3.0 MATERIALS AND METHOD

The objective of the study was to determine and compare the rate and extent of absorption of single dose of olopatadine hydrochloride 10 mg extended release tablet (two formulations) of Ranbaxy Laboratories Limited with two doses of Allelock® 5 mg tablets (each dose containing olopatadine hydrochloride 5 mg administered 12 hourly; total dose 10 mg) of Kyowa Hakko Kogyo Co. Ltd., in healthy, adult, human male subjects under fed condition.

The final version of the protocol and informed consent form (ICF) of the study were reviewed and approved by Jamia Hamdard Institutional Review Board (JHIRB), IRB approval letter is appended as annexure II. All the subjects provided the written informed consent after attending an oral presentation about study and after thoroughly reading the final version of the ICF. Protocol summary and ICF (English and Hindi) are appended as annexure III, IV & V. Clinical part of the study was carried out at Ranbaxy Clinical Pharmacology Unit (CPU) Majeedia Hospital New Delhi. Analytical part of the study was carried out at Dept. of Clinical Pharmacology and Pharmacokinetics (CPP), Ranbaxy Research Laboratory, Gurgaon, India. The experimental section has been divided into five parts:

- Clinical study methodology
- Bioanalytical methodology (method development and method validation)
- Analysis of clinical study samples
- Pharmacokinetic analysis
- Statistical analysis
3.1 CLINICAL STUDY METHODOLOGY

3.1.1 Products Evaluated

➢ **Reference (R):** Allelock® 5 mg tablet manufactured and distributed by Kyowa Hakko Kogyo Co. Ltd., Japan

➢ **Test (Two formulations A & B):** Olopatadine hydrochloride 10 mg extended release tablet manufactured by Ranbaxy Laboratories Limited, India

<table>
<thead>
<tr>
<th>Product</th>
<th>Batch. No.</th>
<th>Expiry date</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allelock® (Reference)</td>
<td>109AIH</td>
<td>August 2012</td>
<td>Kyowa Hakko Kogyo Co. Ltd., Japan</td>
</tr>
<tr>
<td>Test A</td>
<td>RV(4430)01</td>
<td>February 2012</td>
<td>Ranbaxy Laboratories Limited, India</td>
</tr>
<tr>
<td>Test B</td>
<td>RV(4430)05</td>
<td>February 2012</td>
<td>Ranbaxy Laboratories Limited, India</td>
</tr>
</tbody>
</table>

3.1.2 Study Design

The study was designed as an open label, balanced, randomized, three-treatment, three-period, three-sequence, crossover comparative bioavailability study comparing single dose of olopatadine hydrochloride 10 mg extended release tablet (two formulations) of Ranbaxy Laboratories Limited with two doses of Allelock® 5 mg tablets in healthy, adult, human male subjects under fed condition.

3.1.3 Number of Subjects

Adequate healthy, adult, human male subjects were enrolled and finally 15 subjects were admitted in study to allow dosing in all the three periods of the study. Subsequent drop-outs were not replaced. Data is presented on all the completed subjects.
3.1.4 Selection of Subjects
Adequate numbers of subjects were selected randomly from the volunteer bank of the clinical pharmacology unit (CPU) and all the subjects underwent a standardized screening procedure.

- **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 underwent physical examination and the laboratory tests of hematologic, hepatic and renal functions as listed in below table:

**Table 3.2: Laboratory tests carried out during screening of subjects**

<table>
<thead>
<tr>
<th>TEST</th>
<th>PARAMETERS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematology</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemoglobin</td>
</tr>
<tr>
<td></td>
<td>Total Leukocyte Count</td>
</tr>
<tr>
<td></td>
<td>Differential Leukocyte Count</td>
</tr>
<tr>
<td></td>
<td>Platelet count</td>
</tr>
<tr>
<td>Biochemistry</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Blood urea Nitrogen (BUN)</td>
</tr>
<tr>
<td></td>
<td>Creatinine</td>
</tr>
<tr>
<td></td>
<td>Total Bilirubin</td>
</tr>
<tr>
<td></td>
<td>Alkaline Phosphatase (ALP)</td>
</tr>
<tr>
<td></td>
<td>Aspartate aminotransferase (AST)</td>
</tr>
<tr>
<td></td>
<td>Alanine aminotransferase (ALT)</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td></td>
<td>Cholesterol</td>
</tr>
<tr>
<td>Urinalysis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Routine Examination</td>
</tr>
<tr>
<td></td>
<td>Microscopic Examination</td>
</tr>
<tr>
<td></td>
<td>Colour</td>
</tr>
<tr>
<td></td>
<td>RBC</td>
</tr>
<tr>
<td></td>
<td>Appearance</td>
</tr>
<tr>
<td></td>
<td>WBC</td>
</tr>
<tr>
<td></td>
<td>PH</td>
</tr>
<tr>
<td></td>
<td>E. cells</td>
</tr>
<tr>
<td></td>
<td>Specific Gravity</td>
</tr>
<tr>
<td></td>
<td>Crystals</td>
</tr>
<tr>
<td></td>
<td>Protein</td>
</tr>
<tr>
<td></td>
<td>Casts</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
</tr>
<tr>
<td>Additional Tests</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Human Immunodeficiency Virus (HIV I and II)</td>
</tr>
<tr>
<td></td>
<td>Hepatitis B Antigen (HBsAg)</td>
</tr>
<tr>
<td></td>
<td>Hepatitis C virus (HCV)</td>
</tr>
<tr>
<td></td>
<td>Venereal Disease Research laboratory (VDRL)</td>
</tr>
<tr>
<td></td>
<td>ECG</td>
</tr>
</tbody>
</table>
Only medically healthy subjects with clinically normal laboratory profiles were selected who met following inclusion criteria. 15 healthy, adult, human male subjects were selected based on the following inclusion and exclusion criteria.

➢ **Inclusion criteria**

- Be in the age range of 18-45 years
- Be neither overweight nor underweight for his height as per the Life Insurance Corporation of India height/weight chart for non-medical cases
- Have voluntarily given written informed consent to participate in this study
- Have hemoglobin ≥ 13.0 g/dL
- 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

➢ **Exclusion criteria**

The subjects were excluded who had any of the following exclusion criteria and finally fifteen healthy, adult, human male subjects were selected.

- History of hypersensitivity to olopatadine and any other related drugs
- History of chronic headache
- History of abdominal discomfort, diarrhea and/or nausea in the preceding week
- History of hepatic function disorder and/or jaundice
- History of any sleep disorder
- History of drug induced skin rashes and or/or pruritis
- Any evidence of organ dysfunction or any clinically significant deviation from the normal, in physical or clinical determinations
- Clinically abnormal ECG and/or laboratory test parameter(s), which is/are outside acceptable limits and is judged clinically significant by investigator
- History of serious medical illness including but not limited to gastrointestinal, hepatic, renal, cardiovascular, pulmonary, neurological or hematological disease, diabetes, glaucoma, any serious, potentially life-threatening illness
- Inability to communicate well (i.e. language problem, poor mental development, psychiatric illness or poor cerebral function) that may impair the ability to provide, written informed consent
- Regular smoker, who smokes more than 10 cigarettes daily or has difficulty abstaining from smoking for the duration of all the three periods of the study
- 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 the study
- Use of any medication within 30 days prior to admission of the study
- Participation in a clinical trial within 90 days preceding admission of the study (except for subjects who dropped out/was withdrawn from the previous study prior to period I dosing)

3.1.5 Study Schedule

- Period I of the study was conducted between dates 06 August 2010 to 08 August 2010.
- Period II of the study was conducted between dates 12 August 2010 to 14 August 2010.
- Period III of the study was conducted between dates 19 August 2010 to 21 August 2010.

During all the three periods of the study, subjects reported to Ranbaxy CPU at least 12 hours before dose administration on day 1. After sampling for 36 hours post dose as per schedule, subjects were discharged on the evening of day 2.

Detailed schematic representation of the study schedule is presented in the table 3.3.
### Table 3.3: Schematic representation of study design

#### For Treatment Test (A & B)

<table>
<thead>
<tr>
<th>AE, CE &amp; Vitals</th>
<th>Dinner</th>
<th>Vitals</th>
<th>Breakfast</th>
<th>Dosing</th>
<th>Vitals</th>
<th>Lunch</th>
<th>Snacks</th>
<th>Vitals</th>
<th>Dinner</th>
<th>Vitals</th>
<th>Breakfast</th>
<th>Lunch</th>
<th>Snacks</th>
<th>Dinner</th>
<th>Discharge, AE, CE &amp; Vitals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1300</td>
<td>2100</td>
<td>0700</td>
<td>0730</td>
<td>0815</td>
<td>0900</td>
<td>1100</td>
<td>1300</td>
<td>1800</td>
<td>1900</td>
<td>2015</td>
<td>2300</td>
<td>0700</td>
<td>0900</td>
<td>1300</td>
<td>1800</td>
</tr>
<tr>
<td>-21 hr (approx.)</td>
<td>-12 hr</td>
<td>-2.0 hr</td>
<td>-1.5 hr</td>
<td>0 hr</td>
<td>2 hr</td>
<td>4 hr</td>
<td>9 hr</td>
<td>10 hr</td>
<td>11.25 hr</td>
<td>14 hr</td>
<td>22 hr</td>
<td>24 hr</td>
<td>28 hr</td>
<td>33 hr</td>
<td>35 hr</td>
</tr>
</tbody>
</table>

**Admission**

Pre dose blood sample

Olopatadine hydrochloride 10 mg extended release tablet

AE monitoring was done at admission, prior to dosing and approximately at 2, 10, 14, 22 and 36 hrs post dose.

Post dose samples at 0.250, 0.500, 0.750, 1.000, 1.500, 2.000, 2.500, 3.000, 3.500, 4.000, 5.000, 6.000, 7.000, 8.000, 10.000, 12.000, 16.000, 24.000, 30.000 and 36.000 hours post-dose in each period.

AE: Adverse Event, CE: Clinical Examination

**Note:** Dosing and subsequent sampling timings were suitably staggered.

#### For Treatment Reference (R)

<table>
<thead>
<tr>
<th>AE, CE &amp; Vitals</th>
<th>Dinner</th>
<th>Vitals</th>
<th>Breakfast</th>
<th>1st Dose</th>
<th>Vitals</th>
<th>Lunch</th>
<th>Snacks</th>
<th>Vitals</th>
<th>Dinner</th>
<th>1nd Dose</th>
<th>Vitals</th>
<th>Breakfast</th>
<th>Lunch</th>
<th>Snacks</th>
<th>Dinner</th>
<th>Discharge, AE, CE &amp; Vitals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1300</td>
<td>2100</td>
<td>0700</td>
<td>0730</td>
<td>0815</td>
<td>0900</td>
<td>1100</td>
<td>1300</td>
<td>1800</td>
<td>1900</td>
<td>2015</td>
<td>2300</td>
<td>0700</td>
<td>0900</td>
<td>1300</td>
<td>1800</td>
<td>2000</td>
</tr>
<tr>
<td>-21 hr (approx.)</td>
<td>-12 hr</td>
<td>-2.0 hr</td>
<td>-1.5 hr</td>
<td>-0.75 hr</td>
<td>0 hr</td>
<td>2 hr</td>
<td>4 hr</td>
<td>9 hr</td>
<td>10 hr</td>
<td>11.25 hr</td>
<td>12 hr</td>
<td>14 hr</td>
<td>22 hr</td>
<td>24 hr</td>
<td>28 hr</td>
<td>33 hr</td>
</tr>
</tbody>
</table>

**Admission**

Pre dose blood sample

Allelock® 5 mg tablet (Morning dose)

Allelock® 5 mg tablet (Evening dose)

AE monitoring was done at admission, prior to dosing and approximately at 2, 10, 14, 22 and 36 hrs post dose.

Post dose samples at 0.167, 0.250, 0.333, 0.500, 0.667, 0.833, 1.000, 1.333, 1.667, 2.000, 2.500, 3.000, 4.000, 6.000, 8.000, 10.000, 12.000, 12.167, 12.250, 12.333, 12.500, 12.667, 12.833, 13.000, 13.333, 13.667, 14.000, 14.500, 15.000, 16.000, 18.000, 20.000, 24.000, 30.000 and 36.000 hours post morning dose in each period.

AE: Adverse Event, CE: Clinical Examination

**Note:** Dosing and subsequent sampling timings were suitably staggered.
3.1.6 Fasting/Meals

Post-admission to Ranbaxy CPU during all the three periods of the study, all subjects were fasted overnight for at least 10 hours before the high-fat high-calorie breakfast. Morning dose was administered with 240 mL of drinking water at ambient temperature, 45 minutes after starting of high fat high calorie breakfast in all the three periods of the study. In case of reference product, evening dose was administered with 240 mL of drinking water at ambient temperature, 45 minutes after starting of high fat high calorie dinner in all the three periods of the study.

All the subjects received standard meals-lunch, snacks, dinner, breakfast, lunch and snacks at 4, 9, 11.25, 24, 28 and 33 hours post morning dose. During housing, all meal plans were identical for all the three periods of the study. In case meals and blood sample collection coincide, samples were collected before meals are provided. Drinking water was not allowed from 1 hour before dosing until 2 hours post-dose. Thereafter, it was allowed at all times. No food was allowed for at least 4 hours post-dose. For detailed meal menus, refer to annexure VI.

3.1.7 Assignment to Treatment

The order of receiving study treatments for each subject during the three periods of the study was determined according to the SAS-generated balanced randomization schedule (Annexure VII). Dosing of subjects as per randomization schedule was done during all the three periods of the study under supervision of a trained medical officer.

**Reference (R):** Two oral doses of Allelock® 5 mg (each dose containing olopatadine hydrochloride 5 mg administered 12 hourly; total dose 10 mg). Morning dose was
administered 45 minutes after the start of high-fat high-calorie breakfast. Evening dose was administered 45 minutes after the start of high-fat high-calorie dinner during all the three periods of the study.

**Test (A & B):** A single oral dose of Olopatadine hydrochloride 10 mg extended release tablet was administered 45 minutes after the start of high-fat high-calorie breakfast during all the three periods of the study.

### 3.1.8 Washout Period

There was 6 days washout period between the administrations of study drugs in each period.

### 3.1.9 Assessment of Compliance

Compliance was assessed by conducting a thorough examination of the oral cavity by trained study personnel after dosing in all the three periods and by measurement of plasma olopatadine (during the analytical phase of the study).

### 3.1.10 Restrictions

- **Medications**

  All the subjects were instructed not to take any other medications including vitamins and over the counter (OTC) medications during the 30 days prior to the onset of the study. They were instructed during the screening process not to take any prescription, OTC medications and vitamins until the completion of the study.

- **Diet**

  All the subjects abstained 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 all the three periods of the study. Subjects also abstained from tea, coffee, cigarette and any other xanthine containing beverages, during in-house stay in all the three periods of the study.

➔ Activity

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

3.1.11 Blood Sampling

Blood samples from each subject were collected in prechilled K$_3$ EDTA vacutainers during the course of the study through indwelling heparinized cannulae placed in forearm veins. 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 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 heparinized blood and heparin solution from the tubing. The minimum blood sample volume required for analytical purpose was 4 mL in this study.

The blood samples from subjects who received test drug were collected pre-dose (in duplicate) and at 0.250, 0.500, 0.750, 1.000, 1.500, 2.000, 2.500, 3.000, 3.500, 4.000, 5.000, 6.000, 7.000, 8.000, 10.000, 12.000, 16.000, 24.000, 30.000 and 36.000 hours post-dose in all the three periods of the study.
The blood samples from subjects who received reference drug were collected pre-dose (in duplicate) and at 0.167, 0.250, 0.333, 0.500, 0.667, 0.833, 1.000, 1.333, 1.667, 2.000, 2.500, 3.000, 4.000, 6.000, 8.000, 10.000, 12.000, 12.167, 12.250, 12.333, 12.500, 12.667, 12.833, 13.000, 13.333, 13.667, 14.000, 14.500, 15.000, 16.000, 18.000, 20.000, 24.000, 30.000 and 36.000 hours post morning dose in all the three periods of the study.

A total of 81 (including three pre-dose duplicate blood samples), 4 mL blood samples were collected from each subjects during the course of the study and for each subject, the total volume of blood drawn, including 16 ml for screening, 08 mL for safety analysis at the end of the study, and 34.5 mL ‘discarded’ blood did not exceed 382.5 ml (except for subject no. 12 who was withdrawn from the study due to adverse event & subject no. 08 who was withdrawn from the study due to non-compliance to protocol requirements).

The pre-dose blood samples in all the three periods of the study were collected within a period of approximately 1.5 hours before the morning dose and the post-dose samples were generally collected within 2 minutes of the scheduled time (except for subject no 03; in period II 6.000 hrs post dose sample was delayed by 3 minutes due to difficulty in vein). After collection, the blood samples were centrifuged at a speed of 4000 RPM for duration of 15 minutes and at a temperature of 4 ± 2 °C under refrigeration as soon as possible to separate plasma. All post dose plasma samples were divided into two aliquots and transferred to suitably labeled tubes and rechecked to ensure the transfer of plasma to the correct tube. The plasma samples were then stored at below -50°C, pending transfer to the analytical facility for assay.
3.1.12 Safety Assessment

- Clinical Safety Measurements

Vital signs of oral temperature, sitting blood pressure and radial pulse were measured after admission, prior to dosing and at 2, 10, 14, 22 and 36 hours (within 2 hours of scheduled time) after administration of morning dose in all the three periods of the study. Brief clinical examination of the subjects was conducted by a qualified medical designate on duty at subject admission and at discharge in all the three periods of the study. In the event of detection of any abnormality during measurement of vital signs and/or clinical examination, the clinical investigator was consulted for necessary action. Laboratory parameters were repeated at the end of the study.

- Adverse events

The clinical investigator or a medical officer was available at the site of investigation until 36 hours post morning dose during all the three periods of the study. Subjects were monitored throughout the study period for adverse events. Subjects were informed to bring to the notice of the nurse or the physician, if any adverse event that may occur during their stay at the site of investigation. Subjects were also specifically asked about any adverse events at the time of admission, prior to dosing and approximately at 2, 10, 14, 22 and 36 hours post morning dose in all the three periods of the study.

3.1.13 Discharge

All the subjects were discharged 36 hours after administration of the study drug in all the three periods of the study.
3.1.14 Ethical Considerations

➢ Basic principles

This research was carried out in accordance with the basic principles defined in US 21CFR Part 320, the ICH Guidance for Good Clinical Practice (62 FR25692, 09 May 1997), Indian council for medical research ethical guidelines for biomedical research on human participants, CDSCO guidance for Good Clinical Practices for Clinical Research in India and the principles enunciated in the Declaration of Helsinki (WMA General Assembly, Seoul, 2008) (19, 22, 77, 78, 79).

➢ Institutional Review Board

This protocol and the corresponding informed consent form (ICF) used to obtain informed consent of study subjects were reviewed and approved by the Jamia Hamdard Institutional Review Board and the study was only started after the board approved the protocol and the ICF, as submitted or with modifications.

➢ Informed Consent

The purpose of the study, procedures to be carried out, potential hazards and rights of the subjects were described to the subjects in non-technical terms before the subjects were admitted to the Ranbaxy CPU for Period I. All the subjects provided formal written consent after attending an oral presentation about study and after thoroughly reading the informed consent form.
Drop-out/ Withdrawal of Subjects from Study

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

- The subject suffers from significant inter-current illness or undergoes surgery during the course of the study.
- The subject experiences adverse event and withdrawal is in the best interest of the subjects.
- The subject fails to comply with the requirements of the protocol. This would include pre-study directions regarding alcohol and drug use, fasting or if the subject is uncooperative during the study.
- The subject requires concomitant medications which may interfere with the pharmacokinetics of study drug.
- The subject has an episode of vomiting at any time during 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.

Volunteer Compensation

The subjects were adequately compensated on account of their participation in the study as per the guidelines issued by the JHIIRB. In case of drop-out/withdrawal of a subject before completion of the study, the subjects were compensated on pro rata basis as per ICF. The compensation for this study was Rs. 7250/- per completed subject in the study.
3.1.15 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 CPU, 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.
3.2 BIOANALYTICAL METHODOLOGY

A liquid chromatography mass spectroscopy (LC-MS/MS) method for the estimation of olopatadine in human plasma was developed and validated by using Olopatadine-d3 as internal standard (ISTD).

3.2.1 Method Development

- **Selection of Column**

On the basis of physiochemical properties of the drug, reverse phase chromatography with Zorbax Eclipse XDB C18 column was preferred. Various columns of different manufacturer like Hypersil, Nucleosil, Bondapak, Novapak and Zorbax Eclipse were tried. Desired retention time and good peak shape was obtained with Zorbax Eclipse XDB C18, 100 x 4.6 mm, 3.5 µM column.

- **Selection of Mobile Phase**

Buffers like (Ammonium acetate, Potassium dihydrogen orthophosphate, Ammonium formate and formic acid) at different pH with varying concentration of organic phases (methanol, acetonitrile) were tried. Optimum resolution and appropriate retention time of both IS and analyte were obtained with Formic Acid:Methanol (40:60) (v/v) solution.

- **Instrumentation and Chromatographic Conditions**

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>Zorbax Eclipse XDB C18, 100 x 4.6mm,3.5µM column</td>
</tr>
<tr>
<td>HPLC system</td>
<td>Shimadzu Prominence</td>
</tr>
<tr>
<td>Mass spectrometer</td>
<td>API 4000, MDS Sciex, Applied Biosciences</td>
</tr>
<tr>
<td>Weighing balances</td>
<td>Mettler Toledo</td>
</tr>
</tbody>
</table>
**Chapter 3**

**Materials & Methods**

- **Solid phase extraction unit**: Orochem Ezypress 48
- **Ultra sonicator**: S.V. Scientific
- **Vortex**: Spinix
- **Water purification system**: Milli-Q-Synthesis, Millipore
- **Freezer & Refrigerator**: Thermofisher Scientific
- **Micropipettes, Stepper pipettes**: Eppendorf, Thermofisher
- **Mobile Phase**: 0.02% Formic Acid Solution:Methanol (40:60) (v/v)
- **Flow Rate**: 1mL/min
- **Volume of Injection**: 5 µL
- **Retention Time**: Drug: 1.25±0.5 minute, ISTD: 1.25±0.5 minute
- **Run Time**: 2.50 minute
- **Column Oven Temperature**: 30°C
- **Autosampler Temperature**: 4°C

**Contents of custom injector program for Shimadzu prominence**

- **Rinse Volume**: 800 µL
- **Needle Stroke**: 52 minute
- **Rinse Speed**: 32 µL/sec
- **Rinse Mode**: Before and after aspiration
- **Needle rinsing solution**: Methanol
Multiple Reaction Monitoring (MRM) Conditions

Mass spectrometer  
API 4000

Ion source  
Turbo ion spray

Polarity  
Positive ion mode

Resolution  
Q1- unit, Q3- unit

Spray needle set point  
5/5

Detection ion

Olopatadine  
338.1 m/z (parent), 165.1 m/z (product)

Olopatadine-d3  
341.1 m/z (parent), 165.1 m/z (product)

Chromatography data acquisition and analysis was done by using Applied Bio-system/MDS SCIEX analyst version 1.4.2. The operating system used was windows XP professional (version 2002 with SP3). Electrospray positive ion mass spectrum for olopatadine and olopatadine-d3 are presented in annexure VIII and IX.

➢ Reagents

• Olopatadine working standard

• Olopatadine-d3 working standard (ISTD)

• Methanol (HPLC grade)

• Formic Acid (GR Grade)

• Water (generated from Mill-Q system; HPLC grade)

• Matrix: Human plasma (K3 EDTA-Anticoagulant)

• Oasis HLB cartridge 1 cc (30 mg)
➢ Preparation of Solutions

- Formic acid solution (0.02%)

0.200 mL formic acid was transferred into a 1000 mL volumetric flask. The volume was made up with 1000 mL of HPLC grade water. This solution was stored at room temperature and used within 3 days from the date of its preparation.

- Mobile Phase

Mobile phase was prepared by mixing 60 parts of methanol and 40 parts of 0.02 % formic acid solution and sonicated for 5 minutes. This solution was stored at room temperature and used within 3 days from the date of its preparation.

- Methanol Solution (60%)

60% methanol solution was prepared by mixing 60 parts of methanol and 40 parts of water. The solution was stored at room temperature and used within 3 days from the date of its preparation.

- Methanol Solution (5%)

5% methanol solution was prepared by mixing 5 parts of methanol and 95 parts of water. The solution was stored at room temperature and used within 3 days from the date of its preparation.

➢ Preparation of Stock Solutions

- Olopatadine Calibration Curve Stock Solution

Olopatadine (5.132 mg) was weighed accurately and transferred into a 5 mL volumetric flask. To this flask, 5 mL of methanol was added up to the mark, dissolved to get a final
concentration of 913.99 µg/mL. The final concentration of olopatadine was corrected accounting for its potency, molecular weight and the actual amount weighed. The stock solution was stored in refrigerator (2-8°C). This stock solution (SS ID 1) was used for preparing the calibration curve (CC) standard stock dilutions for method validation and also for preparing comparison quality control (LQC & HQC) dilutions for stability experiments.

For stability experiments, 5.114 mg of olopatadine was weighed accurately and transferred into a 5 mL volumetric flask. To this flask, 5 mL of methanol was added up to the mark, dissolved to get a final concentration of 910.78 µg/mL. This stock solution was used for preparing the fresh calibration curve standard stock dilutions for stability experiments and also used to establish short term and long term stock solution stability. The stock solution was stored in refrigerator (2-8°C). Calibration curve stock solution and all further dilutions from above stock were prepared under low light conditions.

- **Olopatadine Quality Control Stock Solution**

Olopatadine (5.255 mg) was weighed accurately and transferred into a 5 mL volumetric flask. To this flask, 5 mL of methanol was added up to the mark, dissolved to get a final concentration of 935.89 µg/mL. The final concentration of olopatadine was corrected accounting for its potency, molecular weight and the actual amount weighed. The stock solution was stored in refrigerator (2-8°C). This stock solution (SS ID 2) was used for preparing the quality control (QC) sample dilutions for method validation. Quality control stock solution and all further dilutions from above stock were prepared under low light conditions.
• **Olopatadine-d3 (ISTD) Stock Solution**

Olopatadine-d3 (10.57 mg) was weighed accurately and transferred into a 5 mL volumetric flask. To this flask, 5 mL of methanol was added up to the mark, dissolved to get a final concentration of 933.74 µg/mL. The final concentration of olopatadine-d3 was corrected accounting for its potency, molecular weight and the actual amount weighed. The stock solution was stored in refrigerator (2-8°C). This stock solution (SS ID 3) was diluted further with 60% methanol solution to obtain desired ISTD concentration as and when required. Stock solution and all further dilutions from above stock were prepared under low light conditions.

➢ **Sample Preparation**

The calibration curve (CC) standards and quality control (QC) samples were withdrawn from the freezer and allowed to thaw at room temperature. These samples were processed as follows:

Exactly 0.200 mL of sample was transferred into a polypropylene vial and 50.0 µL of ISTD solution (500.17 ng/mL) was added, except for standard blank, and in standard blank 50.0 µL of diluent (60% methanol solution) was added and vortexed. To this 0.200 mL of 0.02% formic acid solution was added and vortexed. Oasis HLB cartridge 1CC (30 mg) was conditioned with 1 mL methanol followed by 1 mL of water. The sample was loaded on to the preconditioned cartridge and washed with 1 mL of water followed by 1 mL of 5% methanol solution. The samples were eluted with 0.300 mL of methanol into a polypropylene tubes containing 0.100 mL of 0.02% formic acid solution and vortexed. A 5 µL of the sample was injected into the LC-MS/MS system in split mode of 90:10.
3.2.2 Analytical Method Validation

The validation of this procedure was performed in order to evaluate the method in terms of selectivity, linearity, precision, accuracy, sensitivity, recovery and stability (48). The linearity, precision and accuracy evaluations were performed on three batches of spiked plasma samples.

➢ Autosampler Carryover Test

The experiment was carried out by injecting reconstitution solution followed by extracted limit of quantification (LOQ) sample, aqueous mixture (higher concentration of analyte with ISTD) and then once again reconstitution solution followed by aqueous mixture and reconstitution solution injections. It was considered to having no carryover if the interference peak obtained at the retention time (RT) of analyte is not more than or equal to 20% of extracted lower limit of quantification (LLOQ) sample response or interference at the RT of ISTD is less than or equal to 5% of internal standard response in extracted LLOQ, respectively.

➢ Selectivity

The selectivity of the present method was established by checking the blank plasma (without spiking with olopatadine) obtained from different blood donors. Ten different batches of plasma were screened including two sources of lipemiic and two sources of haemolyzed matrix. All the blank matrices along with their respective LOQ samples were processed and analyzed. The blank matrices were considered to free of interference if the responses for measured peak at the RT of analyte was less than 20% of extracted LOQ response and that at the RT of ISTD was less than 5% of extracted ISTD. At least 80% of the screened matrix
should have less than 20% interference at the analyte for acceptance. Human plasma batches, free of interferences were used to prepare calibration standards and quality control samples.

- **Preparation of Calibration Curve Standards, Quality Control Samples and Internal Standard Dilution**

Spiking dilutions for calibration standards were prepared from olopatadine stock solutions (SS Id 1) in 60% methanol solution (v/v). Following are the concentrations of spiking solutions: 20.046 ng/mL (STD 1), 40.092 ng/mL (STD 2), 100.23 ng/mL (STD 3), 200.46 ng/mL (STD 4), 801.85 ng/mL (STD 5), 1603.7 ng/mL (STD 6), 3207.4 ng/mL (STD 7) and 4009.2 ng/mL (STD 8). The concentration of olopatadine in human plasma was in the range of 1.0023 ng/mL to 200.46 ng/mL. The spiked calibration curve (CC) standards were prepared as presented in table 3.4.

**Table 3.4: Calibration curve standard**

<table>
<thead>
<tr>
<th>CC ID</th>
<th>Final spiking solution concentration (ng/mL)</th>
<th>Spiking solution volume (mL)</th>
<th>Final volume made up with plasma (mL)</th>
<th>Olopatadine concentration in plasma (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STD 8</td>
<td>4009.2</td>
<td>0.500</td>
<td>10</td>
<td>200.46</td>
</tr>
<tr>
<td>STD 7</td>
<td>3207.4</td>
<td>0.500</td>
<td>10</td>
<td>160.37</td>
</tr>
<tr>
<td>STD 6</td>
<td>1603.7</td>
<td>0.500</td>
<td>10</td>
<td>80.185</td>
</tr>
<tr>
<td>STD 5</td>
<td>801.85</td>
<td>0.500</td>
<td>10</td>
<td>40.093</td>
</tr>
<tr>
<td>STD 4</td>
<td>200.46</td>
<td>0.500</td>
<td>10</td>
<td>10.023</td>
</tr>
<tr>
<td>STD 3</td>
<td>100.23</td>
<td>0.500</td>
<td>10</td>
<td>5.0115</td>
</tr>
<tr>
<td>STD 2</td>
<td>40.092</td>
<td>0.500</td>
<td>10</td>
<td>2.0046</td>
</tr>
<tr>
<td>STD 1</td>
<td>20.046</td>
<td>0.500</td>
<td>10</td>
<td>1.0023</td>
</tr>
<tr>
<td>Blank+IS</td>
<td>0.00000</td>
<td>1.250</td>
<td>25</td>
<td>0.00000</td>
</tr>
<tr>
<td>Blank</td>
<td>0.00000</td>
<td></td>
<td></td>
<td>0.00000</td>
</tr>
</tbody>
</table>
For blank and blank+IS samples, instead of spiking solutions, 60% methanol solution (v/v) was added.

Spiking dilutions for quality control samples were prepared from olopatadine stock solution (SS ID 2) in 60% methanol solution (v/v). Following are the concentrations of spiking solutions: 21.358 ng/mL (LOQ QC), 58.243ng/mL (LQC), 499.08 ng/mL (LMQC), 1497.4 ng/mL (MQC) and 2994.8 ng/mL (HQC). The spiked QC samples were prepared as presented in table 3.5.

**Table 3.5: Quality control standard**

<table>
<thead>
<tr>
<th>QC ID</th>
<th>Final spiking solution concentration (ng/mL)</th>
<th>Spiking solution volume (mL)</th>
<th>Final volume made up with plasma (mL)</th>
<th>Olopatadine concentration in plasma (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HQC</td>
<td>2994.8</td>
<td>2.500</td>
<td>50</td>
<td>149.74</td>
</tr>
<tr>
<td>HQC</td>
<td>2994.8</td>
<td>0.500</td>
<td>10</td>
<td>149.74</td>
</tr>
<tr>
<td>MQC</td>
<td>1497.4</td>
<td>2.500</td>
<td>50</td>
<td>74.870</td>
</tr>
<tr>
<td>LMQC</td>
<td>499.08</td>
<td>2.500</td>
<td>50</td>
<td>24.954</td>
</tr>
<tr>
<td>LQC</td>
<td>58.243</td>
<td>2.500</td>
<td>10</td>
<td>2.9122</td>
</tr>
<tr>
<td>LQC</td>
<td>58.243</td>
<td>0.500</td>
<td>10</td>
<td>2.9122</td>
</tr>
<tr>
<td>LOQ QC</td>
<td>21.358</td>
<td>0.500</td>
<td>10</td>
<td>1.0679</td>
</tr>
<tr>
<td>DI QCS</td>
<td>5989.7</td>
<td>0.500</td>
<td>10</td>
<td>299.49</td>
</tr>
</tbody>
</table>

The method was validated in human plasma in the concentration range of 1.0023 ng/mL to 200.46 ng/mL using eight non zero concentrations and five quality control samples at 1.0679 ng/mL (LOQ QC), 2.9122 ng/mL (LQC), 24.954 ng/mL (LMQC), 74.870 ng/mL (MQC) and 149.74 ng/mL (HQC) levels. The concentration of the internal standard solution prepared throughout the method validation period was 500.17 ng/mL.
➢ **Selection of Weighing Factor**

During pre-method validation, three precision and accuracy runs were performed and weighing factor was established.

➢ **Sensitivity**

The sensitivity of the method was determined in terms of LLOQ. The sensitivity established by processing six different lots of matrices spiked with LLOQ and one set of CC standards. The lowest standard was accepted as the 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).

➢ **Matrix Effect**

The matrix effect experiment was performed with six different lots of blank plasma. LQC and HQC level concentration was spiked in six different lots of plasma in duplicate, processed along with a CC standards and samples were analyzed. The acceptance criteria of the mean % nominal for LQC and HQC should be between 85-115% and % CV at both LQC and HQC should be less than 15%.

➢ **Limit of Detection**

The limit of detection for olopatadine was established at S/N = 3, which was equivalent to 0.077100 ng/mL.

➢ **Linearity**

The linearity of the method was determined by weighted \((1/x^2)\) least square regression analysis of standard plot associated with eight point calibration curve for olopatadine. Three batches of calibration curve standards were processed and analyzed to check linearity of the
method. The calibration curve was linear for the standards ranging from 1.0023 ng/mL to 200.46 ng/mL of olopatadine. The standard curve linearity was determined on three standard curves and slope, intercept and correlation coefficient were determined. For calculation of the standard curve plots, area ratio of analyte and internal standard area against concentration were determined. For acceptance of linearity of calibration curves, at least 75% or a minimum of 6 non-zero standards including LOQ (lower limit of quantification) and ULOQ (upper limit of quantification) must meet the following passing criteria:

- Accuracy of standards (% nominal): within ± 15% of their nominal values (within ± 20% for LOQ).
- Precision of calibrates (% CV): ≤ 15% (≤ 20% for LOQ)
- In case first injected LOQ or ULOQ standard failed, lost or exhibited bad chromatography, their duplicates were passing the above criteria.
- Linear coefficient of correlation: ≥ 0.98.

➢ 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 8 (ULOQ); each in duplicate and standard - 2, 3, 4, 5, 6, 7 and six replicates of LOQQC (1.0679 ng/mL), LQC (2.9122 ng/mL), LMQC (24.954 ng/mL), MQC (74.870 ng/mL) and HQC (149.74 ng/mL) samples, interspersed within each other, were processed and analyzed according to the proposed method. Ruggedness of the method was assessed by processing and analyzing new PA batch on same LC-MS/MS system using different column (same type) by different analyst and using fresh solutions and calculating the precision and accuracy.
The back calculated concentration olopatadine in each quality control sample was calculated using linear regression parameters of the corresponding calibration curve. The mean olopatadine 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).

➢ **Recovery**

- **Recovery of Olopatadine**

The percentage recoveries were determined by comparing the peak areas of the prepared plasma quality control samples against corresponding aqueous standards.

- **Recovery of Olopatadine-d3 (ISTD)**

The percentage recoveries were determined by comparing the peak areas of the prepared extracted ISTD against corresponding aqueous ISTD (unextracted) at the concentration level intended for use during analysis of the study samples.

Below mentioned formula was used for calculation of recovery.

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

➢ **Stability**

Stability studies at various conditions were conducted as described below for replicate LQC and HQC samples, along with a freshly spiked calibration curve standards and QC samples (LQC and HQC) which were prepared from two different stock solutions. The freshly spiked QC samples were used as “comparison QC samples”. The mean concentrations of the
stability QC samples were compared with mean concentration of comparison QC samples. The stability calculations were done as follows:

\[
\% \text{Nominal} = \frac{\text{Mean concentration of stability QC at each level} - \text{Mean concentration of comparison QC at each level}}{\text{Mean concentration of comparison QC at each level}} \times 100
\]

\[
\text{Correction factor} = \frac{\text{Stock concentration used for comparison QC’s}}{\text{Stock concentration used for stability QC’s}}
\]

\[
\% \text{Stability (\% bias)} = \% \text{Nominal} \times \text{Correction factor}
\]

- **Freeze-thaw Stability**

The freeze-thaw stability of analyte in matrix was assessed by freezing the samples in freezer (below -50°C) and thawing them. Replicate numbers of QC samples were thawed after 24 hr freezing and were replaced into freezer by identifying them as freeze-thaw V cycle samples “FT-5”. After minimum of every 12 hr of freezing, along with FT-5 samples, replicate number of QC samples was allowed to thaw and after freezing for minimum 12 hr, were identified as freeze-thaw IV cycle sample “FT-4”. The “FT-5” samples, which underwent five freeze and thaw cycles were processed and analyzed. The percentage stability was determined by comparing mean concentration of stability QC samples against the mean concentration of comparison QC samples at each concentration level. Samples were deemed stable after three freeze thaw cycles if % stability was within ± 15 % of nominal values and % CVs ≤ 15 % at both LQC and HQC levels.
• **Bench-top Stability**

Bench-top stability of replicate quality control samples was determined by allowing the samples to thaw and maintaining for 18 hrs 16 min at room temperature on bench. The percentage stability was determined by comparing mean concentration of stability QC samples against the mean concentration of comparison QC samples at each concentration level. Samples were deemed to be stable for the specified bench top period if % stability was within ± 15 % of nominal values and % CVs ≤ 15 % at both LQC and HQC levels.

• **In-injector (Autosampler) Stability**

In-injector stability of replicate QC samples was determined by placing the extracted QC samples, immediately after preparation, into autosampler at until analyzed for 29 hr 22 minutes. The percentage stability was determined by comparing mean concentration of stability QC samples against the mean concentration of comparison QC samples at each concentration level. In-injector stability of ISTD was determined at HQC concentration level by comparing the mean area ratio of stability samples with comparison samples (Area ratio = Peak area of ISTD/Peak area of analyte). The samples were considered to be stable for the specified in-injector stability period if % stability was within ± 15 % of nominal values and % CVs ≤ 15 % at both LQC and HQC levels.

• **Wet Extract Stability**

The stability of olopatadine in reconstituted extract of plasma sample (wet extract) (6 replicates of LQC and HQC), was determined by storing the samples in autosampler vials at room temperature. After 18 hr 41 minutes, samples were loaded into autosampler tray and analyzed. The percentage stability was determined by comparing mean concentration of
stability QC samples against the mean concentration of comparison QC samples at each concentration level. The samples were considered to be stable for the specified time period if % stability was within ± 15 % of nominal values and % CVs ≤ 15 % at both LQC and HQC levels.

- **Sample Stability During Processing**

This experiment was performed to ensure stability and integrity of samples during the different steps of sample processing at room temperature. Stability during processing was determined by allowing the samples to thaw, giving a gap between each processing step and then running the samples. The percentage stability was determined by comparing mean concentration of stability QC samples against the mean concentration of comparison QC samples at each concentration level. Samples were deemed stable for the specified time period if % stability was within ± 15 % of nominal values and % CVs ≤ 15 % at both LQC and HQC levels.

- **Long Term Stability in Plasma**

Long term stability of replicate quality control samples was determined by storing the samples at -50°C in deep freezer for 60 days and then analyzing the samples. The percentage stability was determined by comparing mean concentration of stability QC samples against the mean concentration of comparison QC samples at each concentration level. Samples were deemed stable for the specified long term stability period if % stability was within ± 15 % of nominal values and % CVs ≤ 15 % at both LQC and HQC levels.
• **Stock Solution Stability**

1. Short-term stock solution stability: Stock solutions of olopatadine (910.78 µg/mL) and ISTD (933.74 µg/mL) was stored at room temperature for 6 hr 30 minutes. A middle level concentration of olopatadine as well as ISTD at working concentration level was prepared and replicate injections were performed. The stability was established by comparing the area of drug and ISTD with the corresponding area at 0 hr.

2. Long-term stock solution stability: Stock solutions of olopatadine (935.89 µg/mL) prepared and stored at 2 – 8°C for 20 days. A middle level concentration solution of analyte and ISTD was prepared on 21st day and replicate injections were performed. The stability was established by comparing the area of drug and ISTD with the corresponding area of the freshly prepared respective stock solutions.

\[
\text{Correction factor} = \frac{\text{New stock concentration (µg/mL)}}{\text{Old stock concentration (µg/mL)}}
\]

\[
\% \text{ Stability} = \% \text{ Nominal} \times \text{Correction factor}
\]

The solutions were considered stable for specified period if percent stability was within the range of 90-110 %.

➢ **Dilution Integrity**

To assess the dilution integrity (1:2 and 1:4), samples were prepared by spiking the plasma with olopatadine at a concentration of 299.49 ng/mL during bulk spiking. Replicate samples were prepared by further diluting these samples to two times and four times with blank plasma and processed. The precision ad accuracy for dilution integrity (1:2 and 1:4) was determined by measuring the concentrations of diluted samples against bulk spiked CC
standards. The integrity of the samples were considered to be maintained if % stability was within ± 15 % of nominal values and % CVs ≤ 15 % for both dilutions.

- **Haemolysis Effect**

Haemolysis effect was studied by processing six replicates of LQC and HQC concentrations spiked in haemolyzed plasma along with the calibration standards prepared in normal plasma. At least five out of six normal matrix batches along with haemolyzed matrix batches should meet the following acceptance criteria. The acceptance criteria was same as discussed under precision and accuracy.

- **Ruggedness**

Ruggedness was assessed by analyzing a sample precision and accuracy batch (P & A run) performed by using the same instrument, different analyst and different column. The same acceptance criteria was utilized as mentioned under precision and accuracy.

- **Effect of Concomitant Medication**

The effect of concomitant medication on olopatadine was checked with caffeine, paracetamol, cetirizine, diclofenac and aspirin. It was studied by processing 3 replicates of LQC and HQC concentrations spiked with above mentioned drug along with the calibration standards prepared in normal plasma. The acceptance criteria was same as discussed under precision and accuracy.

- **Matrix Factor**

The matrix factor experiment was performed with six independent lots of blank plasma. LQC and HQC level concentration of analyte along with ISTD spiked to the extracted blanks in duplicate (i.e. two LQC and two HQC for each lot of plasma) and prepared post extraction.
spiked samples. Reference solution of analyte at LQC and HQC level concentration was prepared along with ISTD in reconstitution solution, representing 100% of extracted samples, referred to as unextracted sample. Six replicates of unextracted LQC and HQC samples and the post extraction spiked LQC and HQC samples were injected. The acceptance criteria of the mean % nominal for LQC and HQC should be between 85-115% and % CV at both LQC and HQC should be less than 15%.

➢ **Reinjection Reproducibility**

To assess the reinjection reproducibility, one set of CC standards and six sets of QC samples (LQC and HQC) were injected. The QC samples along with CC standards were reinjected after storing for a period of 27 hrs 51 minutes in the autosampler. The reinjected QC samples were measured against initial CC standards and reinjected CC standards. The same acceptance criteria was utilized as mentioned under precision and accuracy.

➢ **Analytical Run Size Evaluation**

The analytical run size evaluation experiment was carried out with 112 samples and the run was found to be meeting the acceptance criteria.

3.2.3 **Standardizations and Calculations**

The chromatographic data were acquired and analyzed by using Applied Bio-system/MDS SCIEX analyst version 1.4.2. The operating system used was windows XP professional (version 2002 with SP3). The best-fit curves using weighted \((1/X^2)\) linear least square regression analysis were obtained by peak area ratio of olopatadine to olopatadine d-3 (ISTD). The concentration of olopatadine in the plasma samples was calculated using linear regression parameters of the corresponding calibration curve.
3.3 CLINICAL STUDY SAMPLE ANALYSIS

3.3.1 Objective

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

3.3.2 Sample Collection and Storage

The blood samples for period I, II and III were collected on 07th and 08th August 2010; 13th and 14th August 2010; 20th and 21st August 2010 respectively. Blood samples from each period were centrifuged to separate plasma, divided into 2 aliquots and stored at below –50°C in a Haereus deep freezer at the clinical facility on their respective dates of collection. All plasma samples from three periods were then packed properly using dry ice, transported to the analytical facility on 26th August 2010 (Ist aliquots) and 30th August 2010 (IInd aliquots), and stored at below -50°C until analysis.

3.3.3 Sample Analysis

As per the protocol, a total number of 1170 samples supposed to be collected from 15 subjects in over three periods. Due to two withdrawn subjects (subject no. 08 and 12), a total of 1071 samples were collected during the whole study and 1050 samples were used for analysis. For sample analysis by LC-MS/MS, one analytical batch consisting of total 98 samples, which include all the samples of one subject (78) in three periods along with the calibration standards (14) and quality control samples (two each of LQC, MQC and HQC) were processed and analyzed.
3.3.4 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.

- **Calibration Curve Acceptance Criteria**

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

- Accuracy of calibrators: within ± 15 % of their nominal values (within ± 20 % for LOQ).
- At least 75% or a minimum of 6 calibrators including LOQ and ULOQ meet the above criteria.
- In case first injected LOQ or ULOQ standard failed, lost or exhibited bad chromatography, their duplicates were passing the above criteria.
- Linear coefficient of correlation: ≥0.98.

- **Blank and Blank + Internal Standard Acceptance Criteria**

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

- Peak area responses of the blanks at the retention time of the olopatadine were < 20 % of the peak area response of the LOQ standard
- 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.

- **Quality Control Sample Acceptance Criteria**

Batch acceptance required that back calculated concentrations of at least 50% of each QC sample (LQC, MQC and HQC) and 67 % overall were within ± 15 % of their nominal values.
Repeat Analysis

Samples were subjected to repeat analysis using following criteria:

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

Protocol Deviations

There were no significant protocol deviations during the analysis of the clinical samples.
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 olopatadine using WinNonlin Node version 5.0.1 from Pharsight:

- AUC<sub>0-t</sub>: 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<sub>0-24</sub>: The area under the plasma concentration versus time curve, from time zero to 24 h.
- AUC<sub>0-∞</sub>: The area under the plasma concentration versus time curve, from time zero to infinity. AUC<sub>0-∞</sub> is calculated as the sum of AUC<sub>0-t</sub> 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<sub>0-∞</sub> - AUC<sub>0-t</sub>) / AUC<sub>0-∞</sub>] * 100
- C<sub>max</sub>: Maximum measured plasma concentration over the time span specified.
- T<sub>max</sub>: Time of the maximum measured plasma concentration. If the maximum value occurs at more than 1 time point, T<sub>max</sub> is defined as the first time point with this value.
- K<sub>el</sub>: 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<sub>1/2</sub>**: The apparent first-order terminal elimination half-life was calculated as \(0.693/K_{el}\).

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

### 3.4.2 Statistical Analyses

Statistical analysis was performed on plasma olopatadine using the WinNonlin PK Software, Version 5.0.1. The analysis included the data from all subjects who has completed the study except subject no. 08, 12. These two subjects were withdrawn from the study.

- **Summary Statistics**

Arithmetic means, standard deviations and coefficients of variation were calculated for the parameters listed in section 3.4.1. Additionally, geometric means and percentage coefficient of variation of geometric means was calculated for \(AUC_{0-t}\), \(AUC_{0-24}\), \(AUC_{0-\infty}\) and \(C_{max}\).

- **Analysis of Variance (ANOVA)**

The log-transformed pharmacokinetic parameters (\(C_{max}\), \(AUC_{0-t}\), \(AUC_{0-24}\), \(\square\) and \(AUC_{0-\infty}\)) for Test (A&B) and Reference (R) formulations 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 were done using the appropriate SAS® procedure.

➢ Ratio Analysis

The ratio of the test (A or B) and reference (R) product averages (least square means) was calculated for olopatadine 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 A vs R and B vs R, so the ratios was of the form A/R and B/R. Ratio of means was expressed as a percentage of the LSM for the reference formulations.