Chapter 5

In-vitro toxicity studies
5.0 In vitro toxicity studies

The absorption of most of lipophilic drugs is limited by poor intestinal wall permeability. Surfactants have often been reported to be effective enhancers of intestinal wall permeability for polar and non polar drugs based on the ionic character (Hoogdalem et al., 1989; Muranishi, 1990). This capability has been exhibited by surfactants with a broad range of structures, including anionic detergents, bile salts, glycerides, lysophospholipids, and others. A major limiting factor in the application of the permeability enhancers approach in the formulation is the potential toxicity of enhancers themselves (Swenson & Curla to, 1992). For example, certain bile salts have been shown to cause acute local damage to the intestinal wall (Dawson et al., 1960; Teem et al., 1972). Likewise, intestinal wall damage has been observed, as the result of treatment with nonionic surfactants (Yonezawa et al., 1977), sodium lauryl sulphate (SDS) and lysolecithin (Tagesson et al., 1985).

In this study, we have developed nonionic surfactants based formulations of CsA, where in one of the formulation contain sodium desoxycholate as the integral component of the system.

The developed formulations are directly used through experimental system (rat perfusion) and evaluated in terms of (biochemical and morphological characteristics) of any damage in intestine locally. Finally the study was carried out to gain insight into the potential short term consequences of this damage.

5.1 Intestinal Perfusion

Single pass intestinal perfusions were carried out using an experiment design similar to that described by (Hoogdalem et al., 1989). Fasted (12hr) male SD rats of 225-250g of body weight, were anesthetized with urethane (1.5g/kg) by intraperitoneal injection. The jejunum is exposed via a midline incision. Sutures were placed 5cm distal to the ligament of treitz in a position which minimally disrupted blood flow and a small incision was made. The intestine was gently washed and rinsed with normal saline to residual contents, then securely fastened with plastic threeway stop cock attached with syringe at both the distal and proximal ends. The perfused segment was moistened with saline and was covered with parafilm. Body
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Temperature is maintained with heating pad. The 22cm intestinal segment was perfused with various formulations containing fixed proportion of nonionic surfactants (total quantity which include, span, stearylamine, cholesterol 100mg/10ml) prepared with phosphate buffer of pH 7.4 isotonic solution. The perfusion solution is maintained at 37°C using a water bath circulator. Perfusion samples were collected into tared vials over 30min, 90min and 120min intervals and used for the estimation of LDH (Lactate dehydrogenase) which reflects membrane integrity in the presence of the nonionic surfactants in the various formulations. The same study is used further for histological studies, but after 1hr of intestinal perfusion.

5.2 Lactate dehydrogenase assay

Traditionally, the toxic effects of unknown compounds have been measured in vitro by counting viable cells after staining with a vital dye. Alternative methods include the measurement of DNA synthesis by radioisotope incorporation, cell counting by automated counters, and other methods that rely on dyes and cellular activity. The lactate dehydrogenase assay is a means of measuring either the number of cells via total cytoplasmic lactate dehydrogenase (LDH) or membrane integrity as a function of the amount of cytoplasmic LDH released into the medium. The lactate dehydrogenase method is simple, accurate and yields reproducible results. The assay is based on the reduction of NAD by the action of LDH. The resulting reduced NAD (NADH) is utilized in the stoichiometric conversion of a tetrazolium dye. The resulting colored compound is measured spectrophotometrically. After different formulation treatments, cell-free aliquots of samples were assayed; then, the amount of LDH activity can be used as an indicator of relative cell viability as well as a function of membrane integrity (Decker and Lohmann-matthes, 1988; Legrand et al, 1992). Assessing the membrane integrity of cells as a function of the amount of LDH leakage into the medium was done on samples obtained from intestine, after perfusion of various formulations, these samples were added into the well plates under sterile area and centrifuged at 250xg for 4 min to form pellet cells then aliquots are transferred to clean flat bottom plate and proceeded with enzymatic analysis.

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25ml of 1X LDH Assay Cofactor solution was prepared by adding 25 ml of tissue culture grade water to bottle of lyophilized cofactors. Store reconstituted cofactor preparation at 0°C (avoid frost-free freezers) in working aliquots to avoid repeated freeze/thaw procedures, next Lactate Dehydrogenate Assay Mixture was prepared by mixing equal amounts of LDH Assay Substrate, Cofactor and Dye Solutions and aliquots of medium for testing (approximately \( \frac{1}{2} \) of the volume of culture medium) were removed further assay mixture in an amount equal to 2X the volume of medium removed for testing to each sample.

Sample plate was covered by opaque material to protect from light (e.g. aluminum foil or a box) and incubated at room temperature for 20-30 minutes and finally reaction was terminated by the addition of 1/10 volume of 1N HCl to each well and absorbance was measured spectrophotometrically at a wavelength of 490 nm. (Lactate dehydrogenase (LDH) was assayed utilizing a kit (LDH TOX-7) supplied by Sigma chemical Co. USA.).

5.3 Histopathological studies

Single pass rat perfusions were carried out as described above. At the end of 1h perfusion, the lumen was filled with 2.5% glutaraldehyde/2% paraformaldehyde in 0.1M cacodylate buffer (pH-7), and fixed in situ. The perfused intestinal segment was then removed and immersed in the same fixative. Cross sections were prepared (according to the protocol as per sectioning of intestine is concerned, it was carried out with the help of technical person who has expertise in sectioning) and further sections were stained with haematoxylin-eosin, and were examined by light microscopy. Various measures of histological abnormality were quantitated on arbitrary scale of 0-3, with 0 indicating no effect and 3 indicating an extensive effect. This evaluation was carried out with the help of an experienced histologist (Dept of Parasitology, Central Drug Research Institute India) in a blinded fashion.
5.4 Results and Discussion

LDH assay is related to measurement of one of the biochemical markers i.e. lactate dehydrogenase (LDH) present in cytoplasm of the cells which releases due to intestinal wall damage. As shown in Figure 50 there is maximum release of lactate dehydrogenase after intestinal perfusion with 1% of Triton x-100, which served as the control considered as 100% release.

When intestinal membranes are treated with plain saline buffer about 49% of LDH was detected in first 30 minutes which was reduced to 21% after 120 minutes compared to positive control. This data suggests that there may be continuous process of shedding off and revival of cells which could give rise to release of LDH to such an extent. However when membranes are treated with 1 % triton x 100 there was abrupt release of LDH within 30 minutes in higher quantum which served as 100% release.

![Figure 50: Indicate the % LDH release at different intervals after treatment of formulations with Intestine upto 2hr. Control- 1% of Triton x100; BL-Bilosomal; NV2-Niosomal; ME-microemulsion; all the values are calculated against control taking as 100% of LDH release. All the values are mean of three determinations (n=3); (mean ± SD)](image-url)
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When intestinal mounds were exposed to B. formulation the amount of LDH released was 45.36% after 30 minutes which was reduced to 25.49% after 120 minutes. It is anticipated that this time do not signify toxicity as this may be related to intrinsic release of LDH due to processes as mentioned above. However, there is a 2-5% reduction in LDH release as a component to positive control. This suggests that the type and amount of estrogen surrogates employed in the formulation is not toxic to intestinal mounds. The intestinal formulation (NVE) released 36.9% in first 30 min and 18.9% of LDH after 2 hours while ME released 27.6% and 16.6% of LDH after 30 and 120 minutes respectively compared to positive control. This study suggests that the components employed in the formulation are safe and none other of them is liable to cause the intestinal damage on oral administration and they are bioequivalent with intestinal mounds.

![Figure 2: Represents the Histological section of Intestine treated with buffer (x40)](image_url)
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Figure 52: Represents the Histological section of intestine treated with 1% of Triton x-100 (40 xs)

Figure 51 shows cross section of rat intestinal segment after perfusion with plain saline buffer made of oxygenated Krebs ringer for 1h. This figure revealed normal intestinal epithelium with well viable aligned viable epithelial cells in the section. Figure 52 represents cross section of rat intestinal segment after perfusion for 1h with 1% of triton x-100 and it served as the positive control. It has been observed that there is infiltration of large quantities of mucus and debris, the villi were contracted and swollen, and the alignment of epithelial cells is disturbed. When rat intestinal segments were perfused with different formulations i.e BL, NV2 and ME there was no significant change in alignment of epithelial cells and the structures of the cells are organized even after 1hour. The Histopathological effect of different formulation in intestine has been represented in figure 53-55 and histological sections were scored for a variety of abnormalities, using a scoring range of 0-3, where 0 represents no effect and 3 represents the severe effect. Scoring was carried out by the single person who was unaware of the identity of the sections.
Figure 53: Represents the Histological section of intestine treated with bilosomal (BL), formulation (100 xs)

Figure 54: Represents the Histological section of intestine treated with niosomal (NV2), formulation (100 xs)
Figure 55: Represents the Histological section of intestine treated with (ME), microemulsion formulation (100 xs)

Table 35, represented, scores for various measures of damage, for intestines perfused with various formulations made of nonionic surfactants. Mucus/debris refers to the presence of basophilic material and lysed cells in the lumen. Villous shortening refers to apparent retraction of villi. Erosion refers to loss of epithelium, exposure of lamina propria, without ulceration. Swollen epithelial cells are an indication of cytoplasmic fluid gain. Flat epithelial cells refer to cells which are short, and have spread laterally in an apparent attempt to cover voids in the epithelium. The concentration of goblet cells is an indication of relative loss of columnar epithelial cells. In general, goblet cells appeared to be more resistant than absorptive enterocytes to surfactant-induced damage. The average total scores of BL and NV2 demonstrate the damage is minimal. The observed rank order of histological damage was similar to that obtained by the study of biochemical markers (lactate dehydrogenase release).
Table 35: Histological evaluation of rat small intestinal mucosa after 1hr perfusion with various formulations and further section stained with haematoxylin-eosin

<table>
<thead>
<tr>
<th>Score* for treatment with</th>
<th>Control</th>
<th>PB</th>
<th>BL</th>
<th>NV2</th>
<th>ME</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of Rats used</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Mucus/debris</td>
<td>2, 2</td>
<td>1, 1</td>
<td>2, 2</td>
<td>1, 2</td>
<td>2, 1</td>
</tr>
<tr>
<td>Villous shortening</td>
<td>2, 1</td>
<td>0, 0</td>
<td>1, 0</td>
<td>1, 0</td>
<td>1, 0</td>
</tr>
<tr>
<td>Erosion</td>
<td>3, 3</td>
<td>0, 0</td>
<td>0, 0</td>
<td>0, 0</td>
<td>0, 0</td>
</tr>
<tr>
<td>Swollen epithelial cells</td>
<td>0, 0</td>
<td>0, 0</td>
<td>0, 0</td>
<td>0, 0</td>
<td>0, 0</td>
</tr>
<tr>
<td>Flat epithelial cells</td>
<td>0, 0</td>
<td>0, 0</td>
<td>0, 0</td>
<td>0, 0</td>
<td>0, 0</td>
</tr>
<tr>
<td>Goblet cells</td>
<td>0, 0</td>
<td>0, 0</td>
<td>0, 0</td>
<td>0, 0</td>
<td>0, 0</td>
</tr>
<tr>
<td>Total</td>
<td>7, 6</td>
<td>1, 1</td>
<td>3, 2</td>
<td>2, 2</td>
<td>3, 1</td>
</tr>
<tr>
<td>Average total score</td>
<td>6.5</td>
<td>1.0</td>
<td>2.5</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*Scores range from 0 (no effect) to 3 (severe effect). PB (Plain buffer); BL (Bilosomal); NV2 (Niosomal); ME (Microemulsion); Control (1% of Triton x-100)

Histological evaluation, though less quantitative, demonstrates that the release of LDH is associated with tissue damage, and is not merely the consequence of increased intestinal wall permeability. Intestinal wall damage by bile salts (Dawson et al, 1960), nonionic surfactants (Yonezawa, 1977) has been reported previously. The present work demonstrates that concentration of nonionic surfactant used in formulations did not cause much change in LDH release and the changes were minimal histologically. A survey of published literature (Yamamoto et al, 1996) on permeability enhancement and toxicity generally indicates a significant decrease in the efficacy or toxicity of nonionic surfactants as the polarity is increased with some threshold HLB.

A report (Florence, 1952) has pointed out that a surfactant with a medium chain-length alkyl group is capable of penetrating the membrane lipid layer due to its aqueous solubility, a medium chain surfactant has greater monomer concentration (higher CMC) than a surfactant with a longer alkyl chain. Increasing the length of surfactant with a longer alkyl chain may improve membrane penetration. It has been
reported that surfactant with lower HLB may be toxic (Macek and Krzeminiski, 1975), however the concentration of low HLB surfactant employed in the formulation is very low and the type of surfactant used in the formulation belongs to GRAS and therefore it can be anticipated that these formulations safer especially in context of oral administration.

The concentration of surfactants used in formulations unambiguously shown that has not caused any damage to intestine, and even corroborated with histologically, and in terms of LDH release into lumen (purely an important biochemical marker that measures the cytoplasmic LDH) or membrane integrity as a function of the amount of cytoplasmic LDH released into the medium.