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4. RESULTS AND DISCUSSION OF ETOPOSIDE NANOFORMULATIONS

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4. RESULTS AND DISCUSSION OF ETOPOSIDE NANOFORMULATIONS

4.1. Preformulation Studies

4.1.1. Solubility studies

The solubility profile of etoposide in water and at different pH conditions (ranging from pH 1.3 to 7.3) was thoroughly investigated. Table 4.1 shows the solubility profile of etoposide in water and different pH conditions. The solubility of etoposide is lowest in water (112.46 μg/mL). As evident from HPLC chromatograms etoposide has undergone extensive degradation at pH 1.3 which precluded the measurement of solubility.

Table 4.1. Solubility of etoposide in water and at different pH conditions

<table>
<thead>
<tr>
<th>Water/ Buffer (pH)</th>
<th>Solubility (μg/mL) Mean±SD&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled Water</td>
<td>112.46±9.36</td>
</tr>
<tr>
<td>0.1 M HCL (1.3)</td>
<td>Extensive degradation of etoposide was observed</td>
</tr>
<tr>
<td>0.1 M Na citrate/citric acid (3.0)</td>
<td>154.38±12.25</td>
</tr>
<tr>
<td>0.1M sodium acetate/ acetic acid (5.0)</td>
<td>142.29±13.41</td>
</tr>
<tr>
<td>0.1M KHPO&lt;sub&gt;4&lt;/sub&gt;/KH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt; (pH 7.4)</td>
<td>118.46±11.84</td>
</tr>
</tbody>
</table>

<sup>a</sup>n=3, 24-h data.

The aqueous solubility of etoposide at 37 °C was 112.46-154.38 μg/mL which was low, and did not vary much over the pH range 3.0-5.0. The drug exhibited a solubility of 154.38 μg/mL, 142.29 μg/mL and 118.46 μg/mL at pH 3.0, 5.0 and 7.4 respectively. Therefore low aqueous solubility and rapid degradation at pH 1.3 may be the reasons for the reported low oral bioavailability of etoposide.

4.1.2. Drug-excipient compatibility studies

The aim of the drug-excipient compatibility studies is to check for any possible interaction between etoposide and other formulation components such as polymers, stabilizer, and cryoprotectant used in the development of etoposide nanoformulations. The mixtures of drug and individual excipients were prepared in equal proportions and were stored in stability chamber at 25±2 °C / 60±5 % RH for four week time period (Suriyaprakash et al., 2011).
After the study period the drug-excipient mixtures were assessed for the changes in physical attributes as well as for any possible chemical interactions using FTIR and DSC studies. The pure etoposide did not show any change in the physical attributes. Similarly etoposide in the presence of other excipients in equal proportions apparently did not show any characteristic change in morphological characteristics. Therefore, the excipients in formulation were found to be compatible with drug substance.

4.1.2.1. Fourier Transform Infra Red (FTIR) spectral studies

Compatibility of excipients with the pure drug was evaluated by analyzing the FTIR spectra of physical mixtures (Figure 4.1). FTIR spectrum of etoposide exhibited a broad absorption band at 3450 cm\(^{-1}\) which corresponds to O-H stretching frequency of secondary alcoholic hydroxyl groups. The presence of carbonyl group is evident from characteristic absorption band at 1764.93 cm\(^{-1}\). Characteristic peaks at 1074.39 and 1030.02 cm\(^{-1}\) corresponds to Ar-OCH\(_3\) and ArO-CH\(_3\) stretching vibrations. IR spectrum of physical mixture of etoposide and excipients in customized proportions showed all the characteristic peaks of the drug without any appreciable shift which indicates that there is no significant interaction between the drug and the excipients. Physical mixture of the drug with excipients such as PCL, PLGA 50:50 PVA, pluronic F-68 and mannitol in equal ratios exhibited peaks corresponding to the drug at 3400-3500 cm\(^{-1}\) (-O-H str), 1735-1753 cm\(^{-1}\) (>C=O str, lactone) and 1080-1082 cm\(^{-1}\) & 1020-1039 cm\(^{-1}\) which corresponds to Ar-OCH\(_3\) (str) and ArO-CH\(_3\) (str) respectively.
Figure 4.1. IR Spectrum of physical mixture of etoposide and excipients with PCL in equal proportions: a) Etoposide b) EPCLP-mixture of etoposide, PCL and PVA c) EPLGAP-mixture of etoposide, PLGA 50:50 and PVA d) EPLGAF-etoposide, PLGA 50:50 and pluronic F-68.
4.1.2.2. Thermogram properties

DSC thermograms of free etoposide (ET) and excipients such as PCL polymer (PC), PLGA 50:50 (PLGA), PVA (PV) or pluronic F-68 and mannitol (MN) were analysed to detect the occurrence of interactions, if any, between the drug and excipients (Figure 4.2). DSC thermogram of pure etoposide exhibited endothermic thermal transitions at 80-115 °C, 180 °C and 295 °C. The endotherm over the range of 80-115 °C was attributed to dehydration of etoposide. The endotherm at 180 °C is due to the melting of anhydrous form of etoposide, which soon begins to crystallize to a different polymorphic form. An exothermic peak was observed at 210 °C which corresponds to the crystallization temperature (Tc) of etoposide polymorphic form. The third endotherm at 295 °C was attributed to the melting point of the newly formed etoposide (Jasti et al., 1995). DSC thermogram of pure PCL polymer only showed an endothermic peak at 65.5 °C which corresponds to its melting temperature (Tm) while PLGA 50:50 showed glass transition temperature (Tg) at 50.5 °C. Thermogram of PVA showed glass transition temperature (Tg) at 100 °C and the melting endotherm was observed at 191.5 °C, while pluronic F-68 exhibited endothermic peak at 55.5 °C which corresponds to its melting temperature (Tm). The DSC thermogram of mannitol exhibited an endothermic peak at 171.5 °C which corresponds to its melting temperature (Tm).

Physical mixture of pure etoposide along with these excipients did not show any significant shift in peaks compared to that of pure drug and pure excipients. The thermogram of EPCLP showed endothermic peaks at 63.84 °C, which is the slight preshifted peak corresponds to melting endotherm (Tm) of PCL. Another endothermic peak was observed at 167.5 °C which was preshifted endothermic melting peak of mannitol. A broad endothermic peak at 295 °C corresponds to the endothermic peaks of etoposide. However crystallization temperature exotherm at 210 °C was disappearing which may be due to the loss in crystallinity of the drug in the presence of excipients. Similarly other physical mixtures like EPLGAP (Figure 4.2c) and EPLGAF (Figure 4.2d) also showed broad endothermic peaks at 295 °C corresponding to the melting endotherm of etoposide. The results indicated no significant interactions between the pure drug and excipients used in the formulation confirming the compatibility between etoposide and formulation components.
Figure 4.2. DSC thermogram of physical mixture of etoposide and excipients with PCL in equal proportions: a) Etoposide b) EPCLP-mixture of etoposide, PCL and PVA c) EPLGAP-mixture of etoposide, PLGA50:50 and PVA d) EPLGAF-etoposide, PLGA 50:50 and pluronic F-68.
4.1.3. Analytical method development and validation of reverse phase HPLC method for estimation of etoposide

4.1.3.1. Analytical method development

Effect of ratio of mobile phase

The effect of ratio of mobile phase, Methanol and Water were studied at 50:50, 60:40 and 70:30 (% v/v). The retention time of etoposide was found to be 8.8, 5.4 and 3.1 min respectively with 50:50, 60:40 and 70:30 (Methanol: Water % v/v).

Effect of flow rate

Flow rate of 1 mL/min was selected as there is no noticeable changes in the retention time as well as good peaks were obtained with the all the different flow rates.

Based on the above optimization parameters, the following chromatographic conditions were selected for the estimation of etoposide by HPLC method consists of:

- Stationary phase: Hypersil C18 ODS column (250 x 4.6 mm, 5μm)
- Mobile phase: Methanol: Water (60:40)
- Run time: Isocratic run for 10 min
- Detection wavelength: 286 nm
- Flow rate: 1 mL/min
- Injection volume: 20 μL
- Temperature: Ambient (around 25 °C)

With the above separation condition, the retention time for etoposide was found to be 5.4 min. The typical standard chromatogram of etoposide is showed in the Fig. 4.3.

Figure 4.3. A representative chromatogram of etoposide
4.1.3.2. Method validation

**Linearity**

The coefficient determination \((r^2)\) for the present method was 0.9994 which indicated that the present method is linear and it is linear in the range from 0.05–50 μg/mL \((y = 11579x + 9122.5)\). Acceptance criteria for linearity, \((r^2)\) is >0.999. Calibration curve is shown in the Figure 4.4.

**Accuracy**

The recoveries at three different levels (80, 100, 120 %) were found to be within the range of 98 to 102 % as per ICH guidelines (Guideline, 2005). Mean % recovery (Mean±SD) was found to be 98.97±0.89, shown in Table 4.2.

**Table 4.2. The recoveries at three different levels (Accuracy)**

<table>
<thead>
<tr>
<th>Accuracy level</th>
<th>Amount added (mg)</th>
<th>Amount recovered (mg)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>80%-1</td>
<td>8.02</td>
<td>7.91</td>
<td>98.63</td>
</tr>
<tr>
<td>80%-2</td>
<td>8.00</td>
<td>7.95</td>
<td>99.38</td>
</tr>
<tr>
<td>80%-3</td>
<td>8.06</td>
<td>7.87</td>
<td>97.64</td>
</tr>
<tr>
<td>100%-1</td>
<td>10.05</td>
<td>10.06</td>
<td>100.10</td>
</tr>
<tr>
<td>100%-2</td>
<td>10.01</td>
<td>9.83</td>
<td>98.20</td>
</tr>
<tr>
<td>100%-3</td>
<td>10.02</td>
<td>9.85</td>
<td>98.30</td>
</tr>
<tr>
<td>120%-1</td>
<td>12.04</td>
<td>12.01</td>
<td>99.75</td>
</tr>
<tr>
<td>120%-2</td>
<td>12.05</td>
<td>11.89</td>
<td>98.67</td>
</tr>
<tr>
<td>120%-3</td>
<td>12.06</td>
<td>12.07</td>
<td>100.08</td>
</tr>
</tbody>
</table>

Mean±SD 98.97±0.89

**Precision**

**Repeatability**

The repeatability of the proposed method was determined from the percentage relative standard deviation of six determinations at 100 % of test concentration. The % RSD was found to be 0.645% which is within the acceptance criteria for the repeatability (<1% RSD).
Intermediate precision

The intermediate precision of the proposed method was determined from the overall percentage relative standard deviation of six determinations at 100 % of test concentration on different days by two different analysts. The overall % RSD was found to be 1.31 % which is within the acceptance criteria for the intermediate precision (<2 % RSD).

Specificity

There was no interaction observed between drug and excipients present in the formulation, which has confirmed the specificity of the method.

Robustness

Robustness of the method was evaluated under following conditions

Set-1: Normal conditions: Wavelength 286 nm; flow rate 1.0 mL/min, Mobile phase composition 60:40.
Set-2 Sample (Mobile phase 50:50)
Set-3 Sample (Mobile phase 70:30)
Set-4 Sample (flow rate 0.9 mL/min)
Set-5 Sample (flow rate 1.1 mL/min)
Set-6 Sample (detection wavelength 291 nm)
Set-7 Sample (detection wavelength 281 nm)

The % RSD and overall %RSD for each set is shown in Table 4.3.

Table 4.3. The % RSD and overall % RSD for each set (Robustness)

<table>
<thead>
<tr>
<th>Conditions</th>
<th>% RSD</th>
<th>Overall % RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set-1</td>
<td>1.32</td>
<td>-</td>
</tr>
<tr>
<td>Set-2</td>
<td>1.56</td>
<td>1.62</td>
</tr>
<tr>
<td>Set-3</td>
<td>1.11</td>
<td>1.31</td>
</tr>
<tr>
<td>Set-4</td>
<td>0.91</td>
<td>1.29</td>
</tr>
<tr>
<td>Set-5</td>
<td>1.21</td>
<td>1.45</td>
</tr>
<tr>
<td>Set-6</td>
<td>1.05</td>
<td>1.26</td>
</tr>
<tr>
<td>Set-7</td>
<td>0.97</td>
<td>1.74</td>
</tr>
</tbody>
</table>

The overall percentage relative standard deviation in the various parameters was found to be less than 2 %. Thus the result indicated that the method was robust.
LOD and LOQ

LOD and LOQ of the present method was found to be 0.015 and 0.05 μg/mL respectively. The percentage RSD for six injections of the LOQ solution was 1.71%.

Summary of the analytical method validation parameters are reported in the Table 4.4.

Table 4.4. Data of analytical method validation of etoposide by HPLC

<table>
<thead>
<tr>
<th>Validation Parameters</th>
<th>Validation results</th>
<th>Acceptance criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity ($r^2$) (0.05 – 50 μg/mL)</td>
<td>0.9994</td>
<td>&gt; 0.999</td>
</tr>
<tr>
<td>Accuracy (% Recovery Mean±SD)</td>
<td>98.97±0.89</td>
<td>98-102 %</td>
</tr>
<tr>
<td>Robustness (% RSD)</td>
<td>&lt; 2.0</td>
<td>&lt; 2.0</td>
</tr>
<tr>
<td>Repeatability (% RSD)</td>
<td>0.645</td>
<td>&lt; 1.0</td>
</tr>
<tr>
<td>Intermediate precision (% RSD)</td>
<td>1.31</td>
<td>&lt; 2.0</td>
</tr>
<tr>
<td>LOD (μg/mL)</td>
<td>0.015</td>
<td>S/N ratio should be 3:1</td>
</tr>
<tr>
<td>LOQ (μg/mL)</td>
<td>0.05</td>
<td>S/N ratio should be 10:1</td>
</tr>
</tbody>
</table>

Figure 4.4. Calibration plot of etoposide by HPLC method
### 4.1.4. Optimized chromatographic conditions of reverse phase HPLC bioanalytical method for estimation of etoposide

Analyses were carried out at ambient temperature on a Hypersil C\textsubscript{18} ODS column (250 x 4.6 mm, 5 µm) coupled with a Phenomenex C\textsubscript{18} (4.0 x 3.0 mm, 5 µm) guard column. The optimal mobile phase was a mixture of methanol, acetonitrile and monopotassium phosphate (0.020 M) (18:19:63, v/v/v) containing 0.007 % triethylamine (TEA) that was adjusted to pH 5.2 with phosphoric acid (5 %, v/v). The mobile phase was filtered using a Millipore filter (0.45 µM), degassed for 30 min. The injection volume was 100 µL and delivered at a flow rate of 1.0 mL/min. Lamotrigine was used as the internal standard and the detection wavelength was set at 285 nm (Saadati \textit{et al.}, 2011). The typical standard chromatograms obtained for etoposide in rat plasma, brain tissue homogenate and CSF is showed in the Figures 4.5a, 4.5b & 4.5c.

#### Specificity

No interference was observed in blank samples using three independent sources of the rat plasma, CSF and tissue homogenates. Blank samples for specificity testing were prepared in the same way as the study samples.

#### Linearity

The calibration curves for plasma, CSF and brain tissue homogenates were linear in the range of the assay, and an excellent correlation coefficient was consistently found over different days. The correlation coefficients ($r^2$) of calibration curves for plasma, CSF and brain tissue homogenates were found to be 0.9981, 0.9979 and 0.9964 respectively. Calibration curves for etoposide estimation in plasma, CSF and brain tissue homogenate is shown in the Figures 4.6a, 4.6b & 4.6c.

#### Extraction Recovery

The recoveries of etoposide from QC biological samples were compared to the directly injected analytes at the same concentrations. Recovery values for etoposide in plasma, CSF and brain tissue homogenate ranged from 90.025–94.427 %.
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Figure 4.5a. The typical standard chromatogram obtained for etoposide in rat plasma

Figure 4.5b. The typical standard chromatogram obtained for etoposide in rat brain

Figure 4.5c. The typical standard chromatogram obtained for etoposide in rat CSF
**Results & Discussion**

**Figure 4.6a.** Calibration plot of etoposide in rat plasma by HPLC method

**Figure 4.6b.** Calibration plot of etoposide in CSF by HPLC method

**Figure 4.6c.** Calibration plot of etoposide in brain tissue homogenate by HPLC method
4.2. Development of Etoposide Nanoformulations

4.2.1. Formulation development and optimization of etoposide-PCL nanoparticles by emulsification solvent evaporation technique

4.2.1.1. Formulation development

In order to optimize the encapsulation efficiency and particle size of etoposide loaded PCL nanoparticles (EB), various batches were formulated varying the experimental conditions. Different methods like emulsification solvent evaporation and nanoprecipitation techniques were studied for preparation of etoposide loaded PCL nanoparticles. Among these methods, better particle size and encapsulation efficiency were obtained with emulsification solvent evaporation technique. Therefore, this method was used for preparation of etoposide PCL nanoparticles. Further this method is well reported for its application in encapsulating hydrophobic drug. Acetone was selected as the solvent for etoposide and PCL was dissolved in dichloromethane based on the results of preliminary studies. Selection was based on the solubility studies of drug and polymer in various organic solvents as well as based on the observation that these solvents gave maximum encapsulation efficiency and minimum particle size. The nanoparticles prepared using optimized parameters had a percentage encapsulation efficiency of 73.07±3.11 % and particle size 518.0±10.2 nm, with a polydispersity index and zeta potential of 0.260 ±0.018 and -20.6±2.1 mV respectively. The particle size, PDI, zeta potential and percentage encapsulation efficiency results of all batches are presented in Table 4.5.

4.2.1.1.1. Entrapment efficiency (EE %)

Various batches were prepared using different ratios of drug to polymer in order to accomplish optimum loading of drug in the polymer. Drug to polymer ratio of 1:2.5 showed an optimum encapsulation efficiency of 73.07±3.11 %. The EE % of all designed batches of etoposide PCL nanoparticles are reported in Table 4.5. An increase in entrapment efficiency and decrease in particle size were observed as the drug: polymer ratio increased from 1:1 to 1:10. Nevertheless further increase in drug: polymer ratio resulted in a decrease in the drug entrapment and increase in the particle size of the nanoparticles. Increase in encapsulation efficiency with increase in polymer concentration is a well reported phenomenon and this is because with high polymer concentration, drug loss can be minimised (Hoffart et al., 2002; Galindo-Rodriguez et al., 2004; Averineni et al., 2012).
4.2.1.2. Particle size, polydispersity index and zeta potential

Average particle size, polydispersity index and zeta potential of all batches of EBs are reported in Table 4.5. The polydispersity index (PDI) being a measure of the extent of particle size distribution, was determined to confirm the narrowness of the particle size distribution. Particle size distribution of optimized batch EB-4 is depicted in Figure 4.7. The PDI value for EB-4 was found to be 0.260±0.018. Particle size and size distribution pattern of particles has an important role in determining the efficacy of developed formulation. These are valuable parameters which influence the drug release behaviour and their possibility for intranasal administration. Smaller size of the particles enables them to amass at the tumour sites whose uptake into the tumour cells are brought about by the enhanced permeation and retention (EPR) property of the tumour vasculature. In addition, particles smaller than 1 μm are capable of passing through the nasal cavities along with the inspired air, which reveals the possibility of a route to deliver nano sized particles (Illum et al., 1987). The zeta potential for optimized formulation EB-4 was -20.6±2.1 mV, which could be due to the negative charge of the PCL (Figure 4.8). Zeta potential values in the range -15 to -30 mV are common for stabilized nanoparticles (Musumeci et al., 2006). High negative values of the zeta potential are found to be ideal as aggregation of particles are prevented due to the electrostatic repulsion between them thus yielding a stable nanoparticulate dispersion (Feng et al., 2001). The optimized formulation (EB-4) with desirable properties was used for the further studies.

![Size Distribution by Intensity](image)

**Figure 4.7.** Particle size distribution of etoposide PCL nanoparticles (EB-4)
Results & Discussion

Figure 4.8. Zeta potential profile of etoposide PCL nanoparticles (EB-4)

Table 4.5: Formulation of etoposide PCL nanoparticles

<table>
<thead>
<tr>
<th>Batch</th>
<th>Drug: Polymer ratio</th>
<th>Stabilizer</th>
<th>Average particle size $^a$ (nm)</th>
<th>Zeta Potential $^a$ (mV)</th>
<th>PDI $^a$</th>
<th>% Encapsulation Efficiency $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EB-1</td>
<td>1:1</td>
<td>PVA</td>
<td>1012.2±20.12</td>
<td>-17.5±3.4</td>
<td>0.675±0.115</td>
<td>12.87±2.37</td>
</tr>
<tr>
<td>EB-2</td>
<td>1:2.5</td>
<td>PVA</td>
<td>889.1±13.16</td>
<td>-13.5±2.9</td>
<td>0.484±0.102</td>
<td>26.8±5.79</td>
</tr>
<tr>
<td>EB-3</td>
<td>1:5.0</td>
<td>PVA</td>
<td>756.5±11.24</td>
<td>-19.7±3.5</td>
<td>0.389±0.053</td>
<td>68.36±8.34</td>
</tr>
<tr>
<td>EB-4</td>
<td>1:10</td>
<td>PVA</td>
<td>518.0±10.02</td>
<td>-20.6±2.1</td>
<td>0.260±0.018</td>
<td>73.07±3.11</td>
</tr>
<tr>
<td>EB-5</td>
<td>1:15</td>
<td>PVA</td>
<td>712.1±16.56</td>
<td>-22.7±1.8</td>
<td>0.295±0.039</td>
<td>68.68±7.58</td>
</tr>
<tr>
<td>EB-6</td>
<td>1:20</td>
<td>PVA</td>
<td>788.4±14.39</td>
<td>-25.6±5.1</td>
<td>0.352±0.085</td>
<td>60.19±4.32</td>
</tr>
<tr>
<td>EB-7</td>
<td>1:30</td>
<td>PVA</td>
<td>1134.3±21.47</td>
<td>-23.7±4.4</td>
<td>0.785±0.108</td>
<td>58.65±5.55</td>
</tr>
<tr>
<td>EB-8</td>
<td>1:40</td>
<td>PVA</td>
<td>1065.9±19.28</td>
<td>-15.5±3.2</td>
<td>0.693±0.111</td>
<td>53.46±7.25</td>
</tr>
<tr>
<td>EB-9</td>
<td>1:10</td>
<td>F-68</td>
<td>775.6±12.75</td>
<td>-24.5±2.7</td>
<td>0.425±0.029</td>
<td>56.43±6.42</td>
</tr>
<tr>
<td>EB-10</td>
<td>1:10</td>
<td>F-127</td>
<td>794.8±10.98</td>
<td>-19.7±3.3</td>
<td>0.381±0.062</td>
<td>47.32±2.67</td>
</tr>
<tr>
<td>EB-11</td>
<td>1:10</td>
<td>SC</td>
<td>634.5±11.35</td>
<td>-26.9±4.8</td>
<td>0.286±0.053</td>
<td>54.71±5.85</td>
</tr>
</tbody>
</table>

$^a$These results are mean±standard deviation. (n=3).
4.2.1.1. 3. Effect of Drug: Polymer ratio on encapsulation efficiency and particle size

The effect drug: polymer ratio on encapsulation efficiency and particle size was studied at different drug: polymer ratios like 1:1, 1:2.5, 1:5, 1:10, 1:15, 1:20, 1:30 and 1:40. The results are shown in table 4.5 and figure 4.9 & 4.10. An increase in entrapment efficiency and decrease in particle size was observed when the drug: polymer ratio was increased from 1:1 to 1:10. Nevertheless, further increase in drug: polymer ratio resulted in a decrease in the drug entrapment and increase in the particle size of the nanoparticles (Budhian et al., 2007; Shah et al., 2009). The viscosity of organic phase was increased due to an increase in the concentration of the polymer which prompted the rapid diffusion of organic phase into the aqueous phase. Higher concentrations of polymer yielded coarse dispersions with higher particle size. This may be due to the lack of sufficient amount of stabilizer in the aqueous phase to match the amount of polymer.

**Figure 4.9.** Effect of drug: polymer ratio on encapsulation efficiency of etoposide PCL nanoparticles

**Figure 4.10.** Effect of drug: polymer ratio on particle size of etoposide PCL nanoparticles
4.2.1.4. Effect of stabilizers on encapsulation efficiency and particle size

Stabilizers are used in formulation of nanoparticles to provide stability of the dispersion in aqueous medium (Mainardes et al., 2005). Various surfactants like PVA, pluronic F-68, pluronic F-127 and sodium cholate (SC) were tried to study the influence of type of surfactant on physicochemical properties of etoposide nanoparticles. The results are shown in Table 4.5. PVA was selected as the stabilizer as it gave lower particle size and maximum encapsulation efficiency without particle aggregation. Various concentrations of PVA ranging between 0.25% w/v and 1.5% w/v were tested. At a higher concentration (1.5% w/v) of PVA, there was an increase in particle size and decrease in entrapment efficiency. This could be due to more solubility of the drug in aqueous medium (Joshi et al., 2010). An optimum concentration (1.0 % w/v) of stabilizer yielded smaller sized particles, but insufficient amount of stabilizer caused particle aggregation as the amount of stabilizer was not appropriate to stabilize the nanoparticles by covering the dispersed nanoparticles completely. (Feng et al., 2001). Hence, 1.0 % w/v PVA concentration was considered optimum.

4.2.1.5. Effect of homogenization speed and sonication amplitude

High speed homogenization technique was employed to obtain etoposide PCL nanoparticles. To optimize the homogenization conditions, homogenization was carried out at four different speed levels (5000, 10000, 150000, 20000 rpm) by keeping other formulation parameters constant. Decrease in particle size was observed with increase in homogenization speed and sonication amplitude. However increase in homogenization speed above 20000 rpm and sonication amplitude above 80 % did not show any decrease in particle size. It is well reported that higher homogenization speed can cause particle agglomeration due to high energy build up (Nafee et al., 2007).

4.2.1.6. Effect of cryoprotectants during freeze drying process of etoposide PCL nanoparticles

Various cryoprotectants such as sucrose, fructose, dextrose and mannitol with suitable concentration were tested for their effect on freeze drying process of etoposide polymeric nanoparticles. Among these, mannitol yielded completely dry and free flowing powders with lowest particle size. The concentration of mannitol was varied at different levels such as 2.5, 5 and 10 % w/v to study their influence on physicochemical properties of nanoparticles. 2.5 % w/v mannitol produced a particle size of 693 nm with slight broad range of particles. However
lower particle size was observed with 5 and 10 % w/v mannitol displaying a particle size distribution in the range of 515-585 nm. Therefore 5 % w/v mannitol was found to be sufficient to obtain nanoparticles of desirable properties.

### 4.2.1.2. Characterization of optimized etoposide PCL nanoparticles

#### 4.2.1.2.1. Fourier Transform Infrared spectroscopic studies

FTIR spectrum of etoposide loaded PCL nanoparticle (EB-4) (Figure 4.11) was analysed to prove the encapsulation of etoposide in nanoparticles and to detect the occurrence of interactions, if any, between the drug and excipients in the nanoparticles. The presence of characteristic peaks of pure drug etoposide (Figure 4.1a) in etoposide loaded PCL nanoparticle (EB-4) can be confirmed by the typical bands at 3437.15 cm\(^{-1}\) (-O-H str), 2945.30, 2866.22 cm\(^{-1}\) (aliphatic -C-H str), 1730.15 cm\(^{-1}\) (>C=O, lactone) and 1045.42 & 1107.14 cm\(^{-1}\) (Ar-OCH\(_3\) & ArO-CH\(_3\)). Thus FTIR spectrum of etoposide loaded PCL nanoparticles (EB-4) showed all the characteristic peaks of the drug without any appreciable shift which confirms the encapsulation of drug and also that there is no significant interaction between the drug and the excipients during the formation of nanoparticles.

![FTIR spectrum of EB-4](image)

**Figure 4.11:** FTIR spectrum of EB-4

#### 4.2.1.2.2. Thermogram properties

DSC thermograms of PCL polymer (PC), free etoposide (ET), PVA (PV), mannitol, (MN) and etoposide loaded PCL nanoparticle (EB-4) were analyzed to detect the occurrence of
Results & Discussion

interactions, if any, between the drug and excipients in the nanoparticles (Figure 4.12). DSC thermogram of pure etoposide exhibited endothermic thermal transitions at 80-115 °C, 180 °C and 295 °C. The endotherm over the range of 80-115 °C was attributed to a dehydration of etoposide. The endotherm at 180 °C was due to the melting of anhydrous form of etoposide, which soon begins to crystallize to a different polymorphic form. An exothermic peak was observed at 210 °C which corresponds to the crystallization temperature (Tc) of etoposide polymorphic form. The third endotherm at 295 °C was attributed to the melting point of the newly formed etoposide (Bhaskara RJ et al., 1995). DSC thermogram of pure polymer PCL only showed an endothermic peak at 65.5 °C which corresponds to its melting temperature (Tm), while PVA showed glass transition temperature (Tg) at 100 °C and the melting endotherm was observed at 191.5 °C. The DSC thermogram of mannitol exhibited an endothermic peak at 171.5 °C which corresponds to its melting temperature (Tm). The thermograms of etoposide loaded PCL nanoparticles exhibited endotherms at 63.84 °C and 171.53 °C which corresponds to melting endotherms of PCL polymer and mannitol respectively. This thermogram did not show any detectable endotherm corresponding to the melting temperatures of free drugs between 200–300 °C. This is due to the conversion of drug from crystalline to amorphous form. Thus it is understood that the etoposide in PCL nanoparticles existed in amorphous form of a molecular dispersion or solid solution state in the polymer matrix (Jain et al., 2011). This can lead to increased solubility and finally to an improved biological activity (Dhanaraju et al., 2010).

Figure 4.12. DSC thermogram of etoposide (ET), PCL polymer (PC), PVA (PV), mannitol (MN), and EB-4 (etoposide loaded PCL nanoparticles).
4.2.1.2.3. Scanning electron microscopy (SEM)

The SEM photomicrograph of optimized etoposide PCL nanoparticles were obtained before and after gold sputtering is shown in the Figure 4.13a & 4.13b. The SEM photomicrograph confirmed their spherical shape. The particles exhibited moderate uniformity and all the particles were discrete entities without any aggregation. They were homogeneous with smooth surface and without any rupture.

Figure 4.13a. Scanning electron microscopy of etoposide loaded PCL nanoparticles after gold sputtering (EB-4).

Figure 4.13b. Scanning electron microscopy of etoposide loaded PCL nanoparticles without gold sputtering (EB-4).
4.2.1.3. Evaluation of optimized etoposide PCL nanoparticles

4.2.1.3.1. In vitro release study

In vitro release study was performed on all prepared batches but results are reported only for EB-3, EB-4 and EB-5 (Figure 4.14). The in vitro release profiles for all the formulations EB-3, EB-4 and EB-5 showed biphasic behaviour consisting of initial burst release followed by a sustained release phase for the period of 72 h. The initial burst release of drugs may be because of the release of etoposide loosely bound on the surface of the nanoparticles. Sustained release may be due to slow diffusion of the drug from the highly lipophilic polymeric matrix. The release of entrapped etoposide from nanoparticles with lower concentration of polymer was more rapid than those with higher polymer concentration. This could be due to the fact that the polymer matrix formed was thick and less porous when the polymer concentration was high.

To study the drug release kinetics the data obtained from in vitro drug release studies of optimized batch EB-4 was fitted to the different models like zero-order, first-order and Higuchi’s model. The regression coefficient (R²) of zero-order, first-order and Higuchi’s model was found to be 0.9384, 0.9709 and 0.9976, respectively. Thus, in vitro drug release of EB-4 formulation was best explained by Higuchi kinetic diffusion control mechanism, as the plot showed the highest linearity (Jain et al., 2009).

To study the mechanism of drug release, data obtained from in vitro drug release studies were fitted to the Korsmeyer–Peppas model. The regression coefficient for the plot of log cumulative percentage drug release vs log time of the Korsmeyer–Peppas equation for etoposide PCL nanoparticle formulation EB-4 was found to be 0.9893. The release exponent (n) value was found to be 0.5089. As the n value is >0.5, it can be concluded that the release of etoposide from PCL nanoparticle was by non-Fickian diffusion (diffusion coupled with erosion) (Peppas, 1984; Vaghani et al., 2010).
Figure 4.14. *In vitro* % cumulative drug release profile of etoposide PCL nanoparticle formulations.

4.2.1.3.2. *Ex vivo* permeation studies of etoposide PCL nanoparticles through sheep nasal mucosa

The drug permeation pattern through sheep nasal mucosa reflected the release pattern as shown for *in vitro* release studies, but the amount of drug permeated was lower when compared to that in release studies. The permeation of etoposide from nanoparticles through nasal mucosa was found to be low when compared with that through dialysis membrane. This resistance to the penetration of drug molecules could be attributed to the complexity of the composition of the mucosa during the diffusion process (Tas et al., 2004). Among the three formulations (EB-3, EB-4 & EB-5) selected for the study, formulation EB-4 exhibited better permeation across the sheep nasal mucosa. The cumulative percentage of etoposide permeated from EB-4 after 8 h was found to be 16.421±1.61 %, after triplicate determinations. Permeation profile is shown in Figure 4.15.

**Histopathological studies**

Histopathological studies were carried out to examine the histological changes in nasal mucosa caused by etoposide PCL nanoparticles. The cross section of the sheep nasal mucosa used for permeation before (untreated control, Figure 4.16a) and after the permeation (treated)
was stained by hematoxylin–eosin (HE) and observed under light microscope. Cross section of control nasal mucosa showed ciliated respiratory epithelium and normal goblet cells (Mahajan et al., 2012). The effect of etoposide PCL nanoparticles (EB-4) on sheep nasal mucosa, 8 h after permeation studies (treated, Figure 4.16) showed no severe damage on the integrity of nasal mucosa when compared to untreated control. Only a mild epithelial disruption of nasal mucosa was observed.

**Figure 4.15.** Percentage cumulative drug permeated from etoposide PCL nanoformulations through nasal mucosa

**Figure 4.16.** Histopathology of sheep nasal mucosa. a) Nasal mucosa of untreated control (20X) b) Nasal mucosa permeated with EB-4 (20X).
4.2.1.3.3 Nasal absorption studies of etoposide PCL nanoparticles by in situ nasal perfusion in male Wistar rats

Nasal absorption of optimized etoposide loaded nanoparticle formulation (EB-4) and commercial etoposide formulation (FY) were studied by in situ nasal perfusion in male Wistar rats. Both the commercial formulation and nanoparticle formulation of etoposide showed progressive nasal absorption with time. A plot of percentage drug remaining in the perfusing solution versus time is shown in Figure 4.17. Etoposide loaded PCL nanoparticles exhibited better nasal absorption compared to the commercial etoposide formulation (FY). The first order nasal absorption rate constants for commercial etoposide formulation (FY) and optimized etoposide loaded nanoparticle formulation (EB-4) were calculated from the slopes of first-order plots of the percentage drug remaining in the perfusing solution versus time. The first order nasal absorption rate constants for FY and EB-4 were found to be $3.455 \times 10^{-3}$ and $4.606 \times 10^{-3}$ min$^{-1}$ respectively (Shinichiro et al., 1981; Huang et al., 1985). There is a statistically significant ($p < 0.05$) increase in first order nasal absorption rate constant value for optimized etoposide PCL nanoparticle (EB-4) when compared to that of commercial etoposide formulation (FY). This increase in nasal absorption for etoposide loaded PCL nanoparticles may be due to smaller particle size (518.0 nm) and bioadhesive nature of PCL polymer, as it binds to nasal mucosa and increase the residence time which consequently increases the absorption (Dondeti et al., 1996; Ugwoke et al., 2001).

![Figure 4.17](image_url)

Figure 4.17. Percentage of etoposide remaining in the perfusate versus time after nasal perfusion of rats with etoposide commercial formulation (FY) and etoposide PCL nanoparticles (EB-4). (n=3)
4.2.1.3.4. *In vitro* cytotoxicity study (MTT assay)

In the *in vitro* cytotoxicity study, cells were incubated with various concentrations of both pure drug and etoposide loaded PCL nanoparticles for 48 h and 96 h separately. Percentage cell viability (Y-axis) was plotted against concentration of the etoposide nanoparticles (X-axis). IC\textsubscript{50} value (concentration of drug that reduces the viability of cell to 50 \%) was determined for etoposide and etoposide nanoparticles, after 48 h and 96 h treatments (Table 4.6). Results revealed a dose dependent reduction in the percentage cell viability after treatment with etoposide nanoparticle and pure etoposide (Figure 4.18). At shorter incubation time (48 h) cytotoxicity of etoposide nanoparticles were low at all tested concentrations. While for longer incubations (96 h) etoposide nanoparticle (EB-4) showed better cytotoxicity with all tested concentrations than that of pure etoposide. The result suggests that polymeric nanoparticle release the drug slowly and have increased retention in the cells as compared to etoposide alone (Averineni *et al.*, 2012). Thus the IC\textsubscript{50} value was significantly (*p* < 0.05) decreased with etoposide loaded nanoparticles compared to free etoposide, following 96 h of incubation indicating enhanced antitumor activity of nanoparticles against human glioblastoma cells LN229.

![Figure 4.18. In vitro cytotoxicity studies of etoposide nanoparticles in human glioblastoma cells.](image-url)
Table 4.6. *In vitro* cytotoxicity studies of etoposide loaded PCL nanoparticles in human glioblastoma cells

<table>
<thead>
<tr>
<th>Sample code</th>
<th>IC_{50}±SEM μg/mL After 48 hours</th>
<th>IC_{50}±SEM μg/mL After 96 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETP pure</td>
<td>4.64±0.27</td>
<td>3.46±0.21</td>
</tr>
<tr>
<td>EB-4</td>
<td>11.48±0.51</td>
<td>2.07±0.14*</td>
</tr>
</tbody>
</table>

*P<0.05 versus ETP pure (after 96 h).

4.2.1.3.5. Accelerated stability studies

Stability studies of optimized batch of freeze-dried etoposide nanoparticles EB-4 was carried out and stability of formulation was evaluated on the basis of physical appearance, particle size, zeta potential, and encapsulation efficiency as main parameters (Kalaria *et al.*, 2009). The freeze dried nanoparticles did not show any change in visual appearance and has also retained its free flowing properties. The results showed a small increase in average particle size and minor decrease in the values of zeta potential and % encapsulation efficiency, upon three-month storage. As there is no significant alteration in average particle size, zeta potential and encapsulation efficiency it can be concluded that the etoposide nanoparticle formulation EB-4 is stable at 25 ± 2 °C / 60 ± 5 % RH for a total period of 3 months (Table 4.7).

Table 4.7. Accelerated stability studies of optimized batch of etoposide PCL nanoparticles

<table>
<thead>
<tr>
<th>Stability parameter</th>
<th>Test period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 month</td>
</tr>
<tr>
<td>Particle size a (nm)</td>
<td>518.0±10.2</td>
</tr>
<tr>
<td>Zeta Potential a (mV)</td>
<td>-20.6±2.1</td>
</tr>
<tr>
<td>% EE a</td>
<td>73.07±3.11</td>
</tr>
</tbody>
</table>

*a These results are mean±standard deviation. (n=3).
4.2.2. Formulation development of etoposide PLGA 50:50 nanoparticles by nanoprecipitation method

4.2.2.1. Formulation optimization of etoposide PLGA 50:50 nanoparticles

In order to optimize the particle size and entrapment efficiency of etoposide-PLGA 50:50 nanoparticles, various batches were formulated varying the experimental conditions. Different methods like emulsification solvent evaporation and nanoprecipitation techniques were studied for preparation of etoposide loaded PLGA 50:50 nanoparticles. Among these methods, better particle size and encapsulation efficiency was obtained with nanoprecipitation method. Therefore, this method was used for preparation of etoposide PLGA 50:50 nanoparticles. Further, nanoprecipitation is the preferred method for incorporating hydrophobic drug into polymeric nanoparticles (Fessi et al., 1989; Vauthier-Holtzscherer et al., 1991). It is highly challenging to develop a method which permits the precipitation of nanoparticles avoiding the possibility or reducing the extent of diffusion of drug along with the solvent, thus increasing the encapsulation efficiency. Selection criteria for the solvent were encapsulation efficiency and particle size. Based on the solubility studies of drug and polymer in various organic solvents, acetone was selected as the solvent for etoposide and PLGA 50:50, which gave maximum encapsulation efficiency and minimum particle size. Formation of colloidal particles as a result of nanoprecipitation is determined mostly by the organic/aqueous phase ratio (Quintanar-Guerrero et al., 1998; Barichello et al., 1999). Hence it is essential to study the influence of organic/aqueous phase ratio on encapsulation efficiency of drugs in nanoparticles prepared by this method.

In the development of etoposide loaded PLGA 50:50 nanoparticles by nanoprecipitation method, it was observed that when the volume of external aqueous phase was increased from 1:2 to 1:3, there was a decrease in the encapsulation efficiency of etoposide in PLGA 50:50 nanoparticles and also a significant decrease in the nanoparticles size. When organic to aqueous phase ratio was 1:2 encapsulation efficiency was reasonably good with low particle size. Therefore organic/aqueous phase ratio selected was 1:2. Based on the observations it is rational to understand that as a result of formation of nanodroplets, surface area was increased which facilitated the diffusion of drug to the external phase along with the solvent resulting in lower encapsulation efficiency (Fonseca et al., 2002). It is also possible that the capacity of
the polymer matrix to encapsulate the drug become lower as the particle size decreases. The physicochemical characteristics of nanoparticles such as size and surface charge, has high influence on their physical stability and significantly affect their interaction with the biological systems after in vivo administration. This method is ideal for the preparation of etoposide PLGA 50:50 nanoparticles due to the ease of preparation as well as the instantaneous and reproducible nature of nanoparticle formation characterized by particle size lower than 200 nm and homogeneous size distribution indicated by the low polydispersity index values. The nanoparticles prepared using optimized parameters had a percentage encapsulation efficiency of 88.57±5.95 % and particle size 143.4±10.05 nm, with a polydispersity index and zeta potential of 0.095±0.017 and -33.1±2.55 mV respectively. The particle size, PDI, zeta potential and % encapsulation efficiency results of all batches are presented in Table 4.8.

4.2.2.1.1. Entrapment Efficiency (EE %)

Percentage encapsulation efficiency of all batches of etoposide PLGA 50:50 nanoparticles were found to be in the range of 20.69±1.83 to 88.57±5.95 (Table 4.6). To obtain optimum encapsulation of drug, various batches were prepared by varying the drug : polymer ratios. The EE % of all designed batches of etoposide PCL nanoparticles are reported in Table 4.8. As the drug: polymer ratio increased from 1:5 to 1:10 using 0.5 % w/v of PVA(H) as stabilizer, entrapment efficiency and particle size increased (Hoffart et al., 2002; Galindo-Rodriguez et al., 2004; Averineni et al., 2012). The drug: polymer ratio of 1:10 showed a desirable particle size below 200 nm with reasonable percentage encapsulation efficiency of 45.67. Therefore further formulation optimization with respect to type of the stabilizer and its concentration was carried out using drug: polymer ratio of 1:10. Drug : polymer ratio of 1:10 using 0.5 % w/v of pluronic F-68 as stabilizer showed an optimum encapsulation efficiency of 88.57±5.95 %. The optimized formulation (EPG-23) with highest encapsulation efficiency was further used for in situ nasal perfusion studies and in vivo pharmacokinetic studies.

4.2.2.1.2. Particle size, polydispersity index and zeta potential

The average particle diameter of all the prepared batches of etoposide-PLGA 50:50 nanoparticles ranged from 140.9±10.13 to 693.3±15.78 nm and the polydispersity varied from 0.088±0.006 to 0.316±0.043, implying narrow particle size distribution in all samples, whereas
the zeta potential of etoposide-PLGA 50:50 nanoparticles were found to be in the range of -12.12±0.94 to -34.6±2.81 mV (Table 4.8). The negative zeta potential values for etoposide PLGA 50:50 nanoformulations may be due to the ionization of carboxyl groups of PLGA (Moreno-Vega et al., 2012). The polydispersity index (PDI) being the measure of homogeneity of particle size, was studied to determine the extent of the particle size distribution. Particle size distribution of optimized batch EPG-23 is depicted in Figure 4.19. The PDI value for EPG-23 was found to be low (0.095±0.017), indicating a homogeneous size distribution. Particle size and its distribution pattern are critical factors which determine the feasibility of administration through nasal route and its release behaviour. Small size of the nanoparticles enables them to pass through the nasal cavities by various mechanisms such as paracellular and receptor/carrier mediated transports. (Illum et al., 1987). Smaller size of the particles enables them to amass at the tumour sites whose uptake into the tumour cells are brought about by the enhanced permeation and retention (EPR) property of the tumour vasculature. Zeta potential values are valuable indicators of stability of nanoparticles in dispersions. The zeta potential for optimized formulation EPG-23 was found to be -33.1±2.55 mV. The negative charge of zeta potential was indicative of the negatively charged groups present in the polymer (Figure 4.20). Stabilized nanoparticles exhibit zeta potential values in the range -15 to -30 mV (Musumeci et al., 2006). High negative values of zeta potential are considered to be ideal as it brings about electrostatic repulsion between particles which will prevent their aggregation and there by stabilize the nanoparticulate dispersion (Feng et al 2001).

![Size Distribution by Intensity](image)

**Figure 4.19.** Particle size distribution of optimized batch EPG-23
4.2.2.1.3. Effect of drug: polymer ratio on encapsulation efficiency and particle size

Etoposide loaded PLGA 50:50 nanoparticles were formulated by varying the PLGA concentration to different weight ratio levels, with respect to the drug. The effect of drug: polymer ratio on encapsulation efficiency and particle size was studied at different drug: polymer ratios like 1:5, 1:10, 1:20, 1:40, 1:60, 1:80, 1:100 and 1:110. The particle size and % encapsulation efficiency for these batches ranged between 140.9 to 693.3 nm and 20.69 to 68.71 % respectively (Table 4.8). Entrapment efficiency and particle size were found to increase as the drug: polymer ratio increased from 1:5 to 1:10, (Figure 4.21 & 4.22). Effect of drug: polymer on particle size obtained during nanoprecipitation method can be directly correlated with the viscosity of the organic phase. Increase in polymer concentration increases the organic phase viscosity causing an increase in mass transfer resistance that reduces the diffusion of polymer into the aqueous phase might have resulted in increased particle size (Averineni et al., 2012). On the other hand, the increase in encapsulation efficiency with increase in drug: polymer ratio may be due to the fact that the amount of drug loss can be drastically reduced during the encapsulation process involving high polymer concentrations (Averineni et al., 2012). Furthermore, extreme low concentration of polymer may not be sufficient to encapsulate the drug.

Figure 4.20. The zeta potential of optimized formulation EPG-23
Table. 4.8. Formulation optimization of etoposide PLGA 50:50 nanoparticles

<table>
<thead>
<tr>
<th>Batch</th>
<th>Drug: Polymer ratio</th>
<th>Stabilizer</th>
<th>Stabilizer Concentration (% w/v)</th>
<th>Average particle size (\text{a} ) (nm)</th>
<th>Zeta Potential (\text{a} ) (mV)</th>
<th>PDI (\text{a} )</th>
<th>% Encapsulation Efficiency (\text{a} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPG-1</td>
<td>1:5</td>
<td>PVA(H)</td>
<td>0.5</td>
<td>140.9±10.13</td>
<td>-17.81±1.21</td>
<td>0.112±0.016</td>
<td>20.69±1.83</td>
</tr>
<tr>
<td>EPG-2</td>
<td>1:10</td>
<td>PVA(H)</td>
<td>0.5</td>
<td>176.6±13.78</td>
<td>-18.83±1.55</td>
<td>0.104±0.027</td>
<td>45.67±3.21</td>
</tr>
<tr>
<td>EPG-3</td>
<td>1:20</td>
<td>PVA(H)</td>
<td>0.5</td>
<td>210.8±15.42</td>
<td>-19.81±1.47</td>
<td>0.095±0.016</td>
<td>51.69±3.97</td>
</tr>
<tr>
<td>EPG-4</td>
<td>1:40</td>
<td>PVA(H)</td>
<td>0.5</td>
<td>389.7±9.34</td>
<td>-20.53±2.13</td>
<td>0.115±0.019</td>
<td>56.78±4.59</td>
</tr>
<tr>
<td>EPG-5</td>
<td>1:60</td>
<td>PVA(H)</td>
<td>0.5</td>
<td>403.4±10.29</td>
<td>-21.21±2.11</td>
<td>0.152±0.012</td>
<td>59.96±5.12</td>
</tr>
<tr>
<td>EPG-6</td>
<td>1:80</td>
<td>PVA(H)</td>
<td>0.5</td>
<td>515.6±12.41</td>
<td>-12.12±0.94</td>
<td>0.191±0.015</td>
<td>64.34±5.71</td>
</tr>
<tr>
<td>EPG-7</td>
<td>1:100</td>
<td>PVA(H)</td>
<td>0.5</td>
<td>676.7±13.57</td>
<td>-21.56±2.55</td>
<td>0.259±0.021</td>
<td>66.76±4.64</td>
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<tr>
<td>EPG-8</td>
<td>1:110</td>
<td>PVA(H)</td>
<td>0.5</td>
<td>693.3±15.78</td>
<td>-22.59±2.71</td>
<td>0.316±0.043</td>
<td>68.71±5.15</td>
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<td>EPG-9</td>
<td>1:10</td>
<td>PVA(H)</td>
<td>0.25</td>
<td>180.3±8.65</td>
<td>-19.56±1.29</td>
<td>0.111±0.014</td>
<td>46.37±3.72</td>
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<tr>
<td>EPG-10</td>
<td>1:10</td>
<td>PVA(H)</td>
<td>1.0</td>
<td>321.5±10.52</td>
<td>-24.54±2.17</td>
<td>0.097±0.008</td>
<td>43.76±3.17</td>
</tr>
<tr>
<td>EPG-11</td>
<td>1:10</td>
<td>PVA(H)</td>
<td>1.5</td>
<td>404.0±13.21</td>
<td>-26.2±2.85</td>
<td>0.134±0.017</td>
<td>41.44±2.95</td>
</tr>
<tr>
<td>EPG-12</td>
<td>1:10</td>
<td>PVA(H)</td>
<td>2.0</td>
<td>422.7±11.34</td>
<td>-27.3±2.28</td>
<td>0.088±0.006</td>
<td>40.11±3.15</td>
</tr>
<tr>
<td>EPG-13</td>
<td>1:10</td>
<td>PVA(H)</td>
<td>2.5</td>
<td>440.5±14.27</td>
<td>-32.86±2.88</td>
<td>0.129±0.015</td>
<td>39.25±3.11</td>
</tr>
<tr>
<td>Batch</td>
<td>Drug: Polymer ratio</td>
<td>Stabilizer</td>
<td>Stabilizer Concentration (% w/v)</td>
<td>Average particle size (nm)</td>
<td>Zeta Potential (mV)</td>
<td>PDI</td>
<td>% Encapsulation Efficiency</td>
</tr>
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<td>---------</td>
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<td>---------------------</td>
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</tr>
<tr>
<td>EPG-14</td>
<td>1:10</td>
<td>PVA (L)</td>
<td>0.25</td>
<td>250.9±12.12</td>
<td>-20.3±1.78</td>
<td>0.141±0.018</td>
<td>46.72±3.79</td>
</tr>
<tr>
<td>EPG-15</td>
<td>1:10</td>
<td>PVA (L)</td>
<td>0.5</td>
<td>300.9±15.21</td>
<td>-22.9±2.31</td>
<td>0.137±0.022</td>
<td>42.79±3.85</td>
</tr>
<tr>
<td>EPG-16</td>
<td>1:10</td>
<td>PVA (L)</td>
<td>1.0</td>
<td>342.2±12.86</td>
<td>-23.8±1.64</td>
<td>0.155±0.031</td>
<td>31.67±2.11</td>
</tr>
<tr>
<td>EPG-17</td>
<td>1:10</td>
<td>PVA (L)</td>
<td>1.5</td>
<td>378.5±13.71</td>
<td>-28.5±2.39</td>
<td>0.149±0.028</td>
<td>26.91±2.34</td>
</tr>
<tr>
<td>EPG-18</td>
<td>1:10</td>
<td>PVA (L)</td>
<td>2.0</td>
<td>426.5±15.79</td>
<td>-27.8±2.16</td>
<td>0.161±0.011</td>
<td>27.23±1.99</td>
</tr>
<tr>
<td>EPG-19</td>
<td>1:10</td>
<td>PVA (L)</td>
<td>2.5</td>
<td>479.8±14.29</td>
<td>-31.5±2.77</td>
<td>0.145±0.024</td>
<td>24.31±3.85</td>
</tr>
<tr>
<td>EPG-20</td>
<td>1:10</td>
<td>F-68</td>
<td>2.0</td>
<td>211.7±13.45</td>
<td>-34.6±2.81</td>
<td>0.099±0.011</td>
<td>39.60±3.12</td>
</tr>
<tr>
<td>EPG-21</td>
<td>1:10</td>
<td>F-68</td>
<td>1.5</td>
<td>171.7±11.18</td>
<td>-33.1±2.95</td>
<td>0.105±0.019</td>
<td>44.44±3.75</td>
</tr>
<tr>
<td>EPG-22</td>
<td>1:10</td>
<td>F-68</td>
<td>1.0</td>
<td>166.6±9.54</td>
<td>-32.9±2.47</td>
<td>0.091±0.012</td>
<td>65.19±4.89</td>
</tr>
<tr>
<td>EPG-23</td>
<td>1:10</td>
<td>F-68</td>
<td>0.5</td>
<td>143.4±10.05</td>
<td>-33.1±2.55</td>
<td>0.095±0.017</td>
<td>88.57±5.95</td>
</tr>
<tr>
<td>EPG-24</td>
<td>1:10</td>
<td>F-68</td>
<td>2.5</td>
<td>254.6±12.73</td>
<td>-30.5±2.83</td>
<td>0.110±0.019</td>
<td>33.45±2.74</td>
</tr>
<tr>
<td>EPG-25</td>
<td>1:10</td>
<td>F-68</td>
<td>0.25</td>
<td>164.6±11.71</td>
<td>-29.5±2.14</td>
<td>0.097±0.024</td>
<td>87.45±5.31</td>
</tr>
</tbody>
</table>

* These results are mean ± standard deviation. (n=3).

PVA(H): Polyvinyl alcohol high molecular weight; PVA(L): Polyvinyl alcohol low molecular weight
4.2.2.1.4. Effect of stabilizers on encapsulation efficiency and particle size of etoposide PLGA 50:50 nanoparticles

Stabilizers are used in formulation of nanoparticles so as to provide stability of the dispersion in aqueous medium (Mainardes et al., 2005). Various surfactants like PVA (L) (low molecular weight - M.W. 11,000-31,000), PVA (H) (high molecular weight M.W. 30,000–70,000) and Pluronic F-68 were tried to study the influence of type of surfactant on physicochemical properties of etoposide nanoparticles. Pluronic F-68 was selected as the stabilizer as it gave lower particle size, better zeta potential and maximum encapsulation efficiency without particle aggregation and was studied at various concentrations ranging from 0.25 % w/v to 2.5 % w/v. The effect of stabilizer concentration on entrapment efficiency and particle size is shown in Figure 4.23 & 4.24.
As the stabilizer concentration was increased from 0.5 to 2.5% w/v there was a decrease in entrapment efficiency which may be due to the increase in solubility of the drug in aqueous medium (Joshi et al., 2010). Increase in stabilizer concentration caused an increase in particle size possibly due to increase in viscosity of the aqueous medium (Murakami et al., 1999). The optimum concentration was found to be 0.5 % w/v. Lower concentration of stabilizer could not yield nanoparticles free of aggregation. This may be because the concentration of stabilizer was not sufficient to cover the dispersed nanoparticles completely, failing to stabilize and causing aggregation, leading to larger nanoparticles (Feng et al., 2001). Hence, 0.5 % w/v F-68 concentration was considered optimum.

4.2.2.1.5. Effect of cryoprotectants during freeze drying process of etoposide PLGA 50:50 nanoparticles

Various cryoprotectants such as sucrose, fructose, dextrose, mannitol with suitable concentration were tested for their effect on freeze drying process of etoposide PLGA 50:50 nanoparticles. Among them, mannitol yielded completely dry and free flowing powders with lowest particle size. The concentration of mannitol was varied at different levels such as 2.5, 5 and 10 % w/v to study their influence on physicochemical properties of nanoparticles. 2.5 % w/v mannitol produced a particle size of 241 nm with a little variation. However lower particle size was observed with 5 and 10 % w/v mannitol displaying a particle size distribution in the range of 143-181 nm. Therefore 5 % w/v mannitol was found to be sufficient to obtain nanoparticles of desirable properties.

![Figure 4.23: The effect of stabilizer concentration on entrapment efficiency of etoposide PLGA 50:50 nanoparticles.](image)
Results & Discussion

Figure 4.24: The effect of stabilizer concentration on particle size of etoposide PLGA 50:50 nanoparticles.

4.2.2.2. Characterization of etoposide PLGA 50:50 nanoparticles

4.2.2.2.1. Fourier Transform Infrared spectroscopic studies

FTIR spectrum of optimized batch of etoposide loaded PLGA 50:50 nanoparticles (EPG-23) (Figure 4.25) was analysed to prove the encapsulation of etoposide in nanoparticles and to detect the occurrence of interactions, if any, between the drug and excipients in the nanoparticles. The presence of characteristic peaks of pure drug etoposide in etoposide loaded PLGA 50:50 nanoparticle (EPG-23) can be confirmed by the typical bands at 3394.72 cm\(^{-1}\)(-O-H str), 2947.23, 2904.80 cm\(^{-1}\)(aliphatic -C-H str), 1741.72 cm\(^{-1}\)(>C=O, lactone) and 1018.41 & 1082.07 cm\(^{-1}\) (Ar-OCH\(_3\) & ArO-CH\(_3\)). Thus FTIR spectrum of etoposide loaded PLGA 50:50 nanoparticle (EPG-23) showed all the characteristic peaks of the drug without any appreciable shift which confirms the encapsulation of drug and also that there is no significant interaction between the drug and the excipients during the formation of nanoparticles.

Figure 4.25: FTIR spectrum of optimized batch of etoposide loaded PLGA 50:50 nanoparticles (EPG-23)
4.2.2.2. Thermogram properties

DSC thermograms of PLGA 50:50 polymer (PG), free etoposide (ET), pluronic F-68, mannitol (MN) and etoposide loaded PLGA nanoparticles (EPG-23) were analysed to detect the occurrence of interactions, if any, between the drug and excipients in the nanoparticles (Figure 4.26). DSC thermogram of pure etoposide exhibited endothermic thermal transitions at 80-115 °C, 180 °C and 295 °C which correspond to transitions due to dehydration of etoposide, melting of anhydrous form of etoposide and melting temperature of newly formed crystalline etoposide respectively (Jasti et al., 1995). DSC thermogram of pure polymer PLGA 50:50 and pluronic F-68 exhibited endothermic peaks at 43.61 °C and 55.5 °C corresponding to their glass transition temperature (Tg) and melting temperature (Tm) respectively. The DSC thermogram of mannitol exhibited an endothermic peak at 171.5 °C which corresponds to its melting temperature (Tm). The thermogram of optimized etoposide loaded PLGA 50:50 nanoparticles (EPG-23) exhibited a very weak endotherm at 43.60 °C which corresponds to glass transition temperature (Tg) of PLGA 50:50 polymer, while endotherm at 171.53 °C corresponds to melting endotherm of mannitol. This thermogram did not show any detectable endotherm corresponding to the melting temperature of free drug which is between 200 and 300 °C. If the drug is present in a molecular dispersion in the polymeric nanoparticles loaded with smaller amount of drug, then the endotherm is not detected. Thus, it is understood that the etoposide in PLGA 50:50 nanoparticles existed in amorphous form of a molecular dispersion or solid solution state in the polymer matrix (Jain et al., 2011). This can lead to increased solubility and finally to an improved biological activity (Dhanaraju et al., 2010).

Figure 4.26: DSC thermogram of etoposide (ET), PLGA 50:50 polymer (PG), pluronic F-68, mannitol (MN), and EPG-23 (etoposide loaded PLGA 50:50 nanoparticles).
4.2.2.2.3. Scanning electron microscopy (SEM)

The SEM photomicrograph of optimized etoposide PLGA 50:50 nanoparticles (EPG-23) were obtained before and after gold sputtering is shown in the Figure 4.27a & 4.27b. The SEM photomicrograph confirmed their spherical shape. They possessed homogeneous, smooth surface without any rupture. The particles possessed adequate homogeneity and the particles existed as discrete entities without any aggregation.

![Figure 4.27a](image1.png)

**Figure 4.27a.** Scanning electron microscopy of etoposide loaded PLGA 50:50 nanoparticles (EPG-23) after gold sputtering.

![Figure 4.27b](image2.png)

**Figure 4.27b.** Scanning electron microscopy of etoposide PLGA 50:50 nanoparticles (EPG-23) without gold sputtering.
4.2.2.3. Evaluation of optimized etoposide PLGA 50:50 nanoparticles

4.2.2.3.1. In vitro release study

The in vitro release study was performed on all prepared batches but results are reported only for EPG-21, EPG-22, EPG-23 and EPG-25 (Figure 4.28). The in vitro release profiles of EPG-21, EPG-22, EPG-23 and EPG-25 showed biphasic behaviour consisting of initial burst release followed by a sustained release phase for the period of 72 h. The initial burst release of drugs may be due to the fraction of etoposide that was adsorbed on the surface of nanoparticles. Sustained release may be due to slow diffusion of the drug from the lipophilic polymeric matrix. The release of entrapped etoposide from nanoparticles with lower concentration of polymer was more rapid than those with higher polymer concentration. This could be due to the fact that increase in polymer concentration, lead to the formation of thick and less porous polymer matrix. Another important factor which influences the release rate of drug from the nanoparticle is its smaller particle size. Smaller the particle size higher will be the surface area; therefore, some amount of drug will be at or near the particle surface which contributes for the initial burst release. Further, smaller particle size reduces distance for diffusion and allows the drug to rapidly diffuse out from the polymer core.

To study the drug release kinetics, the data obtained from in vitro drug release studies of optimized batch EPG-23 was fitted to different models like zero-order, first-order and Higuchi’s model. The regression coefficient (R²) for zero-order, first-order and Higuchi’s model was found to be 0.8196, 0.9397 and 0.9935, respectively. Thus, in vitro drug release of EPG-23 formulation was best explained by Higuchi kinetic diffusion control mechanism, as the plot showed the highest linearity (Jain et al., 2009).

To study the mechanism of drug release, data obtained from in vitro drug release studies was fitted to the Korsmeyer–Peppas model. The regression coefficient for the plot of log cumulative percentage drug released vs log time of the Korsmeyer–Peppas equation for etoposide PLGA 50:50 nanoparticle formulation EPG-23 was found to be 0.9934. The value of release exponent (n) was found to be 0.5241. As the n value is >0.5, it can be concluded that the release of etoposide from PLGA 50:50 nanoparticles was by non-Fickian diffusion (diffusion coupled with erosion) (Peppas, 1984; Vaghani et al., 2010).

When the in vitro drug release profile of optimised batch of etoposide loaded PCL nanoparticles (EB-4) was compared with that of optimized etoposide PLGA 50:50
nanoparticles (EPG-23), a remarkable difference in the release rate was observed. The cumulative amount of etoposide released at the end of 72 h was 82.27 % and 50.18 % for EPG-23 and EB-4 respectively. Thus maximum control release was observed with PCL nanoparticles than that of PLGA 50:50 nanoparticles. This decrease in release may be due to the more hydrophobic nature of PCL than PLGA co-polymers. As etoposide is also hydrophobic, its release from PCL nanoparticles was very slow.

![Graph showing cumulative drug release profile](image)

**Figure 4.28.** *In vitro* % cumulative drug release profile of etoposide PLGA 50:50 nanoparticle formulations.

4.2.2.3.2. *Ex vivo* permeation studies of etoposide PLGA 50:50 nanoparticles through sheep nasal mucosa

The drug permeation pattern through sheep nasal mucosa reflected the release pattern as shown for *in vitro* release studies, but the amount of drug permeated was lower when compared to that of release studies. The permeation of etoposide from nanoparticles through nasal mucosa was found to be low when compared with that through dialysis membrane. This resistance to the penetration of drug molecules could be attributed to the complexity of the composition of the mucosa during the diffusion process (Tas et al., 2004). Among the two formulations (EPG-23 & EPG-25) selected for the study, formulation EPG-23 exhibited better permeation across the sheep nasal mucosa. The
cumulative percentage of etoposide permeated from EPG-23 after 8 h was found to be 40.19±2.01 %, after triplicate determinations. Permeation profile is shown in Figure 4.29.

When the drug permeation pattern through sheep nasal mucosa from the optimised batch of etoposide loaded PCL nanoparticles (EB-4) was compared with that of optimized etoposide PLGA 50:50 nanoparticles (EPG-23), a notable change in the permeation rate was observed. The cumulative amount of etoposide permeated across the sheep nasal mucosa at the end of 8 h was 40.19 % and 16.42 % for EPG-23 and EB-4, respectively. This decrease in permeation of etoposide from PCL nanoparticles may due to the more hydrophobic nature of PCL than PLGA 50:50.

![Figure 4.29. Percentage cumulative drug permeated from etoposide PLGA 50:50 nanoparticles through nasal mucosa.](image)

**Histopathological studies**

Histopathological studies were carried out to examine the histological changes in nasal mucosa caused by etoposide PLGA 50:50 nanoparticles. The cross section of the sheep nasal mucosa used for permeation before (untreated control, Figure 4.30a) and after the permeation (treated) was stained by hematoxylin–eosin (HE) and observed under light microscope. Cross section of control nasal mucosa showed ciliated respiratory epithelium and normal goblet cells. The effect of etoposide PLGA 50:50 nanoparticles (EPG-23) on sheep nasal mucosa, 8h after permeation studies (treated, Figure 4.30b) showed no severe
damage on the integrity of nasal mucosa when compared to untreated control. The observed change on nasal mucosa was only a mild epithelial disruption.

Figure 4.30. Histopathology of sheep nasal mucosa. a) Nasal mucosa of untreated control (20X) b) Nasal mucosa permeated with EPG-23 (20X).

4.2.2.3.3. Nasal absorption studies of etoposide PLGA 50:50 nanoparticles by in situ nasal perfusion in male Wistar rats

Nasal absorption of commercial etoposide formulation (FY) and optimized etoposide loaded PLGA 50:50 nanoparticle formulations EPG-23 & EPG-25 were studied by in situ nasal perfusion in male Wistar rats. The commercial formulation and both nanoparticle formulation of etoposide EPG-23 & EPG-25 showed progressive nasal absorption with time. A plot of percentage drug remaining in the perfusing solution versus time is shown in Figure 4.31. Etoposide loaded PLGA nanoparticles exhibited better nasal absorption compared to the commercial etoposide formulation (FY). The first order nasal absorption rate constants for commercial etoposide formulation (FY) and optimized etoposide loaded nanoparticle formulation (EPG-23) were calculated from the slopes of first-order plots of the percentage drug remaining in the perfusing solution versus time (Huang et al., 1985). The first order nasal absorption rate constants for FY and EPG-23 were found to be $3.455 \times 10^{-3}$ and $6.906 \times 10^{-3}$ min$^{-1}$ respectively. There is a statistically significant ($p < 0.05$) increase in first order nasal absorption rate constant value for optimized PLGA 50:50 nanoparticle (EPG-23) when compared to that of commercial etoposide formulation (FY). This increase in nasal absorption for etoposide loaded PLGA nanoparticles may be due to smaller particle size (143.4 nm) and bioadhesive nature of PLGA polymer, as it binds to nasal mucosa and increase the residence time which consequently increases the absorption (Dondeti et al., 1996; Ugwoke et al., 2001).
Results & Discussion

Figure 4.31. Percentage of etoposide remaining in the perfusate versus time after nasal perfusion of rats with etoposide commercial formulation and etoposide 50:50 nanoparticles EPG-23 & EPG-25, (n=3).

When the nasal absorption of etoposide from etoposide loaded PLGA nanoparticles (EPG-23) was compared with that of etoposide loaded PCL nanoparticles (EB-4), EPG-23 was found to exhibit better nasal absorption. The first order nasal absorption rate constant value for PLGA 50:50 nanoparticles (EPG-23) was better than that of etoposide loaded PCL nanoparticles EB-4. This increased nasal absorption of EPG-23 may be due to comparatively faster release of etoposide from PLGA 50:50 polymeric matrix than from PCL polymeric matrix.

4.2.2.3.4. In vitro cytotoxic study by MTT assay

In the in vitro cytotoxic study, human glioblastoma cells (LN229) were incubated with various concentrations of both pure drug and etoposide loaded in PLGA 50:50 nanoparticles for 48 h and 96 h separately. Percentage cell viability (Y-axis) was plotted against concentration of the etoposide nanoparticle (X-axis). IC$_{50}$ value (concentration of nanoparticles that reduces the viability of cell to 50 %) was determined for pure etoposide and etoposide loaded PLGA nanoparticles (EPG-23) for both 48 h and 96 h treatment (Table 4.9). Results revealed a dose dependent reduction in the percentage cell viability after treatment with etoposide loaded PLGA nanoparticles and pure etoposide (Figure 4.32). At shorter incubation time (48 h) cytotoxicity of etoposide loaded PLGA nanoparticles (EPG-23) were low at all tested concentrations than that of pure etoposide.
Results & Discussion

While for longer incubations (96 h) EPG-23 showed better cytotoxicity at all tested concentrations than that of pure etoposide. The result suggests that polymeric nanoparticles releases the drug slowly and have increased retention in the cells as compared to etoposide alone (Averineni et al., 2012). Thus the IC$_{50}$ value was significantly ($p < 0.05$) decreased with etoposide loaded PLGA nanoparticles (EPG-23) compared to free etoposide, following 96 hours of incubation, indicating an enhanced antitumor activity of nanoparticles against human glioblastoma cells LN229.

When the cytotoxicity of etoposide loaded PLGA nanoparticles (EPG-23) was compared with that of etoposide loaded PCL nanoparticles (EB-4), it was observed that etoposide loaded PLGA nanoparticles (EPG-23) exhibited better cytotoxicity at all tested concentrations both after 48 and 96 h of incubation. This increased cytotoxicity of EPG-23 may be due to comparatively faster release of etoposide from PLGA 50:50 polymeric matrix than from PCL polymeric matrix.

![Graph showing in vitro cytotoxicity of etoposide loaded PLGA 50:50 nanoparticles in human glioblastoma cells.]

**Figure 4.32.** *In vitro* cytotoxicity studies of etoposide loaded PLGA 50:50 nanoparticles in human glioblastoma cells.

**Table 4.9.** *In vitro* cytotoxicity of etoposide loaded PLGA 50:50 nanoparticles in human glioblastoma cells

<table>
<thead>
<tr>
<th>Sample code</th>
<th>IC$_{50}$±SEM µg/mL After 48 h</th>
<th>IC$_{50}$±SEM µg/mL After 96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETP pure</td>
<td>4.64±0.27</td>
<td>3.46±0.21</td>
</tr>
<tr>
<td>EPG-23</td>
<td>6.63±0.37</td>
<td>1.83±0.14*</td>
</tr>
</tbody>
</table>

*P<0.05 versus ETP pure (after 96 h).
4.2.2.3.5. Accelerated stability studies

Stability studies of optimized batch of freeze-dried etoposide nanoparticle EPG-23 was carried out and stability of formulation was evaluated on the basis of physical appearance, particle size, zeta potential, and encapsulation efficiency as main parameters. The freeze dried nanoparticles did not show any change in visual appearance and has also retained its free flowing properties. There was slight increase in average particle size and a small decrease in zeta potential and % EE was observed on three-month storage. As there is no significant alteration in average particle size, zeta potential and % encapsulation efficiency it can be concluded that the etoposide nanoparticle formulation EPG-23 is stable at 25±2 °C / 60±5 % RH for a period of 3 months (Table 4.10).

Table 4.10. Accelerated stability studies of optimized batch of etoposide PLGA 50:50 nanoparticles

<table>
<thead>
<tr>
<th>Stability parameter</th>
<th>Test period</th>
<th>0 month</th>
<th>1 month</th>
<th>2 months</th>
<th>3 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle sizea (nm)</td>
<td></td>
<td>143.4±10.05</td>
<td>151.11±11.3</td>
<td>155.31±10.5</td>
<td>159.25±12.1</td>
</tr>
<tr>
<td>Zeta Potentiala (mV)</td>
<td></td>
<td>-33.1±2.55</td>
<td>-31.4±1.13</td>
<td>-30.1±1.9</td>
<td>-29.7±2.1</td>
</tr>
<tr>
<td>% EEa</td>
<td></td>
<td>88.57±5.95</td>
<td>85.12±4.11</td>
<td>81.41±2.98</td>
<td>79.32±4.17</td>
</tr>
</tbody>
</table>

aThese results are mean±standard deviation. (n=3).

4.2.3. Formulation development and optimization of etoposide nanosuspension by Microprecipitation – High-pressure homogenization technique (Nanoedge)

4.2.3.1. Formulation development and optimization of etoposide nanosuspension

Nanosuspension has emerged as one of the strategies for overcoming the problem of low bioavailability of drugs with low aqueous solubility. Nanosuspension is a colloidal dispersion of drug particles in nanometer size range, produced by a suitable method and is stabilized by an appropriate stabilizer. The dissolution velocity and saturation solubility of drug are increased by the reduced size of nanoparticles which is responsible for an increase in the bioavailability of the poorly water soluble drug (Hintz et al., 1989; Böhm et al., 1999).

In order to optimize the particle size, zeta potential and polydispersity index of etoposide nanosuspension, different parameters were experimented and evaluated during the
formulation development. Techniques such as high-pressure homogenization and wet milling are often used to prepare nanosuspensions of nanosize range. The high pressure homogenization is a widely used technique for effective size reduction of particles. High pressure homogenization method is found to be advantageous over milling techniques as it is a simple and time-saving process (Li et al., 2009). Therefore, this method was used for preparation of etoposide nanosuspension. After preliminary study, methanol was selected as the solvent for etoposide. Selection was based on the solubility studies of drug in various solvents, as this solvent gave lowest particle size. The nanosuspension prepared using optimized parameters had a particle size of 311.4±11.19 nm, with a polydispersity index and zeta potential of 0.191±0.025 and -15.4±2.14 mV respectively. The particle size, PDI and zeta potential results of all batches are presented in Table 4.11.

Figure 4.33: Particle size distribution of ENSP-7

Figure 4.34: Zeta potential of ENSP-7
### Table 4.11. Formulation optimization of etoposide nanosuspension

<table>
<thead>
<tr>
<th>Batch</th>
<th>Stabilizer</th>
<th>Stabilizer Conc. (% w/v)</th>
<th>HPH Pressure (psi); 20 Cycles</th>
<th>Average particle size $^a$ (nm)</th>
<th>Zeta Potential $^a$ (mV)</th>
<th>PDI $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ENSP-1</td>
<td>PVA</td>
<td>0.5</td>
<td>10000</td>
<td>891.9±20.49</td>
<td>-18.2±1.79</td>
<td>0.312±0.038</td>
</tr>
<tr>
<td>ENSP-2</td>
<td>PVA</td>
<td>0.5</td>
<td>15000</td>
<td>810.2±25.19</td>
<td>-17.7±1.69</td>
<td>0.245±0.021</td>
</tr>
<tr>
<td>ENSP-3</td>
<td>PVA</td>
<td>0.5</td>
<td>20000</td>
<td>805.7±21.91</td>
<td>-18.1±1.31</td>
<td>0.215±0.037</td>
</tr>
<tr>
<td>ENSP-4</td>
<td>PVA</td>
<td>1.0</td>
<td>15000</td>
<td>495.4±15.34</td>
<td>-16.9±2.07</td>
<td>0.215±0.031</td>
</tr>
<tr>
<td>ENSP-5</td>
<td>PVA</td>
<td>1.0</td>
<td>10000</td>
<td>541.7±16.79</td>
<td>-17.1±2.71</td>
<td>0.272±0.026</td>
</tr>
<tr>
<td>ENSP-6</td>
<td>PVA</td>
<td>1.0</td>
<td>20000</td>
<td>474.1±17.75</td>
<td>-17.5±1.29</td>
<td>0.199±0.021</td>
</tr>
<tr>
<td>ENSP-7</td>
<td>PVA</td>
<td>1.5</td>
<td>15000</td>
<td>311.4±11.19</td>
<td>-15.4±2.14</td>
<td>0.191±0.025</td>
</tr>
<tr>
<td>ENSP-8</td>
<td>PVA</td>
<td>1.5</td>
<td>20000</td>
<td>321.1±14.82</td>
<td>-12.1±2.69</td>
<td>0.198±0.041</td>
</tr>
<tr>
<td>ENSP-9</td>
<td>PVA</td>
<td>1.5</td>
<td>10000</td>
<td>413.1±18.31</td>
<td>-14.9±2.76</td>
<td>0.221±0.024</td>
</tr>
<tr>
<td>ENSP-10</td>
<td>F-68</td>
<td>0.5</td>
<td>10000</td>
<td>921.7±27.12</td>
<td>-14.3±1.85</td>
<td>0.497±0.058</td>
</tr>
<tr>
<td>ENSP-11</td>
<td>F-68</td>
<td>0.5</td>
<td>15000</td>
<td>904.5±23.39</td>
<td>-16.6±2.01</td>
<td>0.374±0.047</td>
</tr>
<tr>
<td>ENSP-12</td>
<td>F-68</td>
<td>0.5</td>
<td>20000</td>
<td>897.7±21.17</td>
<td>-17.1±2.11</td>
<td>0.368±0.049</td>
</tr>
<tr>
<td>ENSP-13</td>
<td>F-68</td>
<td>1.0</td>
<td>15000</td>
<td>540.39±15.37</td>
<td>-18.8±1.98</td>
<td>0.329±0.041</td>
</tr>
<tr>
<td>ENSP-14</td>
<td>F-68</td>
<td>1.0</td>
<td>10000</td>
<td>610.1±16.15</td>
<td>-20.3±2.17</td>
<td>0.421±0.048</td>
</tr>
<tr>
<td>ENSP-15</td>
<td>F-68</td>
<td>1.0</td>
<td>20000</td>
<td>534.9±14.11</td>
<td>-22.9±2.19</td>
<td>0.307±0.039</td>
</tr>
<tr>
<td>ENSP-16</td>
<td>F-68</td>
<td>1.5</td>
<td>15000</td>
<td>431.9±13.97</td>
<td>-25.8±2.14</td>
<td>0.329±0.053</td>
</tr>
<tr>
<td>ENSP-17</td>
<td>F-68</td>
<td>1.5</td>
<td>20000</td>
<td>426.5±13.71</td>
<td>-26.5±2.75</td>
<td>0.305±0.048</td>
</tr>
<tr>
<td>ENSP-18</td>
<td>F-68</td>
<td>1.5</td>
<td>10000</td>
<td>524.2±15.16</td>
<td>-26.1±2.15</td>
<td>0.391±0.041</td>
</tr>
<tr>
<td>ENSP-19</td>
<td>PVA</td>
<td>2.0</td>
<td>15000</td>
<td>344.7±14.25</td>
<td>-11.3±1.13</td>
<td>0.201±0.031</td>
</tr>
<tr>
<td>ENSP-20</td>
<td>PVA</td>
<td>2.5</td>
<td>15000</td>
<td>371.59±16.29</td>
<td>-9.1±0.99</td>
<td>0.217±0.033</td>
</tr>
</tbody>
</table>

* These results are mean ± standard deviation. (n=3).
4.2.3.1.1. Particle size, polydispersity index and zeta potential

The average particle diameter of the prepared etoposide nanosuspensions ranged from 311.1±14.82 to 921.7±27.12 nm and the polydispersity index varied from 0.191±0.025 to 0.497±0.058, implying narrow particle size distribution in all samples, whereas the zeta potential of prepared batches of etoposide nanosuspension were found to be in the range of -9.1±0.99 to -26.5±2.75 mV (Table 4.11). The optimized batch of etoposide nanosuspension (ENSP-7) exhibited an average particle size of 311.4±11.19 nm. Particle size distribution of optimized batch of etoposide nanosuspension ENSP-7 is depicted in Figure 4.33. The PDI value which is a measure of narrowness of particle size distribution for ENSP-7 was found to be low (0.191±0.025), indicating a homogeneous size distribution. These parameters are critical in determining drug release behaviour and their possibility for intranasal administration. Smaller size of the particles enables them to accumulate at the tumour sites which then gain entry into the tumour cells by the enhanced permeation and retention (EPR) property of the tumour vasculature. The zeta potential for optimized formulation ENSP-7 was -15.4±2.14 mV (Figure 4.34). Zeta potential values in the range -15 to -30 mV are ideal for stabilized nanoformulations (Musumeci et al., 2006). High negative values of the zeta potential suggest that the electrostatic repulsion between particles will be helpful in stabilizing the nanoparticulate dispersion by preventing their aggregation (Feng et al., 2001). The optimized nanosuspension formulation (ENSP-7) with desirable properties was further used for in situ nasal perfusion studies.

4.2.3.1.2. Effect of stabilizers and its concentration on particle size of etoposide nanosuspensions

Stabilizers are used in formulation of nanoparticles so as to provide stability of the dispersion in aqueous medium after high-pressure homogenization technique. Commonly used and safe surfactants such as PVA and pluronic F-68 were selected to study effect of type of surfactant on physicochemical properties of etoposide nanosuspensions. PVA was selected as the stabilizer as it gave lower particle size, zeta potential and better polydispersity index without particle aggregation. Various concentrations of PVA ranging between 0.5% w/v to 2.5 % w/v were used in the development of nanosuspension to study its influence on physicochemical properties and stability of the developed formulation. The effect of stabilizer concentration on particle size is shown in Figure 4.35. A reduction in the size of nanoparticles was observed when the stabilizer concentration was increased.
from 0.5 to 1.5% w/v. This may be due to the fact that insufficient amount of stabilizer was unable to cover the dispersed nanocrystals completely, failing to stabilize and causing aggregation, leading to larger nanocrystals (Feng et al., 2001). Further increase in concentration of stabilizer from 1.5 % w/v to 2.5 % w/v showed slight increase in particle size. This could be due to the increase in viscosity of the solution due to the high concentration of stabilizer which may prevent the transmission of ultrasonic vibration and the diffusion between the solvent and anti-solvent during precipitation (Xia et al., 2010).

An optimum concentration (1.5 % w/v) of stabilizer led to a reduced size of nanoparticles dispersed in nanosuspension with better zeta potential and polydispersity index. Hence, 1.5 % w/v PVA concentration was considered optimum.

**Figure 4.35.** Effect of stabilizer (PVA) concentration on particle size of etoposide nanosuspensions

4.2.3.1.3. Effect of homogenization pressure, cycles and probe sonication amplitude on particle size of etoposide nanosuspensions

Microprecipitation–High-Pressure Homogenization technique was employed to obtain etoposide nanosuspension. Optimization of pre-milling homogenization pressure and cycles were carried out at three different levels such as three cycles each at 4000, 6000 and 8000 psi pressure; six cycles each at 4000, 6000 and 8000 psi pressure and six cycles each at 6000, 8000 and 10000 psi pressure. Based on the results of particle size after pre-milling, optimized pre-milling procedure involves six cycles each at 6000, 8000, and 10000 psi pressure through a high-pressure homogenizer. This pre-milling also avoid blockage of homogenizer gap and chamber. To optimize the final homogenization pressure and cycles, homogenization was carried out at three levels such as 10000, 15000.
and 20000 psi. Number of cycles for each of this homogenization pressure levels ranged from 6 to 25 cycles. The effect of homogenization pressure and cycles on the particle size is shown in Figures 4.36. Smaller etoposide particles were obtained with increased number of HPH cycles between 6 and 20. Nevertheless, when the homogenization cycles were increased from 20 to 25, further reduction in particle size was not observed. Thus 20 cycles were selected as the optimized homogenization condition for preparation of all batches of etoposide nanosuspension (Wang et al., 2010).

![Graph of particle size vs cycles and pressure](image)

**Figure 4.36.** Effect of homogenization pressure and cycles on the particle size of etoposide nanosuspension.

Similarly the impact of probe sonication amplitude on particle size of etoposide nanosuspension was assessed as the part of optimization process. Probe sonication amplitude was optimised by varying the sonication amplitude in the range of 20 to 80 % by keeping the time at 5 minutes. Maximum reduction in particle size was observed at 80 % amplitude for 5 minutes. Further increase in amplitude was not desirable as it leads to heating of the sample. Therefore probe sonication amplitude of 80 % for 5 min was found to be optimum to reduce particle size in the present formulation batches of etoposide nanosuspension.

4.2.3.2. Characterization of etoposide nanosuspension

4.2.3.2.1. Fourier Transform Infrared spectroscopic studies

FTIR spectrum of optimized batch of etoposide nanosuspension (ENSP-7, Figure 4.37) was analysed to prove the presence of etoposide in nanosuspension and to detect the
occurrence of interactions, if any, between the drug and excipients in the formulation. The presence of characteristic peaks of pure drug etoposide in etoposide nanosuspension (ENSP-7) can be confirmed by the typical bands at 3443.05 cm\(^{-1}\) (O-H str), 2920.32, 2854.74 cm\(^{-1}\) (aliphatic -C-H str), 1737.92 cm\(^{-1}\) (>C=O, lactone) and 1030.02 and 1080.17 cm\(^{-1}\) (Ar-OCH\(_3\) & ArO-CH\(_3\)). Thus FTIR spectrum of etoposide nanosuspension (ENSP-7) showed all the characteristic peaks of the drug without any appreciable shift which confirms the presence of drug and also that there is no significant interaction between the drug and the excipients during the preparation of nanosuspension.

![FTIR spectrum of etoposide nanosuspension](image)

**Figure 4.37.** FTIR spectrum of optimized batch of etoposide nanosuspension (ENSP-7)

### 4.2.3.2.2 Thermogram properties

DSC thermograms of PVA-low molecular weight (PV), free etoposide (ET) and etoposide nanosuspension (ENSP-7) were analysed to detect the occurrence of interactions, if any, between the drug and excipients in the formulation (Figure. 4.38). DSC thermogram of pure etoposide exhibited endothermic thermal transitions at 80-115 °C, 180 °C and 295 °C which corresponds to transitions due to dehydration of etoposide, melting of anhydrous form of etoposide and melting temperature of newly formed crystalline etoposide respectively. (Jasti *et al.*, 1995). The DSC thermogram of PVA exhibited an endothermic peak at 191.5 °C which corresponds to its melting temperature (Tm). The thermogram of optimized etoposide nanosuspension (ENSP-7) exhibited endotherms at 189 °C and 295 °C which correspond to melting temperatures of etoposide nanocrystals, which indicated that the crystalline state of etoposide was apparently unaltered during formulation of nanosuspension.
Results & Discussion

Figure 4.38. DSC thermogram of etoposide (ET), PVA (PV) and ENSP-7 (etoposide nanosuspension).

4.2.3.2.3. Scanning electron microscopy (SEM)

The SEM photomicrograph of optimized etoposide nanosuspension (ENSP-7) was obtained after gold sputtering is shown in the Figure 4.39. The SEM photomicrograph confirmed their regular spindle shaped nanocrystals. The nanocrystals have moderate uniformity and all the particles were discrete entities without any aggregation.

Figure 4.39. Scanning electron microscopy of etoposide nanosuspension (ENSP-7) exhibiting etoposide nanocrystals.

4.2.3.3. Evaluation of optimized etoposide nanosuspension

4.2.3.3.1. In vitro release studies of etoposide nanosuspension

This *in vitro* release study was performed on all prepared batches of etoposide nanosuspensions but results were reported only for ENSP-7, ENSP-8, ENSP-19 and ENSP-20. (Figure 4.40). *In vitro* release profiles of ENSP-7, ENSP-8, ENSP-19 and
ENSP-20 batches showed similar release pattern showing more than 80% cumulative drug release at the end of 12 h. There is no significant difference between *in vitro* release profiles of ENSP-7 & ENSP-8 as the average particle size for these formulations are very close to each other. In general faster release was observed with decreasing particle size of the etoposide nanosuspension.

When the *in vitro* drug release profile of optimised batch of etoposide nanosuspension (ENSP-7) was compared with that of optimized batch of etoposide polymeric nanoparticles (EB-4 & EPG-23) a difference in the release rate was observed. The cumulative amount of etoposide released from ENSP-7 at the end of 12 hours was 88.31% while for EB-4 and EPG-23 it was only 20.76% and 51.44% respectively.

![Graph showing cumulative drug release vs time for ENSP-7, ENSP-8, ENSP-19, and ENSP-20](image)

**Figure 4.40.** *In vitro* release studies of etoposide nanosuspensions

### 4.2.3.3.2. *Ex vivo* permeation studies of etoposide nanosuspension through sheep nasal mucosa

The drug permeation pattern through sheep nasal mucosa reflected the release pattern as shown for *in vitro* release studies, but the amount of drug permeated was lower when compared to that of release studies. The permeation of etoposide from nanosuspension through nasal mucosa was found to be low when compared with that through dialysis membrane. This resistance to the penetration of drug molecules could be attributed due to the complexity of the composition of the mucosa during the diffusion process (Tas *et al.*, 2004). Among the two formulations ENSP-7 and ENSP-8 selected for the study, formulation ENSP-7 exhibited better permeation across the sheep nasal mucosa. The
cumulative percentage of etoposide permeated from ENSP-7 after 8 h was found to be 73.35±3.98 %, after triplicate determinations. Permeation profile is shown in Figure 4.41.

When the drug permeation pattern through sheep nasal mucosa from the optimized batches of etoposide polymeric nanoparticles (EB-4 & EPG-23) was compared with that of optimized etoposide nanosuspension (ENSP-7), a notable change in the permeation rate was observed. The cumulative amount of etoposide permeated across the sheep nasal mucosa at the end of 8 h was 40.19 % and 16.42 % for EPG-23 and EB-4 respectively, while for optimized batch of etoposide nanosuspension amount of etoposide permeated across the sheep nasal mucosa at the end of 8 h was 73.35 %. This increase in permeation of etoposide from etoposide nanosuspension could be due of lack of polymeric layer covering the etoposide nanocrystals.

![Figure 4.41](image_url)

**Figure 4.41.** *Ex vivo* permeation studies of etoposide nanosuspension through sheep nasal mucosa

**Histopathological studies**

Histopathological studies were carried out to examine the histological changes in nasal mucosa caused by etoposide nanosuspension. The cross section of the sheep nasal mucosa used for permeation before (untreated control, Figure 4.42a) and after the permeation (treated, Figure 4.42b) was stained by hematoxylin–eosin (HE) and observed under light microscope. Cross section of control nasal mucosa showed ciliated respiratory epithelium and normal goblet cells. The effect of etoposide nanosuspension (ENSP-7) on sheep nasal mucosa, 8h after permeation studies (treated, Figure 4.42b) showed no severe damage on
the integrity of nasal mucosa when compared to untreated control. A slight epithelial disruption was observed on nasal mucosa after 8 h of permeation study.

Figure 4.42. Histopathology of sheep nasal mucosa. a) Nasal mucosa of untreated control (20X) b) Nasal mucosa permeated with ENSP-7 (20X).

4.2.3.3. Nasal absorption studies of etoposide nanosuspension by in situ nasal perfusion in male Wistar rats

Nasal absorption of commercial etoposide formulation (FY) and optimized etoposide nanosuspension (ENSP-7) was studied by in situ nasal perfusion in male Wistar rats. The commercial formulation and nanosuspension formulation of etoposide (ENSP-7) showed progressive nasal absorption with time. A plot of percentage drug remaining in the perfusing solution versus time is shown in Figure 4.43. Etoposide nanosuspension (ENSP-7) exhibited better nasal absorption compared to the commercial etoposide formulation (FY). The first order nasal absorption rate constants for commercial etoposide formulation (FY) and optimized etoposide nanosuspension formulation (ENSP-7) were calculated from the slopes of first-order plots of the percentage drug remaining in the perfusing solution versus time (Shinichiro et al., 1981; Huang et al., 1985). The first order nasal absorption rate constants for commercial etoposide formulation (FY) and ENSP-7 were found to be $3.455 \times 10^{-3}$ and $4.376 \times 10^{-3}$ min$^{-1}$ respectively. There is a statistically significant ($p < 0.05$) increase in first order nasal absorption rate constant value for optimized etoposide nanosuspension (ENSP-7) when compared to that of commercial etoposide formulation (FY). This increase in nasal absorption for etoposide nanosuspension may be due to enhanced permeation because of smaller average particle size (311.4±11.19 nm).
Results & Discussion

Figure 4.43. Percentage of etoposide remaining in the perfusate versus time after nasal perfusion of rats with etoposide commercial formulation (FY) and etoposide nanosuspension ENSP-7 (n=3).

When the nasal absorption of etoposide from etoposide polymeric nanoparticles (EPG-23 & EB-4) was compared with that of etoposide nanosuspension (ENSP-7), better nasal absorption was observed for EPG-23 (Figure 4.44). The first order nasal absorption rate constant value for PLGA 50:50 nanoparticles (EPG-23) was better than that of EB-4. This increased nasal absorption of EPG-23 may be due to comparatively faster release of etoposide from PLGA 50:50 polymeric matrix than from PCL polymeric matrix. Among the three etoposide nanoformulations, etoposide nanosuspension (ENSP-7) exhibited slower nasal absorption, this may be due to the fact that absence of polymeric coating lowered the residence time on nasal mucosa due to lack of bioadhesion.

Figure 4.44. Percentage of etoposide remaining in the perfusate versus time after nasal perfusion of rats with etoposide commercial formulation (FY) and etoposide nanoformulations.
4.2.3.3.4. *In vitro* cytotoxic studies (MTT assay) of etoposide nanosuspension

In the *in vitro* cytotoxicity study, human glioblastoma cells (LN229) were incubated with various concentrations of both pure drug and etoposide nanosuspension for 48 h and 96 h separately. Percentage cell viability (Y-axis) was plotted against concentration of the etoposide nanoparticle (X-axis). IC$_{50}$ value (concentration of nanoparticles that reduces the viability of cell to 50%) was determined for pure etoposide and etoposide nanosuspension (ENSP-7) for both 48 h and 96 h treatment. Results revealed a dose dependent reduction in the percentage cell viability after treatment with etoposide nanosuspension and pure etoposide (Figure 4.45). For both shorter and longer incubations (48 & 96 h) cytotoxicity of etoposide nanosuspension (ENSP-7) were low at all tested concentrations than that of pure etoposide (Table 4.12). The result suggests that etoposide nanosuspension (ENSP-7) exhibited an enhanced permeability of etoposide in cancer cells compared to that of pure etoposide (ETP pure) (Averineni *et al.*, 2012). Thus the IC$_{50}$ value was significantly ($p < 0.05$) decreased with etoposide nanosuspension (ENSP-7) compared to pure etoposide, indicating enhanced antitumor activity of nanoparticles against human glioblastoma cells LN229.

When the cytotoxicity of etoposide nanosuspension (ENSP-7) following 96 h of incubation was compared with that of etoposide loaded PCL nanoparticles (EB-4) and etoposide loaded PLGA nanoparticles (EPG-23), polymeric nanoparticles of etoposide (EB-4 & EPG-23) exhibited better cytotoxicity. This increased cytotoxicity of etoposide loaded polymeric nanoparticles may be due to increased retention of etoposide in cancer cells.

**Figure 4.45.** *In vitro* cytotoxicity studies of etoposide nanosuspension in human glioblastoma cells.
Table 4.1. *In vitro* cytotoxicity of etoposide nanosuspension in human glioblastoma cells

<table>
<thead>
<tr>
<th>Sample code</th>
<th>IC₅₀±SEM µg/mL After 48 hours</th>
<th>IC₅₀±SEM µg/mL After 96 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETP pure</td>
<td>4.64±0.27</td>
<td>3.46±0.21</td>
</tr>
<tr>
<td>ENSP-7</td>
<td>3.93±0.25</td>
<td>2.55±0.17*</td>
</tr>
</tbody>
</table>

*P<0.05 versus ETP pure (after 96 h).

4.2.3.3.5. Accelerated stability studies

Stability studies of optimized batch of freeze-dried etoposide nanosuspension (ENSP-7) was carried out and stability of formulation was evaluated on the basis of physical appearance, particle size and zeta potential as main parameters. The freeze dried nanoparticles did not show any change in visual appearance and has also retained its free flowing properties. There was slight increase in average particle size and a slight decrease in zeta potential was observed on three-month storage. As there is no significant alteration in average particle size and zeta potential it can be concluded that the etoposide nanosuspension formulation ENSP-7 is stable at 25±2 °C / 60±5 % RH for a period of 3 months (Table 4.13).

Table 4.13. Accelerated stability studies of ENSP-7

<table>
<thead>
<tr>
<th>Stability parameter</th>
<th>Test period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 month</td>
</tr>
<tr>
<td>Particle sizea (nm)</td>
<td>311.4±11.19</td>
</tr>
<tr>
<td>Zeta Potentiala (mV)</td>
<td>-29.3±2.7</td>
</tr>
</tbody>
</table>

*aThese results are mean±standard deviation. (n=3).

4.3. *In vivo* Pharmacokinetic Evaluation of Optimized Etoposide Nanoformulations

Optimized nanoformulations of etoposide (ENSP-7 & EPG-23) with desirable physicochemical properties, better *in vitro* release profile and *in situ* nasal perfusion profiles were selected for *in vivo* pharmacokinetic studies. Pharmacokinetic profiles of these optimised etoposide nanoformulations in plasma, brain and CSF were evaluated following intravenous and intranasal administration at a dose of 5 mg/kg, to separate groups of male Wistar rats.
The plasma concentration-versus-time profile of etoposide obtained after the i.v. administration of the commercial etoposide formulation (FY), optimized etoposide nanosuspension (ENSP-7) and optimized etoposide PLGA polymeric nanoparticles (EPG-23) are shown in Figure 4.46. The plasma pharmacokinetic profiles for these formulations following i.v. administration is given in Table 4.14.

4.3.1. Pharmacokinetic parameters (plasma) after i.v. administration of etoposide nanoformulations

The concentration of etoposide in plasma declined exponentially with time for all tested formulations. The etoposide concentrations in plasma were higher for ENSP-7 and EPG-23 than FY-treated rat at all time points. The systemic exposure (AUC) and MRT obtained for both etoposide nanoformulations were greater than that of etoposide commercial formulation (FY) (Table 4.14). The clearance of etoposide was slower for ENSP-7 and EPG-23 than FY-treated rat. The i.v. administration of etoposide PLGA polymeric nanoparticles (EPG-23) in rats resulted in increased AUC, prolonged etoposide residence in systemic blood circulation (MRT) and lower clearance than etoposide nanosuspension (ENSP-7).

![Figure 4.46](image-url)

**Figure 4.46.** Etoposide plasma concentration versus time after i.v. administration (5 mg/kg) of FY, ENSP-7 and EPG-23 in rats (n=4).
Table 4.14. Pharmacokinetic parameters (plasma) after i.v. administration of etoposide nanoformulations

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameters</th>
<th>FY</th>
<th>ENSP-7</th>
<th>EPG-23</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{AUC}_{(0-12)}$ (µg.h/mL)</td>
<td>6.3±0.654</td>
<td>12.3±1.452*</td>
<td>16.0±2.073*</td>
</tr>
<tr>
<td>$\text{AUC}_{(0-\alpha)}$ (µg.h/mL)</td>
<td>6.8±0.711</td>
<td>15.5±1.675*</td>
<td>22.4±3.065*</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>3.0±0.455</td>
<td>7.0±0.931*</td>
<td>9.7±1.11*</td>
</tr>
<tr>
<td>Vd (mL/kg)</td>
<td>4184.2±354.32</td>
<td>3613.8±241.24*</td>
<td>2998.8±131.37*</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>4.0±0.577</td>
<td>7.8±0.869*</td>
<td>9.3±1.05*</td>
</tr>
<tr>
<td>CL (mL/h/kg)</td>
<td>732.2±28.65</td>
<td>322.5±24.115*</td>
<td>223.1±13.107*</td>
</tr>
<tr>
<td>Kel (1/h)</td>
<td>0.175±0.018</td>
<td>0.089±0.009*</td>
<td>0.074±0.008*</td>
</tr>
</tbody>
</table>

All values reported are mean±S.D. (n=4). * Significantly different (p<0.05) at 95 % confidence interval compared to FY.

4.3.2. Pharmacokinetic parameters (plasma) after i.n. administration of etoposide nanoformulations

The plasma concentration-versus-time profile of etoposide obtained after the i.n. administration of the etoposide commercial formulation (FY), etoposide nanosuspension (ENSP-7) and etoposide PLGA polymeric nanoparticles (EPG-23) is shown in Figure 4.47. The plasma pharmacokinetic profiles for these formulations following i.n. administration is given in Table 4.15.

The concentrations of etoposide in plasma were higher for ENSP-7 and EPG-23 than FY-treated rat at all time points. The $\text{AUC}_{(0-12)}$ and MRT obtained for both etoposide nanoformulations were greater than that of etoposide commercial formulation (FY). The clearance of etoposide was slower for ENSP-7 and EPG-23 than FY-treated rat. The i.n administration of etoposide PLGA polymeric nanoparticles (EPG-23) in rats resulted in prolonged etoposide residence in systemic blood circulation as indicated by higher MRT, $t_{1/2}$ and lower clearance than ENSP-7.
Results & Discussion

Figure 4.47. Etoposide plasma concentration versus time after i.n. administration (5 mg/kg) of FY, ENSP-7 and EPG-23 in rats (n=4).

Table 4.15. Pharmacokinetic parameters (plasma) after i.n. administration of etoposide nanoformulations

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameters</th>
<th>FY</th>
<th>ENSP-7</th>
<th>EPG-23</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (µg/mL)</td>
<td>0.443±0.067</td>
<td>0.621±0.093*</td>
<td>0.563±0.085*</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>1.0</td>
<td>1.0</td>
<td>3.0</td>
</tr>
<tr>
<td>$AUC_{(0-12)}$ (µg.h/mL)</td>
<td>3.1±0.411</td>
<td>5.0±0.743*</td>
<td>5.1±0.622*</td>
</tr>
<tr>
<td>$AUC_{(0-\alpha)}$ (µg.h/mL)</td>
<td>4.0±0.583</td>
<td>7.2±0.931*</td>
<td>7.8±0.948*</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>8.2±1.014</td>
<td>10.1±1.273*</td>
<td>11.6±1.357*</td>
</tr>
<tr>
<td>Vd (mL/kg)</td>
<td>10448.9±699.75</td>
<td>6753.2±367.30*</td>
<td>6968.7±471.83*</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>5.8±0.635</td>
<td>6.8±0.742*</td>
<td>7.6±0.915*</td>
</tr>
<tr>
<td>CL (mL/h/kg)</td>
<td>1238.156±74.41</td>
<td>690.4±41.371*</td>
<td>638.4±33.925*</td>
</tr>
<tr>
<td>Kel (1/h)</td>
<td>0.118±0.015</td>
<td>0.102±0.017*</td>
<td>0.092±0.016*</td>
</tr>
</tbody>
</table>

All values reported are mean±S.D. (n=4). * Significantly different (p<0.05) at 95 % confidence interval compared to FY.
4.3.3. Pharmacokinetic parameters of etoposide nanoformulations in brain and CSF after i.v. administration

The brain and CSF concentration-versus-time profile of etoposide obtained after the i.v. administration of the etoposide solution (FY), etoposide nanosuspension (ENSP-7) and etoposide PLGA polymeric nanoparticles (EPG-23) are shown in Figure 4.48 & 4.49. The brain/CSF pharmacokinetic profiles for these formulations following i.v. administration is given in Table 4.16 & 4.17. The concentration of etoposide reached brain/CSF was higher for ENSP-7 and EPG-23 than FY-treated rat at all time points. The AUC(0–12) and MRT obtained for both etoposide nanoformulations were greater than that of etoposide commercial formulation (FY) (Table 4.16 & 4.17). The clearance of etoposide was slower for ENSP-7 and EPG-23 than FY-treated rat. The overall brain/CSF kinetics of the commercial etoposide formulation (FY) was different than that of etoposide nanoformulations. The i.v. administration of etoposide PLGA polymeric nanoparticles (EPG-23) in Wistar rats resulted in prolonged etoposide residence in brain and CSF. EPG-23 showed higher AUC, MRT, t1/2 and lower clearance than ENSP-7 in both brain and CSF.

**Pharmacokinetic parameters (brain) after i.v. administration of etoposide nanoformulations**

![Figure 4.48](image-url)

**Figure 4.48.** Etoposide brain concentration versus time after i.v. administration (5 mg/kg) of FY, ENSP-7 and EPG-23 in rats (n=4).
Table 4.16. Pharmacokinetic parameters (brain) after i.v. administration of etoposide nanoformulations

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameters</th>
<th>FY</th>
<th>ENSP-7</th>
<th>EPG-23</th>
</tr>
</thead>
<tbody>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg/mL)</td>
<td>0.35±0.041</td>
<td>0.592±0.079*</td>
<td>0.538±0.065*</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (h)</td>
<td>3.0</td>
<td>1.0</td>
<td>3.0</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;(0-12)&lt;/sub&gt; (µg.h/mL)</td>
<td>3.1±0.411</td>
<td>4.5±0.655*</td>
<td>5.2±0.736*</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;(0-α)&lt;/sub&gt; (µg.h/mL)</td>
<td>5.3±0.611</td>
<td>9.0±1.01*</td>
<td>15.9±1.691*</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>13.4±1.577</td>
<td>17.8±1.965*</td>
<td>29.8±2.396*</td>
</tr>
<tr>
<td>Vd (mL/kg)</td>
<td>12160.2±885.38</td>
<td>10072.0±739.95*</td>
<td>9251.5±651.72*</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (h)</td>
<td>9.0±0.856</td>
<td>12.6±1.581*</td>
<td>20.4±2.593*</td>
</tr>
<tr>
<td>CL (mL/h/kg)</td>
<td>937.7±53.78</td>
<td>553.4±39.163*</td>
<td>314.6±25.034*</td>
</tr>
<tr>
<td>Kel (1/h)</td>
<td>0.077±0.004</td>
<td>0.055±0.003*</td>
<td>0.034±0.002*</td>
</tr>
</tbody>
</table>

All values reported are mean±S.D, (n=4). *Significantly different (p<0.05) at 95% confidence interval compared to FY.

Figure 4.49. Etoposide CSF concentration versus time after i.v. administration (5 mg/kg) of FY, ENSP-7 and EPG-23 in rats (n=4).
Table 4.17. Pharmacokinetic parameters (CSF) after i.v. administration of etoposide nanoformulations

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameters</th>
<th>FY</th>
<th>ENSP-7</th>
<th>EPG-23</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ ($\mu$g/mL)</td>
<td>0.31±0.022</td>
<td>0.52±0.051*</td>
<td>0.49±0.050*</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>3.0</td>
<td>1.0</td>
<td>3.0</td>
</tr>
<tr>
<td>$AUC_{(0-12)}$ ($\mu$g.h/mL)</td>
<td>2.8±0.203</td>
<td>4.0±0.312*</td>
<td>4.5±0.331*</td>
</tr>
<tr>
<td>$AUC_{(0-\alpha)}$ ($\mu$g.h/mL)</td>
<td>4.2±0.595</td>
<td>7.5±0.655*</td>
<td>11.2±1.153*</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>11.0±0.924</td>
<td>15.8±1.031*</td>
<td>22.8±1.975*</td>
</tr>
<tr>
<td>Vd (mL/kg)</td>
<td>12520.9±831.73</td>
<td>10673.7±638.93*</td>
<td>9969.2±436.38*</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>7.2±0.572</td>
<td>11.1±1.007*</td>
<td>15.5±1.328*</td>
</tr>
<tr>
<td>CL (mL/h/kg)</td>
<td>1200.8±85.42</td>
<td>669.2±44.37*</td>
<td>445.8±45.28*</td>
</tr>
<tr>
<td>Kel (1/h)</td>
<td>0.096±0.006</td>
<td>0.063±0.005*</td>
<td>0.045±0.003*</td>
</tr>
</tbody>
</table>

All values reported are mean±S.D. (n=4). * Significantly different (p<0.05) at 95% confidence interval compared to FY.

4.3.4. Pharmacokinetic parameters of etoposide nanoformulations in brain and CSF after i.n. administration

The brain and CSF concentration-versus-time profile of etoposide obtained after the i.n. administration of the etoposide commercial formulation (FY), etoposide nanosuspension (ENSP-7) and etoposide PLGA polymeric nanoparticles (EPG-23) are shown in Figure 4.50 & 4.51. The concentration of etoposide reached brain was higher for ENSP-7 and EPG-23 than FY-treated rat at all time points. The $AUC_{(0-12)}$ and MRT obtained for both etoposide nanoformulations were greater than that of etoposide commercial formulation (FY) (Table 4.18 & 4.19). The clearance of etoposide from brain/CSF was slower for ENSP-7 and EPG-23 than for FY-treated rat. The overall brain/CSF kinetics of the etoposide commercial formulation (FY) was different from that of etoposide nanoformulations. The i.n. administration of etoposide PLGA polymeric nanoparticles (EPG-23) in rats resulted in prolonged etoposide residence in brain and CSF. EPG-23
showed higher AUC, MRT, $t_{1/2}$ and lower clearance than ENSP-7. The $T_{\text{max}}$ for ENSP-7 after i.n. administration was 0.5 h while for EPG-23 $T_{\text{max}}$ was 3 h.

**Figure 4.50.** Etoposide brain concentration versus time after i.n. administration (5 mg/kg) of FY, ENSP-7 and EPG-23 in rats (n=4).

**Table 4.18. Pharmacokinetic parameters (brain) after i.n. administration of etoposide nanoformulations**

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameters</th>
<th>FY</th>
<th>ENSP-7</th>
<th>EPG-23</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (µg/mL)</td>
<td>3.1±0.25</td>
<td>6.1±0.75*</td>
<td>5.2±0.63*</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>1.0</td>
<td>0.5</td>
<td>3.0</td>
</tr>
<tr>
<td>$\text{AUC}_{(0-12)}$ (µg.h/mL)</td>
<td>17.4±1.976</td>
<td>27.4±1.539*</td>
<td>35.8±3.921*</td>
</tr>
<tr>
<td>$\text{AUC}_{(0-\infty)}$ (µg.h/mL)</td>
<td>24.5±2.639</td>
<td>44.7±6.642*</td>
<td>65.6±8.927*</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>9.8±0.967</td>
<td>13.3±1.415*</td>
<td>15.5±1.781*</td>
</tr>
<tr>
<td>Vd (mL/kg)</td>
<td>2380.9±81.52</td>
<td>1780.8±62.61*</td>
<td>1229.3±51.49*</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>8.1±0.211</td>
<td>11.0±0.335*</td>
<td>11.2±0.529*</td>
</tr>
<tr>
<td>CL (mL/h/kg)</td>
<td>204.03±12.14</td>
<td>111.94±10.42*</td>
<td>76.26±5.95*</td>
</tr>
<tr>
<td>Kel (1/h)</td>
<td>0.086±0.005</td>
<td>0.063±0.004*</td>
<td>0.062±0.005*</td>
</tr>
</tbody>
</table>

All values reported are mean±S.D. (n=4). * Significantly different (p<0.05) at 95 % confidence interval compared to FY.
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**Figure 4.51.** Etoposide CSF concentration versus time after i.n. administration (5 mg/kg) of FY, ENSP-7 and EPG-23 in rats (n=4).

**Table 4.19. Pharmacokinetic parameters (CSF) after i.n. administration of etoposide nanoformulations**

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameters</th>
<th>FY</th>
<th>ENSP-7</th>
<th>EPG-23</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (µg/mL)</td>
<td>2.18±0.173</td>
<td>4.0±0.297*</td>
<td>3.48±0.211*</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>1.0</td>
<td>0.5</td>
<td>3.0</td>
</tr>
<tr>
<td>AUC$_{(0-12)}$ (µg.h/mL)</td>
<td>13.8±1.522</td>
<td>24.3±1.929*</td>
<td>29.4±2.559*</td>
</tr>
<tr>
<td>AUC$_{(0-a)}$ (µg.h/mL)</td>
<td>15.9±1.273</td>
<td>30.5±2.749*</td>
<td>42.0±3.427*</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>6.1±0.528</td>
<td>7.4±0.553*</td>
<td>9.9±0.731*</td>
</tr>
<tr>
<td>Vd (mL/kg)</td>
<td>1755.2±34.78</td>
<td>1294.2±41.39*</td>
<td>1121.3±47.26*</td>
</tr>
<tr>
<td>$t_{1/2}$ (h)</td>
<td>3.9±0.198</td>
<td>5.5±0.429*</td>
<td>6.5±0.376*</td>
</tr>
<tr>
<td>CL (mL/h/kg)</td>
<td>314.92±15.72</td>
<td>163.96±10.79*</td>
<td>119.09±9.31*</td>
</tr>
<tr>
<td>Kel (1/h)</td>
<td>0.179±0.022</td>
<td>0.127±0.014*</td>
<td>0.106±0.009*</td>
</tr>
</tbody>
</table>

All values reported are mean ±S.D. (n=4). * Significantly different (p<0.05) at 95% confidence interval compared to FY.
4.3.5. Etoposide concentration in brain and CSF following i.v. and i.n. administration: A comparison

Etoposide concentration in brain /CSF following i.v. and i.n. administration of etoposide nanoformulations showed significant differences. Following i.v. administration, the peak brain/CSF concentration of etoposide was far lower than the peak brain/CSF concentration of etoposide following i.n. administration. Following i.n. administration of etoposide nanoformulations (ENSP-7 & EPG-23), etoposide was conveyed to the brain very quickly which could be detected in 5 min in all brain tissues. The profile of etoposide levels in the brain/CSF showed an initial absorption phase and a slow elimination phase. The AUC values in brain tissue /CSF after i.n. administration were all much higher than those obtained after i.v. administration. This also signifies the presence of direct nose to brain pathway which could be convincing for treatment of brain tumour.

4.3.6. Drug targeting effect of etoposide nanoformulations

The degree of etoposide targeting to brain or CSF after intranasal administration can be evaluated by calculating drug targeting index (DTI). Drug targeting index is the ratio of the value of AUC\textsubscript{CSF/brain}\textsubscript{}/AUC\textsubscript{plasma} following intranasal administration to that following intravenous injection. The higher the DTI is, the more the degree of etoposide targeting to brain and CSF after intranasal administration. The DTI values of etoposide nanoformulations to brain and CSF are shown in Figure 4.52. For all etoposide formulations following i.n. administration, the DTI values were far greater than 1.0, which suggested etoposide targeting to the brain and CSF following i.n. administration of etoposide nanoformulations.

![Drug targeting index of etoposide nanoformulations](Figure 4.52)

**Figure 4.52.** Drug targeting index of etoposide nanoformulations after nasal administration. Data represent the mean±S.D.
Among the etoposide nanoformulations etoposide PLGA 50:50 polymeric nanoparticles (EPG-23) showed maximum etoposide targeting to brain after i.n. administration. The DTI of EPG-23 was 2 fold more than that of commercial etoposide formulation (FY). These findings projected the possibility of intranasal route as an alternative transport pathway to the brain and CSF apart from the penetration across the BBB from the systemic circulation. Further etoposide nanoformulations like nanosuspension (ENSP-7) and polymeric nanoparticle (EPG-23) showed increased brain targeting than commercial etoposide solution (FY). Thus etoposide nanoformulation may be helpful for both increasing the brain therapeutic levels and reducing the systemic side effects.

4.3.7. Nose to brain direct transport percentage of etoposide formulations

The uptake of drug into brain from nose is believed to follow two pathways. Some amount of drug reaches brain through systemic circulation by crossing blood brain barrier. While the other pathway is through olfactory region in the nasal cavity directly into CSF and brain tissue (Illum, 2000). Another parameter to assess the direct transport of drug to the brain is, nose to brain direct transport percentage (DTP %). This is calculated using brain tissue distribution data of drug following i.n. and i.v. administration (Zhang et al., 2004; Vyas et al., 2006). The % DTP values of etoposide nanoformulations are shown in Figure 4.53. High % DTP values of etoposide nanoformulations following i.n. administration, suggested that there was a great portion of etoposide targeting to the brain following i.n. administration of etoposide nanoformulations. Among the etoposide nanoformulations etoposide PLGA polymeric nanoparticles (EPG-23) showed maximum nose to brain direct transport of etoposide as indicated by higher % DTP values.

The higher DTI and % DTP of etoposide nanoformulations may be due to reduced clearance compared to etoposide commercial formulation. These results are in agreement with previously reported studies, where nanoformulations were found to enhance direct transport to brain of drugs like risperidone, saquinavir and nimodipine upon i.n. administration (Zhang et al., 2004; Kumar et al., 2008; Mahajan et al., 2014). The drug can be released from these nanoformulations by diffusion and undergo further transport into olfactory bulb and brain stem finally reaching brain or cerebrospinal fluid.
**Summary and Conclusion**

Delivery of drugs to the central nervous system (CNS) has always been a challenge due to the presence of blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier. Many novel drug molecules have failed to reach CNS at therapeutically effective dose due to their high molecular weight and low lipophilicity. Development of a formulation whose particle size is in nanometer range and use of biodegradable polymers which are lipophilic in nature can solve this issue. Apart from this, intranasal route is an attractive non-invasive route that can offer advantages such as rapid absorption, avoidance of liver and gut first-pass metabolism, ease of administration, and self-medication. It is also well established that there exist a direct anatomical connection between the nasal cavity and the CNS by paracellular and transcellular pathway as well as through olfactory and trigeminal neurons which suggests the scope of development of CNS therapeutics for intranasal (i.n.) administration. Therefore this route could be convincing for treatment of brain tumour where the nose to brain pathway might provide a faster and specific therapeutic effect (Illum 2002).

Etoposide is a well-known antineoplastic drug with proven anticancer activity. Many antineoplastic drugs with proven therapeutic efficacy towards a particular cancer cause intolerable toxicological consequences when introduced systemically due to random biodistribution throughout the body, lack of drug specific affinity towards a pathological site, necessity of a large total dose of a drug to achieve high local concentration, non-specific toxicity and other adverse side-effects due to high drug doses. A novel approach to overcome these limitations is to develop new dosage forms which target drugs to their...
site of action and sustains the release of drug for prolonged action at a therapeutically optimal rate and dose regimen. Nanoparticles are thus designed for their benefit of possessing nano-size range of particles which can infiltrate through the sites of inflammation and cancer cells and are mostly taken up by the cells leading to effective drug accumulation at the target sites. Enhanced permeation and retention (EPR) effect also permits the passive targeting of drugs encapsulated in nanoparticles to the tumour site.

Hence advantages of intranasal route as a promising strategy for delivering therapeutics to brain using nanoparticulate drug delivery systems of anticancer drug etoposide is utilized. The present study involves formulation development and evaluation of nanoparticulate drug delivery systems of anticancer drug etoposide intended to be administered through nasal route for the treatment of brain cancer.

4.4.1. Preformulation studies

Preformulation studies are helpful in developing a thorough understanding of physical and chemical properties of the drug candidate alone and in combination with various excipients used in the formulation. These studies help in generating significant data of physicochemical aspects of the drug as well as the formulation under investigation, thus enabling the development of stable dosage forms with appropriate bioavailability over the shelf-life. Solubility studies, drug excipient interaction studies using differential scanning calorimetry and FTIR, analytical method development and validation were planned and executed under preformulation studies.

Solubility of etoposide was evaluated in water and in different buffers of pH ranging from 1.3 to 7.4 by shake flask method. The aqueous solubility of etoposide at 37 °C was 112.46-154.38 μg/mL which was low, and did not vary much over the pH range 3.0-5.0. Low aqueous solubility and rapid degradation at pH 1.3 may be the reasons for the reported low oral bioavailability of etoposide. Drug excipient interaction studies were carried out using DSC and FTIR, on the mixtures of drug and individual excipients in equal proportions after storing in a stability chamber at 25±2 °C / 60±5 % RH for four week time period. The pure etoposide and etoposide in the presence of other excipients apparently did not show any characteristic change in morphological characteristics. IR spectrum of physical mixture of etoposide and excipients in equal proportions showed all the characteristic peaks of the drug without any appreciable shift which indicates that there is no significant interaction between the drug and the excipients. DSC spectra also suggested that there was
no significant shift in the thermogram peaks compared to that of pure drug and pure excipients.

A rapid, sensitive, specific, accurate and precise RP-HPLC method has been developed and validated for determination of etoposide in nanoformulations. The analytical method was successfully applied in analysis of encapsulation efficiency, *in vitro* release studies, *ex vivo* permeation studies and *in situ* nasal perfusion studies. The estimation of etoposide in plasma, CSF and brain tissues by RP-HPLC was carried out using an early reported validated bioanalytical method.

### 4.4.2. Formulation of polymeric nanoparticles of etoposide

Polymeric nanoparticles of etoposide using biodegradable synthetic polymers such as PCL and PLGA 50:50 were carried out with the view of developing nanoformulations to deliver anticancer agents to brain through nasal route. This method is hypothesized to encompass the advantages such as small size of nanoparticles which would help the delivery across BBB, the release pattern and biodegradability of the polymer which would ensure release of drug at the target site without accumulating toxic degradation products of polymer and the use of nasal route which enables nose to brain transport through olfactory and respiratory epithelium.

#### 4.4.2.1. Formulation of etoposide PCL nanoparticles

* Polymeric nanoparticles of etoposide with PCL were prepared by emulsification solvent evaporation method (*o/w* emulsion). In order to arrive at the best possible optimized formulation with respect to particle size and encapsulation efficiency, various ratios of drug to polymer in presence of various surfactants such as PVA, pluronic F-68, pluronic F-127, SC etc. were tried at varying concentrations. An increase in entrapment efficiency and decrease in particle size was observed when the drug: polymer ratio was increased from 1:1 to 1:10. Nevertheless further increase in drug: polymer ratio resulted in a decrease in the drug entrapment and increase in the particle size of the nanoparticles. A 1.0 % w/v solution of PVA was selected as the stabilizer as it gave lower particle size and maximum encapsulation efficiency without particle aggregation.

* High speed homogenization technique was employed to obtain etoposide PCL nanoparticles. Decrease in particle size was observed with increase in homogenization
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speed and sonication amplitude. However increase in homogenization speed above 20000 rpm and sonication amplitude above 80 % did not show any decrease in particle size.

* Various cryoprotectants such as sucrose, fructose, dextrose and mannitol with suitable concentrations were tested for their effect on freeze drying process of etoposide polymeric nanoparticles. Among these, mannitol yielded completely dry and free flowing powders with lowest particle size. 5 % w/v mannitol was found to be sufficient to obtain nanoparticles of desirable properties.

* The optimized batch selected based on the results was EB-4 which showed a particle size of 518.0±10.02, zeta potential value -20.6±2.1, PDI 0.260±0.018 and encapsulation efficiency 73.07±3.11%.

* Characterization of the optimized batch of etoposide PCL nanoparticles EB-4 was carried out by studying the FTIR, thermogram properties and scanning electron microscopic studies.

* FTIR spectrum of etoposide loaded PCL nanoparticle (EB-4) showed all the characteristic peaks of the drug without any appreciable shift, which confirms the encapsulation of drug and also that there is no significant interaction between the drug and the excipients during the formation of nanoparticles.

* The thermogram of etoposide loaded nanoparticles exhibited endotherms at 63.84 °C and 171.53 °C which correspond to melting endotherms of PCL polymer and mannitol respectively. This thermogram did not show any detectable endotherm corresponding to the melting temperatures of free drugs between 200–300 °C. This is due to the conversion of drug from crystalline to amorphous form. Thus it is understood that the etoposide in PCL nanoparticles existed in amorphous form of a molecular dispersion or solid solution state in the polymer matrix.

* The SEM photomicrograph of optimized etoposide PCL nanoparticles obtained before and after gold sputtering confirmed their spherical shape. The particles were homogeneous with smooth surface without any rupture and exhibited moderate uniformity without any aggregation.

* Evaluation of release profile, ex vivo permeation through nasal mucosa, in situ nasal absorption, in vitro cytotoxicity and stability studies were carried out on the optimized batch of etoposide PCL nanoparticles.
The *in vitro* release profiles of formulations EB-3, EB-4 and EB-5 showed biphasic behaviour consisting of initial burst release followed by a sustained release phase for the period of 72 h. The initial burst release of drugs may be because of the release of etoposide loosely bound on the surface of the nanoparticles. Sustained release may be due to slow diffusion of the drug from the highly lipophilic polymeric matrix. *In vitro* drug release kinetics of optimized etoposide PCL nanoparticles (EB-4) was best explained by Higuchi kinetic diffusion control mechanism. Further, the release of etoposide from PCL nanoparticle (EB-4) was by non-Fickian diffusion (diffusion coupled with erosion).

The drug permeation pattern through sheep nasal mucosa reflected the release pattern as shown for *in vitro* release studies, but the amount of drug permeated was lower when compared to that in release studies. Among the three formulations (EB-3, EB-4 & EB-5) selected for the study, formulation EB-4 exhibited better permeation across the sheep nasal mucosa. The cumulative percentage of etoposide permeated from EB-4 after 8 h was found to be 16.421±1.61%, after triplicate determinations. The examination of sheep nasal mucosa, after 8h permeation studies showed no severe damage on the integrity of nasal mucosa when compared to untreated control.

The *in situ* nasal perfusion studies showed a statistically significant (*p* < 0.05) increase in first order nasal absorption rate constant value for optimized etoposide PCL nanoparticle (EB-4) when compared to that of commercial etoposide formulation (FY). This increase in nasal absorption for etoposide loaded PCL nanoparticles may be due to smaller particle size (518.0 nm) and bioadhesive nature of PCL polymer, as it binds to nasal mucosa and increase the residence time which consequently increases the absorption.

Results of *in vitro* cytotoxicity studies on LN229 cells by MTT assay revealed a dose dependent reduction in the percentage cell viability after treatment with etoposide nanoparticles or pure etoposide. At shorter incubation time (48 h) cytotoxicity of etoposide nanoparticles were low at all tested concentrations. While for longer incubations (96 h) etoposide nanoparticle (EB-4) showed better cytotoxicity at all tested concentrations than that of pure etoposide.

Stability studies of optimized batch of freeze-dried etoposide nanoparticle EB-4 was carried out and stability of formulation was evaluated on the basis of physical
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appearance, particle size, zeta potential, and encapsulation efficiency as main parameters. As there is no significant alteration in average particle size, zeta potential and encapsulation efficiency it can be concluded that the etoposide nanoparticle formulation EB-4 is stable at 25±2 °C / 60±5 % RH for a total period of 3 months.

4.4.2.2. Formulation of etoposide PLGA 50:50 nanoparticles

* Nanoprecipitation method was used for the preparation of etoposide loaded PLGA 50:50 nanoparticles using acetone as the solvent, as better particle size and encapsulation efficiency was achieved by this method.

* When organic to aqueous phase ratio was 1:2, encapsulation efficiency was reasonably good with low particle size. The nanoparticles prepared using optimized parameters had a percentage encapsulation efficiency of 88.57±5.95 % and particle size 143.4±10.05 nm, with a polydispersity index and zeta potential of 0.095±0.017 and -33.1±2.55 mV respectively. Percentage encapsulation efficiency of all batches of etoposide PLGA 50:50 nanoparticles was found in the range of 20.69±1.83 to 88.57±5.95.

* Various surfactants like PVA (L) (low molecular weight -M.W. 11,000-31,000), PVA (H) (high molecular weight M.W. 30,000–70,000) and pluronic F-68 were tried to study the influence of type of surfactant on physicochemical properties of etoposide nanoparticles. An optimum concentration of 0.5 % w/v pluronic F-68 was selected as the stabilizer as it gave lower particle size, better zeta potential and maximum encapsulation efficiency without particle aggregation.

* As the drug: polymer ratio increased from 1:5 to 1:10 using 0.5 %w/v of PVA(H) as stabilizer, entrapment efficiency and particle size increased. Drug to polymer ratio of 1:10 using 0.5 %w/v of pluronic F-68 as stabilizer showed an optimum encapsulation efficiency of 88.57±5.95 % and particle size 143.4±10.05 nm. The optimized formulation (EPG-23) with highest encapsulation efficiency was further used for in situ nasal perfusion studies and in vivo pharmacokinetic studies.

* Various cryoprotectants such as sucrose, fructose, dextrose and mannitol with suitable concentrations were tested for their effect on freeze drying process of etoposide PLGA nanoparticles. Among them mannitol yielded completely dry and free flowing powders with lowest particle size. 5 % w/v mannitol was found to be sufficient to obtain nanoparticles of desirable properties.
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* The optimized batch selected based on the results was EPG-23 which showed a particle size of 143.4±10.05 nm, zeta potential value -33.1±2.55, PDI 0.095±0.017 and encapsulation efficiency 88.57±5.95 %.

* Characterization of the optimized batch of etoposide PLGA 50:50 nanoparticles EPG-23 was carried out by studying the FTIR and thermogram properties and scanning electron microscopic studies.

* The FTIR spectrum of etoposide loaded PLGA 50:50 nanoparticle (EPG-23) showed all the characteristic peaks of the drug without any appreciable shift which confirms the encapsulation of drug and also that there is no significant interaction between the drug and the excipients during the formation of nanoparticles.

* The DSC thermogram of EPG-23 did not show any detectable endotherm corresponding to the melting temperature of free drug between 200-300 °C indicating that the etoposide in PLGA 50:50 nanoparticles existed in amorphous form of a molecular dispersion or solid solution state in the polymer matrix.

* The SEM photomicrograph with and without gold coating confirmed the spherical shape, smooth surface and homogeneity of EPG-23 nanoparticles.

* Evaluation of release profile, ex vivo permeation through nasal mucosa, in situ nasal absorption, in vitro cytotoxicity and stability studies were carried out on the optimized batch of etoposide PLGA 50:50 nanoparticles.

* The in vitro release profiles of EPG-21, EPG-22, EPG-23 and EPG-25 showed biphasic behaviour consisting of initial burst release followed by a sustained release phase for the period of 72 h. The initial burst release of drugs may be due to the fraction of etoposide that was adsorbed on the surface of nanoparticles. Sustained release may be due to slow diffusion of the drug from the lipophilic polymeric matrix. In vitro drug release kinetics of optimized etoposide PLGA 50:50 nanoparticles (EPG-23) was best explained by Higuchi kinetic diffusion control mechanism and the release of etoposide from PLGA 50:50 nanoparticles (EPG-23) was by non-Fickian diffusion (diffusion coupled with erosion) as the Korsmeyer–Peppas release exponent value was found to be is >0.5.

* The drug permeation pattern of EPG-23 through sheep nasal mucosa reflected the release pattern as shown for in vitro release studies, but the amount of drug permeated
was lower when compared to that in release studies. Further no severe damage on the integrity of nasal mucosa was observed when compared to untreated control after 8h permeation studies.

* When the drug permeation pattern through sheep nasal mucosa from the optimized batch of etoposide loaded PCL nanoparticles (EB-4) was compared with that of optimized etoposide PLGA 50:50 nanoparticles (EPG-23), a notable change in the permeation rate was observed. The cumulative amount of etoposide permeated across the sheep nasal mucosa at the end of 8 h was 40.19% and 16.42% for EPG-23 and EB-4 respectively. This decrease in permeation of etoposide from PCL nanoparticles may be due to more hydrophobic nature of PCL than PLGA co-polymers.

* Nasal absorption of etoposide from commercial etoposide formulation (FY) and optimized etoposide loaded PLGA 50:50 nanoparticles (EPG-23) were evaluated by in situ nasal perfusion in male Wistar rats. The first order nasal absorption rate constants for FY and EPG-23 were found to be $3.455 \times 10^{-3}$ and $6.906 \times 10^{-3}$ min$^{-1}$ respectively. The increase in nasal absorption for etoposide loaded PLGA nanoparticles may be due to smaller particle size (143.4 nm) and bioadhesive nature of PLGA polymer, as it binds to nasal mucosa and increase the residence time which consequently increases the absorption.

* When the nasal absorption results of EB-4 and EPG-23 were compared, it was observed that the nasal absorption of EPG-23 was high which could be due to comparatively faster release of etoposide from PLGA 50:50 polymeric matrix than from PCL polymeric matrix.

* In vitro cytotoxicity studies on LN229 cells revealed that at shorter incubation time (48 h) cytotoxicity of etoposide loaded PLGA nanoparticles (EPG-23) were low at all tested concentrations than that of pure etoposide, while for longer incubations (96 h) EPG-23 showed better cytotoxicity at all tested concentrations than that of pure etoposide.

* When the in vitro cytotoxicity results of EB-4 and EPG-23 were compared, it was observed that etoposide loaded PLGA nanoparticles (EPG-23) exhibited better cytotoxicity at all tested concentrations both after 48 and 96 h of incubation. This increased cytotoxicity of EPG-23 may be due to comparatively faster release of etoposide from PLGA 50:50 polymeric matrix than from PCL polymeric matrix.
Results & Discussion

* Stability studies of optimized batch of freeze-dried etoposide nanoparticle EPG-23 showed a slight increase in average particle size and a slight decrease in zeta potential and % EE was observed on three-month storage. As there is no significant alteration in average particle size, zeta potential and % encapsulation efficiency it can be concluded that the etoposide nanoparticle formulation EPG-23 is stable at 25±2 °C / 60±5 % RH for a period of 3 months.

4.4.3. Formulation of etoposide nanosuspension

* Nanosuspension is a promising strategy for overcoming the problem of low bioavailability of poorly water soluble drugs. High pressure homogenization method was used for the preparation of etoposide nanosuspension using methanol as the solvent. The nanosuspension prepared using optimized parameters had a particle size of 311.4±11.19 nm, with a polydispersity index and zeta potential of 0.191±0.025 and -15.4±2.14 mV respectively.

* Surfactants such as PVA, and pluronic F-68 were studied and PVA was selected as the stabilizer as it gave lower particle size, better zeta potential and polydispersity index without particle aggregation. A reduction in the size of nanoparticles was observed when the stabilizer concentration was increased from 0.5 to 1.5% w/v. An optimum concentration (1.5 % w/v) of stabilizer led to a reduced size of nanoparticles dispersed in nanosuspension with better zeta potential and polydispersity index.

* In order to attain maximum reduction in the particle size, HPH cycles and pressure as well as sonication time and amplitude were varied and the effects were studied. Smaller etoposide particles were obtained with increased number of HPH cycles between 6 and 20. But, when the homogenization cycles were increased from 20 to 25, further reduction in particle size was not observed. Thus 20 cycles was selected as the optimized homogenization condition for preparation of all batches of etoposide nanosuspension. Probe sonication amplitude of 80% for 5 minutes was found to be optimum to reduce particle size in the present formulation batches of etoposide nanosuspension.

* FTIR spectrum and DSC thermogram confirmed that there is no significant interaction between the drug and the excipients during the preparation of nanosuspension. Appearance of melting endotherm of etoposide in the thermograph of optimized
etoposide nanosuspension (ENSP-7) confirmed the crystalline state of etoposide in the formulation, which was also evident in SEM studies.

* The cumulative drug release from ENSP-7 at the end of 12 h was found to be 88.31% which was very high when compared to that of EB-4 & EPG-23 which were 20.76% and 51.44% respectively. Similar results were also obtained in ex vivo nasal permeation studies which indicate that the high release profile and permeation rate is due to the lack of polymer layer.

* There was an increase in nasal absorption of etoposide from etoposide nanosuspension than the commercial formulation, which could be due to the enhanced permeation because of smaller average particle size (311.4±11.19 nm).

* The results of in vitro cytotoxicity studies using LN229 glioblastoma cells suggested an enhanced permeability of etoposide in cancer cells from ENSP-7 compared to that of pure etoposide, which was evident from low IC$_{50}$ values.

* Accelerated stability studies also suggested the suitability of storing nanosuspension in the freeze dried form for a period of three months.

4.4.4. **In vivo pharmacokinetic evaluation of optimized etoposide nanoformulations**

* Optimized nanoformulations of etoposide (ENSP-7 & EPG-23) with desirable physicochemical properties, better in vitro release profile and in situ nasal perfusion profiles were selected for in vivo pharmacokinetic studies. Pharmacokinetic profiles of these optimised etoposide nanoformulations in plasma, brain and CSF were evaluated following intravenous and intranasal administration at a dose of 5 mg/kg to separate groups of male Wistar rats.

* Comparison of etoposide concentration in brain/CSF following i.v. and i.n. administration of etoposide nanoformulations showed significant differences. Following i.v. administration, the peak brain/CSF concentration of etoposide was far lower than the peak brain/CSF concentration of etoposide following i.n. administration. Following i.n. administration of etoposide nanoformulations (ENSP-7 & EPG-23), etoposide was conveyed to the brain very quickly which could be detected in 5 min in all brain tissues. The profile of etoposide levels in the brain/CSF showed an initial absorption phase and a slow elimination phase. The AUC values in brain tissue/CSF after i.n. administration were all much higher than those obtained
after i.v. administration. This also signifies the presence of direct nose to brain pathway which could be convincing for treatment of brain tumour.

* Etoposide nanoformulations such ENSP-7 and EPG-23 showed increased brain targeting than commercial etoposide solution (FY) as indicated by higher Drug Targeting Index (DTI) values. Among the etoposide formulations etoposide PLGA polymeric nanoparticles (EPG-23) showed maximum etoposide targeting to brain after i.n. administration. Thus etoposide nanoformulation may be helpful for both increasing the brain therapeutic levels and reducing the systemic side effects.

* Another parameter to assess the direct transport of drug to the brain is, nose to brain direct transport percentage (DTP %). This is calculated using brain tissue distribution data of drug following i.n. and i.v. administration

* High % DTP values of etoposide nanoformulations following i.n. administration, suggested there was a great portion of etoposide targeting to the brain following i.n. administration of etoposide nanoformulations. Among the etoposide nanoformulations etoposide PLGA polymeric nanoparticles (EPG-23) showed maximum nose to brain direct transport of etoposide as indicated by higher % DTP values.

In conclusion, the etoposide nanoformulations such as etoposide loaded PCL and PLGA 50:50 nanoparticles and nanosuspension with desirable properties were successfully developed and optimized. The optimized etoposide loaded polymeric nanoparticles exhibited sustained release and followed non-Fickian diffusion based release kinetics. The optimized nanoformulations of etoposide exhibited better antitumor activity than that of pure etoposide against human glioblastoma cells LN229. The results of in situ nasal perfusion studies, demonstrate that optimized etoposide nanoformulations showed better nasal absorption compared to that of commercial etoposide formulation (FY). The outcomes of in vivo pharmacokinetic studies showed the brain targeting efficiency of developed etoposide nanoformulations and among the developed etoposide nanoformulations, etoposide loaded PLGA 50:50 nanoparticles (EPG-23) exhibited maximum brain targeting as showed by higher values of drug targeting index and nose to brain direct transport percentage (DTP %). Pharmacokinetic profiles of these optimized etoposide nanoformulations in plasma, brain and CSF following intravenous and intranasal administration confirms the suitability of developed etoposide nanoformulations for improved brain delivery by nasal route.
4.5. References


Results & Discussion


