3.0 MATERIALS AND METHODS

Clinical part of the study was carried out at Ranbaxy Clinical Pharmacology Unit (CPU) Fortis Hospital Noida. Analytical part of the study was carried out at Clinical Pharmacology and Pharmacokinetics, Ranbaxy Research Laboratory, Gurgaon, India. The experimental design was divided into five parts:

- Clinical Study Methodology
- Bioanalytical Method Development
- Bioanalytical Method Validation
- Analysis of Clinical Study Samples
- Pharmacokinetic and Statistical Analysis

3.1 CLINICAL STUDY METHODOLOGY

3.1.1 Objectives

To determine and compare the rate and extent of absorption of olmesartan between two batches of test formulation olmesartan medoxomil 40 mg tablet of Ranbaxy Laboratories Limited and the reference formulation OLMETEC® 40 mg film coated tablet of Daichii Sankyo UK Ltd in healthy, adult, human subjects under fasting condition.

The final version of the protocol and informed consent form for this study were reviewed and approved by Fortis Hospital Institutional Review Board (FHIRB). All the subjects provided the written informed consent by designated person after attending an oral presentation and after thoroughly reading the final version of the informed consents form (ICF).

3.1.2 Products Evaluated

Reference (R)

OLMETEC® 40 mg film coated tablet (containing olmesartan 40 mg) manufactured by Daichii Sankyo UK Ltd.

Test (A & B)

Olmesartan medoxomil 40 mg tablet manufactured by Ranbaxy Laboratories Limited, India.
3.1.3 Study Design

An open label, balanced, randomized, three-treatment, three-period, three-sequence, single-dose, crossover bioavailability study comparing two batches of olmesartan medoxomil 40 mg tablet of Ranbaxy Laboratories Limited with OLMETEC® 40 mg film coated tablet (containing 40 mg olmesartan medoxomil) of Daichii Sankyo UK Ltd in healthy, adult, human subjects under fasting condition.

3.1.4 Study Site

The study was conducted at Ranbaxy Clinical Pharmacology Unit (CPU) Fortis Hospital Noida - 201301

3.1.5 Selection of Subjects

Adequate numbers of subjects (12) were randomly selected from the volunteer bank of Clinical Pharmacology Unit who underwent a standardized screening procedure.

3.1.5.1 Screening Assessments

Medical histories and demographic data, including name, sex, age, body weight (kg), height (cm) and tobacco use (including number of cigarettes smoked per day) were recorded. Each subject had undergone physical examination including ECG and the laboratory tests of hematology, hepatic and renal functions as listed below (Table 3.1).

Table 3.1 Laboratory parameters assessed for each volunteers during screening procedure

<table>
<thead>
<tr>
<th>HEMATOLOGY</th>
<th>BIOCHEMISTRY</th>
<th>URINALYSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin</td>
<td>BUN</td>
<td>Routine Examination</td>
</tr>
<tr>
<td>Total leukocyte count</td>
<td>Creatinine</td>
<td>- Colour</td>
</tr>
<tr>
<td>Differential leukocyte count</td>
<td>Total bilirubin</td>
<td>- Appearance</td>
</tr>
<tr>
<td>Platelet count</td>
<td>ALP</td>
<td>- PH</td>
</tr>
<tr>
<td></td>
<td>AST</td>
<td>- Specific gravity</td>
</tr>
<tr>
<td></td>
<td>ALT</td>
<td>- Protein</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>- Glucose</td>
</tr>
<tr>
<td></td>
<td>Cholesterol</td>
<td>- Microscopic Examination</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- RBC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- WBC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Epithelial Cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Crystals</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Casts</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Others</td>
</tr>
<tr>
<td>HIV I &amp; II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HBsAg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VDRL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnancy test (For female volunteers)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Only medically healthy subjects with clinically normal laboratory profiles were selected who met following inclusion criteria. Twelve healthy male human subjects were selected based on the following inclusion and exclusion criteria.

### 3.1.5.2 Inclusion Criteria

- Be in the age range of 18-45 years.
- Be neither overweight nor underweight for his/her height as per the Life Insurance Corporation of India height/weight chart for non-medical cases.
- Have voluntarily given written informed consent to participate in this study.
- Be of normal health as determined by medical history and physical examination of the subjects performed within 28 days prior to the commencement of the study.

Subject meeting one of the following criteria (in case of female only):

- Of childbearing potential and practicing an acceptable method of birth control for the duration of the study as judged by the investigator (i.e. condoms, foams, jellies, diaphragm, intrauterine device, abstinence); or
- Is postmenopausal for at least 1 year; or
- Is surgically sterile (bilateral tubal ligation, bilateral oophorectomy, or hysterectomy)

### 3.1.5.3 Exclusion Criteria

- History of hypersensitivity to Olmesartan medoxomil or other related drugs.
- History of drug induced skin rashes or pruritis.
- Individuals with systolic blood pressure <100 mmHg or >140 mmHg diastolic blood pressure < 60 mmHg or >90 mmHg, on the day of admission in period I.
- Any evidence of organ dysfunction or any clinically significant deviation from the normal in physical or clinical determinations.
- Clinically abnormal ECG, or hematological and biochemical parameters defined in Section 11 which is/are outside acceptable limits and is judged clinically significant by investigator.
• Investigations with blood samples of the subject shows presence of disease markers of HIV 1 or 2, Hepatitis B or C Viruses of syphilis infection.

• Investigations with urine samples of the subject shows clinically abnormal chemical and microscopic examination of urine defined as presence of RBC, WBC (>4 /HPF), glucose (Positive) or Protein (Positive).

• History of serious medical illnesses including but not limited to gastrointestinal, hepatic, renal, cardiovascular, pulmonary, neurological or haematological disease, diabetes, glaucoma, any serious, potentially life-threatening illness.

• Inability to communicate well (i.e. language problem, poor mental development, psychiatric illness or poor cerebral function) that may impair the ability to provide, written informed consent.

• Regular smoker, who smokes more than 10 cigarettes daily or has difficulty abstaining from smoking for the duration of each study period.

• Subject has history of drug dependence or excessive alcohol intake on a habitual basis or has difficulty in abstaining or found positive in alcohol breath test before admission in period I.

• Subject meeting any of the following criteria (in case of female only):
  o Demonstrating a positive pregnancy test at screening
  o Is currently breastfeeding.

• Subject has used any medication within 30 days prior to admission of this study.

• Participation in a clinical trial within 90 days preceding admission of this study (except for the subjects who dropout/withdrawn from the previous study prior to period I dosing).

• Use of grapefruit juice and/or grape fruit supplements containing products for 48 hours prior to admission.

• Subject has donated and/or lost more than 350 mL of blood in the past 3 months (90 days), including blood loss in this study.
Chapter 3 Materials and Methods

- Problem(s) in complying with the study protocol.

“No waiver was permitted with respect to inclusion and exclusion criteria.”

3.1.6 Study Medication

3.1.6.1 Handling, Storage and Accountability Procedures

The drug products along with Certificate of Analysis (COA) were procured by the investigator in an appropriate package deemed to maintain the integrity of the products. The drug products were stored by registered pharmacist under prescribed storage conditions. The investigator was accountable for the study drug products. The drug products were dispensed according to the randomization schedule.

3.1.6.2 Assignment to Treatment Sequences

The order of receiving the alternate treatments for each subject during the three periods of the study was determined according to the SAS generated balanced randomization schedule.

3.17 Assessment of Compliance

Compliance was assessed by conducting on the spot thorough examination of the oral cavity by trained study personnel after dosing in each period and by measurement of olmesartan in the plasma (during the analytical phase of the study).

3.1.8 Number of Subjects

Enough healthy adult male subjects were enrolled to allow dosing of Twelve (12) subjects in the first period of the study. Subsequent dropouts/withdrawals were not replaced. It was a pilot study and according to FDA, Canadian and European guidelines a minimum of 12 subjects are recruited for conducting bioequivalence.

3.1.9 Admission and Stay

Subjects were admitted and housed in the Clinical Pharmacology Unit from at least 11 hours before dose administration and were discharged 24 hours after the dose administration during each period, if the subjects do not suffer from any adverse drug reaction. In case of an adverse event, the subjects were monitored until the event subsides. After discharge at 24 hours post dose, the subject made three visits to the Clinical Pharmacology Unit for collection of further blood samples at 36, 48 and 72 hours post dose.
3.1.10 Dose

Either test (A/B) or reference (R) products of Olmesartan, was administered during each period of the study under the supervision of trained study personnel.

3.1.10.1 Reference (R)

A single oral doses of reference product (R) OLMETEC® 40 film coated tablet (containing 40 mg olmesartan medoxomil) of Daiichi Sankyo UK Ltd, was administered with 240 ml of drinking water at an ambient temperature, after an overnight fast of at least 10 hours during each period.

3.1.10.2 Test (A or B)

A single oral dose of olmesartan medoxomil 40 mg tablet of Ranbaxy Laboratories, Limited, India was administered with 240 ml of drinking water at ambient temperature after an overnight fast of at least 10 hours during each period.

3.1.11 Fasting/Meals

The drug product was administered after an overnight fast of at least 10 hours and the fasting were continued until 4-hours post-dose in each period. All subjects received Lunch snacks and dinner at 4, 9 and 13 hours post-dose respectively. During housing, all meal plans were identical for each period. Information on the amount of meal consumed and the time taken for consuming the meal were recorded in the appropriate clinical raw data sheets. Drinking water was not allowed from 1 hour before dosing until 2 hours post-dose except 240 ml of water given during administration of the dose. Thereafter, it was allowed at all times.

3.1.12 Sampling Schedule

The blood samples were collected in 4 ml K3-EDTA vacutainers at the following time points after administration of test/ reference formulation in the each period.

Reference (R) : Pre-dose (duplicate), 0.250, 0.500, 0.750, 1.000, 1.333, 1.667, 2.000, 2.333, 2.667, 3.000, 3.500, 4.000, 5.000, 6.000, 8.000, 10.000, 12.000, 16.000, 24.000, 36.000, 48.000 and 72.000 hours post dose.

Test (A&B) : Pre-dose (duplicate), 0.250, 0.500, 0.750, 1.000, 1.333, 1.667, 2.000, 2.333, 2.667, 3.000, 3.500, 4.000, 5.000, 6.000, 8.000, 10.000, 12.000, 16.000, 24.000, 36.000, 48.000 and 72.000 hours post dose.
A total of seventy two (72), 4-ml blood samples were drawn from each subject in K3 EDTA vacutainers. The pre-dose blood sample (in duplicate) in each period was collected within a period of 1.5 hours before dosing. All post-dose blood samples were collected within ±2 minutes (during their in-house stay) of the scheduled collection time and within ±1 hr of the scheduled collection time (during each ambulatory). The actual end-point time of collection of each blood sample was recorded.

The total volume of blood drawn including 16 ml for screening, 08 ml for safety analysis at the end of the study, and 28.5 ml 'discarded' blood prior to venous cannula collections, was not exceeded 340.5 ml.

All the pre-dose and post-dose samples were collected in pre-chilled vacutainers and placed in ice cold water bath. All samples were collected, processed and analyzed under low light conditions.

3.1.13 Sample Separation and Storage

After collection, blood samples from all the subjects at each time-point, were centrifuged as soon as possible at a speed of 4000 rpm, for 15 minutes duration and at temperature (4 ± 2°C) under refrigeration to separate plasma. Thereafter, all post-dose plasma samples were divided into 2 aliquots and transferred to suitably labeled tubes and re-checked to ensure transfer of plasma to the correct tube. The plasma was stored at below –15°C until analysis. All samples were collected, processed and analyzed under low light conditions.

3.1.14 Wash-out Period

There was a washout period of Ten (10) days between each study period.

3.1.15 Restrictions

3.1.15.1 Medications

Subjects did not receive any medication during the 30 days period prior to the onset of the study. They were instructed during screening not to take any prescription and over the counter (OTC) medications until the completion of the study.

3.1.15.2 Diet

All subjects abstained from any xanthine containing food or beverages or alcoholic products for 48 hours prior to dosing and during in-house stay in each period.
3.1.15.3 **Activity**

All subjects were dosed while seated and remained seated or ambulatory for the first two hours following each drug administration. Thereafter, subjects were allowed to engage only in normal activities while avoiding severe physical exertion.

3.1.16 **Clinical Safety Measurements**

3.1.16.1 **Vital Signs Recording**

Vital signs—oral temperature, sitting BP and radial pulse measurements was performed at admission, prior to morning dose (within 2 hours of the scheduled dosing time) and at 2, 8 and 24 hours (within 2 hour of the scheduled time) after administration of morning dose in each period. In the event of detection of any abnormality during measurement of vital signs, the Principal Investigator was consulted for necessary action, which was recorded.

3.1.16.2 **Clinical Examination**

Brief clinical examination of the subjects was conducted by a qualified medical designate on duty after subject admission and prior to discharge. In the event of detection of any abnormality during clinical examination, the Principal Investigator must be consulted for necessary action, which was recorded.

3.1.16.3 **Laboratory Evaluations for Safety**

Laboratory parameters were repeated at the end of the study. Any laboratory parameter outside acceptable limits was termed as laboratory abnormality and followed up until the results are normal/clinically not significant.

3.1.16.4 **Adverse Events**

Subjects were monitored throughout the study periods for adverse events. Subjects were asked to bring to the notice of the nurse or the doctor any adverse event that may occur during their stay at the site of investigation. Subjects was also be specifically asked about any adverse events at admission, pre-dose, 2, 8, and 24, hours post dose and at 36, 48 and 72 hours during ambulatory visit in each period.

Pre-dose adverse events were asked within 2.0 hour prior to dosing and post-dose adverse events asked within ± 2.0 hour of the scheduled time.
3.1.17  Drop-Out/Withdrawal of Subjects from the Study

Subjects were informed that they were free to dropout from the study at any time without stating any reason.

3.1.18  Volunteer Compensation

The subjects were adequately compensated (as per Fortis Hospital Institutional Review Board (FHIRB) approved guidelines) for the inconvenience and discomfort during their participation in the study.

3.1.19  Ethical Considerations

3.1.19.1  Basic Principles

This research was carried out in accordance with the Basic Principles defined in US 21 CFR Part 320, the ICH (62FR 25692, 09 May 1997) 'Guidance for Good Clinical Practice' and the principles enunciated in the Declaration of Helsinki (Edinburgh, October 2000) with notes of Clarification on Paragraph 29 and 30 added by the WMA General Assembly, Washington 2002, and by the WMA General Assembly, Tokyo 2004 respectively.

3.1.19.2  Institutional Review Board

The protocol (Annexure I; Protocol summary) and the corresponding informed consent form (ICF) used to obtain informed consent (Annexure II) of study subjects was reviewed and approved by Fortis Hospital Institutional Review Board (FHIRB). The board was constituted and operated in accordance with the Principles and requirements described in the US Code of Federal Regulations (21 CFR Part 56).

3.1.20  Study Documentation

All data generated during the conduct of the study was directly entered in the raw data recording forms except the analytical data of clinical laboratory of the Clinical Pharmacology Unit, which was transcribed into the study related forms and the raw data retained by the laboratory for their records. The computer-generated chromatograms were also treated as raw data. All raw data and transcribed data forms were completed by the study personnel assisting in the study and were checked wherever applicable for completeness and logistics by the investigator.
3.2 Bioanalytical Method Development

To develop a precise, accurate and reproducible LC-MS/MS method for the simultaneous estimation of Olmesartan in human plasma, various stationary phases, mobile phases and various sample preparation methods were tried. The method was developed according to FDA guidelines (CDER, 2001). Calibration range (4.051-2500.912 ng/mL) was based on human plasma Olmesartan concentrations after single dose oral administration Olmesartan 40 mg tablet.

For the LC/MS method it is recommended to use an internal standard with similar physicochemical properties to that of the analyte in order to narrow the scan range, to increase data acquisition and to obtain a higher sensibility (Georgita et al. 2010). For the LC/MS assay of Olmesartan (molecular weight = 429.94), Olmesartan-d4 was chosen as internal standard since its physicochemical properties is very close to that of Olmesartan. Similar behavior is expected during sample preparation and chromatographic separation.

The analytical method for the determination of Olmesartan in human K3EDTA plasma using Olmesartan-d4 as internal standard was developed and validated at the Department of Clinical Pharmacology and Pharmacokinetics, Ranbaxy Research Laboratories. Sample preparation process was performed by solid phase extraction technique. The processed samples were chromatographed on Synergi MAX-RP 80A 150 × 4.6mm, 4µ column using using Acetonitrile: Buffer-1 (70:30 v/v) as mobile phase. Olmesartan and Olmesartan-d4 (ISTD) were detected by Sciex LC/MS/MS detection. Signals from the detector were captured by computer and processed using Analyst software Version 1.4.1.

3.2.1 Instrumentation & Reagents

3.2.1.1 Instrumentation

HPLC from Agilent, MS from Sciex and Analyst software Version 1.4.1 for data processing were used.

3.2.1.2 Reagents

1. Acetonitrile (HPLC Grade)
2. Olmesartan (Working Standard)
3. Olmesartan-d4 (Working Standard)

4. Water (HPLC Grade)

5. Methanol (HPLC Grade)

6. Formic Acid (Analytical Grade)

7. Liquor ammonia (about 25% v/v)

3.2.2 Chromatographic and Mass Spectrometric Conditions

A summary of the chromatographic and mass spectrometric conditions is as follows:

Column: Synergi MAX-RP 80A (1.50 × 4.6mm, 4µ)

Mobile Phase: Buffer-1: Acetonitrile (30:70, v/v)

Flow Rate: 0.500 mL/minute

Column Oven Temperature: 45ºC ± 1.0ºC

Sample Cooler Temperature: 10ºC ± 1.0ºC

Injection Volume: 20 µL

Retention Time:
- Olmesartan: 2.70 to 3.20 minutes
- Olmesartan-d4: 2.70 to 3.20 minutes

Ion source: Turbo ion spray in negative polarity

Rinsing solution: Acetonitrile: HPLC grade water (80:20, v/v)

Total Run Time: 5 minutes

Table 3.2 Integration Parameters during method development process

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Olmesartan</th>
<th>Olmesartan d4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bunching factor</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Noise threshold (cps)</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Area threshold (cps)</td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>Number of smooth</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Separation width</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>Separation Height</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>RT window (sec)</td>
<td>40</td>
<td>40</td>
</tr>
</tbody>
</table>
### Materials and Methods

**Chapter 3**

#### Detection:
Olmesartan: m/z 445.50 (Q1 Mass) and 149.30 (Q3 Mass)

Olmesartan-d4: m/z 449.20 (Q1 Mass) and 149.30 (Q3 Mass)

#### Source/Gas Parameters:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curtain Gas (CUR)</td>
<td>12'</td>
</tr>
<tr>
<td>Collision Gas (CAD)</td>
<td>8'</td>
</tr>
<tr>
<td>Ion Source Gas-1 (GS1)</td>
<td>60'</td>
</tr>
<tr>
<td>Ion Source Gas-2 (GS2)</td>
<td>40'</td>
</tr>
<tr>
<td>Ion Spray Voltage (IS)</td>
<td>-4500V</td>
</tr>
<tr>
<td>Temperature (TEM)</td>
<td>500°C</td>
</tr>
<tr>
<td>Interface Heater (ihe)</td>
<td>On</td>
</tr>
</tbody>
</table>

#: These are typical values based on setting and scale defined in software application to control gas parameters.

#### Compound parameters:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Olmesartan</th>
<th>Olmesartan-d4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declustering Potential - DP (V)</td>
<td>-70</td>
<td>-60</td>
</tr>
<tr>
<td>Entrance Potential - EP (V)</td>
<td>-10</td>
<td>-10</td>
</tr>
<tr>
<td>Collision Energy -CE (V)</td>
<td>-42</td>
<td>-42</td>
</tr>
<tr>
<td>Collision cell Exit Potential -CXP (V)</td>
<td>-11</td>
<td>-10</td>
</tr>
</tbody>
</table>

Resolution Q1: Unit

Resolution Q3: Unit

#### 3.2.3 Biological Matrix

Human plasma, procured from Yash Laboratory, shop No. 9, Solanki Appt. Louiswadi, Thane (W) using tripotassium ethylene diamine tetra acetic acid (K3 EDTA) was chromatographically screened for interfering substances prior to use. Human plasma batches containing K3 EDTA as anticoagulant, free of significant interferences were used to prepare calibration standard and quality control samples.
3.2.4 Sample Processing

1. Calibration curve standards, quality control samples and plasma samples were withdrawn as per method validation exercises from the freezer room.

2. These were then allowed to thaw in ice-cold water bath.

3. The thawed samples were vortexed to ensure complete mixing of contents.

4. 200µl aliquot of each plasma sample was added into labeled polypropylene tubes.

5. 50 µl of ISTD stock dilution (approximately 500ng/mL of Olmesartan-d4 and) was pipetted out into these polypropylene tubes (except standard blank).

6. 200 µl of solution-1 was added to it and vortexed.

7. The MCX cartridges (Oasis-30 mg/1 cc) were conditioned using 1 mL of methanol followed by 1 mL of HPLC grade water by running centrifuge for 1 minute at 1500 rpm.

8. The samples were loaded onto the cartridge by running the centrifuge for 2 minutes at 1500 rpm.

9. The cartridges were washed with 1 mL of solution-1, followed by 1 mL HPLC grade water, followed by 1 mL Solution-2 by running the centrifuge for 1 minute at 1500 rpm.

10. The samples were eluted with 1 mL of solution-3 twice by running the centrifuge for 1 minute at 1500 rpm.

11. The eluted samples were dried under nitrogen at 50 ± 2ºC at about 15 psi.

12. The dried residue was reconstituted with 500 µL of Reconstitution solution and transferred into vials for analysis.

All sample processing was done under low light conditions. Temperature of the centrifuge was set between 2-10ºC and maintained up to an ambient temperature condition not exceeding 25ºC. Ice-cold water bath was used till loading of the samples on to the cartridge.
3.2.5 Preparations of Solutions

3.2.5.1 Buffer-1

2 ml formic acid was transferred into a 1000 ml volumetric flask. The volume was made up with 1000 ml of HPLC grade water. It was degassed in an ultrasonic bath. This solution was stored at room temperature and used within 5 days from the date of its preparation.

3.2.5.2 Mobile Phase

300 ml of Buffer-1 was transferred into 1000 ml reagent bottle. 700 ml of acetonitrile was added to it and mixed well. It was degassed in an ultrasonic bath. This solution was stored at room temperature and used within 5 days from the date of its preparation.

3.2.5.3 Rinsing Solution

800 ml of acetonitrile was transferred into 1000 ml reagent bottle. 200 ml of HPLC grade water was added to it and mixed well. It was degassed in an ultrasonic bath. This solution was stored at room temperature and used within 5 days from the date of its preparation.

3.2.5.4 Diluent-1

500 ml of methanol was transferred into a reagent bottle. 500 ml of HPLC grade water was added to it. It was mixed well, sonicated and degassed in an ultrasonic bath. This solution was stored at room temperature and used within 5 days from the date of its preparation.

3.2.5.5 Solution-1

2 mL of formic acid was transferred into 100 mL of volumetric flask. The volume was made up with HPLC grade water. It was mixed well, sonicated and degassed in an ultrasonic bath. This solution was stored at room temperature and used within 5 days from the date of its preparation.

3.2.5.6 Solution-2

25 ml of methanol was transferred into a 100 mL volumetric flask. The volume was made up with HPLC grade water. It was mixed well, sonicated and degassed in an ultrasonic bath. This solution was stored at room temperature and used within 5 days from the date of its preparation.
3.2.5.7 **Solution-3**

2 ml of liquor ammonia was transferred into a 100 mL volumetric flask. The volume was made up with methanol and mixed well. This solution was stored at room temperature and prepared freshly every day.

3.2.5.8 **Reconstitution Solution**

500 mL of HPLC grade water was transferred into a reagent bottle. 500 mL of acetonitrile was added to it. It was mixed well, sonicated and degassed in an ultrasonic bath. This solution was stored at room temperature and used within 5 days from the date of its preparation.

3.2.6 **Preparation Of Stock Solutions**

3.2.6.1 **Olmesartan Standard Stock Solution**

- Olmesartan working standard was weighed accurately and transferred to a volumetric flask. It was dissolved in methanol and the volume was made up with the same to prepare a solution of approximately 1 mg/mL.
- The final concentration of Olmesartan was corrected accounting for its potency, molecular weight and the actual amount weighed.
- It was stored in refrigerator between 1-10°C, protected from light in a polypropylene container and used within 13 days from the date of its preparation.
- Stock solution and all further dilutions from above stock were prepared under low light conditions in ice-cold water bath.

3.2.6.2 **Olmesartan-d4 Internal Standard Stock Solution**

- Olmesartan-d4 working standard was weighed accurately and transferred to a volumetric flask. It was dissolved in methanol and the volume was made up with the same to prepare a solution of approximately 1 mg/mL.
- The final concentration of Olmesartan-d4 was corrected accounting for its potency, molecular weight and the actual amount weighed.
- It was stored in refrigerator between 1-10°C, protected from light in a polypropylene container and used within 13 days from the date of its
preparation.

- Stock solution and all further dilutions from above stock were prepared under low light conditions in ice-cold water bath

### 3.3 Bioanalytical Method Validation

Method Validation was performed to evaluate the method in terms of selectivity (normal, haemolysed and lipemic plasma), Selectivity of analyte in presence of concomitant medication (Acetaminophen, Diclofenac, Amoxicillin and Clavulanic acid), sensitivity, linearity of response, carry-over effect in human plasma, precision and accuracy in normal human plasma, recovery, stability, re-injection reproducibility, dilution integrity, matrix effect (normal, haemolysed and lipemic plasma), matrix factor (normal, haemolysed and lipemic plasma), ruggedness and extended precision and accuracy. The sensitivity, linearity, precision and accuracy evaluations were performed on three batches of spiked samples and all of them are reported. Each precision and accuracy batch consisted of one complete calibration curve (comprising of one blank plasma, one blank plasma with internal standard and eight different non-zero concentrations in which LOQ and ULOQ were in duplicate) and six replicates of quality control samples at LOQQC, Low, Middle and High levels. In addition for biostudy analysis in which only Olmesartan is to be determined three precision and accuracy batches for Olmesartan alone were analysed.

#### 3.3.1 System Suitability Test

System suitability test of the LC-MS/MS system was done in order to check the performance of the system. This was carried out prior to validation exercises in order to ensure the system performance. Six injections of aqueous mixture of analyte and internal standard were carried out and % CV of areas ratio (drug/IS) and retention times were calculated. The % CV of the ratio of drug/internal standard should be less than 4%, and % CV for retention time should be less than 10%.

#### 3.3.2 Selectivity

##### 3.3.2.1 Selectivity in Normal, Haemolysed and Lipemic Human Plasma

Eight different blank matrix batches were screened for interference at the retention time (RT) of all peaks of interest. Six batches were from normal matrix, one from hemolyzed matrix and other one from lipemic matrix. From the processed blank
matrix batches, two normal blank matrix batches were identified with minimal or no peak area response at RT of all peaks of interest and pooled in equal proportion to get pooled plasma. Four replicates of the spiked LOQ samples were processed and injected. Interference at the RT of the analyte was evaluated in each blank matrix by comparing the response in the blank matrix against the mean peak area response of analyte in the extracted LOQ samples. Interference at the RT of the internal standard was evaluated in each blank matrix by comparing the response in the blank matrix against the mean peak area response of the internal standard in the extracted LOQ samples. Percentage interference for each blank matrix was calculated using the following formula.

\[
\% \text{ Interference of analyte} = \frac{\text{Peak area response at RT of analyte in blank matrix}}{\text{Mean Peak area response of the analyte in extracted LOQ sample}} \times 100
\]

\[
\% \text{ Interference of Internal Standard} = \frac{\text{Peak area response at RT of Internal Standard in blank matrix}}{\text{Mean Peak area response of the Internal Standard in extracted LOQ sample}} \times 100
\]

At least five out of six normal matrix batches along with both hemolyzed and lipemic matrix batches should meet the following acceptance criteria.

**Acceptance Criteria**

Response of interfering peaks at the retention time of analyte must be ≤ 20% of the mean peak area response of the analyte in LOQ samples. Response of interfering peaks at the retention time of internal standard must be ≤ 5% of the mean peak area response of the internal standard in LOQ samples. % C.V. should be ≤ 20% for both analyte area and internal standard area in the LOQ samples.

### 3.3.2.2 Selectivity of Analyte in Presence of Concomitant Medication

LOQ solution of analyte with internal standard was prepared at a concentration approximately equivalent to validated LOQ concentration assuming actual recovery. Stock solution of 1mg/mL was prepared for the **concomitant medications** (Acetaminophen, Diclofenac, Amoxicillin and Clavulanic acid) in the solvents as specified below in Table 3.2 followed by stock dilution stock dilution of each drug to prepare a concentration of approximately 1.0 mcg/mL using solution used for injecting analyte in the mass spectrometer. Stock dilution of each drug was injected in duplicate.
into the LC/MS/MS system along with six injections of LOQ solution using chromatographic conditions of analyte.

**Acceptance Criteria**

As described in section 3.3.2.1

**Table 3.3 Selectivity of Analyte in Presence of Concomitant Medication**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Solvent for Stock Solution</th>
<th>Stability Duration (Days)</th>
<th>Method SOP No.</th>
<th>Storage condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>HPLC Grade Methanol</td>
<td>14</td>
<td>PK-M215</td>
<td>Refrigerator between 1-10ºC, protected from light</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>HPLC Grade Methanol</td>
<td>24</td>
<td>PK-M274</td>
<td>Refrigerator between 1-10ºC, protected from light</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>HPLC Grade Water</td>
<td>10</td>
<td>PK-M364</td>
<td>Refrigerator between 1-10ºC and protect from light</td>
</tr>
<tr>
<td>Clavulanic acid</td>
<td>HPLC Grade Water</td>
<td>10</td>
<td>PK-M362</td>
<td>Below -50ºC and protect from light</td>
</tr>
</tbody>
</table>

**3.3.3 Sensitivity**

S/N ratio for all accepted normal blank matrix batches and LOQ samples of selectivity exercise was calculated. First mean of S/N ratio for accepted normal blank matrix samples was calculated and then ratio of S/N ratio of each LOQ sample and mean S/N ratio of normal blank matrix samples was calculated. As acceptance criteria ratio of S/N ratio of each LOQ sample and mean S/N ratio of normal blank matrix samples should be ≥ 5 for all LOQ samples.

\[
\frac{S}{N} \text{ of LOQ sample} \geq \frac{\text{Mean}\ S/N \text{ of normal blanks}}{5}
\]

The lowest standard was accepted as the limit of quantification (LOQ) of the method if: between batches precision (%C.V) at the LOQQC was ≤ 20%. (Taken from PA batches) and between batches accuracy (% nominal) at the LOQQC was between 80-120%. (Taken from PA batches).
3.3.4 Weighing Factor Optimization

Three standard curves were used for weighing factor optimization. Three weighing factors none, 1/amount and 1/amount² were used and the accuracy (% nominal) at each concentration level of the standard curve was back calculated. Weighing factor was optimized with \( |\sum \% \text{dev}| + \sqrt{\sum \% \text{dev}^2} \) with minimum value and was used for further validation exercises.

3.3.5 Linearity and Standard Curve

Three batches of calibration curve standards were processed and analyzed to check linearity of the method. The standard curve range of the Olmesartan was 4.055 - 2496.641 ng/ml. The linearity was determined by weighted least square regression analysis of standard plot associated with eight point standard curve respectively.

Each batch of spiked plasma includes: One complete calibration curve (consisting of one blank plasma, one blank plasma with internal standard and eight different non-zero concentration samples).

The best regression algorithm was statistically determined using the following formula (Eq. 3.1).

\[
|\Sigma \% \text{dev}| + \sqrt{( \% \text{dev})^2}
\] (3.1)

Where, \% dev represents the per cent deviation for each non-zero calibrant, i.e.: (Back calculated value - Nominal value)/ Nominal value X 100

These calculations were performed using the linear regression algorithm (without any weighting) for each of the three validation batches and sum of the results was obtained. Same calculations were repeated using weighting 1/X and 1/X² linear regression algorithms.

Lowest summed value was indicative of the regression algorithm (1/X²) to be used throughout validation of the analytical method and in the clinical sample analysis.

The linear equation describing the relationship between concentration ratio and peak area ratio of analyte to internal standard was determined by least-squares weighted (1/concentration²) regression method (Eq. 3.2)

\[
Y = m \frac{1}{X^2} + c
\] (3.2)

Where,
Y = chromatographic response in terms of peak area ratio of (analyte) / (internal standard - IS)

X = concentration ratio of analyte / IS

m = slope of the calibration curve

c = Y-axis intercept at zero concentration.

The concentration of analyte in calibration curve was calculated using above linear regression parameters of the corresponding calibration curve. The mean analyte concentration, SD, % CV (precision) and % nominal (accuracy) for each non-zero calibration standard were calculated. The other calibration curve parameters like slope, intercept and coefficient of correlation were also tabulated.

% CV and % nominal were calculated using Eq. 3.3 and 3.4, respectively:

\[
\% \text{ CV (Coefficient of variation)} = \text{Standard deviation} \times 100 \quad (3.3)
\]

Mean concentration

\[
\% \text{ Nominal} = \text{Mean calculated value} \times 100 \quad (3.4)
\]

Nominal value

The standard curve linearity was determined on three standard curves and slope, intercept and correlation coefficient were determined. For calculation of the standard curve plots, area ratio of analyte and internal standard area against concentration were determined.

For acceptance of linearity of calibration curves, at least 75% or a minimum of 6 non-zero standards including LOQ (lower limit of quantification) and ULOQ (upper limit of quantification) must meet the following passing criteria:

Accuracy of standards (%Nominal): within ± 15% of their nominal values (within ± 20% for LOQ).

Precision of calibrates (% CV): ≤ 15% (≤ 20% for LOQ)

In case first injected LOQ or ULOQ standard failed, lost or exhibited bad chromatography, their duplicates were passing the above criteria.

Linear coefficient of correlation: ≥ 0.98.
3.3.6 Precision and Accuracy

Three Precision and Accuracy batches (PA batches) each consisting of a reference standard solution (aqueous mix), 14 calibration standards (standard blank, standard zero, standard A (LOQ), standard H (ULOQ); each in duplicate and standard-B, C, D, E, F, G and six replicates of each QC set (containing one LOQQC, LQC, MQC and HQC) quality control samples, interspersed within each other, were processed and analyzed according to the proposed method. Ruggedness of the method was assessed by processing and analyzing new PA batch on same LC-MS system using different column (same type) by different analyst and using fresh solutions and calculating the precision and accuracy.

The back calculated concentration of Olmesartan in each quality control sample was calculated using linear regression parameters of the corresponding calibration curve. The mean Olmesartan concentration, SD, % CV (precision) and % nominal (accuracy) for each QC level of the three PA batches were calculated. For the acceptance, between, intraday (using two PA batches run on the same day) and within batch CVs for QC samples should be ≤ 15% (≤ 20% for LOQ QC) and % Nominal of QC samples should be within ± 15% (within ± 20% for LOQ QC).

3.3.7 Ruggedness

One precision and accuracy batch (containing one calibration curve and six sets of quality control samples at LOQQC, LQC, MQC and HQC) was processed by a different analyst as per the standard method. Using the same instrument, a new column was used for analysis. All the solutions were prepared by different analyst. This was done to check the ruggedness of the method.

The acceptance criteria were same as discussed under precision and accuracy.

3.3.8 Recovery

Standard aqueous quality control stock of Olmesartan each at low, medium, and high levels were spiked in plasma and HPLC water separately, the latter being considered as unextracted quality control samples. Six replicates of each quality control plasma samples were processed as usual and analyzed along with six replicates of unextracted standard quality control samples by applying correction factor to nullify dilution of extracted samples during plasma processing. Six replicates of aqueous Olmesartan-d4
were also run for the recovery of Olmesartan-d4. The mean response in terms of the peak area of Olmesartan and IS, SD and % CV were calculated at each QC level. % recovery was calculated as follow:

\[
\text{% Recovery} = \frac{\text{Mean peak area of extracted QC sample}}{\text{Mean peak area of unextracted QC samples}} \times 100
\]

% CV for recovery was calculated between the three QC levels. The recovery was deemed acceptable if CV is ≤ 20 % for % recovery between low, middle and high quality control concentrations and means % recovery between low, middle and high quality control concentrations was ≤ 115 %.

3.3.9 Stability and Integrity Evaluation

3.3.9.1 Stock Solution Stability

Stock solution stability ensures stability of analyte and internal standard in the respective solvents at their storage conditions for certain duration.

\[
\text{% Stability} = \frac{\text{Mean Nominal of Stability Samples}}{\text{Mean Nominal of Comparison Samples}} \times 100
\]

% CV and % stability for peak area ratio obtained from stability and comparison stock dilutions were calculated. The solutions were considered stable for specified period; if percent stability was within the range of 90-110 %.

3.3.9.2 Bench Top Stability

Four replicates of each low and high concentration quality control samples (LQC and HQC) were taken from cold room, thawed at room temperature, kept unprocessed for an intended duration (stability sample), then processed along with freshly spiked calibration standards and freshly spiked LQC & HQC samples (comparison sample) and analyzed by back calculation using regression equation obtained. The % CV and % nominal were calculated at both LQC and HQC levels.
3.3.9.3 Freeze-Thaw Stability

Four replicates of each low and high concentration quality control samples (LQC and HQC) were removed from deep freezer, thawed unassisted at room temperature, refrozen for 24 hours under the same conditions (stability sample). The freeze-thaw cycle was repeated two more times. Samples were then processed along with freshly spiked calibration standards and freshly spiked LQC & HQC samples (comparison sample) and then analyzed by back calculation using regression equation obtained. The % CV and % nominal were calculated at both LQC and HQC levels. Samples were deemed stable after three freeze-thaw cycles if % stability was within ±15 % of nominal values and % CVs ≤ 15 % at both LQC and HQC levels.

3.3.9.4 In-Injector Stability/Auto Sampler Stability

Four replicates of each low and high concentration quality control samples were processed and kept in the auto-sampler for 96 hours (stability sample). Samples were then run and analyzed along with freshly spiked calibration standards & freshly spiked LQC & HQC samples (comparison sample) and analyzed by back calculation using regression equation obtained. Same process was repeated for internal standard. The % CV and % nominal were calculated at both LQC and HQC levels. The samples were considered to be stable for the specified in injector stability period if % stability was within ±15 % of nominal values and % CVs ≤ 15 % at both LQC and HQC levels.

3.3.9.5 Long-Term Stability

Four replicates of each low and high concentration quality control samples were stored at -50°C in D-freezer along with subject samples (stability sample). Samples were taken after 40 days, processed along with freshly spiked LQC & HQC samples (comparison sample) and freshly spiked calibration standards, and then analyzed by back calculation using regression equation obtained. The % CV and % nominal were calculated at both LQC and HQC levels. The samples were considered to be stable for the specified long term stability period if % stability was within ±15 % of nominal values and % CVs ≤ 15 % at both LQC and HQC levels.

3.3.9.6 Dilution Integrity

This exercise ensures integrity of analyte in those samples which are beyond upper limit of the standard curve and need to be diluted. A fresh stock of Olmesartan was prepared and spiked in plasma to get a concentration level of 1.8 times of standard H of the usual
calibration standard. It was then diluted 2 times and 4 times with the blank plasma (dilution integrity samples). Six replicates of both dilutions were processed along with freshly spiked calibration standards and freshly spiked LQC & HQC samples (comparison sample) and then analyzed by back calculation using regression equation obtained. The % CV and % nominal were calculated for both dilutions. The integrity of the samples were considered to be maintained if % stability was within ± 1.5 % of nominal values and % CVs ≤ 15 % for both dilutions.

3.3.10 Matrix Effect

Matrix effect was evaluated by taking six different batches of drug free plasma. Aqueous LQC and HQC were spiked in each batch of plasma and processed in duplicates as per the standard method. One calibration curve was also processed and all quality control samples were run against the calibration curve.

The acceptance criteria of the mean % nominal for LQC and HQC should be between 85-115% and % CV at both LQC and HQC should be less than 1.5%.

3.3.11 Carry Over Effect In Matrix

- Carry over effect was performed by re-injecting blank, LOQ and ULOQ samples as per the sequence given below from any of the accepted calibration curve.
  - Blank matrix sample (First injection)
    - LOQ sample in duplicate from the same vial
    - ULOQ sample in duplicate from the same vial
    - Blank matrix sample (Second injection from the same vial used for first injection)

- Interference at the RT of the analyte was evaluated by comparing response in first blank matrix against the mean peak area response of analyte in the processed LOQ samples.

- Interference at the RT of the internal standard was evaluated by comparing response in the first blank matrix against the mean peak area response of internal standard in the processed LOQ samples.
Carryover effect for analyte and internal standard was calculated as per following formula.

\[
\text{Carry over effect for analyte} = \left( \frac{\text{Response of interfering peak at RT of analyte in second blank sample} - \text{Response of interfering peak at RT of analyte in first blank sample}}{\text{Mean peak area response of the analyte in processed LOQ samples}} \right) \times 100
\]

\[
\text{Carry over effect for internal standard} = \left( \frac{\text{Response of interfering peak at RT of internal standard in second blank sample} - \text{Response of interfering peak at RT of internal standard in first blank sample}}{\text{Mean peak area response of the internal standard in processed LOQ samples}} \right) \times 100
\]

Acceptance Criteria

- The response of interfering peak at RT of analyte in first blank sample should be \( \leq 20\% \) of the mean peak area response of the analyte in processed LOQ sample.

- The response of interfering peak at RT of internal standard in first blank sample should be \( \leq 5\% \) of the mean peak area response of the internal standard in processed LOQ sample.

- Carryover effect should be \( \leq 20\% \) for analyte and \( \leq 5\% \) for internal standard.
3.3.12 Standardization and calculations

The chromatographic data were acquired and processed using computer based Analyst software Version V 1.4.1. The best-fit curves using weighted \( \frac{1}{\text{amount}^2} \) linear least square regression analysis were obtained by peak area ratio of Olmesartan to Olmesartan-d4 (ISTD). The concentrations of Olmesartan in plasma samples were calculated using linear regression parameters of the corresponding calibration curve.

Weighting factor \( \frac{1}{\text{amount}^2} \) was selected after weighting factor evaluation using three precision and accuracy batches.
3.4 Analysis of Clinical Study Samples

3.4.1 Objective

The objective was to analyze the clinical study samples using a validated LC-MS/MS method for the determination of Olmesartan.

3.4.2 Sample Collection and Storage

Blood samples from each period were centrifuged to separate plasma and stored at --15°C in a Heraeus deep freezer at the clinical facility on their respective dates of collection. All plasma samples from five periods were then packed properly using dry ice, transported to the analytical facility and stored at -15°C until analysis.

3.4.3 Sample Analysis

As per the protocol, a total number of 864 samples had to be collected from 12 subjects in over three periods. One subject (subject no. 07) was dropped out of the study due to changes in the personal situation before the start of period I, therefore, a total of 792 samples were collected during the whole study for analysis. For sample analysis by LC-MS/MS, one analytical batch consisted of total 92 samples which include all the samples of one subject (72) in three periods along with the calibration standards (14) and quality control samples (two each of LQC, MQC and HQC) were processed and analyzed.

3.4.4 Batch Acceptance Criteria

All the batches were evaluated rigorously and considered for the repeat analysis if failed with respect to any of the following criteria.

3.4.4.1 Calibration Curve Acceptance Criteria

All the calibration curves were evaluated for the following passing criteria:

1. Accuracy of calibrators: within ± 15% of their nominal values (within ± 20% for LOQ).

2. At least 75% or a minimum of 6 calibrators including LOQ and ULOQ meet the above criteria.

3. In case first injected LOQ or ULOQ standard failed, lost or exhibited bad chromatography, their duplicates were passing the above criteria.

4. Linear coefficient of correlation: ≥0.98.
3.4.4.2 Blank and Blank plus Internal Standard Acceptance Criteria

At least one blank and one blank + IS: free from significant interference. i.e.

1. Peak area responses of the blanks at the retention time of the Olmesartan were < 20 % of the peak area response of the LOQ standard and
2. Peak area responses of the blanks at the retention time of the internal standard were < 5 % of the mean response of internal standards used in the calibration curve.

3.4.4.3 Quality Control Sample Acceptance Criteria

Batch acceptance required that back calculated concentrations of at least 50 % of each QC sample (LQC, MQC and HQC) and 67 % overall were within ± 15 % of their nominal values.

3.4.4.4 Repeat Analysis

Samples were subjected to repeat analysis using following criteria.

1. Bad chromatography.
2. Batch failure.
3. Internal standard variation (< 40 % and more than 180 % of mean IS response of standards).
4. Sample lost in analysis/processing.
3.5 Pharmacokinetic and Statistical Analysis

3.5.1 Pharmacokinetic Analysis

The concentration data obtained from analytical study was entered in WinNonlin pharmacokinetic software for further processing.

The following pharmacokinetic parameters were calculated for Olmesartan using WinNonlin Node version 5.0.1 or above from Pharsight:

- **AUC**<sub>0-t</sub>: The area under the plasma concentration versus time curve, from time zero to the last measurable concentration, as calculated by the linear trapezoidal method.

- **AUC**<sub>0-∞</sub>: The area under the plasma concentration versus time curve, from time zero to infinity. **AUC**<sub>0-∞</sub> is calculated as the sum of **AUC**<sub>0-t</sub> plus the ratio of the last measurable plasma concentration to the elimination rate constant.

- **AUC % Extrapolation**: It is the percentage of extrapolated area under the plasma concentration versus time curve from the last measurable concentration to infinity. It was calculated as $\left(\frac{\text{AUC}_{0-\infty} - \text{AUC}_{0-t}}{\text{AUC}_{0-\infty}}\right) \times 100$

- **C<sub>max</sub>**: Maximum measured plasma concentration over the time span specified.

- **T<sub>max</sub>**: Time of the maximum measured plasma concentration. If the maximum value occurs at more than 1 time point, **T<sub>max</sub>** is defined as the first time point with this value.

- **K<sub>e</sub>**: Apparent first-order terminal elimination rate constant calculated from a semi-log plot of the plasma concentration versus time curve. The parameter was calculated by linear least-square regression analysis using the maximum number of points in the terminal log-linear phase (e.g. three or more non-zero plasma concentrations).
The apparent first-order terminal elimination half-life was calculated as $0.693/K_{el}$.

No value of $K_{el}$, $\text{AUC}_{0-\infty}$ or $T_{1/2}$ was reported for cases that do not exhibit a terminal log linear phase in the concentration versus time profile.

The last three factors fully determine the shape of the plasma concentration-time curve and strategies to compare the shape of the curve itself instead of these 'derived' parameters are of little use.

Plasma concentration-time profile of a drug in a volunteer clearly distinguishes as absorption and elimination phase. When the drug absorbed equals the drug eliminated, $C_{\text{max}}$ is present. Before $C_{\text{max}}$ is reached (before $T_{\text{max}}$) the absorption is higher than the elimination, after $T_{\text{max}}$ the situation is reversed.

Frequently elimination is a so-called first order process, which means that per unit of time a percentage of the drug present in the blood disappears from it. So for example every hour 5% of the drug present in the blood disappears, which means that as the plasma concentration declines, the eliminated drug per time unit also declines.

When elimination is a true first order process, a log transformation of the measured plasma concentrations will render a straight line during the elimination phase.

The sampling schedule should also cover the plasma concentration time curve long enough to provide a reliable estimate of the extent of exposure which is achieved if $\text{AUC}_{0-t}$ covers at least 80.0% of $\text{AUC}_{0-\infty}$. At least three to four samples are needed during the terminal log-linear phase in order to reliably estimate the terminal rate constant.

As discussed earlier, elimination is a first order process and a natural log ($\ln$)-transformation makes it possible to draw a straight line through the elimination phase. The slope of the regression line is now equivalent to $K_{el}$ or the elimination constant.

### 3.5.2 Statistical Analysis

Statistical analysis was performed on plasma Olmesartan using the WinNonlin PK Software, Version 5.0.1 or above. The analysis included data from all subjects those...
who completed the study. The dropouts and / or withdrawals were not replaced and unequal number of subjects per sequence was used.

The data from those subjects who experience emesis post dose during the sample collection schedule was not included for final pharmacokinetic analysis of Olmesartan with below criteria:

- If vomiting (emesis) occurs at or before 2 times median T_{max} of Olmesartan.

- Subject who experience vomiting post dose during the sample collection schedule were not used for median T_{max} calculation

Summary Statistics

Arithmetic means, standard deviations and coefficients of variation was calculated for the parameters listed in section 3.5.1. Additionally, geometric means and percentage coefficient of variation of geometric means was calculated for AUC_{0-t}, AUC_{0-∞} and C_{max}.

3.5.2.1 Ratio Analysis

The ratio of the test (A or B) and reference (R) product averages (least square means) was calculated for Olmesartan by first calculating the differences in the averages (arithmetic means) of the log-transformed data and then taking the antilog of the obtained difference. The comparison of interest was A vs. R and B vs. R, so the ratios were of the form A/R and B/R. Ratio of means was expressed as a percentage of the LSM for the reference formulations.

3.5.2.2 Analysis of Variance (ANOVA)

The log-transformed pharmacokinetic parameters (AUC_{0-t}, AUC_{0-∞} and C_{max}) for Test (A&B) and Reference (R) were analyzed using a mixed effects ANOVA model using Type III sum of squares, with the main effects of sequence, period and formulations as fixed effects and subjects nested within sequence as random effect. A separate ANOVA model was used to analyze each of the parameters. The sequence effect was tested at the 10% level of significance using the subjects nested within sequence mean square as the error term. All other main effects were tested at the 5% level of significance against the residual error (mean square error) from the ANOVA model as the error term. Each analysis of variance included calculation of least-squares means, the difference between the adjusted formulation means and the standard error associated with the difference. The above analyses were done using the appropriate SAS® procedure.