3.0 MATERIALS AND METHODS

The clinical part of the study was carried out at the Ranbaxy Clinical Pharmacology Unit (CPU) Majeedia Hospital, New Delhi, India. The analytical part of the study was carried out at Clinical Pharmacology and Pharmacokinetics, Ranbaxy Research Laboratories, Gurgaon, India.

The experimental design was divided into six parts;

- Clinical study methodology
- Bioanalytical method development
- Bioanalytical method validation
- Analysis of clinical study samples
- Pharmacokinetic analysis
- Statistical analysis

3.1 CLINICAL STUDY METHODOLOGY

3.1.1 Objective

The objective of this study was to compare oral bioequivalence of a single oral dose of Levetiracetam 1000 mg extended release tablet manufactured by Ranbaxy Laboratories Limited with two oral doses of a Keppra™ 500 mg tablet (each tablet containing levetiracetam 500 mg) of UCB Pharma Inc., administered twelve hourly, in healthy, adult, male, human subjects under fed condition.

3.1.2 Study design

An open label, balanced, randomized, two-treatment, two-period, two-sequence, crossover bioequivalence study comparing a single oral dose of Levetiracetam 1000 mg extended release tablet manufactured by Ranbaxy Laboratories Limited with two oral doses of a Keppra™ 500 mg tablet (each tablet containing levetiracetam 500 mg) of UCB Pharma Inc., administered twelve hourly, in healthy, adult, male, human subjects under fed condition.

3.1.3 Number of subjects

The estimation of sample size in this study was done in consultation with a biostatistician according to the method outlined in research article on “Sample Size determination for bioequivalence assessment by means of confidence intervals (Diletti et al, 1991)”. 
Sample size estimation was based on study data on Levetiracetam Extended Release Tablet. Considering an anticipated intra-subject coefficient of variation of approximately 20% and assuming a Test / Reference ratio in the range of 95-105%, the study was expected to yield a power of at least 80% to show bioequivalence under bioequivalence assumptions with 18 subjects. However, in order to allow for a wider range, higher intra-subject coefficient of variation and possible dropouts and/or withdrawals during the conduct of the study, a sample size of 24 subjects was decided for this study.

Adequate healthy adult male human subjects were enrolled and finally 24 subjects were admitted in the study to allow dosing in both periods of the study. Data has been presented on all 24 completed subjects.

3.1.4 Study Site

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

3.1.5 Selection of subjects

Adequate number of healthy, adult, human male subjects were selected randomly from the Volunteer Bank of Ranbaxy Clinical Pharmacology Unit and underwent a standardized screening procedure.

3.1.5.1 Screening assessments-

Complete medical history (including the history of 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) and demographic data, (including name, sex, age, height, weight and number of cigarettes smoked per day), were recorded. Each subject then underwent physical examination and the laboratory tests of hematological, hepatic and renal functions as listed below (Table 5). Only medically healthy subjects with clinically normal laboratory profiles were enrolled in the study.

All the samples collected during screening were analyzed at clinical laboratory at Ranbaxy Laboratories Limited. Dr. Lal Pathlabs or Super Religare Laboratories were to be used as a back-up laboratory for sample analysis whenever the in-house clinical laboratory was out of stock of lab kits or whenever there was a malfunction in laboratory instruments.
In addition, scan for drugs of abuse (cannabinoids and opioids) by card/cassette method and breath test for alcohol were carried out prior to admission in each period of the study.

**Schematic representation of Study Design (From Screening till Inclusion in the study)**-

![Schematic diagram]

**Table 5(a). Screening Assessments**

<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>
<tr>
<td></td>
<td>- Urine drug screen</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Cannabinoids</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Opioids</td>
<td></td>
</tr>
<tr>
<td>SEROLOGY</td>
<td></td>
<td><strong>Microscopic Examination</strong></td>
</tr>
<tr>
<td>- HIV I &amp; II</td>
<td></td>
<td>- RBC</td>
</tr>
<tr>
<td>- HBsAg</td>
<td></td>
<td>- WBC</td>
</tr>
<tr>
<td>- HCV</td>
<td></td>
<td>- Epithelial Cells</td>
</tr>
<tr>
<td>- VDRL</td>
<td></td>
<td>- Crystals</td>
</tr>
<tr>
<td>OTHER Assessments</td>
<td></td>
<td>- casts</td>
</tr>
<tr>
<td>- ECG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Chest X-Ray</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Twenty-four (24) healthy, adult, human male subjects were selected based on the following inclusion and exclusion criteria and documented in the CRITERIA CHECK form (Appendix 2).

3.1.5.2 Inclusion Criteria-

- Be in the age range of 18-45 years.
- Be neither overweight nor underweight for his/her height as per the Life Insurance Corporation of India height/weight chart for non-medical cases.
- Have voluntarily given written informed consent to participate in this study.
- Be of normal health as determined by medical history and physical examination of the subjects performed within 21 days prior to the commencement of the study.

3.1.5.3 Exclusion Criteria-

- Subject having history of hypersensitivity to levetiracetam or any other related drug.
- Subject having history of headache associated with dizziness.
- Subject having history of asthenia and/or fatigue affecting normal routine activities.
- Subject having history and/or family history of depression and behavioral abnormalities.
- Subject having history of excessive somnolence.
- Subject having history of drug-induced rash and/or pruritus.
- Subject having history of fever, upper respiratory tract infections and/or ataxia in the week preceding the study.
- Subject having any evidence of organ dysfunction or any clinically significant deviation from the normal, in physical or clinical determinations.
- Subject having laboratory test parameter(s), as per Annexure IV, which is/are outside acceptable limits and is judged clinically significant.
- Subject having 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 having 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.
- Subject having history of drug dependence or excessive alcohol intake on a habitual basis or has difficulty in abstaining or found positive in alcohol breath test before admission in period I.
- Subject having used any medication within 30 days prior to admission of this study.
• Subject having 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).
• Subjects who, through completion of this study, would have donated and/or lost more than 350 mL of blood in the past 3 months.

“No waivers were permitted with respect to inclusion and exclusion criteria.”

3.1.6 Study schedule

• Period I of the study was conducted between dates 23 April 2010 to 26 April 2010.
• Period II of the study was conducted between dates 27 April 2010 to 30 April 2010.

The duration of treatment was two times for reference and one time for test, single dose. Each subject was dosed with test (T) or reference (R) product once in each period as per the randomization schedule. Period I and II was separated by a washout period of 04 days.

During each study period, all the subjects reported to the Ranbaxy Clinical Pharmacology Unit (CPU) at least 11 hours before dose administration on day 1. After sampling for 36 hours post dose as per schedule, the subjects were discharged from the Clinical Pharmacology Unit on the evening of day 2 of each study period. The subjects made one visit to the Clinical Pharmacology Unit for AE monitoring and collection of further blood sample at 48 hours post-dose in each period.

3.1.7 Fasting/Meals

For Reference: Post admission to the Ranbaxy CPU during each study period, after an overnight fast of at least 10 hours, subjects started the high fat high calorie breakfast, 45 minutes prior to serving of the morning dose. Study subjects were required to eat this meal in 30 minutes or less. The dose was administered 45 minutes after start of the meal with 240 mL of water.

The second dose was administered at 12.00 hrs after the morning dose. The high fat high calorie meal was also served at 45 minutes prior to the second dose. Study subjects were required to eat this meal in 30 minutes or less. The dose was administered 45 minutes after start of the meal with 240 mL of water. No food will be allowed for at least 4 hours post each dose.

For Test: Post admission to Ranbaxy CPU during each period, after an overnight fast of at least 10 hours, subjects started the high fat high calorie breakfast, 45 minutes prior to administration of the study drug. Study subjects were required to eat this meal in 30 minutes or less. The dose was administered 45 minutes after start of the meal with 240 mL of water.
Chapter 3

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The high fat high calorie meal was served at 11.25 hours post-dose also. No food was allowed for at least 4 hours post-dose.

Apart from the above meals, all subjects received Lunch at 4 hours post dose on dosing day, and Breakfast, Lunch and Snacks at 24, 28 and 32 hours post-dose, respectively. Drinking water was not allowed from 1 hour before dosing until 2 hours post-dose (for Test Product and for first and second dose of Reference Product) except 240 ml of water given during administration of the dose. Thereafter, it was allowed at all times.

3.1.8 Treatments

3.1.8.1 Treatments Administered-

Either test (T) or reference (R) products, containing a total dose of 1000 mg of Levetiracetam, were administered during each period of the study under the supervision of trained study personnel.

Reference (R)-

Two oral doses of reference product (R) [Keppra™ tablets 500 mg, each containing levetiracetam 500 mg of UCB Pharma Inc.] was administered with an interval of twelve hours between the two doses. Each dose was administered with 240 mL of water at an ambient temperature, at least 45 minutes after serving of a high-fat high-calorie meal, during each period.

Test (T)-

A single oral dose of test product (T) [Levetiracetam 1000 mg extended release tablet manufactured by Ranbaxy Laboratories Limited] was administered with 240 mL of water at an ambient temperature, at least 45 minutes after serving of a high-fat high-calorie breakfast, during each period.

Both test and reference products were administered to all the study subjects, once in each period. Order of receiving the test (T) or reference (R) products for each subject during each periods of the study was determined according to a SAS-generated randomization schedule.

3.1.8.2 Identity of Investigational Products -

The study formulations were received at the clinical facility on 15 April 2010 and on 21 April 2010. The formulations were stored in a drug store with controlled temperature and restricted access.
Study formulations are described as follows:

<table>
<thead>
<tr>
<th>Table 5 (b). Investigational Products Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reference (R)</strong></td>
</tr>
<tr>
<td>Product Name: KEPPRÂ® (levetiracetam) 500 mg tablets</td>
</tr>
<tr>
<td>Manufactured for: UCB, Inc. Smyrna, GA 30080</td>
</tr>
<tr>
<td>Lot No.: 48448</td>
</tr>
<tr>
<td><strong>Test (T)</strong></td>
</tr>
<tr>
<td>Product Name: Levetiracetam Extended Release Tablets 1000 mg</td>
</tr>
<tr>
<td>Manufactured By: Ranbaxy Laboratories Limited, India</td>
</tr>
<tr>
<td>Batch No.: DM(169D)04</td>
</tr>
</tbody>
</table>

3.1.8.3 *Assignment to treatment*-

The order of receiving the test (T) or reference (R) products for each subject during the 2 periods of the study was determined according to a SAS-generated randomization schedule [Annexure VI]. The randomization was balanced.

A working copy of the same was provided to study personnel responsible for dosing. The Investigator, Registered Pharmacist, personnel involved in dispensing of study drugs and the dosing were accountable for ensuring compliance to randomization schedule.

**Blinding**-

Although this was an open-label study, laboratory analysts did not have access to the randomization scheme.

3.1.9 *Washout period*

There was a washout period of at 04 days between the administrations of study drugs in each period of the study.

3.1.10 *Assessment of compliance*

Compliance was assessed by conducting a thorough examination of the oral cavity by trained study personnel after dosing in each period and by measurement of plasma levetiracetam (during the analytical phase of the study). The investigator ensured dosing compliance.
3.1.11 Restrictions

3.1.11.1 Medications (Prior and Concomitant Medication Procedure)-

Subjects were required not to have received any medication, including vitamins and over the counter (OTC) medications, during a period of 30 days prior to the start of the study. They were instructed during screening not to take any prescription, OTC medications and vitamins until the completion of the study.

In the interest of subject’s safety and acceptable standards of medical care the investigators or medical officers were permitted to prescribe treatment(s) at their discretion.

3.1.11.2 Diet-

Subjects were instructed to abstain from any alcohol / products containing alcohol and grapefruit juice and or grapefruit supplements for 48 hours prior to admission and till last sample collection for pharmacokinetic analysis during each period. They were also instructed to abstain from tea, coffee, cigarette and any other xanthine containing food or beverages, during in-house stay in each period.

3.1.11.3 Activity-

All subjects were dosed while seated and were instructed to remain seated or ambulatory for the first 2 hours following each drug administration. Thereafter, subjects were allowed to engage only in normal activities while avoiding severe physical exertion. However, there was a provision to place the subjects in an appropriate position or to lie down on their right side if any adverse/medical event occurred at any time during the housing of the subjects.

3.1.12 Selection and Timing of Dose for Each Period in the Study

Approval of Levetiracetam 1000 mg extended release tablet by DCGI, New Delhi requires demonstration of bioequivalence with two doses of reference listed product KEPPRA® 500 mg tablets under fed condition.

This study was designed based on the known pharmacokinetics of the study drug, Levetiracetam on the generally accepted standards for the conduct of bioequivalence studies. As it was a bioequivalence study, a single oral dose of either test (T) or reference (R) was administered during each period of the study.
Table 5 (c). Drug Administration Details

<table>
<thead>
<tr>
<th>Period</th>
<th>Date</th>
<th>Time of Drug Administration</th>
<th>No. of Subjects Dosed</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>24 April 2010</td>
<td>Dose 1 0900 hr to 0914 hr [For Reference (R) or Test (T)]</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dose 2 2100 hr to 2114 hr [For Reference (R)]</td>
<td>12</td>
</tr>
<tr>
<td>II</td>
<td>28 April 2010</td>
<td>Dose 1 0900 hr to 0914 hr [For Reference (R) or Test (T)]</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dose 2 2100 hr to 2112 hr [For Reference (R)]</td>
<td>12</td>
</tr>
</tbody>
</table>

3.1.13 Blood sampling

Intravenous indwelling cannulae were kept *in situ* as long as possible (until 24 hours post-dose) for the collection of blood samples. The cannulae were maintained patent by injection of upto 1 mL of 5 IU/mL of heparin in normal saline solution. In such cases, blood samples were collected after discarding the first 0.5 mL of heparinised blood and heparin solution from the tubing.

All post dose blood samples collected early/later than 2 minutes (during their in-house stay) of the scheduled collection time and early/later than 1 hr of the scheduled collection time (at ambulatory) were reported as protocol deviations and the deviation in collection were adjusted during pharmacokinetic and statistical calculations.

After collection of blood samples from all the subjects at each time-point, one of the study personnel or an attendant transferred all the collection tubes to a sample processing room at the Clinical Pharmacology Unit. Thereafter, the blood samples were centrifuged as soon as possible at a speed of 4000 rpm, for 15 minutes duration and at temperature (4 ± 2°C) under refrigeration to separate plasma. All post-dose plasma samples were divided into 2 aliquots and transferred to suitably labelled tubes and re-checked to ensure transfer of plasma to the correct tube. The plasma samples were stored below –15°C, pending transfer to the analytical facility for assay.

All post-dose samples were collected and processed under low light conditions.
3.1.14 Safety

3.1.14.1 Clinical safety measurements (Vitals signs recording)-

Vital Signs (oral temperature, sitting BP and radial pulse) measurements were performed at admission, prior to dosing and at 2, 6, 10, 24 and 36 hours post dose, within 2.0 hours of scheduled time in each period.

3.1.14.2 Clinical examination-

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

3.1.14.3 Laboratory Evaluations for Safety -

Laboratory parameters were repeated at the end of the study as per the in-house Standard Operating Procedure on “Evaluation of clinical laboratory tests conducted for screening of volunteers, during the conduct of clinical study and at end of study safety assessment”. Any laboratory parameter(s) outside acceptable limits (Appendix 4) were termed as laboratory abnormality and followed up until the results were normal /clinically not significant.

3.1.15 Adverse events

3.1.15.1 Definitions-

Adverse event-

Any untoward medical occurrence in a subject or clinical investigation subjects administered a pharmaceutical product, which does not necessarily have a causal relationship with this treatment.

An adverse Event (AE) can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding, for example), symptom, or disease temporally associated with the use of a medicinal product, whether or not considered related to the medicinal product.

Medical Event-

Any event, which occurs in a subject after signing the Informed Consent Form (ICF) and prior to dosing in period I.
Laboratory adverse event-

A laboratory abnormality, after being evaluated by a physician for clinical significance, if any one of the following conditions is met:

(i) When the abnormal lab report is accompanied with associated symptoms,

(ii) When medical/surgical intervention is required.

(iii) When it is considered by clinical/principal investigator as an adverse event

3.1.15.2 Adverse Event Monitoring, Recording and Reporting-

The Clinical Investigator or a Medical Officer was available at the site of investigation from admission until 36 hours post-dose during each period. A medically qualified designate was on call for the remaining period of study. Subjects were monitored throughout the study period for adverse events. Subjects were informed to bring to the notice of the nurse or the doctor any adverse event that may occur during their stay at the site of investigation.

Subjects were specifically asked about any adverse events along with vital sign recordings and during ambulatory visits at 48 hours post-dose. Adverse event monitoring was done within two hours of the specified times. In case of subject reporting as unwell at ambulatory visits, vital signs were recorded and necessary action was taken. Treatment of any adverse events was done by a physician, either at the site of investigation or at a nearby hospital.

All adverse events were to be followed up at regular intervals at a frequency as decided by the Clinical Research Physician/ Clinical Investigator/ Principal Investigator depending upon the condition. All unresolved adverse events till end of study, were to be followed up for at least 30 days from the start of the adverse event or until resolution of adverse event or determination that no further medical intervention is deemed necessary or the adverse event is otherwise explained or the subject is lost to follow-up.

Adverse events experienced by subjects, including any serious adverse events, were to be reported as per the in-house Standard Operating Procedure on “Monitoring, Management & reporting of Adverse Events during a Clinical Study”.

3.1.16 Discharge

All subjects were discharged 36 hours after administration of the study drug during each period.
3.1.17 Ethical Considerations

3.1.17.1 Basic principles-

This research was carried out in accordance with the Basic Principles defined in US 21 CFR Part 320, the ICH (62FR 25692, 09 May 1997) ‘Guidance for Good Clinical Practice’, 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.

3.1.17.2 Institutional Review Board-

This protocol and the corresponding informed consent form (ICF) used to obtain informed consent of study subjects was reviewed by the Jamia Hamdard Institutional Review Board and the study subjects were not to be dosed until the Board had approved the protocol and the ICF, as submitted or with modifications. The Board is constituted and operates in accordance with the Principles and requirements described in the US Code of Federal Regulations (21 CFR Part 56).

The version 01 and version 02 of the protocol and the informed consent form (English and Hindi) for this study were reviewed by the Jamia Hamdard Institutional Review Board (JHIRB) on 12 March 2010 and 22 April 2010, respectively. Details are listed below:

<table>
<thead>
<tr>
<th>Review/Approval Date</th>
<th>Document(s) Reviewed/Approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 March 2010</td>
<td>Protocol (Version 01) and ICF (English &amp; Hindi, Version 1) dated 26 February 2010</td>
</tr>
</tbody>
</table>

3.1.17.3 Informed Consent-

The purpose of the study, procedures to be carried out, potential hazards and rights of the subjects were described to the subjects in non-technical terms in an oral presentation by the Clinical Investigator or his designate. There was also a ‘Medical Query Resolution’ where any queries of the volunteers as regards the study drug were satisfactorily resolved by the Clinical Investigator or his designate Clinical research Physician.
Subjects were required to understand and sign a consent form summarizing the discussion prior to admission for the study in Period I. All the subjects provided formal written consent after attending an oral presentation and after thoroughly reading the informed consent form [Annexure III].

3.1.17.4 Drop-out/ Withdrawal of Subjects from Study-

Subjects were informed that they were free to dropout from the study at any time without stating any reason. The decision of the Investigator of withdrawing of a subject from the study was considered for any of the following reasons:

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

(ii) The subject experienced an 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) If the subject required any concomitant medications, the decision to continue or discontinue the subject was based on the following

- The pharmacology and pharmacokinetics of the non-study medication.
- The likelihood of a drug-drug interaction, thereby affecting pharmacokinetic comparison of the study medication.
- The time of administration of the non-study medication.

(v) If the subject was found positive for breath alcohol test/urinary screen testing of drugs of abuse (opiates or cannabinoids) on the day of admission of any period or had a history of alcohol consumption during the period 48 hrs prior to admission till last sample collection in each period.

(vi) If the subject experienced vomiting at any time during the sample collection schedule.

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

3.1.17.5 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 Laboratories Limited and the study subjects. The compensation in this study was Rs.6,200/- per completed
subject. From this, a sum of Rs. 500/- was given to the subject after satisfactory resolution of the end of the study safety assessment.

3.1.17.6 Medical Treatment for Injury-
In case of research related injury, first aid was available at the Ranbaxy Clinical Pharmacology Unit and treatment of adverse reactions requiring hospitalization was to be undertaken at a nearby hospital and the expenses were to be borne by Ranbaxy Laboratories Limited.

3.1.18 Study documentation
All data generated during the conduct of the study were directly entered in the raw data recording forms as governed by the SOPs of Department of Clinical Pharmacology and Development & Clinical Pharmacology and Pharmacokinetics, Ranbaxy Research Laboratories except the analytical data of clinical laboratory of the Clinical Pharmacology Unit, which were transcribed into the study related forms and the raw data retained by the laboratory for 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 or his designate Scientist for clinical data and the Laboratory Supervisor for the bioanalytical data. The Clinical Investigator and the Laboratory Supervisor were responsible for supervising compilation of data until ready for archiving.

3.1.19 Confidentiality of data
The data identifying each study subject by name was kept confidential and was accessible to the study personnel, Ranbaxy Corporate Quality Assurance Auditor during audits and if necessary, to the Jamia Hamdard Institutional Review Board and various regulatory agencies.

3.1.20 Study monitoring
The study was monitored by the delegated and trained clinical monitor. Monitoring was done by on-site visits from the monitor reviewed study related raw data, standard procedures and activities and ensured that the clinical part of the study was conducted according to the protocol and applicable regulatory requirements.
3.2 BIO-ANALYTICAL METHOD DEVELOPMENT

The analytical method for the determination of Levetiracetam in human K3EDTA plasma using Didanosine as internal standard was developed and validated at the Department of Clinical Pharmacology and Pharmacokinetics, Ranbaxy Research Laboratories, Gurgaon, India.

Sample preparation process was performed by solid phase extraction technique. The processed samples were chromatographed on Acquity UPLC BEH C18 (2.1 X 50 mm, 1.7 µm) column using Methanol: Acetonitrile: Buffer 1 (60:30:10 v/v/v) + 0.2 mL of Formic acid/litre as mobile phase. Levetiracetam and Didanosine (ISTD) were detected by LC/MS/MS detection. Signals from the detector were captured by computer and processed using Masslynx V4.1 software.

3.2.1 Instrumentation

Waters Acquity UPLC MS (Ultra Performance Liquid Chromatography Mass Spectrometry) and Masslynx software V4.1 for data processing were used.

3.2.2 Reagents

1. Acetonitrile (HPLC Grade)
2. Ammonium Acetate (Analytical Grade)
3. Formic Acid (Analytical Grade)
4. Levetiracetam (Working Standard)
5. Didanosine (Working Standard)
6. Water (HPLC Grade)
7. Methanol (HPLC Grade)

3.2.3 Preparation of Standard Stock Solutions

3.2.3.1 Levetiracetam Standard Stock Solution

1. Levetiracetam working standard was weighed accurately and transferred to a volumetric flask. It was dissolved in methanol and the volume was made up with the same to prepare a solution of approximately 6 mg/mL.
2. The final concentration of Levetiracetam was corrected accounting for its potency, molecular weight and the actual amount weighed.
3. It was stored in refrigerator between 1-10°C in a polypropylene container protected from light and used within 6 days from the date of its preparation.
3.2.3.2 Didanosine Internal Standard Stock Solution-

1. Didanosine working standard was weighed accurately and transferred to a volumetric flask. It was dissolved in HPLC grade water and the volume was made up with the same to prepare a solution of approximately 1 mg/mL.
2. The final concentration of Didanosine was corrected accounting for its potency, molecular weight and the actual amount weighed.
3. It was stored in refrigerator between 1-10⁰C in a polypropylene container protected from light and used within 6 days from the date of its preparation.

Note: Stock solution and all further dilutions from the above stocks were prepared under low light condition.

<table>
<thead>
<tr>
<th>Name of Standard</th>
<th>Lot No.</th>
<th>Purity/Potency</th>
<th>Expiry</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levetiracetam</td>
<td>038K4722</td>
<td>97.5% by HPLC</td>
<td>Feb. 2011</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Levetiracetam</td>
<td>118K47022</td>
<td>97.5% by HPLC</td>
<td>07 Apr. 2011</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Didanosine</td>
<td>FOG088</td>
<td>99.7% on as is basis</td>
<td>Current lot was used</td>
<td>USP</td>
</tr>
</tbody>
</table>
Table 5 (f). Source and Type of Biological Matrix

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Lot No.</th>
<th>Anticoagulant</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Plasma</td>
<td>060306, 060301,</td>
<td>K₃ EDTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100309, 160306,</td>
<td></td>
<td>Yash Laboratory, Shop no. 09, Solanki Apartment,</td>
</tr>
<tr>
<td></td>
<td>100307, 020410-12</td>
<td></td>
<td>Louiswadi, Thane (W), Mumbai, India</td>
</tr>
<tr>
<td>Human Haemolysed</td>
<td>240309, 240308,</td>
<td>K₃ EDTA</td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>180201, 240307</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human Lipemic Plasma</td>
<td>240305, 240302,</td>
<td>K₃ EDTA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>240301, 240304</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.2.4 Preparation of Solutions

3.2.4.1 Buffer-1:

- 0.385 ± 0.015 gm of ammonium acetate was transferred into 1000 mL of reagent bottle.
- 1000 mL of HPLC grade water was added to it and dissolved by sonicating in an ultrasonic bath to get approximately 5.0 ± 0.2 mM ammonium acetate buffer.
- This solution was stored at room temperature and used within 5 days from the date of its preparation.
3.2.4.2 Mobile Phase:
- 100 mL of Buffer-1 was transferred into 1000 mL of reagent bottle.
- 600 mL of methanol was added to it followed by addition of 300 mL of acetonitrile and 200 µL of formic acid.
- It was mixed well and degassed in an ultrasonic bath.
- This solution was stored at room temperature and used within 3 days from the date of its preparation.

3.2.4.3 Weak Wash Solution:
- 100 mL of HPLC grade water was transferred into 1000 mL of reagent bottle.
- 600 mL of methanol was added to it followed by addition of 300 mL of acetonitrile.
- It was mixed well and degassed in an ultrasonic bath.
- This solution was stored at room temperature and used within 7 days from the date of its preparation.

3.2.4.4 Strong Wash Solution:
- 100 mL of HPLC grade water was transferred into 1000 mL of reagent bottle.
- 900 mL of methanol was added to it and mixed well.
- It was degassed in an ultrasonic bath.
- This solution was stored at room temperature and used within 7 days from the date of its preparation.

3.2.4.5 Diluent-1:
- 500 mL of methanol was transferred into a reagent bottle.
- 500 mL of HPLC grade water was added to it.
- It was mixed well, sonicated and degassed in an ultrasonic bath.
- This solution was stored at room temperature and used within 7 days from the date of its preparation.
Table 5 (f). Calibration curve standards and Quality control samples

<table>
<thead>
<tr>
<th>Type of Standards</th>
<th>Nominal Concentration (µg/mL)</th>
<th>Preparation Date</th>
<th>Storage Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration curve standards in pooled human plasma</td>
<td>0.482, 0.985, 1.971, 3.244, 5.406, 9.010, 18.020 and 36.221</td>
<td>17 Apr. '10</td>
<td>Below -15°C in freezer room (CR-01)</td>
</tr>
<tr>
<td>Quality control samples in pooled human plasma</td>
<td>LOQC – 0.486&lt;br&gt; LQC – 0.991&lt;br&gt; MQC – 15.014&lt;br&gt; HQC – 30.028</td>
<td>17 Apr. ‘10</td>
<td>Below -15°C in freezer room (CR-01)</td>
</tr>
</tbody>
</table>

Note:

1. Matrix IDs [060306, 060301, 100309 and 100307] were used for bulk spiking to prepare calibration curve standards and quality control samples in human plasma.
2. QC IDs MV 429/LQC, HQC #131-170 were kept in CR-01 (below -15°C) on 17 Apr. ’10 for long-term stability.
3. Calibration curve standards and quality control samples were spiked under low light condition.

3.2.5 Method Description

3.2.5.1 Sample preparation-

1. Plasma samples were withdrawn from the freezer room.
2. These were allowed to thaw at room temperature.
3. The thawed samples were vortexed to ensure complete mixing of contents.
4. 50 µL of internal standard stock dilution (approximately 35 µg/mL of Didanosine) were pipetted out into appropriately labeled polypropylene tubes (except standard blank).
5. 100 µL aliquot of each plasma sample was added into the sample tube.
6. 400 µL of HPLC grade water was added to each tube and vortexed.
7. The cartridges {Oasis HLB; 30 mg (1cc)} were conditioned using 1 mL of methanol followed by 1 mL of HPLC-grade water by running the centrifuge at 1500 rpm for 1 minute after each addition.

8. 100 µL of sample was loaded onto the cartridges by running centrifuge for 1 minute at 1500 rpm.

9. The samples were washed twice with 500 µL of HPLC-grade water by running centrifuge for 1 minute at 1500 rpm.

10. The samples were eluted twice with 500 µL of methanol by running centrifuge for 1 minute at 1500 rpm.

11. Final eluates were dried under nitrogen at 50°C ± 3°C at about 15 psi.

12. The dried residue was reconstituted with 2000 µL of methanol.

13. The samples were then transferred into vials for analysis.

Note:
- The centrifuge was run at temperature 2-10°C.
- All the samples were processed under low light condition.

3.2.6 Chromatographic Conditions

A summary of the chromatographic (Acquity UPLC) and mass spectrometric conditions are as follows:

Column: Acquity UPLC BEH C18 (2.1 X 50 mm, 1.7 µm)

Mobile Phase: Methanol: Acetonitrile: Buffer 1 (60:30:10 v/v/v) + 0.2 mL of Formic acid/Litre

Flow Rate: 0.250 mL/minute

Sample Cooler Temperature: 10°C ± 1.0°C

Column Oven Temperature: 35°C ± 1.0°C

Injection Volume: 2.0 µL

Retention Time: Levetiracetam: 0.2 – 0.9 minutes

Didanosine: 0.2 – 0.9 minutes

Ion source: Electro spray in positive ion mode

Weak Wash Solution: Methanol: Acetonitrile: HPLC grade water (60:30:10 v/v/v)

Strong Wash Solution: Methanol: HPLC grade water (90:10 v/v)
Table 5 (h). Chromatographic Conditions

<table>
<thead>
<tr>
<th>Integration Parameters</th>
<th>Levetiracetam</th>
<th>Didanosine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoothing Iterations</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Smoothing Width</td>
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<td>4</td>
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<tr>
<td>Peak to Peak Baseline Noise</td>
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<td>1</td>
</tr>
<tr>
<td>Peak Width at 5 % Height</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Baseline Start Threshold %</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Baseline end Threshold %</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Threshold Relative Height</td>
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<td>1</td>
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<tr>
<td>Threshold Relative Area</td>
<td>1</td>
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</tr>
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</table>

Table 5 (h). Chromatographic Conditions

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parent (m/z)</th>
<th>Daughter (m/z)</th>
<th>Cone</th>
<th>Collision</th>
<th>Dwell time (Secs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levetiracetam</td>
<td>171.00</td>
<td>126.27</td>
<td>15</td>
<td>20</td>
<td>0.150</td>
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<tr>
<td>Didanosine</td>
<td>236.84</td>
<td>137.12</td>
<td>5</td>
<td>10</td>
<td>0.150</td>
</tr>
</tbody>
</table>
### Chapter 3

**Materials and Methods**

**Pharmaceutical Medicine**

**Jamia Hamdard**

<table>
<thead>
<tr>
<th>Source</th>
<th>Capillary (kV)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Extractor (V)</td>
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<tr>
<td></td>
<td>RF Lens (V)</td>
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<tr>
<td></td>
<td>Source temperature (°C)</td>
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<tr>
<td></td>
<td>Desolvation temperature (°C)</td>
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<td></td>
<td>Cone gas flow (L/Hr)</td>
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<td></td>
<td>Desolvation gas flow (L/Hr)</td>
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</tbody>
</table>

<table>
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<tr>
<th>Analyser</th>
<th>LM Resolution 1</th>
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<tr>
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<td>HM Resolution 1</td>
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<td>Ion Energy 1</td>
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<td>Entrance</td>
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<td></td>
<td>Exit</td>
<td>1.0</td>
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<tr>
<td></td>
<td>LM Resolution 2</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>HM Resolution 2</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>Ion Energy 2</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Collision gas flow (mL/min)</td>
<td>0.35</td>
</tr>
</tbody>
</table>
3.3 BIO-ANALYTICAL METHOD VALIDATION

The analytical method for the determination of Levetiracetam in human K₂EDTA plasma using Didanosine as internal standard was validated at the Department of Clinical Pharmacology and Pharmacokinetics, Ranbaxy Research Laboratories, Gurgaon, India.

Method validation was initiated on 16 Apr. ‘10 and was completed on 30 Apr. ‘10.

3.3.1 Validation Parameters

Validation was performed to evaluate the method in terms of selectivity in normal human plasma, selectivity in haemolysed and lipemic plasma, sensitivity, linearity of response, carry-over effect in human plasma, precision and accuracy in normal human plasma, recovery, stability, re-injection reproducibility, dilution integrity, matrix effect in human plasma, matrix factor, ruggedness and extended precision and accuracy.

The sensitivity, linearity, precision and accuracy evaluations were performed on four batches of spiked samples and all of them are reported. Each precision and accuracy consisted of one complete calibration curve (comprising of one blank plasma, one blank plasma with internal standard and eight different non-zero concentrations) and six replicates of quality control samples at LOQQC, Low, Middle and High levels.

3.3.2 Standardization and Calculation

The chromatographic data were acquired and processed using computer based Masslynx software V4.1. The best-fit curves using weighted (1/Concentration²) linear least square regression analysis were obtained by peak area ratio of Levetiracetam to Didanosine (ISTD).

The concentrations of Levetiracetam in plasma samples were calculated using linear regression parameters of the corresponding calibration curve. Weighting factor 1/Concentration² was selected after weighting factor evaluation using four precision and accuracy batches (with weighting: None, 1/Concentration and 1/Concentration²).

Refer: Table 13 for weighting factor evaluation of Levetiracetam.
3.3.3 **Selectivity**

3.3.3.1 **Selectivity in Normal Plasma**-

Six lots of normal human plasma IDs [060306, 060301, 100309, 160306, 100307 and 020410-12] with K₃ EDTA as an anticoagulant were evaluated.

3.3.3.2 **Selectivity in Haemolysed Plasma**-

Four lots of haemolysed human plasma IDs [240309, 240308, 180201 and 240307], with K₃ EDTA as an anticoagulant were evaluated.

3.3.3.3 **Selectivity in Lipemic Plasma**-

Four lots of Lipemic plasma IDs [240305, 240302, 240301 and 240304] with K₃ EDTA as an anticoagulant were evaluated.

3.3.4 **Sensitivity**

Signal to Noise (S/N) ratio was determined for four replicate samples of each of the followings:

a) Pooled blank plasma samples
b) LOQQC samples

Also mean S/N ratio for pooled blank plasma samples was calculated.

S/N ratio of each LOQQC sample was compared with mean S/N ratio of pooled blank plasma samples.

3.3.5 **Linearity**

Linearity of Levetiracetam was determined by weighted least square regression analysis of standard plot associated with eight-point calibration curve.

Best-fit calibration curves of chromatographic response versus concentrations were determined by weighted least square regression analysis with weighting factor of 1/Concentration².

3.3.6 **Carry-over effect in human plasma**

Carry-over effect was determined by injecting processed blank plasma sample (without addition of internal standard), LOQ and ULOQ samples in the following sequence:

- Blank plasma sample
- LOQ sample in duplicate [from the same vial]
- ULOQ sample in duplicate [from the same vial]
- Blank plasma sample [from the same vial which was used for injection of first blank plasma sample]

Carry-over effect for Analyte and Internal Standard (ISTD) was calculated as per the following formula:

\[
\text{Carry-over effect for Analyte / Internal Standard (ISTD)} = \frac{\text{Response of interfering peak at RT of Analyte/ Internal Standard (ISTD) in last blank sample} - \text{Response of interfering peak at RT of Analyte/ Internal Standard (ISTD) in first blank sample}}{\text{Mean peak area response of the Analyte/ Internal Standard (ISTD) in processed LOQ samples}} \times 100
\]

3.3.7 Precision and Accuracy Batch

Each Precision and Accuracy batch contained:
- Reference solution containing Levetiracetam and Didanosine (ISTD)
- Blank plasma
- Blank plasma with internal standard
- Spiked calibration standards (One set of eight non-zero concentrations)
- Limit of Quantitation QC (LOQ QC - 6 samples)
- Low QC (LQC - 6 samples)
- Middle QC (MQC - 6 samples)
- High QC (HQC - 6 samples)

3.3.8 Accuracy

Accuracy of the method was evaluated as degree of closeness of the calculated values to their respective nominal values, expressed as percentage.

3.3.9 Precision

Precision of the method was measured by the percent coefficient of variation at concentrations of LOQ QC, Low, Middle and High quality control samples.
3.3.10 Recovery

3.3.10.1 Recovery of Levetiracetam-

The percentage recovery of Levetiracetam was determined by comparing the mean peak area response of six replicates of extracted quality control samples (LQC, MQC and HQC) against the mean peak area response of six replicates of aqueous (unextracted) quality control samples prepared at concentrations representing 100% extraction of quality control samples at low, middle and high concentration.

3.3.10.2 Recovery of Didanosine (ISTD)-

The percentage recovery of Didanosine (ISTD) was determined by comparing the mean peak area response of Didanosine (ISTD) in extracted quality control samples (MQC) against the mean peak area response of aqueous (unextracted) internal standards solution prepared at concentration representing 100% extraction of internal standard.

3.3.11 Stability

3.3.11.1 Freeze-thaw stability in human plasma-

The stability of spiked plasma samples was determined after three freeze-thaw cycles.

Four replicates of low and high quality control samples of Levetiracetam (stability samples) stored in polypropylene containers were analysed after third freeze-thaw cycle with four replicates of freshly spiked low and high quality control samples of Levetiracetam (comparison samples) against freshly spiked calibration curve [Refer: Table 18(a) for calibration curve]. Stability quality control samples were frozen at a temperature below -15°C and subsequently thawed at room temperature under low light condition.

The percentage stability was determined by comparing the mean concentration of Levetiracetam in stability quality control samples against the mean concentration of Levetiracetam in comparison quality control samples.

3.3.11.2 Bench-top stability-

Bench-top stability in human plasma:

The bench-top stability evaluation involved analysis of four replicates of low and high quality control samples of Levetiracetam (stability samples), which were spiked in human plasma and stored unprocessed in polypropylene container at room temperature under low light condition for
6.45 hours with four replicates of freshly spiked low and high quality control samples of Levetiracetam (comparison samples) in human plasma against freshly spiked calibration curve [Refer: Table 19(a) for calibration curve].

The percentage stability was determined by comparing the mean concentration of Levetiracetam in stability quality control samples against the mean concentration of Levetiracetam in comparison quality control samples.

**Bench-top stability during extraction:**

The bench-top stability evaluation during sample extraction involved analysis of four replicates of low and high quality control samples of Levetiracetam, which were kept on the bench during processing (stability samples) under low light condition for:

- 2.00 hours after pretreatment
- 2.00 hours before drying
- 2.00 hours after drying

Following the stability duration, the stability quality control samples were analyzed with four replicates of freshly spiked low and high quality control samples of Levetiracetam (comparison samples) against freshly spiked calibration curve [Refer: Table 20(a) for calibration curve].

The percentage stability was determined by comparing the mean concentration of Levetiracetam in stability quality control samples against the mean concentration of Levetiracetam in comparison quality control samples.

3.3.11.3 In-injector stability-

**In-injector stability (post processing stability) of Levetiracetam:**

In-injector stability evaluation involved analysis of processed four replicates of low and high quality control samples of Levetiracetam (stability samples) stored in glass vials in auto injector (10°C ± 1.0°C) for 46.32 hours with four replicates of freshly spiked low and high quality control samples of Levetiracetam (comparison samples) against freshly spiked calibration curve [Refer: Table 21(a) for calibration curve].

The percentage stability was determined by comparing the mean concentration of Levetiracetam in stability quality control samples against the mean concentration of Levetiracetam in comparison quality control samples.
In-injector stability (post processing stability) of Didanosine (ISTD):

In-injector stability of replicate quality control samples was determined. Four replicates of low and high quality control samples stored in auto injector (10°C ± 1.0°C) after processing (stability samples) in glass vials were analyzed after 46.32 hours and the peak area ratio for Didanosine (ISTD) to Levetiracetam was calculated for stability samples and comparison samples (four replicates of freshly spiked low and high quality control samples).

The percentage stability was determined by comparing the mean peak area ratio of Didanosine (ISTD)/ Levetiracetam in stability quality control samples against the mean peak area ratio of Didanosine (ISTD)/ Levetiracetam in comparison quality control samples.

Stock solution stability of Levetiracetam and Didanosine (ISTD):

To determine stock solution stability, analyte [Levetiracetam] and internal standard [Didanosine] stock solutions were prepared (stability stocks) and aliquots of the same were kept at refrigerated temperature (between 1–10°C) protected from light, in polypropylene container for 6 days. Following the storage period, fresh stock solutions of analyte and internal standard were prepared. These served as comparison stocks. Appropriate dilutions from both stability and comparison stocks were prepared and six replicates from each were injected.

Stock solution stability was determined by comparing the mean peak area response of analyte [Levetiracetam] or internal standard [Didanosine] in stability dilutions against mean peak area response of analyte [Levetiracetam] or internal standard [Didanosine] in comparison dilutions.

Short-term stability of Levetiracetam and Didanosine (ISTD):

Short-term stability for Levetiracetam and Didanosine (ISTD), was determined. The stability stock dilutions for Levetiracetam and Didanosine (ISTD) stored at room temperature in glass container for 9.07 hours and 9.05 hours, respectively were analyzed with freshly prepared stock dilutions (comparison stock dilutions) from the same standard stock solution, which was used for preparing the stability dilutions.

Short-term stability was determined by comparing the mean peak area response of analyte [Levetiracetam] or internal standard [Didanosine] in stability dilutions against mean peak area response of analyte [Levetiracetam] or internal standard [Didanosine] in comparison dilutions.
Short-term stability of Reference Solution:
Short-term stability of reference solution containing Levetiracetam and Didanosine (ISTD) was determined. The stability reference dilutions stored at room temperature in glass container for 9.07 hours were analyzed with freshly prepared reference solution dilutions.

Short-term stability was determined by comparing the mean peak area ratio of analyte [Levetiracetam / Internal standard [Didanosine] in stability dilutions against mean peak area ratio of analyte [Levetiracetam] / Internal standard [Didanosine] in comparison dilutions.

Refer: Tables 25(a), 25(b) and 26 for rejected batch of Short-term stability.

For date and time details of rejected batch of short-term stability of Levetiracetam and Didanosine (ISTD), Refer Table 38(b)

For date and time details of rejected batch of short-term stability in reference solution of Levetiracetam, Refer Table 39(b)

Results of the repeated short-term stability exercise are presented in tables 23(a), 23(b) and 24.

3.3.12 Re-injection Reproducibility

Re-injection reproducibility [Batch ID ‘V429-RR.qld’] was evaluated on MS#09 by re-injecting the low, middle and high QC samples (with QC ID: MV429/LQC, HQC # 93–98 and MV429/MQC # 71-76) of the already injected PA Batch-5 ‘V429-5PA.qld’. The re-injected QC samples were quantitated against the calibration curve standards analysed with Precision and Accuracy Batch 5 [Batch ID ‘V429-5PA.qld’].

Refer: Tables 14(a) and 14(b) for Precision and Accuracy Batch 5 originally analysed on MS#09.

Percentage difference between original and re-injected values was calculated as follows:

\[
\% \text{ Difference} = \left( \frac{\text{Absolute (Original Value – Reinjected Value)}}{\text{Original Value}} \right) \times 100
\]
3.3.13 Dilution Integrity

Dilution integrity sample was prepared by spiking Levetiracetam stock dilution into blank plasma sample to get 1.61 times ULOQ concentration. Four replicates of dilution integrity sample were diluted by a factor of 2 and four replicates were diluted by a factor of 4, using similar blank plasma. These diluted samples were processed and analysed against freshly spiked calibration curve [Refer Table 28(a) for calibration curve].

3.3.14 Matrix Effect

Matrix effect was performed by freshly spiking LQC and HQC samples into each of six lots of accepted blank matrices. Two aliquots of LQC and HQC samples from each batch of blank matrix were taken and processed as per method SOP. The values of these QCs were back calculated against freshly spiked calibration curve [Refer Table 29(a) for calibration curve].

3.3.15 Matrix Factor

Matrix Factor at LQC level:

Matrix Factor (MF) was evaluated by preparing reference mixtures of internal standard(s) and analyte(s) at concentrations representing 100% extraction of internal standard and analyte at low QC (LQC) concentrations. These served as ‘reference sample’.

Two replicates of each of the six accepted blank matrix lots were processed without addition of internal standard and were reconstituted with reference sample prepared at LQC level. These samples served as ‘matrix samples reconstituted with reference sample’. These 12 samples (matrix samples reconstituted with reference sample) were injected along with four replicates of ‘reference sample’ and Matrix factor was calculated as per following formula for each blank matrix.

\[
MF = \frac{\text{Mean peak area ratio of matrix samples reconstituted with reference samples}}{\text{Mean peak area ratio of reference samples}}
\]

Matrix Factor at HQC level:

Matrix Factor (MF) was evaluated by preparing reference mixtures of internal standard(s) and analyte(s) at concentrations representing 100% extraction of internal standard and analyte at high QC (HQC) concentrations. These served as ‘reference sample’.
Two replicates of each of the six accepted blank matrix lots were processed without addition of internal standard and were reconstituted with reference sample prepared at HQC level. These samples served as ‘matrix samples reconstituted with reference sample’. These 12 samples (matrix samples reconstituted with reference sample) were injected along with four replicates of ‘reference sample’ and Matrix factor was calculated as per following formula for each blank matrix.

\[
MF = \frac{\text{Mean peak area ratio of matrix samples reconstituted with reference samples}}{\text{Mean peak area ratio of reference samples}}
\]

3.3.16 Ruggedness

Ruggedness of the extraction procedure and chromatographic method was evaluated by analyzing a batch of six replicates of quality control samples at LOQQC, LQC, MQC, HQC level and a set of calibration curve standards on same instrument (MS#09) using a different column [(same type) Serial No. 01613825615741] by different analyst and using fresh solutions prepared by analyst performing the ruggedness activity.

3.3.17 Extended precision and accuracy batch

Forty (40) sets of quality control samples were run for Extended Precision and Accuracy Batch against a single calibration curve (MV429/CC6) for Levetiracetam.

Refer: Table 32(a) for calibration curve.
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 waters LC/MS/MS method for the determination of Levetiracetam in K$_3$EDTA human plasma.

3.4.2 Sample collection and storage
In this clinical study, the blood samples for period I and II were collected on 24$^{th}$ and 25$^{th}$ April 2010 and 28$^{th}$ and 29$^{th}$ April 2010 respectively.

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 transferred to suitably labeled polypropylene tubes and re-checked to ensure transfer of plasma to the correct tubes and were stored in suitably labeled tubes below -15°C, pending transfer to the analytical facility for assay.

Plasma samples of period I and period II were packed with dry ice and transferred to the analytical facility on 10 May 2010 & 13 May 2010, respectively.

3.4.3 Sample analysis
As per the protocol, a total number of 1704 blood samples (excluding pre-dose duplicate samples) were to be collected from 24 subjects in two periods. However, since Subject No. 19 did not report for giving his 48.0 post-dose blood sample in period I, a total of 1703 blood samples were collected for the estimation of drugs in plasma and were used for analysis.

During the study, the assigned doses were administered under the supervision of trained study personnel. The oral cavity was checked after dosing to ensure compliance with the treatment. Compliance was also ensured by measuring plasma Levetiracetam (during the analytical phase of the study).

3.4.4 Batch Acceptance Criteria
All the batches were evaluated rigorously and considered for the repeat analysis if failed with respect to any of the following criteria.
Chapter 3

Materials and Methods

3.4.4.1 Calibration curve acceptance criteria-

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

1. Accuracy of the LOQ in the standard curve was within ± 20% of the nominal value and within ±15% for other concentrations.
2. At least 75% or a minimum of six calibration standards meet the above criteria, including LOQ and ULOQ standard.
3. Linear coefficient of correlation ≥ 0.98.
4. In case the first injected LOQ or ULOQ standard fails/is lost/exhibits bad chromatography, its duplicate can be considered for linear regression analysis.

3.4.4.2 Blank and blank plus internal standard acceptance criteria-

1. Batch acceptance required that one Blank and one Blank+ISTD be free of significant interference at the retention times of the analyte and internal standard.
2. Interference was deemed significant if the peak area response at the retention time of the analyte in the blank, blank+ISTD is > 20% of the peak area response of the LOQ standard.
3. Interference was deemed significant if the peak area response at the retention time of the internal standard in the blank is > 5% of the mean ISTD response of the standards used in the calculation of calibration curve.

3.4.5 Protocol deviations

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

PHARMACOKINETIC AND STATISTICAL ANALYSES

Pharmacokinetic and statistical analyses were performed on data from all 24 subjects who completed both periods of study. Pharmacokinetic analyses was performed using WinNonlin PK software, Version 5.0.1 while the statistical analyses was performed using the SAS system for Windows, release 9.1.3 (SAS Institute Inc., USA).
3.5 PHARMACOKINETIC ANALYSIS

The concentration data obtained from analytical study was entered in WinNonlin pharmacokinetic software for further processing. The Non-Compartmental Analysis for deriving pharmacokinetic parameters was performed with WinNonlin version 5.0.1.

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

- \( \text{AUC}_{0\rightarrow t} \): The area under the plasma concentration versus time curve, from time zero to the last measurable concentration, as calculated by the linear trapezoidal method.
- \( \text{AUC}_{0\rightarrow \infty} \): The area under the plasma concentration versus time curve, from time zero to infinity.
- \( \text{AUC}_{0\rightarrow \infty} \) was calculated as the sum of \( \text{AUC}_{0\rightarrow t} \) plus the ratio of the last measurable plasma concentration to the elimination rate constant.
- \( \text{AUC}_{0\rightarrow 24} \): The area under the plasma concentration versus time curve, from time zero to 24 h.
- AUC % 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 \([\frac{\text{AUC}_{0\rightarrow \infty} - \text{AUC}_{0\rightarrow t}}{\text{AUC}_{0\rightarrow \infty}}] \times 100\)
- \( C_{\text{max}} \): 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, \( T_{\text{max}} \) was 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).
- \( T_{1/2} \): The apparent first-order terminal elimination half-life was calculated as \( 0.693/K_{\text{el}} \).

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

3.6 STATISTICAL ANALYSIS

Statistical analyses was performed on plasma Levetiracetam using the SAS system for Windows, release 9.1.3 or above or WinNonlin PK Software, Version 5.0.1 or above. The analysis included data from all 24 subjects who completed the study.
3.6.1 Summary Statistics

Arithmetic means, standard deviations and coefficients of variation were calculated for the parameters listed in section 3.5 on Pharmacokinetic analysis. Additionally, geometric means and percentage coefficient of variation of geometric means were calculated for $C_{\text{max}}$, $\text{AUC}_{0\rightarrow t}$, $\text{AUC}_{0\rightarrow 24}$ and $\text{AUC}_{0\rightarrow \infty}$.

3.6.2 Analysis of Variance (ANOVA)

The log-transformed pharmacokinetic parameters ($C_{\text{max}}$, $\text{AUC}_{0\rightarrow t}$, $\text{AUC}_{0\rightarrow 24}$ and $\text{AUC}_{0\rightarrow \infty}$) 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 included calculation of least-squares means, the difference between the adjusted formulation means and the standard error associated with the difference.

The above analyses was done using the appropriate SAS® procedure or the WinNonlin PK Software, Version 5.0.1 or above

3.6.3 90% Confidence Intervals and Ratio Analysis

90% confidence interval for the ratio of the test and reference product averages (least square means) for $\text{AUC}_{0\rightarrow t}$ and $\text{AUC}_{0\rightarrow 24}$ was calculated for Levetiracetam by first calculating the 90% confidence interval for the differences in the averages (arithmetic means) of the log-transformed data and then taking the anti-logs of the obtained confidence limits. The comparison of interest is T vs R, so the ratios was in the form:- Test/Reference.

Ratio of means was calculated using the LSM for log-transformed $C_{\text{max}}$, $\text{AUC}_{0\rightarrow t}$, $\text{AUC}_{0\rightarrow 24}$ and $\text{AUC}_{0\rightarrow \infty}$. Ratio of means was expressed as a percentage of the LSM for the reference formulations.