Chapter Nine

SAFETY ASSESSMENT STUDIES
9. SAFETY ASSESSMENT OF DEVELOPED NANOFORMULATIONS

In order to confirm the safety of the optimised and selected nanoemulsion formulations, In vivo toxicity evaluation of ANE and AMNE was carried out in Wistar rats.

Following studies carried out for safety assessment of the developed formulations:

1. In-vivo toxicity studies:
   a. Mortality Count
   b. Nasal mucosal histology
   c. Brain Histology

2. Nasal cavity (mucosa) temperature measurement using NIR camera

3. Neurotoxicity studies by Rotarod method

4. In-vitro safety assessment

Methods:

9.1. In vivo toxicity

*In vivo* toxicity evaluation of ANE/AMNE was carried out to assess the mortality (if any) followed by nasal and brain histology studies (after 14 days treatment) at an equivalent dose respective to amiloride 0.16 mg/kg. The toxicity study was carried out on optimised and selected formulations using rats as animal models as per the reference taken from national toxicology programme ([http://ntp.niehs.nih.gov/go/9987](http://ntp.niehs.nih.gov/go/9987)).

*Animals used:*

Rats (n=6 per group) were used as experimental animals because they are most suitable, easily available and widely used for research especially *in vivo* tissue toxicity studies. Specific-pathogen-free, healthy, adult Wistar rats of either sex (3 months old; 200-250 g) were used in the tissue toxicity study. Tap water and rats food pellets were available *ad libitum* throughout the study. They were maintained in a room that was kept at 25° ± 2°C with relative humidity of 50 ± 5%.
Sample Preparation:
The samples were prepared and used as such in case of ANE/AMNE and administered the dosages of amiloride for rats except control group.

Methodology:
The rats were dosed once daily in morning (between 9.00 am to 10.00 am) with 50 μL of prepared ANE/ANME equivalent to 0.16 mg/kg of amiloride, by intranasal route for 14 days. Prior to treatment, every day the animals were examined for any abnormal behaviour, mortality and morbidity. For each formulation six rats (n=6) were used and divided in the following groups;

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Group</th>
<th>Treatment</th>
<th>Number of Animals</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Group I</td>
<td>Normal Saline</td>
<td>6</td>
<td>50 μL</td>
</tr>
<tr>
<td>2</td>
<td>Group II</td>
<td>ANE</td>
<td>6</td>
<td>0.16 mg/kg</td>
</tr>
<tr>
<td>3</td>
<td>Group III</td>
<td>AMNE</td>
<td>6</td>
<td>0.16 mg/kg</td>
</tr>
<tr>
<td>4</td>
<td>Group IV</td>
<td>Placebo</td>
<td>6</td>
<td>50 μL</td>
</tr>
</tbody>
</table>

*Amount administered 50 μL (20-25 μL in each nostril by keeping rat in supine position)*

After the completion of study period of 14 days, the rats were sacrificed by keeping them in desiccators containing diethyl ether for inhalation anaesthesia. Brain and Nasal mucosa were dissected out, fixed in 10% neutral buffered formalin solution. This prevented the post-mortem changes such as putrefaction and autolysis and preserved the cell-constituents in as life-like manner as possible. Transverse sections (T.S.) of the tissues were stained with hematoxylin and eosin and were examined microscopically for the severity of mucosal irritancy or brain tissue damage loss and atrophy. To evaluate any potential toxic effects of excipients used in the formulation on the nasal mucosa, the nasal mucosa of was dissected and microscopically evaluated for the toxic effects.

9.2. NASAL CAVITY (MUCOSAL) TEMPERATURE MEASUREMENT USING IR CAMERA

Infrared (IR) imaging was shown to be a useful method to diagnose the signs of certain diseases by measuring the local skin temperature (Herman et al., 2011;
Inflammation in human and animals is marked by several parameters such as pain swelling, immobility, and a rise in temperature of the affected part. Since human skin, irrespective of its pigmentation, is an almost perfect radiator of infrared radiation, there is a direct relationship between the temperature and emissivity of this organ (Collins & Ring, 1972).

By using the above mentioned hypothesis that skin/mucosal irritation or sensitization leads to the inflammation and this inflammation leads to localized temperature change, hence forth nasal cavity (mucosal) temperature measurements were obtained using an infrared camera (IR Camera (Extech i5 Infrared camera)) placed at an angle of approximately 90° from the surface, 25 cm from the plantar region of the nose of the animal and 75 cm above the floor post treatment every day (0-8h) for 7 days. This camera has a thermal sensitivity of approximately 0.08°C, with an error of 2°C. The images were digitally recorded at 30-second intervals. The thermographic procedures were conducted in a room at a constant temperature of 23°C (±1°C) and humidity between 30 and 50% (Thermo/hydro/clock, MT-230, Minipa, Brazil). Surface temperature was measured from 9:00 to 12:00 a.m.

9.3. NEUROTOXICITY STUDIES BY ROTAROD METHOD

The rotarod test according to Lima et al. (1993) was used to determine the effect of developed formulation on motor coordination. The integrity of the motor system was evaluated with the rotarod test. Briefly, the rotarod apparatus consists of a rod 30-cm long and 3 cm in diameter that is subdivided into three compartments by discs 24 cm in diameter. The rod rotates at a constant speed of 10 rpm. The trained animals were then evaluated for motor coordination at 7 and 14th days after i.n. administration of 0.16 mg/kg ANE/AMNE everyday for 14 days schedule. The fall off time of each animal was recorded. Grouping for neurotoxicity studies would be as per above mentioned Table 39.

9.4. IN-VITRO SAFETY ASSESSMENT

In-vitro safety of optimized formulation was assessed by observing their effect on histology of goat nasal mucosa which was used for permeation study. Out of three nasal mucosa pieces, one mucosa was used as control (0.6 mL water), the other was
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treated with 0.6 mL of optimized formulation and the last one was treated with KCl solution.

9.5. RESULTS AND DISCUSSION

1. In Vivo toxicity Studies: a. Mortality Count

Table 40: In-Vivo toxicity studies for Amiloride Nanoformulations: Mortality Counts

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Group</th>
<th>Treatment</th>
<th>Number of Animals</th>
<th>Dose</th>
<th>Upon completion of studies (14 Days) No. of Animals survived</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Group I</td>
<td>Normal Saline</td>
<td>6</td>
<td>50 μL</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Group II</td>
<td>ANE</td>
<td>6</td>
<td>0.16 mg/kg</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>Group III</td>
<td>AMNE</td>
<td>6</td>
<td>0.16 mg/kg</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>Group IV</td>
<td>Placebo</td>
<td>6</td>
<td>50 μL</td>
<td>6</td>
</tr>
</tbody>
</table>

*Amount administered 50 μL (20-25 μL in each nostril by keeping rat in supine position*

b. Nasal mucosa histology

![Photomicrographs showing the T.S. of rats’ nasal mucosa for vehicle control (control group), Placebo, ANE, AMNE treated groups after 14 days](image)

**Fig. 63:** Photomicrographs showing the T.S. of rats’ nasal mucosa for vehicle control (control group), Placebo, ANE, AMNE treated groups after 14 days
c. Brain Histology

Fig. 64: Photomicrographs showing the T.S. of rats' brain for vehicle control (control group), Placebo, ANE, AMNE treated groups after 14 days.
2. Nasal cavity (mucosa) temperature measurement using IR camera

Table 41: Nasal Cavity Temperature (°C) Measurement IR Camera for Amiloride mucoadhesive nanoemulsion:

<table>
<thead>
<tr>
<th>Time</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>AMNE</td>
<td>Control</td>
<td>AMNE</td>
<td>Control</td>
<td>AMNE</td>
<td>Control</td>
</tr>
<tr>
<td>0 h</td>
<td>32.2 ±1.63</td>
<td>32.5 ±2.94</td>
<td>32.9 ±1.44</td>
<td>31.9 ±1.96</td>
<td>33.2 ±1.31</td>
<td>30.4 ±2.91</td>
<td>33.2 ±2.14</td>
</tr>
<tr>
<td>0.5 h</td>
<td>31.5 ±2.83</td>
<td>33.4 ±1.83</td>
<td>33.2 ±2.14</td>
<td>32.4 ±1.34</td>
<td>32.6 ±2.16</td>
<td>32.6 ±1.87</td>
<td>33.6 ±3.03</td>
</tr>
<tr>
<td>2 h</td>
<td>32.4 ±1.96</td>
<td>33.1 ±2.02</td>
<td>32.4 ±1.96</td>
<td>34.0 ±4.82</td>
<td>31.7 ±1.98</td>
<td>33.7 ±3.71</td>
<td>30.4 ±5.33</td>
</tr>
<tr>
<td>4 h</td>
<td>33.7 ±2.04</td>
<td>32.6 ±1.84</td>
<td>33.1 ±1.62</td>
<td>33.1 ±2.62</td>
<td>30.5 ±2.06</td>
<td>32.4 ±2.12</td>
<td>32.1 ±2.62</td>
</tr>
<tr>
<td>6 h</td>
<td>32.3 ±1.93</td>
<td>33.1 ±2.02</td>
<td>32.9 ±2.00</td>
<td>30.8 ±3.26</td>
<td>32.5 ±2.72</td>
<td>30.2 ±3.51</td>
<td>30.9 ±0.96</td>
</tr>
<tr>
<td>8 h</td>
<td>31.96 ±4.02</td>
<td>33.4 ±2.94</td>
<td>32.9 ±2.63</td>
<td>32.4 ±1.95</td>
<td>31.8 ±2.44</td>
<td>32.5 ±2.10</td>
<td>33.4 ±1.15</td>
</tr>
</tbody>
</table>
3. Neurotoxicity studies by Rotarod method

![Fig. 65](image_url): Effect of i.n. administration of AMNE on rotarod test endurance time in seconds at different time intervals 7 & 14 days (experiment performed post 1 h of treatment).

4. In-vitro Safety Assessment

![Fig. 66](image_url): Histology of goat nasal mucosa; treated with (a) distilled water, (b) optimized formulation and (c) KCl solution

There were no mortalities of rats observed in any of the groups during the 14-day treatment period with intranasal administration of developed formulation (ANE/AMNE). Clinical examination of the rats’ brain tissues prior to and after administration of each (ANE/AMNE) formulation for 14 days revealed no signs of irritation or tissue damage for all the rats as compared to the vehicle control groups.
Macroscopic examination of the brain tissues exposed to the polymeric (ANE/AMNE) formulation, vehicle also did not show any change in the morphology or tissue microstructure. As compared to vehicle control, the (ANE/AMNE) formulation treated groups showed no visible sign of inflammation or necrosis demonstrating the safety of (ANE/AMNE) formulation (Fig. 64).

The Fig. 63 shows the dissected nasal mucosa treated with various treatments, showed no nasociliary damage and the nasal membrane remained intact. In placebo group, no damage to nasal mucosa in the form of intact ciliated pseudo stratified nasal epithelium (no cilia erosion) could be observed, thus substantiating the safety of the excipients used in the formulations. The mucosal histology images for formulations treated with chitosan containing nanoemulsion (AMNE) showed presence of unaltered tight junctions which is similar to non chitosan based formulation treated nasal mucosa (ANE) supporting that chitosan reversibly alter the mucosal permeability by opening the tight cellular junction for increase drug permeability but not altering cellular structure permanently. These findings corroborate observations reported by Gavini and co-workers that on exposure of nasal mucosa to formulation containing mucoadhesive agent showed opened tight junctions (Gavini et al., 2005).

From the neurotoxicity studies it can be concluded that amiloride mucoadhesive nanoformulations does not cause any neurotoxicity or motor coordination impairment.

The histology of goat nasal mucosa in control, treated with optimized formulation and treated with KCl solution is shown in Fig. 66. The microscopic observations indicate that with the optimized formulation, surface epithelium lining and the granular cellular structure of the nasal mucosa were totally intact, whereas KCl causes major changes in the ultrastructure of mucosa. This indicates amiloride loaded mucoadhesive nanoemulsion formulations are non toxic on goat nasal mucosa as well and can be given by intranasal route for effective treatment of epilepsy.

9.6. CONCLUSION

The short-term (14 days) toxicity studies, repeated intranasal administration of the amiloride loaded nanoformulations to rats caused no significant inflammation, or tissue toxicity. These pre-clinical studies proved the safety of developed brain-targeted amiloride nanoformulations in rats; however clinical data is needed to evaluate the risk vs. benefit ratio.