8. **IN VITRO PERMEABILITY AND IN VIVO BIOAVAILABILITY**

8.1. **Introduction:**

The body contains many biological barriers that serve to protect its interior from a variety of external invaders and toxins. The skin is the largest such obstacle, while the blood-brain barrier forms the tightest barrier to penetration of molecules from the blood stream to the brain. Similarly, for a drug molecule to be orally bioavailable, it has to traverse the epithelial layer of the gastrointestinal tract. Thus, many factors for enhancing the delivery of molecules through this intestinal mucosal barrier must be considered.\(^{(277)}\) The intestine is the most important site for drug absorption and regulates the extent of orally administered drug that reaches the circulation.

Several different obstacles must be overcome for the delivery of drugs through the intestinal mucosa or the blood-brain barrier. These obstructions to drug delivery can be categorized as physiological, biochemical, and chemical barriers. The physical barrier arises from cell membranes and the intercellular junctions between the cells (e.g. tight junctions). Permeation of drugs across the intestinal epithelium is restricted to paracellular and transcellular pathways depending on their physicochemical properties (e.g. size, charge, lipophilicity, and conformation). Most large molecules that are hydrophilic are prevented from passing across the cell membranes unless some specific membrane proteins are involved to serve as channels, carriers, or transporters. Only lipophilic molecules may directly pass across the lipid bilayer of the cell membranes by passive diffusion. In addition to the physical barrier, the intestinal epithelium also possesses various metabolic enzymes (e.g. intestinal peptidases, cytochrome P450) and polarized efflux systems (e.g. \(p\)-glycoprotein, \(P\)-gp) which act as biochemical barriers.
further limiting drug absorption in the intestine. Consequently, many drug candidates are
restricted from oral dosing in clinical development owing to this biological barrier.
Finally, the drug has to have optimal physiochemical properties for its permeation across
the biological barriers. Thus, these various barriers have to be taken into account when
designing drugs with improved absorption characteristics.\(^{(278)}\)

Lipid based formulation is an effective approach for optimization of oral drug
delivery. For commercial success and enhance the development potential of lipid based
formulation, it is essential to develop confidence amongst the industry for these delivery
system.\(^{(279)}\) To fulfill this objective, it is necessary to established *in vitro/ in vivo*
correlations that shortened drug development period and improved product quality.
Determining the dissolution, solubility, lipolysis of lipid excipients, intestinal membrane
techniques (isolated animal tissue and cell culture models) are various *in vitro* techniques
that can be used to asses lipid based formulations.\(^{(280)}\) These techniques provide
information about specific aspects of the formulation only not about *in vivo* interaction
and performance of these systems.

On the other hand, *in vivo* studies performed with humans and laboratory animals
are expensive, time consuming and often even unethical, *in vitro* methods, as accurate as
possible, are needed in screening of new drug candidates. Immortalized, often of cancer
origin, animal and human cell cultures have been used for estimation and prediction of
human drug absorption. Several possible *in vitro* human cell models are available for this
purpose, one of which is the Caco-2 cell model, a well characterized cell line. According
to Biopharmaceutics Classification System (BCS) and FDA approval, Caco-2 cells can be
used as a screening method for new drug candidates during drug discovery and
For the suitability and reliability of the method, permeability of several model compounds with known intestinal absorption in humans has to be demonstrated. FDA recommends the use of compounds with high, low, and zero permeability, passive and active transport, and use of efflux markers for this purpose. The simultaneous use of model compounds requires that they do not express cytotoxicity, do not interact with each other during permeation, and that they are easily detected. Therefore, the use of different sets of model compounds has to be validated before the actual experiments with drug candidates can be performed.

### 8.1.1. **In vitro Caco-2 method**

Various *in vitro* methods are listed in United States FDA guidelines, acceptable to evaluate the permeability of a drug substance, includes monolayer of suitable epithelial cells. One such epithelial cell line that has been widely used as a model system of intestinal permeability is the Caco-2 cell line. Since most drugs are known to absorb via intestines without using cellular pumps, passive permeability models have came into the limelight. In the 1990s membrane-based drug assays led to the passage of drugs through the intestinal mucosa and an important Caco-2 assay emerged in pharmaceutical research.\(^{(283)}\)

In a typical Caco-2 experiment, a monolayer of cells is grown on a filter separating two stacked micro well plates. The permeability of drugs through the cells is determined after the introduction of a drug on one side of the filter. The entire process can be automated, and when used in conjunction with chromatography and/or mass spectroscopy detection, it enables any drug’s permeability to be determined.
The Caco-2 cell line, which exhibits a well-differentiated brush border on the apical surface and tight junctions, and which expresses typical small-intestinal microvillus hydrolases and nutrient transporters, has proven to be the better in vitro model for the following reasons: (a) to rapidly assess the cellular permeability of potential drug candidates (b) to elucidate pathways of drug transport (e.g., passive versus carrier mediated) (c) to assess formulation strategies designed to enhance membrane permeability (d) to determine the optimal physicochemical characteristics for passive diffusion of drugs,(e) to assess potential toxic effects of drug candidates or formulation components on this biological barrier.

Since differentiated Caco-2 cells express various cytochrome P450 isoforms and phase II enzymes such as UDP-glucuronosyltransferases, sulfotransferases and glutathione-S-transferases, this model could also allow the study of presystemic drug metabolism.

The Caco-2 cell model has the advantages of simplicity and reproducibility. US FDA recognizes Caco-2 to measure permeability as part of the bioequivalence waiver process.

8.1.2. In vivo method

In spite of tremendous innovations in the field of drug delivery and the acquisition of detailed knowledge about promising alternative routes of administration, it is estimated that 90% of all medicine usage is in oral form and oral drug delivery systems comprise more than half the drug delivery market.(284) Thus, oral bioavailability plays an imperative role for successful therapy by this route. Oral bioavailability depends on number of factors like aqueous solubility, dissolution rate, residence time, drug
permeability, presystemic metabolism, first pass metabolism and susceptibility to efflux mechanisms. In addition, different characteristics of drugs such as size, density, pH, diffusion, swelling, adhesion, and degradation can also be modified to enhance the oral bioavailability. Thus, only in vitro evaluation will not be able to predict exact role of nanoparticles in improving bioavailability. The impact of excipients on the bioavailability and pharmacokinetic profile of drug can be estimated by designing appropriate in vivo studies.

Bioavailability is the ratio of the area under curve (AUC) after administration by the route of interest and after administration of the same amount of drug direct into the systemic circulation, usually by intravenous injection. Bioavailability is one of the principal pharmacokinetic properties of drugs. It is a subcategory of absorption and it is the processes that are involved in transferring the drug in solution from the site of administration to the venous blood. Relative bioavailability or bioequivalence is the most common measure for comparing the bioavailability of one formulation of the same drug to another. The mean responses such as $C_{\text{max}}$ and AUC are compared to determine relative bioavailability. The AUC refers to the extent of bioavailability while $C_{\text{max}}$ refers to the rate of bioavailability.

8.2. Methodology

8.2.1. In vitro Intestinal Permeability

Possible intestinal absorption enhancement of drug incorporated with liposome, SLN and SNEDDS were assessed with drug transport studies. Test system for permeability study is shown in Figure 8.1.
8.2.1.1. **Drug transport measurements across Caco-2**

Transport across intestinal epithelial cells was tested using model drug compounds Berberine. Cells were seeded at $2 \times 10^6$ cells/mL and cultured on 24 well plates Transwell® permeable supports (0.4 um pore size) for 27 days. Cell culture medium was changed every other day. On day 27, cell culture medium was removed from both the apical and basolateral compartments, and cells were rinsed once with HBSS. For the experiment with Caco-2 monolayers, berberine and its formulation with and without berberine in HBSS were added to the apical compartment of Transwell plate. The formulations were added at a dose equivalent to 100 µM berberine. The basolateral compartment solution was replaced with HBSS. The cells were exposed to formulation for 2 hours inside the incubator at 37°C. After three hours, samples were taken from basolateral compartment and analyzed for drug content using a HPLC. All the experiments were conducted in triplicate. Apparent permeability coefficient, $P_{app}$, of drug for each formulation was calculated according to the following equation 8.1:

$$P_{app} = \frac{(dQ/dt)}{(1|AC_0 \times 60)}$$

where $dQ/dt$ is the cumulative transport rate (µmol/min, nmol/min or µg/min) defined as the slope obtained by linear regression of cumulative transported amount as a function of time (min), $A$ is the surface area of...
the monolayers, \( C_0 \) is the initial concentration of the compounds on the donor side (\( \mu \text{mol/mL} \), nmol/mL or \( \mu \text{g/mL} \)), and 60 is the coefficient when minutes are converted to seconds.

The concentration of the transported drug was measured from A-B and B-A, i.e., \( P_{\text{app}} \text{(AB)} \) and \( P_{\text{app}} \text{(BA)} \), respectively, and the efflux ratio (ER) was calculated from the following Eq. 8.2.

\[
\text{ER} = \frac{P_{\text{app(AB)}}}{P_{\text{app(BA)}}} \quad \text{(Eq. 8.2)}
\]

Absorption enhancement ratio (\( R \)) was calculated by equation 8.3:

\[
R = \frac{P_{\text{app(Sample)}}}{P_{\text{app(Control)}}} \quad \text{(Eq. 8.3)}
\]

**8.2.1.2. Monolayer Integrity test**

At the end of the experiment, the monolayer integrity test was done by analyzing the concentration of Lucifer Yellow (LY) in the apical and basolateral compartments. An initial stock solution of LY (50 mM) was prepared and diluted to 100 µM working solution. Four hundred microliter of the 100 µM working solution of LY was added to the apical side of Caco-2 cell monolayer (in the wells in which drug transport study was performed), and 800 µL of HBSS buffer was added to the basolateral side. The plate was then kept in an incubator at 37°C. After 120 min, 700 µL and 300 µL of the samples were withdrawn from the basolateral side and apical side, respectively. The samples were analyzed by fluorescence spectroscopy at an excitation wavelength (\( \lambda_{\text{ex}} \)) of 485 nm and emission wavelength (\( \lambda_{\text{em}} \)) of 535 nm using a Spectrofluorophotometer (RF-5301-PC, Shimadzu, Kyoto, Japan).
8.2.2. Bioavailability Study

Thirty six male Wistar rats weighing 220–240 g were fasted overnight for at least 12 h, with free access to water, and randomly divided into three groups for oral administration. The drug suspension (control), formulation I (SLN) and formulation II (SNEDDS) were administered by oral gavage at a dose of 50 mg/kg. The rats will be anaesthetized using ether anesthesia. Blood samples (approximately 0.3 mL) will be withdrawn from the retro-orbital plexus at 0 (pre-dose), 0.25, 0.5, 0.75, 1, 2, 3, 4, 6, 8, 12, 24 and 36 h. The blood was collected into a 2 mL heparinized Eppendrof tubes and centrifuged at 4000 rpm for 10 min. The supernatant plasma was collected and stored at -20°C for later analysis. The analysis of samples of pharmacokinetic studies was performed as per the HPLC method given in section 4.1.7.

8.2.3. High fat diet induced hyperlipidemia

Twenty four Wistar rats of either sex (200-250 g), will be placed in four groups (n=6). Negative control, toxic control, standard and test will receive distilled water, cholesterol, drug suspension along with cholesterol and formulation along with cholesterol, respectively. Hyperlipidemia will be induced by the use of high fat diet containing 200mg of cholesterol suspended in 2 mL of coconut oil for 14 days. Treatment will be given orally, using 18-gauge oral feeding needle, 2 h after the administration of high fat diet. After fourteen days of treatment, the rats will be anaesthetized using light ether anesthesia and blood samples (0.5 mL, once) will be withdrawn from retro orbital plexus. The biochemical parameters such as serum lipid level like total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density
lipoprotein cholesterol (LDL-C) level was estimated by standard diagnostic kit (SPAN Diagnostic and Crest Biosystem, India).

8.3. Results and Discussion

8.3.1. *In vitro* Intestinal Permeability

Intestinal absorption enhancement of drug incorporated with liposome, SLN and SNEDDS were assessed with *in vitro* transport studies (Fig. 8.2). It was observed that the permeability coefficient for plain BER was $0.74 \times 10^{-6}$ cm/s in the absorptive direction (A→B), whereas it was $2.84 \times 10^{-6}$ cm/s in the secretory transport (B→A). The efflux ratio was 3.84 indicates the low absorption of BER from intestine suggesting the P-gp efflux of drug. However, permeability coefficient for BER loaded liposome, SLN and SNEDDS were higher than for plain BER in both direction. This is consistent with the presence of several excipients in lipid based formulations indirectly inhibit P-gp through effects on the lipid membrane and thus enhance the intestinal membrane permeability and oral absorption of the substrate drug. Increased drug absorption through the intestinal mucosa is often associated with damage caused to the intestinal cells and to their barrier function. The effect of different formulations on the monolayer integrity was examined by measuring the permeability of the paracellular leakage marker, Lucifer yellow across the monolayers. The apparent permeability coefficient ($P_{\text{app}}$) for Lucifer yellow was more than $1 \times 10^{-6}$ cm/s for formulations while it was less in plain BER. This implied that formulations may affect the paracellular route through the opening of tight junctions and thus reduce the cell integrity of Caco-2 cells. However, $P_{\text{app}}$ values measured 48 h after transport experiment (recovery) revealed that all the monolayers fully recovered. This
indicated that although the formulations affected the tightness of the cell monolayer, it reversibly recovered after the experiment.

Figure 8.2. $P_{app}$ of plain berberine and berberine loaded liposome, SLN and SNEDDS

8.3.2. Bioavailability Study

Based on the permeability study, SLN and SNEDDS were selected for in vivo bioavailability study. The results of single dose bioavailability studies showed $C_{\text{max}}$ for SLN and SNEDDS was found to be 192.32 ± 5.25 ng/mL and 391.12 ± 22.64 ng/mL respectively, which was significantly higher than the plain drug solution 66.88 ± 2.15 ng/mL (Table 8.1). A higher $C_{\text{max}}$ for formulations could be achieved as drug loaded in SLN and SNEDDS was capable to bypass hepatic first pass metabolism and able to reach directly to systemic circulation by virtue of size and surface properties of nanocarrier system. $T_{\text{max}}$ for SLN and SNEDDS were found to be 2 and 1.5 h respectively, while for plain drug solution was found to be 2 h (Fig. 8.3). AUC$_{0-36}$ for SLN and SNEDDS were found to be 1383.44 ± 139.68 ng.h/mL and 2921.74 ± 319.9 ng.h/mL, which is significantly (P<0.05) higher than AUC$_{0-36}$ for plain drug solution; 334.41 ± 44.35
ng.h/mL (Table 8.1). Improvement in bioavailability could be attributed to ability of lipid based formulations to reach the oral lymphatic region after absorption and reaching to systemic circulation. Thus, lipid based formulations could play important role in enhancement of its bioavailability. From this data it can be concluded that the SNEDDS are effective tool for enhancing bioavailability of BER.

Table 8.1. Pharmacokinetic parameters for single dose oral bioavailability of plain BER, SLN and SNEDDS

<table>
<thead>
<tr>
<th></th>
<th>C&lt;sub&gt;max&lt;/sub&gt;± SD (ng/mL)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>AUC&lt;sub&gt;0-36&lt;/sub&gt;± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plain Drug</td>
<td>66.88±2.15</td>
<td>2</td>
<td>334.41±44.35</td>
</tr>
<tr>
<td>SLN</td>
<td>192.32±5.25**</td>
<td>2</td>
<td>1383.44±139.68**</td>
</tr>
<tr>
<td>SNEDDS</td>
<td>391.12±22.64 **</td>
<td>1.5</td>
<td>2921.74±319.9**</td>
</tr>
</tbody>
</table>

**P<0.01
8.3.3. Anti-hyperlipidemic activity

The selected formulation was used to check the effect of formulation on high fat diet induced hyperlipidemia in rats. Induction of hyperlipidemia was confirmed from the increase in TC, TG, LDL and decreased in level of HDL in control. Treatment with BER loaded SNEDDS (100 mg/kg) significantly ameliorate the level of TC, TG, HDL and LDL compared to hyperlipidemic control (Fig. 8.4). These results indicate that the prepared SNEDDS was more efficient in controlling hyperlipidemia as compared to plain

*P<0.05, ***P<0.0001, ns=non significant

**Figure 8.4.** Effect of berberine loaded SNEDDS on plasma lipid levels of high-fat diet induced hyperlipidemic rats
drug and this can be attributed to enhance bioavailability. Hence BER loaded SNEDDS can be exploited as an antihyperlipidemic therapeutic agent or adjuvant in existing therapy for the treatment of hyperlipidemia.