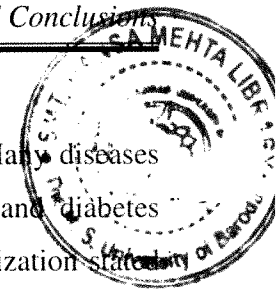
CHAPTER 10

SUMMARY AND CONCLUSIONS



10 SUMMARY AND CONCLUSIONS

Pain is escalating health problem globally affecting 19% of the population. Many diseases like cancer, multiple sclerosis, herpes zoster infection, accidental surgeries and diabetes patients suffer from different types of pain. A survey by World Health Organization states that more than 50% of patients still suffer severe and intolerable pain after surgery and trauma. To live everyday with any type of pain is extremely adverse experience that challenges every fibre of an individual's being. The pain disorder brings very high direct and indirect costs to patients and society in terms of suffering and lost productivity. Pain is able to alter a patient's quality of life by interfering with mood, sleep and emotional well-being.

Opioid analgesics are increasingly being prescribed for the treatment of multiple and diverse acute and chronic painful conditions. However, their role of opioids in the treatment of pain is also influenced by the fact that these potent analgesics are associated with a significant number of side effects and complications. Common side effects of opioid administration include sedation, dizziness, nausea, vomiting, constipation, physical dependence, tolerance, and respiratory depression. Physical dependence and addiction are clinical concerns that may prevent proper prescribing and in turn inadequate pain management.

Non-opiods based drugs are normally used for postoperative and non–cancer pain. Patients are not frequently receiving the appropriate drug therapy with inadequate drug dosing and are frequently dissatisfied with treatment. Analgesics very effective for acute inflammatory traumatic pain, are not very helpful in treating the majority of chronic pain conditions. Most current therapies have come about from efficacy noted in non-pain formulations from anti-epileptics and depressants. Unfortunately, the level of efficacy reaches only 5-30% for any particular drug. Advance pain treatments, such as, regional or local nerve blocks, epidural steroid injections, spinal cord stimulators, and acupuncture have no rational basis in terms of efficacy or outcome studies and are associated with serious complications.

Tramadol (TMD) is a central acting analgesic which has been shown to be effective and well tolerated, and likely to be of value for treating several pain conditions (step II of the World Health Organization ladder) where treatment with strong opioids is not recommended. Due to absence of clinically relevant effects on respiratory or cardiovascular functions and negligible tendency of abuse, Tramadol, is a drug of choice for alleviation of post operative/ moderate-to-severe pain. However high frequency of drug administration (4-6 hourly), short half-life (5-6 h), low bioavailability of 68% and dose dependent side effects like GI disorders, pruritus poses challenge for its clinical use. Adverse effects are dose-dependent and therefore

considerably more likely to appear if the loading dose is high. Transporting Tramadol in enhanced concentration to the brain would result in enhanced bioavailability for effective pain management and reduction in drug exposure to other organs and thereby reducing the side effects.

Anticonvulsant drugs gain importance in neuropathic pain treatment because of advantages of lesser side effects as compared to opioids and anti-depressants. Lamotrigine (LTG), a sodium and calcium channel blocker, has demonstrated efficacy for the treatment of neuropathic pain in multiple, randomized, controlled trials. However there is a risk of dose dependent severe rashes, as well as Stevens-Johnson syndrome, a potentially fatal epidermal necrosis associated with high dose and prompt dose escalation. High dose, variable brain permeability, dose dependent side effects and severe skin rashes associated with poses challenge for their effective clinical use in neuropathic pain. With IR formulations, the drug peak serum concentration may be associated with considerable adverse effects. Enhancing the drug concentration to the brain would there by lead to reduction in systemic exposure and resulting in reduced side effects. Moreover, such concentration curve helps to avoid a drug trough level, which makes pain control more efficient.

However, drug delivery to brain is challenged by a variety of formidable obstacles like blood brain barrier (BBB), brain cerebrospinal fluid barrier and brain tumour barrier. The BBB comprising of the endothelial cells forming tight junctions separates brain from the systemic circulation, thereby restricting delivery of therapeutics to brain. Several approaches are employed to enhance drug delivery across BBB.

The BBB is provided with active transport mechanisms like carrier mediated transport, adsorption mediated transport and receptor mediated transport for nutrient supply to the brain. The transport of essential nutrients across the BBB using interaction of ligand with the receptors located at the luminal membrane is known as Receptor mediated transport (RMT). The movement of free iron into cells is essentially mediated via a family of non-heme iron binding glycoproteins termed Transferring (Tf), Lactotransferrin (Lf) and melanotransferrin, and their respective cognate membrane receptors.

Transferrin and Transferrin family receptors are expressed on the luminal membrane of brain endothelial cells and mediate the internalization of iron-saturated Tf through RMT. The Tf receptors are of particular interest because their substantial expression in brain capillaries. On the other hand they suffer shortcoming that Tf receptors are almost saturated under

physiologic conditions because of high endogenous plasma Tf concentration. Nevertheless, the receptor-mediated endocytosis of Tf from blood to brain is well documented.

Lf is a multifunctional protein to which several physiological roles have been attributed which are mediated by Lf receptors. Lf has been demonstrated to cross the BBB via receptor-mediated transcytosis. However there are only few citations from different authors signifying the role of Lf as brain delivery vector. Therefore, it was also of interest to determine whether which is better ligand among Tf or Lf for brain delivery.

Surface engineering of nanoparticles (NPs) with ligand like Transferrin and Lactoferrin offers promising tool for brain delivery of otherwise inaccessible drugs. Several researchers across the globe have successfully targeted drugs across BBB by incorporation into the nanocarrier and surface modifying the NPs with Transferrin ligand. Therefore, it was of interest to determine whether Lf conjugated drug loaded PLGA can be transported to the extent of Tf conjugated drug loaded PLGA, into the brain across the BBB in vivo.

By incorporating Tramdol and Lamotrigine in ligand conjugated NPs the higher amount of drug can be delivered to the site of action with lesser systemic exposure leading to reduced side effects. There is possibility of dose reduction which will minimize dose dependent side effects. The drug delivered form PLGA NPs in sustained manner provide improved patient compliance. The proposed delivery system is also useful for maintaining therapeutic effect for prolonged period of time.

Many advanced and effective approaches to the CNS delivery of drugs have emerged in recent years. Intranasal drug delivery is one of the focused delivery option for brain targeting as brain and nose compartments are connected to each other via olfactory/ trigeminal route via peripheral circulation. Realization of nose to brain transport and the therapeutic viability of the route can be traced from the ancient times and has been successfully investigated for rapid and effective transport in last two decades.

Intranasal route is noninvasive mode of drug administration in comparison to the other routes of administration. Intranasal drug delivery delivers the drug directly to the brain by circumventing BBB and reduces drug delivery to non targeted sites. Direct transport of drugs to the brain may lead to the administration of lower doses and in turn can reduce toxicity. Systemic dilution effect and first pass metabolism are also avoided. Direct transport could result rapid and/or higher uptake in brain, which provides an alternative option of self-medication in management of emergencies. Among the novel systems for brain delivery through intranasal route, microemulsions gained considerable interest for their simple

formulation with more stability and optical clarity and efficient to across the biological membranes, biocompatibility, biodegradability, easy to prepare and handle and most importantly solubilization capacity for both water and oil soluble drugs. Microemulsion demonstrates a possible alternative to i.v. administration and a promising approach for rapid onset delivery of CNS medications.

Microemulsions (ME) are equilibrium systems (i.e. thermodynamically stable), while nanoemulsions (NE) are non-equilibrium systems with a spontaneous tendency to separate into the constituent phases. Nevertheless, NEs may possess a relatively high kinetic stability, even for several years. The foremost advantage of nanoemulsion system is it consists of lesser amount of surfactants suggesting suitability for multiple dosing without affecting nasal mucosal epiltelium. Also, use of lecithin as surfactant ensures biocompatibility and safety for chronic treatment. However, drug loading is lesser compared to microemulsion.

By formulating Tramadol and Lamotrigine in microemulsion and nanoemulsion formulations, delivers drug directly to brain by passing BBB which is useful for episodic and emergency pain treatment. The drug will be delivered to brain circumventing BBB in brisk manner establishing immediately, minimum effective concentration required for therapeutic response. Direct transport of drugs to the brain may lead to the administration of lower doses, reduce the toxicity and avoids systemic dilution effect and first pass metabolism.

The objective of the study is to incorporate therapeutic pain alleviating drugs into nanoconstructs for their enhanced & selective brain uptake after parenteral or nasal administration for prolonged and rapid drug delivery respectively in pain treatment. For parenteral administration, nanoconstructs were be surface modified with surfactants for long circulation and attached with brain selective ligand such as Tf/ Lf for enhancing brain bioavailability and reducing systemic toxicity. For intranasal administration, nanocostructs formulated as microemulsion and nanoemulsion could result rapid and higher uptake in brain, which provides an alternative option of self-medication in management of emergencies.

TMD and LTG were incorporated into polymeric PLGA NPs and the surface of the NPs was engineered by conjugation of Transferrin (Tf) and Lactoferrin (Lf) and further characterized for Particle size (PS), Zeta potential (ZP), entrapment efficiency (%EE), surface morphology (TEM) and in vitro drug release. Ligand conjugated NPs were assessed for their pharmacokinetic and pharmacodynamic performance for brain drug delivery in mice/rat after i.v. administration. It is also the objective to compare the targeting capability of NPs after conjugation with Lf and Tf. It was hypothesized that incorporation of the drugs in the NPs

will alter the pharmacokinetics of the drug making it long circulating and due to the presence of surface attached Tf/Lf lead to targeted and enhanced delivery to the brain after i.v. administration.

TMD and LTG were formulated as microemulsion/ nanoemulsion and further characterized for globule size (GS), zeta potential (ZP), globule size, % transmittance, drug content, pH, viscosity, nasal mucosa tissue compatibility, in vitro diffusion studies across nasal mucosa. Microemulsion and nanoemulsion were assessed for their pharmacokinetic and pharmacodynamic performance for brain drug delivery in mice/rat after i.n. administration. It is also the objective to compare the efficacy, safety and stability of microemulsion with nanoemulsion. It is also hypothesized that, the intranasal microemulsion and nanoemulsion may provide comparatively faster and higher uptake via direct nose to brain transport of drug by brain by circumventing BBB and reducing drug delivery to non targeted sites, avoiding systemic dilution effect and first pass metabolism.

NANOPARTICLES

Analytical methods

The UV spectrophotometric method was employed for estimation of drug content in NPs. The calibration curve of TMD and LTG was established in acetonitrile by UV spectrophotometry at 278 and 307 nm respectively. The linearity of TMD and LTG in acetonitrile was found to be 12.5-150 µg/ml (R2=0.9993) and 1-30 µg/ml (R2=0.9996) respectively. The recovery study of TMD and LTG for acetonitrile was performed at 37.5, 75, 112.5 µg/ml and 7.5, 15, 22.5 µg/ml to check accuracy of developed method. % accuracy was found to be more than 99% for developed methods of TMD and LTG. Intraday and inter day precision of developed method for TMD and LTG was performed at low, medium and high concentration of drug. %RSD of developed method for TMD and LTG was found to be less than 1. Results of accuracy and precision are indicates the reliability of the developed method for both the drugs. The method was validated for linearity, accuracy and precision. The validation parameters were complies with USP and % RSD was found less than 1%.

The estimation of Tf and Lf conjugation was carried out using BCA protein estimation. The calibration curve was established at 12.5-175 µg/ml (R2=0.9990). The amount of PVA associated with NPs was determined by a colorimetric method based on the formation of a colored complex between two adjacent hydroxyl groups of PVA and an iodine molecule. A

standard plot for known concentrations of PVA was established at 10-200 µg/ml. (R2=0.9998)

Preparation, Optimization and Characterization

The drug loaded NPs were prepared by nanoprecipitation technique. Acetone was selected as organic phase. The rate of addition of organic phase to the aqueous was kept constant at 0.5ml/min throughout the entire experimentation. The major process parameter effecting the formation of NPs 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 uniform NPs dispersion. Based on preliminary investigations drug: polymer ratio, PVA concentration in aqueous phase (%w/v) and the ratio of the organic: aqueous phase were found to influence the major variables of Particle Size (PS) and Entrapment Efficiency (% EE). Hence, drug: polymer ratio (represented as polymer concentration, as the amount of the drug was kept constant), PVA concentration in aqueous phase (%w/v) and the ratio of the organic: aqueous phase (represented in decimal form) were kept as independent variables to find optimized condition to obtain lowest PS (<150nm) with highest %EE (dependent variables).

Twenty-seven batches for each TMD and LTG NPs were prepared by nanoprecipitation method using 33 factorial design varying three independent variables %w/v PVA concentration (X1), drug: polymer ratio (X2) & organic: aqueous phase ratio (X3).

The PS and entrapment were strongly influenced by the independent variables.

- PS of NPs decreased with the increase of PVA concentration. With increase in concentration more PVA can be oriented at organic solvent/water interface thereby reducing interfacial tension efficiently (Galindo-Rodriguez S et al., 2004), which promoted the formation of smaller emulsion droplets. Thus, at sufficient concentration, PVA cover the droplets completely and avoid coalescence of droplets during the removal of organic solvent thereby forming NPs with smaller size. In addition, a large number of hydroxyl groups extending into the continuous phase forms hydrated layer at the surface hinder NPs aggregation. The decrease in EE with the increase of PVA concentration was probably due to decrease in PS.
- The increase in the concentration of PLGA resulted in the increase in the PS of the NPs. The viscosity of PLGA appears to affect the size of NPs due to hindrance in rapid dispersion of PLGA solution of higher viscosity into the aqueous phase resulted

in increase in the NPs size. Availability of PVA on the surface of NPs prevents the aggregation of NPs during solvent evaporation but in case of higher concentrations of PLGA, deposition of PVA on the particle surface may not be uniform and sufficient leading to increase in PS. However, increase in concentration of PLGA increases the EE. It may be due to increase in drug entrapping polymer and resultant decrease in the diffusion of the drug towards the aqueous phase The increase in PS with the increasing PLGA concentration, can increase the length of diffusion pathways of drugs from the organic phase to the aqueous phase, thus reduce the drug loss through diffusion and increase EE.

- The PS and EE were found to be inversely proportional to the organic: aqueous phase ratio. As the organic: aqueous phase ratio was increased, the PS and drug EE were decreased. The increase in the organic phase ratio leads to increased evaporation time causing slower polymer precipitation, and thereby formation of small particles. Due to the increased evaporation time and slower polymer precipitation, the tendency of the drug to escape in the aqueous phase before polymer precipitation increases leading to lower drug EE.

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 PS and entrapment values for the 27 batches for TMD-NPs showed a variation starting from a minimum of 108.6 nm to maximum of 185.3 nm and minimum of 49.2% to maximum of 78.3% respectively. Similarly, for LTG-NPs the PS and entrapment values for the 27 batches ranged from minimum of 101.0 nm to maximum of 170.7 nm and minimum of 52.4% to maximum of 83.4% respectively.

The reduced model was used for plotting the contour plots for PS and entrapment efficiency. The contour plots were made by keeping the minor contributing variable (PVA concentration) fixed at -1, 0, +1 the contours were constructed between the other independent variables (Concentration of PLGA and volume of organic phase) for PS and drug entrapment efficiency separately. The contour plots demonstrate clearly the relationship between the independent variable and dependent variables. The overlay of contours was used to predict the PS and % entrapment efficiency. Three checkpoints were selected from contour plots, and

the predicted values of PS and % EE were compared with the experimental values using student t test. The difference was 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 NPs of TMD and LTG of predetermined PS and EE (%). Response surface plots were plotted by keeping the factor X_1 at fixed levels (-1, 0 and 1). Response surface plots are very helpful in learning about both the main and interaction effects of the independent variables.

For TMD-NPs, the batch with PS of 141.1 ± 3.7 nm and drug entrapment efficiency of 73.57 ± 2.1 % prepared at 0 level of X1 (1% w/v PVA in aqueous solution), +1 level of X_2 (100 mg polymer 5 mg drug) and +1 level of X_3 (organic: aqueous phase of 1:2, i.e. 5 ml of organic phase and 10ml of aqueous phase) was considered optimum based on the criteria of PS <150 nm with highest drug entrapment efficiency.

Similarly for LTG-NPs, the batch with 133.9 ± 3.6 nm PS and 78.28 ± 1.7 % drug entrapment efficiency was considered to be optimum at the at 0 level of X_1 (1% w/v PVA in aqueous solution), +1 level of X_2 (100 mg polymer 5 mg drug) and +1 level of X_3 (organic: aqueous phase of 1:2, i.e. 5ml of organic phase and 10ml of aqueous phase). Hence, 10 mg of drug and 100 mg of PLGA was dissolved in 5ml of acetone and this solution was added to 10 ml of 1%w/v PVA aqueous solution under constant moderate stirring.

The prepared TMD-NPs and LTG-NPs were conjugated with Tf and Lf. Tf/Lf was conjugated to the NPs surface in two steps involving the activation of the NPs in the presence of catalyst zinc tetrafluoroborohydrate [Zn(BF4)2] with epoxy compound (SR-4GL, hexa epoxy) which acts as linker, followed by attachment of Tf/Lf to the NPs at the other end of the epoxy compound. PVA cross links with PLGA and is not removed despite several washings. In the conjugation process, at least one of the epoxy of SR-4GL would have conjugated to the hydroxyl group of PVA and the other epoxy groups to the amine group of Tf. The amount of the activating agent (epoxy compound) and Tf/Lf were optimized based on their influence on the PS and surface Tf/Lf density. The amount of the catalyst zinc tetrafluoroborohydrate [Zn (BF4)2] was kept constant at 10 mg throughout the experiment.
The influence of the amount of epoxy compound on the density of surface Tf and PS was evaluated keeping the amount of NPs and the amount of Tf/Lf constant at 75 mg and 1mg respectively. The amount of epoxy compound was varied at 5 mg, 10 mg and 20 mg. With the increase in the amount of the epoxy from 5 to 10 mg, the surface Tf density for Tf-TMD-NPs increased from 6.3 µg/mg to 10.6 µg/mg and the PS increased from 151.3 nm to 157.5

nm while for Tf-LTG-NPs Tf density increased from 6.7 µg/mg to 10.7 µg/mg and the PS increased from 146.9 nm to 151.0 nm. Increasing further the epoxy compound to 20 mg did not considerably increase the surface Tf density. However, the PS increased from 157.5 to 175.1 nm and 151.0 to 168.7nm for Tf-TMD-NPs and Tf-LTG-NPs respectively. Similar results were observed for Lf conjugation. The epoxy compound amount at 5, 10 and 20 mg resulted in the surface Lf density of 6.5 µg/mg, 11.4 µg/mg and 11.4 µg/mg respectively, with corresponding PS of 150.9 nm, 158.8 nm and 173.4 nm for Lf-TMD-NPs. The epoxy compound amount at 5, 10 and 20 mg resulted in the surface Lf density of 6.9 µg/mg, 11.4 µg/mg and 11.9 µg/mg respectively, with corresponding PS of 144.2 nm, 150.4 nm and 166.5 nm for Lf-LTG-NPs. Conjugation efficiency of Tf and Lf to TMD-NPs at 10 mg SR-4GL, were 79.5 and 83.3 respectively. Conjugation efficiency of Tf and Lf to LTG-NPs at 10 mg concentration of SR-4GL, were 80.3 and 85.5 respectively. The increase in the surface Tf/Lf density may be due to the increase in the number of the epoxy molecules reacting with hydroxyl of PVA and thereby increase in the availability of the epoxy groups for conjugation of Tf/Lf. The association of epoxy and ligand with NPs is believed to have resulted in the increase in the PS. Increasing the epoxy amount from 10 mg to 20 mg resulted in much increase in the PS but the amount of the ligand conjugated did not increase significantly. Hence, the epoxy amount was optimized at 10 mg for both Tf and Lf conjugation with TMD-NPs and LTG-NPs.

The influence of the amount ligand on the density of surface Tf/Lf and PS was evaluated keeping the amount of NPs and the amount of SR-4GL constant at 75 mg and 10 mg respectively.

The amount of Tf was varied from 0.25 mg to 1.5 mg. With increase in the amount of Tf from 0.25 mg to 1.0 mg, the surface Tf density for TMD-NPs increased from 3.2 µg/mg to 10.6 µg/mg and the PS increased from 149.2 nm to 157.5 nm and for LTG-NPs, Tf density increased from 3.3 µg/mg to 10.7 µg/mg and the PS increased from 140.2 nm to 151.0 nm. Further increasing the amount of Tf from 1.0 mg to 1.5 mg, the Tf density increased from 10.6 µg/mg to 13.1 µg/mg and 10.7 µg/mg to 13.0 µg/mg for TMD and LTG NPs respectively. But the PS increased from 157.5 to 168.9 nm and 151.0 nm to 163.5 nm for TMD and LTG NPs respectively. Similarly, for Lf conjugation surface Lf density and PS were found to increase from 3.3 µg/mg to 13.5 µg/mg and 152.6 nm to 171.9 nm respectively when Lf amount was varied from 0.25mg to 1.5 mg. With increase in the amount of Tf/Lf added for conjugation, the increase in the surface Tf/Lf density could have been due to the increase in the Tf molecule density available for conjugation. The increase in the PS could

have been due to increased surface Tf/Lf density. At the highest amount of Tf added for conjugation i.e. 1.5 mg, the PS increased, probably due to the cross linking of Tf/Lf molecule with the epoxy groups of the neighbouring molecules. Also, conjugation efficiency for TMD-NPs was around 65-67% at 1.5 mg ligand compared to 80-83% at 1.0 and for LTG-NPs conjugation efficiency was around 65-69% at 1.5 mg ligand compared to 80-85 % at 1.0. For, i.v. administration, the preferable PS is below 200 nm and hence considering the size and conjugation efficiency, 1.0 mg of Tf/Lf was considered as optimized amount.

The conjugation amino of Tf and Lf with the methylene of epoxy compound was confirmed by 1H-NMR. For TMD-NPs and LTG-NPs the peaks at around 2.2 to 2.3 ppm were observed representing the conjugation of amino group of Tf and Lf to the methylene group of epoxy compound.

The optimized batch of TMD-NPs was subjected to lyophilization using sucrose, mannitol and trehalose as lyoprotectant at 1:1, 1:1.5 and 1:2 (NPs: lyoprotectant). The redispersibility of the lyophilized product and PS of the NPs was measured after lyophilization. The redispersibility of NPs with sucrose was poor and was only possible after sonication and show significant increase in PS. The increase in the PS could have been due to the cohesive nature of the sucrose. With mannitol, the redispersion was possible only after vigorous shaking and the PS of the NPs increased on lyophilization. Possibly, the polyalcohol structure tends to form of a crystalline mass during lyophilisation process. With trehalose, all the lyophilized cakes were snow-like, voluminous and easy to reconstitute. The increase in PS was not significant as indicated by Sf/Si values which were 2.52, 1.52, and 1.01 for 1:1, 1:1.5 and 1:2 NPs: trehalose. Also the tyndal effect observed with NPs was retained after redispersion of the NPs lyophilized using trehalose. Therefore, trehalose at a ratio of 1:2 (NPs: trehalose) was used as lyoprotectant for lyophilization of optimized batch of NPs. Also, it was found satisfactory for LTG-NPs without any significant change observed against initial. Further it was found satisfactory at 1:2.25 ratios for conjugated NPs of TMD. The superior lyoprotective effect may be attributed to the ability of trehalose to form a glassy amorphous matrix around the particles, through hydrogen-bonding with polar groups of the product, preventing the particles from sticking together during removal of water. Therefore, trehalose at a ratio of 1:2.5 (NPs: trehalose) was used as lyoprotectant for lyophilization of optimized batch of conjugated NPs for further studies.

The prepared NPs were characterized for PS, ZP, %EE and in vitro drug release. The surface morphology of the unconjugated and conjugated NPs was assessed using Transmission

Electron Microscopy. Residual PVA was determined using colorimetric reaction. Differential scanning calorimetry was performed for TMD, TMD-NPs, LTG, LTG- NPs, PLGA and PVA to assess the state of drug present in the NPs.

Surface conjugation with Tf and Lf leads to change in the particle characteristics. Conjugation with Tf and Lf increased the PS 157.5 ± 4.2 nm and 158.8 ± 3.9 nm respectively from 141.1 ± 3.7 nm for TMD-NPs. The ZP changed from -10.32 ± 0.48 mV to -11.06 ± 0.41 mV and -9.35 ± 0.29 mV for Tf and Lf conjugated NPs respectively. Conjugation with Tf and Lf increased the PS 151.0 ± 3.8 nm and 150.4 ± 4.0 nm respectively from 133.9 ± 3.6 nm for LTG-NPs. The ZP changed from -12.07 ± 0.33mV to -12.88 ± 0.46 mV and -11.21 ± 0.35 mV for Tf and Lf conjugated LTG-NPs respectively.

The % EE for TMD-NP, Tf-TMD-NPs and Lf-TMD-NPs was determined to be 73.57 ± 2.1 %, 71.49 ± 1.6% and 71.16 ± 2.8 respectively. For LTG-NPs, Tf-LTG-NPs and TL-LTG-NPs the %EE was determined to be 78.28 ± 1.7%, 76.05 ± 1.8 and 76.74 ± 1.3% respectively. The reduction in the % EE after conjugation could be due to depletion of the surface associated drug, during the conjugation process. The residual PVA associated with the NPs surface was 5.8 ± 0.4% & 5.6 ± 0.3% w/w of NPs for unconjugated TMD and LTG NPs respectively.

Transmission Electron Microscopy images of the unconjugated and conjugated NPs showed spherical NPs. DSC thermogram reveled that TMD had an endothermic peak of melting at 84-86°C whereas drug-loaded NPs had no such peak indicating molecularly dispersed drug in polymer matrix. Similarly LTG has endothermic peak at 225-227°C, drug-loaded NPs had no such peak indicating molecularly dispersed drug in polymer matrix. PLGA shows a Tg rather than Tm (melting point), indicating the presence of the polymer in amorphous form.

The release studies of TMD and LTG were conducted in PBS pH 7.4 + 2% Tween-80 and PBS pH 7.4 + 1% SLS respectively. An initial burst release of approximately 20% was observed within 4 h, which may be attributed to the presence of TMD or LTG at the surface of the NPs. More than 50 % of drug was released within one day for all NPs formulations. After this phase, prolonged release was observed up to 5 days, showing a typical sustained drug release indicative of drug diffusion and matrix erosion mechanisms.

Regression coefficients of all formulations in different orders were compared and it was found that the release pattern of TMD and LTG from the formulation follow Korsmeyer peppas model. The release of the drug from PLGA is by the degradation of polymer which occurs by hydrolysis of its ester linkages in presence of water. The general mechanism by which an active agent is released from a delivery vehicle is a combination of diffusion of an

active agent from the polymer matrices, bulk erosion of the polymer, swelling and degradation of the polymer. The degradation of PLGA is slow, therefore the release of TMD and LTG from NPs may depend on drug diffusion and PLGA surface and bulk erosion or swelling.

Stability Studies

The stability studies were conducted in accordance with ICH guidelines for drug products intended to be stored in a refrigerator. The stability of NPs formulations in terms of PS, ZP, drug content and redispersibility was conducted for 6 M at 5 ± 3°C and 25 ± 2°C/60 ± 5 % RH.

It was observed that conjugated NPs of both TMD and LTG shows no significant change ($P>0.05$) in PS, ZP, drug content and redispersibility at 5 ± 3°C for 6 M and 25 ± 2°C / 60 ± 5% RH for 3M. The storage of the conjugated NPs of TMD and LTG at 25 ± 2°C / 60 ± 5% RH, led to increase in the PS. The increase in the PS was not significant during the first 3 months, however became significant after 6 M. Increase in PS may be due to the evaporation of residual acetone which might have migrated to the surface of the NPs and dissolved the PLGA on the superficial layers causing coalescence of the NPs. The increase in the PS can also be attributed to the absorption of the moisture by lyophilized NPs resulting in the coalescence of the small NPs forming particles larger in size.

At 25 ± 2°C / 60 ± 5% RH, the ZP of the NPs shifted towards the zero for conjugated NPs and after 6M increased significantly towards 0 due to the degradation of PLGA. The lowered ZP values also might have contributed toward the aggregation of particles. In addition, the NPs displayed poor redispersibility after 6 M. This may be due to the degradation of PLGA. Also, the Tf and Lf conjugated NPs demonstrated difference in the colour than the initial powder after 6 M at 25 ± 2°C / 60 ± 5% RH. This could be indicative of the degradation of the surface Tf and Lf. The drug content of the conjugated NPs was not altered up to 6M at 5 ± 3°C. However, the drug content was reduced after 6 M storage at 25 ± 2°C / 60 ± 5% RH. The release profile of the drug from the NPs was not affected upon storage at 5 ± 3°C. The similarity factor calculated between the initial and the 6 M samples show values greater than 80, indicating high similarity between the initial and 6 M release profile.

From the above study, we can demonstrate that the conjugated PLGA NPs of TMD and LTG when stored at 25 ± 2°C / 60 ± 5% RH for 6 M show instability reflected by change in physical appearance, increase in the PS, ZP and reduction in the drug content. Hence, we can

conclusively specify that conjugated NPs of TMD and LTG were stable and can be stored 5°C ± 3°C for 6 M retaining its original formulation characteristics.

Radiolabeling of Formulations

The in vivo evaluation of the drug in solution and NPs form was performed after Radiolabeling with 99mTc. The drug solution and NPs formulations were labeled with 99mTc with high labeling efficiency using direct labeling method. The radiolabeling was optimized for quantity of stannous chloride, incubation time. The quantity of stannous chloride for solution, unconjugated and conjugated NPs was optimized at 150 µg/ml. Incubation time for TMDS and LTGS was optimized at 10 and 15 min. Optimized incubation time for unconjugated and conjugated NPs of TMD and LTG is 30 min. pH was kept around 6.5 for all formulations of TMD and LTG. The labeling efficiency for TMDS, TMD-NPs, Tf-TMD-NPs and Lf-TMD-NPs was found to be 98.09%, 96.67%, 97.28% and 96.82% respectively. The labeling efficiency for LTGS, LTG-NPs, Tf-LTG-NPs and Lf-LTG-NPs was found to be 98.16%, 97.14%, 96.87% and 97.53% respectively. The radiolabelled complex show high stability in rat serum with radiolabeling efficiencies measured, greater than 90%.

Pharmacokinetics and Biodistribution studies

The *in-vivo* biodistribution studies were performed on healthy swiss mice weighing between 25-30 g. The drug solution, unconjugated NPs and conjugated NPs labeled with ^{99m}Tc were administered intravenously. The studies were conducted for 48 h and at different time intervals the radioactivity was measured in tissue/organ. The radioactivity in each tissue/organ was determined as fraction of administered dose per gram of the tissue (%A/g). The radioactivity measured in the blood for unconjugated and conjugated NPs were significantly higher than the drug solution. TMD and LTG blood circulation time gets significantly enhanced after its incorporation in PLGA NPs. The plasma AUC ($0\rightarrow\infty$), Mean Residence Time (MRT), and $T_{1/2}$ values shown by TMD and LTG NPs are similar and significantly higher than their respective solutions. The $T_{1/2}$ and MRT of TMD after incorporation in NPs improves more than two folds. Similarly, $T_{1/2}$ and MRT of LTG after incorporation in NPs improves by around 1.5 folds. Clearance (Cl) of TMD and LTG NPs was significantly lesser than TMDS and LTGS respectively.

The results shows extended residence time and lower blood clearance of drug from NPs. Better pharmacokinetic profile of NPs may be attributed to slow opsonisation from blood due

to smaller size of NPs (< 200 nm). and presence of PVA on the NPs surface providing hydrophilic covering around the particles. Nano-sized PLGA NPs have easy accessibility in the body and transported to different parts of body via systemic circulation, while hydrophilic surface of PVA provide prolonged circulation time for tissue distribution. The internalisation of unconjugated NPs occurs probably by non-specific process.

The distribution of drug in brain is key focus of the present study. Conjugated and unconjugated NPs of TMD and LTG demonstrated higher brain deposition than their respective solution. The AUC (0→48) brain for Tf-TMD-NPs and Lf-TMD-NPs were found to be 5.90 folds and 8.88 folds higher than TMDS after i.v. administration. Similarly, The AUC (0→48) brain for Tf-LTG-NPs and Lf-LTG-NPs were found to be 3.43 folds and 5.05 folds higher than LTGS after i.v. administration.

Higher brain concentrations of NPs can be attributed to prolonged systemic circulation and superior brain transport. $T_{1/2}$ and MRT, indicative parameters for the retention of the drug delivery system in the brain revealed more than 2 folds higher values for TMD conjugated NPs against TMDS. Similarly, $T_{1/2}$ and MRT of LTG conjugated NPs in the brain revealed more than 1.8 folds higher values than LTGS.

The AUC (0→48) brain/AUC (0→48) blood for TMDS, TMD-NPs, Tf-TMD-NPs and Lf-TMD-NPs were found to be 0.0233, 0.0266, 0.0633 and 0.1025 respectively. TMD-NPs show marginally higher targeting than TMDS. Tf-TMD-NPs and Lf-TMD-NPs were found to have 2.72 and 4.40 folds higher brain targeting than TMDS and, 2.38 and 3.85 folds higher brain targeting than TMD-NPs. Thus, Tf and Lf ligand conjugation confer preferential uptake by brain endothelial cells and suggest enhanced brain transport. Moreover targeting achieved with Lf conjugation was 1.62 folds higher than Tf conjugation.

The AUC (0→48) brain/AUC (0→48) blood for LTGS, LTG-NPs, Tf-LTG-NPs and Lf-LTG-NPs were found to be 0.0350, 0.0393, 0.0747 and 0.1206 respectively. LTG-NPs show marginally higher targeting than LTGS. Tf-LTG-NPs and Lf-LTG-NPs were found to have 2.14 and 3.45 folds higher brain targeting than LTGS and, 1.90 and 3.07 folds higher brain targeting than LTG-NPs. Thus, Tf and Lf ligand conjugation confer preferential uptake by brain endothelial cells and suggest enhanced brain transport. Moreover targeting achieved with Lf conjugation was 1.61 folds higher than Tf conjugation.

The Tf and Lf conjugated NPs could have gained an access across the BBB through receptor mediated endocytosis/transcytosis on the membrane. The superior uptake of Lf conjugated NPs against Tf conjugated NPs could be primarily because of low circulating concentration

of endogenous Lf, approximately 5 nM against higher Kd for brain affinity, thereby avoiding the competitive uptake of endogenous Lf to Lf-conjugated NPs. The membrane preparations of mice brain have high affinity binding site with Kd of about 10.61 nM and the low affinity binding site is with a Kd of about 2228 nM.

Second, the relatively cationic nature of Lf imparts higher affinity towards the negatively charged cellular membranes. Third, Lf exhibit unidirectional transport across the BBB from the apical to the basolateral side, which leads to higher accumulation of Lf-conjugated drug delivery system formulation in the neuron, compared to Tf counterpart. It was demonstrated that Lf receptors exhibited at least two classes of binding sites, with high or low affinity to Lf, in the BBB and brain tissues. One of the published report showed that exogenous gene expression of Lf-modified NPs in brains was about 2.3 folds higher than that of Tf-modified NPs.

The major amount of injected dose was distributed to organs of the RES, such as liver, spleen, and lung. TMDS, LTGS and NPs exhibited significant hepatic and spleenic uptake. The hepatic accumulations ascertained by AUC(0→48) indicate 1.51 and 1.18 folds higher deposition for TMDS than Tf-TMD-NPs and Lf-TMD-NPs respectively. Similarly, the hepatic accumulations of LTGS demonstrated 1.47 and 1.19 folds higher deposition than Tf-LTG-NPs and Lf-LTG-NPs respectively.

In liver, the major reason for distribution could be opsonisation and filtration barrier formed by splenic and hepatic cord. Small sterically stabilized particles can distribute mainly to the parenchymal cells of the liver after i.v. administration. The low accumulation of TMD and LTG NPs compared to their respective drug solution may be due to the hydrophilicity associated with the surface of NPs, as mentioned earlier and higher accumulation of ligand conjugated NPs against unconjugated NPs could be because of presence of Tf and Lf receptors in liver.

Lf and Tf conjugated NPs revealed higher spleen deposition than their respective drug solutions. The Tf conjugated NPs displayed slightly higher accumulation than Lf conjugated NPs possibly because of modest presence of LfR against TfR. The phagocytes present in the red pulp of spleen engulf and remove the NPs from systemic circulation. The radioactivity measured indicates higher accumulation of TMDS and LTGS in the lungs than NPs formulation. The conjugated NPs of TMD and LTG shows approx. 1.7 folds lower AUC(0→48) values than TMDS and LTGS respectively for lung. Also the values of radioactivity distribution in heart indicate significantly higher values for TMDS and LTGS

than the NPs at all time points. In case of kidney, conjugated NPs demonstrated relatively higher deposition than unconjugated NPs and drug solutions. The moderately higher uptake of Lf conjugated NPs was in agreement with higher uptake observed for Lf distribution in mouse

Receptor-mediated endocytosis/transcytosis was considered as the main mechanism of uptake of Lf by organs/cells. The biodistribution data of Tf conjugated NPs are in agreement with the earlier published reports that that mouse TfR are expressed in liver, spleen and kidney. Similarly LfR has been identified in many tissues, including monocytes, lymphocytes and liver. In addition, preferential uptake of Lf by liver paranchymal cells is well reported. The expression of LfR in lymphocyte, monocytes and liver is in accordance with the high accumulation of Lf conjugated NPs in spleen and liver observed in the present study.

To ascertain the organ deposition following i.v. administration of TMDS and 99mTC TMD loaded NPs, gamma scintigraphy was performed and scintigrams after 2 h post i.v. injection were taken. The major radioactivity deposition was seen in liver and spleen for all formulations, in confirmation to the Tissue /Organ distribution studies.

Pharmacodynamic Studies

Antinociception produced by the i.v. administration of the drug solution and different NPs formulations was tested using hot plate method in mice. Analgesic effect of TMD NPs was determined as paw withdrawal latency. Conjugated NPs formulation of TMD and LTG displayed significantly higher antinociceptive effect at all time point except 0.5 h when compared against unconjugated NPs as well drug solution. Also, the antinociceptive effect of conjugated NPs was sustained for period of 48 h. Tf-TMD-NPs and Lf-TMD-NPs showed MPE (maximum possible effect) of 70.69% and 84.50 respectively after 2 h of administration and antinociceptive effect was sustained for period of 48 h.

Antinociception produced by the i.v. administration of the drug solution and different NPs formulations was tested using radiant heat method in neuropathic rats. Analgesic effect of LTG NPs formulations was determined as paw withdrawal latency. Conjugated NPs formulation of LTG displayed significantly higher antinociceptive effect at all time point except at 0.5 h, when compared against unconjugated NPs as well drug solution. Also, the antinociceptive effect of conjugated NPs was sustained for period of 48 h. Tf-LTG-NPs and Lf-LTG-NPs showed MPE 64.53 and 74.85% respectively after 1 h of administration and antinociceptive effect was sustained for period of 48 h. The antinociceptive effect of TMD

and LTG NPs formulations was in agreement with blood and brain distribution of the formulations observed in biodistribution studies.

MICROEMULSION AND NANOEMULSION

Analytical methods

The estimation of TMD and LTG in ME/NE and in diffusion sample was performed by UV spectrophotometry. For the estimation of TMD and LTG in ME and NE, method was developed in methanol. Calibration curve of TMD and LTG was established in methanol by UV spectrophotometry at 278 and 307 nm respectively. The linearity of TMD and LTG was found to be 12.5-150 µg/ml (R2=0.9998) and 2.5-35 µg/ml (R2=0.9994) respectively. The recovery study of TMD and LTG was performed at 37.5, 75, 112.5 µg/ml and 10, 20, 30 µg/ml respectively to check accuracy of developed method.

The in vitro drug diffusion study of ME/NE containing TMD and LTG was performed using PBS pH 5 + 2% Tween-80 and PBS pH 5 + 1% SLS as diffusion media respectively. For the estimation of TMD and LTG in diffusion sample, method was developed in PBS pH 5 + 2% Tween-80 and PBS pH 5 + 1% SLS using UV spectrophotometry at 278 and 307nm respectively. The linearity of TMD and LTG was found to be 25-200 µg/ml (R2=0.9992) and 2.5-35 µg/ml (R2=0.9995) respectively. The recovery study of TMD and LTG was performed at 50, 100, 150 µg/ml and 10, 20, 30 µg/ml respectively to check accuracy of developed method.

% accuracy was found to be more than 99% for developed methods of TMD and LTG. Intraday and inter day precision of developed methods for TMD and LTG was performed at low, medium and high concentration of drug. %RSD of developed methods for TMD and LTG was found to be less than 1. Results of accuracy and precision are indicating the reliability of the developed methods for both the drugs. There was no interference observed with any excipients used. The methods were validated for linearity, accuracy and precision. The validation parameter complies with USP and % RSD was found less than 1%.

Preparation, Optimization and Characterization

Based upon the solubility studies, IPM and Capmul MCM was selected as an internal oil phase for the preparation of TME and LME respectively. The selection of surfactant and cosurfactant was on the basis of HLB values, drug solubility, safety and stability profile.

Non-ionic surfactants are known to be least toxic and chemically highly stable. Labrasol/Tween-20 and Tween-20/Transcutol were selected as surfactant/cosurfactant for the formulation of TME and LME respectively.

MEs of TMD and LTG were successfully prepared using titration method followed by construction of pseudo ternary phase diagram. Different ratios of surfactant:cosurfactant for TME (1:1 to 3:1) and for LME (1:1 to 4:1) were studied in the phase diagram construction. The phase study revealed that increasing the S_{mix} ratio from 1:1 to 3:1, the ME region increased toward water-oil axis. This indicates that increasing surfactant concentration the maximum amount of oil can be solubilised/ emulsified. However surfactant concentration should be used at minimum. The increased oil content may provide opportunity for the solubilisation of the drug.

The formulation of TME containing varying oil content from 2.5%v/v to 7.5%v/v and Smix (2:1) from 20%v/v to 50%v/v were prepared and globule size (GS) and zeta potential (ZP) were measured as the responses. Formulation of LME containing varying oil content from 3%v/v to 9%v/v and S_{mix} (3:1) from 20%v/v to 50%v/v were prepared measuring GS (GS) and zeta potential (ZP) as the responses. In TME up to 5% v/v of oil was emulsified by 50% of the S_{mix} while, in LME up to 6% v/v of oil was emulsified by 50% of S_{mix}. From the results of GS and ZP values, TME was optimized containing 5% v/v oil and 30%v/v S_{mix} as final formulation of TMD. Similarly, from the GS and ZP results batch LME containing 6% v/v oil and 40% v/v S_{mix} was selected as a final optimized formulation of LTG.

Lipid NE of drug was prepared by ultrasonication method, which has been successfully used to reduce oil droplet size of NE below 250 nm. Based up on solubility study oil used in NE formulation was selected. Oil concentration in NE formulation was decided by considering drug loading in formulation and upon solubility of drug in oil provided it form physically stable NE system. However, oil concentration up to 20% are used in marketed NE. (Celepid fat emulsion) Suitable surfactant was selected based upon literature Drug loading in formulation was decided from dose to be administered. TNE was consisting of Isopropyl myristate (IPM) (17.5%), soya lecithin, poloxamer and TMD (9.75mg/ml) while, LNE contains Capmul MCM (15%), soya lecithin, poloxamer and LTG (6.5mg/ml).

Surfactant concentration is major formulation variable affecting NE characteristics. Selection of Surfactant, concentration of drug and oil used was decided as describe earlier. Concentration of surfactant has to be optimized.

Soya lecithin concentration was optimized for TNE by preparing formulations containing varying concentrations of Soya lecithin (1%w/v, 1.5%w/v, 2%w/v & 2.5%w/v) and keeping IPM 17.5%v/v, poloxamer 1.5% and water q.s. as constant ingredients. For LNE Soya lecithin concentration was optimized by preparing formulations containing varying concentrations of Soya lecithin (1%w/v, 1.25%w/v, 1.5%w/v & 2%w/v) and keeping Capmul MCM 15%v/v, poloxamer 1.5% and water q.s. as constant ingredients.

Poloxamer concentration was optimized for TNE by preparing formulations containing varying concentrations of poloxamer (1%w/v, 1.5%w/v, 2%w/v & 2.5%w/v) and keeping IPM 17.5%v/v, Soya lecithin 2% and water q.s. as constant ingredients. For LNE poloxamer concentration was optimized by preparing formulations containing varying concentrations of poloxamer (1%w/v, 1.5%w/v, 2%w/v & 2.5%w/v) and keeping Capmul MCM 15%v/v, Soya lecithin 1.5% and water q.s. as constant ingredients.

Formulations were evaluated for GS and PDI. GS and PDI of formulation were also measured after 15 days. PDI represents uniformity of particle size distribution. Higher value of PDI indicates non-uniform distribution of particle size. PDI less than 0.2 is desirable. PDI values lower than 0.25 indicate a close size distribution providing good stability of nanoemulsions due to the reduced Ostwald ripening. Optimization of surfactant concentration was carried out based on initial and 15th day data for GS and PDI.

Optimized Soya lecithin concentration for TNE and LNE was 2% and 1.5%w/v respectively. Optimized concentration of poloxamer for both TNE and LNE was 2% w/v. Thus, from the results optimized formulation of TNE containing IPM (17.5%v/v), LTG (9.75mg/ml), Soya lecithin (2%w/v), poloxamer (2%) and water (q.s.) while optimized formulation of LNE composed of Capmul MCM (15%v/v), LTG (6.5mg/ml), Soya lecithin (1.5%w/v), poloxamer (2%) and water (q.s).

MEs and NEs of TMD and LTG were characterized for GS, PDI, ZP, % assay, pH, viscosity, %T and TEM. ME formulations have GS less than 20 nm, while NE formulations have GS less than 150 nm. Low PDI values suggest narrow size distribution. ZP were lesser than -8.0 indicating stability against globule-globule aggregation. The pH of the formulations was found in the range of 5 to 6, which is compatible with nasal mucosa. ME demonstrated higher viscosity when compared against NE. NE contains lesser ratio of surfactant/co-surfactant and hence have water like consistency. The %T of ME was found to be more than 99% and shows that the prepared TME and LME are isotropic in nature. % assay of all formulations was more than 98%.

TEM images shows globules of uniform size without aggregation. The in vitro diffusion study through excised sheep nasal mucosa was performed with an aim to assess the drug release through a biological membrane simulating the actual in vivo barrier to drug diffusion. The in vitro diffusion studies for TMD and LTG formulations were performed in PBS pH 5 with 2% Tween-80 and PBS pH 5 with 1% SLS respectively. The % cumulative drug diffused across nasal mucosa from TMD and LTG loaded formulations were calculated. The kinetic pattern of the diffusion was studied by fitting % drug diffused in given time in different order kinetics like zero order, first order, higuchi, Hixon Crowell, and Korsmeyer peppas. Regression coefficients of all formulations in different orders were compared and found that the release pattern of TMD and LTG from the formulation across the nasal mucosa followed Korsmeyer peppas order except for LNE which follows first order. This was concluded by higher regression coefficient value in curve fitting. However, apart from Korsmeyer peppas model, zero, first and Higuchi models shows Regression coefficients higher than 0.9 indicating suitability of the said models.

The results show that flux and diffusion coefficients are in the order of ME > NE > DS, which clearly confirms the permeation improvement with ME and NE systems. ME permit drug loading at saturation solubility and increase their thermodynamic activity favouring partition/permeation into biological membrane. Also, amount of surfactants in ME may lead to tight epithelial junction opening in nasal membrane thereby increasing net flux. However toxicity of ME on nasal epithelial membrane needs to be evaluated. NE demonstrated lower flux than ME however it was more than two times compared to control (DS) indicating suitability for nasal delivery. NE was composed of lecithin as surfactant which is natural body component present in lipid bilayer thus favouring permeation into biological membrane without affecting normal functioning.

The prepared formulations were subjected to nasal toxicity study to evaluate the safety of the ingredients used in the formulation. The optical microscopy images of nasal mucosa treated with formulations were evaluated for toxicity. The nasal mucosa treated with PBS pH 6.4 showed intact epithelial layer without any damage while mucosa treated with isopropyl alcohol (mucociliary toxic agent) showed complete destruction of epithelial layer and even deeper tissues.

Mucosa treated with microemulsions (TME and LME) were found to intact with slighter damage of the epithelial layer after 1h. However, after 2 h treatment damage become prominent with loss of epithelial layer. This may be due to high amount of surfactants (30-

40%) present in MEs. For Mucosa treated with nanoemulsions (TNE and LNE), epithelium layer was intact and there were no alterations in basal membrane and superficial part of submucosa even after 2 h of treatment as compared with phosphate buffer (pH 6.4) treated mucosa. Thus, the developed NE formulations seem to be safe with respect to nasal administration for repeated administrations. MEs can be use for single use and repeated dosing can affect integrity of nasal epithelium. However further toxicity studies have to be conducted prior to clinical application of the prepared formulations.

Stability Studies

In long term stability study, the MEs and NEs containing TMD and LTG were packed in the borosil screw capped vials. MEs were kept at room temperature (25°C/60%RH) and accelerated temperature (40°C/75% RH) while NEs were kept at refrigeration temperature (5°C) and room temperature (25°C/60% RH) conditions. During the storage period, ME and NE systems were assessed for their GS, ZP, assay, pH and %T (in case of ME). Over the time period there was an increment in GS and ZP and change in assay, %T, pH. However, the changes observed were non-significant when no visual indications of physical instability of MEs and NEs of TMD and LTG were seen. Irrespective of the storage conditions, the ME and NE system remained stable for 3 months duration at 40°C/75%RH and 25°C/60%RH respectively.

In order to assess the thermodynamic stability, the accelerated stability studies were done by subjecting the formulations for centrifugation, freeze-thaw cycle and heating cooling cycle. Before and after each treatment, GS, ZP and %T (in case of ME) of the formulations were determined. The parameters after accelerated stability conditions were found to be nonsignificant which clearly indicates that the prepared MEs and NEs (TME, LME, TNE, and LNE) systems were thermodymically stable.

Radiolabeling

The in vivo evaluation of the drug in solution, ME and NE form was performed after radiolabeling with 99mTc. The drug solution, ME and NE formulations were labeled with 99mTc with high labeling efficiency using direct labeling method. The radiolabeling was optimized for quantity of stannous chloride, incubation time. The optimum quantity of stannous chloride for high labeling efficiency and low free and reduced/hydrolyzed 99mTc, was found to be 250 µg, 200 µg and 150 µg for ME/NE formulations and drug solutions (TS

and LS) respectively. The incubation time was optimized at 30 min for ME and NE formulations. TS and LS require incubation of 10 and 15 min respectively. The pH of all the formulations was kept at around 6.5. The labelling efficiency for TS, TME and TNE was found to be 98.18%, 97.32% and 96.28 % respectively. The labelling efficiency for LS, LME and LNE was found to be 97.22%, 98.13% and 97.19 % respectively. The radiolabelled complex show high stability in rat serum with radiolabeling efficiencies measured, greater than 90%.

Pharmacokinetics and Biodistribution Studies

The in-vivo biodistribution studies were performed on healthy swiss mice weighing between 25-30 g. The drug solution, Microemulsion and Nanoemulsion labeled with 99mTc were administered intranasally. The studies were conducted for 48 h and at different time intervals the radioactivity was measured in tissue/organ. The radioactivity in each tissue/organ was determined as fraction of administered dose per gram of the tissue (%A/g).

Reports in the literature reveal that the drug uptake into the brain from the nasal mucosa mainly occurs via three different pathways. One is the systemic pathway by which some of the drug is absorbed into the systemic circulation and subsequently reaches the brain by crossing BBB. The others are the olfactory pathway and the trigeminal neural pathway by which partly the drug travels directly from the nasal cavity to CSF and brain tissue. We can conclude that the amount of drug reaches in the brain tissue after nasal administration is attributed to these three pathways. Therefore, DTP (%) represents the percentage of drug directly transported to the brain via the olfactory pathway and the trigeminal neural pathway.

The pharmacokinetic parameters Cmax, Tmax, AUC(0→48), half life, MRT and nasal bioavailability were calculated for TMD and LTG formulations. Cmax and AUC(0→48) of blood for TSin are respectively more than 27 and 15 folds lower than TSiv. However, brain concentration for TME and TNE was significantly higher. Similarly, Cmax and AUC(0→48) of blood for LSin are respectively more than 33 and 16 folds lower than LSiv. However, brain concentration for LME and LNE was significantly higher. Drug transport from nose to brain, for all i.n formulations, as reflected by DTP values, was contributing more 90%. This is attributed to preferential nose to brain transport following nasal administration. The brain/blood ratios of drug at all time points were found to be higher following i.n. administration of the formulations than i.v. solution. This further confirms direct nose to brain transport.

AUC (0→48) brain for TME and TNE increased by 2-3 folds as compared to their respective solutions after i.n. administration. The enhancement of AUC in brain followed by i.n. ME and NE are in congruence with the observations reported by and that microemulsion enhances the transport of drug across nasal mucosa. Tmax, values observed in brain for solutions, MEs and NEs of TMD and LTG was 0.5 h. It is indicative of rapid transport from nose to brain. Half life and MRT of TSin, TME and TNE for both blood and brain are similar to that of TSiv. Similarly, half life and MRT of LSin, LME and LNE for both blood and brain are similar to that of LSiv.

Targeting efficiency of formulations is reflected by %DTE. Targeting achieved with TME and TNE was around 3.6 times higher than that of solution and 50 times higher than TSiv. Targeting of achieved with LME and LNE was around 2 times higher than that of solution and 37 times higher than LSiv. The higher concentration of drug in brain following i.n administration of TME, TNE, LME and LNE demonstrates the suitability and capability of microemulsion as an effective delivery system across the nasal membrane and a larger extent of selective transport of drug from nose to brain. This is in agreement with published reported stating unique connection between the nose and brain and drug transport to brain circumventing the BBB after i.n. administration. Nasal bioavailability of TME and TNE was 6 folds higher than TSiv while nasal bioavailability of LME and LNE was 2-3 folds higher than LSin. Though ME system for TMD and LTG demonstrated higher targeting efficiency than their respective solution but difference was nonsignificant ($P > 0.05$).

Organ distribution data shows that only minor amount of ME and NE goes to liver, spleen, kidney and lungs compared to their respective solution administered intravenously. In case of stomach and kidney, AUC(0→48) for ME and NE were higher than solution administered intravenously. This may be due to fraction of dose entered GI tract from nasal cavity after i.n. administration.

Pharmacodynamic Studies

Antinociception produced by the i.n. (intranasal) administration of the TMD drug solution, ME and NE formulations was tested using hot plate method in mice. Analgesic effect of was determined as paw withdrawal latency. TME and TNE formulation displayed significantly higher antinociceptive effect at all time point except 24 h when compared against drug solution administered intravenously and intranasally. TME demonstrates higher antinociceptive effect (except at 1 h) than TNE, however difference was non-significant at

p>0.05. TME and TME showed MPE 85.38 and 76.20% respectively after 0.5 h of administration. This prompt effect signifies the role of for ME and NE formulations for episodic and emergency treatment of pain.

Antinociception produced by the i.n. administration of the drug solution, ME and NE formulations was tested using radiant heat method in neuropathic rats. Analgesic effect of ME and NE formulations, determined as paw withdrawal latency. LME and LNE formulation displayed significantly higher antinociceptive effect at all time points when compared against drug solution administered intravenously and intranasally. Also LME demonstrates higher antinociceptive effect than LNE (except at 1 h), however difference was non-significant at p>0.05.The data obtained after converting the paw withdrawal latency into % MPE LME and LNE showed MPE 69.02 and 60.13% respectively after 0.5h of administration. This prompt effect signifies the role of for ME and NE formulations for episodic and emergency treatment of pain. The antinociceptive effects of formulations were in agreement with blood and brain distribution of the formulations observed in biodistribution studies.

Conclusions

To conclude, Tramadol and Lamotrigine loaded nanoparticles were successfully prepared using nanoprecipitation method and conjugated with Transferring and Lactoferrin. The characterization of NPs demonstrated small PS (< 200nm) suitable for i.v. administration and drug release was found to be prolonged. The prepared NPs were stable for 6M at refrigerated condition (2-8oC) and demonstrated no significant change in PS, drug content and invitro drug release compared to the initial. The results of pharmacodynamic indicates that Tf and Lf conjugated NPs were efficient in brain targeting and sustaining the drug release as evidenced by the prolonged analgesic effect.

Pharmacokinetic and biodistribution studies revealed that Tramadol and Lamotrigine exhibited higher brain uptake after incorporation in PLGA NPs as compared to solution. Nano-sized PLGA NPs have easy accessibility in the body and transported to different parts of body via systemic circulation, while hydrophilic surface of PVA provide prolonged circulation time for tissue distribution. Significant improvement in brain uptake was observed following i.v. administration of conjugated NPs compared to unconjugated NPs due to receptor mediated intracellular endocytosis through Transferring (Tf) and Tactoferrin (Lf) receptors present in the blood brain barrier. Functionalization of the NPs with Lf was proved to be superior to Tf, for facilitating their translocation into the brain tissue after i.v. administration. Though Tf and Lf exhibit structural similarity and homology, the distribution of their respective receptors being different, it directs the difference in the tissue distribution of Tf and Lf conjugated PLGA NPs. Primarily the low endogenous concentration and additionally, cationic charge and unidirectional transport as observed with Lf are supposed to be the major reason for enhanced uptake of Lf conjugated NPs in brain when compared against Tf conjugated NPs. The expression of LfR varied among different animals, hence further, more animal studies followed by extensive toxicological evaluation are necessary to confirm the role of Lf conjugated NPs for brain delivery.

The findings of these investigations suggest that drugs in the form of Transferrin and Lactoferrin conjugated NPs can be effectively delivered to brain and may find a possible role in the treatment of postoperative and neuropathic pain. The studies also suggest that delivering Tramadol and Lamotrigine in the form of Tf and Lf conjugated NPs enhance the brain uptake of the drug. The higher t1/2 combined with prolonged release from NPs can lead to possible reduction in the dose/frequency of dosing along with systemic side effects. It also minimizes the dose dependent and peak-trough related side effects of Lamotrigine. However, more animal studies followed by extensive toxicological and clinical studies are necessary to

confirm the role of Tf/Lf conjugated Tramadol/Lamotrigine loaded PLGA NPs for the treatment of pain.

To conclude, Microemulsions (MEs) and Nanoemulsions (NEs) of TMD and LTG were successfully prepared and demonstrated brisk delivery to brain and in large quantities following i.n administration. Studies under this investigation demonstrates direct nose to brain delivery of TMD and LTG and significant enhancement in antinociceptive effect. Hence, these studies confirm role of intranasal Tramadol and Lamotrigine, administered as microemulsion or nanoemulsion, in effective management of episodic and emergency pain treatment possibly by reducing dose and dose dependent side effects, minimizing side effects associated with the systemic exposure. The nasal toxicity study demonstrated suitability of nanoemulsion against microemulsion formulation. Efficacy of However, clinical studies with special focus on toxicity evaluation on chronic use of the developed formulations is necessary for establishing suitability in clinical practice in the treatment of pain and neuropathy.