Chapter 8: Summary and Conclusions

8. SUMMARY AND CONCLUSIONS

Brain is a uniquely protected organ residing within the bony confines of the skull, thus making systemic delivery of drugs difficult. However, the mechanisms that protect it from exogenous molecules also inhibit the entry of medicaments into the brain, rendering debilitating brain disorders almost untreatable. Blood-brain-barrier represents the strictest barrier to the brain drug delivery inhibiting the ingress of nearly all large-molecule drugs and more than 98% of small-sized therapeutics. Intranasal drug delivery is one of the focused and rapidly budding delivery option for brain targeting as brain and nose compartments are connected via the olfactory/trigeminal route and peripheral circulation. Intranasal drug delivery delivers drug directly to the brain circumventing BBB and reduces drug delivery to the non targeted sites. This may result in reduction in dose, systemic dilution and first pass metabolism of the drug. Direct nose to brain transport results into rapid and/ of higher uptake in the brain, which provides an alternative option of self medication in the management of emergencies.

Microemulsions and nanoparticles are widely employed for administering drugs intranasally to the brain. The observation that BBB receptor mediated transcytotic systems mediate the brain uptake of circulating endogenous peptides or proteins, such as insulin or transferrin, provided the basis for an approach to brain drug targeting called chimeric peptide technology. Nanoparticles and surface modified nanoparticles have been explored as intranasal drug delivery vectors but without any basis and not specifically for nose to brain drug delivery. Since long, certain receptors have been known to be expressed on the olfactory bulb of nose in comparatively higher abundance than others such as human insulin receptor (HIR) in humans and transferrin receptors in rodents. Therefore, it was hypothesized that a drug loaded nanoconstruct having ligand, specific for these receptors, conjugated to its surface will be transported selectively to the brain across the olfactory or trigeminal nerves on intranasal delivery through receptor mediated transcytosis (RMT). While with microemulsions, prolonged residence time of the formulations can be achieved by tailoring its viscosity by the addition of various grades and types of mucoadhesive agents.

Despite enormous advances in brain research, brain and central nervous system disorders remain the world's leading cause of disability, and account for more hospitalizations and prolonged care than almost all other diseases combined. Patients suffering from fatal/and/or devastating CNS disorders such as neurogenerative disorders like alzheimer's disease, Parkinson's disease, epilepsy, migraine, cerebrovascular diseases and HIV encephalopathy, far outnumber of those victimized from several types of systemic cancers and heart diseases. Beyond the loss of life, this broad category of disorders can have an overwhelming effect on the quality of life for the surviving patient and can lead to serious social and economic burdens on society. In developed nations, stroke is the third leading cause of death, only surpassed by heart disease and cancer. Despite the tremendous mortality and morbidity of stroke, treatment options remain limited. The most plausible reason for this failure is the multiplicity of mechanisms involved in causing neuronal damage during ischemia. At present the only FDA approved treatment is to provide tissue plasminogen activator to reopen occluded blood vessels, and secondary stroke prevention includes antiplatelet, antihypertensive, and lipid-lowering therapies. The therapeutics selected for the study i.e hydergine and nicergoline may prove to be an effective treatment for cerebral ischemia following intranasal administration, as will exert their anti-ischemic activity by virtue of vasodilatation, anti-platelet aggregability and antioxidant properties.

Obesity is a growing health problem in many of the richest nations of the world reaching epidemic proportions. It has now been explored that the body has a homeostatic mechanism for controlling body fat and that the central nervous system (CNS) is involved. The anti-obesity drug selected for the study, sibutramine, was a clinically approved orally administered, centrally acting anti-obesity drug and produced its therapeutic effects by norepinephrine, serotonin and dopamine reuptake inhibition. It has been withdrawn due to serious peripheral side effects such as cardiovascular disturbances, gastrointestinal disturbances, etc arising from unwanted tissue distribution. In the light of above facts, an alternative drug delivery system is needed which can selectively target the candidate drugs to the brain. Thus, the objectives of the proposed work were to develop drug delivery system to deliver nootropics nicergoline and hydergine, and centrally acting anti-obesity drug sibutramine effectively to the brain via the nasal route for the effective treatment of CNS disorders like cerebral ischemia, and related disorders like obesity.

Hence, NG, HG and SB were formulated into suitable intranasal nanoparticulate and microemulsion systems. The drugs were incorporated into polymeric PLGA nanoparticles and the surface of the nanoparticles was engineered by conjugation with anti-transferrin receptor monoclonal antibody and further characterized for particle size, zeta potential, entrapment efficiency, surface morphology and invitro drug release. While, drug containing microemulsions and mucoadhesive microemulsions were also formulated and characterized for globule size, zeta potential, assay, pH, viscosity, in-vitro drug diffusion and in-vitro drug release. It was hypothesized that incorporation of the drugs in the nanoparticles will alter the pharmacokinetics of the drug making it more brain selective, due to attached antibody, following intranasal administration. The efforts were made to elucidate the brain targeting efficiencies of the two different formulations developed viz. antibody conjugated nanoparticles and microemulsions.

The estimation in the nanoparticles, microemulsions and in-vitro release studies for NG, HG and SB was performed by UV spectrophotometry. The calibration curve of NG was established in acetonitrile for nanoparticles by 2^{nd} derivative UV spectrophotometry at 320nm. The linearity of NG was found to be 15-45µg/mL (R^2=0.999). The recovery studies for accuracy and precision were carried out at 10, 30 and 50µg/mL and the recovery was found to be more than 90%, indicating the reliability of the method. To determine the amount of drug entrapped nanoparticles were dissolved in acetonitrile. The resulting system was centrifuged to remove the precipitated components and the supernatant was diluted with the solvent system and subjected to analysis. While, the calibration curve of NG was established in methanol for microemulsions by 2^{nd} derivative UV spectrophotometry at 320nm. The linearity was found to be 15-45µg/mL (R^2=0.999). The recovery studies for accuracy and precision were carried out at 10, 30 and 50µg/mL and the recovery was found to be more than 90%, indicating the reliability of the method. To determine the amount of drug in microemulsions, formulations were dissolved in methanol. The resulting system was centrifuged to remove the precipitated components and the supernatant was diluted with the solvent system and subjected to analysis. The drug was estimated accordingly for in-vitro drug release studies in respective solvents for respective formulation.

For HG also, calibration curve was established in acetonitrile for nanoparticles by 2nd derivative UV spectrophotometry at 298.5nm. The linearity of HG was found to be 20-80µg/mL (R^2=0.999). The recovery studies for accuracy and precision were carried out at 10, 30 and 50µg/mL and the recovery was found to be more than 90%, indicating the reliability of the method. To determine the amount of drug entrapped nanoparticles were dissolved in acetonitrile. The resulting system was centrifuged to remove the precipitated components and the supernatant was diluted with the solvent system and subjected to analysis. While, the calibration curve of HG was established in methanol for microemulsions by 2nd derivative UV spectrophotometry at 292.8nm. The linearity was found to be 20-80µg/mL (R^2=0.999). The recovery studies for accuracy and precision were carried out at 10, 30 and 50µg/mL and the recovery was found to be more than 90%, indicating the reliability of the method. To determine the amount of drug in microemulsions, formulations were dissolved in methanol. The resulting system was centrifuged to remove the precipitated components and the supernatant was diluted with the solvent system and subjected to analysis. The drug was estimated accordingly for in-vitro drug release studies in respective solvents for respective formulation.

Similarly, for SB the calibration curve was established in acetonitrile for nanoparticles by 2nd derivative UV spectrophotometry at 282.2nm. The linearity of SB was found to be 250-850µg/mL (R^2=0.997). The recovery studies for accuracy and precision were carried out at 400, 600 and 800µg/mL and the recovery was found to be more than 90%, indicating the reliability of the method. To determine the amount of drug entrapped nanoparticles were dissolved in acetonitrile. The resulting system was centrifuged to remove the precipitated components and the supernatant was diluted with the solvent system and subjected to analysis. While, the calibration curve of SB was established in methanol for microemulsions by 2nd derivative UV spectrophotometry at 277nm. The linearity was found to be 250-850µg/mL (R^2=0.999). The recovery studies for accuracy and precision were carried out at 400, 600 and 800µg/mL and the recovery was found to be more than 90%, indicating the reliability of the method. To determine the amount of drug in microemulsions, formulations were dissolved in methanol. The resulting system was centrifuged to remove the precipitated components and the supernatant was diluted with the solvent

system and subjected to analysis. The drug was estimated accordingly for in-vitro drug release studies in respective solvents for respective formulation.

The estimation of antibody conjugation was carried out using spectrofluorometry at an excitation and emission wavelengths of 488nm and 578 nm respectively in PBS 7.4. The calibration curve was established at 20-80ng/mL (R^2=0.999).

For the preparation of sibutramine loaded nanoparticles or microemulsions, SB was extracted from SBHM using chloroform. The yield of SB from SBHM was found to be 85±5% and the melting point was observed to be 50°±1°C and also confirmed by the DSC analysis.

The drug loaded nanoparticles were prepared by the emulsion solvent evaporation technique. The major process parameters effecting the formation of nanoparticles were rate of addition of primary emulsion, the speed of the high speed stirrer (or cyclomixer), the probe sonication time and the stirring time. Evaluation of the parameters was carried out at slow, moderate and high levels. Moderate specification was optimized as the best suitable for the preparation of uniform nanoparticle dispersion with the rate of addition of primary emulsion been optimized as 1.5mL/min, the speed of the high speed stirrer as 1200rpm, the probe sonication time as 90sec and the stirring time as 30mins. Based on the preliminary investigations drug to polymer ratio, total lutrol concentration (%w/v) and the organic to aqueous phase volume ratio were found to influence the major variables of particle size and entrapment efficiency. Hence, drug to polymer ratio (keeping the amount of the drug constant), total lutrol concentration (%w/v) and the organic to aqueous phase volume ratio (keeping the volume of the organic phase constant) were kept as independent variables to find optimized condition to obtain lowest particle size (<230nm; of unconjugated nanopartciles) with highest percentage drug entrapment efficiency (dependent variables). The traverse of nanoparticles across the nasal mucosa decreases with increasing size. However, the particle size cut off for drug containing nanoparticles was kept as 230 (such that PS on antibody conjugation remains below 250nm) to have maximum %EE to accommodate nanoparticles equivalent to the dose of the drug in small intranasally administrable volume of 300µL.

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The particle size and entrapment were strongly influenced by the independent variables.

- The increase in the amount of PLGA (keeping the drug amount constant) resulted in the increase in the particle size of the nanoparticles. The viscosity of the organic phase in which the PLGA is dissolved appears to affect the nanoparticles size due to hindrance in rapid dispersion of PLGA solution into the aqueous phase and resulted in increased droplet and nanoparticle size. Increasing the polymer amount also increased the entrapment efficiency and may be due to increase in drug entrapping polymer and a decrease in the diffusion of the drug towards the aqueous phase.
- Increase in the lutrol concentration led to a decrease in the particle size and entrapment efficiency of the nanoparticles. The decrease in size may be due to formation of a more homogenous and stable primary emulsion and a more uniform distribution of the primary emulsion in the aqueous phase. However, the decrease in drug entrapment may be attributed to the increased surfactant in the aqueous phase resulting in increased leaching of drug into the aqueous phase.
- Increase in the volume of the aqueous phase leads to a decrease in the particle size and entrapment efficiency. An increase in the aqueous phase results in availability of more microenvironment for uniform distribution of primary emulsion as smaller droplets and hence, resulting in smaller particle size. While, the drug leached more due to availability of more aqueous microenvironment leading to decreased drug entrapment.

Twenty-seven batches for each NG, HG and SB nanoparticles were prepared by nanoprecipitation method by using 3^3 factorial design varying three independent variables polymer to drug ratio (X_1), lutrol concentration (X_2) & the ratio of aqueous phase to organic phase volume (X_3). The PS and %EE were subjected to multiple regression analysis and mathematical modeling was done using second order polynomial equations (full model). Reduced model equations were achieved after neglecting the nonsignificant terms ($P>0.05$). Results of analysis of variance (ANOVA) of full and reduced model demonstrated that the terms omitted from full model to achieve reduced model, were nonsignificant. The particle size and

entrapment values for the 27 batches for NNp showed a wide variation starting from a minimum of 124.3nm to maximum of 273.1nm and minimum of 70.2% to maximum of 85.4% respectively. Similarly, for HG the particle size and entrapment values for the 27 batches ranged from minimum of 159.7nm to maximum of 301.6nm and minimum of 48.3% to maximum of 69.7% and for SB from 138.6nm to maximum of 310.1nm and minimum of 49.3% to maximum of 68.8%respectively.

The reduced model was used for plotting the contour plots for particle size and entrapment efficiency. The contour plots were made by keeping the major contributing (variable with highest coefficient value) variable fixed. For NG, the contour plots were made at fixed levels of -1, 0, and 1 of X_3 and the values of X_1 and X_2 between -1 to 1 for particle size and entrapment efficiency were computed. Similarly, for HG and SB, the contours were plotted at fixed levels of -1, 0 and 1 of major contributing variable X_3 with X_1 and X_2 varied between -1 to 1 for particle size and X_2 for entrapment efficiency. The contour plots for both particle size and percentage entrapment efficiency of all the three drugs demonstrate a non linear relationship between the two variables X_1 and X_2 and the responses respectively. Both the variables have a negative impact on the responses i.e. both particle size and percentage entrapment efficiency decrease with increasing volume of aqueous phase and lutrol F-68 concentration respectively. The contour plots were used to predict the particle size and percentage entrapment efficiency of nanoparticles at known values of X_1 and X_2. Three checkpoints were selected from contour plots, and the predicted values of particle size and percentage entrapment efficiency were compared with the experimental values using student t test. The difference between the predicted and experimental values 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 nanoparticles of NG, HG and SB of predetermined PS and %EE.

For NG, the batch with particle size of 173.6nm and drug entrapment efficiency of 81.2%w/w prepared at 0 level of X_1 (30mL of aqueous phase per mL of organic phase), 0 level of X_2 (total 0.32%w/v Lutrol F-68 in aqueous phase) and 0 level of X_3 (20:1 polymer to drug weight ratio) was considered optimum based on the criteria of particle size <250nm with highest drug entrapment efficiency.

Similarly for HG, the batch with 198.6nm particle size and 65.8%w/w drug entrapment efficiency was considered to be optimum at 0 level of X_1 (30mL of aqueous phase per mL of organic phase), 0 level of X_2 (total 0.32%w/v Lutrol F-68 in aqueous phase) and 0 level of X_3 (20:1 polymer to drug weight ratio).

Similarly for SB, the batch with 210.3nm particle size and 62.5%w/w drug entrapment efficiency was considered to be optimum at 0 level of X_1 (30mL of aqueous phase per mL of organic phase), 0 level of X_2 (total 0.32%w/v Lutrol F-68 in aqueous phase) and 0 level of X_3 (20:1 polymer to drug weight ratio).

The optimized batch was subjected to lyophilization using sucrose, mannitol and trehalose as cryoprotectant at 1:2, 1:3 and 1:4 (nanoparticle: cryoprotectant). The redispersibility of the lyophilized product and particle size of the nanoparticles was measured after lyophilization. The redispersibility of nanoparticles with sucrose was poor and was only possible after sonication and show significant increase in particle size. The increase in the particle size could have been due to the cohesive nature of the sucrose. With mannitol, the redispersion was possible only after vigorous shaking and the particle size of the nanoparticles increased on lyophilization. This effect may be due to the low solubility of mannitol in water i.e. 0.18 part of mannitol soluble in 1 parts of water. With trehalose as cryoprotectant, the lyophilized nanoparticles were redispersed easily and the increase in particle size was not significant as indicated by Sf/Si of 1.08 for 1:4 of nanoparticles:trehalose. Also, the tyndall effect was retained with use of trehalose as cryoprotectant. Hence, trehalose at a ratio of 1:4 (nanoparticles:trehalose) was used as cryoprotectant for lyophilization of optimized batch of nanoparticles for further studies.

The prepared NG nanoparticles (NNp), HG nanoparticles (HNp) and SB nanoparticles (SNp) were conjugated with phycoerythrin tagged anti-CD71 anti-mouse monoclonal antibody (PE-mAb-Tfr). The antibody was conjugated to the nanoparticle surface by the two step carbodiimide process, which involved the activation of the carboxyl groups on the nanoparticle surface by an EDC/NHS mixture, followed by binding of the activated carboxyl groups to the amino groups in the antibody. The amount of the activating agent (EDC-HCl/NHS concentration), reaction pH, reaction temperature, reaction time and antibody concentration were optimized based on the particle size

and antibody conjugation efficiency keeping other factors constant. The antibody conjugation efficiencies for PE-mAb-Tfr-NNp, PE-mAb-Tfr-HNp and PE-mAb-Tfr-SNp were found to be 43.5 ± 1.2, 42.6 ± 1.4 and 44.1 ± 1.1 which is much more times than the one reported in previous studies and may be attributed to the precise optimization of the various process parameters.

The activation pH was varied between 5-7 and at pH 5 highest conjugation efficiency was observed with a concurrent decrease in percentage drug entrapment efficiency (%EE) which may be due to accelerated hydrolysis of PLGA at this pH. EDC-HCl is generally used as an activating agent in the 4.0-6.0 pH range and hence a low conjugation efficiency was observed at a pH 7 followed by pH 6. Hence, the activation pH was optimized as 5 for nanoparticle activation followed by antibody conjugation effected at pH 7.4 to avoid denaturation of protein at lower pH. The activation pH did not significantly affect the mean particle size of the antibody conjugated nanoparticles.

At a reaction temperature of 45°C both the conjugation efficiency amd %EE were observed to be the lowest and may be attributed to a low glass transition temperature of PLGA of 45°C resulting in increased drug leaching from nanoparticles and availability of less surface carboxyl group to effect conjugation. The same may explain for a low conjugation efficiency and %EE at 30°C. Also, carbodiimide coupling has been reported to be efficient at room temperature. While both conjugation efficiency and %EE were observed to be high at a temperature of 15°C and hence, was optimized as reaction temperature.

It was observed that increasing the concentration of activating agent from 7.3 to 7.8 μM there was an increase in conjugation efficiency from 29.9±1.6% to 40.9±2.3% with no appreciable effect on mean particle size or %EE of antibody conjugated nanoparticles due to activation of more surface carboxyl groups by the increased activating agent. While, there was no significant increase in antibody conjugation efficiencies of nanoparticles with increase in EDC-HCl/NHS concentration from 7.8 to 8.3 μM indicating nanoparticle surface saturation with unavailability of surface carboxyl groups for more antibody attachment.

The conjugation efficiency of antibody to nanoparticles was observed to be significantly low with a reaction time of 1 hr (half an hour each for activation and conjugation) with no significant effect on particle size and %EE indicating the time to be insufficient to achieve maximum antibody conjugation. However, there was no significant increase in antibody conjugation, mean particle size and %EE with increase in reaction time from 2 to 3 hr. Thus, the reaction time was standardized as 2 hr.

The amount of antibody (PE-mAb-Tfr) was varied from 10µg to 500µg and with an increase in the amount of antibody the conjugation efficiency increased with no significant increase in the particle size. This is because by increasing the antibody concentration from 10µg to 500µg no surface saturation was observed for PLGA nanoparticles with respect to the antibody attached as has not been used in molar ratios.

The conjugation of amino group of the antibody with the carboxyl group (of the polymer) on the nanoparticle surface was confirmed by the ^{1}H-NMR for nicergoline containing nanoparticles. Peak at 5.1 in NMR spectra of antibody conjugated NG nanoparticles with respect to unconjugated NG nanoparticles demonstrate antibody conjugation to the nanoparticles.

The prepared nanoparticles were characterized for particle size, zeta potential, entrapment efficiency, in-vitro drug release. The surface morphology of the unconjugated and conjugated nanoparticles was assessed using Transmission Electron Microscopy. Residual solvents (dichloromethane and chloroform) were determined by gas chromatography. Differential scanning calorimetry was performed for the drugs and respective nanoparticles to assess the state of drug in the nanoparticles.

Surface conjugation with the antibody led to changes in the nanoparticle characteristics. Antibody conjugation increased the particle size from 165±15nm to 178.8±16nm, 198.5±12nm to 207.8±14nm and from 210±13nm to 227.8±11nm for nicergoline, hydergine and sibutramine nanoparticles respectively. Similarly, the zeta potential changed from -28.8±2.3mV to -26.5±1.8mV, -30.8±4mV to -28.6±3.1mV and from -28.6±3.1 to -26.6±2.9 for NG, HG and SB nanoparticles respectively. The

%EE for NNP, HNP and SNP was determined to be 80.5±4%, 68.6±3.1% and 65.8 ±3.9% respectively. For PE-mAb-Tfr-NNp, PE-mAb-Tfr-HNp and PE-mAb-Tfr-SNp the %EE was determined to be 70.3 ± 2.4% and 61.4 ± 2.9% respectively. The reduction in the % EE after conjugation could be due to depletion of the surface associated drug, during the conjugation process. The release studies were conducted in 10% methanolic phosphate buffer saline pH 7.4+2 %w/w polysorbate-80 for NNP while for HNP and SNP in 10% methanolic phosphate buffer saline pH 7.4+2 %w/w polysorbate-80 and 30% ethanolic phosphate buffer saline pH 7.4+2 %w/w polysorbate-80 respectively. For NNP there was an initial burst release of about 25% in 12hrs (0.5 days) and then there was a lag phase and about 85% release resulted in 24 days. The burst effect was absent in PE-mAb-Tfr-NNp and the release in 21 days was found to be about 78%. Similarly, for HNP and SNP the initial burst was about 27% and 24% respectively in 12 hrs (0.5days) and lag phase showing about 83% and 87% release in 24 days. The cumulative drug release for PE-mAb-Tfr-NNp and PE-mAb-Tfr-NNp were found to be devoid of initial burst with about 75% and 81% drug release in 24 days. Transmission Electron Microscopy images of the unconjugated and antibody conjugated nanoparticles showed spherical nanoparticles with smooth surfaces. NG, HG and SB demonstrate sharp melting peak at 136.76-141.26°C, 203.66-213.06°C and 55.05-60.46°C respectively. In the nanoparticles the peak of the drugs were either absent as for NNP and SNP or stretched as with HNP, indicating the presence of the drug in amorphous state or disordered-crystalline phase of a molecular dispersion or a solid solution state in the polymer matrix after nanoparticle formulation.

Residual dichloromethane/chloroform was found to be within the permissible limit for both unconjugated and antibody conjugated nanoparticles. Residual dichloromethane/chloroform of antibody conjugated nanoparticles was less than the unconjugated nanoparticles and can be due to the evaporation/washing of surface dichloromethane/chloroform during antibody conjugation process.

The stability studies were conducted in accordance with ICH guidelines for drug products intended to be stored in a refrigerator. The stability of nanoparticle formulations in terms of particle size, zeta potential and drug content was conducted for 6 M at 5°±3°C and 25°±2°C/60±5 %RH. It was observed that for unconjugated

and antibody conjugated nanoparticles of NG, HG and SB there was no significant change ($P>0.05$) observed in particle size, zeta potential and drug content at 5°C ± 3°C for 6M. The storage of the unconjugated and antibody conjugated nanoparticles of the drugs at 25°C ± 2°C/60% ± 5% RH, led to increase in the particle size. The increase in the particle size was not significant during the first month, however became significant and more prominent after 2, 3 and 6 months. During our analysis of samples, the polydispersity index of the nanoparticle stored at 25°C±2°C/60%±5%RH was found to increase as compared to the initial. The increase in the particle size may be due to the absorption of the moisture by the nanoparticles resulting in the coalescence of the small nanoparticles forming larger particles. The nanoparticles were also observed for physical appearance. After 3 and 6 months the physical appearance was also changed, with loss of the free flowing property followed by the difficulty in redispersibility.

At 25°C±2°C/60%±5% RH, the zeta potential of the nanoparticles shifted towards the zero for both unconjugated and conjugated nanoparticles. This may be due to the acidic conditions produced due to the degradation of PLGA into lactic and glycolic acid. The lowered zeta potential values also might have contributed toward the aggregation of particles. The drug content of the unconjugated and conjugated nanoparticles was not altered upto 6M at 5°C ± 3°C. However, the drug content reduced significantly after 2M storage at 25°C±2°C/60%RH ± 5% RH, with the drug content reducing below 95%. This impact could be due to the moisture absorbed by the nanoparticles upon storage at 25°C±2°C/60%RH±5%RH, possibly resulting in the degradation of the drug and the increased temperature contributing to the rate of degradation.

From the above study, we can demonstrate that the unconjugated and antibody conjugated nanoparticles of NG, HG and SB when stored at 25°C±2°C/60%±5%RH for 6M show instability reflected by change in physical appearance, increase in the particle size, zeta potential and reduction in the drug content. Hence, we can conclusively specify that both unconjugated and antibody conjugated nanoparticles of the drugs were stable and can be stored at 5°C±3°C for 6M retaining its original formulation characteristics.

Microemulsions and mucoadhesive microemulsions of NG, HG and SB were successfully prepared by the water titration method followed by construction of pseudo ternary phase diagram. Based on the drug solubility studies capmul MCM was selected as the internal phase for the preparation of microemulsions as drugs had 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 surfactant for pharmaceutical microemulsion formulation is gradually increasing. Surfactant polysorbate 80/tween 80 was selected for the preparation of microemulsions along with co-surfactants like transcutol and ethanol.

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. This indicates that increasing surfactant concentration, the maximum amount of oil can be solubilised/emulsified. 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%w/w 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. Increasing dilution results in an increase in the globule size due to imbibation of added water in to the globules. 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. The major process parameter effecting the formation of nanoparticles was the speed of the stirring. Evaluation of the variation of the stirring speed was carried out at slow, moderate and high speed. Moderate speed was optimized as the best suitable for the preparation of microemulsions without foaming.

Based on preliminary investigations oil content and surfactant concentration (S_{mix}) were found to influence the major variables of globule size and drug loading. Hence, oil content and surfactant concentration were kept as independent variables to find optimized condition to obtain lowest globule or particle size (<50nm) with highest drug loading (dependent variables).

The particle size and drug loading were strongly influenced by the independent variables.

- The increase in the amount of oil resulted in an increase in the globule size of the microemulsions and may be due to non-uniform emulsification of more oil with less amount of surfactant available . With an increase in the oil concentration the and drug loading of the microemulsions increased and may be attributed to the inherent solubility of the drugs in the oil Capmul MCM.
- Increase in the surfactant concentration also led to a decrease in the particle size and is due to more homogenous emulsification of the oil with increased amount of surfactant. While the drug loading increased with an increase in surfactant concentration and may be attributed to the inherent solubility of the drugs in the surfactants.

Nine batches for each NG, HG and SB microemulsions were prepared by titration method using 3^2 factorial design varying two independent oil content (X_1) and surfactant concentration (X_2). The globule size and drug loading were subjected to multiple regression analysis and mathematical modeling was done using second order polynomial equations (full model). Reduced model equations were achieved after neglecting the non-significant terms ($P>0.05$). Results of analysis of variance (ANOVA) of full and reduced model demonstrated that the terms omitted from full model to achieve reduced model, were significant and hence can not be ommitted. The globule size and zeta potential for the 9 batches of NG 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. Similarly, for HG the globule size and drug loading for the 9 batches ranged from minimum of 12.69nm to maximum of 90.29nm and minimum of 33.8% to maximum of 94.7%, and for SB from minimum of 5.14nm to maximum of 82.43nm and minimum of 21.3% to maximum of 96.3% respectively.

The reduced model was used for plotting the contour plots for globule size and drug loading. The contour plots were made by keeping the major contributing (variable with highest coefficient value) variable fixed. For NG, the contour plots were made at fixed levels of -1, 0, and 1 of X_1 and the values of X_2 between -1 to 1 for globule size and drug loading were computed. Similarly, for HG and SB, the contours were plotted at fixed levels of -1, 0 and 1 of major contributing variable (X_1 for oil concentration and X_2 for surfactant concentration). The contour plots demonstrate a non-linear relationship between the independent variables and the dependent variables. The independent variable X_1 was found to have a positive impact on globule size i.e. globule size increases with increasing oil concentration, while X_2 has a negative impact on globule size i.e. globule size decreases with increasing surfactant concentration. Similarly, both the independent variables X_1 and X_2 have a positive impact on the drug loading i.e. drug loading was found to increase with increasing oil and surfactant concentrations. The contour plots were used to predict the globule size and drug loading of microemulsions containing known concentration of oil and surfactant. Three checkpoints were selected from contour plots, and the predicted values of globule size and drug loading were compared with the experimental values using student t test. The difference between the predicted and experimental values 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.

For NG microemulsion (NME), the batch with globule size of 20.43nm and drug loading of 78.3%w/w prepared at 0 level of X_1 (6%w/w) and 0 level of X_2 (36%w/w) was considered optimum based on the criteria of globule size <50nm with highest drug loading.

Similarly for HG microemulsion (HME), the batch with globule size of 14.23nm and drug loading of 72.9%w/w prepared at 0 level of X_1 (6%w/w) and 0 level of X_2 (36%w/w) was considered optimum based on the criteria of globule/particle size <50nm with highest drug loading.

And for SB microemulsion (SME), the batch with globule size of 9.11nm and drug loading of 81.7%w/w prepared at 0 level of X_1 (6%w/w) and 0 level of X_2 (36%w/w) was considered optimum based on the criteria of globule/particle size <50nm with highest drug loading.

The drug mucoadhesive microemulsions (NMME, HMME and SMME) 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. 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. Chitosan being positively charged was found to increase the zeta potential in the positive side without significantly affecting the globule size.

The prepared microemulsions and mucoadhesive microemulsions were subjected to dilution and dye tests and characterized for globule size, zeta potential, assay, pH, viscosity, conductivity, *in-vitro* drug release and diffusion and nasal toxicity. The surface morphology of the microemulsions was assessed using transmission electron microscopy. Both dilution and dye tests indicated that the prepared microemulsions were of o/w type. 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 with bright surroundings. The sizes were in agreement with the globule size distribution measured using photon correlation spectroscopy.

For NG microemulsions (NME and NMME) drug release and diffusion studies were conducted in 10% methanolic phosphate buffer saline pH 7.4+2 %w/w polysorbate-80, while for HG and SB microemulsions (HME, HMME, SME and SMME) in 10% methanolic phosphate buffer saline pH 7.4+2 %w/w polysorbate-80 and 30% ethanolic phosphate buffer saline pH 7.4+2 %w/w polysorbate-80 respectively. 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. 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 and can 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.

In nasal toxicity study, 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.

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.

The in-vivo pharmacokinetics of the drugs in solution, nanoparticles and microemulsions were performed after radiolabeling with ^{99m}Tc. The drug formulations were labeled with ^{99m}Tc with high labeling efficiency using direct labeling method. The radiolabeling was optimized for quantity of stannous chloride, incubation time. The quantity of stannous chloride and incubation time were optimized at 250μg/mL and 10mins for all the formulations of NG, HG and SB. The pH was kept around 6.5 for the formulations. The labeling efficiency for NS, NNp PE-mAb-Tfr-NNp, NME and NMME was found to be 99.1%, 98.7%, 98.9%, 99.1% and 98.9% respectively.

Similarly, the labeling efficiency for HS, HNp PE-mAb-Tfr-HNp, HME and HMME was found to be 98.9%, 99.1%, 98.8%, 99.0% and 98.7% respectively. And the labeling efficiency for SS, SNp PE-mAb-Tfr-SNp, SME and SMME was found to be 99.0%, 98.9%, 99.1%, 98.7% and 98.6% respectively. The radiolabeled complex show high stability in rat serum and 0.9%w/v sodium chloride with radiolabeling efficiencies measured, greater than 95%. The DTPA challenging test, suggest high stability of the radiolabeled complex, with percent transchelation below 3%w/w at 50mM.

The in-vivo biodistribution studies were performed using balb/c mice weighing between 25-30gms. The drug solutions, unconjugated nanoparticles, antibody conjugated nanoparticles, microemulsions and mucoadhesive microemulsions labeled with ^{99m}Tc were administered intranasally. The unconjugated nanoparticles, antibody conjugated nanoparticles and drug microemulsions were also administered intravenously for comparative evaluation. Also, unconjugated nanoparticles and drug microemulsions were also administered orally for comparisons. The studies were conducted for 24hrs and at different time intervals the radioactivity was measured in brain and blood. The radioactivity in brain and blood was determined as fraction of administered dose per gram of the tissue (%ID/g).

Drug concentrations in brain following intranasal administration of ^{99m}Tc labeled drug formulations were found to be significantly higher at all time points compared to intravenous and oral administrations. Also, at all time points the brain/blood ratios were calculated for the formulations and were found to be significantly higher for intranasally administered formulations than formulations administered intravenously or oral, demonstrating intranasal delivery a promising strategy to deliver drugs quickly and effectively to the brain. Also, the brain/blood ratios for ^{99m}Tc-PE-mAb-Tfr-HNp (i.n.) at all time points were found to be more than 10 folds compared to ^{99m}Tc-PE-mAb-Tfr-HNp (i.v.). The significantly high brain/blood ratios for ^{99m}Tc-PE-mAb-Tfr-HNp (i.n.) compared to ^{99m}Tc-HS (i.n.) and ^{99m}Tc-HNp (i.n.) demonstrates selective uptake of antibody conjugated nanoparticles into the brain compared to unconjugated nanoparticles and drug solution across transferrin receptors at the olfactory bulb of animals. These receptors are expressed abundantly on the olfactory

bulb of rodents resulting in selective and effective distribution of antibody (specific to transferrin receptors) conjugated nanoparticles into the brain than other formulations.

The pharmacokinetic parameters of NG, HG and SB in mice after intranasal, intravenous and oral administrations of drug solutions, drug loaded nanoparticles, antibody conjugated drug loaded nanoparticles, microemulsions and mucoadhesive microemulsions were calculated using Kinetica. Intranasal antibody conjugated nanoparticles showed highest C_{max} (brain) compared to other intranasally administered formulations. Also, the C_{max} (brain) for intranasal antibody conjugated nanoparticles was found to be approximately 10-folds and more than 50-folds higher when compared to intravenous and oral administrations respectively. C_{max} (brain) and $AUC_{0\rightarrow\infty}$ (brain) of intranasal antibody conjugated nanoparticles is approximately 2.5 folds than other intranasal formulations demonstrating antibody conjugated nanoparticles a suitable formulation to deliver drugs effectively to the brain intranasally and may be attributed the longer T_{max} (brain) of antibody conjugated nanoparticles than other formulations administered intranasally, sustaining the drug for a longer time in brain and resulting in greater AUC (brain).

For intranasally administered formulations brain targeting efficiency was assessed based on the values of drug targeting index (DTI) which represents time average partitioning of drug between brain and blood, and brain drug targeting percentage (DTP%), which represents the percentage of drug directly transported to the brain via the neural pathway. The antibody conjugated nanoparticles showed the highest DTI and DTP% values among all intranasally administered formulations. Higher DTI and DTP% for antibody conjugated nanoparticles may be attributed to the higher brain concentrations of drug following its intranasal administration resulting from selective uptake of antibody conjugated nanoparticles across the transferrin receptors on the murine olfactory bulb to the brain.

Gamma scintigraphy imaging demonstrate high radioactivity in the animal brain for intranasal antibody conjugated nanoparticles compared to other intranasal formulations and formulations administered intravenously and orally and supports selective and effective transport of drug to the brain following intranasal

administration of antibody conjugated nanoparticles. Thus, gamma scintigraphy images are consistent with the biodistribution data.

SB being a centrally acting anti-obesity drug was evaluated for its utility through the intranasal route that delivers drug quickly and in higher amounts to the brain avoiding first pass metabolism, unwanted systemic exposure and side effects (gastrointestinal disturbances) and bypassing the BBB. The anti-obesity activity of SB results was determined with respect to body weight, serum lipids, leptin and white adipose tissue mass following intranasal and oral drug administrations reespectively. The differences between the control and drug treated groups were found to be significant ($P<0.05$). Also, the difference between the intranasal SB and oral SB treated groups were found to be significant and can be attributed to the significantly improved brain uptake of drug SB following intranasal administration of brain selective antibody conjugated SB nanoparticles. Thus, treatment with intranasal SB was found to be more effective for the management of obesity than oral administration when the drug has a central mechanism of action.

Intravenous delivery has limited utility in managing ischemic stroke due to the presence of BBB and restriction of blood supply to the brain due to thrombus or embolism limiting drug ingress effectively in to the brain illustrating intranasal route to be advantageous in such circumstances. Nicergoline and hydergine have almost similar mechanism of action with nicergoline having added neuroprotectives properties and therefore the neuroprotectives effect was evaluated for nicergoline. Significantly high brain GSH levels were observed following treatment with nicergoline 30mins after induction of transient global ischemia compared to ischemic control while the difference between the pre-treated group and ischemic control was insignificant. Also, the infarct volume and brain oedema were found to be significantly less for post-treated group than pre-treated or ischemic control group. Also, the pretreated group demonstrated less infarct volume and brain oedema and higher GSH levels than the ischemic control illustrating a possibility of nicergoline to be advantageous if taken prophylactically. These demonstrate the neuroprotective effect of nicergoline in cerebral ischemia with intranasal delivery enhancing their magnitude to many times by virtue of selective and effective delivery of NG to the brain following intranasal administration of antibody conjugated nicergoline

nanoparticles. The neuroprotectives effect of NG is attributed to its multiple mechanisms of action viz. antioxidant activity, antiplatelet activity and increasing cellular ATP in brain.

Conclusions

Nanoparticulate and microemulsion formulations of nicergoline, hydergine and sibutramine were prepared and subjected to characterization, pharmacokinetic and pharmacodynamic studies. The antibody conjugated drug nanoparticles showed higher brain uptake compared to drug solution, unconjugated nanoparticles, microemulsions and mucoadhesive microemulsions following intranasal administrations. When compared to drug solution, protection of the drug from degradation and/or efflux back into the nasal cavity may be the reason for improved brain uptake of drug nanoparticles on intranasal administration. Nanoparticles ($\approx$ 20 nm) are thought to pass transcellularly (apical to basolateral transport through epithelial cell) in nose-to-brain drug delivery through a number of different molecular mechanisms including macropinocytosis, clathrinmediated, clathrin-independent, caveolin-mediated, caveolinindependent, receptor-meditated endocytosis and phagocytosis. However, transcellular transport is a slower process than paracellular transport which is thought to be mainly responsible for quick nose to brain delivery of drugs as microemulsions or mucoadhesive microemulsions. The same is also evident by the longer T_{max} for antibody conjugated nanoparticles than drug microemulsions and mucoadhesive microemulsions. Receptor-meditated endocytosis is a term used to describe a group of endocytic mechanisms where the ligand is thought to stimulate the endocytic event by complementing a receptor on the cell membrane and is a highly specific mechanism. Thus, the selectivity conferred by the attached antibody (specific for transferrin receptors on murine nasal olfactory bulb) may have contributed to the improved (two times higher) nose to brain drug uptake compared to microemulsions and mucoadhesive microemulsions. This is also supported by the higher (two times higher) C_{max} of antibody conjugated nanoparticles than microemulsions and mucoadhesive microemulsions. Also, it was evident from the kinetic studies that the drug brain concentrations declined comparatively faster for drug microemulsions and mucoadhesive microemulsions than antibody conjugated drug nanoparticles or unconjugated drug nanoparticles resulting in lower (two times lower) $AUC_{0\rightarrow\infty}$ (brain)

for microemulsion formulations than nanoparticle formulations and may be due to the protection of the drug from degradation in nanoparticles. The same can explain the higher $AUC_{0\rightarrow\infty}$ (brain) for unconjugated nanoparticles than drug microemulsions. Thus, the selectivity conferred by the attached antibody (specific for transferrin receptors on murine nasal olfactory bulb), higher C_{max}, longer T_{max} and slower clearance justifies the enhanced nose to brain uptake of drug as antibody conjugated nanoparticles than unconjugated nanoparticles or microemulsion or mucoadhesive microemulsion.

Also, intranasal administration demonstrated significantly high brain concentrations of drugs when compared to their intravenous and oral counterparts. This improved brain drug uptake may be attributed to the unique connection between the nasal cavity and the brain tissue conferred by the olfactory and trigeminal nerves. Almost similar results were observed for the three drugs. Thus, antibody conjugated drug nanoparticles were used for the further pharmacodynamic studies.

Intravenous or oral delivery has limited utility in managing brain and brain related disorders like ischemic stroke and obesity due to the presence of BBB and first pass metabolism, and limiting drug ingress effectively in to the brain in therapeutic concentration illustrating intranasal route to be advantageous in such circumstances. Significant difference was observed between the control and drug treated groups when assessing the anti-obesity effect of sibutramine with intranasal treatment been significantly more effective than oral and can be attributed to improved brain uptake of sibutramine following intranasal administration.

Similarly, the neuroprotective effect of nicergoline was evaluated following intranasal administration within 30mins of stroke onset. The presence of BBB and the occluded blood vessels restrict effective drug delivery to the brain following intravenous administration during stroke management, illustrating the advantage of intranasal delivery in such circumstances delivering drugs quickly and effectively to the brain bypassing the BBB. During evaluation of the neuroprotective effect of nicergoline in cerebral ischemia insignificant difference was observed between the ischemic control and drug treated group receiving drug 30mins before stroke onset (drug pretreated

group). However, a significant difference was observed between the ischemic control and drug treated group receiving drug within 30mins after stroke onset (drug post-treated group) demonstrating the neuroprotective effect of nicergoline. Thus, intranasal nicergoline may felicitate the stoke treatment from home itself before hospitalization after stroke onset restricting the damage to the minimum and providing a possibility of the reversal of the neural damage. Also, intranasal nicergoline, simultaneously or after initial treatment with thrombolytics, may add to the narrow therapeutic time window of 4.5hr of stroke therapy initiation and may have a synergistic effect taking care of neural damage and secondary mechanisms of excitotoxicity, oxidative stress, inflammation and apoptosis.

The findings of the investigation suggest that nicergoline, hydergine and sibutramine in the form of antibody conjugated nanoparticles can be delivered effectively to the brain following intranasal administration and may find a possible role in clinical practice of cerebral ischemia and obesity. Antibody conjugation to drug carriers was proved to be effective in selective improvement in brain uptake of drugs after intranasal administration and the concept may be used to deliver drugs to the brain which otherwise pose a challenge in brain uptake in therapeutic concentrations. However, the clinical use of the concept requires strengthening of this vital finding through more animal followed by clinical experimentations. Similarly, regional distribution of drug within the brain may provide vital clue to drug delivery scientists to incorporate a drug for better therapeutic outcome.