CHAPTER – 5
MATERIALS AND METHODS

5.1. MATERIALS

5.1.1 Chemicals

All chemicals used in the study were of analytical grade and supplied commercially.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Risperidone</td>
<td>Torrent Pharmaceuticals, Baddi, India</td>
</tr>
<tr>
<td>Olanzapine</td>
<td>Ranbaxy Labs, Paonta Sahib, India</td>
</tr>
<tr>
<td>Eudragit RL100</td>
<td>Rohm Pharma, Germany</td>
</tr>
<tr>
<td>Eudragit RS100</td>
<td>Rohm Pharma, Germany</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>S.D. Fine chemicals Ltd.</td>
</tr>
<tr>
<td>Di-n-butylphthalate</td>
<td>S.D. Fine chemicals Ltd.</td>
</tr>
<tr>
<td>Sodium lauryl sulphate</td>
<td>S.D. Fine chemicals Ltd.</td>
</tr>
<tr>
<td>Tween 80</td>
<td>S.D. Fine chemicals Ltd.</td>
</tr>
<tr>
<td>Benzalkonium chloride</td>
<td>S.D. Fine chemicals Ltd.</td>
</tr>
<tr>
<td>Span 20</td>
<td>S.D. Fine chemicals Ltd.</td>
</tr>
<tr>
<td>Olive oil</td>
<td>Rajesh Chemicals, Mumbai</td>
</tr>
<tr>
<td>Jojoba oil</td>
<td>Rajesh Chemicals, Mumbai</td>
</tr>
<tr>
<td>Groundnut oil</td>
<td>Rajesh Chemicals, Mumbai</td>
</tr>
<tr>
<td>Chloroform (AR)</td>
<td>S.D. Fine chemicals Ltd.</td>
</tr>
<tr>
<td>Ethanol, AR</td>
<td>Bengal chemicals</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>S.D. Fine chemicals Ltd.</td>
</tr>
<tr>
<td>Di-Sodium hydrogen phosphate</td>
<td>S.D. Fine chemicals Ltd.</td>
</tr>
</tbody>
</table>
n - Octanol S.D. Fine chemicals Ltd.

Isopropanol S.D. Fine chemicals Ltd.

Acetonitrile, HPLC grade E Merck Ltd.

Methanol HPLC grade E Merck Ltd.

Triethylamine S.D. Fine chemicals Ltd.

Carboxy methyl cellulose S.D. Fine chemicals Ltd.

Adhesive tape Johnson & Johnson, India

Gluteraldehyde S.D. Fine chemicals Ltd.

Acetone S.D. Fine chemicals Ltd.

Sodium chloride S.D. Fine chemicals Ltd.

Isoamylacetate S.D. Fine chemicals Ltd.

Phosphoric acid S.D. Fine chemicals Ltd.

Hexane S.D. Fine chemicals Ltd.

Sodium bicarbonate S.D. Fine chemicals Ltd.

Potassium phosphoric acid S.D. Fine chemicals Ltd.

ONZA® tablet Manufactured by Nicholas Piramal

RISPID® tablet Manufactured by Panacea Biotec Ltd.

5.1.2 Animals

Male/female swiss white mice (25-30g), wistar rats (150-250g) and white rabbits (1.5-2.5 kg) obtained from Central Animal House facilities of the Rayat Institute of Pharmacy, Railmajra were employed in the studies. The animals were kept in polycrystalline cages with wire mesh top and soft bedding. They were kept under standard husbandary conditions of 12/12 hr light/dark cycle with food and water ad libitum and maintained at 22±2°C and a humidity of 50-60%. The experimental
protocols were approved by IAEC (Institutional Animal Ethics Committee) as per guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

### 5.1.3 Equipments used along with their manufacturers

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>U.V.Spectrophotometer</td>
<td>Shimadzu (UV-1700 PharmaSpec)</td>
</tr>
<tr>
<td>Electronic weight balance</td>
<td>Afoset, Mumbai (ER-182A)</td>
</tr>
<tr>
<td>pH meter</td>
<td>Systronic electronics, Ahmedabad, India</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Remi equipments, Mumbai</td>
</tr>
<tr>
<td>Magnetic stirrer</td>
<td>Remi equipments, Mumbai</td>
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<tr>
<td>Incubator</td>
<td>Narang Scientific Works Pvt. Ltd., Delhi</td>
</tr>
<tr>
<td>Diffusion Cell Apparatus</td>
<td>Fabricated by CIL, Panjab University, Chandigarh</td>
</tr>
<tr>
<td>Shaking incubator</td>
<td>Remi equipments, Mumbai</td>
</tr>
<tr>
<td>FT Infra Red Spectroscopy</td>
<td>Perkin Elmer, USA</td>
</tr>
<tr>
<td>Hot air oven</td>
<td>Thermotech TH012</td>
</tr>
<tr>
<td>Dissolution apparatus</td>
<td>Electrolab TDL-08L</td>
</tr>
<tr>
<td>HPLC system</td>
<td>Perkin Elmer, USA</td>
</tr>
<tr>
<td>Sonicator</td>
<td>Remi equipments, Mumbai</td>
</tr>
<tr>
<td>Micrometer</td>
<td>Micron Instrument Co., Pune</td>
</tr>
<tr>
<td>Tensile strength</td>
<td>Fabricated in Rayat Institute of Pharmacy, Railmajra</td>
</tr>
<tr>
<td>Rota rod apparatus</td>
<td>Inco Instruments, Ambala</td>
</tr>
<tr>
<td>Weighing balance</td>
<td>Mettler Toledo, Mumbai</td>
</tr>
<tr>
<td>Humidity Chamber</td>
<td>Newtronics, Mumbai</td>
</tr>
<tr>
<td>Scanning Electron Microscope</td>
<td>JSM 6100 JEOL, Tokyo, Japan</td>
</tr>
</tbody>
</table>
5.2 METHODOLOGY

Preformulation studies of risperidone and olanzapine including tests for identification, solubility studies, partition coefficient determination, melting point determination and other studies were carried out and compared with the specification as per literature.

5.2.1 Preformulation studies

5.2.1.1 Ultraviolet Absorption Maxima (λmax): The organic molecules in solution form when exposed to light in the ultra-violet region of the spectrum absorb light of particular wavelength depending on the type of electronic transition associated with the absorption. 10 µg/ml solutions of the risperidone and olanzapine in dichloromethane, methanol, n-octanol, phosphate saline buffer (PBS) pH 7.4 and buffer with Tween 80 were scanned between 200-400 nm for determination of absorption maxima.

5.2.1.2 Partition coefficient: 10 mg of drug was accurately weighed and taken in stoppered vial containing 10 ml each of two immiscible phases, n-octanol and aqueous phase (PBS 7.4). The vials were placed on water bath shaker for 24 h. Phases were separated using separating funnel and both the phases were analyzed for the amount of drug after suitable dilution spectrophotometrically. The partition coefficient was calculated by following formula:

\[ K_{o/w} = \frac{C_o}{C_w} \]

Where \( K_{o/w} \) = Partition coefficient

\( C_o \) = concentration of drug in n-octanol phase

\( C_w \) = concentration of drug in aqueous phase

This procedure was followed for both the drugs separately.

5.2.1.3 Validation of UV spectrophotometric method: The UV spectrophotometric method was validated for determination of risperidone and olanzapine in the formulation with respect to linearity and range, accuracy and precision, selectivity and robustness (Shahi et al., 2008).

Linearity and Range

The prepared aliquots in dichloromethane (2-20 µg/ml) were scanned for absorbance at \( \lambda_{max} \) value 254 and 248 nm for risperidone and olanzapine respectively.
Accuracy and Precision

Accuracy and precision were investigated by analyzing three concentrations of risperidone (i.e. 1, 2 and 4 mg tablet) olanzapine (i.e. 5, 10 and 20 mg tablet) in three independent replicates on the same day (Intra-day accuracy and precision) and on three consecutive days (Inter-day accuracy and precision).

Robustness and selectivity

Repeatability was validated based on the results of the method operating over short time interval under same conditions.

5.2.1.4 Preparation of calibration curves: The calibration curves of risperidone and olanzapine were prepared in different fluids (methanol, dichloromethane, n-octanol, PBS 7.4 and PBS 7.4 containing various concentrations (0.25%, 0.5%, 0.75%, 1% w/v) of Tween 80. The method estimated the drug concentration in the range of 2-20 μg/ml in all media and it followed the Beer’s Lambert law in the same concentration range.

10 mg accurately weighed drug was dissolved in 100 ml with the different media resulting in a stock solution of 100 μg/ml. From the stock solution, aliquots of 0.2, 0.4, ……, 1.8, 2.0 ml were withdrawn in a series of 10 ml volumetric flasks and diluted to 10 ml with media. This gave a concentration range of 2, 4…… 18, 20 μg/ml. The absorbance of each solution was measured in UV/Vis spectrophotometer at λ_{max} (Table 6.2 and 6.19).

5.2.1.5 Solubility studies: Receptor fluid for permeation studies was selected based on solubility data of risperidone and olanzapine at 37°C in various fluids. Various fluids used were PBS 7.4 and buffer containing various concentrations (0.25%, 0.5%, 0.75%, 1%) of Tween 80. Risperidone and olanzapine were added individually in excess amount in each fluid and excess drug containing phosphate buffer flasks were agitated in water bath shaker for 48 h at 37°C. After 48 hrs solutions were analyzed spectrophotometrically and drug concentrations were calculated.

5.2.1.6 Drug polymer interaction studies: FTIR spectra of pure drug risperidone, olanzapine, eudragit RL 100 (ERL100), eudragit RS 100 (ERS100) and mixture of both the drugs with eudragits in the same ratio as used for formulation were taken using Perkin Elmer FTIR spectrophotometer (RXIFT-IR system). One part of sample was mixed with three parts of potassium bromide in a mortor and tritutrated. The
triturated sample was placed in pellet maker and compressed using hydraulic press.
The pellet was kept in a sample holder and scanned from 450 cm\(^{-1}\) to 4000 cm\(^{-1}\).

### 5.2.2 Formulation of transdermal patches

Transdermal patches of risperidone and olanzapine were prepared by solvent casting technique in a glass mould fabricated locally. To determine the optimum combination of polymers, plasticizer and solvent, placebo patches were formulated. On the basis of preliminary studies, the optimized polymers ERL 100 and ERS 100 in different ratios were mixed to a total weight of 500 mg and dissolved in 10 ml of isopropanol-dichloromethane (60:40) solvent system using magnetic stirrer. Drug (20% w/w of polymer weight) was added slowly to the polymer solution and mixed thoroughly to obtain a homogenous solution and di-n-butyl phthalate (30% w/w of polymer) was used as plasticizer. Different permeation enhancers (BC, SLS, span 20, olive oil, groundnut oil and jojoba oil) were added in three different concentrations i.e. 1%, 5% and 10% w/w of polymer weight for each. The resulting polymeric solution was poured in circular aluminium foil cups placed in circular glass mould (internal diameter 3.57 cm and thickness 1 cm) and dried at 35 °C in dust free environment. After 24 h, the films were collected and peeled off. A circular USP adhesive tape of internal diameter 5 cm was attached on the patch. A backing film made up of aluminium was applied with the help of adhesive and a release liner (wax paper) was applied on other side of the film to complete the TDDS.

#### Fabrication of mould for making films

A circular mould with flat surface having internal diameter 3.57 cm was fabricated in the lab. The glass surface of the mould was labeled properly, so as to obtain films having uniform thickness.

### 5.2.3 Characterization of transdermal patches

#### 5.2.3.1 Weight variation and Thickness: Weight variation of the prepared films was studied by individually weighing 10 randomly selected patches and thickness was determined by micrometer at five random points on the films. Such determination was performed for each formulation (Damodharan et al., 2009).

#### 5.2.3.2 Drug content determination: An accurately weighed portion of the film (100 mg) was dissolved in 100 ml of dichloromethane and then the solution was shaken continuously for 24 h in shaker incubator. After sonicating and filtering, concentration
of drug was estimated spectrophotometrically (at 325 nm) by appropriate dilution.

**5.2.3.3 Flatness:** For flatness determination, one strip was cut from the centre and two from each side of patches. The length of each strip was measured and variation in length was measured by determining percent constriction. Zero percent constriction is equal to 100% flatness (Chandak and Verma, 2009).

\[
\text{%constriction} = \frac{l_1 - l_2}{l_1} \times 100
\]

where:
- \(l_1\) is the initial length of each strip
- \(l_2\) is the final length of each strip

**5.2.3.4 Folding endurance:** Folding endurance of the film was determined repeatedly folding the film at the same place until it break. The number of times the film could be folded at the same place without breaking was the folding endurance value (Chandak and Verma, 2009).

**5.2.3.5 Tensile strength:** A small film strip was cut on a glass plate with a sharp blade. One end of the film was fixed between adhesive tapes to give support to film when placed in the film holder. Another end of the film was fixed between the adhesive tapes with a small pin sandwiched between them to keep the strip straight while stretching. A small hole was made in the adhesive tape near the pin in which a hook was inserted. A thread was tied to this hook, passed over the pulley and a small pan attached to the other end to hold the weights. A small pointer was attached to the thread, which travels over the scale affixed on the base plate. To determine tensile strength, the film was pulled by means of a pulley system. Weights were gradually added to the pan to increase the pulling force till the film was broken.

The elongation was recorded as the distance travelled by the pointer before break of the film on the scale. The weight required to break the film was noted as break force. Tensile strength was calculated as:

\[
\text{Tensile strength} = \frac{\text{break force}}{a \times b \times (1 + \Delta L/L)}
\]

where:
- \(a\), \(b\) and \(L\) were width, thickness and length of strip respectively and \(\Delta L\) is the elongation at break (Gannu et al., 2008)

**5.2.3.6 Moisture content:** The prepared films were weighed individually and kept in desiccators containing calcium chloride at room temperature for 24 h. The films were weighed again and again after specified interval until they show a constant weight. The percent moisture content was calculated using following formula:
% Moisture content = Initial weight - Final weight / Final weight * 100

5.2.3.7 Moisture uptake: Weighed films were taken and exposed to 84% relative humidity using saturated solution of potassium chloride in desiccators until a constant weight is achieved. % moisture uptake was calculated as given below (Gannu et al., 2008):

% Moisture uptake = Final weight - Initial weight / Initial weight * 100

5.2.3.8 Microbial studies: The potential of transdermal patch for promoting growth of micro-organisms was evaluated by bacteriological cultures. The film strips of different formulations were cut into small pieces of 1 cm² and aseptically transferred into each petri plate containing 25 ml of nutrient agar media. These agar plates were incubated at 37±0.5°C for 48 h. After incubation, sample was observed under microscope.

5.2.4 In vitro drug release studies

The in vitro drug release studies were performed by using a modified USP type II dissolution apparatus using 900 ml of PBS 7.4 with Tween 80 (1% w/v for risperidone and 0.75% w/v for olanzapine) as dissolution medium. A circular patch with an internal diameter of 3.57 cm was used for the study and a stainless steel ring was employed to sink the patch at bottom of dissolution apparatus. All dissolution studies were performed at 32±0.5 °C (temperature of skin) at 100 rpm. Samples were withdrawn at predetermined time intervals (replaced with equal volume of fresh dissolution media to maintain sink conditions) and their concentrations were analyzed spectrophotometrically at λ_max of 322 nm for risperidone and 315 nm for olanzapine (Mittal et al., 2009).

To study the release kinetics, data obtained from in vitro drug release studies were fitted in various kinetic models: zero order as cumulative percent of drug released vs. time, first order as log cumulative percentage of drug remaining vs. time and Higuchi’s model as cumulative percent drug released vs. square root of time. To determine the mechanism of drug release, the data were fitted into Korsmeyer and Peppas equation as log cumulative percentage of drug released vs. log time, and the exponent n was calculated from slope of the straight line. For slab matrix, if exponent is 0.5, then diffusion mechanism is fickian; if 0.5<n<1.0, mechanism is non-fickian;
if $n$ is 1.0, mechanism is zero order and if $n > 1.0$, then it is super case II transport (Alam et al., 2009).

5.2.5 **In vitro permeation studies**

5.2.5.1. **Preparation of full thickness rat abdominal skin:** Hairless animal skin and human cadaver skin are generally used for permeation studies. Human cadaver skin may be a logical choice as the skin model because the final product will be used in humans. But it is not easily available. So, in the present study, hairless Wistar rat abdominal skin was used. The experimental protocols were approved by IAEC (Institutional Animal Ethics Committee) as per guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India.

5.2.5.2 **Permeation studies:** 25 rats were sacrificed by excess ether inhalation. Hairs on dorsal skin of animal were removed with animal hair clipper, subcutaneous tissue was surgically removed and dermis side was wiped with isopropyl alcohol to remove residual adhering fat. The skin was washed with PBS pH 7.4. The skin so prepared was wrapped in aluminum foil and stored in a deep freezer at $-20^\circ$C till further use. The skin was defrosted at room temperature when required (Ren et al., 2009).

The *in vitro* permeation studies were carried out in vertical Franz diffusion cell with a capacity of 35 ml, using rat abdominal skin (Jain et al., 2008). The patch was placed on the skin with the drug matrix side towards the donor side and backing membrane on the upper side. PBS 7.4 with Tween 80 was used as receptor fluid as in release studies. The receptor fluid was agitated at 100 rpm by magnetic stirrer and temperature was maintained at $32 \pm 0.5^\circ$C. The samples were withdrawn at different time intervals and replaced with equal amounts of dissolution media. Samples were analyzed for its drug content. The drug permeated per cm$^2$ of patch was calculated and plotted against time and the flux was calculated as drug permeated per cm$^2$ per hour (Gullick et al., 2010).

The steady state flux was determined from the slope of the linear portion of a cumulative amount permeated versus time plot. The lag time ($T_{\text{lag}}$) was determined by extrapolating the linear portion of the cumulative amount permeated versus time curve to the abscissa. Enhancement ratio of the flux ($E_{\text{pen}}$) was calculated as:

$$E_{\text{pen}} = \frac{P_{\text{treatment}}}{P_{\text{control}}}$$
Where $P_{\text{treatment}}$ is flux of formulation containing enhancer and $P_{\text{control}}$ is flux of control group (Without permeation enhancer)

The current oral dosing regimen of risperidone is 2-8 mg/day. As bioavailability of risperidone is 70%, thus anticipated transdermal dose is 1.4-5.6 mg daily. So the target flux required is 5.83-23.33 $\mu$g/cm$^2$/h from a patch having diameter 3.57 cm and surface area 10 cm$^2$.

The current oral dose of olanzapine is 5-10 mg/day. Bioavailability of the drug is 60%. So the target flux required is 12.5-25 $\mu$g/cm$^2$/h from a patch having diameter 3.57 cm and surface area 10 cm$^2$.

5.2.5.3 Scanning Electron Microscopy (SEM) studies: The skin sections obtained before and after permeation studies were fixed in 3% glutaraldehyde phosphate buffer (pH 7.4) and subsequently dehydrated in a series of acetone solution (50% for 20 min, 70% for 20 min, 80% for 20 min, 90% for 20 min, 100% for 50 min) in water and isoamylacetate (100%) : acetone (100%) solutions (1:1) for 20 min followed by isoamylacetate (100%) for 20 min. Sections were further dried using four flushes of liquid CO$_2$ with 100 psi pressure in critical point drier.

The sections of film and skin before and after permeation studies were cut and mounted onto stubs using double sided adhesive tape. The sections were coated with gold palladium alloy using fine coat ion sputter to render them electrically conductive and examined under SEM (JSM 6100 JEOL, Tokyo, Japan) to observe the integrity of skin before and after permeation and distribution of drug in the film and skin (Mukherjee et al., 2005).

5.2.6 In vivo studies
5.2.6.1 Skin irritation studies: Skin irritation studies and histopathological studies were carried out according to Draize technique (Kirwin, 1984) for selected formulations (RE3 and OD3; formulations containing vegetable oil and non ionic surfactant respectively). Rabbits were used to study any hypersensitivity reaction on the skin. Rabbits were divided into 5 groups, each containing 6 animals. The animals of group I were served as normal, without any treatment. One group of animals (group II, control) were applied with marketed adhesive tape (official adhesive tape in USP). Transdermal patches (blank and drug loaded) were applied on to nude skin of animals of III and IV groups respectively. A 0.8% v/v aqueous solution of formalin was
applied as standard irritant (group V). The experiment was carried out for 7 days and the application sites were graded according to a visual scoring scale, by the same investigator (jayaprakash et al., 2010). The scores of erythema and edema was as follows: 0 for none, 1 for slight, 2 for well defined, 3 for moderate and 4 for scar formation and severe erythema and edema. After evaluation of skin irritation, skin samples were processed for histological examination.

### 5.2.6.2 In vivo pharmacodynamic studies:
Risperidone and olanzapine usually cause a state of sedation and motor in-coordination. Rota rod and grip tests were used to assess muscular strength or neuromuscular function in rodents which can be influenced by sedative drugs and muscle relaxant compounds.

Swiss mice were divided into 3 groups, each containing four animals. First group served as control i.e. without drug, second group was administered with oral dose (1mg/kg for risperidone and 10 mg/kg for olanzapine) of marketed formulation (RISPID® tablet by Panacea Biotec and ONZA® tablet by Nicholas Piramal, India) in 0.5% carboxymethyl cellulose (CMC) and third group was treated with selected transdermal formulation (RE3 and OD3) containing equivalent dose as that of oral formulation (Zhang et al., 2007).

For rota rod test, animals were placed on an aluminium rod; revolving at 10 rpm and the time taken to fall of animal from the rod was noted. The test was terminated at 270 s (Samanta et al., 2003).

For grip test, the animals were exposed to a horizontal thin metallic wire suspended about 30 cm in air which they immediately grasp with the 4 paws. The mice were released to hang on with its four limbs. Control animals were able to hold the wire with hind limbs and to climb up within 5 s. After oral or transdermal administration, the animals were not able to hold the wire with the hind limbs within 5 s or fall off from the wire and they were considered to be impaired. The test was continued for 6 h and repeated after every hour. The general behaviours were observed from selected batches in cages and observations noted. Only if their behaviour and their motility in the cages seem to be normal, the disturbance of grasping reflex is considered as caused by central relaxation.

### 5.2.6.3 In vivo pharmacokinetic studies of olanzapine transdermal patches:
Nine healthy white rabbits each weighing 1.5-2.0 kg were selected and divided into three
groups. For single dose study, first group was administered with pure drug dispersed in 0.5% CMC, the second group was administered with marketed tablet formulation (ONZA® tablet by Nicholas Piramal), powdered and dispersed in 0.5% CMC and the third group was applied with test formulation (OD3; TDDS of olanzapine) on rabbit’s pinna following an overnight fast of 12 h. Water was used ad libitum. For the application of TDDS patches, an equivalent square cm piece of patch (calculated as per body weight of rabbits i.e. 10 mg/kg for olanzapine) was fixed with its drug releasing surface in contact with pinna. Immediately after administration of the oral dosage forms or application of TDDS films, 2.5 ml of blood samples were withdrawn from the marginal ear vein (of the other pinna in the case of TDDS application) into heparinized (0.2 ml) glass centrifuge tube and centrifuged immediately in a cooling centrifuge at 0°C. The plasma was separated by centrifugation at 4000 rpm for 15 min and stored at -10 °C. This was considered as blank. Subsequently, the same volume of blood samples was withdrawn at predetermined time intervals of 1, 4, 8, 16, 24, 48 and 72 hr and corresponding plasma was separated accordingly (Hai et al., 2008).

**Analytical methodology**: Plasma concentrations of the olanzapine were measured by high performance liquid chromatography (HPLC) method.

**HPLC instrumentation and chromatographic conditions**

HPLC system consisted of a Perkinelmer instrument series 200 with UV detector (Diode array detector series 200) and quaternary pump series 200 at 270 nm. Separations were achieved on a Merck LiChrosphor 60RP Select B 5 μ column using a mobile phase of 14% acetonitrile in water containing 0.25% H₃PO₄ and 0.05% triethylamine and pumped at a flow rate of 1ml/min.

**Preparation of standard plot for in vitro studies**

10 mg of olanzapine was dissolved in 10 ml of ethanol. Then 0.1 ml of this was diluted to 10 ml to make 10000 ng/ml stock solution. The stock solution was further diluted to get 100 ng/ml, 200 ng/ml, 300 ng/ml, 400 ng/ml and 800 ng/ml samples for calibration curve. All the samples were filtered through 0.44μ membrane filter and injected into HPLC system for calibration curve.

**Preparation of standard curve of olanzapine in plasma samples**

10 mg of olanzapine was dissolved in 10 ml of ethanol. Then 0.1 ml of this was diluted to 10 ml to make 10000 ng/ml stock solution. Combined standard solutions
with concentrations of 200, 600, 1000, 2000 and 14000 ng/ml were prepared from stock solution. Then samples of calibration curve were prepared by adding 50 µl of each standard solution into 1.0 ml of blank plasma. This yielded calibration standard concentrations of 10, 30, 50, 100 and 700 ng/ml. The spiked plasma samples were extracted along with unknown samples.

**Extraction procedure for olanzapine from plasma**

Plasma samples were aliquoted into 15 ml borosilicate glass test tubes to which 500 µl of 0.1 M Na₂CO₃ was added. The tubes were shaken with 10 ml of a solvent mixture consisting of hexane/dichloromethane (85:15) for 5 min, and then centrifuged at 1800 X g for 5 min. The supernatant was transferred to clean 15 ml borosilicate glass tubes and 200 µl of 45 mM KH₃PO₄ (buffered to pH 2.8 with concentrated H₃PO₄) was added. The mixture was shaken for 30 s and then centrifuged as before. The upper organic layer was aspirated to waste and the residue was reconstituted with 500 µl of mobile phase with sonication and an aliquot (80µl) was injected into the HPLC system (Dusci et al., 2002).

**5.2.7 Stability studies**

The stability studies are conducted to investigate the influence of temperature and relative humidity on the drug content in different formulations. The transdermal formulations RE3 and OD3 were subjected to stability studies for 3 months using storage conditions 45 °C / 75% RH as per ICH guidelines. Throughout the course of aging study, triplicate samples were taken at three sampling times (i.e. 0, 1 month and 3 month) and evaluated for physical texture, drug content and in vitro permeation studies as the indicators.

**5.2.8 Statistical analysis**

Graph pad prism 5 was used for statistical analysis. All studies were done in triplicates unless specified and data represents the mean ± SD. The statistical analysis was performed using student’s t-test and ANOVA. A difference below the probability level of 0.05 was considered statistical significant.

Pharmacokinetic parameters were determined using Winnonlin version 5.2.