stirring using NG microemulsion and results tabulated in table 5.3. Effect of dilution was also determined on size and zeta potential of NG microemulsions to standardize globule size and zeta potential estimation procedure and the results recorded in table 5.4.

5.1.1.3.1 Optimisation of microemulsion preparation:

Experimental design (3^2) was applied in the formulation of microemulsion by varying concentrations/ levels of oil and S_{mix} and measuring globule size (GS) and drug loading (DL) as the responses. Nine batches of microemulsions of each system were prepared by titration method according to experimental design. The prepared batches were evaluated for drug loading and globule size. The factorial design of NG microemulsions is shown in the Table 5.5. Similarly, the factorial design of HG and SB microemulsion systems are shown in the Tables 5.6 & 5.7 respectively.

Mathematical modeling of the preparation of microemulsion, multiple regression analysis was carried out by using Eq. 1 to obtain a second order polynomial equation.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2 \quad \text{..... (1)}$$

Where b_0 is the arithmetic mean response of 9 runs and b_1 and b_2 is the estimated coefficients for the factors X_1 and X_2, respectively. The major responses represent the average result obtained by changing one factor at a time from its low to high value. The interaction terms show how the response changes when 2 factors are simultaneously changed. The following equations were was derived by the best-fit method to describe the relationship of the globule size (Y_{GS}) and drug loading (Y_{DL}) with the oil concentration (X_1) and surfactant concentration (X_2). A full model was established after putting the values of regression coefficients in Equation 1.

Equations 2 and 3 represent the full model equations for NME for globule size and drug loading respectively:

$$Y_{GS} = 22.13817 + 27.63828X_1 - 5.78581X_2 + 23.12656X_1^2 + 0.105484X_2^2 - 4.93742X_1X_2 \quad \text{.....(2)}$$

$$Y_{DL} = 0.762366 + 0.151344X_1 + 0.075161X_2 - 0.03231X_1^2 - 0.0271X_2^2 + 0.025484X_1X_2 \quad \text{.....(3)}$$

Equations 4 and 5 represent the full model equations for HME for globule size and drug loading respectively:

$$Y_{GS} = 15.55774 + 33.61258X_1 - 4.27462X_2 + 30.33183X_1^2 + 0.796774X_2^2 - 4.75387X_1X_2 \ldots\ldots(4)$$

$$Y_{DL} = 0.708602 + 0.138978X_1 + 0.129677X_2 - 0.06704X_1^2 + 0.024194X_2^2 - 0.00097X_1X_2 \ldots.(5)$$

Equations 6 and 7 represent the full model equations for SME for globule size and drug loading respectively:

$$Y_{GS} = 11.10118 + 31.48317X_1 - 5.99742X_2 + 27.47134X_1^2 - 2.39355X_2^2 - 7.57226X_1X_2 \ldots\ldots(6)$$

$$Y_{DL} = 0.759032 + 0.274677X_1 + 0.111828X_2 - 0.12898X_1^2 - 0.0571X_2^2 - 0.00452X_1X_2 \ldots\ldots(7)$$

Neglecting nonsignificant ($P > 0.05$) terms from the full model, a reduced model was established, which facilitates the optimization technique by plotting contour plots to establish the relationship between independent and dependent variables. The optimized batches were selected on the basis of lowest globule size with highest drug loading.

Equations 8 and 9 represent the reduced model equations for NME for globule size and drug loading respectively:

$$Y_{GS} = 22.17333 + 25.99833X_1 - 7.44333X_2 + 24.80167X_1^2 \ldots\ldots(8)$$

$$Y_{DL} = 0.724333 + 0.1535X_1 + 0.081833X_2 \ldots\ldots(9)$$

Equations 10 and 11 represent the reduced model equations for HME for globule size and drug loading respectively:

$$Y_{GS} = 15.82333 + 32.07222X_1 - 5.94778X_2 + 32.13778X_1^2 \ldots\ldots(10)$$

$$Y_{DL} = 0.680667 + 0.134X_1 + 0.120667X_2 \ldots\ldots(11)$$

Equations 12 and 13 represent the reduced model equations for SME for globule size and drug loading respectively:

$$Y_{GS} = 10.30333 + 28.82611X_1 - 8.25556X_2 + 29.33056X_1^2 \ldots\ldots(12)$$

$$Y_{DL} = 0.654 + 0.255667X_1 + 0.102333X_2 \ldots\ldots(13)$$

ANOVA:

Analysis of variance (ANOVA) of full model and reduced model was carried out and the F statistic was applied to check whether the nonsignificant terms can be omitted or not, from the full model. Tables 5.8 to 5.10 show results of analysis of variance of full and reduced model for GS and DL of nicergoline, hydergine and sibutramine microemulsions respectively.

Construction of contours:

Two dimensional contour plots were established using the reduced polynomial equations. At fixed levels of -1, 0 and 1 of independent variable with highest coefficient value, values of independent variables were computed for globule size and drug loading and contour plots were established. The contours for nicergoline, hydergine and sibutramine microemulsions are shown in Fig. 5.3 to 5.4, Fig. 5.5 to 5.6 and Fig. 5.7 to 5.8 respectively.

Check point analysis:

A check point experiment was performed to confirm the utility of polynomial equation and established contour plots in the preparation of microemulsion. Three values of independent variables X_1 and X_2 were taken and the values of globule size and drug loading (dependent variable) were calculated by substituting the values in the polynomial equation. Microemulsions were prepared experimentally by taking the amounts of the independent variables X_1 and X_2 on the same checkpoints. Each batch was prepared three times and mean globule size and drug loading values were determined. The check point batches of NG containing formulations were prepared and recorded in Table 5.11. Similarly, the check point batches of HG and SB containing formulations were prepared and recorded in Tables 5.12 and Tables 5.13 respectively.

5.1.1.4 Preparation of mucoadhesive microemulsions

The mucoadhesive microemulsions were prepared by first preparing a microemulsion of the drug using minimum volume of external phase and then adding the required volume of concentrated polymer solution to it such that the required final concentration of the polymer in the mucoadhesive microemulsion was obtained (Sharma et al 2009). 1%w/v chitosan solution was prepared by dispersing chitosan in acetate buffer and allowing to hydrate for 24 hours. After the addition of the mucoadhesive polymer solution to the microemulsion, the system was stirred for 10 minutes to homogenize. The mucoadhesive microemulsions containing drugs NG, SB and HG i.e. NMME, SMME and HMME were prepared respectively and characterized and results recorded in Table 5.14.

5.1.1.5 Preparation of drug solutions

NG solution (NS, 25mg/ml) was prepared by first dissolving NG in minimum volume of transcutol and diluting further with required volume of distilled water. Similarly, SB solution (SS, 25mg/mL) was prepared by first dissolving in ethanol and making the volume with distilled water. While, HG solution (HS, 22.5mg/mL) was prepared by first dissolving in transcutol and making the volume with distilled water.

5.1.2 Characterization of microemulsions

5.1.2.1 Qualitative tests

These tests were used to determine the type of microemulsion.

Dilution test: Dilution tests are based on the fact that the emulsion is only miscible with the liquid that forms its continuous phase. The system is diluted with either the oil or the aqueous phase, whichever is used as the external phase in the microemulsion preparation. Hence, in case of o/w system the microemulsion can be diluted with the aqueous phase while with w/o microemulsion the system is diluted with the oil used.

Dye solubility test: It is also known as the ***staining test.*** Staining tests in which a dye is sprinkled on the surface of the emulsion also indicate the nature of the continuous phase. With an o/w emulsion there is rapid dispersion of a water-soluble dye into the system where as with w/o emulsion the dye forms microscopically visible clumps.

The reverse happens on addition of an oil-soluble dye. These tests essentially identify the continuous phase.

5.1.2.2 Globule size determination:

The globule size was determined (Kaler et al 1982, Roland et al 2003) using photon correlation spectroscopy (PCS) with in-built Zetasizer (model: Nano ZS, Malvern instruments, UK). The instrument is based on the principle of dynamic light scattering (DLS), also sometimes referred to as photon correlation spectroscopy (PCS) or quasi elastic light scattering. DLS is a technique of measuring the size of particles typically in the sub-micron region and is usually applied to the measurement of particle suspended within a liquid. The technique measures particle diffusion due to Brownian motion and relates this to the size of the particles. Brownian motion is the random movement of particles due to the bombardment by the solvent molecules that surrounds them. The parameter calculated is defined as the translational diffusion coefficient. The particle size is then calculated from the translational diffusion coefficient using the Strokes-Einstein equation and recorded in table 5.14 and shown in figures 5.9, 5.11 and 5.13 for NG, HG and SB respectively.

5.1.2.3 Zeta potential determination

Malvern zetasizer Nano ZS was used to measure the zeta potential of the globules based on the electrophoresis and electrical conductivity of the formed microemulsion. The electrophoretic mobility (μm/s) of the particles was converted to the zeta potential by in-built software based on Helmholtz-Smoluchowski equation. Measurements were performed using small volume disposable zeta cell. Average of twenty measurements of each sample was used to derive the average zeta potential. The results are tabulated in table 5.14 and shown in figures 5.10, 5.12 and 5.14 for NG, HG and SB respectively.

5.1.2.4 Transmittance

The %transmittance of the microemulsion was checked against distilled water using UV-Visible spectrophotometer (UV, 1700, Shimadzu, Japan) at 630nm (table 5.14).

5.1.2.5 pH

pH of the formulations were measured using pH meter (Lab India) and tabulated in table 5.14.

5.1.2.6 Assay

Assay of the microemulsions were determined as per the methods described in the analytical sections 3.1.2.2, 3.2.2.2 and 3.3.2.2 for NG, SB and HG respectively and the results recorded in Table 5.14.

5.1.2.7 Viscosity

Viscosity of the formulations were determined using Brookfield cone and plate Rheometer (Model LVDV III) using CPE spindle at the rotational speed of 5rpm, shear rate of 10 at 33.8±0.3°C and the results recorded in tables 5.14.

5.1.2.8 Transmission Electron microscopy (TEM)

TEM is used as a tool to study the morphology and structure of the delivery systems. The TEM images of microemulsions were taken to get idea about the size of microemulsions (Shafiq et al 2007). The images were taken by Tecnai200 with CCD camera operating at 200kV (Philips Instruments, Holland) and capable of point to point resolution. To perform TEM observations, a drop of diluted (1 in 10 dilution) microemulsion, i.e. NME, HME and SME respectively, was directly deposited on the copper grid and observed after drying and the positive image were shown in Fig 5.15.

5.1.2.9 *In vitro* drug release studies

To elucidate the effect of microemulsion and mucoadhesive microemulsion systems on release kinetics of the drugs, release studies were performed for drug solutions (NS, SS, HS), microemulsions (NME, SME, HME), and mucoadhesive microemulsions (NMME, SMME, HMME) using dialysis method. For NS, the dialysing media was 10 % methanolic phosphate buffer saline pH 6.4 + 2 %w/w polysorbate-80, while for HG and SB in 10 % methanolic phosphate buffer saline pH 6.4 + 2 %w/w polysorbate-80 and 30 % ethanolic phosphate buffer saline pH 6.4 + 2 %w/w polysorbate-80 respectively. The cellulose acetate membrane (molecular weight cutoff = 12,000 kDa) was hydrated in the buffer solution for 24 h. One end of pretreated cellulose dialysis tubing (7 cm in length) was tied with thread, and then 0.5mL of each formulation was placed in it along with 1 mL of dialyzing medium.

The other end of the tubing was also secured with thread and was allowed to rotate freely in 50mL of dialyzing medium and stirred continuously at 100 rpm with magnetic bead on magnetic plate at 37°C. Aliquots of 0.5mL were removed at different time intervals and diluted further with methanol. Volume of aliquots was replaced with fresh dialyzing medium each time. These samples were analyzed quantitatively for the drug dialyzed across the membrane using UV-visible spectrophotometer (Shimadzu 1601, Japan) against methanol as blank as described under sections 3.1.2.3, 3.2.2.3 and 3.3.2.3 for NG, SB and HG respectively. The cumulative amount of drug released was calculated for the formulations (Table 5.15) and shown graphically in figure 5.16. The kinetics of the drugs from the test formulations was evaluated by fitting the experimental data to different order kinetics such as zero-order, first order, and Higuchi's model. Each experiment was repeated three times.

5.1.2.10 *In vitro* drug diffusion

The *in vitro* drug diffusion study was performed using Franz diffusion cell of diameter 10 mm mounted with excised sheep nasal mucosa of thickness (height) 0.2 mm for drug solutions (NS, SS, HS), microemulsions (NME, SME, HME), and mucoadhesive microemulsions (NMME, SMME, HMME). Formulations were placed in the donor compartment and recipient compartment contained 25 ml of diffusion medium stirred with Teflon coated magnetic stirrer (120 rpm). The diffusion medium for the respective drugs is the same as under section 5.2.2.9. Samples from the receptor compartment were withdrawn at predetermined time intervals and analyzed spectrophotometrically as described under sections 3.1.2.3, 3.2.2.3 and 3.3.2.3 for NG, SB and HG respectively. Each sample removed was replaced with an equal volume of fresh medium. Each study was carried for a period of 4hr and in triplicate. The cumulative amount of drug released and flux was calculated for the formulations (Table 5.16) and shown graphically in figure 5.17. The kinetics of the drugs from the test formulations was evaluated by fitting the experimental data to different order kinetics such as zero-order, first order, and Higuchi's model. Each experiment was repeated three times.

5.1.2.11 Nasal toxicity study

Freshly excised sheep nasal mucosa, except for the septum part was collected from the slaughter house and immediate transferred to PBS pH 6.4. The mucosa was kept in PBS pH 6.4 for 15 min. Sheep nasal mucosa pieces with uniform thickness were mounted on Franz diffusion cells. One mucosa was treated with 0.5 ml of PBS pH 6.4; the other mucosa with 0.5 ml of isopropyl alcohol and the remaining with microemulsions and mucoadhesive microemulsions 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 pathological slides. The sheep nasal mucosa treated with PBS pH 6.4 and isopropyl alcohol were taken as positive and negative control respectively. The prepared pathological slides were studied under optical microscope for any sign of toxicity and the images were stored in the form of photographs and shown in Fig 5.18.

5.1.3 Stability study

The stability of the prepared microemulsions was assessed by conducting stability study at room temperature (25-35°C) and refrigeration temperature (4°C) for a period of 2 months. Over the time period microemulsion systems were assessed for their zeta potential, globule size, physical stability, assay, and pH and the observations recorded in table 5.17.

5.2.4 Statistical analysis

All data are reported as mean ± SEM, and the difference between the groups were tested using Student's t test at the level of $p<0.05$, and differences greater at $p\leq0.05$ were considered insignificant.

NOTE: TEM, nasal toxicity study and stability studies were performed for optimized formulations only.

5.2 RESULTS

5.2.1. Preparation of microemulsions and mucoadhesive microemulsions:

Table 5.2: Drug solubility study data

Sl No.	Material	Solubility (in mg/ml)		
		Nicergoline	Hydergine	Sibutramine
1	**Capmul MCM (oil)**	**350.17**	**160.27**	**300**
2	Labrafil 2125M (oil)	27.8	11.78	8.65
3	Labrafac PG (oil)	4.76	7.13	4.72
4	Labrafac Lipo (oil)	5.18	10.61	6.21
5	Soyabean oil	5.33	4.82	3.96
6	**Tween-80**	**70.12**	**43.95**	**40.71**
6	Tween-20	31.47	24.31	29.47
7	PEG-400	51.64	46.29	31.76
8	**Transcutol**	**185.72**	**550.27**	**200**
9	Propylene glycol	75.38	480.43	65. 49
10	**Ethanol**	**76.23**	**89.15**	**220**

Table 5.3: Effect of process parameters on the globule size and zeta potential of NG microemulsions

S. No	Capmul MCM (%w/w)	Sur/CoS (T/T) (3:1) (%w/w)	Water (%w/w)	Time of stirring (min)	Speed of stirring (rpm)	Size (nm)	Zeta potential (mV)
1	6%	36%	48%	10	393	54.4 ± 1.3	-6.75 ± 0.9
2	6%	36%	48%	10	550	18.9 ± 0.9	-4.23 ± 0.5
3	6%	36%	48%	10	707	20.1 ± 0.7	-5.4 ± 0.7
4	**6%**	**36%**	**48%**	**5**	**550**	**17.2 ± 1.1**	**-5.42 ± 0.8**
5	6%	36%	48%	10	550	16.4 ± 0.8	-3.12 ± 0.6
6	6%	36%	48%	15	550	16.7 ± 1.2	-5.75 ± 0.9

* The results are mean ± SEM derived from three different experiments. T/T implies Tween-80/Transcutol

Table 5.4: Effect of dilution on globule size and zeta potential of NG microemulsion

S.No	Dilution	Globule size(nm)	Zeta potential (mV)
1	Undiluted	16.53 ± 2.61	-3.38 ± 0.72
2	1 in 5	17.07 ± 3.44	-3.63 ± 0.51
3	1 in 10	24.31 ± 3.65	-4.1 ± 0.84
4	1 in 50	54.3 ± 4.12	-5.4 ± 0.63
5	1 in 100	72.6 ± 4.26	-8.7 ± 0.9

Fig 5.1: Phase diagram of NG and HG microemulsion system (Capmul MCM, Tween 80: Transcutol P, Distilled Water)

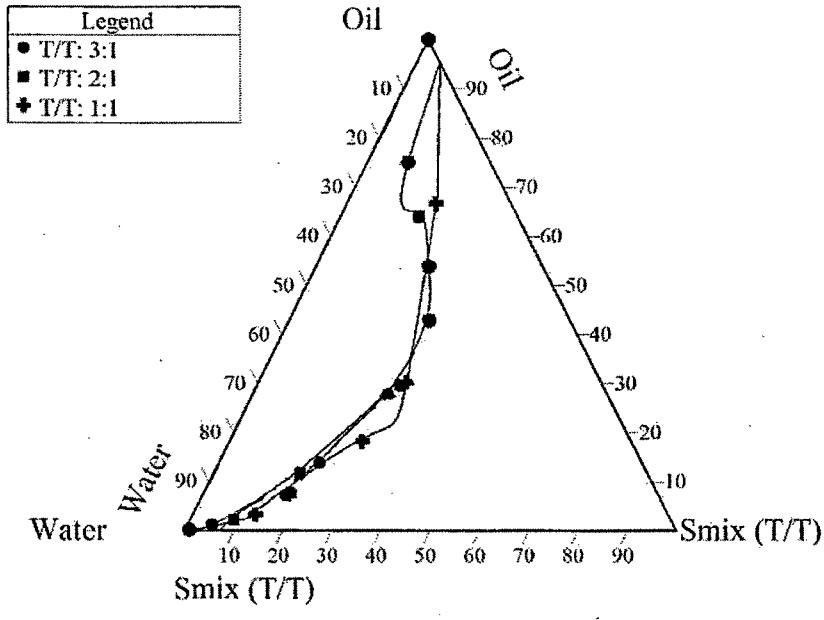


Fig 5.2: Phase diagram of SB microemulsion system (Capmul MCM, Tween 80: Ethanol, Distilled Water)

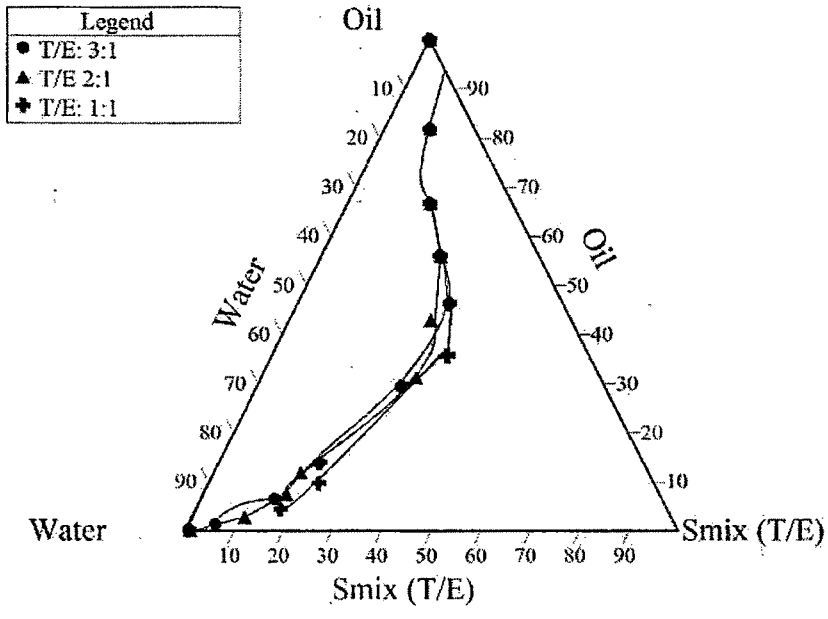


Table 5.5: 3^2 Factorial design for optimization of nicergoline microemulsion

S.No	Formulation	Oil (%w/w)	S_{mix} (T/T) (%w/w)	Water (%w/w)	Globule size* (nm)	Drug loading* (%w/w)
1	N1	2	26	72	20.44	41.2
2	N2	2	36	62	18.29	56.4
3	N3	2	46	52	16.22	62.1
4	N4	6	26	68	28.22	67.6
5	**N5**	**6**	**36**	**58**	**20.43**	**78.3**
6	N6	6	46	48	17.87	81.2
7	N7	10	26	64	84.3	73.6
8	N8	10	36	54	72.43	91.2
9	N9	10	46	44	62.19	95.5

* Globule size was measured for the dispersion of 1 in 5 dilution in distilled water at 25°C

Table 5.6: 3^2 Factorial design for optimization of hydergine microemulsion

S.No	Formulation	Oil (%w/w)	S_{mix} (T/T) (%w/w)	Water (%w/w)	Globule size* (nm)	Drug loading* (%w/w)
1	H1	2	26	72	15.81	33.8
2	H2	2	36	62	13.14	51.5
3	H3	2	46	52	12.69	65.7
4	H4	6	26	68	20.43	59.3
5	**H5**	**6**	**36**	**58**	**14.23**	**72.9**
6	H6	6	46	48	12.81	84.7
7	H7	10	26	64	90.29	69.4
8	H8	10	36	54	78.44	76.1
9	H9	10	46	44	71.37	94.7

* Globule size was measured for the dispersion of 1 in 5 dilution in distilled water at 25°C

Table 5.7: 3^2 Factorial design for optimization of sibutramine microemulsion

S.No	Formulation	Oil (%w/w)	S_{mix} (T/E) (%w/w)	Water (%w/w)	Globule size* (nm)	Drug loading* (%w/w)
1	S1	2	26	72	6.74	21.3
2	S2	2	36	62	8.22	34.6
3	S3	2	46	52	5.14	43.4
4	S4	6	26	68	14.57	58.1
5	**S5**	**6**	**36**	**58**	**9.11**	**81.7**
6	S6	6	46	48	7.23	83.2
7	S7	10	26	64	82.43	73.4
8	S8	10	36	54	68.79	91.5
9	S9	10	46	44	54.16	96.3

*Globule size was measured for the dispersion of 1 in 5 dilution in distilled water at 25°C

Table 5.8: Analysis of variance of full and reduced model for NME

		Df	SS	MS	F	R	R^2	Adj R^2
Regression (GS)	FM	5	5548.2	1109.64	331.5465	0.999397	0.998795	0.995782
	RM	3	5499.076	1833.025	131.3598	0.994963	0.989952	0.982416
Error (GS)	FM	2	6.69372 (E1)	3.34686				
	RM	4	55.81693 (E2)	13.95423				
Regression (Drug loading)	FM	5	0.126519	0.025304	15.48189	0.987327	0.974814	0.911849
	RM	2	0.119249	0.059625	28.28938	0.958542	0.918803	0.886324
Error (Drug loading)	FM	2	0.003269 (E1)	0.001634				
	RM	5	0.010538 (E2)	0.002108				

Number of parameters omitted = 2 (GS); 3 (Drug loading).
†SSE2 – SSE1 = 55.81693-6.69372 =49.12321 (GS); 0.010538-0.003269=0.007269 (Drug loading)
‡MS of error (full model) = 3.34686 (GS); 0.001634 (Drug loading)
§F calculated = (49.12321/2)/ 3.34686 = 6.694 (GS); (0.007269/3)/ 0.001634=1.483 (Drug loading)
F tabulated (2) = 4.303; F tabulated (3) = 3.182
Since, for GS F cal > F tab, the omitted parameters are significant and the hypothesis cannot be accepted. However, since for DL F cal < F tab, the omitted parameters are non significant and the hypothesis is accepted.

Table 5.9: Analysis of variance of full and reduced model for HME

		Df	SS	MS	F	R	R^2	Adj R^2
Regression (GS)	FM	5	8231.331	1646.266	428.2614	0.999533	0.999067	0.996734
	RM	3	8182.479	2727.493	192.9585	0.996563	0.993137	0.987991
Error (GS)	FM	2	7.688138 (E1)	3.844069				
	RM	4	56.54051 (E2)	14.13513				
Regression (Drug loading)	FM	5	0.129675	0.025935	48.239	0.99588	0.991776	0.971217
	RM	2	0.122137	0.061068	35.44988	0.966501	0.934124	0.907773
Error (Drug loading)	FM	2	0.001075 (E1)	0.000538				
	RM	5	0.008613 (E2)	0.001723				

Number of parameters omitted = 2 (GS); 3 (Drug loading).
†SSE2 – SSE1 = 56.54051-7.688138=48.852327 (GS); 0.008613-0.001075=0.007538 (Drug loading)

‡MS of error (full model) = 3.844069 (GS); 0.000538 (Drug loading)
§F calculated = (48.852327/2)/ 3.844069=6.354 (GS); (0.007538 /3)/ 0.000538=4.67 (Drug loading)
F tabulated (2) = 4.303; F tabulated (3) = 3.182
Since, F cal > F tab, the omitted parameters are significant and the hypothesis cannot be accepted.

Table 5.10: Analysis of variance of full and reduced model for SME

		Df	SS	MS	F	R	R^2	Adj R^2
Regression (GS)	FM	5	7096.869	1419.374	201.2894	0.999008	0.998017	0.993059
	RM	3	6984.089	2328.03	73.3914	0.991038	0.982157	0.968774
Error (GS)	FM	2	14.10282 (E1)	7.051409				
	RM	4	126.883 (E2)	31.72074				
Regression (Drug loading)	FM	5	0.353485	0.070697	28.26667	0.992999	0.986047	0.951163
	RM	2	0.310841	0.15542	16.30968	0.931177	0.86709	0.813926
Error (Drug loading)	FM	2	0.005002 (E1)	0.002501				
	RM	5	0.047647 (E2)	0.009529				

Number of parameters omitted = 2 (GS); 3 (Drug loading).
†SSE2 – SSE1 = 126.883-14.10282=112.78018 (GS); 0.047647-0.005002=0.042645 (Drug loading)
‡MS of error (full model) = 7.051409 (GS); 0.002501 (Drug loading)
§F calculated = (112.78018/2)/ 7.051409 = 7.997 (GS); (0.042645/3)/ 0.002501=5.684 (Drug loading)
F tabulated (2) = 4.303; F tabulated (3) = 3.182
Since, F cal > F tab, the omitted parameters are significant and the hypothesis cannot be accepted.

Fig 5.3 Contour plots for globule size of nicergoline microemulsions

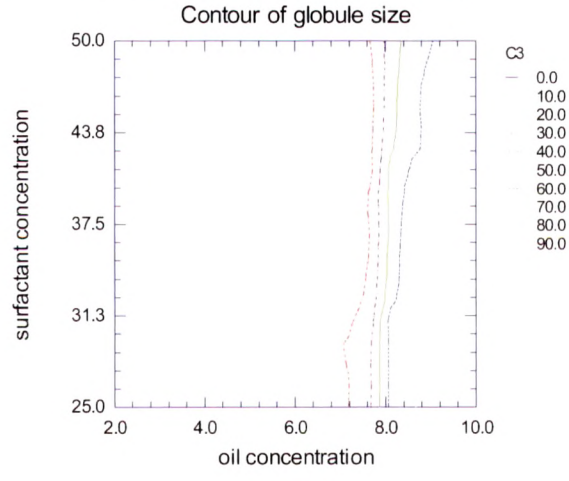


Fig 5.4 Contour plots for drug loading of nicergoline microemulsions

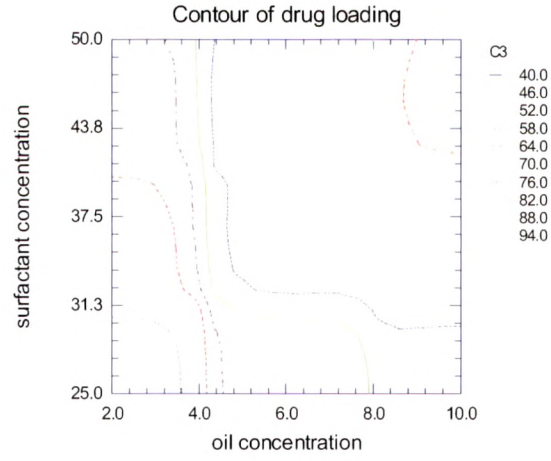


Fig 5.5 Contour plots for globule size of hydergine microemulsions

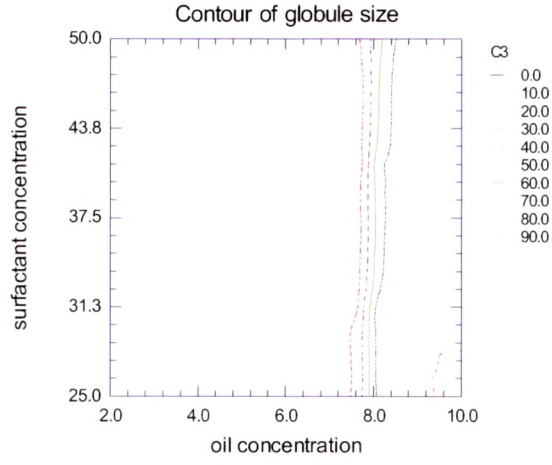


Fig 5.6 Contour plots for drug loading of hydergine microemulsions

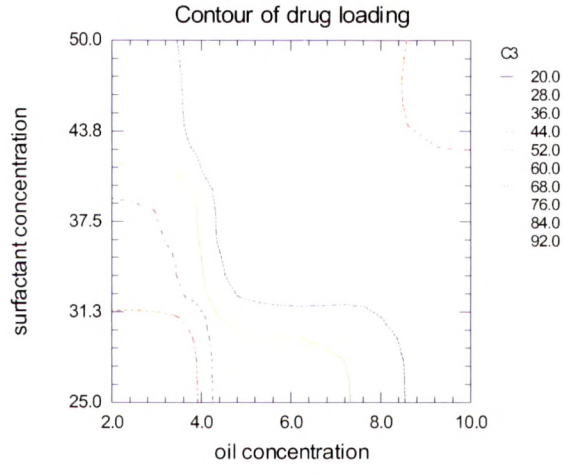


Fig 5.7 Contour plots for globule size of sibutramine microemulsions

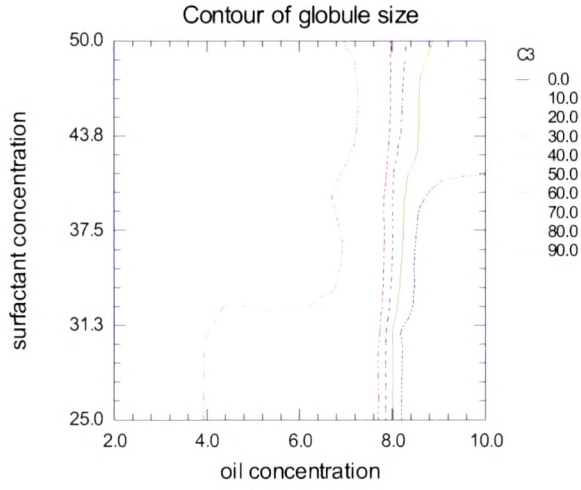


Fig 5.8 Contour plots for drug loading of sibutramine microemulsions

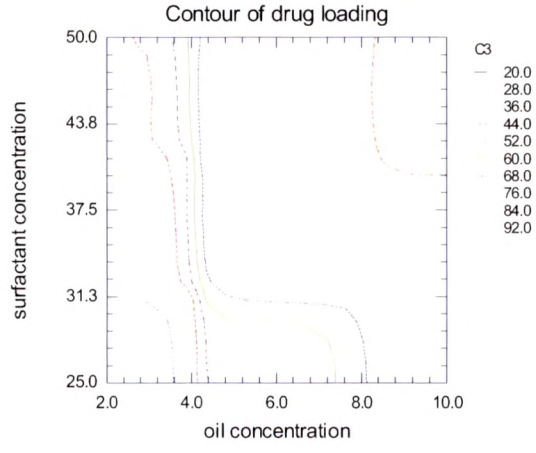


Table 5.11: Checkpoint batches for nicergoline microemulsions

S.No.	Oil (%w/w)	S_{mix} (%w/w)	Predicted GS	Experimental GS**	Predicted DL	Experimental DL**
1.	2.470588	27.94118	20.55593	22.91* ± 3.8	78.0447	75.61* ± 0.93
2.	6.705882	36.76471	20.4406	24.43* ± 2.9	41.17356	38.52* ± 0.78
3.	9.058824	41.17647	64.76737	61.76* ± 2.4	92.70781	86.78* ± 1.12

*Difference between predicted and experimental values were found to be insignificant (P>0.05)

** n = 3

Table 5.12: Checkpoint batches for hydergine microemulsions

S.No.	Oil (%w/w)	S_{mix} (%w/w)	Predicted GS	Experimental GS**	Predicted DL	Experimental DL**
1.	2.470588	41.17647	12.88462	9.84* ± 2.8	59.33355	56.96* ± 0.98
2.	6.235294	30.88235	18.49966	15.21* ± 3.4	64.95266	62.23* ± 0.87
3.	9.058824	25	89.47522	82.93* ± 2.2	68.93077	63.87* ± 1.06

*Difference between predicted and experimental values were found to be insignificant (P>0.05)
** n = 3

Table 5.13: Checkpoint batches for sibutramine microemulsions

S.No.	Oil (%w/w)	S_{mix} (%w/w)	Predicted GS	Experimental GS**	Predicted DL	Experimental DL**
1.	2.941176	45.58824	5.182366	9.47* ± 3.1	43.2971	38.75* ± 1.03
2.	6.705882	33.82353	9.658665	15.09* ± 2.7	80.60085	76.83* ± 0.83
3.	9.529412	29.41176	80.63474	71.89* ± 2.3	74.35214	65.96* ± 1.14

*Difference between predicted and experimental values were found to be insignificant (P>0.05)
** n = 3

5.2.2. Characterization of microemulsions and mucoadhesive microemulsions:

Table 5.14: Composition and characterization of drug containing microemulsions

Test	NME	HME	SME	NMME	HMME	SMME
	Drug microemulsions			Drug mucoadhesive microemulsions		
Oil (%w/w)	6	6	6	6	6	6
Surfactant (%)	27	27	27	27	27	27
Co-surfactant (%)	6 (T)	9 (T)	6 (E)	6 (T)	6 (T)	6 (E)
Aqueous phase (%)	48	48	48	48	48	48
Assay (%w/w)	98.5 ± 0.7	101.2 ± 0.5	99.1 ± 0.9	101.3 ± 0.6	99.3 ± 0.5	99.2 ± 0.9
Transmittance (%)	98.8 ± 0.9	99.1 ± 0.6	98.7 ± 0.8	---	---	---
pH	6.2 ± 0.4	6.4 ± 0.3	6.5 ± 0.4	5.5 ± 0.4	5.7 ± 0.5	5.6 ± 0.4
Conductivity (mS)	0.328 ± 0.09	0.268 ± 0.07	0.272 ± 0.08	3.1 ± 0.3	2.4 ± 0.4	1.94 ± 0.4
Viscosity (Cp)	336.2 ± 7.5	296.2 ± 5.9	325.6 ± 6.8	373.2 ± 7.4	325.8 ± 5.8	354.2 ± 4.45
Zeta potential (mV)	-3.38 ± 0.7	2.29 ± 0.6	2.06 ± 0.5	8.8 ± 0.5	12.7 ± 0.9	11.7 ± 1.1
Globule size (nm)	16.53 ± 3.4	9.03 ± 2.6	8.9 ± 4.6	18.9 ± 2.7	13.1 ± 3.1	14.5 ± 6.9

* The results are mean values ± SEM derived from three different experimental batches. O denotes Oil (Capmul MCM), S the surfactant (polysorbate 80/tween 80) and CoS denotes co-surfactant ('E' for ethanol and 'T' for transcutol) and AQ the aqueous phase (distilled water).

Figure 5.9: Particle size distribution plot of nicergoline microemulsion and mucoadhesive microemulsion

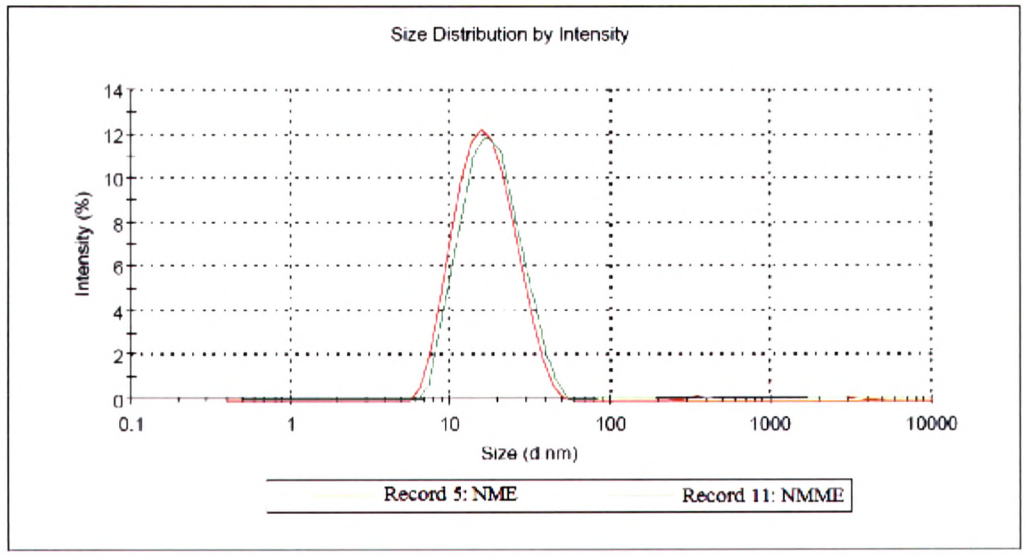


Figure 5.10: Zeta potential plot of nicergoline microemulsion and mucoadhesive microemulsion

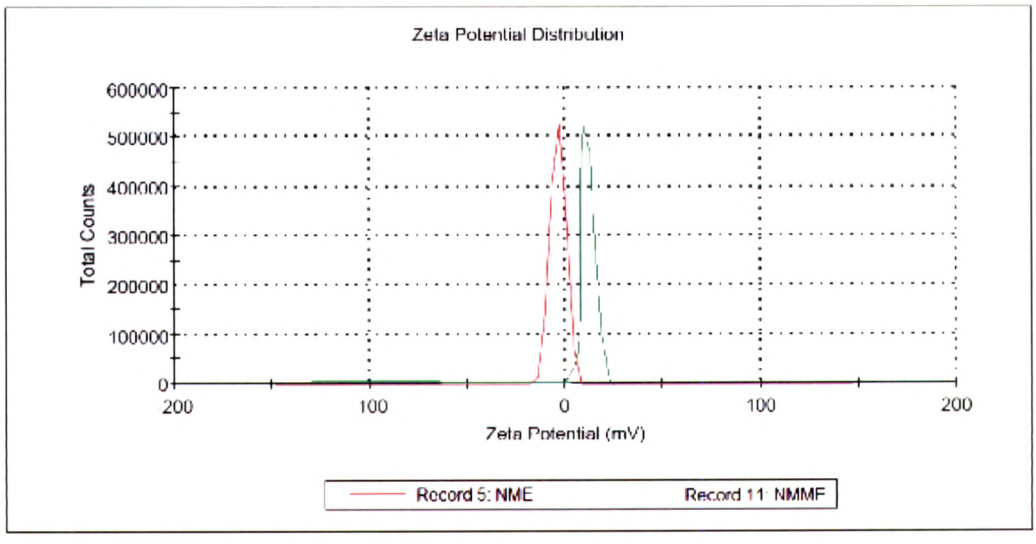


Figure 5.11: Particle size distribution plot of hydergine microemulsion and mucoadhesive microemulsion

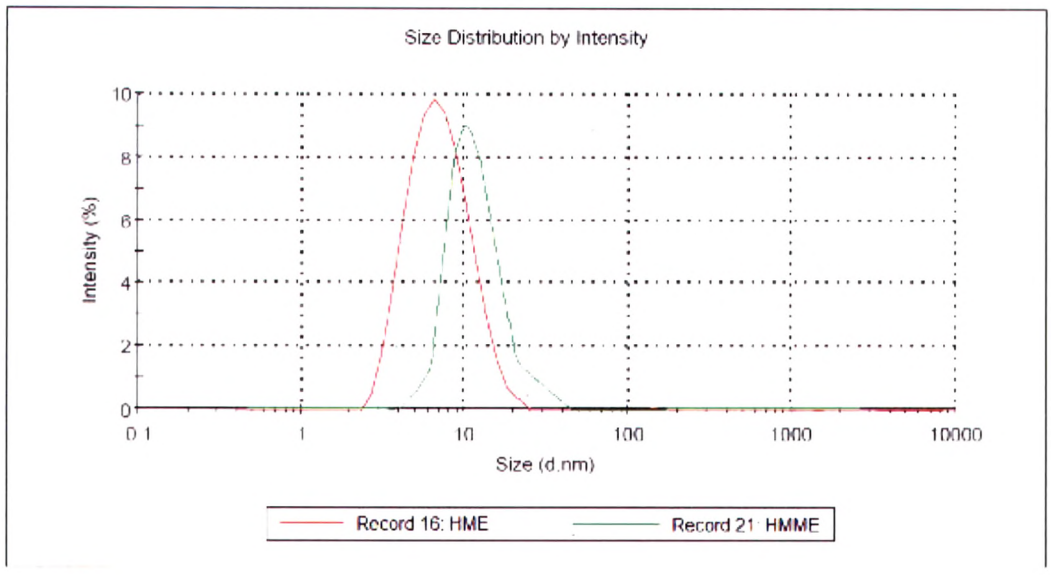


Figure 5.12: Zeta potential plot of hydergine microemulsion and mucoadhesive microemulsion

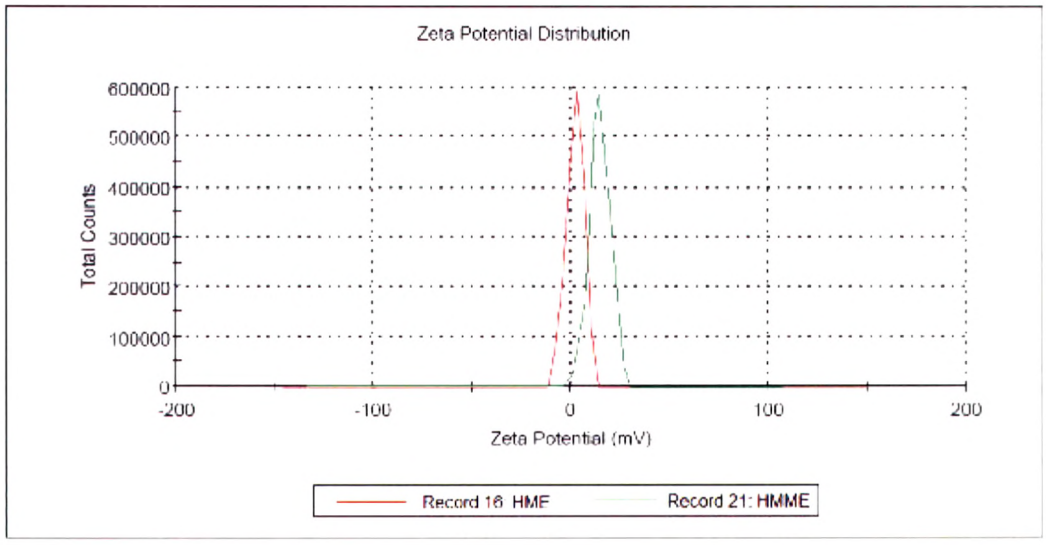


Figure 5.13: Particle size distribution plot of sibutramine microemulsion and mucoadhesive microemulsion

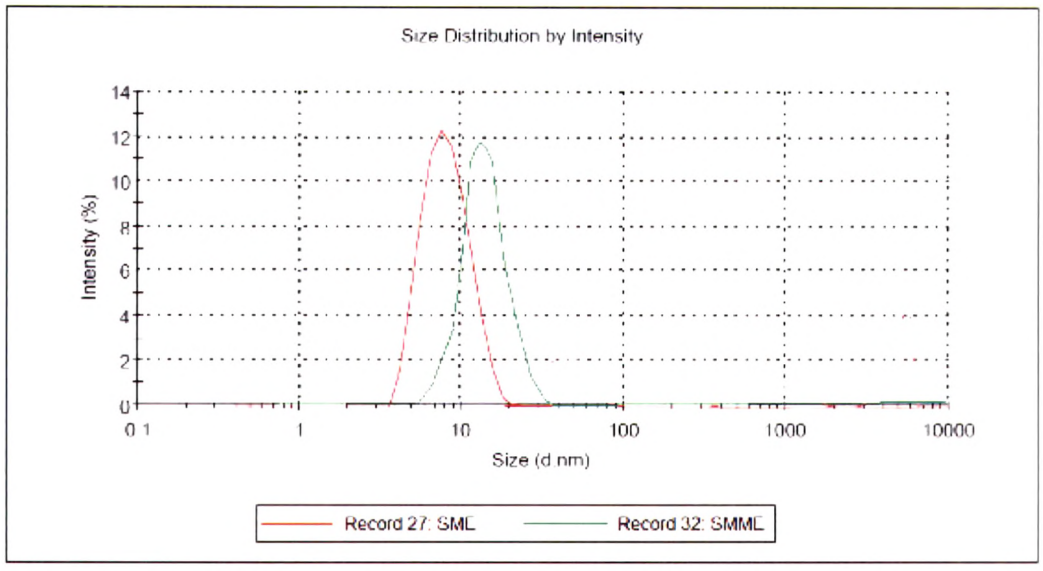


Figure 5.14: Zeta potential plot of sibutramine microemulsion and mucoadhesive microemulsion

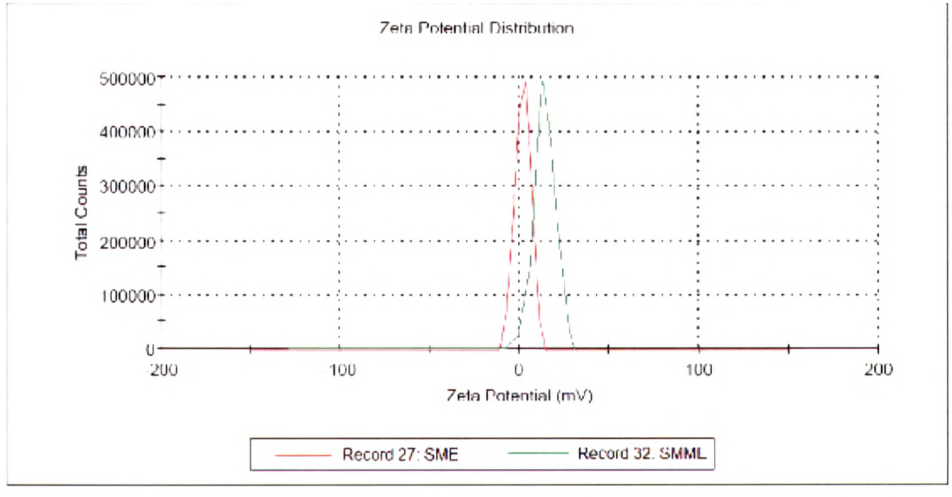
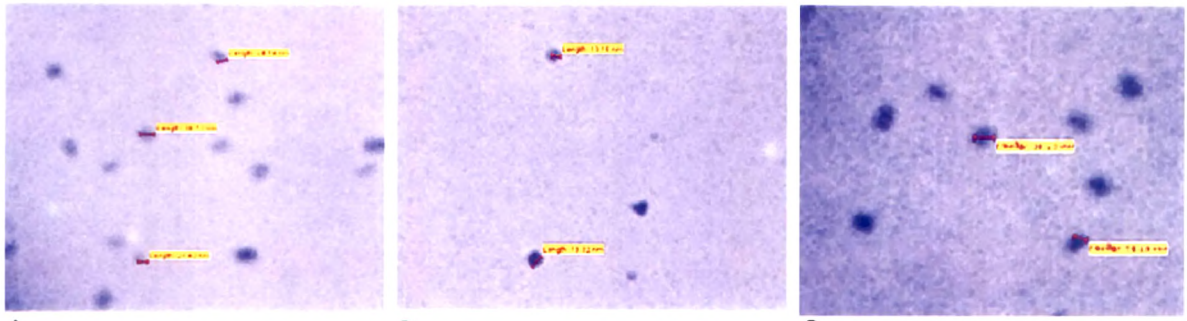


Figure 5.15: TEM images of A NME B HME C SME



A B C

Table 5.15: *In vitro* release study data for drug containing microemulsions

Time (min) ↓ / Root time (min)		Cumulative percentage drug released (%w/w)								
Batch →		NS	NME	NMME	HS	HME	HMME	SS	SME	SMME
15	3.87	22.53 ± 1.13	9.98 ± 0.79	5.93 ± 0.92	24.45 ± 0.76	12.25 ± 0.59	9.27 ± 0.85	13.35 ± 0.93	12.72 ± 0.87	12.16 ± 1.04
30	5.47	30.89 ± 0.81	12.95 ± 1.11	8.95 ± 0.78	31.12 ± 0.91	16.72 ± 1.03	13.14 ± 1.12	19.19 ± 0.82	16.34 ± 1.01	15.78 ± 0.66
60	7.74	38.76 ± 0.93	16.82 ± 0.86	13.53 ± 0.84	37.89 ± 1.11	21.67 ± 0.94	17.64 ± 0.83	27.85 ± 1.1	21.53 ± 0.82	19.26 ± 1.06
90	9.48	45.71 ± 0.89	21.52 ± 1.07	19.15 ± 1.15	44.68 ± 0.94	26.63 ± 0.86	22.16 ± 0.93	35.12 ± 0.79	26.22 ± 0.81	23.18 ± 0.86
120	10.95	58.42 ± 1.01	25.74 ± 0.87	23.52 ± 0.69	50.46 ± 0.91	32.12 ± 1.12	26.25 ± 0.57	42.92 ± 1.07	32.27 ± 0.94	27.79 ± 1.16
150	12.24	67.08 ± 0.67	31.71 ± 0.56	29.93 ± 0.94	59.13 ± 0.65	37.62 ± 1.07	31.48 ± 0.67	49.28 ± 0.92	38.69 ± 1.08	32.14 ± 0.65
180	13.41	75.82 ± 0.78	37.35 ± 0.94	33.57 ± 0.85	66.71 ± 1.17	44.56 ± 0.85	35.62 ± 0.97	56.68 ± 0.84	45.32 ± 0.93	37.42 ± 0.73
210	14.49	84.59 ± 0.97	42.27 ± 1.03	38.17 ± 0.79	73.64 ± 1.05	49.25 ± 0.77	41.57 ± 1.05	63.31 ± 1.02	52.47 ± 0.88	42.91 ± 1.05
240	15.49	96.95 ± 1.12	47.17 ± 1.09	42.57 ± 1.03	81.96 ± 0.96	54.15 ± 0.64	46.73 ± 0.89	70.54 ± 0.95	59.18 ± 1.12	47.12 ± 0.77
R^2 values for different kinetic models										
Zero order		0.965	0.974	0.962	0.935	0.97	0.972	0.976	0.961	0.961
First order		0.532	0.644	0.734	0.485	0.595	0.639	0.599	0.614	0.581
Higuchi's kinetics		0.978	0.98	0.992	0.983	0.979	0.978	0.983	0.978	0.974

Figure 5.16: Cumulative percentage drug released Vs time plot for drug containing microemulsions

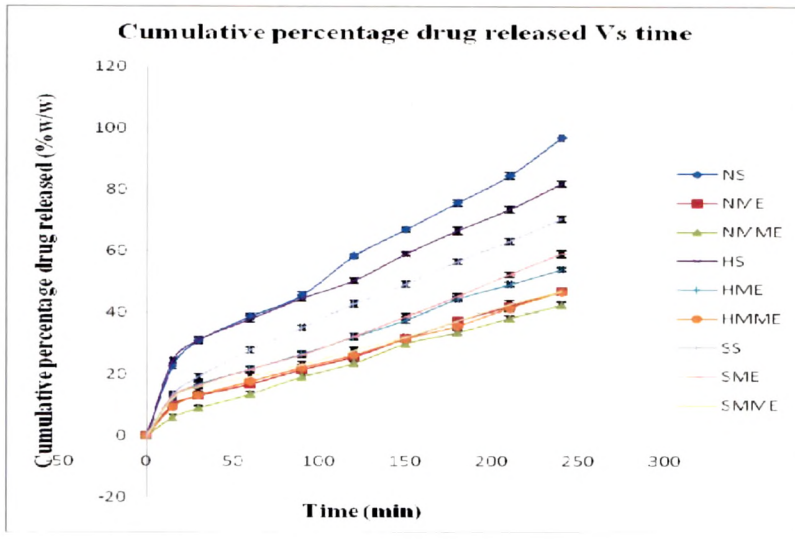


Table 5.16: *In vitro* diffusion study data for drug containing microemulsions

Time (min) ▼	Root time (min)	Cumulative percentage drug diffused (%w/w)								
Batch →		NS	NME	NMME	HS	HME	HMME	SS	SME	SMME
15	3.87	12.72 ± 0.87	14.56 ± 0.68	16.21 ± 0.78	12.17 ± 0.62	12.84 ± 0.81	14.35 ± 0.59	12.08 ± 0.87	12.76 ± 1.04	13.35 ± 0.93
30	5.47	18.34 ± 1.16	15.91 ± 0.74	21.89 ± 0.64	16.85 ± 0.96	17.13 ± 1.05	19.76 ± 0.91	16.34 ± 1.01	18.38 ± 0.85	18.19 ± 0.82
60	7.74	22.65 ± 0.76	23.93 ± 0.84	27.63 ± 0.91	20.45 ± 1.02	23.42 ± 0.77	24.78 ± 0.83	21.53 ± 0.82	24.23 ± 1.14	25.85 ± 1.1
90	9.48	27.48 ± 0.98	29.18 ± 1.05	33.32 ± 0.57	25.18 ± 0.76	27.95 ± 1.05	28.74 ± 0.68	26.22 ± 0.81	30.57 ± 0.75	32.12 ± 0.79
120	10.95	33.81 ± 1.15	35.45 ± 0.79	39.92 ± 1.16	29.42 ± 1.03	33.09 ± 0.68	33.82 ± 0.72	32.27 ± 0.94	36.87 ± 0.96	40.92 ± 1.07
150	12.24	39.43 ± 0.86	43.13 ± 0.72	46.08 ± 0.67	34.11 ± 0.79	38.93 ± 0.94	39.79 ± 0.84	38.69 ± 1.08	44.76 ± 1.02	47.28 ± 0.92
180	13.41	45.14 ± 0.78	49.62 ± 0.69	55.68 ± 0.93	38.94 ± 1.07	44.76 ± 1.05	48.71 ± 1.07	44.32 ± 0.93	51.21 ± 0.89	53.68 ± 0.84
210	14.49	50.67 ± 1.18	56.37 ± 0.84	64.65 ± 1.98	47.17 ± 0.96	53.91 ± 1.14	60.35 ± 0.98	51.47 ± 0.88	57.89 ± 1.14	61.31 ± 1.02
240	15.49	54.97	63.25	70.83	53.75 ±	66.25	68.96 ±	56.78 ±	64.47	67.54

		± 1.09	± 1.13	± 1.05	0.82	± 0.89	1.11	1.12	± 0.84	± 0.95
Flux [(%w/w)/min]										
		0.201 ± 0.012	0.238 ± 0.024	0.26 ± 0.016	0.19 ± 0.009	0.232 ± 0.021	0.246 ± 0.017	0.212 ± 0.025	0.243 ± 0.009	0.258 ± 0.014
R^2 values for different kinetic models										
Zero order		0.96 ± 0.007	0.971 ± 0.011	0.966 ± 0.014	0.958 ± 0.012	0.94 ± 0.022	0.93 ± 0.029	0.969 ± 0.023	0.975 ± 0.011	0.978 ± 0.009
First order		0.562 ± 0.016	0.63 ± 0.022	0.57 ± 0.023	0.585 ± 0.019	0.618 ± 0.028	0.596 ± 0.032	0.605 ± 0.033	0.611 ± 0.027	0.606 ± 0.042
Higuchi's kinetics		0.98 ± 0.009	0.983 ± 0.01	0.97 ± 0.006	0.962 ± 0.013	0.97 ± 0.017	0.964 ± 0.021	0.976 ± 0.011	0.979 ± 0.016	0.979 ± 0.018

Values are expressed as mean ± SEM of three estimations.

Figure 5.17: Cumulative percentage drug diffused Vs time plot for drug containing microemulsions

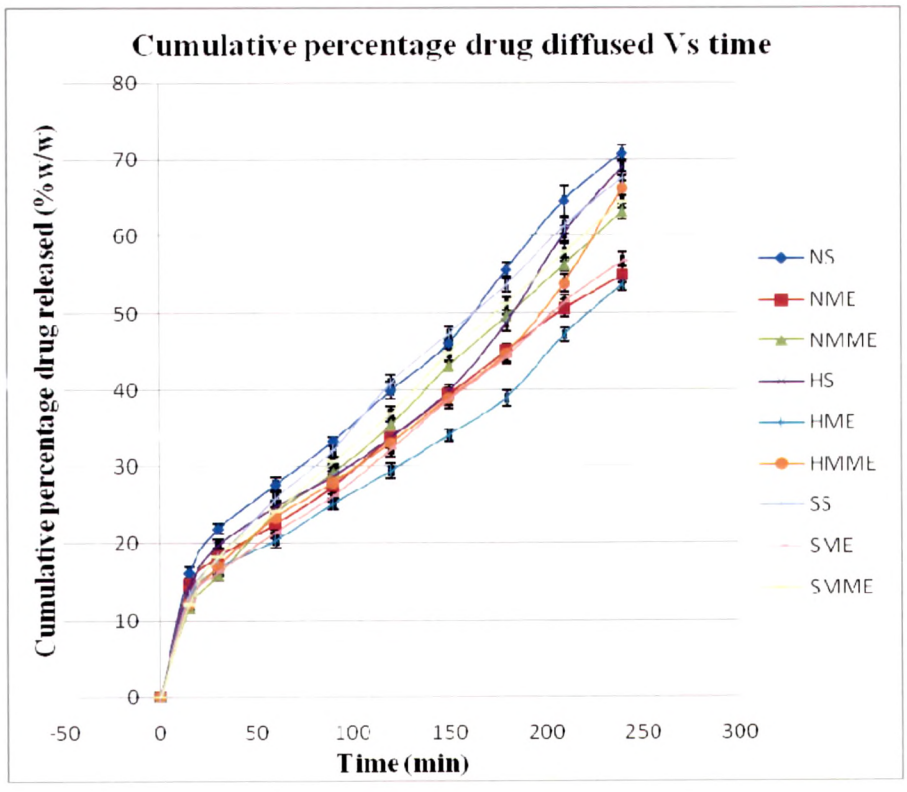
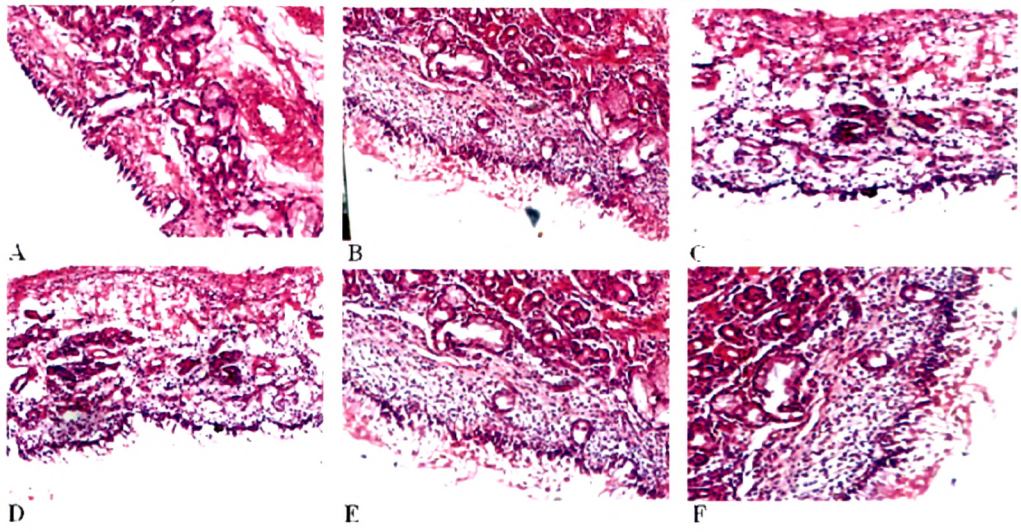


Figure 5.18: Optical microscopy of drug containing microemulsions treated nasal mucosa for nasal toxicity study: A PBS-6.4 treated nasal mucosa **B** Isopropyl alcohol treated nasal mucosa **C** NME (Tween-80/transcutol microemulsion) treated nasal mucosa **D** NMME treated nasal mucosa **E** SME (Tween-80/ethanol microemulsion) treated nasal mucosa **F** SMME treated nasal mucosa



5.2.3 Stability study:

Physical stability:

- ***Precipitation of drug-*** No precipitation of drug was observed during storage period.
- ***Phase separation-*** Was not observed.
- ***Centrifugation test-*** The batches of formulations were found to be stable and no phase separation was observed even after two months.

Table 5.17: Results of stability study

Test		At refrigeration temperature						At room temperature					
		NME	HME	SME	NME	HME	SME	NME	HME	SME	NME	HME	SMME
Assay (%w/w)	Initial	101.6 ± 0.7	99.3 ± 0.5	100.8 ± 0.8	99.2 ± 0.4	102.1 ± 0.4	99.5 ± 0.5	99.8 ± 0.7	100.2 ± 0.6	101.4 ± 0.4	100.4 ± 0.7	99.6 ± 0.5	99.9 ± 0.6
	After 2 months	99.3 ± 0.5	97.6 ± 0.7	98.3 ± 0.6	97.6 ± 0.5	99.1 ± 0.4	96.9 ± 0.6	92.5 ± 0.6	91.4 ± 0.5	94.1 ± 0.8	93.2 ± 1.1	91.6 ± 1.4	90.9 ± 0.9
pH	Initial	6.4 ± 0.08	6.3 ± 0.11	6.4 ± 0.13	5.9 ± 0.12	5.6 ± 0.09	5.5± 0.12	6.3 ± 0.14	6.2 ± 0.21	6.3 ± 0.17	6.0 ± 0.11	5.5 ± 0.14	5.4 ± 0.13
	After 2	6.2 ± 0.13	6.0 ± 0.09	6.1 ± 0.15	5.7 ± 0.12	5.6 ± 0.13	5.3 ± 0.09	5.8 ± 0.21	5.6 ± 0.19	5.7 ± 0.17	5.2 ± 0.26	5.1 ± 0.18	4.9 ± 0.24

	months												
Trans-mittance (%)	Initial	99.1 ± 0.8	99.8 ± 1.0	99.6 ± 0.9	---	---	---	99.1 ± 0.7	98.9 ± 1.0	99.6 ± 0.9	---	---	---
	After 2 months	97.4 ± 1.2	98.5 ± 0.9	97.9 ± 0.7	---	---	---	88.1 ± 1.0	87.8 ± 0.9	86.6 ± 0.7	---	---	---
Zeta potential (mV)	Initial	-3.1 ± 0.7	2.4 ± 0.4	2.9 ± 0.5	5.8 ± 0.8	7.2 ± 0.6	7.8 ± 0.5	-2.8 ± 0.6	2.1 ± 0.5	2.6 ± 0.8	5.5 ± 0.6	6.9 ± 0.4	7.3 ± 0.8
	After 2 months	-2.7 ± 0.5	2.1 ± 0.7	2.6 ± 0.6	5.2 ± 0.6	6.9 ± 0.4	7.5 ± 0.7	0.7 ± 0.3	0.4 ± 0.6	0.9 ± 0.5	2.5 ± 0.4	4.1 ± 0.3	3.9 ± 0.5
Globule size (nm)	Initial	22.8 ± 3.9	14.1 ± 4.7	13.2 ± 3.8	22.5 ± 4.1	12.8 ± 2.9	15.1 ± 3.7	21.4 ± 4.1	12.6 ± 3.6	11.9 ± 2.9	23.5 ± 3.7	13.4 ± 3.1	15.7 ± 2.9
	After 2 months	20.4 ± 4.3	11.4 ± 5.6	10.8 ± 4.8	21.1 ± 3.8	10.8 ± 4.1	13.7 ± 4.6	28.6 ± 3.4	21.7 ± 4.2	23.1 ± 3.6	42.3 ± 3.2	25.6 ± 2.9	28.2 ± 3.4

Values are expressed as mean ± SEM of three estimations. *Difference between initial values and values after 2 months was found to be significant ($P<0.05$)

5.3 DISCUSSION

5.3.1 Preparation of microemulsions and mucoadhesive microemulsions

Microemulsions of drugs NG, HG and SB were successfully prepared using titration method followed by construction of pseudo ternary phase diagram. Based on the solubility study data shown in table 5.2, capmul MCM was selected as an internal phase for the preparation of microemulsions for drugs having maximum solubility in it. The selection of surfactant and co-surfactant mixture was on the basis of drug solubility, safety and stability profile. Non-ionic surfactants are known to be least toxic and chemically highly stable and hence, use of non-ionic surfactants for pharmaceutical microemulsion formulation is gradually increasing. Surfactant polysorbate 80/tween 80 was selected for the study along with co-surfactants like transcutol and ethanol being respective drugs having maximum solubility in these.

Different ratios of surfactant and cosurfactant (1:1 to 3:1) were studied in the phase diagram construction. The phase study revealed that increasing the S_{mix} ratio from 1:1 to 3:1, the microemulsion region increased toward water-oil axis (Fig. 5.1 and 5.2).

This indicates that increasing surfactant concentration, the maximum amount of oil can be solubilised/emulsified (Lianli et al 2002, Zhang et al 2004). The increased oil content may provide opportunity for the solubilisation of the drug. For NG and HG microemulsion system containing capmul MCM, tween 80:transcutol (3:1), and distilled water was developed. While, for SB system containing capmul MCM, tween 80:transcutol (3:1), and distilled water was developed. In both the systems, up to 6% w/w of oil was emulsified by 36% of the S_{mix}.

It was observed that the zeta potential and globule or particle size of microemulsions were influenced by the dilution made for the estimations, as a low interparticle space between the globules results in multiple light scattering leading to a false measurement. Effect of dilution on zeta potential and globule size was studied (Table 5.4), and the dilution and temperature at which the measurements have to be made were kept constant throughout the study for all the systems. Thus, the zeta potential and globule size for the microemulsions were measured at a dilution of 1 in 5 in distilled water at 25°C.

Multiple regression analysis

Nine batches for each of the drug (NG, HG and SB) microemulsions were prepared by the water titration method using 3^2 factorial design varying two independent variables namely oil content/concentration (X_1) from 2%w/w to 10%w/w and surfactant concentration (X_2) from 26%w/w to 46%w/w. The influence of these independent variables on the dependent variables globule size (GS) and drug loading (DL) was evaluated and the results recorded in tables 5.5, 5.6 and 5.7 for NG, HG and SB microemulsions respectively. The GS and DL obtained at various levels of three independent variables (X_1, X_2 and X_3) were subjected to multiple regression. Second order polynomial equations (full model) were obtained. The optimized batches for respective drugs were selected on the basis of highest drug loading and globule size less than 50nm.

The effects of X_1 and X_2 on GS and DL were evaluated by changing one variable at a time from its low to high value. The interaction (X_1X_2) shows how the globule size and drug loading changes when one or more variables were simultaneously changed.

For nicergoline microemulsions, the globule size and drug loading for the 9 batches showed a wide variation starting from a minimum of 16.22nm to maximum of 84.3nm and minimum of 41.2% to maximum of 95.5% respectively as shown in table 5.5. The

coefficients of terms X_2^2 and X_1X_2 ($p>0.05$) in equation 2 are regarded as least contributing to the GS of NME. While, none of the terms X_1^2, X_2^2 or X_1X_2 contributed significantly (having $p<0.05$) in equation 3 to the DL of NME. Hence, these terms were neglected from full model considering non-significant and reduced polynomial equations 8 and 9 were obtained for GS and DL respectively by including significant terms ($p<0.05$) of equations 2 and 3 respectively.

F-statistic of the results of ANOVA of full model and reduced model (as represented in table 5.8) did not confirmed omission of non-significant terms of equations 2 and 3. Since Fcal (6.694) > Ftab (4.303) for GS it was concluded that the neglected terms significantly contribute in predicting globule size and hence, the hypothesis cannot be accepted. However, since Fcal (1.483) < Ftab (3.182) for DL ($a = 0.05$, $v_1 = 2$ and $v_2 = 3$), it was concluded that the neglected terms do not significantly contribute in predicting drug loading and hence, the hypothesis can be accepted. When the coefficient values of two independent key variables (X_1 & X_2) in equation 8 and equation 9 were compared, the value for variable X_1 ($b_1 = 25.99833$ for particle size, $b_1 = 0.1535$ for entrapment efficiency) was found to be maximum and hence the variable X_1 was considered to be a major contributing variable to the globule size and drug loading of NME.

For hydergine microemulsions, the globule size and drug loading for the 9 batches showed a wide variation starting from a minimum of 12.69nm to maximum of 90.29nm and minimum of 33.8% to maximum of 94.7% respectively as shown in table 5.6. The coefficients of terms X_2^2 and X_1X_2 ($p>0.05$) in equation 4 are regarded as least contributing to the GS of HME. While, none of the terms X_1^2, X_2^2 or X_1X_2 contributed significantly (having $p<0.05$) in equation 5 to the DL of HME. Hence,

these terms were neglected from full model considering non-significant and reduced polynomial equations 10 and 11 were obtained for GS and DL respectively by including significant terms ($p<0.05$) of equations 4 and 5 respectively.

F-statistic of the results of ANOVA of full model and reduced model (as represented in table 5.8) did not confirmed omission of non-significant terms of equations 4 and 5. Since Fcal (6.354) > Ftab (4.303) for GS and Fcal (4.67) > Ftab (3.182) for DL ($a = 0.05$, $v_1 = 2$ and $v_2 = 3$), it was concluded that the neglected terms significantly contribute in predicting globule size and drug loading and hence, the hypothesis cannot be accepted. When the coefficient values of two independent key variables (X_1 & X_2) in equation 10 and equation 11 were compared, the value for variable X_1 ($b_1 = 32.07222$ for particle size, $b_1 = 0.134$ for entrapment efficiency) was found to be maximum and hence the variable X_1 was considered to be a major contributing variable to the globule size and drug loading of HME.

For sibutramine microemulsions, the globule size and drug loading for the 9 batches showed a wide variation starting from a minimum of 5.14nm to maximum of 82.43nm and minimum of 21.3% to maximum of 96.3% respectively as shown in table 5.5. The coefficients of terms X_2^2 and X_1X_2 ($p>0.05$) in equation 6 are regarded as least contributing to the GS of SME. While, none of the terms X_1^2, X_2^2 or X_1X_2 contributed significantly (having $p<0.05$) in equation 7 to the DL of SME. Hence, these terms were neglected from full model considering non-significant and reduced polynomial equations 12 and 13 were obtained for GS and DL respectively by including significant terms ($p<0.05$) of equations 6 and 7 respectively.

F-statistic of the results of ANOVA of full model and reduced model (as represented in table 5.10) did not confirmed omission of non-significant terms of equations 6 and 7. Since Fcal (7.997) > Ftab (4.303) for GS and Fcal (5.684) > Ftab (3.182) for DL ($a = 0.05$, $v_1 = 2$ and $v_2 = 3$), it was concluded that the neglected terms significantly contribute in predicting globule size and drug loading and hence, the hypothesis cannot be accepted. When the coefficient values of two independent key variables (X_1 & X_2) in equation 12 and equation 13 were compared, the value for variable X_1 ($b_1 = 28.82611$ for particle size, $b_1 = 0.255667$ for entrapment efficiency) was found to be

maximum and hence the variable X_1 was considered to be a major contributing variable to the globule size and drug loading of SME.

Contours

For NME, two dimensional contour plots for globule size and drug loading are shown in figures 5.3 and 5.4 respectively. The independent variable with highest coefficient was X_1 (oil concentration) for both globule size and drug loading.

Similarly, two dimensional contour plots for globule size and drug loading for HME and SME, are shown in figures 5.5 and 5.6 and figures 5.7 and 5.8 respectively. The independent variable with highest coefficient was X_1 (oil concentration) for both particle size and drug entrapment efficiency.

Check Point Analysis

For NME, three check points were selected. Nanoparticles at these three checkpoints were prepared experimentally using the same procedure keeping the other process variables as constant, with the amounts of X_1 and X_2 at the selected check points. The computed values from the contours at -1, 0 and 1 level and the experimentally determined values for globule size and drug loading are shown in table 5.11. Both experimentally obtained and theoretically computed globule size and drug loading values were compared using student 't' test and the difference was found to be non significant ($p>0.05$).

Similarly for HME, the check point batches were selected from contours plotted and the computed values from contours and the experimental values are recorded in table 5.12 for globule size and drug loading and the difference was found to be non significant ($p>0.05$).

Similarly for SME, the check point batches were selected from contours plotted and the computed values from contours and the experimental values are recorded in table 5.13 for globule size and drug loading and the difference was found to be non significant ($p>0.05$).

This proves the role of a derived reduced polynomial equation and contour plots in the preparation of microemulsions of NG, HG and SB of predetermined globule size and drug loading within the selected range of the independent variables.

For NME, batch N5 with 0 level of oil concentration and 0 level of surfactant concentration was considered optimum having lowest globule size with highest drug loading. Although batch N6 has globule size smaller than N5 but there is a no significant difference in the drug loading. Also, N6 has higher content of surfactant than N5. Hence, N5 was considered optimum. Similarly, for HME and SME batches H5 and S5 respectively were considered optimum.

The addition of mucoadhesive polymer chitosan tends to influence the zeta potential and viscosity of the microemulsions being ionic, by adsorbing on the interface and influencing zeta potential considerably (Cui et al 2006). Chitosan being positively charged was found to increase the zeta potential in the positive side without significantly affecting the globule size (Table 5.14).

5.3.2 Characterization of microemulsions and mucoadhesive microemulsions

The optimized drug loaded microemulsions and mucoadhesive microemulsions were characterized for their qualitative test, zeta potential, globule size, transmittance, pH, assay, conductance and viscosity, and the results recorded in Table 5.14. When the microemulsion systems were diluted with water, it was readily miscible with water. When methyl orange was added to the microemulsions, they resulted into colored solutions without any clumps. These both dilution and dye tests indicated that the prepared microemulsions were of o/w type. The globule size distribution plot and zeta potential plot for drug containing microemulsions and mucoadhesive microemulsions are illustrated in figures 5.9 to 5.14. The pH of the formulations was found to be within the range of nasal cavity secretions and hence would not cause nasal irritation on application. Microemulsions were found to possess low viscosity and exhibited newtonian behaviour. In the TEM images of microemulsions, globules appeared dark and the surroundings were bright (Fig 5.15). Some globule sizes measured by TEM

were capable of point to point resolution. The sizes were in agreement with the globule size distribution measured using photon correlation spectroscopy.

The prepared formulations of NG, HG and SB were subjected to *in vitro* release and diffusion studies through dialysis membrane and sheep nasal mucosa respectively for

4 hrs. The percentage cumulative drug released and diffused were calculated and recorded in tables 5.15 and 5.16 and shown graphically in figures 5.16 and 5.17. The kinetic pattern of the release and diffusion was studied by fitting percentage drug diffused and released in given time in different order kinetics like zero order, first order and higuchi. Regression coefficients of all formulations in different orders were compared and found that the release pattern of drug from the formulation across the nasal mucosa followed higuchi's kinetics rather than zero order and first order. This was concluded by higher regression coefficient value in curve fitting. There was a controlled release of drugs from microemulsions and mucoadhesive microemulsions as demonstrated by low percentage drug released when compared to respective drug solutions and is attributed to the inclusion of mucoadhesive polymer. However, the chitosan containing mucoadhesive microemuslions showed highest percentage drug diffused and drug flux across nasal mucosa than drug containing microemulsions and solutions. This may be explained by the bioadhesive and absorption enhancement property of chitosan across the mucosal membrane by opening tight epithelial junctions of the mucosal membranes like nasal membrane and intestinal membrane (Ugwoke et al 2001).

The prepared drug microemulsions were subjected to nasal toxicity study to evaluate the safety of the ingredients used in the formulation. The optical microscopy images of formulation treated nasal mucosa are shown in Fig 5.18. The nasal mucosa treated with isopropyl alcohol (mucociliary toxic agent) showed complete destruction of epithelial layer with no cilia visible while nasal mucosa treated with drug microemulsions and mucoadhesive microemulsions and subsequent washing were found to be intact without much damage of the epithelial layer and intact cilia. Thus, the prepared formulations were found to be comparatively safe on nasal mucosa than isopropyl alcohol. However, further toxicity studies need to be conducted prior to clinical application of the prepared formulations.

Prepared microemulsions were subjected to globule size and zeta potential measurements after two months of storage and the results are recorded in table 5.17. The microemulsions were found to be stable for two months at refrigeration temperatures as no phase separation or flocculation was observed during storage.

However, microemulsions stored at room temperature were found to be unstable due to significant difference between the initial and final values of the various parameters determined. The results were found to be satisfactory.

5.4 CONCLUSION

The drug loaded microemulsions and mucoadhesive microemulsions were successfully prepared and were found to be stable and suitable for further pharmacokinetic studies.

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