Chapter 6

Evaluation of the Bioavailability of Major Withanolides of Ashwagandha
6.1. Introduction

Therapeutic efficacy and stability of WRF was evaluated and reported in the previous chapters of this thesis. It was noted that the content of withanolides in roots are very important for the therapeutic activity of Ashwagandha. Many researchers have reported that individual withanolides have pharmacological activity. It means that individual withanolides can be used as therapeutic drug. The estimation of bioavailability of compounds is the most important initial step in drug designing and drug development. Therefore, it becomes essential to evaluate the bioavailability of individual withanolides. In vitro cell culture systems may therefore be a good option for rapid bioavailability testing of withanolides.

The U.S. Food and Drug Administration (FDA) define bioavailability as "the rate and extent to which the active drug ingredient is absorbed from a drug product and becomes available at the site of drug action". In practice it is rare that drug concentrations can be determined at the site of action (Dalton and Yates, 2007). Hence, usually bioavailability refers to the absorption of a drug from an in vitro absorption model gastrointestinal tract following oral administration of a dosage form. The dosage form may be any type of product, including a solution, suspension, tablet, capsule or powder. Bioavailability can also refer to other types of dosage form, such as intramuscular injections, ointments and other topical preparations, transdermal patches, and implants, which also require an absorption step prior to reaching the systemic circulation (Sachan et al., 2009; Makanikar and Parekh, 2011). Although many compounds show a promising in vitro therapeutic potential, they cannot be used in vivo due to bioavailability problems. The intestinal absorption of a compound may occur via passive diffusion (paracellularly or transcellularly), or via carrier-mediated active transport (eg. D-glucose, dipeptides). The intestinal absorption of a compound may be hindered by P-glycoprotein (P-gp), an ATP-
dependent multidrug for the permeation of compounds across the MDCK cell monolayer (Fig. 6.1). The membrane transport properties of novel compounds can thereby be assessed using these differentiated cell monolayers (Engman et al., 2001).

The intestinal epithelium plays an important role in drug absorption and transportation. Thus it becomes important to determine the bioavailability of a drug in the intestine. *In vitro* cell culture may therefore be useful model system to quickly assess the bioavailability of the given drug (Pang et al., 1996; Owens et al., 1976). A Sino-Veda MDCK *in vitro* cell culture system shows characteristics similar to *in vivo* intestinal epithelium (Tam et al., 2000). In view of this for the evaluation of bioavailability profiles of major withanolides, Sino-Veda MDCK cell culture system was used.
Fig. 6.1: Mode of absorption of compounds in intestinal lumen.
Chapter 6: Bioavailability of Major Withanolides

6.2. Materials and Methods

6.2.1. Standard Withanolides

The authentic withanolides described in section 2.2.1 of Chapter 2 were used for bioavailability study. The purity of the standards was established by HPLC analysis (Fig. 6.2).

The bioavailability studies were facilitated through active collaboration with Sino-Veda (Canada). They had developed a patented method, standardized with Ginseng for in vitro bioavailability studies. Therefore in order to carry out bioavailability of withanolides, standard withanolides were sent to Sino-Veda laboratory (Edmonton, Canada) and the results are presented below.

6.2.1. HPLC-MS Analysis

For evaluating the permeability of major withanolides, standard authentic individual withanolides were diluted with Hank’s buffered saline and tested for permeability by the Sino-Veda’s proprietary cell culture system (Fig. 6.3). For determining the concentration of withanolides, high performance liquid chromatography (HPLC) coupled to diode array absorbance detection (DAD) and positive mode electrospray ionization mass spectroscopy (ESI) was employed. A Phenomenex Luna 3µ C18 (2) 100A 15 cm X 4.60 mm column equipped with a guard column (security guard C18) and column heater set at 40°C was used. For HPLC-MS analysis mobile phase A was 5 mM ammonium acetate (pH 3.0 made with formic acid) in 18 mega Ω water, and mobile phase B was HPLC grade acetonitrile. Based on withanolides behavior in cell culture system the mobile phase gradient program was modified to obtain better resolution. Flow rate was 0.7 mg/mL and solvent gradient program for WF A, WN A, WN B, WNN and 1,2 DWM was 0-15 min A: B (60: 40%), 15-20 min A: B (15: 85%) and 20-25 min A: B (60: 40%). For 0-15 min A: B (70: 30%), 15-20 min A: B (15: 85%) and 20-25 min A: B (70: 30%). Mobile phase program for WS V was 0-15 min A: B (70: 30%),
**Fig. 6.2:** The major withanolides of Ashwagandha and their HPLC elution profile.
Fig. 6.3: The cell culture chamber.

Samples to be tested for permeability are incubated under control conditions and allowed to pass through the cell monolayer. Samples collected from apical and basal chambers are analyzed by HPLC-MS for ascertaining permeability of different withanolides.
15-20 min A: B (75: 25%) and 20-25 min A: B (70: 30%). DAD detector conditions specific signals were collected at 205 nm (bandwidth 16), 210 nm (bandwidth 8), 254 nm (bandwidth 16), 270 nm (bandwidth 16) and 280 nm (bandwidth 16). All spectra were scanned from 190 nm to 400 nm with 2 nm step.

Electrospray mass spectrometer conditions were; Positive mode, gas temp 350°C, drying gas 13 L/min, neb pressure 60 psig, vaporizer 350°C and capillary voltage 3000 V. Scan conditions were low mass 150, high mass 600, fragmentary 70, gain 1, threshold 150, step size 0.20. Selected ion mode signals monitored at various ranges. Electrospray mass spectrometer selected ion mode signals monitored at 471.3, 488.4 (WN A), 455.4, 472.4 477.4 (WN B), 471.2, 453.2, 493.2 (WNN), 471.3, 493.2 (WF A, 1, 2 DWM) and 407.4, 425.4, 443.4, 767.4, 784.6, 789.4 (WS V) except WS V was the high mass is 850.

Madin Darby Canine Kidney cells (MDCK) were used in the proprietary Sino-Veda cell culture system. The MDCK cells show absorption characteristics similar to human intestinal epithelium and suited for rapid permeability screening (Irvine et al., 1999; Hugger et al., 2002). Cells were cultured for 3 days and monolayers with Trans Epithelial Electric Resistance (TEER) between 80 to 120 ohm.cm² were used in the study. Stock solutions of standards were prepared in methanol and then further diluted with Hank’s buffered saline supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) to desired concentrations. Lucifer Yellow was added to the test solution as an indicator for monitoring the integrity of membrane monolayer. Incubation was carried out on a shaker (50 – 70 rpm) at 37°C for one hr. Samples were collected from apical donor side before incubation, and form basal receiver side after incubation (Fig. 6.3). All withanolides were tested at 2 µg/mL to ascertain that they did not disrupt membrane integrity and the chemical concentrations at the receiver side were
above their quantifiable limits. Apical chamber sample was diluted 1: 20 and the injection volume was 20 µL for LCMS analysis. For basal chamber samples no dilution was necessary and 40 µL was injected.

6.3. Results

The bioavailability of standard withanolides was assessed with the help of standardized proprietary cell cultured system mentioned above (Fig. 6.3). Under these HPLC conditions the mean retention times (Rt) for withanolides WF A, 1, 2 DWM, WN A, WS V, WNN and WN B, respectively, were 6.8, 7.75, 9.01, 9.2, 9.31 and 13.1 min. (Table 6.1). Fig. 6.4 and 6.5 shows representative mass spectrometric selected ion mode chromatograms for the individual withanolides which were collected from apical and basal permeability chambers. A single peak was observed in all chromatograms except for WNN and WNB which showed an extra peak possibly representing a degraded product. WNN and WNB and the unidentified degraded /derivatized products were highly permeable. By contrast, WS V seems to be partially permeable. A standard curve was generated and amounts quantified in the experiments were well within the range of standard curve. Permeability was measured in terms of efflux pump (cm/sec) and was calculated as follows:

$$P_{eff} = \frac{\text{Receiver Volume}}{\text{Membrane Area} \times \text{Donor Concentration}} \times \frac{\text{Receiver Concentration}}{\text{Incubation Time}}$$

$P_{eff}$ values of WN A, WNN, 1, 2 DWM, WN B, WS V and WF A were 4.05 X 10^{-05}, 2.06 X 10^{-05}, 1.97 X 10^{-05}, 1.80 X 10^{-05}, 3.03 X 10^{-06} and 3.30 X 10^{-07} respectively (Table 6.1).
Table 6.1: $P_{\text{eff}}$ values and permeability of Ashwagandha standard.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Rt (min)</th>
<th>$P_{\text{eff}}$ (sec/cm) Mean</th>
<th>Permeability</th>
</tr>
</thead>
<tbody>
<tr>
<td>WN A</td>
<td>9.01</td>
<td>$4.05 \times 10^{-05}$</td>
<td>High</td>
</tr>
<tr>
<td>WNN</td>
<td>9.31</td>
<td>$2.06 \times 10^{-05}$</td>
<td>High</td>
</tr>
<tr>
<td>1, 2 DWM</td>
<td>7.75</td>
<td>$1.97 \times 10^{-05}$</td>
<td>High</td>
</tr>
<tr>
<td>WN B</td>
<td>13.1</td>
<td>$1.80 \times 10^{-05}$</td>
<td>High</td>
</tr>
<tr>
<td>WS V</td>
<td>9.2</td>
<td>$3.03 \times 10^{-06}$</td>
<td>Low</td>
</tr>
<tr>
<td>WF A</td>
<td>6.8</td>
<td>$3.30 \times 10^{-07}$</td>
<td>Impermeable</td>
</tr>
</tbody>
</table>
**Fig. 6.4:** Apical and Basal distribution pattern for highly permeable withanolides. Degradation product is denoted by D.
Fig. 6.5: Apical and Basal distribution pattern for low and impermeable withanolides.
6.4. Discussion

Biological activities of some individual withanolides of Ashwagandha have been evaluated by in vitro assays and it was noted that withanolides have therapeutic activity (Yang et al., 2007; Sen et al., 2007; Mohan et al., 2007; Devi and Sharada, 1995; Sabina et al., 2008; Kobuyama et al., 2005; Malik et al., 2007; Kour et al., 2009; Nakayama and Tohada, 2007). The detailed pharmacological activities of major withanolides have been described in section 1.4.6 of Chapter 1.

In vivo bioavailability study of WN A and WF A has been carried out by Patil et al. 2013. However, in vitro assay for evaluation of bioavailability of withanolides have not been carried out. In vitro cell culture systems may therefore be a useful model to study the absorption behavior of withanolides (Engman et al., 2001; Pang et al., 1996). In view of this the bioavailability of withanolides was carried out by using proprietary Sino-Veda (Canada) MDCK cell culture system (Lin et al., 2004; Tam et al., 2000).

It has been reported that among all withanolides WN A is most stable and bioavailable under in vivo condition (Patil et al., 2010, 2013); the present investigation has shown similar results. Several researchers studied biological activity under in vitro culture for WN A (Kobuyama et al., 2005; Malik et al., 2007; Kour et al., 2009), it indicates that WN A is permeable under in vitro conditions, and this is in accordance with the present observation. Cells grown under the proprietary conditions have a narrow range of paracellular permeability (1 X 10^{-06} to 1 X 10^{-05} cm/sec). In present study the P_{eff} value of WN A was 4.05 X 10^{-05} which indicates high permeability. The permeability observed in these cells correlates directly to the permeability in human system (Sun et al., 2002). In the present investigation it was noted that WNN, 1, 2 DWM, and WN B are highly permeable (Fig. 6.4). Fig. 6.4 also shows that significant metabolic and or degradation product appeared in WNN and WN B.
Apparently, of the various withanolides tested in the present investigation only WNN and WNB seem to be modified and the rest seems to be stable. Putative modified products of WNN and WNB are unidentified and need further investigations. In present investigation $P_{\text{eff}}$ value of WS V was $3.03 \times 10^{-06}$ observed, signifying their low permeability as compared to other above mentioned withanolides. WS V have glucose moiety at C$_3$ position (Fig. 6.2), thus they are polar and also have higher molecular weight. It is known that hydrophobic interior of the cell membrane- the lipid bilayer- serves as a barrier to the passage of polar and high molecular weight molecules (Alberts et al., 2002). The gut has glucosidases which could hydrolyze the glucose moiety, thus facilitating the absorption of such compounds. In view of this it may be suggested that further studies on these lines are required.

Several researchers have reported that WF A is a highly biologically active compound (Yang et al., 2007; Sen et al., 2007; Mohan et al., 2007; Devi and Sharada, 1995; Sabina et al., 2008). After oral administration of aqueous extract of Ashwagandha, the WF A was more bioavailable compared to WNA (Patil et al., 2013). Surprisingly and paradoxically it was observed that the $P_{\text{eff}}$ value of WF A was the lowest ($3.30 \times 10^{-07}$) implying that it may be impermeable in the in vitro model i.e. in the MDCK cells or that it may be metabolized as it passes through the cell layer and we are unable to measure the metabolite. However, as mentioned above, after oral administration of aqueous extract of Ashwagandha to mice there was significant absorption resulting in high concentration of WF A in the plasma (Patil et al., 2013). It seems that the process of WF A absorption is more complex and that MDCK cells in vitro model possibly does not provide the exact in vivo environment. However, this possibility needs to be verified further by direct experiments using different cell culture system such as Human Epithelial Colorectal Adenocarcinoma (Caco-2) cell
monolayer (Irvine et al., 1999). Although both CaCo2 cells and MDCK cells are predictors for passive properties and lack of active transporters, the discrepancy seen in the present studies could likely be attributed to the lack of active transporters or species difference.

6.5. Conclusions

Based on the Sino-Veda absorption model the absorption characteristics of the tested withanolides, it may be concluded that WN A, WNN, 1, 2 DWM and WN B were highly permeable; whereas WS V showed low permeability. Surprisingly WF A, the highly biologically active withanolide was found to be either impermeable or WF A metabolized on passing through the cell layer. It is likely that WFA in vivo absorption is more complex and needs further in depth investigations. The study demonstrates the usefulness of the technology for rapid testing of bioavailability of drugs.