IN-SITU NASAL GEL OF LEVODOPA FOR BRAIN TARGETING USING CHITOSAN – THIOGLYCOLIC ACID CONJUGATE AND MUSK KETONE BY EFFLUX TRANSPORT MODULATION

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ABSTRACT

Conventional levodopa therapy has problems like peripheral oxidation, poor penetration through blood brain barrier and efflux through the P-gp efflux pump that limits the drug bioavailability. The rationale of project was aimed to formulate and evaluate pluronic thermosensitive nasal gel of levodopa for brain targeting using chitosan-thioglycolic acid conjugate as P-gp efflux transport inhibitor and nitromusk (musk ketone) as fragrance compound that stimulates the sensation and thus permeability in the nasal cavity. Synthesised thiolated chitosan was used for preparation of levodopa nanoparticles (NPs) by ionic gelation method and Pluronic i.e. PF127 was used for the purpose of thermoreversible gel formation. The optimised thiolated chitosan NPs showed 222.6 nm particle size, 0.296 PDI and +27.91mV zeta potential, confirming the stability of formulation and drug entrapment efficiency of NPs was found to be 76±1.2 %. The release of the drug from the thiolated chitosan NPs in PBS pH 6.4 showed non-fickian diffusion. The release profile indicated the suitability of CS-TGA derivative in nasal pH range of 5.5. In-vivo pharmacokinetic studies with the levodopa in saline, levodopa in CS-TGA NPs, levodopa in CS-TGA NPs in pluronic gel and gel with musk ketone showed enhanced bioavailability in brain (AUC-1.4 times) with more MRT (2.3 times) in case of CS-TGA nanoparticles dispersed in thermosensitive formulation with musk ketone in comparison to plain drug solution. However CS-TGA NPs without gel formulation showed more bioavailability (1.15 times) of drug level in brain compare to NPs in gel in presence of musk ketone but drug retention time in brain was 2 times less, because of low efflux through the P-glycoprotein in presence of musk ketone and...
pluronic gel. The results justified our rationale in selecting thiolated chitosan in presence of *in-situ* pluronic gel and musk ketone for enhanced bioavailability of levodopa in brain through nasal route.

**KEYWORDS:** Levodopa, Chitosan-thioglycolic acid conjugate, Musk ketone, Parkinson’s disease, Thermosensitive gel.

**INTRODUCTION**

The brain drug delivery is required in some diseases of the central nervous system (CNS) such as schizophrenia, meningitis, migraine, Parkinson’s disease (PD) and Alzheimer’s disease.\(^1\) There are so many challenges in drug delivery to brain by crossing blood brain barrier (BBB) like few fenestrations in the microvascular endothelial cells as well as pinocytic vesicles, numerous metabolic enzymes and membrane efflux transporters like P-glycoproteins (P-gp).\(^2\) Parkinson disease, a neurodegenerative disorder of the central nervous system affects 1-2% of general population over the age of 65 years and symptoms include impaired motor skills, cognitive processes, leading to tremor, rigidity, bradykinesia and postural instability.\(^3\) Levodopa (L-DOPA) is the drug of choice for Parkinson’s disease.\(^4\) The main problems associated with the levodopa therapy are its degradation in peripheral nervous system by dopa-decarboxylase (DDC) enzyme and poor penetration through BBB (<5% if alone, along with carbidopa, 4 fold reduction in dose).\(^5\) Even this combination therapy suffered from major drawbacks like uncontrollable movements, fluttering heart beat, hearing things that are not real, loss of appetite, faintness, dizziness, black and bloody stools etc. Intranasal route for brain targeting is gaining much attention since last three decades due to several advantages such as non-invasive rapid systemic absorption, fast onset of action, avoidance of first-pass metabolism, increasing drug bioavailability, and less systemic side effects.\(^6\) Nasal delivery thus through olfactory route may offer targeted delivery of the drug to the brain for the treatment of Parkinson disease. This route though explored by various scientists is also associated with failure in levodopa therapy due to mucociliary clearance, enzymatic barriers like glutathione transferase, aldehyde dehydrogenase etc. and P-gp efflux system.\(^7\) Graff and Pollack (2003), however, found that uptake into the brain was enhanced when drugs were administered in combination with the P-gp efflux inhibitor, rifampin.\(^2\) The bioavailability of drugs through nasal route may be enhanced by either using mucoadherence, or use of penetration enhancers or The incorporation of mucoadhesive polymers into nasal formulation have shown improved the
brain penetration of a hydrophilic peptide due to increase in the mucosal contact time and prolonged residence in the nasal cavity.\cite{8} Chitosan, being non-toxic, mucoadhesive and permeation enhancer in nature have shown enhanced the brain bioavailability of intranasally administered nerve growth factor by a 14-fold.\cite{9} Thiolated chitosan, where chitosan’s amino group is modified with thioglycolic acid, showed improved the permeability of the drug through blood brain barrier.\cite{8} The thiolated chitosan can act as P-glycoprotein efflux inhibitor and the mechanism behind this is that thiomers seem to enter in the channel of P-gp and likely form subsequently one or two disulfide bonds with one or both cysteine subunits located within the channel. Due to this covalent interaction the allosteric change of the transporter being essential to move drugs outside of the cell might be blocked. Moreover the transport of polar drugs can be enhanced by use of thiolate chitosan polymer. Also it is compatible with intranasal cavity pH 5.5-6.5.

Thiomers enhance the permeability of drugs with the potential advantage of not being absorbed through the nasal mucosa compared to low molecular weight permeation enhancers. Thus their permeation enhancing effects can be maintained over a longer period of time while excluding systemic toxic effects.\cite{24} Thiomers tend to cause reversible opening of the tight junctions with glutathione as permeation mediator. Thiolated polymers display in situ gelling properties due to the oxidation of thiol groups at physiological pH-values, which results in the formation of inter- and intramolecular disulfide bonds.\cite{12} This increases the viscosity of the formulation coupled with extensive crosslinking due to formation of disulphide bonds with the nasal mucosa, which increases the residence time of the formulation tremendously.

Penetration enhancers are used to improve the permeability and bioavailability of the drug upon contacting the nasal mucosa. The bioavailability of nerve growth factor in the brain could be enhanced by intranasal administration of peppermint oil (Vaka and Murthy, 2010). Intranasal administration of hexarelin, a growth hormone releasing neuropeptide for nose-to-brain targeting, was also enhanced by N-tridecyl-beta-D-maltoside as a permeation enhancer (Yu and Kim, 2009). Nitromusks (Musk ketone), a fragrance compound activates the signals throughout from nasal cavity to brain and increases the sensitivity. Hence it increases the uptake from the nasal cavity and also found to be P-gp efflux inhibitor.\cite{9}

Poloxamers are said to contain thermoreversible property and will convert from a liquid to a gel at body temperature, thus, causing in situ gelation at the site of interest\cite{1} preventing the drug to be removed from the nasal cavity due to mucociliary clearance. This vastly improves
the bioavailability of the drug administered. One of the most promising poloxamer is Poloxamer 407 (Pluronic F127) because of its low toxicity, high solubility, bioadhesion characteristics, and acceptability as drug delivery vehicle. Controlled release nasal formulations of propranolol have been made using a combination of poloxamers and other mucoadhesive polymers for such as Carbopol 934P. By controlling the release of the drug and by increasing its residence time in the nasal cavity, there was a significant increase in bioavailability of the drug.[52,51]

Colloidal systems like nanoparticles have high drug loading capacity and can be used to encapsulate, entrap, or absorb the drug on the surface or bound by covalent linkage. Especially, entrapping the drug within the particle matrix helps to protect the drug against degradation from various factors like enzymes etc. and enables complete release of the drug.[11] Intranasal drug delivery of didanosine-loaded chitosan nanoparticles for brain targeting has shown increased drug delivery to the brain (Al-Ghananeem et al., 2010). Because of sufficiently small size, nanoparticles could potentially carry drug to brain via axons through the olfactory bulb.[12] (Mistry et al., 2009).

In view of above, it was proposed to synthesise CS-TGA conjugated polymer and formulate its nanoparticles loaded with levodopa for enhanced mucoadhesion as well as better penetration through nasal cavity and finally dispersing these drug loaded nanoparticles into in-situ gel solution of pluronic PF127 for sustained release of drug in presence of permeation enhance, musk ketone. The project aimed to inhibit the P-gp pump system and enhanced penetration of levodopa in brain with more retention time.

RESULTS

Synthesis of chitosan-thioglycolic acid conjugate

FTIR analysis
The thiolation of chitosan was confirmed using IR spectroscopy as shown in Figure 4.6. The characteristic vibration peaks for chitosan were at 3440 cm$^{-1}$, assigned to -OH and -NH stretching vibration, and the weak peak was at 2920 cm$^{-1}$, attributed to -CH stretch. The distinctive absorption peaks were appeared at 1635.26, 1590 and 1380cm$^{-1}$, attributed to the amide I, -NH$_2$ bending and amide III.[20] In contrast the spectra of thiolated chitosan derivative showed, distinct absorption peak at 1732 cm$^{-1}$ corresponding to the carbonyl groups of thioglycolic acid and the decreased peak at 1590 cm$^{-1}$ assigned to -NH bending implied that the carbonyl groups of thioglycolic acid were successfully introduced into the
chemical structure of chitosan derivative. The additional peaks of newly formed amide bond and peaks of thiol groups (from TGA) were observed: 1,250 cm\(^{-1}\) (S-C bond), 1,527 cm\(^{-1}\) (C=O double bonds of the amido bond), and 2,635 cm\(^{-1}\) (H-S bond). Both CS and TCS had absorption band at 2923 cm\(^{-1}\) peak corresponds to C-H bond. During reaction process, the amino groups in chitosan reacted with the carboxyl groups of TGA, resulted in an amide bond, the additional peaks of this newly formed amide bond. TCS has three characteristic peaks at 1156, 1527 and 2635, corresponding to the vibration of the S-C and C=O bonds of the amido bond and H-S bond respectively, while the peak at 2926 is attributed to stretching of the C-H bond.

![Figure 4: IR spectra of CS-TGA derivative](image)

**Figure 4: IR spectra of CS-TGA derivative**

**Determination of thiol content with Ellman’s reagent:** The degree of modification in chitosan was determined by quantifying the thiolated chitosan with 5, 5'-dithiobis (2-nitrobenzoic acid) (Ellman’s agent).

**Table 1: Showing the amount of thiol in the CS-TGA derivative**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Amount of Thioglycolic acid added</th>
<th>Absorbance</th>
<th>Thiol content</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS-TGA</td>
<td>30 ml in 100 ml</td>
<td>1.853</td>
<td>13.62 \times 10^{-3}</td>
</tr>
</tbody>
</table>

**Morphology**

Optimized formulation was visualized under SEM for morphology of thiolated chitosan nanoparticles. Figure 2 and 3 showed the SEM images of nanoparticles.
Particle size, Surface Morphology, and Zeta Potential Measurement of Nanoparticles

The particle size and zeta potential of the chitosan nanoparticles were analyzed by zetasizer. The nanoparticles were round in shape with a smooth appearance as shown in Figure 2 and 3. The values for the average particle size, zeta potential, and poly-dispersity index are tabulated in Table 2. CS-TGA NPs showed the minimum average particle size of 222.6 nm and a polydispersity index of 0.296, respectively, which can be taken into account as optimized in terms of particle size and polydispersity index. The zeta potential of the optimized formulation was found to be +27.8 mV.

Table 2: Optimization of amount of drug and entrapment efficiency

<table>
<thead>
<tr>
<th>Code</th>
<th>CS-TGA:TPP</th>
<th>Amount of Drug (mg)</th>
<th>Sonication Time (min)</th>
<th>Size (nm)</th>
<th>PDI</th>
<th>Zeta Potential (mV)</th>
<th>E.EFF (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>3:1</td>
<td>40</td>
<td>3 min</td>
<td>294.1</td>
<td>0.268</td>
<td>-39.0</td>
<td>20</td>
</tr>
<tr>
<td>N2</td>
<td>3:1</td>
<td>50</td>
<td>3 min</td>
<td>286.0</td>
<td>0.311</td>
<td>18.17</td>
<td>37</td>
</tr>
<tr>
<td>N3</td>
<td>3:1</td>
<td>60</td>
<td>3 min</td>
<td>222.6</td>
<td>0.296</td>
<td>27.8</td>
<td>76</td>
</tr>
<tr>
<td>N4</td>
<td>3:1</td>
<td>70</td>
<td>3 min</td>
<td>299.5</td>
<td>0.351</td>
<td>22.14</td>
<td>71</td>
</tr>
<tr>
<td>N5</td>
<td>3:1</td>
<td>80</td>
<td>3 min</td>
<td>285.3</td>
<td>0.151</td>
<td>-4.8</td>
<td>69</td>
</tr>
<tr>
<td>N6</td>
<td>3:1</td>
<td>90</td>
<td>3 min</td>
<td>289.0</td>
<td>0.299</td>
<td>52.0</td>
<td>45</td>
</tr>
</tbody>
</table>

Entrapment Efficiency

Entrapment efficiency of the levodopa-loaded thiolate chitosan nanoparticles was analyzed and the data are shown in Table 2. CS-TGA NPs showed an average drug entrapment efficiency of 76.4 ± 3.1%, which is higher amongst the other formulations.

Pluronic F127 concentration in gel and nanoparticles in gel

Table 3 represents the optimization of pluronic gel on the basis on gelation time, gelation temperature and mucoadhesivity strength.\(^{[14]}\)
Table 3: Gelation time, Gelation temperature and Mucoadhesivity strength of gel and nanoparticles in gel at different concentrations of PF 127

<table>
<thead>
<tr>
<th>CODE</th>
<th>Chitosan: TPP</th>
<th>Amount of drug (mg)</th>
<th>Conc. Of PF127 (%)</th>
<th>Gelation Time (Sec)</th>
<th>Gelation Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>3:1</td>
<td>60</td>
<td>16</td>
<td>60 Sec</td>
<td>70-75</td>
</tr>
<tr>
<td>G2</td>
<td>3:1</td>
<td>60</td>
<td>18</td>
<td>30 Sec</td>
<td>38-40</td>
</tr>
<tr>
<td>G3</td>
<td>3:1</td>
<td>60</td>
<td>20</td>
<td>12 Sec</td>
<td>30-32</td>
</tr>
<tr>
<td>G4</td>
<td>3:1</td>
<td>60</td>
<td>22</td>
<td>15 Sec</td>
<td>33-35</td>
</tr>
</tbody>
</table>

Mucoadhesive strength of gel (dynes/cm²): 874.6 dynes/cm²
Mucoadhesive strength of optimized formulation (dynes/cm²): 1105.67 dynes/cm²

Rheology of gel

Gel and nanoparticles in gel exhibited different rheological behavior at 30 ºC and 35 ºC which were represented in Figure 5 and 6 respectively.

Figure 5: Rheological behavior of gel at 30 ºC
Figure 6: Rheological behaviour of NPs in gel at 30 ºC

Figure 5 and 6 showed that initially viscosity rises drastically showing the phase transition from sol to gel and after that at particular point the viscosity remained constant at 30°C after complete phase transition from sol to gel. Also with increase in shear rate the viscosity remained constant. Hence there was a decrease in viscosity as we increased the shear rate and stress.
Figure 7: Rheological behavior of gel at 35 ºC Figure 8: Rheological behaviour of NPs in gel at 35 ºC

Figure 7 and 8 showed the rheology of gel at 35 ºC when system was already in the gel form because the study temperature was above gelation temperature i.e. 30 ºC. This showed that at 35 ºC with increase in shear rate viscosity tended to decrease which indicated that system was showing pseudoplastic behavior. This was may be due to some intramolecular interactions within the gel system at higher temperatures and hence may be responsible for shear thinning.[18]

In-vitro drug release studies

In-vitro drug release was performed using dialysis bag as mentioned in section 3.6. In-vitro drug release profile of entrapped drug into PBS (pH 6.8) across the dialysis bag has been shown in figure 9 representing comparative dissolution profile of thiolated nanoparticles and thiolated nanoparticles in gel in PBS buffer in shaking incubator. Table 4 showed kinetic analysis of drug release from nanoparticles in saline and nanoparticles in gel. Both followed Higuchi model which was graphically represented between % release vs. square root of time based on r² value (0.9592 and 0.9656 respectively). From the graph it could be inferred that drug is released rapidly in the initial phase by burst release followed by release through diffusion mechanism from nanoparticles. When % release between drug in gel and drug in saline was compared, it was observed that from both formulations the release was almost complete in 8 hours. The value of n on applying Korsmeyer Peppas model on drug release data thiolated nanoparticles and thiolated nanoparticles in gel showed 0.23 and 0.32 value for respectively (n<0.45) that indicated the non-fickian diffusion as shown in table 4.12. On comparison of pharmacokinetic data of thiolated chitosan nanoparticles and nanoparticles in gel were compared and found that the gel showing the sustained release.
Table 4: Different models and $r^2$ value in case of NPs and NPs in gel

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Model</th>
<th>$r^2$ in case of NPs</th>
<th>$r^2$ in case of NPs in gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Zero order</td>
<td>0.7722</td>
<td>0.9025</td>
</tr>
<tr>
<td>2</td>
<td>First order</td>
<td>0.9138</td>
<td>0.9329</td>
</tr>
<tr>
<td>3</td>
<td>Higuchi</td>
<td>0.9592</td>
<td>0.9656</td>
</tr>
<tr>
<td>4</td>
<td>Korsmeyer-peppas</td>
<td>0.7044, $n = 0.23$</td>
<td>0.8479, $n = 0.32$</td>
</tr>
</tbody>
</table>

$NPs$ $in$ $Phosphate$ $Buffer$

$Nanoparticles$ $in$ $Gel$

Figure 9: Comparative % release of nanoparticles in saline, drug in gel, nanoparticles in gel and drug in saline.

The highest values of regression coefficient suggested that both optimized formulations, in the phosphate buffer, followed Higuchi model release kinetics, which was describing the drug dissolution from sustained release gel carrier delivery system. The Korsmeyer Peppas model was indicating that the value of $n$ was less than 0.45; it means it was following the non-fickian diffusion. Such release occurs by the usual molecular diffusion of the drug due to a chemical potential gradient indicating diffusional controlled drug release. Fickian release was indicating that polymer relaxation followed by diffusion of the drug. This may lead to reduction in frequency of dosing and minimize the blood level oscillations, dose related adverse effects, ultimately improve the patient compliance and drug efficiency.

**In-vivo pharmacokinetic studies**

For the determination of percentage amount of levodopa in brain, standard curve in rat brain was made with $r^2$ value of 0.992 and slope of 0.039.

Brain homogenate studies were carried out for plain drug, nanoparticles in saline, nanoparticles in gel and nanoparticles in gel with musk ketone. Figure 11 and 12 represents % Drug in Brain with respect to time.
Pharmacokinetic parameters in plasma

Table 5: Different pharmacokinetic parameters in case of different formulations in plasma

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Plain drug solution</th>
<th>CS-TGA NPs</th>
<th>NPs loaded Gel</th>
<th>NPs loaded Gel +Musk Ketone</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/ml)</td>
<td>189</td>
<td>360</td>
<td>186</td>
<td>132</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (hours)</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (hours)</td>
<td>5.80779</td>
<td>3.21955</td>
<td>9.861467</td>
<td>13.301175</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;total&lt;/sub&gt; (µg/ml/min)</td>
<td>1614.14</td>
<td>1871.58</td>
<td>2949.11</td>
<td>2753.49</td>
</tr>
<tr>
<td>MRT</td>
<td>8.6</td>
<td>5.0</td>
<td>14.6</td>
<td>19.5</td>
</tr>
<tr>
<td>K&lt;sub&gt;e&lt;/sub&gt; (hr&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.119</td>
<td>0.215</td>
<td>0.05</td>
<td>0.07</td>
</tr>
</tbody>
</table>

Pharmacokinetic parameters in brain homogenate

Table 6: Different pharmacokinetic parameters in case of different formulations in brain homogenate

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Plain drug solution</th>
<th>CS-TGA NPs</th>
<th>NPs loaded Gel</th>
<th>NPs loaded Gel +Musk Ketone</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/ml)</td>
<td>498</td>
<td>623</td>
<td>236</td>
<td>257</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (hours)</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (hours)</td>
<td>2.28</td>
<td>2.55</td>
<td>5.36</td>
<td>5.49</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;total&lt;/sub&gt; (µg/ml/min)</td>
<td>1642.254</td>
<td>2664.808</td>
<td>2174.094</td>
<td>2308.417</td>
</tr>
<tr>
<td>MRT</td>
<td>3.832294</td>
<td>4.150078</td>
<td>8.419320</td>
<td>8.587015</td>
</tr>
<tr>
<td>K&lt;sub&gt;e&lt;/sub&gt; (hr&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.302</td>
<td>0.271</td>
<td>0.129</td>
<td>0.126</td>
</tr>
</tbody>
</table>
Figure 11: Showing the percentage amount of plain drug and NPs in brain with respect to time
Where * represents P< 0.001 in comparison to plain drug concentration

Figure 12: Showing percentage amount of NPs in gel and Gel with musk ketone in brain with respect to time
Where *** represents the P< 0.001, ** represents the P<0.01 and ns represents the P>0.05.

The pharmacokinetic data obtained after administering the drug to the wistar rats as in figure 11, 12, 13 and 14 through intranasal route for levodopa in-saline, levodopa in CS-TGA NPs, CS-TGA NPs in pluronic gel and levodopa in CS-TGA NPs in pluronic gel along with musk ketone showed high flux of NPs in plasma as well as brain homogenate. Indicating the suitability of CS-TGA nanoparticulate delivery system for high permeability of drug in comparison to plain drug solution (P<0.001).
Figure 13: Showing %age amount of plain drug and CS-TGA NPs in plasma with respect to time
Where *** represents the P<0.001 and * represents the P<0.05

Figure 14: Showing %age amount of NPs in gel and Gel with musk ketone in plasma with respect to time
Where *** represents the P<0.001 and ns represents the P>0.05

The pharmacokinetic data of brain homogenate and plasma for all formulations was shown above in table 5 and 6. The concentration of thiolated chitosan nanoparticles was found to be more (72.7 ± 2.27 %) both in brain and plasma i.e approximately 50% more as compared to plain drug. This can be explained as follows: thiolated chitosan nanoparticles have improved adhesion with nasal mucosal tissue as the nasal mucosa contains mucin which has significant proportion of sialic acid and at physiological pH it carries a net negative charge and consequently has strong electrostatic interactions between them. This proves that thiolated chitosan as a bioadhesive material significantly reduces the clearance time of formulation and
provides more time for drug absorption.\cite{7} The AUC of thiolated chitosan nanoparticles was found to be more than the plain drug but the MRT was less in the case of thiolated chitosan nanoparticles in plasma. But it was found to be more in brain. Hence from this it could be said that maximum drug concentration reached into the brain than the plasma, so maximum drug targeting can be achieved with nanoparticles. But low MRT value was indicating that drug may be effluxed from the P-glycoprotein efflux pump. It means that the permeation with thiolation got increased\cite{7} but efflux could not be minimized. In-situ gelling formulation was also observed to maintain higher percentage of levodopa in brain by sustaining the release for longer period of time. This can be explained as follows, poloxamers have the property to convert from sol to gel at physiological temperature (~37 °C).\cite{17} Hence at this nasal temperature gel adheres to the nasal cavity and reduces the clearance thereby controlling the release. That’s why the thiolated chitosan nanoparticles were incorporated into the gel. With this high MRT value was achieved that was indicating here that the drug was released by sustained release effect and for long duration. The value of MRT was found to be maximum (almost double) in case of nanoparticles loaded gel in comparison to the thiolated chitosan nanoparticles. Moreover, PF127 has the property to inhibit P-glycoprotein efflux pump by reducing the availability of ATP required by the mitochondria. Hence increase in the absorption of drug into the brain was observed. This effect got increased by adding of musk ketone into the gel formulation as it is a fragrance compound and also acts as P-glycoprotein efflux inhibitor. When nanoparticles were incorporated into the gel it showed control release into the brain due to enhanced mucoadhesive behavior. In this case the drug has to cross double barrier i.e. the nanoparticles and gel respectively. On addition of musk ketone into this formulation the P-glycoprotein efflux got inhibited that was proved from the pharmacokinetic results.\cite{9} As the value of MRT got increased in brain and elimination rate got decreased.

Hence in the last it was concluded that the musk ketone with nanoparticles loaded gel was successful in achieving the maximum concentration in the brain and to minimize the efflux from the brain through efflux pump by inhibiting P-gp.

Figure 15 (Plain drug), 16 (CS-TGA NPs loaded with drug), 17 (Drug loaded NPs in gel) and 18 (Drug loaded NPs in gel with musk ketone) showing the comparative release in brain and plasma with respect to time of all formulations.
From the above graphs it was concluded that the drug reaches maximum in the brain as compared to the plasma this may be due to maximum absorption and permeation through the nasal cavity to the brain via thiolated chitosan nanoparticles.

**DISCUSSION**

According to the literature, chitosan nanoparticles can be prepared by several techniques such as the microemulsion method, the ionotropic gelation method, and the solvent emulsification diffusion method. Ionotropic gelation requires a simple laboratory set-up and was used in the
present study by varying the amount of polymer (CS-TGA), cross-linking agent (sodium tripolyphosphate i.e TPP). Ionotropic gelation yielded remarkably high drug entrapment (76 ± 3.1%) and small particle size (222.6 nm).

The shape and surface morphology of the levodopa-loaded thiolated chitosan nanoparticles were visualized by scanning electron microscopy (SEM) and revealed a spherical shape and with a smooth surface (Figure 1). Furthermore, thiolated chitosan nanoparticles were nearly uniform in their size distribution. This may be attributed to the optimum selection of drug-polymer ratio and stirring speed. The polymer solution was not too viscous, thus a high stirring speed (3000rpm) could easily break down the formed droplets. As reported in the literature, a higher concentration of polymer results in more viscous solutions which may resist particle breakdown by stirring and lead to an increase in particle size.[21] A smaller size helps in targeting and increasing the drug’s penetration of biological membranes.[22] The measurement of the zeta potential allows predictions of storage stability of colloidal dispersions. In general, particle aggregation is less likely to occur in cases of high zeta potential due to electric repulsion.[23] The mean zeta potential was found to be +27.9 mV which may be attributed to the positive charges on the polymer’s matrices and surfactant’s mixture provides steric stabilization of the nanoparticles.[24]

The drug entrapment was relatively high in the formulation CNP4 amounting to 76 ± 3.1 %, as compared to the other formulations. Effective entrapment depends on the type of polymer and solubility of the drug in the polymer. Since thiolated chitosan is a hydrophilic polymer and levodopa is also freely soluble in water, more of the drug could be entrapped into the polymer matrices. Apart from the hydrophilicity of the drug and polymer, the concentration of polymer (medium level) was high enough to entrap high amounts of drug, which led to increased entrapment.[21] If the concentration of the polymer is further increased, a decrease in entrapment is observed, which may be due to a higher viscosity of the polymeric solution which hinders diffusion of the drug into the polymer. Despite the optimum concentration of chitosan, the concentration of the cross-linking agent played a major role in entrapment efficiency. Higher concentrations of the cross-linking agent could gelate a higher amount of polymer, thereby increasing the amount of drug entrapped into the nanoparticles.[25]

Upon fitting the in vitro release data into different equations, the optimized formulation followed by Higuchi’s equation. The value of release component ‘n’ obtained using the Korsmeyer-Peppas equation is 0.32 which appears to indicate the anomalous, non- Fickian
diffusion suggesting that the drug release was controlled by more than one process i.e. superposition of both the phenomena, the diffusion-controlled and swelling-controlled.

CONCLUSION
Parkinson is a neurodegenerative disorder in which levodopa is used as a drug of choice.\(^5\) But levodopa therapy for Parkinson treatment needs a novel route so that maximum drug concentration can be achieved in the brain with more MRT in brain also with minimum efflux through P-glycoprotein.\(^3\) Hence this thermosensitive gel loaded with nanoparticles was proved to be the best for Parkinson treatment.\(^7\) As the CS-TGA nanoparticles formed by ionotropic gelation method\(^{10}\) was successful in enhancing permeation, mucoadhesion. Pharmacokinetic data obtained from the study showed that the concentration of thiolated nanoparticles loaded with drug in brain was almost double than plain drug formulation. The pluronic PF127 in-situ gel was made by cold method\(^{14}\) and showed very good thermosensitive properties. The musk ketone that is a fragrance compound added into the thermosensitive gel was proved to be very useful in enhancing the concentration in the brain by increased sensitivity of the nasal cavity along with this it was successful in inhibition in efflux of levodopa through P-glycoprotein efflux pump in the brain.\(^9\) Hence more MRT was achieved by using musk ketone as compared to simple formulation. The optimized final formulation was following the Higuchi model based on \(r^2\) value i.e through diffusion pathway. While the Korsmeyer Pappas model was found to be followed the non-fickian diffusion (\(n < 0.45\)).

So, it could be concluded that the thiolated chitosan nanoparticles have more potential to be used as carrier system for intranasal route. Nanoparticles loaded thermosensitive gel with musk ketone stimulates the nasal cavity and hence increases the Cmax in the brain through intranasal delivery.

EXPERIMENTAL
Levodopa was gifted by Divis laboratory, Musk ketone was gifted by Maschemiejer (Hyderabad), chitosan, pluronic PF127 and thioglycolic acid was purchased from Sigma-Aldrich, EDAC (1-Ethyl-3-(dimethyl amino propyl) Carbodiimide Hydrochloride was purchased from Himedia. All the reagents used for analysis were of HPLC or analytical grade.
Synthesis of chitosan-TGA conjugate

To prepare thiolated chitosan, 500mg of chitosan and 100mg of EDAC was taken in completely dried 100ml of volumetric flask. The chitosan and EDAC mixture was dissolved in 50ml of 1.0% glacial acetic acid with continuous stirring. To the above prepared solution 30ml of thioglycolic acid was added drop wise. The pH of the solution was maintained at 4.5 using 3N NaOH. EDAC was added to facilitate the thiolation process. The reaction was carried out in fuming chamber. The reaction mixture against 5mM HCl over a period of 3 days in dark to remove the free thiol group. The reaction is showing in the figure 1.

![Figure 1: Synthesis of thiolated chitosan from chitosan](image)

Determination of thiol content

The degree of modification was determined by quantifying the thiolated chitosan with DTNB i.e 5, 5’- dithiobis-2-nitrobenzoic acid (Ellman’s agent).

**Working DTNB reagent:** - It was prepared by adding 50µl of the DTNB solution and 100 µl Tris (also known as tromethane) solution into 840 µl deionized water.

**Procedure:** - 10 µl of sample solution was mixed with 990 µl of working DTNB reagent and incubated for 5 min at 37°C. The absorbance values (optical density) of the sample were immediately measured at 412 nm using UV/ VIS-spectrophotometer. Thiol content was then determined by the following formula:

\[
\text{Thiol Content} \left(\frac{\text{Sample}}{}\right) = \text{Total Volume} \times \text{OD at 412 nm} \div 13600
\]

Preparation of levodopa loaded nanoparticles: The nanoparticles were prepared by ionic gelation method (Ionotropic gelation was based on the ability of polyelectrolytes to cross link
in the presence of counter ions to form hydrogel beads also called as gelispheres. Gelispheres were spherical crosslinked hydrophilic polymeric entity capable of extensive gelation and swelling in simulated biological fluids and the release of drug through it controlled by polymer relaxation.\cite{13} 10ml of TH- Ch was taken in a completely dried 50ml beaker. L-DOPA was dissolved in above polymer solution with sonication. While sonicating the above solution, 5ml of sodium tripolyphosphate (dissolved in water) was added drop wise. Sonication was done for 3 min and at amplitude of 70 watts. The resulting mixture contained L-DOPA loaded nanoparticles and kept for refrigeration at 2 to 8°C for further use.

**Preparation and optimization of thermosensitive gel**

Pluronics are known to form thermo reversible gels that have the capacity to make, break, and modify the bonds responsible for holding the network together. This characteristic has allowed PF-127 to be used as a carrier for most routes of administration including oral, topical intranasal, vaginal, rectal, ocular and parenteral routes.\cite{22} Different concentration of Pluronic F-127 was used in the preparation of in-situ gelling liquids. Mediated in-situ gelling liquids were prepared on a mass basis using modified cold method. For preparation of formulation, Pluronic F-127 was mixed and dissolved with 0.8% drug in cold phosphate buffer of pH 5. Benzalkonium chloride, concentration 0.01%, was used as preservative. Partially dissolved pluronic solution were stored in refrigerator at 4°C overnight and stirred periodically until clear homogenous solutions were obtained.\cite{14}

**Characterization of formulation**

**Probe sonication time, Polymer:TPP ratio and Amount of drug**

Particle size of nanoparticles was affected considerably by time length of ultrasonication. To optimize ultrasonication time, varied time length of ultrasonication was applied i.e. 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 minutes. During this process, homogenization time, speed, CS-TGA:TPP ratio were kept constant at 10 min, 2000rpm and 3:1 respectively. Formulations were evaluated on the basis of the size and PDI of CS-TGA nanoparticles.

**CS-TGA: TPP ratio considerably affects the size, PDI and the entrapment efficiency of the particulate systems. To optimize CS-TGA:TPP ratio, ratio was varied as 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 1:2 and 1:3 respectively. During the entire process homogenization time and sonication time were fixed at 10 min and 3.0 min respectively. Formulations were evaluated on the basis of the size and PDI of nanoparticles.**
The effect of the proportional amount of drug substance on the entrapment efficiency was studied by varying the amount of levodopa. The different concentrations of levodopa were taken varied from 40 mg to 90 mg. All parameters like sonication time, ratios were fixed. Also, because of the increased drug concentration inside the nanoparticles, the osmotic pressure difference builds between the outer and inner phases; this make it easier for the drug substance to escape from the inner phase. By keeping all the parameters constant like ratio and sonication time the amount of drug was studied. Further studied for entrapment efficiency.\textsuperscript{[15]}

**Entrapment efficiency of levodopa loaded nanoparticles**

Amount of levodopa entrapped in nanoparticles was estimated by passing the formulation through a sephadex Column. The first 5 ml of formulation was taken and centrifuged at 14,000 rpm for 20 min in refrigerated centrifuge maintained at 4\textdegree{}C. Nanoparticles settled down and the supernatant contained free drug. The supernatant was analyzed in UV Spectrometer at \(\lambda\)max of 280nm.\textsuperscript{[16]}

\[
\text{% Drug Entrapped} = \frac{\text{Total Amount of drug added} - \text{Amount of drug in supernatant}}{\text{Total amount of drug added}} \times 100
\]

Study was done in triplicate and their mean values were reported.

**Gelling time and gelling temperature**

Gelling time may be defined as the interval of time required for a colloidal solution to become a solid or semisolid jelly or gel. Thermoreversible gels are characterized by their ability to gel at a specified temperature. Gelling time must be adequate for the required formulation. To determine the gelling time the colloidal solution was kept at its gelation temperature in water bath and then the time required for the transformation to take place was observed and this corresponds to the gelling temperature.\textsuperscript{[17]} The temperature at which the liquid phase transforms into the gel is determined as the gelling temperature. To carry out the study various concentrations of pluronics were made i.e. 16, 18, 20, 22 and 24 % respectively in water. The temperature at which the colloidal system formed gel when kept in a water bath was noted with thermometer. At gelling temperature system loses its fluidity.\textsuperscript{[17]}

**Mucoadhesive strength**

Nasal tissue was isolated from the rat after cervical dislocation. 1 cm\(^2\) area was isolated and attached to the mucoadhesive force determination apparatus. To the tissue polymeric solution
was applied on the lower side and on the other side weights were applied till the tissue detached from the polymeric material.\[^{17}\] The weight required for the detachment was calculated and mucoadhesive strength was determined according to the formula given below:

\[
\text{Mucoadhesive Strength} = \frac{\text{Force of Adhesion}}{\text{Area}}
\]

Wherein Force of Adhesion = \((m \text{ (grams)} \times g \text{ (acceleration due to gravity)}) / 1000

**Rheological studies**

Rheology is the study of the flow of matter, primarily in the liquid state, but also as ‘soft solids’ or solids under conditions in which they respond with plastic flow rather than deforming elastically in response to an applied force. Rheometers are instruments used to characterize the rheological properties of materials, typically fluids that are melts or solution. These instruments impose a specific stress field or deformation to the fluid, and monitor the resultant deformation or stress. Instruments can be run in steady flow or oscillatory flow, in both shear and extension. Gels generally exhibit non-newtonian flow. The viscosity (resistance to deformation or other forces) of non-Newtonian fluids is dependent on shear rate or shear rate history.\[^{18}\]

**In-Vitro release studies**

*In-vitro* drug release profile of entrapped drug from nanoparticles was studied in saline PBS (pH 6.4) using dialysis bag. Dialysis membrane was exposed to running water for 12 hours to remove glycerin based contents. The sulphur based contents were removed by treating tube with 0.3 % (w/v) sodium sulphite at 70 ºC for 20 min. Dialysis membrane was washed with hot water at 70ºC for 2 min and exposed to a 0.2 % (v/v) solution of sulphuric acid for 5 min. This acidification was followed by treatment with hot water to remove excess acid. Treated membrane was kept in alcohol until used for *in-vitro* drug release studies.\[^{7}\] *In vitro* drug release studies were performed by dialysis bag method using shaking incubator at rotation speed of 100 rpm. saline phosphate buffer (pH 6.4) was used as dissolution medium. Each dialysis bag (pore size: 12 KD, Sigma Chemical Co., USA) was loaded with 2 ml nanoparticles previously passed through the sephadex column G-50. Volume and temperature of dissolution medium were 50 ml, and 37.0 ± 0.2 ºC respectively. At predetermined time interval samples (4 ml) were withdrawn, replaced with same volume of fresh media, filtered and assayed for drug content at 280 nm against blank using UV-Visible spectrophotometer. Mean results of triplicate measurements and standard deviation were reported.
In-Vivo brain homogenate studies

UV method development for brain homogenate studies

To study levodopa in brain homogenate, a modified UV-Vis Spectrophotometer method was developed in our lab[12] briefly, Rat was sacrificed by cervical dislocation and the brain was removed. The brain was washed with phosphate buffer and brain sample was homogenized and immediately centrifuged (5000-10000 rpm) for 10 minutes at an ambient temperature. After centrifugation, supernatant of brain homogenate was transferred into clean, fresh eppendorf tubes. 0.1 ml of organ homogenate was withdrawn and to this 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 ml of different concentrations of levodopa in phosphate buffer were added respectively to obtain concentrations of 10, 20, 30, 40, 50, 60, 70 and 80 mcg/ml. To this 0.1 ml acetonitrile was added and the volume was made upto 10 ml with phosphate buffer at pH 5. The pH is maintained same as that of nasal cavity pH (5.5). The sample was analyzed in UV at λmax of 280 nm. Standard curve was plotted using the absorbance values to be used and standard for successive analysis of sample.[19]

Animal study protocol

Wistar albino rats of either sex (200-250 gm), fasted overnight and for 8 hour after the dosing, were used to study pharmacokinetics of various formulation of levodopa i.e levodopa in saline, levodopa in thiolated nanoparticles, levodopa nanoparticles in gel and levodopa nanoparticles in gel with musk ketone after intranasal administration. Each rat was anaesthized with ether and sacrificed, decapitated and the whole brain was eased out of the skull. Drug-free (i.e. blank) brain tissue and drug containing brain tissue were obtained from the control rats and from formulation induced rats. After removal of brain, it was rinsed with cold saline (0.9 % NaCl, g/ml) then surface vasculature ruptured, blotted with dry gauze and weight was taken. The whole brain is homogenized within 1 hour of collection in phosphate buffer (pH 6.8 M) (3 ml per rat brain) with a hand-held glassteflon homogenizer in an ice-cold bath. To induce precipitation of protein, 30 μl of acetonitrile was subsequently added to each brain homogenate and the organic layer (upper layer) was separated by centrifugation and the supernatants was directly used for analysis.[19]

Same procedure is followed for plasma studies by anaesthizing rats with ether and plasma (Retro) was collected from the eyes of the rats. This plasma was further taken into eppindrof’s and then homogenized for 10 min at 4°C temperature.
Statistics: Statistical analysis was performed using GraphPad Prism version 5.01 for Windows (Graph Pad Software, San Diego CA). ANOVA was used with bonferroni posttests to compare the data groups with control.

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Authors’ Statement

Competing Interests
- No conflict of interest.

Informed Consent, Ethical Approvals
The experiments were conducted with as per CPCSEA (Committee for Prevention, Control and Supervision of Experimental Animals, approval No. ISF/CPCSEA/IEAC/2012/31) guidelines. The animals were procured from animal house of ISF College of Pharmacy, Moga, India and were housed under standard laboratory conditions with free access to food and water.

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