Formulation and Evaluation of Lamotrigine Nanoparticles Incorporated In-Situ Gel for Epilepsy

Keywords: Lamotrigine, Epilepsy, Nanoparticles, Carboxymethyl chitosan, Optimization

ABSTRACT

The objective of this study was to develop a nanoscale delivery system to improve the solubility and brain penetration of the AEDs. The present study is concerned with the development and characterization of a novel nanoparticulate system containing hydrophobic AED, Lamotrigine for brain targeting to improve the bioavailability, patient compliance and reducing the strength of the drug. Epilepsy is a neurological disorder which requires quick action. The pharmacoresistance is the major cause of failure of anticonvulsant therapy. To overcome, using Carboxymethyl chitosan (CMC) nanoparticles as a carrier. So, Lamotrigine loaded CMC nanoparticles were prepared by a solvent evaporation method. The formulations were optimized by using Design Expert Software and the optimized formula was selected and evaluated. The drug-loaded nanoparticle is mainly integrated into an in-situ gel for active targeting to the brain and for easy administration, thus providing quick action prepared formulation were subjected to characterization in-vitro and in-vivo drug release studies.
INTRODUCTION

In the past few decades, considerable attention has been focussed on the development of novel drug delivery system for the treatment of epilepsy. Novel drug delivery attempts to sustain drug action at a predetermined rate with concomitant minimization of undesirable side effects. Epilepsy is a common neurological disorder characterized by recurrent episodes of paroxysmal neural discharge. Its symptoms can be controlled by using Anti-epileptic drugs (AEDs). Lamotrigine (LTG) is a 2nd generation anticonvulsant drug used in the treatment of epilepsy and bipolar disorder. It also acts as a mood stabilizer which exhibits a broad spectrum of efficacy and tolerability, being effective against partial and generalized tonic-clonic seizure either alone or adjunctive therapy. Epilepsy requires quick management in order to avoid the risk of permanent brain damage. Pharmacoresistance is considered one of the major causes underlying the failure of anticonvulsant therapy, demanding the development of alternative and more effective therapeutic approaches. The drug efflux transporters in the blood-brain barrier (BBB) restrict the access of various CNS drugs to the target site. To overcome this situation, we propose carboxymethyl chitosan nanoparticles (CMC NPs) as a carrier to deliver the LTG across the BBB.

Nanoparticles are solid colloidal carrier particles ranging 1 to 1000 nm in size. They consist of macromolecular materials in which the active principle is dissolved, entrapped or encapsulated or to which the active principle is adsorbed or attached. Nanoparticles have become an important area of research in the field of drug delivery because they have the ability to deliver a wide range of drugs to a varying area of the body for a sustained period of time. NPs used as an enhancer of the drug across the BBB and it delivers a high concentration of the pharmaceutical agent to the desired location. The nano-sized property helps to cross the BBB easily. Nasal route is the preferred and noninvasive route for brain targeting. Because brain and nose compartments are connected with each other via olfactory, trigeminal nerves, the vasculatures, the cerebrospinal fluid, and lymphatic system. Nasal cavity consists of vascularised epithelium, large surface area, and lower enzymatic activity when compared to GIT. This pathway of a nose to brain deliver the drugs directly to CNS without first pass metabolism and provide faster and maximum therapeutic effect. Generally, the intravenous route is given for immediate relief from status epilepsy due to good bioavailability. But it produces pain, irritation, local systemic adverse effect, and produces
precipitation and tissue necrosis. For status epilepsy, the Nasal route is an alternative route to parenteral since it has good bioavailability and less side effect\[8\].

Due to the particular anatomical features of the nasal cavity, intranasal administration has been explored as a means of preferential drug delivery to the brain. Lamotrigine was directly transferred to the brain via the olfactory neuronal pathway, circumventing the blood-brain barrier. Therefore, it seems that intranasal route can be assumed as a suitable and valuable drug delivery strategy for the chronic treatment of epilepsy, also providing a promising alternative approach for a prospective management of pharmacoresistance\[3\].

MATERIALS AND METHODS

Chemical and reagents

Lamotrigine and Chitosan were purchased from Yarrow ChemPvt. Ltd Mumbai. Monochloro acetic acid was obtained from Chem dyes corporation, Rajkot. All other chemical solvents and reagents used were of analytical grade.

Synthesis of carboxymethyl chitosan (CMC)\[9\]

Chitosan (10 g) and sodium hydroxide (15 g) were suspended in isopropanol (100 ml) to swell and alkalize at room temperature for 1 h. The monochloro-acetic acid (10 g) was dissolved in 20 ml isopropanol, and added to the reaction mixture drop-wise within 30 min and reacted for 4 h at 55 °C. Then the reaction was stopped by removing the reaction mixture from heat and discarding the isopropanol. Ethyl alcohol (80%) was added and the solid product was filtered and rinsed with 80%~90% ethyl alcohol to desalt and dewater, and vacuum-dried at 50 °C. The product yield of CMC from the synthetic scheme was calculated by the following formula:

\[
\text{Product yield} = \frac{(\text{Amount of CMC} \times \text{Molecular weight of CS})}{(\text{Amount of CS} \times \text{Molecular weight of CMC})} \times 100 \%
\]

FT-IR Spectroscopy\[9\]

FT-IR spectrum of CMC was taken and compared with the FT-IR spectrum of chitosan for the verification of the chemical structure of synthesized CMC.
Degree of substitution (DS)\[^{10}\]

A 100 mg of CMC in 10 mL of 0.12 M NaOH was prepared. The mixture was stirred 30 min. at room temperature. Methyl red as an indicator was added and the mixture was titrated with 0.13 M HCl until the mixture became reddish.

\[
DS = \frac{MW \times M \times (B - S)}{1000 \times W}
\]

Where,

- MW - Molecular weight of monomer chitosan (g/mL)
- B - Volume of HCl blank (mL)
- S - Volume of HCl sample (mL)
- M - Molarity of HCl (mol/L)
- W - Mass of a sample (g)

Synthesis of Lamotrigine loaded CMC Nanoparticles\[^{6,9}\]

The Lamotrigine loaded CMC nanoparticles were prepared by the solvent evaporation method. LTG was dissolved in one part of ethanol, and the CMC was separately dissolved in one part of 0.1 N HCl. The LTG solution was added dropwise into the polymer solution with the magnetic stirrer. The resultant solution was added to the aqueous phase containing surfactant like sodium lauryl sulfate under high homogenization to form an emulsion. After the formation of a stable emulsion, the organic solvent was evaporated or removed by continuous stirring for 22 hr. The nanosuspension produced is freeze-dried using 5% mannitol as a cryoprotectant to obtain a fine powder of nanoparticles.

OPTIMIZATION OF LAMOTRIGINE LOADED CMC NANOPARTICLES\[^{9,11,12}\]

Optimization was performed to determine the relative significance of a number of variables and their interactions. The 3\(^2\) factorial design was used for optimization. CMC Concentration and Stirring speed were taken as the main factors. The nine batches of LTG NPs were prepared for optimization.
DEVELOPMENT OF THE OPTIMUM BATCH

Based on the statistical evaluations the software suggested one optimum batch from each Lamotrigine loaded CMC nanoparticles formulations. This optimum batch of the formulation was used for further studies.

Physicochemical and Morphological Characterization

The resultant NPs were critically analyzed for physicochemical parameters such as Polydispersity index (PDI), and Zeta potential. The PDI and Zeta potential of NPs were determined by Dynamic Light Scattering using zetasizer (MALVERN ZETASIZER VER. 7.01). Surface morphology was determined by Scanning Electron Microscopy (SEM). Nanoparticles were mounted on a metal stub and stub was then coated with conductive gold with sputter coater attached to the instrument. The photographs were taken using a scanning electron microscope\[13\].

Entrapment Efficiency\[9\]

The entrapment efficiency was determined by centrifugation method. 1ml of LTG-NPs was centrifuged at 2500 rpm for 1 hr and the clear supernatant solutions were obtained. After dilution with ethanol, concentrations of LTG in the supernatant (free drug) were determined by UV spectrometer at \(\lambda_{max} 305\) nm. The entrapment efficiency (EE %) could be calculated by the following formula:

\[
EE (\%) = (C_{total} –C_{free})/ C_{total} \times 100\%
\]

Where \(C_{free}\) was the LTG concentration in the supernatant after centrifugation while \(C_{total}\) was the initial amount of drug added during the preparation of LTG-NPs.

Drug Content\[14\]

An aliquot (0.5 mL) of the prepared nanosuspension was evaporated to dryness. The residue was dissolved in ethanol and filtered with a 0.45 \(\mu\)m filter. Total drug content was determined by UV spectrophotometer at \(\lambda_{max} 305\) nm.
In-vitro drug release study\textsuperscript{[12]}

Dialysis bag technique was used to study the in-vitro release of drug from NPs suspension. The formulation (2mL) was then placed in the dialysis bag, hermetically sealed and immersed into a 100 mL beaker containing 50 mL of release media (1\%\text{v/v} tween 80 in phosphate buffer pH 7.4) maintained at 37±0.5\textdegree C and stirring at 100 rpm. Aliquots of 1 mL were withdrawn at predetermined intervals and immediately restored with the same volume of fresh media. The amount of released LTG was measured by UV spectrophotometer at 305 nm after suitable dilution.

PREPARATION OF LTG NPs INCORPORATED NASAL IN-SITU GEL\textsuperscript{[15]}

In-situ gel was prepared by the modified hot method. Here, gellan gum was used as a gelling agent and HPMC as a viscosity increasing agent. Gellan gum was dissolved in one part of distilled water by increasing the temperature and HPMC was dissolved in another part of distilled water separately with stirring. Then allow standing for 2 hrs until a clear solution was obtained. Gellan gum solution was added slowly into the HPMC solution with stirring. An accurately weighed amount of optimum batch of Lamotrigine loaded CMC nanoparticles were suspended in distilled water and added to the polymer solution. Finally adjust the volume up to 100 mL with distilled water, filled into the glass vial and stored.

CHARACTERIZATION OF NASAL IN SITU GEL

Clarity\textsuperscript{[16]}

The clarity of the solution was done by visual inspection under the black and white background.

\textit{pH}\textsuperscript{[16]}

pH of each formulation was determined by using Digital pH meter (Systronic Digital pH meter) was previously calibrated by pH 4 and pH 7. The pH values were recorded immediately after preparation.
**Gelling Capacity**[^16]

The gelling capacity of the formulation was determined by placing one drop of the prepared formulation into a vial containing 2 mL of freshly prepared nasal saline solution. Gelation was assessed visually and noted the time for gelation and the time taken for the gel formed to dissolve.

**Viscosity**[^17]

A viscosity of the prepared formulation was determined by Ostwald viscometer. Filled the viscometer with purified water to mark A and fixed it on a burette stand and calculated the time required for water to pass from mark A to B. Repeated the experiment thrice and replace the water with the prepared formulation and repeated the same procedure.

**Drug content**[^18]

Drug content of formulation was determined by using double beam UV visible spectrophotometer. 1mL of the formulation was taken in the capacity of 10 mL volumetric flask, diluted with distilled water and volume adjusted to 10 mL. From this solution, 1 mL of the solution was taken and again diluted to 10 mL with distilled water. Finally, the absorbance of the prepared solution was measured at 305 nm by using UV visible spectrophotometer.

**In-vitro drug release study**[^18]

The drug release from the gel was determined by using Franz diffusion cell. The dialysis membrane was soaked in the receptor medium (phosphate buffer pH 6.4) for 24 hrs. Diffusion cell was filled with 21 mL of PBS pH 6.4 and dialysis membrane was mounted on a cell. 2 mL of prepared solution was placed on to donor chamber and release medium was maintained at 37°C with stirring speed 100 rpm. Samples of 1 mL were withdrawn at different time intervals and replaced with the same volume of fresh solution. The amount of released LTG was measured by UV spectrophotometer at 305 nm after suitable dilution.
**Nasal Ciliotoxicity study** [19]

The fresh nasal mucosa was carefully removed from the nasal cavity of mice and was stored in 10% of formalin solution. The mucosa exposed and treated with 0.5 mL of *in situ* gel for 1 hr and the mucocilia was examined with an optical microscope. Saline and isopropanol were used as a negative and positive control.

**Pharmacokinetic study** [3]

Male mice 20-30 gm were randomly divided into two experimental groups of 4 animals each. One of the groups received IN formulation whereas the other group was treated with the IV dosage form. At predetermined time points after LTG dosing, the mice were sacrificed by cervical dislocation followed by decapitation. Brain tissues were quickly removed and weighed. Mice brain tissues were homogenized with 0.1M sodium phosphate buffer pH 5.0 (4 mL per gram of tissue). Tissue homogenates were centrifuged at 2400 rpm for 30 min and the resultant supernatants were also frozen at -30°C until analysis.

The concentration of LTG in brain tissues were determined by using a liquid-liquid extraction followed by High-performance liquid chromatography.

**Stability study** [20]

The optimized nanoparticle incorporated *in-situ* gel formulation underwent stability evaluation for 60 days in refrigerated (4±2°C) conditions and analyzed for clarity, drug content, gel capacity and *in-vitro* release study.

**RESULTS AND DISCUSSIONS**

**SYNTHESIS OF CARBOXYMETHYL CHITOSAN AND ITS IDENTIFICATION**

Carboxymethyl chitosan was prepared successfully by etherification method using chitosan and monochloroacetic acid in a NaOH – isopropanol system. The prepared CMC was shown in fig.1. A beige colour powder was obtained and increases the water solubility. The amount of CMC was synthesized from this method was 28.162 gm and the product yield was found to be 83.50%.

The FT-IR spectrum of CMC was shown in fig.2. As compared with the FT-IR spectrum of chitosan IP, two new bands at 1591.00 cm⁻¹ & 1414.60 cm⁻¹ were observed in the spectrum of
the CMC, indicating the existence of two functional group COO$^- \& \text{CH}_2$ in the carboxymethylated derivatives. This confirmed the conversion of chitosan to carboxymethyl chitosan. The degree of substitution of a CMC is the number of substituent group attached per base unit. The degree of substitution of CMC was found to be 0.6124.

**FORMULATION AND OPTIMIZATION OF LTG LOADED CMC NPs**

Lamotrigine loaded CMC nanoparticles were prepared by the solvent evaporation method using a different concentration of CMC with different stirring speed. All the prepared formulations were taken for further evaluation studies like entrapment efficiency and *in-vitro* drug release. The entrapment efficiency and *in-vitro* drug release of 9 formulations are given in table 1.

A three level, two factorial designs were used for the optimization. In the preliminary studies, it was found out that the two most important factors affecting % entrapment efficiency and *in-vitro* drug release are CMC concentration and stirring speed. Hence these two factors were selected as independent variables. The levels (-1, 0, +1) of these parameters were selected from the preliminary studies. All other parameters kept constant throughout the study. To elucidate the influences of the decision variables on the response variables, a two factor, three level, nine runs factorial design was employed. Design Expert Software 10.03.1 (statease, Minneapolis USA) trial package was used for the generation and evaluation of statistical experimental design. After generating the polynomial equation relating to the dependent and independent variables, the formulation was optimized for the responses. The optimum values of the variables were obtained by the numerical analysis based on the criterion of desirability. Therefore a new batch of nanoparticles with the predicted levels of formulation factors was prepared to confirm the validity of the optimization procedure. The contour plot and response surface plot for the effect of factors on the variables are given in fig. 3-6. From the figures concluding that, % entrapment efficiency was inversely proportional to stirring speed & polymer conc. The *in-vitro* drug release of the formulation was directly proportional to CMC conc. & stirring speed.

The final equation in terms of actual factors:

Entrapment efficiency $Y_1$

$$= - 279.90000 - 2.30244\times\text{Conc. of CMC} + 0.727067\times\text{Stirring speed}$$
In vitro drug release $Y_2$

$$= +4.89333 + 0.634889 \times \text{Conc. of CMC} + 0.043033 \times \text{Stirring speed}$$

The equation in terms of actual factors can be used to make predictions about the response for given levels of each factor. Here, the levels should be specified in the original units for each factor. This equation should not be used to determine the relative impact of each factor because the coefficients are scaled to accommodate the units of each factor and the intercept is not at the centre of the design space.

Based on the statistical evaluations the software suggested a good number of combinations of which we selected formulation F5 as optimum batch i.e. 30 mg CMC & 1100 rpm stirring speed. The predicted % entrapment efficiency was 69.720 % and % in-vitro drug release was 71.277 % and its desirability was found to be 1.

Fig.1: Prepared CMC                                        Fig.2: FT-IR spectrum of CMC
Table 1: The factors and responses of prepared formulations (F1-F9)

<table>
<thead>
<tr>
<th>FORMULATION CODE</th>
<th>FACTORS</th>
<th>RESPONSES</th>
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<tbody>
<tr>
<td></td>
<td>X1: CMC Concentration (mg)</td>
<td>X2: Stirring speed (rpm)</td>
</tr>
<tr>
<td>F1</td>
<td>15</td>
<td>1000</td>
</tr>
<tr>
<td>F2</td>
<td>15</td>
<td>1000</td>
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<tr>
<td>F9</td>
<td>45</td>
<td>1200</td>
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</table>

Fig. 3: Contour plot for the effect of CMC Conc.  Fig. 4: Response surface plot for the effect of CMC Conc. and Stirring speed on the Entrapment efficiency
CHARACTERIZATION OF OPTIMIZED FORMULATION

The characterization of the optimized formulation of LTG loaded CMC NPs was done by evaluated the % drug content, % entrapment efficiency, \textit{in-vitro} drug release, particle morphology by SEM analysis and Zeta potential and particle size distribution by DLS. The % drug content of the formulation obtained after suitable dilution with ethanol and it found to be 73.02 %. The % entrapment efficiency was determined by centrifugation method and the % amount of drug enter into the nanoparticles was 71.62 %. About $\frac{3}{4}$ of the drug was entraped into the nanoparticles. The drug release profile of the optimized formulation of lamotrigine loaded CMC nanoparticles were performed in PBS pH 7.4. % cumulative drug release of LTG from NPs at 6$^{\text{th}}$ hr was 75.65%. The drug release profile of LTG was shown in graph 1.

\textit{SEM Analysis}

The particle morphology and particle size of the optimized NPs were performed by Scanning Electron Microscopy. The smooth surface with round shaped particles shown in SEM image (fig.7) and its particle size was mentioned, which is in the nano-size range.

\textit{Dynamic Light Scattering}

The Zeta potential and particle size distribution of the optimized LTG NPs were done by using dynamic light scattering. Zeta average diameter of the SLN of the optimized batch was found to be 461.1 nm. The zeta potential analysis revealed the stability of prepared
nanoparticles and it found to be 14 mV and is shown in graph 2. The system still is stable even though the zeta potential is only 10 mV – 20 mV. PDI value used to characterize the monodispersed or polydispersed nature of NPs. PDI value 0.416 was obtained, which indicate the low level of nonuniformity. PDI was shown in graph 3.

Graph 1: *In-vitro* drug release profile of optimum batch of nanoparticles.

Graph 2: Particle size distribution by intensity

Graph 3: Zeta potential

![Graph 1](image1)

![Graph 2](image2)

![Graph 3](image3)
SYNTHESIS AND CHARACTERIZATION OF LTG NPs INCORPORATED IN SITU GEL

The lamotrigine loaded carboxymethyl chitosan nanoparticle incorporated *in situ* gel was prepared by the hot method and evaluated the clarity, pH, gelling capacity, viscosity, drug content, *in-vitro* drug release study etc. The nasal *in-situ* gel prepared successfully and its appearance is clear without any turbidity. The pH of the *in situ* gel was found to be 5.9. Which is in the nasal pH range i.e. 4.5 – 6.4. The gelling capacity of the prepared formulation was coded as +++ i.e. the gelation immediate and for the extended period. The viscosity of the prepared nasal in situ gel was determined by Ostwald viscometer and the viscosity was found to be 2.6832 Cp. Which has low viscosity because it appeared as a solution. The 90.06% of the drug present in the formulation. The *in-vitro* drug diffusion profile of *in situ* gel were performed in PBS pH 6.4 using Franz diffusion cell. The % cumulative drug release at 6th hr was found to be 73.06 %. The percentage drug released as a function of time was shown in graph 4.

*Nasal Ciliotoxicity Study*

The microscopic observation indicates that the formulation has no any significant effect on the microscopic structure of mucosa. There is no cell destruction observed in mucosa treated with saline & *in situ* gel but it showed in mucosa treated with isopropyl alcohol. The microscopic observation of nasal mucosa after treated with saline, *in situ* gel and isopropyl alcohol were shown in fig.8, 9 & 10 respectively.

*Pharmacokinetic Study*

The results showed that *in-vivo* administration of LTG NPs incorporated *in-situ* gel remarkably increased the LTG concentration in the brain when compared to concentration found after intravenous administration of LTG solution. The concentration Vs time graph is shown in Graph 5. The AUC after intranasal administration was found about 195.6339 Min. *µg/mL* which is 4.25 times higher than intravenous administration of LTG solution. The pharmacokinetic parameters were shown in table 2. The results of *in-vivo* studies showed that the nanoparticulate system based on CMC was able to promote a rapid drug absorption through the nasal mucosa and remarkably to improve the bioavailability of lamotrigine.
Stability Study

Formulated NPs loaded nasal in-situ gel were subjected to short-term stability studies by storing it in a glass vial at the refrigerated condition for a period of 60 days. After 60 days the samples were analyzed for clarity, drug content, gelling capacity & the in-vitro drug release. There were no considerable changes in these parameters. Stability parameters of the prepared formulation shown in table 3.

Graph 4: In-vitro drug release of nasal in-situ gel

Fig.8: Nasal mucosa treated with saline (Negative control)

Fig.9: Nasal mucosa after application of in-situ gel

Fig.10: Nasal mucosa treated with isopropyl alcohol (Positive control)
Graph 5: Concentration Vs time profile

Table 2: Pharmacokinetic parameters of LTG after Intranasal & Intravenous administration

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>CONCENTRATION OF LAMOTRIGINE</th>
<th>AFTER IN administration of LTG NPs</th>
<th>AFTER IV administration of LTG solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>T max (Min.)</td>
<td>60</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>C max (µg/mL)</td>
<td>0.693</td>
<td>0.291</td>
<td></td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-∞&lt;/sub&gt; ( Min. µg/mL )</td>
<td>195.6339</td>
<td>45.9279</td>
<td></td>
</tr>
<tr>
<td>MRT (Min.)</td>
<td>246.3108</td>
<td>146.5178</td>
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Table 3: Stability parameters of LTG NPs loaded nasal in-situ gel

<table>
<thead>
<tr>
<th>PARAMETERS</th>
<th>BEFORE STABILITY</th>
<th>AFTER STABILITY</th>
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<tbody>
<tr>
<td>Clarity</td>
<td>Clear without any turbidity</td>
<td>Clear without any turbidity</td>
</tr>
<tr>
<td>Gelling Capacity</td>
<td>++ +</td>
<td>++ +</td>
</tr>
<tr>
<td>Drug Content</td>
<td>97.83 %</td>
<td>96.52 %</td>
</tr>
<tr>
<td>In-vitro drug release at 6th hr.</td>
<td>73.06 %</td>
<td>72.71 %</td>
</tr>
</tbody>
</table>

*+++ - Immediate gelation for extended period

CONCLUSION

Lamotrigine loaded CMC nanoparticle incorporated in situ gel was successfully formulated and resulted in fast and pronounced absorption across BBB by bypassing the first pass metabolism. The nasal in situ gel having improved stability, penetrability, patient compliance, and enhanced bioavailability. Lamotrigine was directly transferred to the brain.
via the olfactory neuronal pathway, circumventing the BBB. Therefore, it seems that the intranasal route can be assumed as a suitable and valuable drug delivery strategy for chronic treatment of epilepsy, also providing a promising approach for a prospective management of pharmacoresistance.

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