3.0 MATERIALS AND METHODS

Objective

This study was planned to determine and compare the rate and extent of absorption of Rasagiline between test formulation of Rasagiline 1 mg (containing rasagiline mesylate equivalent to 1mg of rasagiline base; total dose rasagiline 2mg) of Ranbaxy Laboratories Limited, with Azilect tablets 1 mg (containing rasagiline mesylate equivalent to 1mg of rasagiline base; total dose rasagiline 2mg) of Teva Neuroscience, Inc., in healthy, adult, human male subjects under fasting condition.

Experimental Design

Clinical part of the study was carried out at Ranbaxy Clinical Pharmacology Unit (CPU), HAH Centenary Hospital New Delhi. Analytical part of the study was carried out at Clinical Pharmacology and Pharmacokinetics, Ranbaxy Research Laboratory, Gurgaon, India. The project was divided into following sections:

3.1 Protocol Preparation and Approval
3.2 Clinical Study Methodology
3.3 Bioanalytical Methodology
   o Bioanalytical Method Development
   o Bioanalytical Method Validation
   o Analysis of Clinical Study Samples
3.4 Pharmacokinetic and Statistical Analysis

3.1 Protocol Preparation and Approval

The protocol and informed consent form for this study was prepared as per global regulatory guidelines on BA/BE studies. Final version of the protocol (Annexure I) along with ICF (Annexure II) was submitted to Jamia Hamdard Institutional Review Board (JHIRB). All the subjects provided the written informed consent after attending an oral presentation and after thoroughly reading the final version of the informed consents form (ICF). Clinical, bioanalytical, PK/SAS was done as per the approved protocol.
3.2 Clinical Methodology

The clinical phase of the study was initiated after the final approval of protocol by IRB.

3.2.1 Products Evaluated

Reference (R)

AZILECT 1mg tablet containing (rasagiline mesylate equivalent to 1 mg of rasagiline base) manufactured by Teva Pharmaceutical Industries Ltd. Kfar Saba, 44102 Israel, marketed by Teva Neuroscience, Inc., Kansas city, MO 64131.

Test (T)

Rasagiline 1 mg tablet (containing rasagiline mesylate equivalent to 1 mg of rasagiline base) of Ranbaxy Laboratories Limited, India.

3.2.2 Study Design

An open label, balanced, randomized, single-dose, two-treatment, two-period, two-sequence, crossover, bioequivalence study comparing two tablets of rasagiline 1 mg tablet (each containing rasagiline mesylate equivalent to 1 mg of rasagiline base; total dose rasagiline 2 mg) of Ranbaxy Laboratories Limited with two tablets of AZILECT 1 mg (each containing rasagiline mesylate equivalent to 1 mg of rasagiline base; total dose rasagiline 2 mg) of Teva Neuroscience, Inc. in healthy, adult, human, male subjects under fasting condition.

3.2.3 Study Site

The study was conducted at Ranbaxy Clinical Pharmacology Unit, 2nd Floor HAH Centenary Hospital New Delhi-110 062.

3.2.4 Selection of Subjects

Eighteen (18) healthy, adult, human, male subjects were admitted in the first period of the study. Subsequent dropouts and/or withdrawals were not replaced. Data was presented on all completed subjects. As per the CDSCO bioequivalence guideline minimum 16 completed subjects are required. However in order to take care of dropouts and withdrawals a sample size of 18 subjects was taken.
3.2.5 Screening Assessments

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

Table 3.1 Laboratory parameters assessed for each volunteers during screening procedure

<table>
<thead>
<tr>
<th>HEMATOLOGY</th>
<th>BIOCHEMISTRY</th>
<th>URINALYSIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Haemoglobin</td>
<td>• BUN</td>
<td>• Routine Examination</td>
</tr>
<tr>
<td>• Total leukocyte count</td>
<td>• Creatinine</td>
<td>- Colour</td>
</tr>
<tr>
<td>• Differential leukocyte</td>
<td>• Total bilirubin</td>
<td>- Appearance</td>
</tr>
<tr>
<td>count</td>
<td>• ALP</td>
<td>- PH</td>
</tr>
<tr>
<td>• Platelet count</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>SEROLOGY</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• HIV I &amp; II</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• HBsAg</td>
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<td></td>
</tr>
<tr>
<td>• HCV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• VDRL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OTHER</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• ECG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

3.2.6 Inclusion Criteria

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

3.2.7 Exclusion Criteria

• Subject has history of hypersensitivity to rasagiline mesylate and/or other related drugs like MAO inhibitors.
• Subject has history of hypertensive crisis precipitated by intake of tyramine rich foods and/or medications.
• Subject has history of arthalgia.
• Subject has history of dyspepsia.
• Subject has history of depression.
• Subject has clinically abnormal ECG or Chest X-ray.
• Subject has history of recurrent episodes of headache.
• Subject has history of drug-induced rash and/or pruritis.
• Presence of disease markers of HIV 1 or 2, Hepatitis B or C viruses or syphilis infection.
• Presence of values which are significantly different from normal reference ranges and/or judged clinically significant for haemoglobin, total white blood cells count, differential WBC count or platelet count.
• Positive for urinary screen testing of drugs of abuse (opiates or cannabinoids) and/or breath alcohol test.
• Subject has history of serious gastrointestinal, hepatic, renal, cardiovascular, pulmonary, neurological or haematological disease, diabetes or glaucoma.
• Subject has history of any psychiatric illness which may impair the ability to provide written informed consent.
• Subject has any evidence of organ dysfunction or any clinically significant deviation from the normal, in physical or clinical determinations.
• 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 has history of drug dependence or excessive alcohol intake on a habitual basis or has difficulty in abstaining or found positive in alcohol breath test before admission in period-I of study.
• Subject has used any medication within 30 days prior to admission of this study.
• Subject has participated in a clinical trial within 90 days preceding admission of this study (except for subjects who dropout / are withdrawn from the previous study prior to period-I dosing).

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

3.2.8 Study Medication

3.2.8.1 Handling, Storage and Accountability Procedures

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

3.2.8.2 Assignment to Treatment Sequences

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

3.2.9 Number of Subjects

Enough healthy adult male subjects were enrolled to allow dosing of eighteen (18) subjects in the first period of the study. Subsequent dropouts/withdrawals were not replaced.

3.2.10 Admission and Stay

Subjects were admitted and housed in the Clinical Pharmacology Unit from at least 11 hours before dose administration and were discharged 24 hours after the dose administration during each period, if the subjects do not suffer from any adverse drug reaction. In case of an adverse event, the subjects were monitored until the event subsides.

3.2.11 Dose

The dosing of test and reference was carried out as per randomization schedule. The total dose of test and reference formulation was 2 mg.
Reference (R)

A single oral dose of two tablets of AZILECT 1 mg (each containing rasagiline mesylate equivalent to 1 mg of rasagiline base; total dose rasagiline 2 mg) of Teva Neuroscience Inc. was administered with 240 mL drinking water at ambient temperature after an overnight fast of at least 10 hours.

Test (T)

A single oral dose of two tablets of rasagiline 1 mg (each containing rasagiline mesylate equivalent to 1 mg of rasagiline base; total dose rasagiline 2 mg) of Ranbaxy Laboratories Limited, India, was administered with 240 mL drinking water at ambient temperature after an overnight fast of at least 10 hours.

3.2.12 Fasting/Meals

All subjects were on overnight fast after admission for at least 10 hours before the morning dose and for 4 hours post-dose. They received standard meals – lunch snacks and dinner at 4, 9 and 13 hours after drug administration. During housing, all meal plans were identical for all periods. Information on the amount of meal consumed and the time taken for consuming the meal was recorded in the appropriate clinical raw data sheets. In case meals and blood sample collection coincide, sample was collected before meals. Drinking water was not allowed from 1 hour before dosing until 2 hours post-dose. Thereafter, it was allowed at all times.

3.2.13 Sampling Schedule

The minimum blood sample volume required for analytical purpose in this study is 4-mL. The blood samples were collected in 4 ml K3-EDTA vacutainers at the following time points after administration of test/ reference formulation in the each period.

**Reference (R)**: Pre-dose (duplicate), 0.083, 0.167, 0.250, 0.333, 0.417, 0.500, 0.583, 0.667, 0.750, 0.833, 1.000, 1.250, 1.500, 1.750, 2.000, 2.500, 3.000, 4.000, 6.000, 8.000, 10.000, 12.000, 16.000, 20.000 and 24.000 hours post dose in each period. Samples were collected and processed under low light condition.
Test (T) : Pre-dose (duplicate), 0.083, 0.167, 0.250, 0.333, 0.417, 0.500, 0.583, 0.667, 0.750, 0.833, 1.000, 1.250, 1.500, 1.750, 2.000, 2.500, 3.000, 4.000, 6.000, 8.000, 10.000, 12.000, 16.000, 20.000 and 24.000 hours post dose in each period.

A total of fifty two (52) 4 mL blood samples (excluding duplicate pre-dose samples) were collected in K3 EDTA pre-chilled vacutainers maintained in wet ice bath from each subject during the course of the study through indwelling cannulae placed in forearm veins. The pre-dose blood sample (in duplicate) in each period was collected within a period of 1.5 hours before dosing. All post-dose blood samples were collected within ±2 minutes (during their in-house stay) of the scheduled collection time and within ±1 hr of the scheduled collection time (during each ambulatory). The actual end-point time of collection of each blood sample was recorded.

Thus, for each subject, the total number 4-mL blood samples processed was 54 (including two pre-dose duplicate blood samples) and the total volume of blood drawn- including 16 mL for screening, 8 mL for predose duplicate sample, 25 mL 'discarded' blood prior to venous cannula collections and 8 mL for safety analysis at the end of the study- did not exceed 265 mL.

3.2.14 Sample Separation and Storage

After collection, blood samples from all the subjects at each time-point, was centrifuged at under refrigeration as soon as possible to separate plasma. All plasma samples were transferred to suitably labeled tubes and re-checked to ensure transfer of plasma to the correct tube. The plasma was stored at below −15°C under low light condition until analysis.

3.2.15 Wash-out Period

There was a washout period of at least 07 days between the administrations of study drugs in each period.

3.2.16 Restrictions

3.2.16.1 Medications

Subjects did not receive any medication (except vitamins preparations) including OTC medications during the 2 weeks period prior to the onset of the study. They were instructed
during screening not to take any prescription and over the counter (OTC) medications until the completion of the study.

3.2.16.2 Diet

All subjects abstained from alcohol / caffeine / xanthine and grapefruit juice and/or supplements containing any of these products for 48 hours prior to dosing and during in-house stay in each period.

Instructions were given to the subjects to avoid tyramine rich products (all aged cheese products or nonpasturized dairy products) 48 hours prior to dosing up till 14 days from the day of receiving the last dose.

3.2.16.3 Activity

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

3.2.17 Clinical Safety Measurements

3.2.17.1 Vital Signs Recording

Vital signs– oral temperature, supine and standing BP and radial pulse measurements was performed at admission, prior to morning dose (within 1.5 hours of the scheduled dosing time) and at 2, 6, 12 and 24 hours (within 1.5 hour of the scheduled time) after administration of morning dose in each period. In the event of detection of any abnormality during measurement of vital signs, the Principal Investigator was consulted for necessary action, which was recorded.

3.2.17.2 Clinical Examination

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

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

3.2.18 Adverse Events

Subjects were monitored throughout the study periods for adverse events. Subjects were asked to bring to the notice of the nurse or the doctor any adverse event that may occur during their stay at the site of investigation.

3.2.19 Drop-Out/Withdrawal of Subjects from the Study

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

3.2.20 Volunteer Compensation

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

3.2.21 Ethical Considerations

3.2.21.1 Basic Principles

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

3.2.21.2 Institutional Review Board

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

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

3.3 Bioanalytical Methodology

This phase of the study was further divided in following parts-

3.3.1 Bioanalytical Method Development

3.3.2 Bioanalytical Method Validation

3.3.3 Analysis of Samples

3.3.1 Bioanalytical Method Development

A precise, accurate and reproducible LC-MS/MS method was developed for the simultaneous estimation of Rasagline mesylate in human plasma. The method was developed according to FDA guidelines.\textsuperscript{131} Stable labeled Rasagiline was used as the internal standard in this assay. The structure of Rasagiline is shown below.

\[
\text{Rasagiline: Molecular Weight: 267.34; Chemical Formula: } \text{C}_{13}\text{H}_{17}\text{NO}_{3}\text{S}
\]
3.3.1.1 Materials and Equipment

A. Analytical Column

Phenomenex: Luna C-18 (2), 150 x 4.6mm

B. Chemicals and Reagents

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Company</th>
<th>Batch No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile HPLC grade</td>
<td>Fisher</td>
<td>CAT # A998-4</td>
</tr>
<tr>
<td>Methanol HPLC grade</td>
<td>Fisher</td>
<td>CAT # A452-4</td>
</tr>
<tr>
<td>Methyl-tert Butyl Ether</td>
<td>J.T.Baker</td>
<td>CAT 9034-03</td>
</tr>
<tr>
<td>Water HPLC grade</td>
<td>Milli-Q® purified water prepared in-house</td>
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</tr>
<tr>
<td>Ammonium Hydroxide 28-30%</td>
<td>J.T.Baker</td>
<td>CAT 9721-01</td>
</tr>
<tr>
<td>Formic Acid</td>
<td>Acros</td>
<td>CAT # 200-579-1</td>
</tr>
<tr>
<td>Human Plasma</td>
<td>Bio-Reclamation, NY</td>
<td>BRH 307394-400</td>
</tr>
</tbody>
</table>

C. Consumables

<table>
<thead>
<tr>
<th>Product</th>
<th>Company</th>
</tr>
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<tbody>
<tr>
<td>16x125 mm screw top tube</td>
<td>Fisher</td>
</tr>
<tr>
<td>16x100 culture tubes</td>
<td>Fisher</td>
</tr>
<tr>
<td>Micro-vials (polypropylene) with fused conical inserts</td>
<td>Sun Int.</td>
</tr>
<tr>
<td>Snap cap, 11 mm, silicone/PTFE</td>
<td>Sun Int.</td>
</tr>
<tr>
<td>Combi-tips 5, 10, 25 and 50 mL for repeater pipette</td>
<td>Eppendorf</td>
</tr>
</tbody>
</table>
Chapter 3
Materials and Methods


<table>
<thead>
<tr>
<th>Dry Ice for flash freezing</th>
<th>In-house supply</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipette Tips</td>
<td>Fisher</td>
</tr>
</tbody>
</table>

D. Auxiliary Equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single-tube vortex</td>
<td>VWR Scientific Products</td>
</tr>
<tr>
<td>Centrifuge</td>
<td>Beckman; model GRR</td>
</tr>
<tr>
<td>Analytical Balance</td>
<td>Mettler; model AE163</td>
</tr>
<tr>
<td>Shaker</td>
<td>Janke and Kunkle Gmbh; model HS-500</td>
</tr>
<tr>
<td>Turbovap® LV Evaporator</td>
<td>Zymark Corp.</td>
</tr>
<tr>
<td>Milli-QTM Water Purification System</td>
<td>Millipore Corp.</td>
</tr>
<tr>
<td>Repeater pipette</td>
<td>Eppendorf; Repeater Plus®</td>
</tr>
<tr>
<td>Pipettes</td>
<td>Rainin edp2 100 and 250 μl</td>
</tr>
</tbody>
</table>

E. Reference Standards

Compound: Rasagiline Mesylate

Lot number: 16-ANR-169-1

Source: TRC

Purity: 98.0%

Internal Standard: Rasagiline-13C3 Mesylate

Lot number: 6-THT-99-2
F. Preparation of Stock Solutions

1. Rasagiline Standard Solution

- Rasagiline reference standard was weighed accurately and transferred to a volumetric flask.
- It was dissolved in acetonitrile: methanol (1:1 v/v) and the volume was made up with the same to prepare a solution of approximately 1 mg/ml.
- The final concentration of Rasagiline was corrected accounting for its potency, molecular weight and the actual amount weighed.
- It was stored in refrigerator between 4°C, protected from light in a polypropylene container and used within 7 days from the date of its preparation.
- Stock solution and all further dilutions from above stock were prepared under low light conditions.

2. Rasagiline Internal Standard Solution

- The stable label internal standard was weighed accurately and transferred to a volumetric flask.
- It was dissolved in methanol (HPLC grade) and volume was made up with the same to prepare a solution of approximately 50 ng/ml.
- The final concentration of Rasagiline IS was corrected accounting for its potency, molecular weight and the actual amount weighed.
- It was stored in refrigerator between 4°C, protected from light in a polypropylene container and used within 7 days from the date of its preparation.

G. Preparation of Reagents and Internal Standard Solutions

1. Mobile Phase: Mobile phase consists of solution A and solution B.

Solution A: Acetonitrile

Solution B: 0.1 % Formic Acid
Solution B was prepared by adding 1.0 mL of formic acid per 1L of purified water.

2. Makeup Flow Auxiliary “AUX” Solution: Make up flow “AUX” solution consists of 50% methanol, 50% 0.1 % Formic Acid. ”AUX“ is prepared by adding 500mL of methanol and 500mL of 0.1% formic acid in water. Solution is premixed, degassed and delivered using an auxiliary pump.

3. Autosampler Wash Solution: The autosampler wash solution is the same as the makeup flow “AUX” solution.

4. Internal Standard Spiking Solution: The stable label IS spiking solution consists of Rasagiline (Internal Standard) at 50 ng/mL. The spiking solution is prepared in methanol and was stored at 4°C.

5. 0.5 % Ammonium Hydroxide Buffer Solution: This solution is prepared by adding 1.25mL of ammonium hydroxide to 250 mL of purified water.

6. Recostitution Reagent: The recostitution reagent consists of Acetonitrile: Water: 0.1 % Formic Acid (70:30:1,v/v/v). This reagent is prepared by adding 700mL of acetonitrile, 300 mL of purified water, and 1.0 mL of formic acid.

H. Sample Preparation

1. All standards, QC’s and samples were kept at room temperature.

2. 500 μL of standard, QC, and study sample was pipetted out directly into a 16x125 tube.

3. 50 μL of IS solution was added into the tube.

4. 200 μL of buffer was added into tubes and tubes were vortexed for proper mixing.

5. 6.0 mL of Methyl-tert Butyl Ether was added and then tubes were capped and further shaked @ 200spm for 10 min.

6. Thereafter centrifugation was done for approximately 5 min at 3600 rpm @ 5°C.

7. Aqueous layer was flash freezeed by placing test tubes in dry ice/methanol (or acetone) for approximately 1-3 minutes. Organic layer was transfered to a clean tube by pouring out the organic layer.
8. Solvent was evaporated under a gentle stream of nitrogen in a water bath at 23°C.

9. Solution was reconstituted with 300uL of reconstitution reagent (3.7.6), then vortexed, and transferred to a micro-vial.

10. Vials were capped and centrifuged again for 5 min @ 3000rpm.

11. Finally sample analysis was done by LC/MS/MS.

**I. Instrumentation**

**1. Analytical System**

Detector: PE Sciex API-3000 Mass Spectrometer

HPLC Pumps: PE series 200 dual Micropump, gradient system

Auxiliary Pump: PE series 200 LC Pump

Flow diverter: Valco switching valve

Autosampler: PE series 200 autosampler

Data System: Analyst Version ver. 1.3.2.

**2. Mass Spectrometer Conditions**

Ion Source: Turbo Ion Spray

Scan Mode: MRM

Polarity: Positive Ion Mode

Auxiliary gas: Air at 8L/min, flow rate

Nebulizer gas: Nitrogen at setting 10

Curtain gas: Nitrogen at setting 8

CAD gas: Nitrogen at setting 10
3. MS Voltages

IS Voltage: 5000v
Temperature: 400°C

Data Acquisition: Duration 5 min.

Volatges: DP: 10, FP: 70, EP: 6, CE: 15, CXP: 10

Ion Channels (m/z) \(Q_{1} \rightarrow Q_{3}\) Dwell Time (msec)

Rasagiline: 172.2 → 117.2 300
Rasagiline IS: 175.2 → 117.2 150

Resolution Offset: Q1 Unit, Q3 Unit
Settling Time: 0 msec
Pause time: 5 msec

4. Chromatographic Conditions

a. HPLC Settings:

Analytical Column: Phenomenex: Luna 5μm, 150 x 4.6 mm with C18 guard column
Column Temperature: 45°C
Back Pressure: 650psi
Mobile Phase: Solutions A & B

b. Flow Diversion: Column flow was directed to mass spectrometer between 1.5 – 3.0 minutes after injection. During the rest of the run the column flow was diverted to waste while an auxiliary pump provided makeup flow to the ion source at 350 μL/min (Flow diversion times may vary depending on retention times).

c. Retention Times

Rasagiline and IS: 2.3 minutes.
d. Autosampler settings:

Loop Volume: 50 μL
Injection Volume: 20 μL
Needle Level: 25%
Air Cushion: 10 μL
Excess Volume: 10 μL
Autosampler Temperature: 5°C
Flush Volume: 0.5 mL each for a total of 5 washes (1 pre and 4 post) at very slow speed.

5. Quantitation

LC/MS/MS data was acquired using Analyst v1.3.2 software. The calibration curve for rasagiline was constructed as a function of the weighted (1/x^2) linear regression of the theoretical concentration versus response ratio (where response ratio = ratio of analyte peak area to internal standard peak area).

3.3.2 Bioanalytical Method Validation

The validation of this procedure was performed in order to evaluate the method in terms of selectivity, linearity, precision, accuracy, sensitivity, recovery and stability (CDER, 2001). The linearity, precision and accuracy evaluations were performed on three batches of spiked plasma samples. Each batch of spiked plasma samples included one complete set of calibration standards (Blank, blank plus internal standard, LLOQ (Std 1), ULOQ (Std 8) each in duplicate and Std-2, 3, 4, 5, 6 and 7) and six replicates of each quality control samples namely limit of quantification quality control- QC_LLOQ, lower quality control- QC_Low, medium quality control- QC_Mid and higher quality control- QC_High.

3.3.2.1 System Suitability Test

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

The % CV of the ratio of drug/internal standard should be less than 4%, and % CV for retention time should be less than 10%.

3.3.2.2 Carryover

Sample to sample carryover was evaluated by running a blank or wash after the highest standard and assessing the relative peak area of this blank versus the signal at LLOQ.

The difference in the area of first injection and the injection after system suitability should not be more than 0.1%.

3.3.2.3 Selectivity in Normal, Haemolysed and Lipemic Human Plasma

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

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

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

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

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

3.3.2.4 Bulk Spiking

3.3.2.4.1 Preparation of Calibration Standards Samples

Calibration standards for Rasagiline were prepared by serial dilution with analyte free plasma to obtain 7 concentration levels ranging nominally from 0.04 to 39.0 ng/mL.

<table>
<thead>
<tr>
<th>Table 3.2: Calibration Standards in K3 EDTA Human Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Designations</td>
</tr>
<tr>
<td>Target Conc. (ng/ml)</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>1000.0</td>
</tr>
<tr>
<td>40.0</td>
</tr>
<tr>
<td>20.0</td>
</tr>
<tr>
<td>5.0</td>
</tr>
<tr>
<td>1.0</td>
</tr>
<tr>
<td>0.5</td>
</tr>
<tr>
<td>0.1</td>
</tr>
<tr>
<td>0.04</td>
</tr>
</tbody>
</table>

- Aliquots of S1 were also used as LLOQ during validation.
- The S8 was used for dilution purposes only and was not used in the calibration curve.
- Standards were stored at -15°C.
- Samples were prepared and stored in polypropylene tubes.
3.3.2.4.2 Preparation of Quality Control Samples

Quality control samples (QCs) for Rasagiline in human plasma were similarly prepared, to obtain 3 concentration levels per analyte designated as QC_Low, QC_Mid, and QC_High. (see table 3.3)

Table 3.3: Quality Control’s in K3 EDTA Human Plasma

<table>
<thead>
<tr>
<th>Designations</th>
<th>Dilutions</th>
<th>Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target Conc. (ng/ml)</td>
<td>Level Name</td>
<td>Take Amount (ml)</td>
</tr>
<tr>
<td>1200</td>
<td>Dil</td>
<td>25 µL</td>
</tr>
<tr>
<td>33.0</td>
<td>High</td>
<td>0.88</td>
</tr>
<tr>
<td>6.0</td>
<td>Mid</td>
<td>4.50</td>
</tr>
<tr>
<td>0.12</td>
<td>Low</td>
<td>0.50</td>
</tr>
</tbody>
</table>

QCs were stored at -15°C.
Samples were prepared and stored in polypropylene tubes.

3.3.2.5 Sensitivity

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).

3.3.2.6 Weighing Factor Optimization

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

Calibration range was selected on the basis of earlier published reports so that it can be effectively utilized for the bioavailability study analysis. The calibration range for rasagiline was selected to be 0.041 to 39.023 ng/ml. The calibration curve linearity was determined on three standard curves and slope, intercept and correlation coefficient were determined. For calculation of the standard curve plots, area ratio of analyte and internal standard area against concentration were determined.

3.3.2.8 Precision and Accuracy

Three Precision and Accuracy batches (PA batches) each consisting of a reference standard solution (aqueous mix), 14 calibration standards (standard blank, standard zero, standard 1 (LOQ), standard 7 (ULOQ); each in duplicate and standard-2, 3, 4, 5, 6, 7 and six replicates of each limit of quantification (0.041 ng/ml), low (0.110 ng/ml), middle (5.592 ng/ml) and high (32.933 ng/ml) quality control samples, interspersed within each other, were processed and analyzed according to the proposed method.

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

For the acceptance, between, intraday (using two PA batches run on the same day) and within batch CVs for QC samples should be ≤ 15 % (≤ 20 % for LOQ QC) and % Nominal of QC samples should be within ± 15 % (within ± 20 % for LOQ QC).

3.3.2.9 Dilution Integrity

In order to test for dilution bias when diluting samples with concentrations above the upper limit of the calibration curve, a QC level with high concentration (1015.6µg/ml) was prepared (designated as QC_Dil). Dilution consisted of transferring 50-µL of the QC_Dil sample to a 16x100 tube and mixing with 450-µL of blank plasma for a dilution factor of 10. Then 100-µL of the 1:10 diluted QC_Dil sample was transferred to a 16x125 tube and mixing with 400-µL of blank plasma for a total dilution factor of 50. Sample workup followed the normal procedure. Six replicates of QC_Dil sample were analyzed by back calculation using regression equation obtained. The % CV and % nominal were calculated for dilutions.
The integrity of the samples were considered to be maintained if % stability was within $\pm 15\%$ of nominal values and % CVs $\leq 15\%$ for dilutions.

### 3.3.2.10 Extraction Recovery

Standard aqueous quality control stock of rasagiline each at low, medium, and high levels were spiked in plasma and solvent separately, the latter being considered as nonextracted quality control samples. Three replicates of each quality control plasma samples were processed as usual and analyzed along with three replicates of nonextracted 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 rasagiline, SD and % CV were calculated at each QC level. % recovery was calculated as follow:

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

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

### 3.3.2.11 Matrix Effect

Matrix effect was evaluated by taking six different batches of blank plasmas spiked with the analyte at a low concentration (0.106 ng/mL). The matrix effect samples were prepared by diluting the S4 standard (1.063ng/ml) 1:10 with 6 independent blank plasmas and quantitated against a standard curve.

The acceptance criteria of the mean % nominal for QC-Low and QC-High should be between 85-115% and % CV at both QC-Low and QC-High should be less than 15%.
3.3.2.12 Stability Evaluation

A- Bench Top Stability

Three replicates of each low and high concentration quality control samples (QC-Low and QC-High) were taken from cold room, thawed and kept in ice cold water bath, kept unprocessed for 4 hours (stability sample), then processed along with freshly spiked calibration standards and analyzed by back calculation using regression equation obtained. The % CV and % nominal were calculated at both QC-Low, QC-Mid and QC-High levels. *Samples were deemed stable after specified bench top period if % stability was within ± 15 % of nominal values and % CVs ≤ 15 % at QC-Low, QC-Mid and QC-High levels.*

B- Freeze-Thaw Stability

Three replicates of each low, mid and high concentration quality control samples (QC-Low, QC-Mid and QC-High) were removed from deep freezer, thawed unassisted in ice cold water bath, refrozen at -15°C 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 then analyzed by back calculation using regression equation obtained. The % CV and % nominal were calculated at both QC-Low, QC-Mid and QC-High levels. *Samples were deemed stable after three freeze thaw cycles if % stability was within ± 15 % of nominal values and % CVs ≤ 15 % at QC-Low, QC-Mid and QC-High levels.*

C- In-Injector Stability/Auto Sampler Stability

Three replicates of each low, mid and high concentration quality control samples were processed and kept in the auto-sampler at 5°C for at least 72 hours (stability sample). Samples were then run and analyzed along with freshly spiked calibration standards and analyzed by back calculation using regression equation obtained. The % CV and % nominal were calculated at QC-Low, QC- Mid and QC-High 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 QC-Low, QC-Mid and QC-High levels.*
D- Stock Solution Stability

Stock solution stability ensures stability of analyte and internal standard in the respective solvents at their storage conditions for certain duration. The stock solution stability was evaluated by injecting three replicates of each low, mid and high concentration quality control samples (stability sample) stored in refrigerator (1-10°C) and comparing the response with freshly prepared stock solution (comparison sample). The stock solution stability of the internal standard stock was evaluated by same process.

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

% CV and % stability for peak area ratio obtained from stability and comparison stock dilutions were calculated.

*The samples were considered to be stable for the specified period if % stability was within ± 15 % of nominal values and % CVs ≤ 15 % at QC-Low, QC-Mid and QC-High levels.*

E-Long-Term Stability

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

3.3.2.13 Standardization and calculations

The chromatographic data were acquired and processed using computer based Analyst software. The best-fit curves using weighted (1 /amount^2) linear least square regression analysis were obtained by peak area ratio of Rasagiline to Rasagiline IS. The concentrations
of Rasagiline in plasma samples were calculated using linear regression parameters of the corresponding calibration curve.

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

3.3.3 Analysis of Clinical Study Samples

3.3.3.1 Objective

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

3.3.3.2 Sample Analysis

As per the protocol, all the samples collected from 18 subjects in over two periods were used for analysis. For sample analysis by LC-MS/MS, one analytical batch consisted of samples which include all the samples of one subject (54) in two periods along with the calibration standards (14) and quality control samples (three each of QC-Low, QC-Mid and QC-High).

Batch Acceptance Criteria:

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

A- Calibration Curve Acceptance Criteria

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

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

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

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

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

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

1. Peak area responses of the blanks at the retention time of the rasagiline were < 20% of the peak area response of the LOQ standard and

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

C- Quality Control Sample Acceptance Criteria

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

D- Repeat Analysis

Samples were subjected to repeat analysis using following criteria.

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

3.4 Pharmacokinetic and Statistical Analysis

3.4.1 Pharmacokinetic Analysis

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

The following pharmacokinetic parameters were calculated for rasagiline using WinNonlin Node software.

\[
\text{AUC}_{0-t}:
\]

The area under the plasma concentration versus time curve, from time zero to the last measurable concentration, as calculated by the linear trapezoidal method.
AUC\textsubscript{0-24}: The area under the plasma concentration versus time curve, from time zero to 24 h.

AUC\textsubscript{0-\infty}: The area under the plasma concentration versus time curve, from time zero to infinity. AUC\textsubscript{0-\infty} is calculated as the sum of AUC\textsubscript{0-t} 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 was calculated as \[(AUC\textsubscript{0-\infty} - AUC\textsubscript{0-t})/AUC\textsubscript{0-\infty}] * 100

\text{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.

Ke\textsubscript{L}: 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/Ke\textsubscript{L}.

No value of Ke\textsubscript{L}, AUC\textsubscript{0-\infty} or T_{1/2} was reported for cases that do not exhibit a terminal log linear phase in the concentration versus time profile.
3.4.2 Statistical Analysis

Statistical analysis was performed on plasma rasagiline using the WinNonlin PK Software. The analysis included the data from all subjects who had completed the study.

3.4.3 Summary Statistics

Arithmetic means, standard deviations and coefficients of variation was calculated for the parameters listed in section 3.4.1. Additionally, geometric means and percentage coefficient of variation of geometric means was calculated for $AUC_{0-t}$, $AUC_{0-\infty}$ and $C_{\text{max}}$.

3.4.4 Ratio Analysis

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

3.4.5 Analysis of Variance (ANOVA)

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