CHAPTER 9

PHARMACODYNAMIC STUDIES

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9.1 Introduction

Experimental models of pain sensitivity (nociception) include tests of response thresholds to high-intensity stimuli (acute pain tests) and of changes in spontaneous or evoked behavioural responses in animals with peripheral injury or inflammation (persistent pain models). Acute thermal pain is modelled by the hot-plate and tail-flick tests. Persistent pain produced by peripheral tissue injury and inflammation can be modelled by the formalin test. In addition to these pain tests, models exist to study changes in thermal and mechanical thresholds that occur after injury to a peripheral nerve (neuropathic pain). There is evidence that the neurotransmitters and neuromodulators mediating pain responses in each of these models may differ. As a result, different pharmacological agents may be effective in attenuating pain in the different models. A basic understanding of the mechanisms involved in pain transmission is important in order to choose the model most appropriate for examining the analgesic properties of specific agents or classes of compounds.

This chapter describes antinociceptive testing; acute pain using the hot-plate and neuropathic pain using radiant heat method, preparation of animals (mice or rats) for the tests, administration of a compound being tested for its analgesic properties, and data collection and interpretation. Proper experimental design, which includes the application of cut-off times to limit the exposure to noxious stimuli, will minimize the amount of pain and distress experienced by experimental animals. Tramadol (TMD) being centrally acting analgesic hot plate test was employed for evaluation of its formulations. For lamotrigine (LTG), being indicated for neuropathic pain, partial sciatic nerve ligation injury model (PSI) was used. To access effect of formulation in the above model radiant heat method was employed.

9.2 Methods

9.2.1 Antinociceptive Testing for Tramadol formulations

All experiments conducted on animals were approved by the Committee for the purpose of control and supervision of experiments on animals, Ministry of social Justice and Empowerment, government of India, NewDelhi, India.The protocol was approved by Institutional Animal Ethics Committee, Pharmacy Dept., The M. S. University of Baroda.All experiments were conformed to ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann M, 1983).

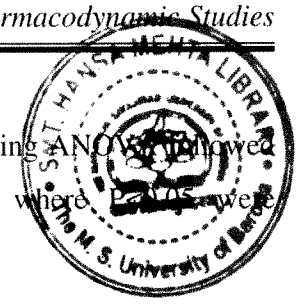
9.2.1.1 Animals

The experiments were performed on adult mice (either sex) weighing 25-30 g obtained from Food and Drug Laboratory, Vadodara, Gujarat. Animals were housed 6-8 per cage in a room maintained at 22 ± 1°C with an alternating 12 h dark/12 h light cycles. Standard laboratory chow and tap water were available *ad libitum*. The animal in each group were used for maximum of 2-4 experiments, with each experiment using a different formulation regimen. A recovery period of at least 7 days was allowed between experiments. Animals were accustomed to testing conditions for 5 days and each animal was trained on hot plate apparatus before the actual start of experiment in order to minimize the stress induced by handling.

9.2.1.2 Evaluation of antinociceptive effect

Nociceptive threshold was measured by the hot-plate test using mice as animal model, based on the method of Woolfe and Macdonald (Woolfe HG and Macdonald AD, 1944). The mice were placed on Eddy's hot plate thermostatically maintained at 55 ± 0.2°C. Licking of their hindpaws or jumping (whichever occurred first), was used as the end point for the determination of pain response latencies. Failure to respond within 25 s resulted in the termination of the test (cutoff). Animals presenting training latencies higher than 10 s were excluded. Mice were divided into five groups namely saline control, TMDS, TMD-NPs, Tf-TMD-NPs and Lf-TMD-NPs for intravenously administered formulations. The dose administered were 6.10 (mg/kg) and 4.88 (mg/kg) for TMDS and NPs formulations respectively. Mice were divided into four groups namely saline control, TS, TME and TNE for intranasally administered formulations. 4/5 µL of formulation was administered in the each nostril using micropipette (10 µl) fixed with low density polyethylene tube having 0.10 mm internal diameter at the delivery site. The mice were held from the back in slanted position during nasal administration (Jogani VV et al., 2008).The dose administered were 6.10 (mg/kg) for TSiv and 4.88 (mg/kg) for TSin, TME and TNE formulations respectively.

Each mouse was then tested before and 0.5, 1, 2, 4, 8, 16, 24, 36 and 48 after administration of above mentioned formulations. TME and TLE were evaluated for 24 h period. Paw withdrawal latencies ia calculated for each time point. The hot plate response was converted to maximum possible effect (%MPE) calculated as [(T1 - T0)/(T2 - T0)]×100. Where, T0 and T1 paw withdrawal latency before and after administrationof formulation or saline and T2 was the cut-off time. The data were expressed as mean of six determinations.



9.2.1.3 Statistical Analysis

All data are reported as mean ± SD and all groups were compared using ANOVA followed by Bonferroni multiple comparison test and differences greater where $P<0.05$ were considered significant.

9.2.2 Antinociceptive Testing for LTG formulations

9.2.2.1 Animals

The experiments were performed on adult wistar rats weighing 200-250 g obtained from Food and Drug Laboratory, Vadodara, Gujarat. Animals were housed4 per cage in a room maintained at 22 ± 1 °C with an alternating 12 h dark/12 h light cycles. Standard laboratory chow and tap water were available *ad libitum*. Each animal in each group was used for maximum of 2-4 experiments, with each experiment using a different formulation regimen. A recovery period of at least 7 days was allowed between experiments. However, animals undergoing surgical procedures were not used for any other study. Animals were accustomed to testing conditions for for 5 days and each animal was trained for facing radiant heat before the actual start of experiment in order to minimize the stress induced by handling.

9.2.2.2 Evaluation of antinociceptive effect

Surgical Procedure

The partial sciatic nerve injury model was employed for inducing nerve injury in rats (Seltzer Z et al., 1990). Rats were anaesthetised with ketamine (70 mg/kg, intraperitoneal) along with xylazine (7 mg/kg, intramuscular). The skin of the lateral left thigh was incised and the overlying musculature separated to expose the sciatic nerve. Silk ligature was tied tightly approximately 1/3 to 1/2 of the diameter around sciatic nerve. The muscle was then closed in layers and the skin sutured together. Topical antibiotic powder was applied to the wound and the animals were housed individually. The total period of anaesthesia did not exceed 30 minutes. The procedure for the sham surgery was identical except that the spinal nerves were exposed but not ligated. Animals were allowed to recover from surgical trauma. Responses to thermal stimuli were measured before and 6 h, 1, 3, 7 and 14 days after the surgical procedure. Non-injured paw (contra-lateral) was used as control. Appropriate time was decided for evaluation of formulation based on the above results for thermal hypaeralgesia.

Antinociceptive effect of LTG formulation

The thermal nociceptive threshold is measured according to the method of (Hargreaves et al. 1988). The rats are placed beneath a clear plastic cage (10 × 20 × 24 cm) upon an elevated floor of clearglass. A radiant heat source (halogen projector lamp) is placed beneath the glass floor on a movable holder and positioned such that is focuses at the plantar area of one hind paw. The time interval between the application of the light beam and the brisk hind paw withdrawal response is measured. The experimental cut-off to prevent damage to the skin was set at 20s. Licking of their hind paws or jumping (whichever occurred first), was used as the end point for the determination of pain response latencies. Failure to respond within 20s resulted in the termination of the test. Animals (PSI) presenting training latencies higher than 8s were excluded. Rats were divided into five groups namely saline control, LTGS, LTG-NPs, Tf-LTG-NPs and Lf-LTG-NPs for intravenously administered formulations. The dose administered were 3.4 (mg/kg) and 2.25 (mg/kg) for LTGS and NPs formulations respectively. Rats were divided into four groups namely saline control, LS, LME and LNE for i.n. administered formulations. 4/5 µL of formulation was administered in the each nostril using micropipette (10 µl) fixed with low density polyethylene tube having 0.10 mm internal diameter at the delivery site. The rats were held from the back in slanted position during nasal administration (Jogani VV et al., 2008).The dose administered were 3.4 (mg/kg) for LSiv and 2.25 (mg/kg) for LSin, LME and LNE formulations respectively.

Each rat was then tested before and 0.5, 1, 2, 4, 8, 16, 24, 36 and 48 h after administration of above mentioned formulations. TME and TLE were evaluated for 24 h period. Paw withdrawal latency is calculated for each time point. The response was converted to maximum possible effect (%MPE) calculated as [(T1 - T0)/(T2 - T0)]×100. Where, T0 and T1 were paw withdrawal latency before and after administration of formulation or saline and T2 was the cut-off time. The data were expressed as mean of six determinations.

9.2.2.3 Statistical Analysis

All data are reported as mean ± SD and all groups were compared using ANOVA followed by Bonferroni multiple comparison test and differences greater where $P<0.05$ were considered significant.

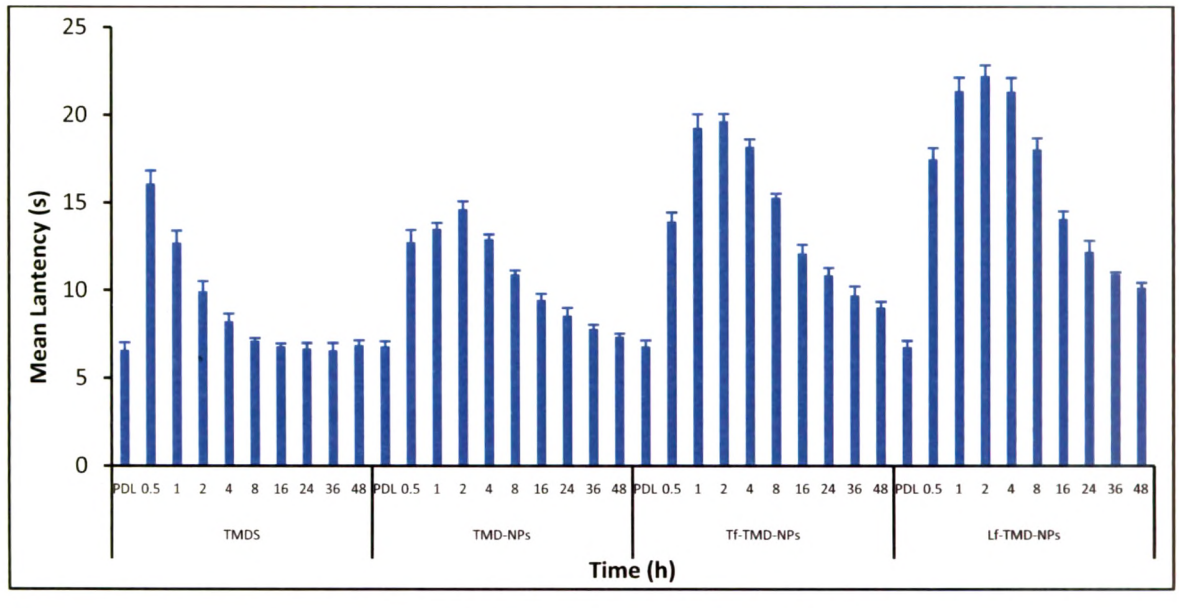
9.3 Results and Discussion

9.3.1 Antinociceptive Testing for Tramadol formulations

Tramadol Nanoparticles formulations (TMD NPs)

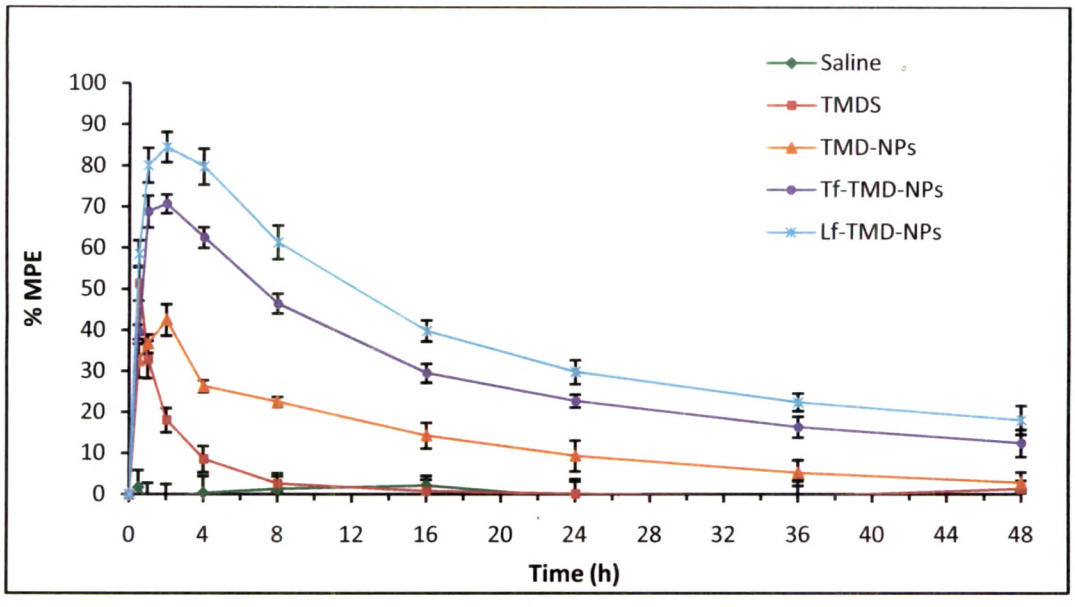
Antinociception produced by the i.v. administration of the drug solution and different NPs formulations was tested using hot plate method in mice. Analgesic effect of TMD NPs formulations, determined as paw withdrawal latency, at different time point is shown in Fig. 9.1.Conjugated NPs formulation of TMD 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 48h. The data obtained after converting the paw withdrawal latency into % MPE is shown in Fig. 9.2. Tf-TMD-NPs and Lf-TMD-NPs showed MPE 70.69% and 84.50 respectively after 2 h of administration and antinociceptive effect was sustained for period of 48h.The antinociceptive effect of TMD solution and NPs formulation was in agreement with blood and brain distribution of the formulations observed in biodistribution studies.

Figure 9.1: Influence on paw withdrawal latency measured in hot plate test by i.v. administration of TMDS, TMD-NPs, Tf-TMD-NPs and Lf-TMD-NPs in mice



The data are expressed as mean of six experiments ± SEM (bars). PDL stands for Pre-Drug Latency.

Figure 9.2: Antinociceptive effect exerted by i.v. administration of TMDS, TMD-NPs, Tf-TMD-NPs and Lf-TMD-NPs in mice



The data are expressed as mean of six experiments ± SEM (bars)
TMD-NPs vs TMDS <0.05 at 0.5, 2,4 and 8h; Tf-TMD-NPs and Lf-TMD-NPs vs TMDS <0.05 at all time points except 0.5h; Tf-TMD-NPs vs TMD-NPs <0.05 at 1,2,4,8 and 16h; Lf-TMD-NPs vs TMD-NPs <0.05 at all time points; Lf-TMD-NPs vs Tf-TMD-NPs <0.05 at 0.5,4 and 8h.

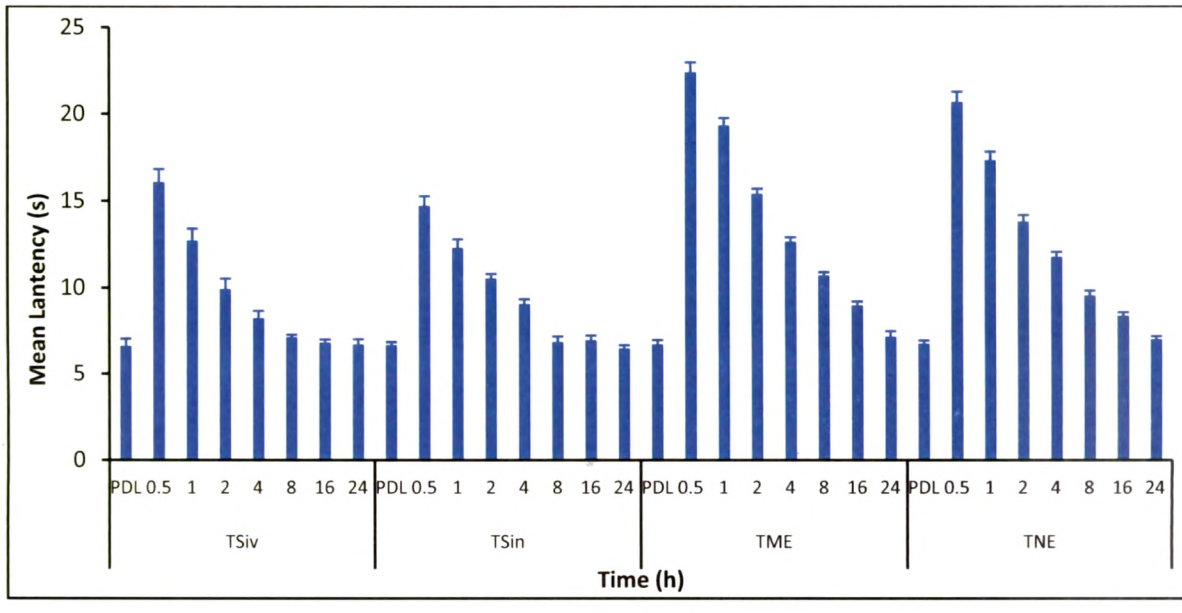
The improved efficacy of conjugated NPs formulation shows possibility of dose reduction with achieving desired therapeutic response. The study reveals enhanced efficacy of ligand conjugated NPs against unconjugated NPs and Lf conjugated NPs against Tf conjugated NPs. The receptor mediated endocytosis of Tf was well established and proven in comparison with Lf. However, conjugation with Lf proves to be superior against its Tf counterpart. The basic reason may be low plasma concentration of endogenous Lf, approximately 5 nM (Talukder MJ et al., 2003) against higher Kd of 6.8 nM and 4815 nM for high and low affinity sites respectively of Lf receptors in brain endothelial capillary cells (Huang RQ et al., 2007). Thus, it avoids the competitive inhibition of endogenous Lf to exogenous Lf-conjugated formulations. In addition, unidirectional transport of Lf from endothelial monolayer from the apical to the abluminal surface may aid to the higher deposition of Lf in neurons (Fillebeen C et al., 1999).

Tramadol Microemulsion and Nanoemulsion (TME and TNE)

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 ME and NE formulations, determined as paw withdrawal latency, at different time point is shown in Fig. 9.3. TME and TNE formulation displayed significantly higher antinociceptive effect at all time point except 24h when compared against drug solution administered intravenously and intranasally. TME demonstrates higher antinociceptive effect (except at 1h) than TNE, however difference was non-significant at $p>0.05$.The data obtained after converting the paw withdrawal latency into % MPE is shown in Fig. 9.4. 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.

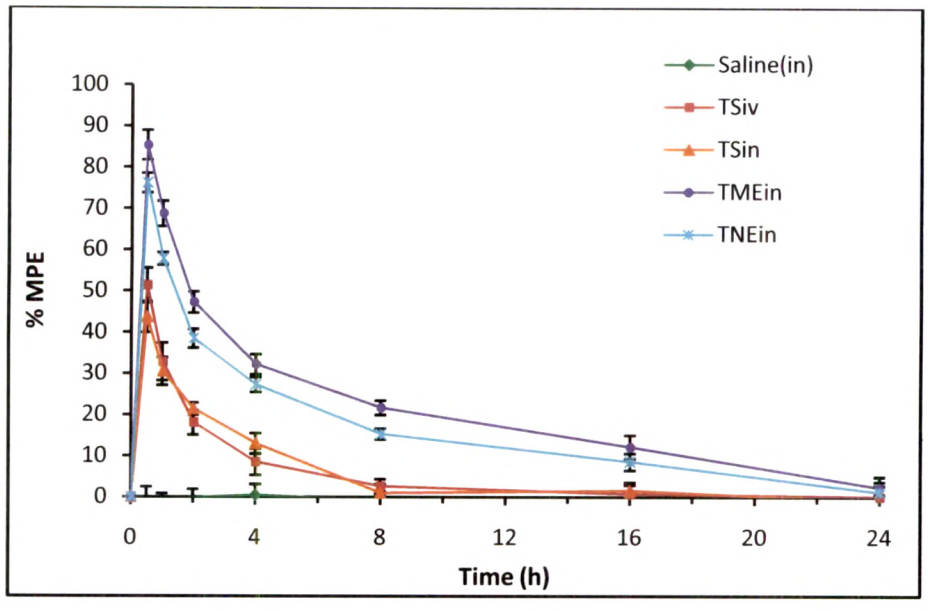
The antinociceptive effect of formulation was in agreement with blood and brain distribution of the formulations observed in biodistribution studies. The higher antinociceptive effect following i.n administration of TME and TNE demonstrates the suitability and capability of microemulsion and nanoemulsion as an effective delivery system across the nasal membrane (Lawrence MJ and Rees GD, 2000) and a larger extent of selective transport 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. (Behl CR et al., 1998; Illum L, 2000).

Figure 9.3: Influence on paw withdrawal latency measured in hot plate test by TSiv, TSin, TME and TNE in mice



The data are expressed as mean of six experiments ± SEM (bars). PDL stands for Pre-Drug Latency.

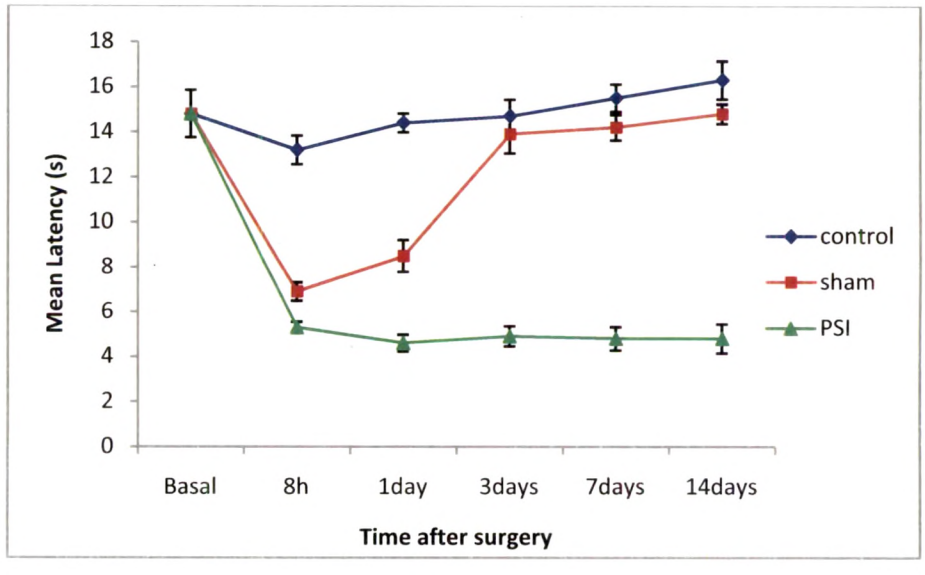
Figure 9.4: Antinociceptive effect exerted by administration of TSiv, TSin, TME and TNE in mice



The data are expressed as mean of six experiments ± SEM (bars)
TSin vs TSiv < 0.05 for none; TME vs TSin and TSiv < 0.05 at all except 24h; TNE vs TSin and TSiv < 0.05 at all except 16 and 24h; TME vs TNE < 0.05 at 1h.

9.3.2 Antinociceptive Testing for Lamotrigine formulations

Figure 9.5: Time course of thermal hyperalgesia in control, sham operated and neuropathic rats (PSI)



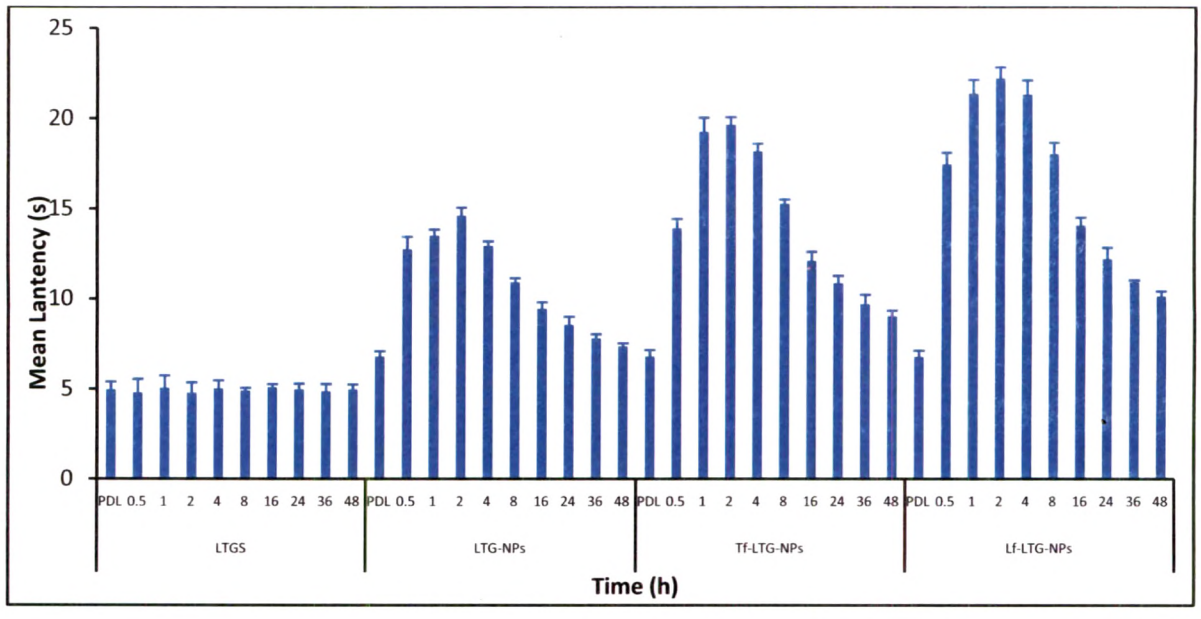
Partial ligation of the sciatic nerve in rats produced a profound and prolonged decrease of thermal thresholds (Fig. 9.5). Neither threshold changed in the sham-operated animals. Within 1 day of the nerve injury, significant reduction of the paw withdrawal latency to the heat stimulus compared to the contralateral (control) side was observed. At 3 days after the nerve injury, there was a significant decrease of the latency (increased sensitivity) to thermal stimulation on the nerve-injured side compared to both the contralateral side and to sham-operated control mice. The latency to thermal stimulation remained constant 7 and 14 days after neuropathy. Hence, formulations were evaluated 5 days after surgery.

Lamotrigine Nanoparticles formulations (LTG NPs)

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, determined as paw withdrawal latency, at different time point is shown in Fig. 9.6. 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. The data obtained after converting the paw withdrawal latency into % MPE for LTG-NPs is shown in Fig. 9.7. 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 48h.

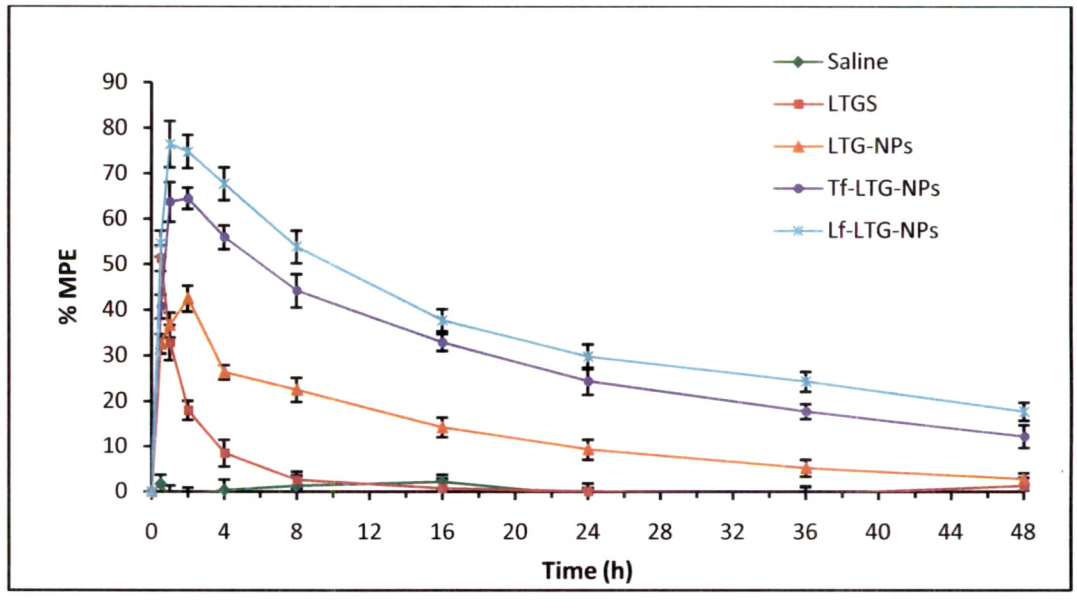
The study reveals enhanced efficacy of ligand conjugated NPs against unconjugated NPs and Lf conjugated NPs against Tf conjugated NPs. The reason was discussed in detail in TMD section. The improved efficacy of conjugated NPs formulation shows possibility of dose reduction with achieving desired therapeutic response. The antinociceptive effect of solution and NPs formulations was in agreement with blood and brain distribution of the formulations observed in biodistribution studies.

Figure 9.6: Influence on paw withdrawal latency measured in hot plate test by i.v. administration of LTGS, LTG-NPs, Tf-LTG-NPs and Lf-LTG-NPs in rats



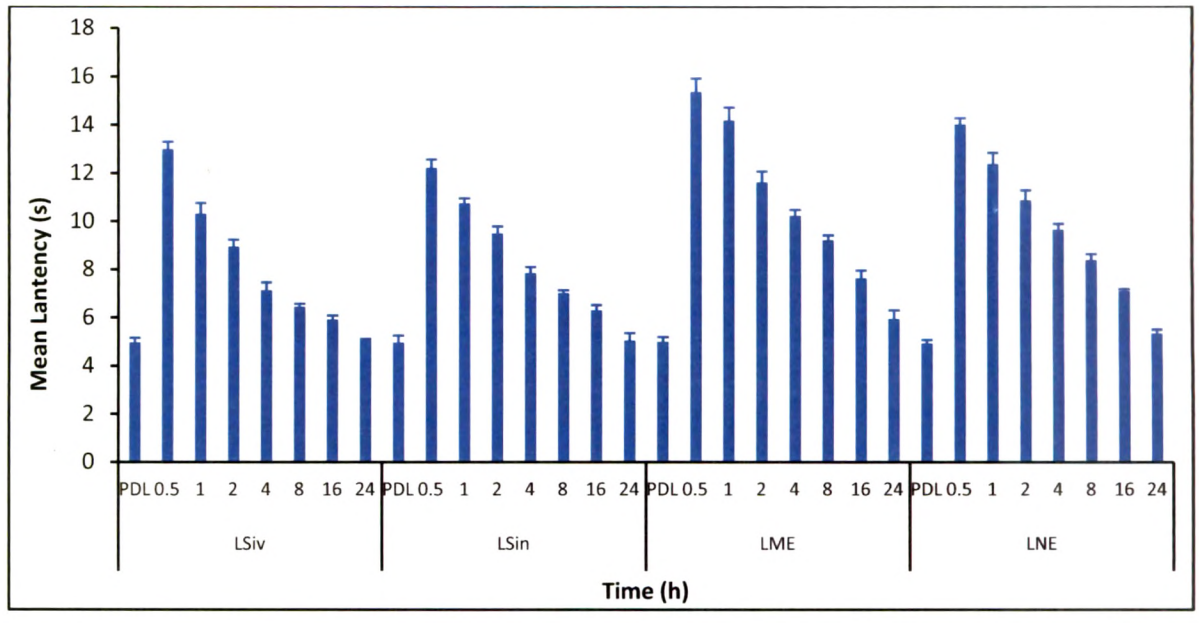
The data are expressed as mean of six experiments ± SEM (bars). PDL stands for Pre-Drug Latency.

Figure 9.7: Antinociceptive effect exerted by i.v. administration of LTGS, LTG-NPs, Tf-LTG-NPs and Lf-LTG-NPs in rats



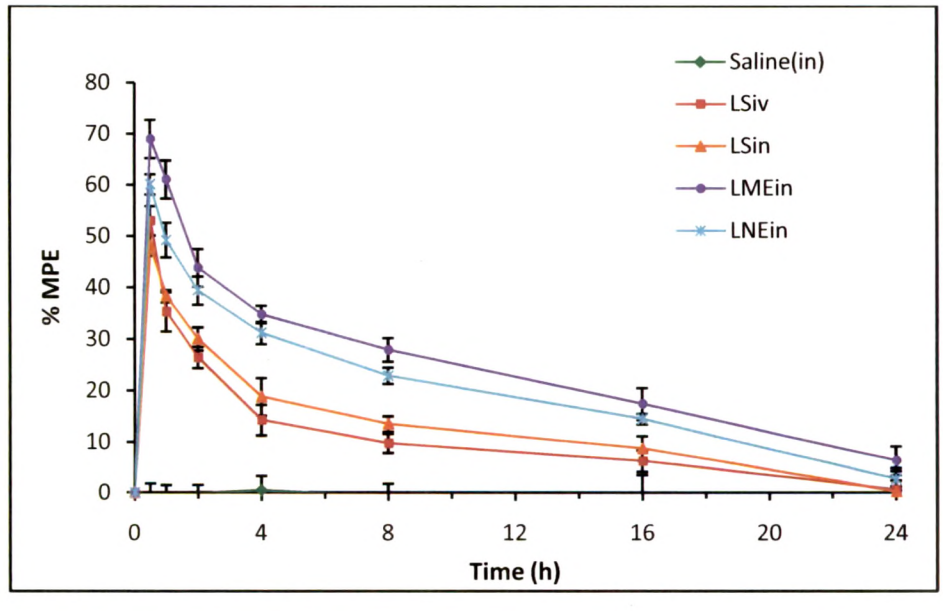
The data are expressed as mean of six experiments ± SEM (bars)
LTG-NPs vs LTGS < 0.05 at all except 0.5h; Tf-LTG-NPs and Lf-LTG-NPs vs LTGS < 0.05 at all except 0.5h; Tf-LTG-NPs vs LTG-NPs< 0.05 at 1,2,4,8 and 48h; Lf-LTG-NPs vs LTG-NPs< 0.05at all time points; Lf-LTG-NPs vs Tf-LTG-NPs< 0.05 at 0.5,1 and 4h

Figure 9.8: Influence on paw withdrawal latency measured in hot plate test by LSiv, LSin, LME and LNE in rats



The data are expressed as mean of six experiments ± SEM (bars). PDL stands for Pre-Drug Latency.

Figure 9.9: Antinociceptive effect exerted by administration of LSiv, LSin, LME and LNE in rats



The data are expressed as mean of six experiments ± SEM (bars)
LSin vs LSiv < 0.05 for none; LME vs LSiv < 0.05 at all except 24h; LNE vs LSiv < 0.05 at all except 0.5, 16 and 24h; LME vs LSin < 0.05 at all except 16 and 24h; LME vs LSin < 0.05 at 0.5, 1 and 4h; LME vs LNE < 0.05 at 1h.

Lamotrigine Microemulsion and Nanoemulsion (LME and LNE)

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, at different time point is shown in Fig. 9.8. 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 is shown in Fig. 9.9. 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 effect of formulation was in agreement with blood and brain distribution of the formulations observed in biodistribution studies. The higher antinociceptive effect following i.n administration of LME and LNE demonstrates the suitability and capability of microemulsion and nanoemulsion as an effective delivery system across the nasal membrane (Lawrence MJ and Rees GD, 2000) and a larger extent of selective transport 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. (Behl CR et al., 1998; Illum L, 2000).

9.4 Conclusion

Antinociceptive effect of drug when formulated in NPs displayed significant improvement in their efficacy. TMD NPs and LTG NPs displayed significant enhancement in their antinociceptive effect as compared to drug solution in mice for hot plate method and in neuropathic rats for radiant heat method respectively. The prolonged and enhanced antinociceptive effect of ligand conjugated NPs of TMD and LTG proves their role in effective management of pain. Similarly, intranasally administered TME/TNE and LME/LNE displayed significant enhancement in their antinociceptive effect as compared to drug solution in mice for hot plate method and in neuropathic rats for radiant heat method respectively. The rapid and enhanced antinociceptive effect of MEs and NEs of TMD and LTG proves their role in effective management of episodic and emergency pain treatment.The studies under this investigation support the biodistribution studies.

9.5 References

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