3.0 MATERIALS AND METHODS

Clinical part of the study was carried out at Ranbaxy Clinical Pharmacology Unit (CPU) Majeedia Hospital. 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:

- 3.1 Clinical Study Methodology
- 3.2 Bioanalytical Method Development
- 3.3 Bioanalytical Method Validation
- 3.4 Analysis of Clinical Study Samples
- 3.5 Pharmacokinetic and Statistical Analysis

3.1 CLINICAL STUDY METHODOLOGY

3.1.1 Institutional Review Board (IRB) Approval

The version 01 of the protocol dated 08 October 2009, was reviewed by the Jamia Hamdard Institutional Review Board (JHIRB) at the convened meeting held on October 19, 2009. An expedited review of the version 02 of protocol for this study was done by the Jamia Hamdard Institutional Review Board (JHIRB) and the same was approved on February 22, 2010.

A copy of the final protocol summary, complete IRB information and sample ICF are included (Annexure 1, 2 and 3).

3.1.2 Ethical Conduct of the Study

This research was carried out in accordance with the basic principles defined in European Union Directive 2001/20/EC, US 21 CFR Part 320, the ICH (62FR 25692, 09 May 1997) ‘Guidance for Good Clinical Practice’, ICMR ‘ethical guidelines for biomedical research on human participants (2006)’, CDSCO ‘guidance on Good Clinical Practices for Clinical Research in India’ and the principles enunciated in the Declaration of Helsinki (WMA General Assembly, Seoul 2008) respectively.
3.1.3 Subject Information & Informed Consent

Informed consent was obtained from all the subjects for the purpose of inclusion into the study. The purpose of the study, the procedures to be carried out and the potential hazards that may be encountered during the conduct of the study were described to the subjects in non-technical terms before the subjects were admitted to the Ranbaxy Clinical Pharmacology Unit for study. All the subjects enrolled provided formal written informed consent after attending an oral presentation and after reading the Version 02 of the informed consent form (ICF) (Annexure 3).

3.1.4 Objective

To assess the single oral dose bioequivalence of the test formulation i.e. Fixed dose combination tablet of Atorvastatin 10 mg (as Atorvastatin Calcium) and Metformin HCl 500 mg ER of Ranbaxy Laboratories Limited, with Lipitor® tablets (containing Atorvastatin 10 mg as Atorvastatin calcium) of Pfizer and Glucophage® XR tablets (containing Metformin HCl 500 mg) of Bristol-Myers Squibb company, administered concurrently in healthy, adult, human male subjects under fed condition.

3.1.5 Product Evaluated

<table>
<thead>
<tr>
<th>Reference (R)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Product Name</strong></td>
</tr>
<tr>
<td>Manufactured By</td>
</tr>
<tr>
<td>Distributed By</td>
</tr>
<tr>
<td>Batch No.</td>
</tr>
<tr>
<td>Expiry Date</td>
</tr>
<tr>
<td><strong>Product Name</strong></td>
</tr>
<tr>
<td>Distributed By</td>
</tr>
<tr>
<td>Lot No.</td>
</tr>
<tr>
<td>Expiry Date</td>
</tr>
</tbody>
</table>
### Test (T)

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Metformin hydrochloride extended release and Atorvastatin Calcium Tablets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manufactured By</td>
<td>Ranbaxy Laboratories Limited</td>
</tr>
<tr>
<td>Batch No.</td>
<td>AG (4265)15</td>
</tr>
<tr>
<td>Expiry Date</td>
<td>Nov 2011</td>
</tr>
</tbody>
</table>

#### 3.1.6 Study Design

The study was conducted as an open label, balanced, randomized, two-treatment, two-period, two sequence, single dose, crossover, bioequivalence study comparing fixed dose combination tablet of Atorvastatin 10 mg (as Atorvastatin calcium) and Metformin HCl 500 mg ER of Ranbaxy laboratories limited, with Lipitor® tablets (containing Atorvastatin 10 mg as Atorvastatin calcium) of Pfizer, and Glucophage® XR tablets (containing Metformin HCl 500 mg) of Bristol-Myers Squibb company administered concurrently, in healthy, adult, human male subjects under fed condition.

The study design was schematically represented (Annexure 5).

#### 3.1.7 Study Site

The study was conducted at Ranbaxy Clinical Pharmacology Unit, 2nd Floor Majeedia Hospital, New Delhi-110062.

#### 3.1.8 Selection of Study Population

According to the design of the study 40 healthy, adult, male human subjects, who met the inclusion and exclusion criteria as described in the protocol, were enrolled into the study, from the volunteer bank of Clinical Pharmacology Unit, underwent a standardized screening procedure from February 03, 2010 to February 22, 2010. Disposition of the subjects are presented (Table 3.1).
3.1.9 Screening Assessments

Subjects were judged to be medically healthy based on their medical history and demographic data (which included age, sex, body weight, height and smoking status), physical examination, vital signs, 12-lead ECG, chest X-ray and laboratory tests for haematological parameters, hepatic and renal functions, and disease markers for syphilis, HIV and hepatitis B and C and urine analysis including drug of abuse. Only subjects who had clinically normal laboratory profiles as well as fulfilled the inclusion and exclusion criteria were enrolled in the study. The screening procedures took place from February 03, 2010 to February 22, 2010 and involved the following laboratory tests presented (Table 3.2).
Table 3.2: Laboratory Test

<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></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>SEROLOGY</th>
<th>Microscopic Examination</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV I &amp; II</td>
<td>Urine drug screen</td>
</tr>
<tr>
<td>HBsAg</td>
<td>- Cannabinoids</td>
</tr>
<tr>
<td>HCV</td>
<td>- Opioids</td>
</tr>
<tr>
<td>VDRL</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>OTHER Assessments</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECG</td>
</tr>
<tr>
<td>Chest X Ray</td>
</tr>
</tbody>
</table>

All the subjects were subjected to breath test for alcohol and test for drug of abuse (cannabinoids and opioids) in urine prior to admission in each period.

Subjects were included in the study on the basis of the following inclusion and exclusion criteria:

**3.1.10 Inclusion Criteria**

Subjects Included:

- Were in the age range of 18-45 years.

- Were neither overweight nor underweight for his height as per the Life Insurance Corporation of India height/weight chart for non-medical cases.
- Had voluntarily given written informed consent to participate in this study.

- Were of normal health as determined by medical history and physical examination of the subjects performed within 21 days prior to the commencement of the study.

- Had a non-vegetarian diet habit

- Had hemoglobin concentration of ≥13 gm%.

There were no deviations in this regard.

### 3.1.11 Exclusion Criteria

- History of allergy or hypersensitivity to Atorvastatin, Metformin or any other related drugs.

- History of diarrhea, nausea, vomiting, abdominal pain, loss of appetite, taste disturbance in the week preceding the study.

- History of chronic headache, migraine and/or dizziness.

- History of muscle cramps, muscle pain and aches or muscle weakness, myopathy or rhabdomyolysis.

- History of tremor, vertigo.

- History of pancreatitis.

- History/evidence of jaundice or any hepatic or gall bladder disease.

- History of acute infection in the week preceding the study.

- History of trauma or seizures.

- History of drug-induced rash and/or pruritus.

- Any evidence of organ dysfunction or any clinically significant deviation from the normal, in physical or clinical determinations,
- Subject had laboratory test parameter(s) which was/were outside acceptable limits and was judged clinically significant.

- Subject had 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 might impair the ability to provide, written informed consent.

- Subject was a regular smoker, who smoked more than 10 cigarettes daily or had difficulty abstaining from smoking for the duration of each study period.

- Use of Grapefruit juice and or grape fruit supplements containing products for 48 hrs prior to admission.

- Subject had history of drug dependence or excessive alcohol intake on a habitual basis or had difficulty in abstaining for the duration of each study period or has consumed alcohol 48 hrs prior to admission.

- Subject had used an enzyme modifying drugs within 30 days prior to admission of this study.

- Subject had participated in a clinical trial within 12 weeks preceding admission of this study (except for subjects who dropout / are withdrawn from the previous study prior to period 1 dosing)

NOTE: No waivers were permitted with respect to the inclusion/ exclusion criteria.

There were no deviations in this regard.

3.1.12 Removal of Subjects from Therapy or Assessment

Subjects were informed that they were free to drop-out from the study at any time without stating any reason. The investigator had the right to withdraw a subject from the study if:
1. The subject suffered from significant inter-current illness or undergoes surgery during the course of the study.

2. The subject experienced adverse event, when withdrawal would be in the best interest of the subjects.

3. The subject failed to comply with the requirements of the protocol. This would include pre-study directions regarding alcohol and drug use, fasting or if the subject was uncooperative during the study.

4. The subject required concomitant medications which might interfere with the pharmacokinetics of the investigational products.

5. If the subject experienced vomiting at any time during the sample collection schedule.

Details of reasons for withdrawal and drop out of subjects were recorded and have been included in this report.

3.1.13 Study Medication

3.1.13.1 Handling, Storage and Accountability Procedures

The drug products were procured by the investigator in an appropriate package deemed to maintain the integrity of the products. The drug products were stored under prescribed storage conditions. The investigator was accountable for the study drug products. The drug products were dispensed according to the randomization schedule (Annexure 4).

3.1.13.2 Assignment to Treatment Sequences

The order of receiving the test and reference products for each subject during both the periods of the study was determined according to a SAS-generated randomisation schedule. The randomisation was balanced and the code was kept under controlled access in the drug store. A working copy of the same was provided to study personnel responsible for dosing. The investigator, pharmacy in-charge, personnel involved in dispensing of study drugs and the dosing were accountable for ensuring compliance to randomization schedule. The randomization schedule was generated by the Statistical
Services Department of CPP using SAS software Version 9.1 (SAS Institute Inc., USA). Detailed randomization codes are presented (Annexure 4).

### 3.1.14 Assessment of Compliance

Compliance to the treatment was assessed by conducting a thorough examination of the oral cavity by trained study personnel after dosing in each period and by measuring Metformin and Atorvastatin in the plasma (during the analytical phase of the study). The investigator ensured dosing compliance.

### 3.1.15 Admission and Stay

Subjects were admitted and housed in the Clinical Pharmacology Unit from at least 60 hrs before dose administration and were discharged 24 hrs 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. The subjects made three ambulatory visits to the Clinical Pharmacology Unit for vitals, AE monitoring and collection of further blood sample at 36, 48 and 72 hrs post-dose in each period

### 3.1.16 Dose

A single oral dose of fixed dose combination tablet of Metformin hydrochloride Extended release and Atorvastatin Calcium Tablets [500 mg + 10 mg] of either test or Lipitor® tablet 10 mg and Glucophage® XR 500 mg tablet administered concurrently of reference formulation was administered during each period of the study, along with 240 ml of 20% glucose solution in drinking water at ambient temperature, under the supervision of trained study personnel, 45 minutes after starting high-fat high-calorie breakfast. The breakfast was provided after an overnight fast of at least 10 hr.

Both test and reference products were administered to all the study subjects, one in each period (except for subject numbers 05, 07, 08, 16, 24 and 25). The order of receiving the test and reference products for each subject during both the periods of the study was determined according to a SAS-generated randomization schedule.
3.1.17 Fasting/Meals

All subjects received standard meals after admission- Dinner, Breakfast, Lunch, snacks, dinner, Breakfast, Lunch, snacks, dinner at approximately -60, -48, -44, -39, -36, -24, -20, -15, and -12 hrs pre-dose, respectively.

Following an overnight fast of at least 10 hrs, subject started the recommended meal (breakfast) 45 minutes prior to administration of drug product. The same was to be consumed within 30 minutes of serving in each period. Lunch, snacks and dinner at 4, 9 and 13 hrs post-dose, respectively. Drinking water was allowed as desired except for one hr before and two hr after drug administration.

3.1.18 Sampling Schedule

The blood samples were collected in prechilled K3-EDTA vacutainers placed in wet ice bath during the course of study through indwelling cannulae placed in forearms veins at the following time points after administration of test/ reference formulation in the each period.

Blood samples were collected at pre-dose and at 0.333, 0.667, 1.000, 1.333, 1.667, 2.000, 2.333, 2.667, 3.000, 3.500, 4.000, 4.500, 5.000, 5.500, 6.000, 6.500, 7.000, 8.000, 10.000, 12.000, 16.000, 20.000, 24.000, 36.000, 48.000 and 72.000 hrs post-dose in each period under low light condition in pre-chilled vacutainers placed in wet ice bath. The pre-dose blood samples in each period were collected in duplicate (2 x 7 ml), within a period of 1.5 hr before dosing. Post-dose samples were generally collected within 2 minutes of the scheduled time till discharge. Ambulatory blood samples were collected within 60 minutes of scheduled time.

For any subject who completed both periods of the study, the total number of 54, 7 ml blood samples (excluding pre-dose duplicate samples) were drawn. The total volume of blood drawn, including 16 ml for screening, 14 ml for duplicate sample at pre-dose, 23 ml 'discarded' blood prior to venous cannula collections and 8 ml for safety analysis at the end of the study did not exceed 439 ml.

3.1.19 Sample Separation and Storage

All the collected blood samples in collection tubes were transferred by one of the study personnel or an attendant to a sample processing room at the Clinical
Pharmacology Unit. Thereafter, the blood samples were centrifuged at 4000 RPM for duration of 15 minutes and at temperature 4 °C under refrigeration as soon as possible to separate plasma. All plasma samples were divided into 4 aliquots and transferred to suitably labelled polypropylene tubes and re-checked to ensure transfer of plasma to the correct tubes and were stored in suitably labelled tubes below -50 °C, until transfer to the analytical facility for assay.

The samples were processed under low light condition. All aliquot 1, 2, 3 & 4 of plasma samples of period I and period II were packed with dry ice and transferred to the analytical facility for analysis.

3.1.20 Wash-Out Period

There was a washout period of at least ten days between each study period.

3.1.21 Restrictions

3.1.21.1 Medications

Subjects did not receive any medication including vitamin preparations and over the counter medications (OTC) 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. If drug therapy other than that specified in the protocol was required during the study or in the washout period, decisions to continue or discontinue the subject was based on the following:

i) The pharmacology and pharmacokinetics of the non-study medication.

ii) The likelihood of a drug-drug interaction, thereby affecting pharmacokinetic comparison of the study medication.

iii) The time of administration of the non-study medication.

3.1.21.2 Diet

All subjects were instructed to abstain from any alcohol and grapefruit juice and or grape fruit supplements containing products for 48 hrs prior to admission and during the course of the study till last sample collection for pharmacokinetic analysis. They were also abstaining from tea, coffee, cigarette and any other any xanthine containing
Chapter 3  Materials & Methods

food or beverages during in-house stay in each period. If volunteer consumes alcohol / grapefruit juice and or grape fruit supplements or products containing alcohol or grapefruit during 48 hrs prior to admission or in the washout period, decisions to continue or discontinue the subject was based on the following:

1) The likelihood of an interaction with drug, thereby affecting pharmacokinetic comparison of the study medication.

2) The time of such consumption

3.1.21.3 Activity

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

3.1.22 Clinical Safety Measurements

3.1.22.1 Vital Signs Recording

Vital signs of sitting blood pressure, radial pulse and oral temperature were measured and recorded during admission, at -46, -38, -22, -14 hrs, pre dose (within 2.0 hrs) and at 2, 6 and 24 hrs after administration of study drug (within 2.0 hrs) in both periods of the study. In the event of detection of any abnormality during measurement of vital signs, the Principal Investigator was consulted for necessary action.

3.1.22.2 Clinical Examination

A brief clinical examination of the subjects was performed 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.

3.1.22.3 Laboratory Evaluations for Safety

Laboratory parameters of hematology and biochemistry 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.
3.1.22.4 Adverse Events

The study subjects were monitored throughout the study period for adverse events. Subjects were specifically asked about any adverse event during admission, at -46, -38, -22, -14, pre-dose, and at 2, 6, 24, 36, 48 and 72 hrs after dosing 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.

3.1.23 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.

3.1.24 Volunteer Compensation

The subjects were adequately compensated (as per Jamia Hamdard IRB approved guidelines) for the inconvenience and discomfort during their participation in the study.

3.1.25 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

The analytical methods for the determination of Atorvastatin and Metformin in human plasma were developed and validated at the Bioanalytical Section, Fortis Clinical Research Limited, Faridabad, India.

3.2.1 Estimation of Atorvastatin

The conduct of this method deals with the development of a specific LC-MS/MS procedure for determination of Atorvastatin, p-Hydroxy Atorvastatin and o-Hydroxy Atorvastatin in human plasma.

3.2.1.1 Reagents/Materials

All solvents and reagents used were of AnalR or HPLC grade. Working standards of Atorvastatin was procured from Ranbaxy Laboratories Limited, Pharma Manufacturing, Dewas, Indore and working standards of p-Hydroxy Atorvastatin, o-Hydroxy Atorvastatin, Atorvastatin D5, p-Hydroxy Atorvastatin D5, o-Hydroxy Atorvastatin D5 were procured from Varda Biotech (P) Ltd, Kartik Complex, New link Road, Andheri (W), Mumbai-400 053, India.

HLB Oasis cartridges were purchased from Waters Corporation Bangalore, India, (A subsidiary of Waters Corporation, Milford, Massachusetts, USA). The AR grade Formic acid and Orthophosphoric Acid was used and the HPLC grade Methanol and Water was used. Water was prepared using a Milli-Q system (Millipore, Mosheim Cedex, France). Drug free K3 EDTA (ethylene diamine tetraacetic acid) human plasma was procured from Yash Laboratory, shop No. 9, Solanki Appt., Louiswadi, Thane.

3.2.1.2 Instrumentation

HPLC System (Shimadzu Tokyo, Japan), LCMS - API-4000 (MDS Sciex Toronto, Canada) and Analyst software Version 1.5 (MDS Sciex Toronto, Canada) for data processing were used. The column used was Ascentis Express C18, 75 x 4.6 mm, 2.7 µm using mobile phase (Solution A: 0.1% formic acid solution- 65: 35 v/v) at a flow rate of 1ml/min. Column oven temperature was kept at 40±2 °C.
3.2.1.3 Preparation of Standard Stock Solutions

a) Atorvastatin Standard Stock Solution

Approximately 1 mg/ml of Atorvastatin working standard solution was prepared using methanol. The final concentration for Atorvastatin was corrected and accounted for its potency and the actual amount weighed. It was stored in a refrigerator (2-10 °C), protected from light.

b) p-Hydroxy Atorvastatin Standard Stock Solution

Approximately 1 mg/ml of p-Hydroxy Atorvastatin working standard solution was prepared using methanol. The final concentration for p-Hydroxy Atorvastatin was corrected and accounted for its potency and the actual amount weighed. It was stored in a refrigerator (2-10 °C), protected from light.

c) o-Hydroxy Atorvastatin Standard Stock Solution

Approximately 1 mg/ml of o-Hydroxy Atorvastatin working standard solution was prepared using methanol. The final concentration for o-Hydroxy Atorvastatin was corrected and accounted for its potency and the actual amount weighed. It was stored in a refrigerator (2-10 °C), protected from light.

d) Atorvastatin D5 Internal Standard Stock Solution (IS)

Approximately 1 mg/ml of Atorvastatin D5 working standard solution was prepared using methanol. The final concentration for Atorvastatin D5 was corrected and accounted for its potency and the actual amount weighed. It was stored in a refrigerator (2-10 °C), protected from light.

e) p-Hydroxy Atorvastatin D5 Internal Standard Stock Solution (IS)

Approximately 1 mg/ml of p-Hydroxy Atorvastatin D5 working standard solution was prepared using methanol. The final concentration for p-Hydroxy Atorvastatin D5 was corrected and accounted for its potency and the actual amount weighed. It was stored in a refrigerator (2-10 °C), protected from light.
f) o-Hydroxy Atorvastatin D5 Internal Standard Stock Solution (IS)

Approximately 1 mg/ml of o-Hydroxy Atorvastatin D5 working standard solution was prepared using methanol. The final concentration for o-Hydroxy Atorvastatin D5 was corrected and accounted for its potency and the actual amount weighed. It was stored in a refrigerator (2-10 °C), protected from light.

3.2.1.4 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.

3.2.1.5 Preparation of Solutions

a) Diluent Solution

500 ml of methanol and 500 ml of water were transferred into a 1000-ml reagent bottle. It was mixed well and degassed at ultrasonic bath. Freshly prepared solution was used daily.

b) 1% Formic Acid Solution

1 ml of formic acid was transferred into a 100-ml volumetric flask containing 50 ml of water. Volume was made up to the mark with water. It was mixed well. Freshly prepared solution was used daily.

c) 0.1% Formic Acid Solution

1 ml of formic acid was transferred into a 1000-ml volumetric flask containing 250 ml of water. Volume was made up to the mark with water. It was mixed well. Freshly prepared solution was used daily.

d) Solution A

400 ml of acetonitrile and 600 ml of methanol were transferred into a 1000-ml reagent bottle. It was mixed well and degassed at ultrasonic bath. Freshly prepared solution was used daily.
e) **Rinsing Solution**

Pure methanol was used as rinsing solution.

f) **Washing Solution**

300 ml of methanol and 700 ml of water were transferred into a 1000-ml reagent bottle. It was mixed well and degassed at ultrasonic bath. Freshly prepared solution was used daily.

**g) Reconstitution Solution**

20 ml of methanol, 20 ml of acetonitrile and 60 ml of 0.1% formic acid were transferred into a 100-ml reagent bottle. It was mixed well and degassed at ultrasonic bath. Freshly prepared solution was used daily.

h) **25 % Orthophosphoric Acid Solution**

25 ml of orthophosphoric acid was transferred into a 100-ml, of volumetric flask containing 50 ml of water. Volume was made up to the mark with water. It was mixed well. Freshly prepared solution was used daily.

3.2.1.6 **Sample Preparation**

The required number of calibration curve standards and quality control samples were withdrawn from the storage equipment (cold room/deep freezer) and allowed to thaw in ice cold water bath. Thawed samples were vortexed to ensure complete mixing of contents. 500 µL of each sample was aliquotted into micro-centrifuge tube. 50 µL of internal standard dilution (approximately 50 ng/ml of Atorvastatin D5, 65 ng/ml of p-Hydroxy Atorvastatin D5 and 45 ng/ml of o-Hydroxy Atorvastatin D5) were added to it and vortexed. 400 µL of 1% formic acid solution was added and vortexed to mix well. Till then, sample preparation was done in ice cold water bath.

The samples were centrifuged at 12000 rpm for 2 min at 5 °C. Required numbers of HLB (Ice, 30 mg) extraction cartridges were placed in solid phase extraction assembly for sample preparation. The HLB cartridges were conditioned with 1 ml of methanol followed by 1 ml of water. The vortexed samples were loaded into the cartridges and passed through the cartridges under a constant pressure. It was washed with 1 ml of washing solution followed by 1 ml of water and eluted with 1 ml of
methanol. The samples were dried on nitrogen evaporator at 50 °C, 20 psi until complete dryness. Reconstituted with 0.300 ml of reconstitution solution. 25 μL of the solution was injected into LC-MS/MS system for analysis. Sample preparation was carried out under low-light condition.

### 3.2.2 Estimation of Metformin

The conduct of this method deals with the development of a specific LC-MS/MS procedure for determination of Metformin in human plasma.

#### 3.2.2.1 Reagents/Materials

All solvents and reagents used were of AnalR or HPLC grade. The following reagents were used.

- i. Acetonitrile (HPLC Grade)
- ii. Ammonium Acetate (AR Grade)
- iii. Formic Acid (AR Grade)
- iv. Metformin (working standard)
- v. Methanol (HPLC Grade)
- vi. Ranitidine (working standard)
- vii. Water (HPLC grade)

HLB Oasis cartridges were purchased from Waters Corporation Bangalore, India, (A subsidiary of Waters Corporation, Milford, Massachusetts, USA). Water was prepared using a Milli-Q system (Millipore, Mosheim Cedex, France).

#### 3.2.2.2 Instrumentation

HPLC System (Shimadzu Tokyo, Japan), MS - API-3000 (MDS Sciex Toronto, Canada) and Analyst software Version 1.4.1 (MDS Sciex Toronto, Canada) for data processing were used.

The column used was Hypurity Advance, 50 x 4.6 mm, 5μm using 80% of acetonitrile and 20% of 5 mM ammonium acetate solution as mobile phase at a flow rate of 1.2 ml/min. Column oven temperature was kept at 35±2 °C.
3.2.2.3 Preparation of Standard Stock Solutions

a) Metformin Standard Stock Solution

Approximately 1 mg/ml of Metformin working standard solution was prepared using diluents solution. The final concentration for Metformin was corrected and accounted for its potency and the actual amount weighed. It was stored in a refrigerator (2-10 °C), protected from light.

b) Ranitidine Internal Standard Stock Solution (IS)

Approximately 1 mg/ml of ranitidine working standard solution was prepared using diluent solution. The final concentration for ranitidine was corrected and accounted for its potency and the actual amount weighed. It was stored in a refrigerator (2-10 °C), protected from light.

3.2.2.4 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.

3.2.2.5 Preparation of Solutions

a) Diluent Solution

500 ml of methanol and 500 ml of water was transferred into a 1000-ml reagent bottle. It was mixed well and degassed at ultrasonic bath. This solution was used within five days from the date of preparation.

b) Rinsing Solution

Use diluent solution as rinsing solution.

c) Mobile Phase

800 ml of acetonitrile and 200 ml of 5 mM ammonium acetate solution was transferred into a 1000-ml reagent bottle.
It was mixed well and degassed at ultrasonic bath. This solution was used within five days from the date of preparation.

d) 5 mM Ammonium Acetate Solution

385 mg of ammonium acetate was transferred into a 1000-ml volumetric flask and 100 ml of water was added to it. Sonicate to mixed well and volume was make up with water up to the mark. This solution was used within five days from the date of preparation.

e) Acidified Acetonitrile Solution

500 ml of acetonitrile and 0.5 ml of formic acid solution was transferred into a 1000 ml reagent bottle. It was mixed well and degassed at ultrasonic bath. This solution was used within five days from the date of preparation.

3.2.2.6 Sample Preparation

The required number of calibration curve standards and quality control samples were withdrawn from the cold room and thawed in ice cold water bath. Thawed samples were vortexed to ensure complete mixing of contents. 100 µL of each sample was aliquotted into micro-centrifuge tubes and 50 µL of internal standard (5000 ng/ml) solution was added to it and vortexed to mix well. 1000 µL of acidified acetonitrile solution was added and vortexed for 30 seconds. The tubes were centrifuged in a refrigerated centrifuge at 8500 rpm for 5 minutes at 5 °C. Supernatant solution was transferred into clean auto-sampler vials. 10 µL of the solution was injected for analysis. Sample preparation was done under low-light condition.
3.3 BIOANALYTICAL METHOD VALIDATION

The validation was performed in order to evaluate the method in terms of selectivity, linearity of response, sensitivity, accuracy, precision, recovery, stability, dilution integrity, matrix effect and ruggedness.

3.3.1 Method Validation for Estimation of Atorvastatin

The linearity, sensitivity, precision and accuracy evaluations were performed on three precision and accuracy batches and all of them were reported.

3.3.1.1 Selectivity

The selectivity of the method was done by checking the interference of endogenous compounds in human plasma at the retention time of the analytes (Atorvastatin, o-Hydroxy Atorvastatin, p-Hydroxy Atorvastatin) and internal standard (Atorvastatin D5, o-Hydroxy Atorvastatin D5 and p-Hydroxy Atorvastatin D5). Twelve lots of plasma with tripotassium EDTA (ethylene diamine tetra acetic acid) were used to screen. Interference at the RT of the analyte in each blank matrix was evaluated by comparing the response in the blank matrix against the mean peak area response of the extracted (LOQ) samples.

Matrix was said to be selective when interference at the retention time of analyte and internal standard was less than 20% and 5% respectively of the average area of six replicates of the spiked LOQ. At least 80% of the screened matrix should meet the above acceptance criteria.

Note: Ten calibration curves and one hundred fifty quality control samples (LOQQC, LQC, MQC and HQC) were bulk spiked and stored below -50 °C, till further analysis, using drug free K3 EDTA plasma.

3.3.1.2 Standard Curve and Linearity

Three batches of calibration curve standards were processed and analyzed to check linearity of the method. The standard curve range of the Atorvastatin, p-Hydroxy Atorvastatin and o-Hydroxy Atorvastatin were 0.103 to 200.867 ng/ml, 0.104 to 202.48 ng/ml and 0.103-202.172 respectively. The linearity of the, Atorvastatin, o-Hydroxy Atorvastatin and p-Hydroxy Atorvastatin 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).

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

(3.1)

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

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 \(1/X^2\) linear regression algorithms.

Lowest summed value was indicative of the regression algorithm (\(1/X^2\)) 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 \((1/\text{concentration}^2)\) 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:

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

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

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.

3.3.1.3 Sensitivity

Limit of quantification (LOQ) is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy under the stated experimental conditions. To determine limit of quantification, six of LOQQC replicates (concentration equal to lower standard of calibration curve) were prepared and run against a calibration curve. The precision and accuracy was calculated.
The acceptance criteria for the % nominal of mean concentration should be between 80-120% and for %CV should not be more than 20%.

3.3.1.4 Accuracy and Precision

Three precision and accuracy (PA) batches were processed and analyzed as per the standard method, each batch of spiked plasma consist of one complete calibration curve (consisting of one blank plasma, one blank plasma with internal standard and eight different non-zero concentration samples) and six replicate quality control samples (LOQQC, LQC, MQC and HQC). The range for calibration standard for Atorvastatin, p-Hydroxy Atorvastatin and o-Hydroxy Atorvastatin were from 0.103 to 200.867 ng/ml, 0.104 to 202.480 ng/ml and 0.103 to 202.172 ng/ml respectively.

For Atorvastatin, four concentrations of quality control samples were 0.0104 ng/ml (LOQQC), 0.288 ng/ml (LQC), 80.017 ng/ml (MQC) and 160.033 ng/ml (HQC), for o-Hydroxy Atorvastatin four concentrations of quality control samples were 0.104 ng/ml (LOQQC), 0.290 ng/ml (LQC), 80.430 ng/ml (MQC) and 160.859 ng/ml and for p-Hydroxy Atorvastatin four concentrations of quality control samples were 0.105 ng/ml (LOQQC), 0.291 ng/ml (LQC), 80.792 ng/ml (MQC) and 161.584 ng/ml.

The back calculated concentration of Atorvastatin, o-Hydroxy Atorvastatin and p-Hydroxy Atorvastatin for each quality control sample were calculated using linear regression parameters of the corresponding calibration curve. The mean concentration, SD, %CV (precision) and % nominal (accuracy) for each QC level of the three PA batches were calculated.

Accuracy and precision (%CV) less than or equal to 15% are considered acceptable except at LOQ QC where criteria is <20%.

3.3.1.5 Recovery

3.3.1.5.1 Recovery of Atorvastatin, p-Hydroxy Atorvastatin and o-Hydroxy Atorvastatin

The percentage recovery of Atorvastatin, p-Hydroxy Atorvastatin and o-Hydroxy Atorvastatin was determined by measuring the peak area response of spiked (extracted) quality control samples (LQC, MQC and HQC) against the peak area
response of aqueous (unextracted) quality control samples (LQC, MQC and HQC) of equivalent concentrations.

### 3.3.1.5.2 Recovery of Atorvastatin D5 (IS), p-Hydroxy Atorvastatin D5 (IS) and o-Hydroxy Atorvastatin

The percentage recovery of Atorvastatin D5 (IS), p-Hydroxy Atorvastatin D5 (IS) and o-Hydroxy Atorvastatin was determined by measuring the peak area response of spiked (extracted) quality control samples (MQC) against the peak area response of aqueous (unextracted) IS dilution of same concentration.

All the samples were analyzed as per the standard method and per cent recovery was calculated using Eq. 3.5.

\[
\% \text{ Recovery} = \frac{\text{Mean peak area of extracted QC sample}}{\text{Mean peak area of un-extracted QC samples}} \times 100 \tag{3.5}
\]

The % 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.

### 3.3.1.6 Stability

Stability was evaluated by determining following parameters:

#### 3.3.1.6.1 Freeze-Thaw stability

The stability of the spiked plasma samples was determined during three freeze-thaw cycles. For Atorvastatin, p-Hydroxy Atorvastatin and o-Hydroxy Atorvastatin four replicate numbers of LQC and HQC quality control samples were analyzed after third freeze thaw cycle. Low and high concentration quality control samples (LQC and HQC) were removed from deep freezer below -50 °C and allowed to thaw in ice cold water bath under low light condition, refrozen for 24 h under the same conditions. The freeze-thaw cycle is repeated two more times. Samples were then processed along with freshly spiked calibration curve standards and freshly spiked QC samples and analyzed by back calculation using regression equation obtained. The mean, SD,
% CV and % stability were calculated at both LQC and HQC levels. Samples were deemed stable after three freeze thaw cycles if % stability is within ± 15% of stability values and % CV is ≤ 15% at both LQC and HQC levels.

3.3.1.6.2 Bench-Top Stability

The bench top stability was determined by evaluating four replicate samples at lower and higher quality control levels. The samples were processed after keeping them at bench top (in ice cold water bath under low light condition) for about 7.52 hrs and then analyzed against freshly spiked calibration curve standards and freshly spiked QC samples. Samples were deemed stable after three freeze thaw cycles if % stability is within ± 15% of stability values and % CV is ≤ 15% at both LQC and HQC levels.

3.3.1.6.3 In-Injector Stability

For Atorvastatin, o-Hydroxy Atorvastatin and p-Hydroxy Atorvastatin, four replicates of each low and high concentration quality control samples were processed and kept in the auto-injector for 50.38 hr. Samples were then run and analyzed along with freshly spiked calibration curve standards and freshly spiked QC samples and analyzed by back calculation using regression equation obtained. The mean, SD, % CV and % stability were calculated at both LQC and HQC levels. The samples were considered to be stable for the specified in injector stability period if % stability is within ± 15% of stability values and % CV is ≤ 15% at both LQC and HQC levels.

3.3.1.6.4 Stock Solution Stability

The stock solution stability was performed by storing Atorvastatin, p-Hydroxy Atorvastatin, Atorvastatin D5 (IS), p-Hydroxy Atorvastatin D5 (IS) and o-Hydroxy Atorvastatin D5 (IS) stock solutions between 2-10 °C under low light condition. The evaluation of stability was done by assaying 6 replicate injections of appropriately prepared dilution from fresh stock solutions of Atorvastatin, p-Hydroxy Atorvastatin, o-Hydroxy Atorvastatin, Atorvastatin D5 (IS), p-Hydroxy Atorvastatin D5 (IS) and o-Hydroxy Atorvastatin D5 (IS) respectively against 6 replicate injections of appropriately prepared dilutions from stored aliquots of Atorvastatin, p-Hydroxy Atorvastatin, Atorvastatin D5 (IS), p-Hydroxy Atorvastatin D5 (IS) and o-Hydroxy Atorvastatin D5 (IS) stock solutions respectively.
The solutions were considered stable for specified period, if % stability is within the range of 85-115%.

3.3.1.6.5 Long-Term Stability

The long-term stability evaluation of Atorvastatin, p-Hydroxy Atorvastatin and o-Hydroxy Atorvastatin were performed on LQC and HQC following a storage period of three months at temperature below -50 °C, protected from light. After three months of storage period, four replicates of the stored low and high concentration quality control samples were removed from the freezer and allowed to thaw in ice cold water bath under low light condition. The samples were processed and analyzed against freshly spiked calibration curve standards and freshly spiked QC samples. The samples were considered to be stable for the specified long term stability period if % stability is within ± 15% of stability values and % CV is ≤ 15% at both LQC and HQC levels.

3.3.1.7 Dilution Integrity

Dilution integrity was assessed by assaying six replicates of quality control samples spiked with approximately two times of 90% concentration (1.8 times) of highest limit of quantification (ULOQ) and diluted by factor of two and four prior to extraction. All the samples were processed along with freshly spiked calibration standards and analyzed by back calculation using regression equation obtained. The mean, SD, % CV and % nominal were calculated for both dilutions.

The integrity of the samples were considered to be maintained if, % nominal is within ± 15% of nominal values and % CVs ≤ 15 % at both 2 times and 4 times.

3.3.1.8 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 15%.
3.3.1.9 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.

3.3.1.10 System Suitability and Carry Over

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 analytes and internal standard were carried out and % CV of areas ratio (drug/IS) and retention times less than 4% and less than 10% were acceptable respectively. For the carryover, the difference in the area of first injection and the injection after system suitability should not be more than 0.1%.

3.3.1.11 Standardization and Calculations

The chromatographic data were acquired and processed using computer based analyst software version 1.5. The best fit lines using weighted (1/Concentration\(^2\)) linear least square regression analysis were obtained by peak area ratio of Atorvastatin, o-Hydroxy Atorvastatin and p-Hydroxy Atorvastatin to internal standard Atorvastatin D5 (IS) o-Hydroxy Atorvastatin D5 (IS) and p-Hydroxy Atorvastatin D5 (IS) respectively.

The concentrations of Atorvastatin, o-Hydroxy Atorvastatin and p-Hydroxy Atorvastatin in human EDTA plasma samples were calculated using linear regression parameters by corresponding calibration curve.
3.3.2 Method Validation for Estimation of Metformin

For Metformin, the linearity, sensitivity, precision and accuracy evaluations were performed on five batches of spiked samples and four of them are reported.

3.3.2.1 Selectivity

The selectivity of the method was done by checking the interference of endogenous compounds in human plasma at the retention time of the analyte (Metformin) and internal (Ranitidine). Twelve lots of plasma with tripotassium EDTA (ethylene diamine tetra acetic acid) were used to screen. Interference at the RT of the analyte in each blank matrix was evaluated by comparing the response in the blank matrix against the mean peak area response of the extracted (LOQ) samples.

Matrix was said to be selective when interference at the retention time of analyte and internal standard was less than 20% and 5% respectively of the average area of six replicates of the spiked LOQ. At least 80% of the screened matrix should meet the above acceptance criteria.

Note: Ten calibration curves and one hundred fifty quality control samples (LOQQC, LQC, MQC and HQC) were bulk spiked and stored below -50 °C, protected from light, till further analysis, using drug free K3 EDTA plasma.

3.3.2.2 Standard Curve and Linearity

Four batches of calibration curve standards were processed and analyzed to check linearity of the method. The linearity of the Metformin was determined by weighted least square regression analysis of standard plot associated with eight point standard curve respectively. The standard curve range of the Metformin was 20.6 to 3219.5 ng/ml.

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).
\[ \left| \sum \% \text{dev} \right| + \sqrt{\left(\% \text{dev}\right)^2} \quad (3.6) \]

Where, \% dev represents the per cent deviation for each non-zero calibrant, i.e.: (Back calculated value - Stability value)/ Stability value \times 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/X^2 linear regression algorithms.

Lowest summed value was indicative of the regression algorithm (1/X^2) 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/concentration^2) regression method (Eq. 3.2)

\[ Y = m \frac{1}{X^2} + c \quad (3.7) \]

Where,

\[ Y = \text{chromatographic response in terms of peak area ratio of (analyte) / (internal standard - IS)} \]
\[ X = \text{concentration ratio of analyte / IS} \]
\[ m = \text{slope of the calibration curve} \]
\[ c = \text{Y-axis intercept at zero concentration.} \]

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 % stability (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 % Stability were calculated using Eq. 3.3 and 3.4, respectively:

\[ \% \text{ CV (Coefficient of variation)} = \frac{\text{Standard deviation}}{\text{Mean concentration}} \times 100 \quad (3.8) \]

\[ \% \text{ Stability} = \frac{\text{Mean calculated value}}{\text{Stability value}} \times 100 \quad (3.9) \]
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.

3.3.2.3 Sensitivity

Limit of quantification (LOQ) is defined as the lowest concentration of analyte that can be determined with acceptable precision and accuracy under the stated experimental conditions. To determine limit of quantification, six of LOQQC replicates (concentration equal to lower standard of calibration curve) were prepared and run against a calibration curve. The precision and accuracy was calculated.

The acceptance criteria for the % nominal of mean concentration should be between 80-120% and for %CV should not be more than 20%.

3.3.2.4 Accuracy and Precision

Precision and accuracy were calculated from the data obtained from four precision and accuracy (PA) validation batches. The range for calibration standard for Metformin were from 20.6 to 3219.5 ng/ml Metformin, four concentrations of quality control samples were 21.0 ng/ml (LOQQC), 60.0 ng/ml (LQC), 1200.2 ng/ml (MQC) and 2400.5 ng/ml (HQC). The back calculated concentration of Metformin for each quality control sample was calculated using linear regression parameters of the corresponding calibration curve. The mean concentration, SD, %CV (precision) and % nominal (accuracy) for each QC level of the three PA batches were calculated.
Accuracy and precision (%CV) less than or equal to 15% are considered acceptable except at LOQ QC where criteria is <20%.

3.3.2.5 Recovery

3.3.2.5.1 Recovery of Metformin (IS)

The percentage recovery of Metformin was determined by measuring the peak area response of spiked (extracted) quality control samples (LQC, MQC and HQC) against the peak area response of aqueous (unextracted) quality control samples (LQC, MQC and HQC) of equivalent concentrations.

3.3.2.5.2 Recovery of Ranitidine (IS)

The percentage recovery of Ranitidine (IS) was determined by measuring the peak area response of spiked (extracted) quality control samples (MQC) against the peak area response of aqueous (unextracted) IS dilution of same concentration.

All the samples were analyzed as per the standard method and per cent recovery was calculated using Eq. 3.10.

\[
% \text{ Recovery} = \frac{\text{Mean peak area of extracted QC sample}}{\text{Mean peak area of un-extracted QC samples}} \times 100 \quad (3.10)
\]

The % 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.

3.3.2.6 Stability

Stability was evaluated by determining following parameters:

3.3.2.6.1 Freeze-Thaw Stability

For Metformin six replicate numbers of LQC and HQC samples (stability samples) were analyzed after third freeze thaw cycle against freshly spiked calibration curve standards and freshly spiked QC samples (comparison samples). Low and high concentration quality control samples (LQC and HQC) were removed from deep freezer below -50ºC and allowed to thaw in ice cold water bath under low light.
condition. Samples were deemed stable after three freeze thaw cycles if % stability is within ± 15% of stability values and % CV is ≤ 15% at both LQC and HQC levels.

### 3.3.2.6.2 Bench Top Stability

The bench-top stability (Short-term stability in matrix) determined by analyzing six replicate of low and high QC stability samples, which had been kept at room temperature for a designated time (15.62 hrs) against the freshly spiked calibration curve standards and freshly spiked QC samples (comparison samples). Samples were deemed stable after three freeze thaw cycles if % stability is within ± 15% of stability values and % CV is ≤ 15% at both LQC and HQC levels.

### 3.3.2.6.3 In-Injector Stability

For Metformin six replicates of each low and high concentration quality control samples were processed and kept in the auto-injector for 43.80 hr. Samples were then run and analyzed along with freshly spiked calibration curve standards and freshly spiked QC samples and analyzed by back calculation using regression equation obtained. The samples were considered to be stable for the specified in injector stability period if % stability is within ±15% of stability values and % CV is ≤ 15% at both LQC and HQC levels.

### 3.3.2.6.4 Stock Solution Stability

The stock solution stability was performed by storing Metformin and Ranitidine (IS) stock solutions between 2-10 °C under low light condition. The evaluation of stability was done by assaying 6 replicate injections of appropriately prepared dilution from fresh stock solutions of Metformin and Ranitidine (IS) respectively against 6 replicate injections of appropriately prepared dilutions from stored aliquots of Metformin and Ranitidine (IS).

The solutions were considered stable for specified period; if % stability is within the range of 85-115%.

### 3.3.2.6.5 Long-Term Stability

The long-term stability evaluation of Metformin was performed on LQC and HQC following a storage period of 50 days at temperature below -50 °C with K3 EDTA as
an anticoagulant. After 50 days of storage period, six replicates of the stored low and high concentration quality control samples were removed from the freezer and allowed to thaw in ice cold water bath under low light condition. The samples were processed and analyzed against freshly spiked calibration curve standards and freshly spiked QC samples. The samples were considered to be stable for the specified long term stability period if % stability is within ± 15% of stability values and % CV is ≤ 15% at both LQC and HQC levels.

3.3.2.7 Dilution Integrity

Dilution integrity was assessed by assaying six replicates of quality control samples spiked with approximately two times of 90% concentration (1.8 times) of highest limit of quantification (ULOQ) and diluted by factor of two and four prior to extraction. All the samples were processed along with freshly spiked calibration curve standards and freshly spiked QC samples and analyzed by back calculation using regression equation obtained.

The integrity of the samples were considered to be maintained if, % nominal is within ± 15% of nominal values and % CVs ≤ 15% at both 2 times and 4 times.

3.3.2.8 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 15%.

3.3.2.9 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.

### 3.3.2.10 System Suitability and Carry Over

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 analytes and internal standard were carried out and % CV of areas ratio (drug/IS) and retention times less than 4% and less than 10% were acceptable respectively. For the carryover, the difference in the area of first injection and the injection after system suitability should not be more than 0.1%.

### 3.3.2.11 Standardization and Calculations

The chromatographic data were acquired and processed using computer based analyst software version 1.4.1. The best fit lines using weighted (1/Concentration^2) linear least square regression analysis were obtained by peak area ratio of Metformin to internal standard (Ranitidine).

The concentrations of Metformin in plasma samples were calculated using linear regression parameters by corresponding calibration curve.
3.4 ANALYSIS OF PLASMA SAMPLES

The present study deals with the analysis of plasma samples using a validated LC-MS/MS method for the determination of Atorvastatin and Metformin.

3.4.1 Sample Collection and Storage

The blood samples for the periods I and II were collected from February 26, 2010 to March 12, 2010. Blood samples from each period were centrifuged to separate plasma and stored below -50°C in a deep freezer at the clinical facility on their respective date of collection.

All blood samples All aliquot 1, 2, 3 & 4 of plasma samples of period I and period II (in frozen condition) were packed with dry ice and transferred to the analytical facility on April 1, 2010, and stored below -50°C until analysis.

3.4.2 Sample Analysis

As per the protocol a total number of 2160 samples were to be collected from 40 subjects during the study. However, subject numbers 05, 08, 16 and 25 who were withdrawn from the study, subject numbers 07 and 24 who dropped out from the study and subject numbers 09, 19, 24 and 26 who did not report for some of ambulatory visits. A total number of 1900 subject samples were collected and 1833 samples were analyzed from 34 subjects who completed the all period of study.

<table>
<thead>
<tr>
<th>Samples to be Collected:</th>
<th>2160 subject samples from 40 subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects Recruited:</td>
<td>40 subjects</td>
</tr>
<tr>
<td>Completed Subjects:</td>
<td>34 subjects</td>
</tr>
<tr>
<td>Samples Collected:</td>
<td>1900 subject samples</td>
</tr>
<tr>
<td>Analyzable Subject Samples:</td>
<td>1833 [(34 subjects completed study x 27 time points x 2 periods) - 3 missing sample]</td>
</tr>
</tbody>
</table>

Samples were analyzed subject wise using one analytical batch at a time which consisted of an aqueous reference standard dilution, eight non-zero calibration standards (lower and higher standards were taken as duplicates), blank plasma in duplicate, blank plasma with internal standard in duplicate.
For analysis of Atorvastatin, a set of eight non-zero calibration standards and quality control samples were prepared on May 04, 2010 and stored at a temperature below -50 °C in deep freezer of sample storage area at the Bioanalytical Section, Fortis Clinical Research Limited, Faridabad, Haryana, India. The calibration standard range was 0.104 to 199.791 ng/ml. All samples of one subject with two sets of quality control samples (LQC, M1QC, MQC and HQC with concentrations of 0.289 ng/ml, 40.131 ng/ml, 80.262 ng/ml and 160.523 ng/ml, respectively) interspersed between subject samples.

For analysis of Metformin, a set of eight non-zero calibration standards and quality control samples were prepared on April 17, 2010 and stored at a temperature below -50 °C in deep freezer of sample storage area at the Bioanalytical Section, Fortis Clinical Research Limited, Faridabad, Haryana, India. The calibration standard range was 20.7 to 3206.8 ng/ml. All samples of one subject with two sets of quality control samples (LQC, M1QC, MQC and HQC with concentrations of 61.4 ng/ml, 639.4 ng/ml, 1278.8 ng/ml and 2557.6 ng/ml, respectively) interspersed between subject samples.

3.4.3 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.

a) 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.
b) 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 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.

c) 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 Incurred Sample Reanalysis (ISR)

Incurred sample reanalysis was done for both Atorvastatin and Metformin. The order of selection of samples from all the subjects of the study was determined according to a SAS-generated randomization schedule.

\[
\text{% Difference} = \frac{\text{Repeat Value - Initial Value}}{\frac{\text{Repeat Value} + \text{Initial Value}}{2}} \times 100
\]

The incurred sample reanalysis is deemed acceptable if % difference of 67% of incurred sample is within 20% of its original value.

3.4.5 Repeat Analysis

Samples were subjected to repeat analysis using following criteria.

1. Analytical batch fails the batch acceptance criteria.

2. Bad chromatography is observed.

3. Internal standard variation (<40% and more than 180% of mean IS response of standards) is observed.

4. Sample is lost during 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 all pharmacokinetic parameters were calculated for Atorvastatin and Metformin except AUC0-24 only calculated for Metformin, using WinNonlin Node version 5.0.1 from Pharsight:

\[ \text{AUC}_{0-t} : \] The area under the plasma concentration versus time curve, from time zero to the last measurable concentration, as calculated by the linear trapezoidal method.

\[ \text{AUC}_{0-24} : \] The area under the plasma concentration versus time curve, from time zero to 24 h.

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

\[ \text{AUC} \%\text{Extrap}: \] 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 \[ \left( \frac{\text{AUC}_{0-\infty} - \text{AUC}_{0-t}}{\text{AUC}_{0-\infty}} \right) \times 100 \]

\[ C_{\text{max}} : \] 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 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.

### 3.5.2 Statistical Analyses

Statistical analysis was performed on plasma Atorvastatin and Metformin using the SAS system for Windows, release 9.1.3 or above (SAS Institute Inc., USA) or the WinNonlin PK Software, Version 5.0.1 or above. The analysis included the data from all subjects who has completed the study.

#### 3.5.2.1 Descriptive Statistics

Arithmetic means, standard deviations and coefficients of variation were calculated. Additionally, geometric means and percentage coefficient of variation of geometric means were calculated for $C_{\text{max}}$, $\text{AUC}_{0-t}$, $\text{AUC}_{0-24}$ and $\text{AUC}_{0-\infty}$.

#### 3.5.2.2 Analyses of Variance (ANOVA)

The log-transformed pharmacokinetic parameters ($C_{\text{max}}$, $\text{AUC}_{0-t}$, $\text{AUC}_{0-24}$ and $\text{AUC}_{0-\infty}$) 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 was 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 and the WinNonlin PK Software, Version 5.0.1.

#### 3.5.2.3 Ratio Analysis and Confidence Intervals

90% confidence interval for the ratio of the test and reference product averages (least square means) were calculated for Atorvastatin (for log-transformed $C_{\text{max}}$, $\text{AUC}_{0-t}$, $\text{AUC}_{0-t}$, $\text{AUC}_{0-24}$ and $\text{AUC}_{0-\infty}$).
and AUC\(_{0-\infty}\) and Metformin (for log-transformed \(C_{\text{max}},\ AUC_{0-t},\ AUC_{0-24}\) and \(AUC_{0-\infty}\)) by first calculating the 90% confidence interval for the differences in the averages (arithmetic means) of the log-transformed data and then taking the antilog of the obtained confidence limits. The comparison of interest is T vs. R, so the ratios were of the form of Test/Reference. Ratio of means was calculated using the LSM for log transformed \(C_{\text{max}},\ AUC_{0-t},\ AUC_{0-24}\) and \(AUC_{0-\infty}\). Ratio of means was expressed as a percentage of the LSM for the reference formulations.

### 3.5.2.4 Intrasubject Variability

For Atorvastatin, the intrasubject variability for the \(AUC_{0-t},\ AUC_{0-\infty}\) and \(C_{\text{max}}\) pharmacokinetic parameters, which reflects the residue variability after accounting for differences within subjects, periods and formulations, was derived from the analyses of the ln-transformed data.

For Metformin, the intrasubject variability for the \(AUC_{0-t},\ AUC_{0-24},\ AUC_{0-\infty}\) and \(C_{\text{max}}\) pharmacokinetic parameters, which reflects the residue variability after accounting for differences within subjects, periods and formulations, was derived from the analyses of the ln-transformed data.

### 3.5.2.5 Intersubject Variability

For Atorvastatin, the intersubject variability for the \(AUC_{0-t},\ AUC_{0-\infty}\) and \(C_{\text{max}}\) pharmacokinetic parameters, which reflects the residue variability after accounting for differences between subjects, periods and formulations, was derived from the analyses of the ln-transformed data.

For Metformin, the intersubject variability for the \(AUC_{0-t},\ AUC_{0-24},\ AUC_{0-\infty}\) and \(C_{\text{max}}\) pharmacokinetic parameters, which reflects the residue variability after accounting for differences between subjects, periods and formulations, was derived from the analyses of the ln-transformed data.

### 3.5.2.6 Power

For Atorvastatin, power of the test is defined as the probability of correctly concluding bioequivalence i.e. the ratio of the test and reference product averages lying between 80% to 125% at the 5% level of significance for \(AUC_{0-t}\) and \(AUC_{0-\infty}\), and between 75% to 133% at the 5% level of significance for \(C_{\text{max}}\). Power
calculation was performed on log transformed pharmacokinetic parameters $C_{\text{max}}$, $\text{AUC}_{0-1}$, and $\text{AUC}_{0-\infty}$.

For Metformin, Power of the test is defined as the probability of correctly concluding bioequivalence i.e. the ratio of the test and reference product averages lying between 80% to 125% at the 5% level of significance for, $\text{AUC}_{0-1}$, $\text{AUC}_{0-24}$, $\text{AUC}_{0-\infty}$ and $C_{\text{max}}$.

Power calculation was performed on log transformed pharmacokinetic parameters $\text{AUC}_{0-1}$, $\text{AUC}_{0-24}$, $\text{AUC}_{0-\infty}$, and $C_{\text{max}}$

### 3.5.2.7 Formulae

The following formulae were used for the ratio of means, 90% Confidence Interval and intra-subject variability calculations derived from the ANOVA on the In-transformed pharmacokinetic parameters.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio of Means</td>
<td>$100 \times e^{(\text{LSM}_T - \text{LSM}_R)}$</td>
</tr>
<tr>
<td>90% Confidence Interval</td>
<td>$100 \times e^{(\text{LSM}<em>T - \text{LSM}<em>R) \pm t</em>{df, 0.05} \times \text{SE}</em>{T-R}}$</td>
</tr>
<tr>
<td>Intrasubject CV %</td>
<td>$100 \times \sqrt{\frac{\text{MSE}}{1}}$</td>
</tr>
<tr>
<td>Intersubject CV %</td>
<td>$100 \times \sqrt{\frac{\text{MS}_{\text{subject(seq)}} - \text{MSE}}{2}}$</td>
</tr>
<tr>
<td>Power Calculation</td>
<td>$100 \times \text{prob}(\ln(1.25)/ \text{SE}<em>{T-R} - t</em>{df, 0.05} &gt; t_{df})$</td>
</tr>
</tbody>
</table>

Note: $t_{df}$ alpha is the value of the student’s t-distribution with df degrees of freedom (i.e. degrees of freedom for the error term from the analyses of variance) and a right-tail fractional area of alpha.

$\text{LSM}_T$ and $\text{LSM}_R$ are the least-squares mean of the test and reference formulation, respectively, as computed by the LSMEANS statement of the SAS® GLM procedure. $\text{MSE}$ is the mean square error from the analyses of variance.

$\text{SE}_{T,R}$ is the standard error of the difference between the adjusted formulation means, as computed by the estimate statement in the SAS® GLM procedure. For a balanced study, $\text{SE}_{T,R}$ is equal to the square root of $2 \times \text{MSE}/n$, where n is the number of subjects.