3. MATERIALS AND PROTOCOLS

3.1 Sources of Chemicals

Iloperidone (ILO) and **Vardenafil HCL Trihydrate** (VDN) were kindly gifted by Alembic Pharmaceuticals Ltd. (Vadodara, India). Excipients used for formulation development are shown in Table 3.1 and were used as received. Chemicals and reagents used for the preparation of buffers, analytical solutions, and other general experimental purposes are shown in Table 3.2. Reagents and autoclavable plasticwares used for cell line study are shown in Table 3.3. Equipment used at various stages are listed in Table 3.4. Purified HPLC grade water was obtained by filtering double distilled water through nylon filter paper 0.45 µm pore size (Pall Life sciences, Mumbai, India).

Table 3.1 List of Excipients for formulation development

Excipients	**Manufacturer/Supplier**
Acconon C-80	Abitec Corporation, USA
Acconon CC-6	Abitec Corporation, USA
Brij 35	S.D. Fine Chemicals, Mumbai, India
Capmul MCM	Abitec Corporation, USA
Capmul MCM C8	Abitec Corporation, USA
Capmul MCM L8	Abitec Corporation, USA
Capmul PG 8 (Capryol 90)	Gattefosse, France
Capryol PGE 860	Abitec Corporation, USA
Capryol PGMC	Gattefosse, France
Captex 200 (Labrafac PG)	Abitec Corporation, USA
Captex 300 (Labrafac Lipophile WL 1349)	Abitec Corporation, USA
Captex 500	Abitec Corporation, USA
Castor Oil	S.D. Fine Chemicals, Mumbai, India
Cholesterol	Sigma-Aldrich, Mumbai, India
Coconut Oil	S.D. Fine Chemicals, Mumbai, India
Cremophor EL	Sigma-Aldrich, Mumbai, India
Ethyl Oleate	S.D. Fine Chemicals, Mumbai, India
Glycerol	S.D. Fine Chemicals, Mumbai, India

Imwitor 380	SASOL, Germany
Iso-propyl myristate	S.D. Fine Chemicals, Mumbai, India
Kolliphor HS 15	Sigma-Aldrich, Mumbai, India
Kolliphor RH 40	Sigma-Aldrich, Mumbai, India
Kollisolv MCT 70	BASF, Mumbai, India
Labrafac CC	Gattefosse, France
Labrafil M 1944 CS	Gattefosse, France
Labrafil M 2125 CS	Gattefosse, France
Labrasol (Acconon MC8-2)	Gattefosse, France
Lauroglycol 90	Gattefosse, France
Lauroglycol FCC	Gattefosse, France
Maisine L 35	Gattefosse, France
Oleic Acid	S.D. Fine Chemicals, Mumbai, India
Olive Oil	S.D. Fine Chemicals, Mumbai, India
Peceol (Capmul GMO 50)	Gattefosse, France
PEG 200	S.D. Fine Chemicals, Mumbai, India
PEG 400	S.D. Fine Chemicals, Mumbai, India
PEG 600	S.D. Fine Chemicals, Mumbai, India
Plurol Oleique CC 497	Gattefosse, France
Propylene Glycol	S.D. Fine Chemicals, Mumbai, India
Soyabean Oil	S.D. Fine Chemicals, Mumbai, India
Span 20	S.D. Fine Chemicals, Mumbai, India
Span 40	S.D. Fine Chemicals, Mumbai, India
Span 60	S.D. Fine Chemicals, Mumbai, India
Span 80	S.D. Fine Chemicals, Mumbai, India
Sunflower Oil	S.D. Fine Chemicals, Mumbai, India
Transcutol HP	Gattefosse, France
Tween 20	S.D. Fine Chemicals, Mumbai, India
Tween 40	S.D. Fine Chemicals, Mumbai, India

Tween 60	S.D. Fine Chemicals, Mumbai, India
Tween 80	S.D. Fine Chemicals, Mumbai, India

Table 3.2 List of Chemicals, Reagents and Filters used for Analysis

Chemicals/Reagents	**Manufacturer/Supplier**
Acetonitrile, LC/MS grade	Fisher Chemicals, USA
Methanol, LC/MS grade	Fisher Chemicals, USA
Formic Acid, LC/MS grade	Fisher Chemicals, USA
Acetonitrile, HPLC grade	Spectrochem Labs Ltd., Vadodara, India
Methanol, HPLC grade	Spectrochem Labs Ltd., Vadodara, India
Chloroform, HPLC grade	Spectrochem Labs Ltd., Vadodara, India
Hydrochloric Acid, AR grade	SR Enterprise, Vadodara, India
Potassium Dihydrogen Phosphate, GR grade	S.D. Fine Chemicals, Mumbai, India
Triethylamine, GR grade	S.D. Fine Chemicals, Mumbai, India
Orthophosphoric acid, GR grade	S.D. Fine Chemicals, Mumbai, India
Potassium Hydroxide, AR grade	S.D. Fine Chemicals, Mumbai, India
Dimethyl Formamide, GC headspace grade	Fisher Chemicals, USA
Deuterated water	Sigma-Aldrich, Mumbai, India
25 mm, 0.45 µ PTFE syringe filter	Himedia, India
25 mm, 0.45 µ Nylon syringe filter	Himedia, India
47 mm, 0.45µ, Nylon membrane filter	Millipore, India

Table 3.3 Cell Line study useables

Chemicals/Reagents	**Manufacturer/Supplier**
CaCO2 cell line	NCCS, Pune, India
Trypsin-EDTA	Himedia, India
Coumarin 6 dye	Himedia, India
DAPI dye (4',6-diamidino-2-phenylindole)	Himedia, India
MTT dye (Methylthiazolyldiphenyl tetrazolium bromide)	Himedia, India

Minimum Essential Medium	Himedia, India
Penicillin/Streptomycin (10,000 U/mL/ 10,000 µg/mL)	Himedia, India
Fetal Bovine Serum	Himedia, India
Sterile Tissue culture plates (12, 96 well)	BD Falcon, India
Sterile Tissue culture flasks (25, 75 cm2)	BD Falcon, India

Table 3.4 Instruments used

Bath Sonicator	Modern Industrial Corporation, India
Cell imaging system	FLoid® Imaging Station (Thermo Scientific, India)
Centrifuge	PR 24 Centrifuge (Remi Equipments, India)
Confocal Microscope	Zeiss LSM 510 (Zeiss, Gemrany)
Cryo-TEM	Tecnai G2 cryo-TEM (FEI Company Ltd., USA).
Differential Scanning Calorimeter	DSC-60 (Shimadzu, Japan)
Dissolution apparatus	DS 8000 (M/s LabIndia Instruments, India)
Flow Cytometer	FACS Caliber Flow (BD lifesciences, USA)
Gas Chromatography System	Clarus 500 (Perkin Elmer, India)
Trinocular Microscope	Labomed (Nightingale Sales, USA)
Lyophilizer	Heto Powerdry LL1500 (Thermo Scientific, India)
Magnetic Stirrer	Remi, India
Particle Size Analyzer (DLS)	Nano ZS 90 - Malvern Zetasizer (Malvern, UK)
Diffractometer	SANS Diffractometer (BARC, Mumbai)
Probe Sonicator	Labsonic M (Sartorius, Germany)
Rheometer	RST coaxial cylinder rheometer (Brookfield, USA)
Rotary Evaporator	RV-10 Rotavapor (IKA Companies Ltd., India)
RP-HPLC	LC-20AT (Shimadzu, Japan)
Scanning Electron Microscope	JSM-5610LV (JEOL, India)
Solid Phase Extraction Assembly	Ezypress HT 48 (Orochem Laboratories, India)
UPLC-MS	ABSIEX QTRAP 4500 (DHR Holding Ltd., India)
UV-Vis Spectrophotometer	UV 1800 (Shimadzu, Japan)

X-Ray diffractometer	X'Pert (PANnalyticals, India)
Zetasizer	Nano ZS 90 - Malvern Zetasizer (Malvern, UK)
Laminar air flow (HEPA filter)	Weiber vertical laminar air flow (India)
Stability Chamber	S. R. Lab instrument (Mumbai, India)

3.2 General protocols for Cell Cultures

The Caco2 (Homo sapiens colon colorectal cancer cells) cell line was obtained from the cell repository facility of National Center of Cell Sciences, Pune, India. The cell line was maintained at 37˚C in a humidified 5% CO_2 atmosphere (Jouan IGO 150 incubator, Thermo Fisher Scientific, India). The cultures were maintained in MEM with 10% heat inactivated fetal bovine serum (FBS) and 1% antibiotic solution [1].

3.2.1 Media Preparation

Complete media was prepared by mixing MEM with antibiotic solution (1% v/v) and 10% v/v heat inactivated fetal bovine serum (FBS). This complete media was stored in a sterile screw capped bottle in refrigerated condition (NMT 25° C). The bottle was then sealed with parafilm and wrapped with aluminum foil. The process was carried out in a vertical laminar air flow cabinet.

3.2.2 Sub-culturing of cell line

The cells were maintained as monolayer culture in T-25 cell culture flasks, and subcultured twice every week. The Subcultured cell lines were then placed at 37°C in a humidified atmosphere at 95% air and 5% CO2 (Jouan IGO150 CO_2 Incubator, Thermo Fisher Scientific, India) in complete media. Fresh complete medium was replaced every 3 days.

Detailed Procedure:

- The complete medium was pre-warmed water bath at 37°C.
- The cells were taken from incubator and observed for cell growth under the microscope (made sure cells have grown ~80% confluence).
- The medium was removed from flask by aspiration. The cells attached to the flask were washed twice with culture medium without serum.

- 1 mL of trypsin EDTA solution was added and then allowed to stand for 5 min occasionally swirling to dislodge the cells.
- Then 5 mL of complete medium was added to stop the trypsin activity.
- After gently pipetting the cells up and down to disrupt cell clumps, the cells were counted and reseeded in fresh flask at desired seeding density.
- The flasks were then incubated properly.

3.2.3 Cell counting using Hemocytometer

1. Preparing hemocytometer
    - The hemocytometer was cleaned using 70% ethanol.
    - The coverslip was firmly affixed using gentle pressure.
2. Preparing cell suspension
    - After trypsinization of adherent cells, cell suspension was prepared. The trypsin action was deactivated by adding FBS. After centrifugation at 1000 rpm, the pellet was resuspended in a smaller volume of complete media.
3. Counting
    - After gentle vortex, 20 µL of the cell suspension was transferred to the edge of a coverslip.
    - After removing any surplus fluid carefully, cells in 4 corner squares of the grid were calculated.
    - The average number of cells in 4 squares was calculated as:
      Average cell count per square = total number of cells in 4 squares/4
    - Total cell count was calculated as follow:
      Cell Count (Cells/mL) = Average Cell Count * 10^4 * dilution factor

3.3 General procedure for characterization techniques

3.3.1 Size

Particle size and its distribution is one of the most important characteristics for nano-sized formulation as it governs penetration and absorption from git. For size measurement, which is in nano range, scattering techniques are preferred. Scattering techniques can provide information about the structure as well as size of nano-materials [2]. Amongst the commonly used scattering techniques are DLS, SANS, small-angle X-ray scattering (SAXS) and static light scattering (SLS). SLS, SAXS and SANS can be used to find interactions of the particles, structure and size. DLS is based on diffusion of particles in solution, which is related to its hydrodynamic size.

The type of samples that can be studied by scattering techniques, the sample environment that can be applied, the actual length scale probed and the information that can be obtained, all depend on the nature of the radiation employed. For example, SANS with high penetration depth of monochromatic beam of neutrons can easily be applied to opaque samples which cannot be studied by DLS [3]. SANS measures the actual size of the particle, whereas DLS measures the hydrodynamic size of the particle. As the LASER diffracts from the hydration layer around the particles, the hydrodynamic diameter is always larger than the actual. DLS results are biased towards larger length scale present in the system, whereas SANS provides more statistical results. Thus, to a large extent these techniques are complementary to each other, whilst sharing many similarities also.

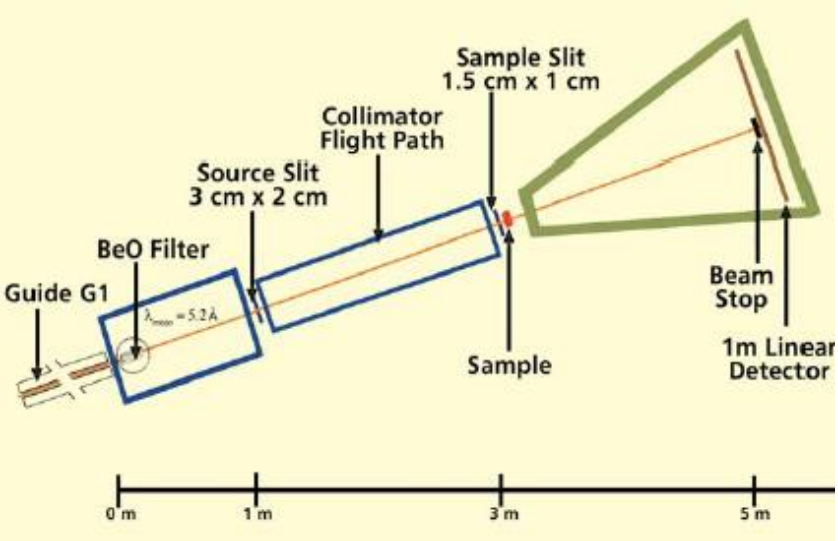
3.3.1.1 Dynamic Light Scattering (DLS)

DLS also known as Photo-Correlation Spectroscopy (PCS) gives hydrodynamic size of the particulate system on the basis of Brownian motion. The diffusion of particles is calculated by Stokes-Einstein equation. For DLS principle-based technique of size determination, zeta sizer Nano-ZS (Malvern Instruments, UK) equipped with 4.0 mW internal laser was used. 1 mL of suitably diluted sample was taken in disposable polystyrene sizing cuvette and measured at 4.65 mm position. Sufficiently diluted samples were used for all measurement to avoid multiple scattering. All measurements were performed at 25°C, at a scattering angle of 173°. The intensity-weighted mean diameter was obtained for each sample in triplicate [4].

3.3.1.2 Small Angle Neutron Scattering (SANS)

In SANS technique, radiation is elastically scattered by a sample and the resulting scattering pattern is analyzed to provide information about the size, shape and orientation of some component of the sample. In SANS study; since D2O is taken as dilution media, the size determination of only core excluding outer hydrophilic part of surfactant is possible due to contrast [5]. The small-angle neutron scattering experiments were performed by SANS diffractometer at Dhruva reactor, Bhabha Atomic Research Centre (BARC), Mumbai, India. The diffractometer was made of beryllium oxide filtered beam having a radiation wavelength of 5.2 Å, with distance of 2 m between sample and detector. The incoming neutrons were monochromatized by mechanical velocity selector. The length of scattering vector Q was from 0.017 to 0.35 Å-1. The scattering from each sample was corrected for electronic background, detector deadtime, scattering from the empty cell and sample transmission. The intensity was converted to differential scattering cross-sections in absolute units (cm−1).

Figure 3.1 Schematic of SANS instrument



3.3.2 Zeta Potential

Zeta potential is a physical property exhibited by colloidal system. It is an index representing the electrostatic stabilization. The liquid layer surrounding the particle exists as two parts; an inner region (Stern layer) where the ions are strongly bound and an outer (diffuse) region where

they are less firmly associated. Within the diffuse layer there is a notional boundary inside which the ions and particles form a stable entity. When a particle moves, ions within the boundary move it. Those ions beyond the boundary stay with the bulk dispersant. The potential at this boundary (surface of hydrodynamic shear) is the zeta potential. Zeta potential was measured using the folded capillary cell in Zetasizer (Nano ZS 90, Malvern Instruments Ltd., Malvern, UK). The measurement was carried out at 25°C in triplicate using multimodal analysis strategy. Smoluchowski approximation was used for zeta potential value determination [6].

3.3.3 Solid state characterization

3.3.3.1 Powder X-ray diffraction (PXRD)

XRD depends on the dual wave and particle nature of X-ray to obtain information about crystallinity of materials. The dominant effect that occurs when an incident beam of monochromatic X-rays interacts with a target material is scattering of those X-rays from atoms within the target material [7]. In materials with crystalline structure, the scattered X-rays undergo constructive and destructive interference which is termed as diffraction. The diffraction of X-rays by crystals is described by Bragg's Law, n(lambda) = 2d sin(theta). The directions of possible diffractions depend on the size and shape of the unit cell of the material. The intensities of the diffracted waves depend on the kind and arrangement of atoms in the crystal structure. The crystal structure of drug and changes in crystallinity (correlated with intensity in XRD graph) were observed using powder X-ray diffractometer (X'Pert Pro, PANalyticals, Singapore) equipped with Ni-filtered Cu-Kα radiation, and voltage diffraction. Scan was performed for around 10-20 mg powder sample placed on sample holder at a 2 – theta range from 5-60° with a scan rate of 3°/min.

3.3.3.2 Fourier Transform Infrared Spectroscopy (FT-IR)

When IR radiation is passed through a sample, some radiation is absorbed by the sample and some passes through (is transmitted). The resulting signal at the detector is a spectrum representing a molecular 'fingerprint' of the sample [8]. The usefulness of infrared spectroscopy arises because different chemical structures produce different spectral fingerprints which are characteristic for each. The compatibility between components can be identified from change in wavenumber. Scanning of samples were performed in the range of 500–4000 cm^{-1}

to obtain FT-IR spectra (FTIR, Bruker; Germany). All solid samples were compressed into KBr disks whereas liquid samples were placed in NaCl cell.

3.3.3.3 Differential Scanning Calorimetry (DSC)

The thermoanalytical technique, DSC measures difference in amount of heat required to increase the temperature of sample and reference as a function of temperature [9]. The instrument (DSC- 60) was calibrated with indium under nitrogen purging to avoid any kind of oxidative degradation. Thermal behavior of drug, its physical mixture with the excipients and formulation was studied. For this, around 5 mg sample was placed in aluminum pan which was heated from 25° to 300° under nitrogen atmosphere at a scanning rate of 10°/min.

3.3.4 Morphology Study

3.3.4.1 Cryo-Transmission Electron Microscopy (Cryo-TEM)

Cryo-TEM was used to study internal structure of ultra-thin cut samples. Appropriately diluted sample was applied on perforated carbon film supported by copper grid, the surface of which was modified for proper adhesion of sample to the grid surface. A thin film of the sample formed when it was put in liquid ethane followed by liquid nitrogen. Blotting parameters such as film thickness and vitrification were kept similar between observed samples. Subsequently, the grid was transferred to cryo holder already maintained at cryo temperature of -170°C and samples were observed for morphology and size using Tecnai G2 cryo-TEM (FEI Company Ltd., Hillsboro, USA) [10].

3.3.4.2 Scanning Electron Microscopy (SEM)

In contrast to TEM, where the electron beam is transmitted through the specimen, in SEM, the beam is scanned across, which creates image of the surface of the sample, with exceptional depth of field. Size and morphology of the sample was studied using SEM - JSM-5610LV (JEOL, India) [11]. For this, samples were mounted on stubs using vacuum compatible double-sided conductive carbon tapes. The acceleration voltage used for analysis was of the order of 10 kV.

References:

1. Freshney, R. I. (2005). Subculture and Cell Lines. In *Culture of Animal Cells: A Manual of Basic Technique* (Vol. 642, pp. 199-216). Hoboken, NJ, USA: Wiley-Liss.

2. Bouanani, F., Bendedouch, D., Teixeira, J., Marx, L., & Hemery, P. (2012). Characterization of a miniemulsion by DLS and SANS. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 404*, 47-51, doi:10.1016/j.colsurfa.2012.04.011.

3. Chu, B., & Liu, T. (2000). Characterization of Nanoparticles by Scattering Techniques. *Journal of Nanoparticle Research, 2*(1), 29-41, doi:10.1023/A:1010001822699.

4. Patel, K., Patil, A., Mehta, M., Gota, V., & Vavia, P. (2013). Medium Chain Triglyceride (MCT) Rich, Paclitaxel Loaded Self Nanoemulsifying Preconcentrate (PSNP): A Safe and Efficacious Alternative to Taxol®. *Journal of Biomedical Nanotechnology, 9*(12), 1996-2006, doi:10.1166/jbn.2013.1710.

5. Shu, F., Ramakrishnan, V., & Schoenborn, B. P. (2000). Enhanced visibility of hydrogen atoms by neutron crystallography on fully deuterated myoglobin. *Proceedings of the National Academy of Sciences of the United States of America, 97*(8), 3872-3877.

6. Kaszuba, M., Corbett, J., Watson, F. M., & Jones, A. (2010). High-concentration zeta potential measurements using light-scattering techniques. *Philosophical transactions. Series A, Mathematical, physical, and engineering sciences, 368*(1927), 4439-4451, doi:10.1098/rsta.2010.0175.

7. X-Ray Diffraction (XRD). https://www.xos.com/XRD. Accessed 22 february 2018.

8. Pavia, D. L., Lampman, G. M., Kriz, G. S., & Vyvyan, J. A. (2008). Infrared Spectroscopy. In *Introduction to spectroscopy* (pp. 13-101). United States: Cengage Learning.

9. Gabbott, P. (2008). A Practical Introduction to Differential Scanning Calorimetry. In *Principles and applications of thermal analysis* (pp. 1-50): John Wiley & Sons.

10. Wolf, L., Hoffmann, H., Talmon, Y., Teshigawara, T., & Watanabe, K. (2010). Cryo-TEM imaging of a novel microemulsion system of silicone oil with an anionic/nonionic surfactant mixture. *Soft Matter, 6*(21), 5367-5374, doi:10.1039/C0SM00049C.

11. SEM - Scanning Electron Microscope A to Z *Basic Knowledge For Using SEM*, 1-32.