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 the department of Clinical Pharmacology and Pharmacokinetics, Ranbaxy Research Laboratory, Gurgaon, India. These are described under five parts:

1. Clinical study methodology
2. Bioanalytical method development
3. Bioanalytical method validation
4. Analysis of clinical study samples
5. Pharmacokinetic and Statistical analysis

3.1 Clinical Study Methodology

3.1.1 Objective

To determine and compare the rate and extent of absorption of esomeprazole between the test formulation, Esomeprazole magnesium for delayed release oral suspension (containing esomeprazole 40 mg) of Ranbaxy Laboratories Limited, India and the reference formulation, NEXIUM® 40 mg (esomeprazole magnesium) for delayed-release oral suspension (containing esomeprazole 40 mg) of AstraZeneca, LP USA in healthy, adult, human male subjects under fasting condition.

3.1.2 Study Design

The study was conducted as an open label, balanced, randomized, two-treatment, two-period, two-sequence, single dose, crossover, bioavailability study comparing esomeprazole magnesium for delayed release oral suspension 40 mg of Ranbaxy Laboratories Limited with NEXIUM® 40 mg for delayed-release oral suspension of AstraZeneca LP, in healthy, adult, human male subjects under fasting condition. Subjects served as their own control in this study.

3.1.3 Number of Subjects

Eighteen (18) healthy, adult, human, male subjects, fulfilling the inclusion and
exclusion criteria as mentioned in the protocol were enrolled in the study.

### 3.1.4 Selection of Subjects

An adequate number of subjects were selected from the volunteer bank of Clinical Pharmacology Unit, who underwent a standardized screening procedure.

#### 3.1.4.1 Screening Assessments

Subjects were judged to be medically healthy based on their medical history and demographic data (which included age, sex, body weight and height), 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 the drugs of abuse.

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.

The screening procedures involved the following laboratory tests:

**Table 3.1 List of laboratory test used for screening of volunteers:**

<table>
<thead>
<tr>
<th>HEMATOLOGY</th>
<th>BIOCHEMISTRY</th>
<th>URINALYSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin</td>
<td>BUN</td>
<td><strong>Routine Examination</strong></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>HIV I &amp; II</td>
<td>AST</td>
<td>- Specific gravity</td>
</tr>
<tr>
<td>HBsAg</td>
<td>ALT</td>
<td>- Protein</td>
</tr>
<tr>
<td>HCV</td>
<td>Glucose</td>
<td>- Glucose</td>
</tr>
<tr>
<td>VDRL</td>
<td>Cholesterol</td>
<td><strong>Microscopic Examination</strong></td>
</tr>
<tr>
<td>OTHER</td>
<td><strong>Urine drug screen</strong></td>
<td>- RBC</td>
</tr>
<tr>
<td>ECG</td>
<td>- Cannabinoids</td>
<td>- WBC</td>
</tr>
<tr>
<td>Chest X Ray</td>
<td>- Opioids</td>
<td>- Epithelial Cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Crystals</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- Casts</td>
</tr>
</tbody>
</table>
Eighteen (18) medically healthy subjects, with clinically normal laboratory profiles, were enrolled in the study who met the following inclusion and exclusion criteria.

### 3.1.4.2 Inclusion Criteria

- Be in the age range of 18-45 years.
- Be neither overweight nor underweight for his 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 21 days prior to the commencement of the study.

### 3.1.4.3 Exclusion Criteria

- Subject has history of hypersensitivity to esomeprazole or any other related drug.
- Recent history of diarrhea, nausea, vomiting or abdominal pain, respiratory tract infection.
- History of recurrent headache.
- Subject has history of drug induced pruritis and/or rash.
- Subject has any evidence of organ dysfunction or any clinically significant deviation from the normal, in physical or clinical determinations.
- Subject has laboratory test parameter(s), as per Section 12.1.1, which is/are outside acceptable limits and is judged clinically significant.
- Subject has 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.
- Subject has inability to communicate well (i.e. language problem, poor mental development, psychiatric illness or poor cerebral function) that may
impair the ability to provide, written informed consent.

- Subject is a regular smoker, who smokes more than 10 cigarettes daily or has difficulty abstaining from smoking for the duration of each study period.
- Use of Grapefruit juice and or grape fruit supplements containing products for 48 hours prior to admission.
- Subject has history of drug dependence or excessive alcohol intake on a habitual basis or has difficulty in abstaining for the duration of each study period or has consumed alcohol 48 hours prior to admission.
- Subject has used an enzyme modifying drugs within 30 days prior to admission of this study.
- Subject has 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).
- Subject has donated and/or lost more than 350 mL of blood in the past 3 months, including blood loss in this study.

### 3.1.5 Treatments

A single oral dose of either test (T) or reference (R) formulation was administered with 240 mL of water at ambient temperature under the supervision of trained study personnel during each period of the study. All subjects were required to fast overnight for at least 10 hours before dose and for 4 hours post-dose during each period of the study.

- **Test (T)**
  Esomeprazole magnesium for delayed release oral suspension (containing esomeprazole 40 mg) of Ranbaxy Laboratories Limited, India.

- **Reference (R)**
  NEXIUM® 40 mg (esomeprazole magnesium) for delayed-release oral suspension (containing esomeprazole 40 mg) manufactured by AstraZeneca AB, Sweden and distributed by AstraZeneca, LP USA.
Both formulations were powder for oral suspension.

**Method for suspension preparation and administration:**

The contents (powder for oral suspension) were emptied into 15 mL of water. It was stirred & left for 5 minutes to thicken. It was stirred and provided to volunteers for drinking within 30 minutes. The dosing cup was then rinsed with about 10 mL of water at least twice and subjects were asked to swallow the rinsing. The subjects were then asked to rinse their mouth with about 40 mL of water and remaining drinking water from 240 mL was administered at an ambient temperature under supervision of trained study personnel. Both test (T) or reference (R) products were administered to all the study subjects.

3.1.6 **Washout Period**

There was a washout period of seven (07) days between the administrations of study drugs between the two periods.

3.1.7 **Assignment to Treatment Sequences**

The order of receiving the test (T) or reference (R) products for each subject during both the periods of the study was determined according to a SAS-generated randomization schedule (Annexure V). The randomization was balanced and the code was kept under controlled access in the drug store. The randomization schedule was generated by the Statistical Services Department of CPP using SAS software Version 9.1.3 (SAS Institute Inc., USA).

3.1.8 **Assessment of Compliance**

Compliance to the treatment was assessed by conducting a thorough examination of the oral cavity by the trained study personnel after dosing in each period. The final confirmation of compliance was done by the measurement of plasma esomeprazole during the analytical phase of the study.

3.1.9 **Fasting/Meals**

All subjects were required to fast overnight after admission for at least 10 hours.
They received standard meals – lunch, snacks and dinner 4, 9 and 13 hours, respectively, after drug administration. During housing, all meal plans were identical for both the 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. Drinking water was not allowed from 1 hour before dosing until 2 hours post-dose during their in-house stay. Thereafter, it was allowed at all times.

### 3.1.10 Sampling Schedule

#### 3.1.10.1 Pharmacokinetic analysis

A total of fifty (50) 4-mL blood samples were collected from each subject in CPDA vacutainers during the course of the study through an indwelling cannulae placed in forearm veins. The blood samples were collected at predose (in duplicate) and at 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 2.75, 3.00, 3.25, 3.50, 3.75, 4.00, 4.50, 5.00, 6.00, 8.00, 10.00, 12.00, 14.00 and 18.00 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 generally within 2 minutes of the scheduled time. The actual end-point time of collection of each blood sample were recorded.

For any subject who completed both periods of the study, the total number of blood samples collected during the study was 52. A total of 900 blood samples were collected for the estimation of esomeprazole in plasma.

All the collected blood samples in collection tubes were transferred by one of the study personnel 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. 50 µL of 0.5 ±0.05 M Sodium Carbonate buffer (pH 11.8 ± 0.1) was added to 950 µL of plasma sample and vortexed before storing the samples in deep freezer. In case of less or more plasma volume, buffer was added in the ratio of 95:5 V/V (plasma: buffer). All plasma samples were divided into 2 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, pending transfer to the analytical facility for assay.

3.1.11 Admission and Stay

Subjects were admitted and housed in the Clinical Pharmacology Unit from at least 12 hours before dose administration and were discharged 24 hours after administration of the test and reference products during each period.

3.1.12 Schedule of Study:

- For period I, 18 subjects were admitted on 13 November 2009 and were housed in the Clinical Pharmacology Unit. They were discharged on 15 November 2009.
- For period II, 18 subjects were admitted on 20 November 2009 and were housed in the Clinical Pharmacology Unit. They were discharged on 22 November 2009.

3.1.13 Restrictions

3.1.13.1 Medications

All subjects were instructed not to take any other medications including vitamins and OTC during the 30 days period prior to the onset of the study.

3.1.13.2 Diet

All subjects were instructed to abstain from alcohol/ grapefruit juice and / or grape fruit supplements containing products for 48 hours prior to admission and during the course of the study till last sample collection for pharmacokinetic analysis. They also abstain from tea, coffee, cigarette and any other xanthine containing food or beverages during in-house stay in 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 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

Medical History

Complete medical history including the past or present cardiovascular, respiratory, musculoskeletal and connective tissue, gastrointestinal, renal, hepatic, genitourinary, neurological, endocrine, psychiatric, lymphatic, dermatological, haematological, metabolic, immune, drug, and surgical history or any other diseases or disorders, was recorded.

A complete medical history was recorded at screening and was updated at clinical examinations during the study.

Vital Signs

Sitting blood pressure, pulse rate and oral temperature were measured and recorded at admission, prior to dosing and at 2, 6 and 24 hours after administration of study drug in each period.

Clinical Examination

The clinical examination of the subjects was performed by a physician and included the following:

General appearance, head, ears, eyes, nose, throat, neck, skin, cardiovascular system, respiratory system, abdominal system, central nervous system including tests for co-ordination of the upper and lower limb, tremor and involuntary movement and others if any.

Clinical examination with medical history was performed at screening and a brief clinical examination was done on admission and at discharge.

Clinical Adverse Events
The study subjects were monitored throughout the study period for adverse
events. Subjects were specifically asked about any adverse event at admission,
prior to dosing and at 2, 6 and 24 hours after administration of study drug in
each period.

The clinical investigator or a medically qualified designate was available at the
study centre throughout the study period. A complete resuscitation facility
inclusive of DC defibrillator and endotracheal intubation was available at the
study centre.

3.1.14.2 Laboratory Evaluations for Safety

Laboratory parameters of hematology and biochemistry were repeated at the end
of the study as per the in-house Standard Operating Procedures. Any laboratory
parameter outside acceptable limits (Appendix IV) were termed as laboratory
abnormality and followed up until the results are normal/clinically not significant.

3.1.15 Ethical Considerations

3.1.15.1 Basic Principles

This research was carried out in accordance with the Basic Principles defined in
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.

3.1.15.2 Institutional Review Board

This protocol and the corresponding informed consent form (ICF) used to obtain
informed consent of study subjects were reviewed by the Jamia Hamdard
Institutional Review Board and the study subjects were enrolled only after the
Board had approved the protocol and the ICF, as submitted. The Board is
constituted and operates in accordance with the Principles and requirements
3.1.15.3 Informed Consent

Informed consent was obtained from all the subjects for screening medically 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, who were enrolled into the study, provided formal written informed consent after attending an oral presentation and after reading the version 01 of the informed consent form (ICF).

3.1.16 Drop-out / Withdrawal of Subjects from Study

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:

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

(ii) The subject experienced adverse event, when withdrawal would be in the best interest of the subject.

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

(iv) The subject requires concomitant medications which may interfere with the pharmacokinetics of investigational product.

(v) The subject who experienced vomiting at any time during sample collection schedule.

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. The compensation in this study was Rs. Four thousand two hundred (Rs.4200/-) per completed subject.

### 3.1.18 Study Documentation

All data generated during the conduct of the study was directly entered in the raw data recording forms as governed by the SOPs of Department of Clinical Pharmacology & Development and Department of Clinical Pharmacology and Pharmacokinetics, Ranbaxy Research Laboratories 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 records. The computer-generated chromatograms will also be 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 Clinical Investigator or his designate Research Scientist for clinical data and the Laboratory Supervisor for the bioanalytical data.

### 3.2 BIOANALYTICAL METHOD DEVELOPMENT

High Performance Liquid Chromatography tandem Mass Spectrometric Method for the determination of Esomeprazole in Human CPDA Plasma using Pantoprazole as Internal Standard. was developed and validated at the Department of Clinical Pharmacology and Pharmacokinetics, Ranbaxy Research Laboratories, Gurgaon, Haryana-122015, India. Sample preparation process was accomplished by liquid-liquid extraction method. The processed samples were chromatographed on Discovery C18 (50 X 4.6mm, 5μ), column-using Acetonitrile :Buffer-2 (90:10, v/v) as mobile phase. Esomeprazole and Pantoprazole (ISTD) were detected by MS/MS detection. Signals from the detector were captured in a computer and processed using Analyst software.

#### 3.2.1 Instrumentation & Reagents

#### 3.2.1.1 Instrumentation

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

3.2.1.2 Reagents

1. Acetic acid Glacial (ExcelaR)
2. Acetonitrile (HPLC grade)
3. Ammonium acetate (AR grade)
4. Esomeprazole (Working Standard)
5. Ethyl Acetate (HPLC grade)
6. Liquor Ammonia Solution (About 25% NH3)
7. Methanol (HPLC grade)
8. Pantoprazole (Working Standard)
9. Sodium Carbonate (Analytical grade)
10. Water (HPLC grade)

3.2.2 Chromatographic and Mass Spectrometric Conditions

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

- **Column**: Discovery C18, 50 X 4.6mm, 5μ
- **Mobile Phase**: Acetonitrile : Buffer-2 :: 90:10, v/v
- **Flow Rate**: 0.7 mL/minute
- **Detection**: Esomeprazole m/z 346.2 (parent) and 198.2 (product)
  - Pantoprazole m/z 384.1 (parent) and 200.1 (product)
- **Sample Cooler Temperature**: 10°C ± 1.0°C
- **Column Oven Temperature**: 35°C ± 1.0°C
- **Injection Volume**: 10 μL
- **Retention Time**: Esomeprazole: 0.6 to 1.4 minutes
  - Pantoprazole: 0.6 to 1.4 minutes
- **Ion source**: Turbo-ion spray in Positive Ion mode
API – 3200 (MS-15)

Source/Gas

Curtain Gas (CUR) : 40#

Collision Gas (CAD) : 3#
Ion spray voltage (IS) : 5500 V
Temperature (TEM) : 5500C
Ion Source Gas 1 (GS1) : 50#
Ion Source Gas 2 (GS2) : 50#

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

Compound parameters:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Esomeprazole</th>
<th>Pantoprazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declustering Potential - DP</td>
<td>25 V</td>
<td>33 V</td>
</tr>
<tr>
<td>Entrance Potential - EP</td>
<td>7 V</td>
<td>7 V</td>
</tr>
<tr>
<td>Collision Energy - CE</td>
<td>18 V</td>
<td>15 V</td>
</tr>
<tr>
<td>Collision cell Exit Potential - CXP</td>
<td>3 V</td>
<td>3 V</td>
</tr>
</tbody>
</table>

Resolution Q1: Unit
Resolution Q3: Unit

3.2.3 Biological Matrix

Human plasma, using CPDA as anticoagulant was chromatographically screened for interfering substances prior to use. Human plasma batches, free of significant interference, were used to prepare calibration curve standards and quality control samples.

3.2.4 Sample Preparation

- Calibration curve standards and quality control samples were withdrawn
from the cold room / freezer.

• Samples were allowed to thaw at room temperature.
• The thawed samples were vortexed to ensure complete mixing of contents.
• 50 µL of internal standard dilution (5000.0 ng/mL) was added into stopper tube.
• 100 µL of each sample was added into these stopper tubes and vortexed.
• 4 ml of Ethyl Acetate was then added and the samples were shaken on a shaker at 100 RPM for 10 min.
• After shaking, the samples were centrifuged at 3000 RPM for 5 minutes.
• 3 mL of the upper organic layer was pipetted out into test tubes and dried at 50 degree C ± 3 degree C and at about 15 psi.
• The dried residue was reconstituted with 500 µl of Reconstitution Solution.
• Samples were transferred to vials for analysis.

Note:
1. Centrifuge was run at temperature 2-10°C.
2. All the samples were processed under low UV light.

3.2.5 Preparations of Solutions

3.2.5.1 Buffer-1

0.7708 ± 0.07 gm of ammonium acetate was weighed and transferred into a reagent bottle. 1000 mL of HPLC-grade water was added to it and it was dissolved by sonicating in an ultrasonic bath to get approximately 10 mM ± 1.0 ammonium acetate buffer. The pH was 6.67 ± 0.20. This solution was stored at room temperature and used within 5 days from the date of its preparation.

3.2.5.2 Buffer-2

Required volume of Buffer-1 was transferred into a reagent bottle and the pH was adjusted with glacial acetic acid to 5.0 ± 0.2. It was sonicated in an ultrasonic bath. This solution was stored at room temperature and used within 5 days from the date of its preparation.

3.2.5.3 Buffer-3
Required volume of Buffer-1 was transferred into a reagent bottle and the pH was adjusted with liquor ammonia solution (about 25% NH3) to $8.0 \pm 0.2$. It was sonicated in an ultrasonic bath. This solution was stored at room temperature and used within 5 days from the date of its preparation.

### 3.2.5.4 Buffer-4

$10.59 \pm 0.1$ gm of sodium carbonate was transferred into a reagent bottle and 200 mL HPLC grade water was added to get approximately 0.5 M sodium carbonate solution. It was sonicated in an ultrasonic bath. The pH was recorded and was $11.8 \pm 0.1$. This solution was stored at room temperature and used within 5 days from the date of its preparation.

### 3.2.5.5 Reconstitution Solution

100 mL of Buffer-3 was transferred into a reagent bottle, 900 mL of HPLC grade acetonitrile was added to it and mixed well. It was sonicated and degassed in an ultrasonic bath. This solution was stored at room temperature and used within 3 days from the date of its preparation.

### 3.2.5.6 Mobile phase

100 mL of Buffer-2 was transferred into a reagent bottle. 900 mL of HPLC grade acetonitrile was added and mixed well. It was sonicated and degassed in an ultrasonic bath. This solution was stored at room temperature and used within 3 days from the date of its preparation.

### 3.2.5.7 Diluent-1

500 mL of methanol was transferred into reagent bottle. 500 mL of HPLC-Grade water was added to it. It was mixed well, sonicated and degassed in an ultrasonic bath. This solution was stored at room temperature and used within 7 days from the date of its preparation.

### 3.2.5.8 Rinsing Solution

800 mL of acetonitrile was transferred into a reagent bottle and 200 mL of HPLC
grade water was added to it. It was mixed well, sonicated and degassed in an ultrasonic bath. This solution was stored at room temperature and used within 7 days from the date of its preparation.

### 3.2.6 Preparation of Stock Solutions

#### 3.2.6.1 Preparation of Esomeprazole Standard Stock Solution

- Esomeprazole working standard was weighed accurately and transferred into a volumetric flask.
- It was dissolved in Methanol and the volume was made up with the same to prepare a solution of approximately 1 mg/mL.
- The final concentration of Esomeprazole was corrected accounting for its potency and the actual amount weighed.
- This solution was stored (protected from light) in refrigerator between 1-10°C and was used within 7 days from the date of its preparation.

#### 3.2.6.2 Preparation of Pantoprazole Internal Standard Stock Solution

- Pantoprazole working standard was weighed accurately and transferred into a volumetric flask.
- It was dissolved in Methanol and the volume was made up with the same to prepare a solution of approximately 1 mg/mL.
- The final concentration of Pantoprazole was corrected accounting for its potency and the actual amount weighed.
- This solution was stored (protected from light) in refrigerator between 1-10°C and was used within 7 days from the date of its preparation.

#### 3.2.6.3 Reference Standards

Esomeprazole Magnesium (Batch No. EMWS-08-007; Purity: 92.82% w/w on as is basis; Expiry Date: Feb 2010) was sourced by Varda Biotech. Pantoprazole Sodium Sesquihydrate (Batch No. 1830414; Purity: 93.0% w/w on as is basis; Revalidation (Date: 21 Jun 2009) was sourced by Ranbaxy Laboratories Limited, Dewas.
3.3 BIOANALYTICAL METHOD VALIDATION

The validation of this procedure was performed in order to evaluate the method in terms of selectivity, sensitivity, linearity of response, accuracy, precision, recovery, stability, dilution integrity, matrix effect, matrix factor and ruggedness in human plasma for Esomeprazole. The sensitivity, linearity, precision and accuracy evaluations were performed on three batches of spiked samples. Each batch of spiked plasma samples included one complete calibration curve (consisting of two blank plasma, two blank plasma with internal standard and eight different non-zero concentrations) and six replicate of quality control samples at LOQQC, LQC, MQC and HQC levels.

3.3.1 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/Concentration²) linear least square regression analysis were obtained by peak area ratio of Esomeprazole to internal standard. Weighting factor (1/Concentration²) was selected after weighting factor evaluation using three Precision and Accuracy batches (with weighting none, 1/Concentration, 1/Concentration²). The concentrations of Esomeprazole in plasma samples were calculated using linear regression parameters by corresponding calibration curve.

3.3.2 System Suitability Test

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

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

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

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

At least 80% of normal matrix batches should meet the following acceptance criteria.

**Acceptance Criteria**

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

### 3.3.4 Sensitivity

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

\[
\frac{S_N \text{ of LOQ sample}}{\text{Mean of } S_N \text{ of normal blanks}} \geq 5
\]

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

3.3.5 Weighing Factor Optimization

Three standard curves were used for weighing factor optimization. Three weighing factors none, 1/amount and 1/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.

3.3.6 Linearity and Standard Curve

Three batches of calibration curve standards were processed and analyzed to check linearity of the method. The linearity was determined by weighted least square regression analysis of standard plot associated with eight point standard curve respectively. Each batch of spiked plasma includes: One complete calibration curve (consisting of one blank plasma, one blank plasma with internal standard and eight different non-zero concentration samples).

The best regression algorithm was statistically determined using the following formula:

\[|\Sigma \% \text{dev}| + \sqrt{(\% \text{dev})^2}\]
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Where, % dev represents the per cent deviation for each non-zero calibrant, i.e.:
(Back calculated value - Nominal value) / Nominal value * 100

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

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.

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)
- Linear coefficient of correlation: ≥ 0.98.

3.3.7 Precision and Accuracy

Three Precision and Accuracy batches (PA batches), each consisting of a reference solution (analyte and internal standard), blank matrix, matrix blank with internal standard, spiked calibration standards (one set of at least six non-zero concentrations and LOQ and ULOQ in duplicate); six replicates of each QC set (containing one LOQQC, LQC, MQC and HQC) quality control samples, interspersed within each other, were processed and analyzed according to the proposed method. Two validation batches were processed and analyzed on the same day and the third batch was analyzed on another day.

The mean concentration, SD, % CV (precision) and % nominal (accuracy) for each QC level of the three PA batches were calculated. Intraday (using two PA batches run on the same day) and Interday (using all three PA batches) precision and
accuracy were assessed.

\[
\% \text{ CV} = \frac{\text{Standard Deviation}}{\text{Mean concentration}} \times 100
\]

\[
\% \text{ Nominal} = \frac{\text{Mean concentration}}{\text{Nominal concentration}} \times 100
\]

For the acceptance, at least 75% of non-zero standards should meet the following criteria, including at least one LOQ and ULOQ standard:

- accuracy of the LOQ in the standard curve should be within ± 20% of the nominal value and within ± 15% for other CC standards

### 3.3.8 Ruggedness

The ruggedness of the extraction procedure and chromatographic method was evaluated by analyzing a batch of six sets of quality control samples at LOQQC, LQC, MQC and HQC and a set of calibration curve standards using a different column (same type) and by a different analyst as per the standard method.

### 3.3.9 Recovery

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

% recovery was calculated as follows:
\[
\text{% Recovery} = \frac{\text{Mean peak area of extracted QC sample}}{\text{Mean peak area of unextracted QC samples}} \times 100
\]

The recovery was deemed acceptable if this was \( \leq 115 \% \).

### 3.3.10 Stability and Integrity Evaluation

#### 3.3.10.1 Stock Solution Stability

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

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

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

#### 3.3.10.2 Bench Top Stability

Four replicates of each low and high concentration quality control samples (LQC and HQC) were taken from cold room, thawed at room temperature, kept unprocessed for 4-24 hours (stability sample), then processed along with freshly spiked calibration standards and freshly spiked LQC & HQC samples (comparison sample) and analyzed by back calculation using regression equation obtained. The % CV and % nominal were calculated at both LQC and HQC levels. Samples were deemed stable after specified bench top period if- % nominal is within \( \leq 15 \% \) and the mean concentration should be \( \pm 15 \% \) at both LQC and HQC levels.

#### 3.3.10.3 Freeze-Thaw Stability

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

### 3.3.10.4 In-Injector Stability/Auto Sampler Stability

Four replicates of each low and high concentration quality control samples were processed and kept in the auto-sampler (stability sample). Samples were then run and analyzed along with freshly spiked calibration standards & freshly spiked LQC & HQC samples (comparison sample) and analyzed by back calculation using regression equation obtained. Same process was repeated for internal standard. The % stability, 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 % stability was within ± 15 % of nominal values and % CVs ≤ 15 % at both LQC and HQC levels. The % stability was deemed acceptable if it was within 85-115% for both low and high QC levels.

### 3.3.10.5 Dilution Integrity

This exercise ensures integrity of analyte in those samples which are beyond upper limit of the standard curve and needs to be diluted. Dilution integrity sample was spiked at a concentration between 160 – 180% of ULOQ. It was then diluted 2 times and 4 times with the blank plasma (dilution integrity samples). Six replicates of both dilutions were processed along with freshly spiked calibration standards and freshly spiked LQC & HQC samples (comparison sample) and then analyzed by back calculation using regression equation obtained. The % CV and % nominal were calculated for both dilutions. The integrity of the samples were considered to be maintained if % stability was within ± 15 % of nominal values and % CVs ≤ 15 % for both dilutions.
3.3.11 **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. Matrix factor (MF) was calculated at low, middle and high QC level for each blank matrix. The mean, SD and %CV of matrix factor were calculated at low, middle and high QC levels.

3.3.12 **Carry Over Effect in Matrix**

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

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

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

Carryover effect for analyte and internal standard was calculated as per following formula.

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

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

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

- Carryover effect should be $\leq 20\%$ for analyte and $\leq 5\%$ for internal standard.

3.4 ANALYSIS OF CLINICAL STUDY SAMPLES

3.4.1 Objective

The objective of the study was to analyze the clinical study samples using a validated LC/MS/MS for the determination of plasma Esomeprazole concentration.

3.4.2 Dates of the sample collection

The plasma samples for period I and II were collected between 14th & 15th November 2009, 3 and 21st & 22nd November 2009 respectively.

3.4.3 Total number of samples

A total of 900 samples were collected during the two periods of the study. For drug
analysis by a validated LC-MS/MS, one analytical batch consisted of all the samples of one subject (50) in two periods along with the calibration standards (14) and quality control samples (2 LQC, 2 MQC and 2 HQC) were processed and analysed.

3.4.4 Clinical Sample Storage Sites

All post dose blood samples were collected and processed under low light condition. The plasma samples were stored at less than –50°C in a Haereus deep freezer at the clinical facility of Ranbaxy Research Laboratories at Majeechia Hospital and were subsequently transferred to bioanalytical facility on 27 November 2009 for analysis.

3.4.5 Protocol deviations

There were no significant protocol deviations during the analysis of clinical samples.

3.5 PHARMACOKINETIC AND STATISTICAL ANALYSES

3.5.1 Pharmacokinetic Analyses

The following pharmacokinetic parameters were calculated for Esomeprazole using WinNonlin PK Software, Version 5.0.1 from Pharsight.

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

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

\[
\text{AUC}_{0 \rightarrow \infty} \quad \text{The area under the plasma concentration versus time curve, from time zero to infinity. AUC}_{0 \rightarrow \infty} \text{ is calculated as the sum of AUC}_{0 \rightarrow t} \text{ plus the ratio of the last measurable plasma concentration to the elimination rate constant.}
\]
AUC % Extrap: It is the percentage of extrapolated area under the plasma concentration versus time curve from the last measurable concentration to infinity. It will be calculated as 
\[
\left(\frac{AUC_{0\rightarrow \infty} - AUC_{0\rightarrow t}}{AUC_{0\rightarrow \infty}}\right) \times 100
\]

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 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\text{1/2}: The apparent first-order terminal elimination half-life will be calculated as 0.693/K\text{el}.

3.5.2 Statistical Analyses

Arithmetic means, standard deviations and coefficients of variation were calculated for the parameters listed in section 3.5.1. Additionally, geometric means and percentage coefficient of geometric means were calculated for C\text{max}, AUC\text{0\rightarrow 24}, AUC\text{0\rightarrow t} and AUC\text{0\rightarrow \infty}.

3.5.3 Analysis of Variance

The log-transformed pharmacokinetic parameters (C\text{max}, AUC\text{0\rightarrow 24}, AUC\text{0\rightarrow t} and AUC\text{0\rightarrow \infty}) were analysed 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 analyse 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 0.05 level of significance against the residual error (mean square error) from the ANOVA model as the error term. Each analysis of variance included calculation of least-squares means, the difference between the adjusted formulation means and the standard error associated with the difference. The above analysis was done using the PROC GLM Procedure.

3.5.4 Ratios analyses and Confidence Intervals

90% confidence interval for the ratio of the test (T) and reference (R) product averages (least’ square means) was calculated for Esomeprazole 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 was T vs R, so the ratios were of the form: - Test/Reference. Ratio of means was calculated using the LSM for log-transformed Cmax, AUC0→t, AUC0→24, and AUC0→∞. Ratios of means were expressed as a percentage of the LSM for the reference formulations.

3.5.5 Intra-subject Variability

The intra-subject variability for Cmax, AUC0→24, AUC0→t, and AUC0→∞ pharmacokinetic parameters, was derived from the analyses of the log-transformed data.

3.5.6 Power of the test

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. Power calculation was performed on log-transformed pharmacokinetic parameters Cmax, AUC0→24, AUC0→t, and AUC0→∞. Based on the available in-house study data of esomeprazole magnesium delayed release capsules and the possible withdrawals/dropouts, sample size of 18 was considered adequate to yield a power of ≥ 80%.