Chapter 4

Experimental
4.1 MATERIALS AND EQUIPMENTS

Table 4-1 and 4-2 list the chemicals/materials and the various instruments used respectively, during the course of study.

Table 4-1. List of materials

<table>
<thead>
<tr>
<th>Name</th>
<th>Purity</th>
<th>Source/Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>HPLC grade</td>
<td>Merck, India</td>
</tr>
<tr>
<td>Ethanol</td>
<td>≥ 99.8%</td>
<td>Merck, Darmstadt, Germany</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>≥ 97%</td>
<td>Merck Schuchardt, Germany</td>
</tr>
<tr>
<td>Stearylamine</td>
<td>~ 97%</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td>Tripalmitin (Glyceryl tripalmitate)</td>
<td>~ 99%</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td>Soya Lecithin</td>
<td>~ 99%</td>
<td>BDH Laboratory, England</td>
</tr>
<tr>
<td>Poloxamer 188 (Pluronic® F-68)</td>
<td>~ 99%</td>
<td>Pluronic® BASF Corp., Sigma, USA</td>
</tr>
<tr>
<td>(+)-α-Tocopherol acetate</td>
<td>~ 95%</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>99.87%</td>
<td>Dabur Pharma Ltd., India</td>
</tr>
<tr>
<td>Pepsin A</td>
<td></td>
<td>Sigma, USA</td>
</tr>
<tr>
<td>Malanaldehyde bis (dimethyl acetal)</td>
<td>99%</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td>2-Thiobarbituric acid</td>
<td>99%</td>
<td>Himedia Labs Pvt. Ltd., India</td>
</tr>
<tr>
<td>MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide)</td>
<td>~ 98%</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td>Human hepatoma cell line, HepG2</td>
<td></td>
<td>National Centre for Cell Sciences (NCCS), Pune, India</td>
</tr>
<tr>
<td>(ATCC No.HB- 8065)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Experimental

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Grade</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Trehalose dihydrate extrapure</td>
<td>AR</td>
<td>SRL Pvt. Ltd., India</td>
</tr>
<tr>
<td>Sodium Taurocholate</td>
<td>AR</td>
<td>CDH, India</td>
</tr>
<tr>
<td>Sodium Glycocholate</td>
<td>AR</td>
<td>CDH, India</td>
</tr>
<tr>
<td>Diethyl Ether</td>
<td>AR</td>
<td>S.D. Fine Chem., India</td>
</tr>
<tr>
<td>Sodium sulfide</td>
<td>AR</td>
<td>CDH, India</td>
</tr>
<tr>
<td>Disodium hydrogen phosphate</td>
<td>AR</td>
<td>Merck, India</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>AR</td>
<td>Qualigens Fine Chemicals, India</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>AR</td>
<td>S.D. Fine Chem., India</td>
</tr>
<tr>
<td>Human Albumin</td>
<td>20%</td>
<td>Ph. Eur ZENA LBR20</td>
</tr>
<tr>
<td>Human Albumin solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg Albumin flakes</td>
<td>AR</td>
<td>S.D. Fine Chem., India</td>
</tr>
<tr>
<td>Tween 80</td>
<td>AR</td>
<td>CDH, India</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>AR</td>
<td>Qualigens Fine Chemicals, India</td>
</tr>
<tr>
<td>Sodium-dodecyl sulphate</td>
<td>99%</td>
<td>Sigma, USA</td>
</tr>
<tr>
<td>Glacial Acetic acid</td>
<td>AR</td>
<td>Qualigens Fine Chemicals, India</td>
</tr>
<tr>
<td>Butanol</td>
<td>AR</td>
<td>SRL Pvt. Ltd., India</td>
</tr>
<tr>
<td>Pyridine</td>
<td>98%</td>
<td>Glaxo Laboratories Ltd., India</td>
</tr>
<tr>
<td>Hydrochloric acid</td>
<td>AR</td>
<td>Qualigens Fine Chemicals, India</td>
</tr>
<tr>
<td>Sodium Hydroxide</td>
<td>AR</td>
<td>S.D. Fine Chem., India</td>
</tr>
<tr>
<td>Dimethyl sulphoxide (DMSO)</td>
<td>99.9%</td>
<td>Fisher scientific, USA</td>
</tr>
</tbody>
</table>
Experimental

High-purity water (Milli-Q) was used throughout and was produced by double reverse osmosis (Milli-Q plus, Millipore, USA) two stages of mixed bed ion exchange, two stages of activated carbon and a final stage involving 0.22μm filtration.

Table 4-2. List of equipments

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Model/ Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifugal Vaccum Concentrator</td>
<td>CHRIST, Germany</td>
</tr>
<tr>
<td>Cooling Centrifuge</td>
<td>Remi Equipments, India</td>
</tr>
<tr>
<td>Cyclo Mixer</td>
<td>CM101, Remi Equipments, India</td>
</tr>
<tr>
<td>Deep Freezer -70°C</td>
<td>925 Thermo Forma, USA</td>
</tr>
<tr>
<td>Deep Freezer -20°C</td>
<td>&quot; New Brunswick Scientific, Germany</td>
</tr>
<tr>
<td></td>
<td>&quot; CC300, Blue Star, India</td>
</tr>
<tr>
<td>Dialysis Tubing Cellulose membrane</td>
<td>D9277, Sigma, USA</td>
</tr>
<tr>
<td>Differential Scanning Calorimeter</td>
<td>Perkin Elmer Pyris 6 DSC, USA</td>
</tr>
<tr>
<td>Double beam UV spectrophotometer</td>
<td>Shimadzu, 1601 UV/VS, Shimadzu, Japan</td>
</tr>
<tr>
<td>Electronic Weighing Balance</td>
<td>&quot; B244, Mettler-Toledo, Switzerland</td>
</tr>
<tr>
<td></td>
<td>&quot; AE240, Mettler, Switzerland</td>
</tr>
<tr>
<td>FT-IR Spectrometer</td>
<td>FTS 135, Bio-Rad Laboratories, USA</td>
</tr>
<tr>
<td>High shear homogenizer</td>
<td>SilentCrusher M, Heidolph instruments GmbH and Co. KG, Germany</td>
</tr>
<tr>
<td>High-vaccum Evaporator</td>
<td>Sputter coater, SCD 020, UK</td>
</tr>
<tr>
<td>Lyophilizer</td>
<td>CHRIST ALPHA 1-2, Vaccubrand Type RZ2, Germany</td>
</tr>
</tbody>
</table>
Magnetic stirrer
pH meter
Scanning Electron Microscope
Semi Autoanalyzer
Shaking Water Bath
Sonicator Bath
Stability Chamber
Tissue Homogenizer
Transmission Electron Microscope
Ultracentrifuge
Vortex Mixer
X-Ray Diffractometer
Zetasizer Nano ZS90

Scientific Apparatus, India
" Mettler Toledo, USA
" DB1011, Widsons Scientific Works, India
LEO 435 VP, Germany
Erba Chem 5 Plus, Transasia, India
NSW133, National Scientific Work, India
Prama Instruments Pvt. Ltd., India
Nirmal International, India
Centrifon motar Type CM120, Widsons Scientific Works, India
FEI Philips, Morgagni 268D, USA
Beckman L-80 equipped with a Ti-70 rotor,
Beckman Instruments Inc., USA
Spinix, India
Bruker D8 ADVANCE, USA
Malvern Instruments, UK

Experimental

4.2 PREPARATION OF STANDARD SOLUTIONS/BUFFERS

4.2.1 Simulated gastric fluid

Dissolved 2.0 g of sodium chloride and 3.2g of pepsin in 7.0 ml of hydrochloric acid and added sufficient water to make 1000 ml. This test solution had a pH of about 1.2. (USP XXIV, pH 1.2, pepsin 0.32% w/v).
4.2.2 Phosphate buffered saline (PBS) pH 7.4

PBS pH 7.4 was prepared as per method given in IP, 1985. 1.38 g of disodium hydrogen phosphate, 0.19 g of potassium dihydrogen phosphate and 8.0 g of sodium chloride were dissolved in sufficient water to produce 1000 ml. Immediately before use the pH was adjusted, if necessary.

4.3 CHARACTERIZATION AND IDENTIFICATION OF PACLITAXEL

The sample of paclitaxel was characterized on the basis of its colour, solubility in water and other organic solvents etc. Melting point, UV, X-ray diffraction and FTIR spectral analysis were carried out on the obtained sample and matched with that of a reference standard. Assay of the drug was done as per the method described in USP XXIV/ NH 19, 2000.

4.4 ANALYTICAL METHODOLOGY FOR PACLITAXEL

High-performance liquid chromatography (HPLC) in conjunction with ultraviolet (UV) detection was used for the development of a sensitive assay for paclitaxel analysis.

4.4.1 Instrumentation

HPLC was done on a LC surveyor system (Thermo Finnigan, USA). System consists of a quartenary LC pumps (LC-10ATVP), an auto sampler (SIL-10AD VP) with a 100μl loop, and degasser unit (DGU-14AM). The system was equipped with a surveyor PDA detector (SPD-M10A VP). For data acquisition and integration, Chromquest software (Thermo Finnigan, USA) was used.

4.4.2 Chromatographic conditions

Chromatographic separations were carried out by slight modification of the method reported by Chen et al., using the LiChroCART® 250-4 LiChrospher® 100, RP-18
Experimental

column purchased from Merck KgaA, Germany (5 μm) and guard column employed was LiChroCART® 4-4 LiChrospher® 100 RP-18 (5 μm) (Merck KgaA, Germany). Mobile phase consisted of acetonitrile (ACN) and purified water (70:30). The mobile phase was prepared by using MilliQ water (Bedford, USA). Mobile phase was filtered through 0.22 μm membrane filter under vacuum before running the experiment and inbuilt degasser present in the system degassed it. The flow rate was kept at 1ml/min and system was maintained at an ambient temperature of 25 ± 1°C and the detection was carried out at λ_max = 227 nm. Injection volume was 20 μl and the data was acquired and processed by Chromquest software (Thermo Finnigan, USA). (Crosasso et al., 2000, Chen et al., 2001).

4.4.3 Preparation of stock solution

Stock solution of paclitaxel was prepared in ACN at 1mg/ml and could be stored in a freezer (−20°C) for a period of four weeks without degradation (Lee et al., 1999). This stock solution was diluted with 50% ACN to obtain the concentrations required for preparation of standard working solutions. All preparations were made in polypropylene microtubes (MCT-175-C, 1.7 ml; MCT-200-C, 2.0 ml, Axygen Scientific, USA) (Wang et al., 2003).

4.4.4 Preparation of standard and quality control solutions

For preparation of calibration curves, an appropriate volume of working standard solution (prepared by diluting the stock solution with 50% ACN in deionized water immediately prior to use) were spiked with phosphate buffer pH 7.4 and blank mouse plasma for buffer and plasma calibration samples. The spiked buffer standards ranged from 66 ng/ml to 66666 ng/ml (66, 166, 333, 666, 1666, 3332, 6664, 33320, 66640 ng/ml) and the
spiked plasma standards ranged from 0.025 μg/ml to 20 μg/ml (25, 100, 500, 1000, 2000, 5000, 10000, 20000 ng/ml).

Quality control solutions were prepared from plasma and buffer in the same manner using a different fresh stock solution at concentrations of 0.025, 1 and 10; and 0.066, 1.66 and 6.66 μg/ml, respectively and stored at -20°C.

4.4.5 Preparation of calibration curve
For calibration curves, the detector response (peak area) on y-axis was plotted against the drug concentrations on x-axis.

4.4.6 Extraction procedures

For assay of in vitro release samples: The buffer standard and sample solutions were extracted with 1ml of dichloromethane-(DCM) and the mixture was vortexed for 60 s. Upon centrifugation at 10000 rpm for 10 min, the organic layer was transferred to microtubes and was evaporated under vacuum using a centrifugal vacuum concentrator (Christ, Germany). The residue was then reconstituted with 200 μl ACN and was mixed on a vortex mixer for 90 s. Then the reconstituted samples were transferred to autosampler vials and 20 μl was injected from each sample onto the HPLC column (Jackson et al., 2004).

For assay of stability study samples: For the estimation of drug content in optimized formulation, stability indicating HPLC method reported by Waugh et al., 1991 was used. The chromatographic conditions were same as mentioned under section 4.4.2.

For assay of in vivo study samples: For the determination of paclitaxel in plasma and tissues a solid phase extraction (SPE) method using SPE cartridges for drug extraction followed by HPLC analysis was used after slight modification of method by Willey et al.
Experimental

(Willey et al., 1993). SPE was processed with C18 SPE columns, 100mg/3ml, Samprep™ (Ranbaxy Fine Chemicals Ltd.). First of all, C18 SPE columns (100mg/3ml, Samprep™) were conditioned by consecutive washings with 1.0 ml Milli Q water, 1.0 ml acetonitrile and 2.0 ml Milli Q water, respectively. Then plasma sample (100 µl) was loaded onto the SPE column; next, the columns were washed with 4.0 ml of Milli Q water. The columns were dried under maximum vacuum for 30 s. Finally the analyte was eluted from the columns with 1.0 ml acetonitrile. The eluent was directly taken for HPLC analysis without vacuum evaporation. Tissue samples (100mg) were homogenized with 200 µl of deionized water using a tissue homogenizer. The tissue homogenates were vortexed and sonicated with 400 µl of ACN and taken for SPE and HPLC analysis.

4.4.7 Validation of analytical method

The developed method was validated as per ICH guidelines for linearity, accuracy and precision, and specificity (ICH Q2A, Q2B, 1996).

4.5 THIOBARBITURIC ACID-REACTIVE SPECIES (TBARS) ASSAY

4.5.1 Preparation of calibration curve of Malondialdehyde (MDA; 1,1,3,3-Tetramethoxypropane (TMP)) (Buege and Aust, 1978; Ohkawa et al., 1979)

4.5.1.1 Solutions prepared

Thiobarbituric acid (TBA)

To prepare 0.8% of TBA solution, 0.2 g of TBA was dissolved in distilled water and made up to 25 ml with distilled water in a amber colored bottle.

Sodium-dodecyl sulphate (SDS)

To prepare 8.1% of SDS solution, 0.81 g of SDS was dissolved in distilled water and made up to 10 ml with distilled water.
*Acetic acid solution*

To prepare 20% of acetic acid solution, 5 ml of acetic acid was dissolved in distilled water and made up to 25 ml with distilled water.

*0.1N Hydrochloric acid (HCl)*

0.45 ml of HCl was made up to 50 ml with distilled water.

*4N Sodium hydroxide (NaOH)*

16 g of NaOH was dissolved in distilled water and made up to 100 ml with distilled water.

*Butanol: Pyridine mixture*

A 15:1 v/v mixture of Butanol: Pyridine was prepared and kept in a amber colored bottle.

*Preparation of MDA (Malondialdehyde) standard solution*

For preparing MDA standard solutions, marketed MDA stock solution (100 µl = 0.1083 g TMP) was used. 10 µl of TMP was made up to 100 ml with 0.1N HCl. After shaking, 1 ml of this solution was diluted up to 10 ml with 0.1N HCl, to give a resultant concentration of 0.66 nmol/10 µl of 0.1N HCl. MDA standards ranged from 0.66 nmol to 13.2 nmol (0.66, 1.32, 2.64, 5.28, 10.56, 13.2 nmol/10 µl of 0.1N HCl). For all the prepared solutions amber colored bottles were used.

4.5.2 Assay Procedure

TBARS assay was based on the method of Ohkawa et al., 1979. For preparation of calibration curve, to the standard MDA solutions (0.2 ml) were added 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with sodium hydroxide, and 1.5 ml of 0.8% aqueous solution of TBA. The mixture was made up to 4.0 ml with
Experimental distilled water, and then heated at 95°C for 60 min. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1, v/v) were added and shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured using UV spectrophotometer. For calibration curves, the obtained absorbances were plotted against respective standard MDA concentrations. All the measurements were done in triplicate.

4.6 PREPARATION OF SOLID LIPID NANOPARTICLES

4.6.1 Preparation of SLN using established Microemulsion technique

Microemulsion based technique for the preparation of SLN first developed by Gasco and co-workers was based on the dilution of microemulsions (Gasco, 1997; Bocca et al., 1998; Tiyaboonchai et al., 2007). The lipid was maintained at 75°C and allowed to melt completely. Separately, double distilled water was heated to 75°C. Typically, surfactants were added to the water under magnetic stirring and allowed to equilibrate at 75°C. Next, the water-surfactant solution was added to the melted lipid and once again allowed to equilibrate at 75°C. Once mixed, the dispersed lipid phase of the emulsion required solidification in order to produce the solid lipid nanoparticles. For this, small emulsion aliquots were added to cold water in ratio 1:10 (microemulsion:water, v/v). The cooling (or dilution) water was in the temperature range of 2–3°C, far below the lipids’ melting temperatures. A transparent, thermodynamically stable system was formed, when the compounds were mixed in the correct ratio for microemulsion formation. The dispersion was ultracentrifuged at 40000 g for 1 h at 4°C (Beckman L-80 ultracentrifuge equipped with a Ti-70 rotor, Beckman Instruments Inc., USA) (Cavalli et al., 2000; Heydenreich et al., 2003). The supernatant was discarded and the SLN were redispersed in a trehalose
solution, used as a cryoprotectant and freeze-dried for 24 h. The lyophilized SLN were resuspended in distilled water prior to evaluation. Solid lipid nanoparticles produced were then characterized by their particle size analysis, zeta potential and surface morphology using photon correlation spectroscopy (PCS) and scanning electron microscopy (SEM), respectively.

4.6.2 Preparation of SLN using modified solvent injection technique

The preparation of SLN was based on the solvent injection method reported by Schubert et al. (Schubert and Goymann, 2003), which was slightly modified and then optimized. The lipid and soya lecithin were dissolved in solvent ether and rapidly injected through an injection needle (single use 30G½ PrecisionGlide Needle) into 20 ml of water with or without emulsifier at 40 ± 2°C, under magnetic stirring. The magnetic stirring was maintained for over 30 min to allow solidification of the nanoparticles at 40 ± 2°C, followed by homogenization at 20000 rpm for 1 hr using SilentCrusher M with dispersion tool 8F. The solvent was allowed to evaporate for another 30 min in a rotary evaporator at 30°C. The dispersion was ultracentrifuged at 40000 g for 1 h at 4°C (Beckman L-80 ultracentrifuge equipped with a Ti-70 rotor, Beckman Instruments Inc., USA) (Cavalli et al., 2000; Heydenreich et al., 2003). The supernatant was discarded and the SLN were redispersed in trehalose solution, used as a cryoprotectant and freeze-dried for 24 h. The lyophilized SLN were resuspended in distilled water prior to evaluation.
Experimental

Melting of the lipid (Stearic acid or Tripalmitin) at 75°C

Preparation of hot aqueous surfactant mixture at 75°C

Dispersing of the hot aqueous surfactant mixture into melted lipid and equilibration of mixture at 75°C.

Hot o/w- nanoemulsion

Solidification of nanoemulsion on addition to cold water

Lyophilization of dispersion for 24 hr

Solid Lipid Nanoparticles (SLN)

Figure 4-1. Flowchart for the preparation of SLN using microemulsion technique
4.7 OPTIMIZATION PARAMETERS FOR MODIFIED SOLVENT INJECTION TECHNIQUE

The novel modified solvent injection method used for the preparation of solid lipid nanoparticles was preceded to optimization by varying various process parameters used in its manufacturing such as amount of injected volume, speed of homogenization, effect of lipid and emulsifier concentration (Ahlin et al., 1998; Schubert and Goymann, 2003). The resultant dispersions were characterized in terms of particle size, zeta potential and surface morphology using SEM and PCS.

The influence of the injected amount of the solvent was investigated, using five different injection volumes i.e. 0.5, 1.0, 3.0, 5.0 and 10.0 ml. The homogenization process was performed at three different homogenization rates: 15000, 20000 and 25000 rpm. The SLN dispersions were prepared with four different concentrations of lipid i.e. 0.12, 0.23, 0.46, 0.92 mmol. The effect of poloxamer 188 concentrations on particle size was studied on the samples that contained 0.1, 0.5, 1.0, 1.5, 2.0% w/v of the emulsifier concentration.

Figure 4-2. Schematic representation of SLN preparation using solvent injection technique (Schubert and Goymann, 2003)
Dissolution of lipids and lecithin in Solvent ether

Preparation of hot aqueous surfactant mixture at 40 ± 2°C

Injecting the Lipid into mechanically stirred water with or without emulsifier at 40 ± 2°C

Homogenization of the mixture at 20000 rpm for 1 hr

Lyophilization of dispersion for 24 hrs.

Solid Lipid Nanoparticles (SLN)

Figure 4-3. Flowchart for the preparation of SLN using modified solvent injection technique
4.8 PREPARATION OF PACLITAXEL LOADED SLN USING MODIFIED SOLVENT INJECTION METHOD

Paclitaxel-loaded SLN formulations were fabricated by the addition of different paclitaxel loads of 0.05, 0.25 and 0.5 mmol dissolved in dichloromethane (DCM) (Ceruti et al., 2000; Xu et al., 2005; Jauhari and Dash, 2006), to ether solution containing lipid (0.23mmol), soya lecithin (0.175 mmol) and α-tocopherol (0.025mmol). The remaining procedure was followed as described for the fabrication of blank SLN.

4.9 DETERMINATION OF DRUG LOADING AND ENTRAPMENT EFFICIENCY (EE)

The method for determining the paclitaxel loading and encapsulation efficiency of SLN was according to the previous workers (Ruan and Feng, 2003; Dong and Feng, 2004). 3 mg of lyophilized SLN was dissolved in 1ml of DCM. The mixture was then vortexed vigorously for 5 min followed by centrifugation at 10000 rpm for 10 min. The DCM layer was evaporated under vaccum using Centrifugal Vacuum Concentrator. The residue was then reconstituted with ACN and was mixed on a vortex mixer for 90 s. A portion (20 μl) of the reconstituted sample was injected into the chromatograph and thus the paclitaxel payload in nanoparticles was determined using HPLC analysis. Data was acquired and processed by Chromquest software (Thermo Finnigan, USA). The concentration of drug in the solution was obtained from the calibration curve, which relates peak areas and concentrations. Results were expressed as the mean of three measurements. The recovery efficiency of this extraction procedure was examined using a known weight of paclitaxel: 0.05, 0.1 and 0.5 mg, mixed with 3mg of drug-free SLN, and the procedure of extraction, described above, was repeated. All the recoveries were
approximately 90%, which revealed that approximately 90% of the original paclitaxel could be extracted by this procedure from the mixture of paclitaxel and SLN. The loading efficiencies of paclitaxel in SLN were corrected accordingly.

Experimental

3 mg of lyophilized SLN

Add 1ml DCM

Vortex (5 min) and Centrifuge (10 min, 10000rpm)

Remove DCM layer

Vacuum evaporated to dryness

Reconstitute with 200μl of CAN and vortexed

Taken for HPLC analysis at λ_{max} 227nm

Figure 4-4. Flow chart for extraction of paclitaxel from SLN for entrapment efficiency determination

The drug loading efficiency (L.E.) and drug entrapment efficiency (E.E.) were defined as follows.

Drug loading efficiency (% w/w)

\[
= \left( \frac{\text{Mass of drug in nanoparticles}}{\text{Mass of nanoparticles}} \right) \times 100
\]

Drug entrapment efficiency (% w/w)

\[
= \left( \frac{\text{Mass of drug in nanoparticles}}{\text{Mass of feed drug}} \right) \times 100
\]
4.10 CHARACTERIZATION OF SOLID LIPID NANOPARTICLES

The blank and paclitaxel loaded SLN produced by microemulsion and modified solvent injection methods were characterized by various state-of-art techniques (Schwarz et al., 1994; Cavalli et al., 2000; Fonseca et al., 2002; Dong and Feng, 2004; Lee et al., 2007).

4.10.1 Surface Morphology

The surface morphology of drug free and drug loaded SLN were investigated by scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

4.10.1.1 Scanning Electron Microscopy (SEM)

In a typical SEM, electrons are thermionically emitted from a tungsten or lanthanum hexaboride (LaB$_6$) cathode and are accelerated towards an anode; alternatively, electrons can be emitted via field emission. The electron beam, which typically has an energy ranging from a few hundred eV to 100 keV, is focused by one or two condenser lenses into a beam with a very fine focal spot sized 0.4 nm to 5 nm. The beam passes through pairs of scanning coils or pairs of deflector plates in the electron optical column, typically in the objective lens, which deflect the beam horizontally and vertically so that it scans in a raster fashion over a rectangular area of the sample surface. When the primary electron beam interacts with the sample, the electrons lose energy by repeated scattering and absorption within a teardrop-shaped volume of the specimen known as the interaction volume, which extends from less than 100 nm to around 5 µm into the surface. The size of the interaction volume depends on the electrons' landing energy, the atomic number of the specimen and the specimen's density. The energy exchange between the electron beam and the sample results in the emission of electrons and electromagnetic radiation, which can be detected to produce an image. SEM requires samples to be imaged under...
Experimental

vacuum, which means that samples that would produce a significant amount of vapor, e.g. biological samples, need to be either dried or cryogenically frozen.

SLN dispersion was dried on supports (Critical point dryer, Jumbo) and coated with gold under an argon atmosphere using a gold sputter module in a high-vacuum evaporator (Sputter coater, SCD 020). Samples were then observed with the scanning electron microscope at 15kV (LEO 435 VP, Germany) (Mu and Feng, 2001; Dubes et al., 2003).

4.10.1.2 Transmission electron microscopy (TEM)

The transmission electron microscope (TEM) operates on the same basic principles as the light microscope but uses electrons instead of light. What you can see with a light microscope is limited by the wavelength of light. TEMs' use electrons as "light source" and their much lower wavelength makes it possible to get a resolution a thousand times better than with a light microscope. A "light source" at the top of the microscope emits the electrons that travel through vacuum in the column of the microscope. Instead of glass lenses focusing the light in the light microscope, the TEM uses electromagnetic lenses to focus the electrons into a very thin beam. The electron beam then travels through the specimen you want to study. Depending on the density of the material present, some of the electrons are scattered and disappear from the beam. At the bottom of the microscope the un-scattered electrons hit a fluorescent screen, which gives rise to a "shadow image" of the specimen with its different parts displayed in varied darkness according to their density. The image can be studied directly by the operator or photographed with a camera. You can see objects to the order of a few angstrom (10^{-10} m).
Experimental

FEI's Morgagni 268D TEM, an adaptable, easy-to-use digital transmission electron microscope with 80 kV voltage and magnification up to 22000x, was used. Programmable settings on the Morgagni allowed virtually automatic nanoscale microscopy sessions. Before measurement, SLN dispersions were diluted with distilled water, stained with a 2% solution of sodium phosphotungstic acid for contrast enhancement and sprayed on copper grids. The air-dried samples were then directly examined under TEM. (Cavalli et al., 2000; Fonseca et al., 2002; Straub et al., 2005)

4.10.2 Size and Zeta Potential Measurements

The Zetasizer Nano ZS90 performed size measurements using a process called Dynamic Light Scattering (DLS). Dynamic Light Scattering (also known as PCS-Photo Correlation Spectroscopy) measures Brownian motion and relates this to the size of the particles. It does this by illuminating the particles with a laser and analyzing the intensity fluctuations in the scattered light. DLS makes use of particles' ability to scatter light and their natural Brownian motion when suspended in fluid, water in this case. Particle size is calculated based on an estimate of the particles' diffusion coefficient while suspended in a medium. The relationship between the size of a particle and its speed due to Brownian motion is defined in the Stokes-Einstein equation. Particle diffusion rates are inversely proportional to particle size. As the particles are constantly in motion, the speckle pattern will also appear to move. As the particles move around, the constructive and destructive phase addition of the scattered light will cause the bright and dark areas to grow and diminish in intensity – or to put it another way, the intensity appears to fluctuate. The Zetasizer Nano system measures the rate of the intensity fluctuation and then uses this to calculate the size of the particles. The time variation of the scattered light is analyzed by examining
their autocorrelation. From this a diffusion coefficient can be derived. The autocorrelation function, \( C(t) \), is

\[
C(t) = Ae^{-2D} + B
\]

\[
T = q^2 D
\]

\[
q = \frac{4\pi n \sin(\frac{\theta}{2})}{\lambda}
\]

\[
D = \frac{k_B T}{3\pi \eta d}
\]

where \( A \) and \( B \) are constants specific to the machine, \( q \) is the scattering vector, \( D \) is the diffusion coefficient, \( n \) is the refractive index, \( \theta \) is the scattering angle, \( \lambda \) is the light wavelength, \( k_B \) is the Boltzmann Constant, \( T \) is the temperature (K), \( \eta \) is the viscosity, and \( d \) is the particle diameter.

For measuring zeta potential, the Zetasizer Nano series determines the electrophoretic mobility and then applies the Henry equation. The electrophoretic mobility is obtained by performing an electrophoresis experiment on the sample and measuring the velocity of the particles using Laser Doppler Velocimetry (LDV) and, from this, the zeta potential.

For both zeta potential and particle size analyses, drug free and drug loaded lipid nanoparticle suspensions were placed in “Size and Zeta potential” Folded Capillary cell (DTS1060). As an estimate for the size of the lipid particles, the intensity weighted mean diameter (often called effective diameter or z-average diameter) and the polydispersity index were determined by PCS (Zetasizer NanoZS90, Malvern Instruments, UK-Malvern; He-Ne-laser of 633 nm) at 25°C under an angle of 90°. All samples were diluted with demineralized particle-free water to an adequate scattering intensity prior to
the measurement. Measurement intensity was allowed to self-adjust, permitting optimum measurement capabilities. Each dimensional information gained by PCS is expressed as median plus/minus range of three experiments, each performed in triplicate. (Freitas and Muller, 1999; Cavalli et al., 2000; Feng and Huang, 2001)

4.10.3 Differential scanning calorimetry (DSC)

Thermograms of drug (paclitaxel), lipids (stearic acid and stearylamine), drug-lipid physical mixtures, and drug loaded stearic acid and stearylamine SLN were obtained using a differential scanning calorimeter (Perkin Elmer Pyris 6 DSC, USA). Standard aluminum DSC pans were used. Drug physical state in the carrier was investigated by determining the presence of drug melting point. The thermograms of representative batches of the prepared SLN were taken. Samples of 5 mg were sealed in aluminum pans. DSC scans were recorded at a heating rate of 10°C/min over a temperature range of 35–100°C for the lipids and 35–300°C for the drug, drug loaded particles, and physical mixture of drug and lipids. An inert atmosphere was maintained by purging with nitrogen. Melting points correspond to the maxima of the DSC curves (Mu and Feng, 2001; Dhanikula and Panchagnula, 2004; Xie et al., 2005).

4.10.4 X-ray diffraction

Samples were investigated by placing the dried lyophilized SLN formulation on a zero-background plate and by measuring the XRD pattern (Bruker D8 ADVANCE). Copper was the source of radiation obtained at 40 mA and 30 kV, passed through nickel filter. The diffractometer was equipped with a 20-compensating slit. The scans were run from 3° to 40° 20, increasing at a step size of 0.050° with a counting time of 2 s for each step. Al2O3 corrandum was the standard used for calibration and PMMA (poly-methyl-methyl
acrylate) was used as the holder. They were round in shape with a round cavity inside 
(Dhanikula and Panchagnula, 2004; Lu et al., 2004; Xie et al., 2005).

4.10.5 Surface chemistry characterization using Fourier transform infra-red 
(FTIR) spectroscopy

Surface chemistry was examined by FTIR. FTIR spectra were obtained using the 
potassium bromide disc method for, paclitaxel, poloxamer 188, drug-free SLN, and 
paclitaxel loaded SLN on BIO-RAD, FTIR spectrometer (Win-IR software). The infrared 
spectra were acquired to draw information on the molecular state of lipids and paclitaxel 
(Dhanikula and Panchagnula, 2004).

4.11 IN VITRO RELEASE KINETICS OF PACLITAXEL FROM SLN

Paclitaxel release kinetics from SLN was determined using the dialysis-bag method 
(Cavalli et al., 2000; Fatouros et al., 2002; D’Souza and DeLuca, 2005).

4.11.1 Preparation of dialysis bag

Dialysis membranes having varying molecular weight exclusion cutoffs (MWCOs) and 
composition have been used in a variety of setups (eg, dialysis bag, diffusion cell) (Park 
et al., 1995; Larsen et al., 2000). Passage of drug occurs through the membrane into the 
bulk media, a sample of which is withdrawn at intermittent intervals to assess drug 
release. A 10 cm long portion of the dialysis tubing i.e. cellulose tubing, MW cut-off 
12000, 10 mm (0.4 in.) avg. flat width (Sigma, USA) and thus freely permeable to 
paclitaxel (MW =853.9) was used. The tubing was made into a dialysis bag by folding 
and tying up one end of the tubing with thread, taking care to ensure that there would be 
no leakage of the contents from inside the sac. Prior to use, removal of glycerol included 
as a humectant was accomplished by washing the tubing in running water for 3-4 hours.
Experimental

The sulfur compounds were removed by treating the tubing with a 0.3% (w/v) solution of sodium sulfide at 80°C for 1 minute. It was then washed with hot water (60°C) for 2 minutes, followed by acidification with a 0.2% (v/v) solution of sulfuric acid, then rinsed with hot water to remove the acid.

4.11.2 Dialysis set up

The dialysis membrane was soaked for 10 min in a phosphate buffered saline (PBS) solution before use. The wet sac was gently opened and washed copiously with PBS. Then it was filled up with PBS and examined for leaks. The sac was then emptied and 1ml of the SLN dispersion to be investigated was accurately transferred into the sac, which thus became the donor compartment. The sac was examined once again for any leaks and then was suspended in screw-capped tube containing 15ml of PBS pH 7.4, which acted as the receptor compartment. The temperature of the contents of the tube was maintained at 37°C using the thermostatically controlled heater of the magnetic stirrer.

The contents of the tube were stirred using a teflon coated bar magnet at 100rpm (Dong and Feng, 2004; Xu et al., 2005; Liu et al., 2005).

4.11.3 Dialysis bag-method and Sampling

In vitro model 1

A preliminary experiment in triplicate was conducted in vitro to mimic the release of paclitaxel from SLN in plasma. In vitro release of paclitaxel from different formulations in the presence of human albumin was monitored by membrane dialysis at 37°C (Santos Magalhaes et al., 1991). The lyophilized powder of paclitaxel loaded SLN system (containing 1 mg paclitaxel) in 1 ml of plasma was dialysed (12KD cut-off) against 1% albumin in PBS (50 ml) as a sink solution at pH 7.4 kept at 37°C. The sample volume in
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Experimental

the dialysis bag was 1 ml. At predetermined intervals of time, 1ml aliquots were withdrawn from the receptor compartment, paclitaxel was extracted using DCM (Fig. 4-5) and subjected to analysis using HPLC at 227 nm. Fresh buffer was used to replenish the receptor compartment. Analysis was carried out immediately after withdrawal (Constantinides et al., 2000; Sparreboom et al., 2003; Lee et al., 2007).

In vitro model 2

In this model, the freeze-dried paclitaxel-loaded SLN were weighed (containing 1 mg paclitaxel) and added in 1ml of PBS buffer (pH 7.4). They were transferred to a dialysis membrane bag with a molecular weight cut-off of 12 000 Da. The release experiment was initiated by placing the end-sealed dialysis bag in 15 ml of PBS at 37°C in screw-capped tubes. The release medium was stirred at a speed of 100 r/min. At specific time intervals, the entire medium was removed and replaced with fresh buffer (Jackson et al., 2004; Liu et al., 2005). Paclitaxel was extracted from the release medium using 2 ml of DCM. The mixture was vigorously vortexed for 5 min followed by centrifugation for 10 min. After phase separation, the organic phase was taken out carefully and vacuum evaporated. The solid samples were reconstituted in ACN and analyzed using HPLC at 227 nm.

In vitro sample

Add 2ml DCM

Vortex (5 min) and Centrifuge (10 min, 10000rpm)

Remove DCM layer

Vacuum evaporated to dryness

120
Experimental

Reconstitute with 200μl of ACN and vortexed

Taken for HPLC analysis at λ_{max} 227nm

Figure 4-5. Extraction of paclitaxel from *in vitro* samples prior to HPLC analysis

4.11.4 Data Analysis

The *in vitro* release data was fitted to zero order (Chen and Hao, 1998), first order (Shah et al., 1987) and Higuchi release model (Higuchi, 1961). The cumulative amount of drug release vs time was plotted for zero-order kinetics (Equation 1). For first order (Equation 2), the log cumulative percent drug remaining vs time was plotted. The Higuchi square root kinetics (Equation 3), shows the cumulative percent drug release vs the square root of time. The regression coefficient (r²) was determined from the graphs thus obtained.

Table 4-3. Release kinetics models for analysis of *in vitro* release data

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Equation</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F=kxt</td>
<td>Zero order</td>
</tr>
<tr>
<td>2</td>
<td>ln F= kxt</td>
<td>First order</td>
</tr>
<tr>
<td>3</td>
<td>F= k√t</td>
<td>Higuchi</td>
</tr>
</tbody>
</table>

(where F is the fraction of drug release, k is the release constant and t is the time)

4.11.5 Stability study in simulated gastric fluid

The specific aim was to investigate the stability of sterically stabilized paclitaxel loaded SLN formulation in gastrointestinal fluids. The *in vitro* optimized paclitaxel loaded SLN
Experimental formulations were incubated at 37°C in simulated gastric medium (USP XXIV, pH 1.2, pepsin 0.32% w/v). Whenever needed, the pH was readjusted during the experiments using NaOH or HCl. Samples were collected at times 0, 1 and 3 h and centrifuged for 5 min at 20000 rpm to precipitate particle aggregates and enzymes. The stability of poloxamer-coated nanoparticles in simulated digestive and intestinal fluids was evaluated in terms of particle aggregation, and drug content (i.e. lipid degradation). Size distribution of the particles in dispersion was measured by PCS. The samples were then subjected to extraction procedure of paclitaxel and analyzed by HPLC to test for the leak/presence of paclitaxel and thus lipid degradation (Muller et al., 1996; Olbrich and Muller, 1999; Zimmermann and Muller, 2001; Garcia-Fuentes et al., 2002).

4.12 STABILITY STUDIES OF OPTIMIZED SLN FORMULATION

Stability of a lipid system refers to the chemical and physical integrity of the system. The developed paclitaxel loaded SLN formulations were subjected to stability studies to evaluate any physical or chemical changes on storage. Also, the chemical stability of lipids used as excipients in the production of SLN was also investigated (Muller et al., 1996; Heiati et al., 1998; Freitas and Muller, 1998, 1999; Buege and Aust, 1978; Janero, 1990; Genot et al., 1999).

The lyophilized SLN were divided into 3 sample sets and subjected to stability studies, in triplicate, as per ICH guidelines. We observed the effects of storage conditions at 4°C ± 2°C (in a refrigerator), 25°C ± 2°C/60% ± 5% relative humidity [RH], 40°C ± 2°C/75% ± 5% RH (in a stability chamber maintained at respective temperature and RH) for a period of 15, 30, 60 and 90 days, on the particle size, shape, zeta potential, drug
Experimental content, and TBARS assay by keeping the SLN in sealed amber-colored vials after flushing with nitrogen.

**Morphology, Particle size and zeta potential measurements**

As above, particle size was evaluated by dynamic light scattering using a Zetasizer Nano ZS90. Particle shape was determined by TEM. The zeta potential of the SLN formulations was determined using demineralized particle-free water (Zetasizer Nano ZS90, Malvern Instruments).

**Residual Drug Content**

The initial drug content was considered to be 100% for each formulation. The drug content at a definite time was determined after extraction of paclitaxel with DCM using stability indicating HPLC method (Waugh et al., 1991).

**Thiobarbituric Acid–Reactive Species Determination**

Thiobarbituric acid–reactive species (TBARS) were measured for lipid peroxidation assay following the method of Ohkawa et al., 1979. To the aliquots (0.2 ml) of SLN dispersion were added 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with sodium hydroxide, and 1.5 ml of 0.8% aqueous solution of TBA. The mixture was made up to 4.0 ml with distilled water, and then heated at 95°C for 60 min. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15:1, v/v) were added and shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured using UV spectrophotometer. TMP was used as an external standard, and the level of lipid peroxides was expressed as nmol of MDA (Ohkawa et al., 1979; Janero, 1990; Genot et al., 1999).
4.13 *IN VITRO CYTOTOXICITY*

The cell line studies of paclitaxel and its SLN based formulations for the evaluation of cytotoxicity and *in vitro* anticancer activity was done using human hepatoma cell line, HepG2 (ATCC No. HB-8065) (Gagandeep et al., 1999; Jeong et al., 2005) by MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay (Fonseca et al., 2002; Mo and Lim, 2005; Xie and Wang, 2005).

**MTT solution**

5 mg/ml MTT in phosphate buffer saline pH 7.4 (PBS). This solution was filtered through a 0.2 μm filter and stored at 2-8°C.

4.13.1 MTT Assay

A human hepatoma cell line, HepG2 (ATCC No. HB- 8065), was cultured in minimum essential medium (MEM) supplemented with Earl’s salt (1.5 gL⁻¹), fetal calf serum (10%), L-glutamine (2mM), sodium pyruvate (1%), streptomycin (100 mgmL⁻¹), penicillin (100 IUmL⁻¹) and amphotericin B (0.25 mgmL⁻¹). The cells were grown at 37°C in a humidified atmosphere containing 5% CO₂.

HepG2 cell lines were used for the assessment of the cytotoxicity of paclitaxel and its formulations. Cells were seeded in 96-well microtitre plates at a density of $1 \times 10^5$ cells per millilitre in serum-containing media and left for 24 h for recovery. Test lipid systems were added (0.001–1 mg/ml) in fresh complete media to microtitre plates and incubated for 72 h. Five hours before completion of the incubation period, 20 μL MTT (5 mg/mL) was added and the incubation was continued. The medium was removed and 100 μL DMSO was added to dissolve the formazan crystals. The optical density was measured at 550 nm using a plate reader (PowerWave X; BIO-TEK Instruments, Inc).
The cell viability in the presence of free paclitaxel and its formulations were expressed as the percentage of viability of cells in the absence of drug. The experiment was carried out in triplicate. The 50% cell cytotoxic concentration (CC50) of paclitaxel and its SLN based formulations against HepG2 at 72 h was determined.

4.14 IN VIVO STUDIES

4.14.1 Preparation of SLN for in vivo studies

The paclitaxel dose used throughout the study for oral administration was 40 mg/kg body weight (Kim et al., 2001; Taraboletti et al., 2005). The initial amount of each drug required for SLN preparation was calculated by the formula: (amount of drug required per animal/mean drug incorporation efficiency) × 100. Once the total drug quantities required were known, equivalent amount of lipid was used in the preparation. The basic procedure for SLN preparation remained the same as discussed above.

4.14.2 Pharmacokinetics and biodistribution studies

Animals

Pharmacokinetic and biodistribution studies were performed using Male Swiss albino mice, weighing 50 g, procured from the Central Experimental Animal Facility of the All India Institute of Medical Sciences (Animal Ethics committee-AIIMS, application no. 360/IAEC/06), New Delhi. They were housed five per cage with free access to diet and water. The animals were maintained as per the All India Institute of Medical Sciences guidelines for experimental work on animals.

Dosing Solutions

The stock solution of paclitaxel for oral and i.v. dosing was prepared by dissolving 30 mg of pure compound in 2.5 ml of ethanol and 2.5 ml of polysorbate 80. This stock solution
Experimental

containing 6 mg/ml of paclitaxel was stable for at least 1 year if stored at 4°C. Before injection, this stock solution was diluted 6-fold with saline to a final concentration of 1 mg/ml and used within 4 h (Crofton et al., 1995; Bardelmeijer et al., 2000).

Paclitaxel loaded SLN formulations for oral administration, were freshly prepared the day before each experiment. A portion of the SLN was accurately weighed and transferred into a glass tube. The lyophilized powder was suspended in 10 ml of water for injection and transferred into a polypropylene tube. The suspension was sonicated for 10 min and kept protected from light and was stirred continuously. The suspension was kept protected from light and was stirred continuously. The final concentration of suspension was 0.8 mg/ml of paclitaxel.

Administration Routes

Oral, free-drug and drug-formulation, administrations were given by gavage. i.v. drug administration were done by injection with a 29-gauge needle into a lateral tail vein using 1 ml syringes. Animals were placed under a heating lamp prior to i.v. drug administration. No anesthetics were used during these procedures.

Groups

All studies followed established protocols approved by the Institutional animal care and use committee of All India Institute of Medical Sciences (AIIMS), New Delhi. The animals were randomly divided into three groups. Group A and C received pure paclitaxel orally and intravenously, respectively. Group B received paclitaxel loaded SLN orally. Each group for every time point (n=5) was marked and housed in one cage. The mice were fasted for 12 h prior to experiments, but water was available at all times. After dose administration, the mice were kept in cages and had free access to water.
The dose level of paclitaxel was 40 mg/kg for group A and B, and 10 mg/kg for group C. Groups receiving oral paclitaxel consisted of five animals/time point, whereas three animals/time point were used when paclitaxel was administered i.v. Blood sampling was performed at the following approximate time points after the administration of paclitaxel: group A and B, 0, 0.25, 0.5, 1, 2, 4, 6 and 24 h; group C, 0.08, 0.25, 0.5, 1, 2, 4, 6, 8 and 12 h (Sparreboom et al., 1997; Ceruti et al., 2000 Bardelmeijer et al., 2000; Xu et al., 2005).

**Sample Collection and Handling**

Following treatment, the animals were sacrificed by cardiac stick exsanguination under isoflurane anesthesia and tissue and blood samples were collected (Gustafson et al., 2005). EDTA was used as an anticoagulant and the plasma obtained after centrifugation (5 min, 5000 rpm) was stored at -20°C until analysis. Liver, spleen, kidney, lungs and brain were removed, frozen in liquid nitrogen, and stored at -80°C prior to extraction and analysis, to determine the organ distribution of the drug at particular time points.

As the animals (mice) were sacrificed with excess anaesthesia, no survival surgery was needed. The sacrificed animals were incinerated in AIIMS incinerator.

**4.15 TOXICITY STUDIES**

For the toxicity study, the mice were randomized into three groups (n=8). First group received pure paclitaxel orally while second and third groups were treated with drug free SLN and paclitaxel loaded SLN formulation, respectively, at a dose of 40 mg/kg of body weight. After 15 days of dosing mice were sacrificed under anaesthesia and blood samples were withdrawn and tested for hematological parameters and liver function test (Jamois et al., 2005). Histology studies were performed to examine the acute toxicity of
Experimental paclitaxel loaded SLN. For histopathological examinations the tissues (liver, lungs, kidney, spleen, brain) were removed, and then placed in a fixative i.e. 10% formalin (10% formaldehyde in water) which stabilizes the tissues to prevent decay until processing (Dhanikula and Panchagnula, 2004). The tissues were washed with normal saline and were seen for:

Gross Changes: Colour changes or the development of any patch was observed.

Histopathological Changes: The processing of tissues of mice receiving paclitaxel loaded SLN involved dehydra
tion through a graded series of alcohols (70%, 80%, 95%, and 100%), followed by xylene and then infiltration with paraffin. For obtaining thin sections (3-5 μm), tissues were embedded on the edge of paraffin blocks and were cut on a rotary microtome. These sections were deparaffinized, rehydrated with graded alcohols (100%, 95%, 80%, and 75%), and stained with hemotoxylin/eosin for microscopic examination. Similarly, sections of drug free SLN and tissue of mouse receiving pure paclitaxel orally were obtained to serve as control.