CHAPTER 3.
MATERIALS AND METHODS
3.0 Materials and Methods

The experimental design is divided into 6 parts

1. Clinical study methodology
2. Analytical methodology
3. Bio-analytical method validation
4. Clinical study sample analysis
5. Pharmacokinetic and Statistical analysis
6. In-vitro analysis

3.1 Clinical study methodology

3.1.1 Objective

To compare single-dose oral bioavailability of three different marketed Metformin Extended release tablets in healthy, adult, human, male subjects under fed conditions.

3.1.2 Products Evaluated

Reference (R)

Cetapin XR 500mg sustained release tablets (containing Metformin 500mg) manufactured by Aventis Pharmaceuticals, India.

Test (A)

Glycomet SR 500mg sustained release tablets (containing Metformin 500mg) manufactured by USV Pharmaceuticals Ltd, India.

Test (B)

Bigomet SR 500mg extended release tablets (containing Metformin 500mg) manufactured by Otsira Genetica Ltd, India.
Table 3.1: Details of drug product for study number 006_JAMHAM_07.

<table>
<thead>
<tr>
<th>Product</th>
<th>Batch No.</th>
<th>Mfg. Date</th>
<th>Expiry Date</th>
<th>Manufacturer</th>
<th>Cost (Rs./10 Tabs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cetapin XR</td>
<td>C17018</td>
<td>Jun. 2007</td>
<td>Nov. 2009</td>
<td>Aventis Pharma Limited</td>
<td>24</td>
</tr>
<tr>
<td>500mg</td>
<td>28000650</td>
<td>Jun. 2007</td>
<td>Dec. 2008</td>
<td>USV Limited</td>
<td>17</td>
</tr>
<tr>
<td>Bigomet SR</td>
<td>2860307</td>
<td>Apr. 2007</td>
<td>Mar. 2009</td>
<td>Otsira Genetica, Aristo Pharmaceuticals Pvt. Ltd.</td>
<td>9.88</td>
</tr>
<tr>
<td>500mg</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.1.3 Study Design

An open label, balanced, randomized, three-treatment, three-period, three-sequence, single-dose, crossover, bioavailability study comparing three different marketed Metformin sustained release in healthy, adult, human male subjects under fed conditions.

The study design has been schematically represented in Appendix I and a detailed summary of the protocol is presented in Appendix II.

3.1.4 Number of Subjects

Eighteen (18) healthy, adult, human male subjects were enrolled in the first period of the study. Subsequent dropouts/withdrawals were not replaced. Data will be presented on all completed subjects.

3.1.5 Selection of Subjects

Adequate number of subjects were selected randomly from the volunteer bank of Clinical Pharmacology Unit and the subjects 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 were recorded. Each subject underwent physical examination and the laboratory tests of hematologic, hepatic and renal functions as listed below.

Table 3.2: Laboratory Tests conducted for each subject

<table>
<thead>
<tr>
<th>HEMATOLOGY</th>
<th>URINALYSIS</th>
<th>ADDITIONAL TESTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>PHYSICAL EXAMINATION</td>
<td>HIV I &amp; II</td>
</tr>
<tr>
<td>• Total Leukocyte Count</td>
<td>Colour</td>
<td>HBsAg</td>
</tr>
<tr>
<td>• Differential Leukocyte Count</td>
<td>Appearance</td>
<td>HCV</td>
</tr>
<tr>
<td>• Platelet count</td>
<td>PH</td>
<td>VDRL</td>
</tr>
<tr>
<td>• Specific Gravity</td>
<td>URINE DRUG SCREEN</td>
<td>Cannabinoids</td>
</tr>
<tr>
<td>BIO-CHEMISTRY</td>
<td>Protein</td>
<td></td>
</tr>
<tr>
<td>• BUN</td>
<td>Glucose</td>
<td>Opioids</td>
</tr>
<tr>
<td>• Creatinine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Total Bilirubin</td>
<td>MICROSCOPIC</td>
<td></td>
</tr>
<tr>
<td>• Alkaline</td>
<td>EXAMINATION</td>
<td></td>
</tr>
<tr>
<td>Phosphatase</td>
<td>• RBC</td>
<td></td>
</tr>
<tr>
<td>• AST</td>
<td>• WBC</td>
<td></td>
</tr>
<tr>
<td>• ALT</td>
<td>• E. cells</td>
<td></td>
</tr>
<tr>
<td>• Glucose</td>
<td>• Crystals</td>
<td></td>
</tr>
<tr>
<td>• Cholesterol</td>
<td>• Casts</td>
<td></td>
</tr>
<tr>
<td>• Others</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Only medically healthy subjects with clinically normal laboratory profiles were selected if they met following inclusion criteria. Eighteen healthy male human subjects were selected based on the following inclusion and exclusion criteria.

3.1.5.2 Inclusion Criteria
- Be male and 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 14 days prior to the commencement of the study.

3.1.5.3 Exclusion Criteria

- History of allergy to metformin or other related antidiabetic biguanide preparations.

- Any evidence of organ dysfunction or any clinically significant deviation from the normal, in physical or clinical determinations.

- Presence of disease markers of HIV 1 or 2, Hepatitis B or C viruses or syphilis infection.

- Presence of values which are significantly different from normal reference ranges (as defined in Appendix 5) and/or judged clinically significant for haemoglobin, total white blood cells count, differential WBC count or platelet count.

- Positive for urinary screen testing of drugs of abuse (opiates or cannabinoids)

- Presence of values which are significantly different from normal reference ranges (as defined in Appendix 5) and/or judged clinically significant for serum creatinine, blood urea nitrogen, serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), serum alkaline phosphatase, serum bilirubin, plasma glucose or serum cholesterol.

- Clinically abnormal chemical and microscopic examination of urine defined as presence of RBC, WBC (>4/HPF), glucose (positive) or protein (positive).
- Clinically abnormal ECG or Chest X-ray.

- History of serious gastrointestinal, hepatic, renal, cardiovascular, pulmonary, neurological or haematological disease, diabetes or glaucoma.

- History of any psychiatric illness which may impair the ability to provide written informed consent.

- Regular smokers who smoke more than 10 cigarettes daily or have difficulty abstaining from smoking for the duration of each study period.

- History of drug dependence or excessive alcohol intake on a habitual basis of more than 2 units of alcoholic beverages per day (1 unit equivalent to half pint of beer or 1 glass of wine or 1 measure of spirit) or have difficulty in abstaining for the duration of each study period.

- Use of any enzyme modifying drugs within 30 days prior to Day 1 of this study.

- Participation in any clinical trial within 12 weeks preceding Day 1 of this study.

- Subjects who, through completion of this study, would have donated and/or lost more than 350 mL of blood in the past 3 months.

3.1.6 Dosing, Admission and Stay

Subjects were admitted and housed in the Clinical Pharmacology Unit from at least 10 hours before dose administration and discharged 24 hours after administration of the test or reference products 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.

Period I:

Period I of the study was conducted between 20 November 2007 and 22 November 2007.
Period II:
Period II of the study was conducted between 30 November 2007 and 02 December 2007.

Period III:
Period III of the study was conducted between dates 07 December 2007 and 09 December 2007.

During each study period, the following procedure was carried out:

The subjects were divided into three groups with 6 subjects in each group. A single oral dose of Metformin 500mg sustained release tablet was administered with 240 mL of drinking water at ambient temperature after standardized breakfast, during each period of the study under supervision of trained study personnel. The order of drug administration were randomized with SAS-generated randomization schedule.

All subjects will be required to fast overnight after admission for at least 10 hours. The study subjects received the study drug 30 minutes after the recommended meal in each period. The breakfast was consumed within 30 minutes. The subjects received standard meals— lunch, snacks and dinner at 4, 9 and 13 hours, respectively, after drug administration. During housing, all meal plans were identical for all periods. Information on the amount of meal consumed and the time taken for consuming the meal were recorded in the appropriate clinical raw data sheets. In case, meals and blood sample collection coincide, samples were collected before meals are provided.

Drinking water was not allowed from 1 hour before dosing until 2 hours post-dose. Thereafter, it was allowed at all times.

3.1.7 Treatments

Metformin Hydrochloride extended release 500 mg tablets were administered once on the day of dosing of each period.

Reference (R)
Cetapin XR 500mg sustained release tablets (containing Metformin 500mg) manufactured by Aventis Pharmaceuticals, India, was administered after an overnight fast of at least 10 hours.

**Test (A)**

Glycomet SR 500mg sustained release tablets (containing Metformin 500mg) manufactured by USV Pharmaceuticals Ltd, India, was administered after an overnight fast of at least 10 hours.

**Test (B)**

Bigomet SR 500mg extended release tablets (containing Metformin 500mg) manufactured by Otsira Genetica Ltd, India, was administered after an overnight fast of at least 10 hours.

### 3.1.8 Washout Period

A washout period of at least seven days was enforced between dosing of each period.

### 3.1.9 Assignment to Treatment Sequences

The order of receiving study treatments for each subject during the 3 periods of the study was determined according to the SAS-generated balanced randomization schedule by the statistician.

### 3.1.10 Assessment of Compliance

Compliance was assessed by conducting a thorough examination of the oral cavity by trained study personnel, after the morning dose at the start of the period. The final confirmation of compliance was done by the measurement of plasma Metformin (during the analytical phase of the study).

### 3.1.11 Fasting/Meals
All subjects will be required to fast overnight after admission for at least 10 hours. The study subjects will receive the study drug 30 minutes after the recommended meal in each period. The breakfast will be consumed within 30 minutes. They will receive standard meals—lunch, snacks and dinner at 4, 9 and 13 hours, respectively, after drug administration. During housing, all meal plans will be identical for both periods. Information on the amount of meal consumed and the time taken for consuming the meal will be recorded in the appropriate clinical raw data sheets. In case, meals and blood sample collection coincide, samples will be collected before meals are provided.

Drinking water will not be allowed from 1 hour before dosing until 2 hours post-dose. Thereafter, it will be allowed at all times.

3.1.12 Sampling Schedule

A total of sixty six, 4-mL blood samples were collected from each subject in EDTA vacutainers during the course of the study through indwelling cannulae placed in forearm veins. The minimum blood sample volume required for analytical purpose was 4-mL in this study. The blood samples were collected Pre-dose (within 1.5 hours of dosing) and at 0, 0.5, 1, 2, 3, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 9, 10, 12, 14, 16, 20, 24 and 48 hours post-dose in each period. The pre-dose blood sample in each period were collected within a period of 1.5 hours before dosing and the post-dose samples were collected within 2 minutes of the scheduled time. The actual end-point time of collection of each blood sample were recorded. For each subject, the total number of blood draws during the study was 66 and the total volume of blood drawn including 16 mL for screening and 30 mL 'discarded' blood prior to venous cannula collections, did not exceed 310 mL.

Intravenous indwelling cannula was kept in situ as long as possible when multiple samples were collected. The cannula was maintained patent by injection of 1 mL of 5 IU/mL of heparin in normal saline solution. At each time point, the blood samples were collected after discarding the first 0.5 mL of heparinised blood and heparin solution from the tubing.

After collection, the blood samples were centrifuged under refrigeration as soon as possible to separate plasma. All plasma samples were transferred to suitable labeled tubes and rechecked.
to ensure transfer of plasma to the correct tube. The plasma samples were then stored at -20°C or lower, pending transfer to the analytical facility for assay.

3.1.13 Restrictions

3.1.13.1 Medications

All subjects were instructed not to take any other medications including OTC during the 2 weeks period prior to the onset of the study. The medication was advised only in case of medical emergencies.

3.1.13.2 Diet

All subjects abstained from any xanthine containing food or beverages or alcoholic products for 48 hours prior to dosing and throughout the sampling schedule during each period.

3.1.13.3 Activity

All subjects were dosed while seated and were asked to remain seated or ambulatory for the first 2 hours following each drug administration in each period. Thereafter, subjects were allowed to engage only in normal activities while avoiding severe physical exertion.

3.1.14 Safety

3.1.14.1 Clinical Safety Measurements

Vital signs of oral temperature, sitting blood pressure and radial pulse were measured during subject admission, prior to each dosing and 2, 6 and 24 hours after administration of study drug and at ambulatory visits in each study period. Vital signs were measured prior to administration of the dose and were taken within 1 hour of the scheduled dosing time. At all other times, vital signs were taken within 30 minutes of the scheduled times.

Brief clinical examination of the subject was conducted by a qualified medical designate on duty after subject admission, prior to dosing of study drug and before discharge.
Laboratory tests (haemoglobin, serum creatinine, blood urea nitrogen, serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), serum alkaline phosphatase (ALP) and serum bilirubin) were repeated at pre-dose in each study period (day 1) and discharge day (6) of all subjects. Any elevations in hepatic enzymes were to be followed up till they subsided.

3.1.14.2 Adverse Events

All the subjects were monitored throughout the study period for adverse events. Subjects were specifically asked about any adverse events on each ambulatory visit (days 1-4), after admission (day 4), before administration of the dose (day 5) and approximately every four hours thereafter discharge in each period.

3.1.15 ETHICAL CONSIDERATIONS

3.1.15.1 Basic Principles

This research was carried out in accordance with the basic principles defined in US 21 CFR Part 312.20, the ICH (62 FR25692, 09 May 1997)’ Guidance for ‘Good Clinical Practice’ and the principles enunciated in the Declaration of Helsinki (Edinburgh, October 2000).

3.1.15.2 Institutional Review Board

This protocol and the corresponding informed consent form (ICF) used to obtain informed consent of study subjects were reviewed and approved by the Jamia Hamdard Institutional Review Board and the study subjects were not dosed until the Board approved the protocol and the ICF, as submitted or with modifications.

The version 2 of the protocol and the ICF for this study were reviewed and approved by the Jamia Hamdard Institutional Review Board on 24th August 2007.

3.1.15.3 Informed Consent

The purpose of the study, procedures to be carried out, potential hazards and rights of the subjects were described to the subjects in non-technical terms before the subjects were admitted to the Ranbaxy CPU for Period I. All the subjects provided formal written consent after
attending an oral presentation and after thoroughly reading the version 2 of the Informed Consent Form.

3.1.16 Drop-out/Withdrawal of Subjects from Study

Subjects were informed that they are free to drop-out from the study at any time without stating any reason. The decision of withdrawal of a subject from the study was considered for any of the following reasons:

(i) The subject suffers from significant intercurrent illness or undergoes surgery during the course of the study.

(ii) The subject experiences adverse event, and withdrawal is in the best interest of the subjects.

(iii) The subject fails to comply with the requirements of the protocol. This would include pre-study directions regarding alcohol and drug use, fasting or if the subject is uncooperative during the study.

Details of reasons for withdrawal of subjects were recorded and reported. Every effort was made to obtain a complete follow-up for any withdrawn subject.

3.1.17 Volunteer Compensation

The subjects were adequately compensated on account of their participation in the study. In case of drop-out/withdrawal of a subject before completion of the study, the guidelines issued by the Jamia Hamdard Institutional Review Board were final and binding on both Ranbaxy Research Laboratories and the study subjects.

3.1.18 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. All raw data and transcribed data forms were completed by the study personnel.
3.2 Analytical methodology

A Liquid Chromatography Mass Spectroscopy method for the determination of Metformin in human plasma was developed and validated. Metformin-D6 was used as an internal standard.

Sample preparation process was accomplished by Liquid-Liquid extraction method. 50 μl of internal standard (1000 μg/ml) was added to 200μl of an aliquot and vortexed in polypropylene tube. To this 4ml of HPLC grade acetone was added. The tubes were placed in a flat bed shaker at 100 rpm for 20 minutes. The tubes were then placed in a centrifuge and run at 4000 rpm for 5 minutes. 3ml of the supernatant organic layer was pipetted into a glass test tube and contents were dried in a nitrogen evaporator at 15 PSI and 50°C. The dried residue was reconstituted with 500 μl of mobile phase, vortexed and contents decanted into glass vials for analysis by the LCMS.

3.2.1 Instrumentation
- LCMS Applied Biosystems (MDS API 3000, +ve mode, Electron spray ionizer)
- LC-10 AD pump (Shimadzu, Japan)
- SIL-HT Auto sampler
- DG4 Degasser (Shimadzu, Japan)
- SCL-10 A data capture system
- CTO-10A VP column Oven
- Analyst software ver 1.4.1

3.2.2 Reagents
i. Acetonitrile (HPLC grade, Spectrochem)
ii. Metformin Hydrochloride working standard
iii. N, N dimethyl d6-diguanide hydrochloride (CDN isotopes, Quebec, Canada)
iv. Methanol (HPLC grade, Qualigens)
v. Ammonium formate buffer 5mM
vi. Formic acid  
vi. Acetone  
ix. HPLC grade Water

3.2.3 Preparation of reagents

Mobile phase

To a 1000ml reagent bottle, 200ml of 5mM ammonium formate buffer (Ph 3.0 ± 0.1) and 800ml of acetonitrile was added. The contents were mixed well and degassed in an ultrasonic bath. Ph of the buffer was adjusted using formic acid.

Rinsing solution

Transferred 500mL of HPLC grade acetonitrile into 1000 mL reagent bottle and to it 500 mL of HPLC grade water was added. This was mixed well and de-gassed in ultrasonic bath for 10 minutes.

Standard stock solution of Metformin

Weighed accurately 5 mg of Metformin working standard and transferred it into a 5 ml volumetric flask. The contents were dissolved in water and made up the volume with the same to make a solution of approximately 1 mg/mL. Corrected the above final concentration of Metformin accounting for its potency, molecular weight and the actual amount weighed.

3.2.4 Procedures

3.2.4.1 Bulk spiking of plasma for calibration curve standards

Just prior to spiking of plasma, serial dilutions of stock solution were prepared in methanol:water (50:50) to obtain aqueous calibration curve standards (1929.3- 101625.2 ng/ml) as shown in Table 3.1. 1 ml of each of the aqueous calibration standard was transferred into 50 ml of volumetric flask and volume was made up with the plasma to achieve calibration standard range (38.6-2032.5 ng/ml). 2 ml of each of the calibration standard was aliquoted into polypropylene tubes, caped and stored at below -15°C until analysis.
### Table 3.3: Preparation of calibration standards of Metformin

<table>
<thead>
<tr>
<th>Stock concentration (ng/ml)</th>
<th>Stock aliquot (ml)</th>
<th>Volume made upto (ml)</th>
<th>Conc. of aqs. dilution (ng/ml)</th>
<th>Final conc. in plasma (ng/ml)</th>
<th>Calibration Standards</th>
</tr>
</thead>
<tbody>
<tr>
<td>81330.14</td>
<td>0.250</td>
<td>2</td>
<td>101625.2</td>
<td>2032.5</td>
<td>STD H</td>
</tr>
<tr>
<td>101625.2</td>
<td>1.500</td>
<td>2</td>
<td>76218.9</td>
<td>1524.4</td>
<td>STD G</td>
</tr>
<tr>
<td>76218.9</td>
<td>1.350</td>
<td>2</td>
<td>51447.7</td>
<td>1029.0</td>
<td>STD F</td>
</tr>
<tr>
<td>51447.7</td>
<td>1.000</td>
<td>2</td>
<td>25723.9</td>
<td>514.5</td>
<td>STD E</td>
</tr>
<tr>
<td>25723.9</td>
<td>1.000</td>
<td>2</td>
<td>12861.9</td>
<td>257.2</td>
<td>STD D</td>
</tr>
<tr>
<td>12861.9</td>
<td>1.000</td>
<td>2</td>
<td>6431.0</td>
<td>128.6</td>
<td>STD C</td>
</tr>
<tr>
<td>6431</td>
<td>1.500</td>
<td>2</td>
<td>4823.3</td>
<td>96.5</td>
<td>STD B</td>
</tr>
<tr>
<td>4823.2</td>
<td>0.800</td>
<td>2</td>
<td>1929.3</td>
<td>38.6</td>
<td>STD A</td>
</tr>
</tbody>
</table>

### 3.2.5 Preparation of quality control samples

Stock solution of metformin was serially diluted with methanol: water (50:50) to obtain range of aqueous dilutions (1933.4-76458.8 ng/ml) as shown in the Table 3.4. 1 ml of each of the aqueous dilution was transferred into 50 ml of volumetric flask and volume was made up with the plasma to achieve quality control samples namely limit of quantification quality control-LOQ QC (38.7 ng/ml), lower quality control-LQC (95.5 ng/ml), middle quality control-MQC (1032.2 ng/ml) and higher quality control-HQC (1529.2 ng/ml). 2 ml of each of the quality control standards were aliquoted into polypropylene tubes, caped and stored at below -15°C until analysis.

### Table 3.4: Preparation of Quality control samples of metformin

<table>
<thead>
<tr>
<th>Stock Conc. (µg/ml)</th>
<th>Stock aliquot (ml)</th>
<th>Volume made upto (ml)</th>
<th>Conc. of aqs. dilutions (µg/ml)</th>
<th>Final conc. in plasma (ml)</th>
<th>Quality control samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>813391.4</td>
<td>0.188</td>
<td>2</td>
<td>76458.8</td>
<td>1529.2</td>
<td>HQC</td>
</tr>
<tr>
<td>76458.8</td>
<td>1.350</td>
<td>2</td>
<td>51609.7</td>
<td>1032.2</td>
<td>MQC</td>
</tr>
<tr>
<td>51609.7</td>
<td>1.000</td>
<td>2</td>
<td>25804.8</td>
<td>516.1</td>
<td>M1QC</td>
</tr>
<tr>
<td>25804.8</td>
<td>0.370</td>
<td>2</td>
<td>4773.9</td>
<td>95.5</td>
<td>LQC</td>
</tr>
<tr>
<td>4773.9</td>
<td>0.810</td>
<td>2</td>
<td>1933.4</td>
<td>38.7</td>
<td>LOQQC</td>
</tr>
</tbody>
</table>
3.2.6 Preparation of Internal Standard (IS) Solution

5 mg of metformin-D6 was weighed accurately, transferred into a 5 ml of volumetric flask, make up the volume with the methanol to produce 1 mg/ml of metformin-D6 stock solution. The final concentration of metformin-D6 was corrected for its potency, actual amount weighed. The solution was stored in refrigerator at 2 - 10°C and used for 15 days from the date of preparation. The stock solution was diluted in methanol: water (50:50).

3.2.7 Sample preparation

The blank human plasma, calibration standards, quality control and unknown samples were retrieved from freezer room, thawed for 30 min and vortexed for 30 sec. 50 μl of internal standard (1000 μg/ml) was added to 200μl of an aliquot of samples and vortexed in polypropylene tube. To this 4ml of HPLC grade acetone was added. The tubes were placed in a flat bed shaker at 100 rpm for 20 minutes. The tubes were then placed in a centrifuge and run at 4000 rpm for 5 minutes. 3ml of the supernatant organic layer was pipetted into a glass test tube and contents were dried in a nitrogen evaporator at 15 PSI and 50°C. The dried residue was reconstituted with 500 μl of mobile phase, vortexed and contents decanted into glass vials for analysis by the LC-MS.

3.2.8 Chromatography Conditions

A summary of the chromatographic conditions was as described below.

Table 3.5: Summary of chromatographic conditions

<table>
<thead>
<tr>
<th>Column</th>
<th>RP Select B (250 x 4.0 mm), 5 μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column Oven Temperature</td>
<td>32°C</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>Acetonitrile : Ammonium formate (80:20) buffer at pH 3.0±0.1</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>0.8 ml/min</td>
</tr>
<tr>
<td>Rinsing Solution</td>
<td>Acetonitrile: Water 50:50 HPLC grade</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>50 μL</td>
</tr>
<tr>
<td>Retention Time (range)</td>
<td>2.70 - 3.30 min</td>
</tr>
</tbody>
</table>
3.3 Bio-analytical Method Validation

The validation of this procedure was performed in order to evaluate the method in terms of selectivity, linearity, precision, accuracy, sensitivity, recovery and stability (CDER, 2001). The linearity, precision and accuracy evaluations were performed on three batches of spiked plasma samples. Each batch consisted of one complete set of calibration standards (Blank, blank plus internal standard, LLOQ (Std A), ULOQ (Std H) each in duplicate and Std-B, C, D, E, F and G) and six replicates of each quality control samples namely limit of quantification quality control-LOQQC (38.7 ng/ml), lower quality control-LQC (95.5 ng/ml), middle quality control-MQC (1032.2 ng/ml) and higher quality control-HQC (1529.2 ng/ml).

3.3.1 System Suitability test

System suitability test of the LCMS 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. Five injections of aqueous mixture of analytes and internal standard were carried out and % CV of areas ratio (drug/IS) and retention times less than 2 % and less than 5 % were acceptable respectively.

3.3.2 Screening and Selectivity

Ten different lots of blank plasma were screened for the interference at the retention times of Metformin and internal standard (d6-metformin). Blank plasma sample with no or minimum interference were spiked with standard metformin to achieve lower limit of quantification (LOQ) level i.e. 38.7 ng/ml in six replicates. These samples were analyzed according to the proposed method and observed for interference.

For the acceptance, response of the interfering peak must be:

1. \( \leq 20\% \) of the mean peak area response of the LOQ and \( \leq 5\% \) of the mean peak area response of the internal standard.

At least 80 % of the matrix should meet the above mentioned acceptance criteria.

3.3.3 Weighing factor optimization

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

### 3.3.4 Linearity and Standard curve

Standard curve range was selected on the basis of earlier published reports so that it can effectively utilize for the bioequivalence study analysis. The standard curve range was selected to be 38.6-2032.5 ng/ml. 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.

### 3.3.5 Precision and Accuracy

Three Precision and Accuracy batches (PA batches) each consisting of a reference standard solution (aqsmix), 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 limit of quantification (38.7 ng/ml), low (95.5 ng/ml), middle (1032.2 ng/ml) and high (1529.2 ng/ml) quality control samples, interspersed within each other, were processed and analyzed according to the proposed method.

The back calculated concentration of metformin in each quality control sample was calculated using linear regression parameters of the corresponding calibration curve. The mean metformin concentration, SD, % CV (precision) and % nominal (accuracy) for each QC level of the three PA batches were calculated. For the acceptance, between 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).

### 3.3.6 Recovery

Standard aqueous quality control stock of metformin each at low, medium, and high levels were spiked in plasma and HPLC water separately, the later 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 metformin-D6 were also run for the recovery of metformin.
The mean response in terms of the peak area of metformin 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 % mean recovery between low, middle and high quality control concentrations.

3.3.7 Stability and Integrity evaluation

**Bench top stability / Short-Term Stability:** Six aliquots of each low and high concentration quality control samples (LQC and HQC) were taken from cold room, thawed at room temperature, kept unprocessed for 6 hours and then processed along with freshly spiked calibration standards and analyzed by back calculation using regression equation obtained. The mean, SD, % CV and % nominal were calculated at both LQC and HQC levels. Samples were deemed stable after specified bench top period if - % nominal is within ± 15 % of nominal values and % CVs ≤ 15 % at both LQC and HQC levels.

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

**Freeze and Thaw Stability:** Six 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. The freeze–thaw cycle is repeated two more times. Samples were then processed along with freshly spiked calibration standards and analyzed by back calculation using regression equation obtained. The mean, SD, % CV and % nominal were calculated at both LQC and HQC levels. Samples were deemed stable after three freeze thaw cycles if - % nominal is within ± 15 % of nominal values and % CVs ≤ 15 % at both LQC and HQC levels.
Post-Preparative Stability / In-injector stability: Six aliquots of each low and high concentration quality control samples were processed and kept in the auto-sampler for 36 hours. Samples were then run and analyzed along with freshly spiked calibration standards and analyzed by back calculation using regression equation obtained. The mean, SD, % 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 - % nominal is within ± 15 % of nominal values and % CVs ≤ 15 % at both LQC and HQC levels.

3.3.8 Standardization and calculations
The chromatographic data were acquired and processed using computer based class VP software supplied by Shimadzu, Japan. During validation, method was evaluated without weighing as well as with weighing factors (1/X and 1/X²). The best line using weighted (1/X²) least square linear regression analysis was obtained by peak area ratio of metformin and metformin-D6 versus concentration ratio of metformin and d6-metformin. The concentration of metformin in the plasma samples was calculated using linear regression parameters of the corresponding calibration curve.

3.4 Clinical Study Sample Analysis

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

3.4.2 Sample collection and storage
The plasma samples for period I, II and III were collected between 21st - 22nd November 2007; 01st - 02nd December 2007 and 08th -9th December 2007 respectively. Blood samples from each period were centrifuged to separate plasma and stored below −15°C in a Haereus deep freezer at the clinical facility on their respective dates of collection. All plasma samples from three periods were then packed properly using dry ice, transported to the analytical facility on 18th December 2007, and stored below −15 °C until analysis.
3.4.3 Sample analysis
As per the protocol, a total number of 1188 samples have to be collected from 18 subjects over in three periods. One subject (subject no. 9) dropped out from the study voluntarily before dosing in period I, so therefore, a total of 1122 samples were collected during the whole study and 1122 samples were used for analysis. For drug analysis by LC-MS/MS, one analytical batch consisting of total 80 samples which includes all the samples of one subject (66) in three periods along with the calibration standards (14) and quality control samples (3 LQC, 3 MQC and 3 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 metformin 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.

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 following pharmacokinetic parameters were calculated for Metformin using WinNonlin-Node 4.0 from Pharsight:

- **AUC\(_{0\rightarrow t}\):** 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\(_{0\rightarrow \infty}\):** The area under the plasma concentration versus time curve, from time zero to infinity. AUC\(_{0\rightarrow \infty}\) is calculated as the sum of AUC\(_{0\rightarrow t}\) plus the ratio of the last measurable plasma concentration to the elimination rate constant.

- **AUC\(_{0\rightarrow t} / AUC_{0\rightarrow \infty}\):** The ratio of AUC\(_{0\rightarrow t}\) to AUC\(_{0\rightarrow \infty}\).

- **MRT:** The average amount of time spent by the drug in the body before being eliminated after each treatment.

- **C\(_{\text{max}}\):** Maximum measured plasma concentration over the time span specified.

- **T\(_{\text{max}}\):** Time of the maximum measured plasma concentration. If the maximum value occurs at more than 1 time point, T\(_{\text{max}}\) is defined as the first time point with this value.

- **K\(_{\text{el}}\):** Apparent first-order terminal elimination rate constant calculated from a semi-log plot of the plasma concentration versus time curve. The parameter will be 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).

- **T\(_{1/2}\):** The apparent first-order terminal elimination half-life will be calculated as 0.693/K\(_{\text{el}}\).
3.5.2 Statistical analysis

Statistical analyses were performed on plasma Metformin using the SAS system for Windows, release 8.2 (SAS Institute Inc., USA). The analyses included data from subjects 1 to 8 and 10 to 18 as these subjects completed the study.

3.5.2.1 Summary Statistics

Arithmetic means, standard deviations and coefficients of variation will be calculated for the parameters listed in section 13.3. Additionally, geometric means and percentage coefficient of variation of geometric means will be calculated for AUC0-t, AUC0-∞) and Cmax.

Analysis of Variance (ANOVA)

The log-transformed pharmacokinetic parameters (Cmax, AUC0-t and AUC0-∞) will be 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 will be used to analyze each of the parameters. The sequence effect will be tested at the 10% level of significance using the subjects nested within sequence mean square as the error term. All other main effects will be 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 will include calculation of least-squares means, the difference between the adjusted formulation means and the standard error associated with the difference. The above analyses will be done using the appropriate SAS® procedure or the WinNonlin PK Software, Version 4.0 or above.

90% Confidence Intervals and Ratio Analysis

90% confidence interval for the ratio of the test and reference product averages (least square means) will be calculated for metformin by first calculating the 90% confidence interval for the differences in the averages (arithmetic means) of the log-transformed data and then taking the
antilogs of the obtained confidence limits. The comparison of interest is A vs R and B vs R, so ratio will be in the form of test/reference. Ratio of means will be calculated using the LSM for log-transformed Cmax, AUC0−t, and AUC0−∞. Ratio of means will be expressed as a percentage of the LSM for the reference formulations.

For Metformin, the 90% confidence interval for the ratio of test and reference product for Cmax, AUC0→t and AUC0→∞ should be between 80% and 125% for the log transformed data.

### 3.6 In-Vitro Dissolution Study

Drug absorption from a solid dosage form after oral administration depends on the release of the drug substance from the drug products, the dissolution or solubilization of the drug under physiological conditions, and the permeability across the gastrointestinal tract. Because of critical nature of the first two of these steps, in vitro dissolution may be relevant to the prediction of in vivo performance. Based on this general consideration, in vitro dissolution specifications are established to ensure batch-to-batch consistency and to signal potential problems with in vivo bioavailability. In the case of a generic drug product, the dissolution specifications are generally the same as the reference listed drug (RLD). The specifications are confirmed by testing the dissolution performance of the generic drug product from an acceptable bioequivalence study (CDER 1997).

#### 3.6.1 Approaches for setting dissolution specifications for generic products

The approaches for setting dissolution specifications for generic products fall into three categories, depending on whether an official compendial test for the drug product exists and on the nature of the dissolution test employed for the reference listed drug (CDER 1997). The three categories are:

- USP drug product dissolution test available
- USP drug product dissolution test not available; dissolution test for reference listed NDA drug product publicly available.
- USP drug product dissolution test not available; dissolution test for reference listed NDA drug product not publicly available.
3.6.2 Dissolution methodology
Dissolution study was performed according to method specified in USP with additional time-points added to capture the complete release profile till 15hrs.

- Apparatus: Distek dissolution system 2100C (Distek, N. Brunswik, New Jersey).
- Medium: Phosphate Buffer
- pH: 6.8 ± 0.1
- Volume: 900 ml
- Rotation Speed: 100 ± 2 rpm
- Time-points: 0, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 15 hrs.
- Temperature: 37 ±0.5°C
- Drug Analysis: UV spectrophotometer @ 223 nm

Procedure: The dissolution medium was maintained at 37±0.5°C. At each time, 5 ml of the solution was withdrawn under test, and passed through a filter having a 45-µm Millipore nylon filter. The absorbance of filtrate was determined by UV Spectrophotometer at 223 nm. The amount of metformin dissolved (% Release) was determined against the standard curve.

Preparation of phosphate buffer:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potassium dihydrogen orthophosphate</td>
<td>6.8 gm</td>
</tr>
<tr>
<td>Sodium hydroxide pellets</td>
<td>0.9 gm</td>
</tr>
<tr>
<td>Purified water q.s.</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

Weighed accurately 6.8 gm of potassium dihydrogen orthophosphate and dissolved it into approx. 500 ml double distilled water in a 1000 ml volumetric flask. Again 0.9 gm sodium hydroxide was dissolved in a 250 ml of distilled water separately and then poured into volumetric flask and make up the volume up to mark (1000 ml) by double distilled water. Adjust the pH 6.8 ±0.1 by orthophosphoric acid at pH meter.

3.6.3 Dissolution profile comparisons
Until recently, single-point dissolution tests and specifications have been employed in evaluating scale-up and post approval changes, such as:

1. scale-up
2. manufacturing site changes
3. component and composition changes, and
4. equipment and process changes.

A changed product may also be a lower strength of a previously approved drug product. In the presence of certain minor changes, the single-point dissolution test may be adequate to ensure unchanged product quality and performance.

For more major changes as in case of generics or multisource products, a dissolution profile comparison performed under identical conditions for the product before and after the change(s) is recommended. Dissolution profiles may be considered similar by virtue of:

1. overall profile similarity
2. similarity at every dissolution sample time point.

The dissolution profile comparison may be carried out using model independent or model dependent methods. A simple model independent approach uses a difference factor ($f_1$) and a similarity factor ($f_2$) to compare dissolution profiles (Moore et al., 1996). The similarity factor ($f_2$) is a logarithmic reciprocal square root transformation of the sum of squared error and is a measurement of the similarity in the percent (%) dissolution between the two curves.

$$f_2 = 50 \cdot \log \left\{ \left( 1 + \left( 1/n \right) \sum_{t=1}^{n} \left( R_t - T_t \right)^2 \right)^{-0.5} \cdot 100 \right\}$$

Where $n = \text{number of sampling time points}$, $\sum = \text{summation over all time points}$, $R_t = \text{dissolution at time point } t \text{ of the reference product}$, and $T_t = \text{dissolution at time point } t \text{ of the test product}$. An $f_2$ value between 50 and 100 suggests that the two dissolution profiles are similar.

The difference factor ($f_1$) calculates the percent (%) difference between the two curves at each time point and is a measurement of the relative error between the two curves:

$$f_1 = \left[ \frac{\sum_{t=1}^{n} |R_t - T_t|}{\sum_{t=1}^{n} R_t} \right] \cdot 100$$
where $n$ is the number of time points, $R$ is the dissolution value of the reference batch at time $t$, and $T$ is the dissolution value of the test batch at time $t$.

A specific procedure to determine difference and similarity factors was as follows:

- The dissolution profile of two products (6 units each) of the test and reference products were determined.
- Using the mean dissolution values from both curves at each time interval, difference factor ($f_1$) and similarity factor ($f_2$) were calculated with the help of above equations.
- For curves to be considered similar, $f_1$ values should be close to 0, and $f_2$ values should be close to 100. Generally, $f_1$ values up to 15 (0-15) and $f_2$ values greater than 50 (50-100) ensure sameness or equivalence of the two curves and, thus, of the performance of the test and reference products.