Chapter 3: Materials & Methods
Chapter 3

MATERIALS AND METHODS

3.0 MATERIALS AND METHODS:

The experimental design included the following parts:

- Clinical study Phase
- Bioanalytical Phase including
  - Method development
  - Method validation
  - Analysis of clinical study samples
- Pharmacokinetic analysis
- Statistical analysis

3.1 CLINICAL STUDY METHODOLOGY

3.1.1 Objective

Primary objective:

To assess the bioequivalence of single oral dose of levofloxacin 500 mg tablets with Cravit 500 mg tablet (containing levofloxacin 500 mg) in healthy, adult, human subjects under fasting conditions.

Secondary objective:

To monitor safety of subjects.

3.1.2 Study design

The study was designed as an open label, balanced, randomized, two-treatment, two-period, two-sequence, single-dose, crossover, bioequivalence study comparing
levofoxacin 500 mg tablets manufactured by Ranbaxy (Malaysia) SDN BHD, Malaysia with Cravit 500 mg tablet (containing levofoxacin 500 mg) manufactured by PT Kalbe Farma Tbk, Indonesia, and marketed by First Pharmaceuticals SDN BHD, Malaysia in healthy, adult, human subjects under fasting conditions.

3.1.3 **Number of subjects**
Adequate (28) healthy adult human subjects were enrolled/admitted and all subjects were dosed in both periods of the study. Data has been presented on all 28 completed subjects.

3.1.4 **Determination of Sample Size**
Sample size estimation was based on available in-house data on Levofoxacin Tablets. Assuming a Test/Reference ratio of 92.35% and Intra-subject CV of approximately 19.7%, 24 subjects were considered sufficient to yield a power of 80% to show bioequivalence under bioequivalence assumptions. However to be conservative and allow for possible dropouts and/or withdrawals, 28 subjects were considered for this study.

3.1.5 **Selection of subjects**
Adequate numbers of volunteers, selected randomly, underwent a standardized screening procedure within 28 days prior to the commencement of the study. Twenty-eight (28) healthy, adult, human, subjects were selected based on the inclusion and exclusion criteria mentioned in sections 3.1.5.2 and 3.1.5.3 respectively.

3.1.5.1 **Screening assessments**
Subject screening procedure included Complete medical history taking (including the history of past or present cardiovascular, respiratory, musculoskeletal and connective tissue, gastrointestinal, renal, hepatic, genitourinary, neurological, endocrine, psychiatric, lymphatic, dermatological, haematological, metabolic, immune, drug, and surgical history or any other diseases or disorders) and demographic data recording, (including name, sex, age, height, weight and number of cigarettes smoked per day).
Each subject then underwent physical examination and the laboratory tests of hematological, hepatic and renal functions as listed below. Only medically healthy subjects with clinically normal laboratory profiles were enrolled in the study.

<table>
<thead>
<tr>
<th>Haematology</th>
<th>Biochemistry</th>
<th>Urinalysis</th>
<th>Serology</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Haemoglobin</td>
<td>• BUN (Blood urea nitrogen)</td>
<td>• Routine Examination</td>
<td>• HIV I &amp; II</td>
</tr>
<tr>
<td>• Total leukocyte count</td>
<td>• Creatinine</td>
<td>• Colour</td>
<td>(Human immunodeficiency virus)</td>
</tr>
<tr>
<td>• Differential leukocyte count</td>
<td>• Total bilirubin</td>
<td>• Appearance</td>
<td>• HBsAg</td>
</tr>
<tr>
<td>• Platelet count</td>
<td>• ALP (Alkaline Phosphatase)</td>
<td>• PH</td>
<td>(Hepatitis B Surface Antigen)</td>
</tr>
<tr>
<td></td>
<td>• AST (Aspartate aminotransferase)</td>
<td>• Specific gravity</td>
<td>• HCV (Hepatitis C Virus)</td>
</tr>
<tr>
<td></td>
<td>• ALT (Alanine aminotransferase)</td>
<td>• Protein</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Glucose</td>
<td>• Glucose</td>
<td>• VDRL (Venereal Disease</td>
</tr>
<tr>
<td></td>
<td>• Cholesterol</td>
<td></td>
<td>Research Laboratory test)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Others</td>
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<tr>
<td></td>
<td></td>
<td>• Microscopic Examination</td>
<td>• ECG (Electro Cardio Gram)</td>
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<tr>
<td></td>
<td></td>
<td>• RBC (red blood corpuscle)</td>
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<tr>
<td></td>
<td></td>
<td>• WBC (white blood corpuscle)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Epithelial Cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Crystals</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Casts</td>
<td></td>
</tr>
</tbody>
</table>

Samples collected during screening were analyzed at clinical laboratory. In addition, scan for drugs of abuse (cannabinoids and opioids) by card/cassette method and breath test for alcohol were carried out prior to admission in each period of the study.
Schematic representation of Clinic Activities from Screening till Inclusion in the study

SCREENING

ENROLLMENT FOR STUDY

RANDOMIZATION

PERIOD I  PERIOD II

RT  Washout  TR

14 Sub-R  14 Sub-R

14 Sub-T  14 Sub-T

Twenty-eight (28) healthy, adult, human subjects were selected based on the following inclusion and exclusion criteria as mentioned below and the same was documented in the criteria check form during the study.

3.1.5.2 Subjects were included in the study on the basis of the following Inclusion And Exclusion Criteria:

Inclusion Criteria

Subjects Included:

- Were in the age range of 18-45 years
- Had Body Mass Index between 18.0 to 30.0 kg/m².
- Had voluntarily given written informed consent to participate in this study
- Were of normal health as determined by medical history and physical examination of the subjects performed within 28 days prior to the commencement of the study

There were no deviations in this regard.
Exclusion Criteria

- Subject had history of hypersensitivity or anaphylactic reaction to levofloxacin or other quinolones or any other related drug.
- Subject had history of tendinitis or tendon rupture.
- Subject had history of convulsion/seizure/insomnia.
- Subject had recurrent history of muscle tenderness and/or weakness.
- Subject had history of alcohol/drug abuse.
- Subject had history of diarrhea preceding last one week from the day of admission in period I.
- Subject had any evidence of organ dysfunction or any clinically significant deviation from the normal in physical or clinical determinations.
- Subject had clinically abnormal ECG or hematological and biochemical parameters which was/were outside acceptable limits and was judged clinically significant by investigator.
- Investigations with blood samples of the subject showed presence of disease markers of HIV 1 or 2, Hepatitis B or C Viruses or syphilis infection.
- Investigations with urine samples of the subject showed clinically abnormal chemical and microscopic examination of urine defined as presence of RBC, WBC (>4 /HPF), glucose (Positive) or Protein (Positive).
- Subject had history of serious medical illnesses including but not limited to gastrointestinal, hepatic, renal, cardiovascular, pulmonary, neurological or hematological disease, diabetes, glaucoma, any serious, potentially life-threatening illness.
- Subject had inability to communicate well (i.e. language problem, poor mental development, psychiatric illness or poor cerebral function) that might impair the ability to provide, written informed consent.
- Subject was a regular smoker, who smoked more than 10 cigarettes daily or had difficulty abstaining from smoking for the duration of each study period.
- Subject had used any medication within 30 days prior to admission of this study.
Subject had participated in a clinical trial within 3 months (90 days) preceding admission of this study (except for the subjects who dropout/withdrawn from the previous study prior to period I dosing).
- Subject had donated and/or lost more than 350 mL of blood in the past 3 months (90 days).
- Subject had consumed alcohol for 48 hours prior to admission.
- Subject had consumed grapefruit juice and or grape fruit supplements containing products in 48 hours prior to admission in each period.
- Subject had problems in complying with the protocol.

There were no deviations in this regard.

3.1.6 Study Schedule

Informed Consent:

Informed Consent was obtained from all the subjects for the purpose of inclusion into the study. For details refer section 3.1.17.2.

Duration of Treatment and Housing: The duration of treatment was one time, single dose for each treatment. Subjects were admitted and housed at the study site from at least 11 hour before drug administration and were discharged approximately 48 hour after dosing during each period of the study. All 28 subjects completed both periods of the study.

3.1.7 Fasting/Meals

In each period, after admission to the Clinical Study Site, subjects were served a standard dinner approximately 12 hours prior to dosing. After dinner the subjects fasted overnight for at least 10 hours pre-dose. A single oral dose of either test / reference formulation was administered with 240 mL of water during each period of the study. After dosing, the subjects continued to fast for 4 hours post-dose. Drinking water was not allowed from 1 hour before dosing until 1 hour post-dose. Thereafter, it was allowed at all times.
The Subjects received standard meals – Lunch, snacks, dinner, breakfast, lunch, snacks and dinner at approximately 4, 9, 13, 24, 28, 33 and 37 hours post-dose, respectively, in each period. During housing, all meal plans were identical for both the periods and Information on the amount of meal consumed and the time taken for consuming the meal was recorded in case report forms. In case, meals and blood sample collection coincided, samples were collected before meals were provided.

3.1.8  Treatments

3.1.8.1 Treatments administered

A single oral dose of either test or reference drug formulation containing levofloxacin 500 mg was administered to all subjects in each period of the study. The formulations were administered along with 240 mL of drinking water at ambient temperature, under low light condition after an overnight fast of at least 10 hour. Dosing was done under the supervision of trained study personnel. Both test and reference formulations were administered to all the study subjects, one in each period.

Assignment to treatment

The order of receiving the test and reference formulations, for each subject during both the periods of the study, was determined according to a SAS-generated balanced randomization schedule [Annexure I]. A working copy of the same was provided to study personnel responsible for dosing. The investigator, pharmacy in-charge, personnel involved in dispensing of study drugs and the dosing were accountable for ensuring compliance to randomization schedule.

3.1.8.2 Identity of Investigational Products

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

Test (T): Levofloxacin 500 mg tablets [Loxof tablets 500 mg] manufactured by Ranbaxy (M) SDN. BHD. Lot 23, Bakar Arang Industrial estate 08000 Sungai Petani, Kedah, Malaysia. Lot No./Batch No. 2272458

3.1.9 Washout period
There was a washout period of at 11 days between the administrations of study drugs in each period of the study.

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

3.1.11 Restrictions
Medications (Prior and Concomitant Medication Procedure) -
Subjects were required not to receive any medication including vitamins, herbal drugs and over the counter medications (OTC) during the 30 days prior to the onset of the study. They were instructed during screening not to take any prescription, herbal drugs and OTC medications until the completion of the study.

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

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

Activity-

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

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

A single oral dose of either test (T) or reference (R) was administered during each period of the study from 0900 hour to 0918 hour. 28 subjects were dosed in each period of the study.

3.1.13 Blood sampling

Intravenous indwelling cannula was kept in situ as long as possible (upto 24 hours post-dose). The cannulae were maintained patent by injection of upto 1 mL of 5 IU/mL of heparin in normal saline solution. In such cases, blood samples were collected after discarding the first 0.5 mL of heparinised blood and heparin solution from the tubing. Blood samples were collected at pre-dose (duplicate) and at 0.167, 0.333, 0.500, 0.667, 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, 24.000, and 36.000 hours post-dose post-dose in each period.

A total of forty-four (44), 4-mL blood samples (including pre-dose duplicate samples) were collected from each subject in K₃EDTA vacutainers during the course of the study under low light condition. The pre-dose blood sample (in duplicate) in each period were collected within a period of 1.5 hours before dosing and the in-house post-dose samples were collected within ± 2 minutes of the scheduled time. The actual end time of
collection of each blood sample was recorded. Total blood volume collected was 219 mL. Extra blood samples were collected only when required for safety sample after end of study.

All post-dose blood samples collected earlier or later than 2 minutes (during in-house stay) were reported as protocol deviations and the actual deviation from the scheduled time in collection were adjusted during pharmacokinetic and statistical calculations. After collection of blood samples from all the subjects at each time-point one of the study personnel or an attendant transferred all the collection tubes to a sample processing room at the study site. Thereafter the blood samples were centrifuged at a speed of 4000 RPM for duration of 15 minutes, at temperature of 4 ± 2°C under refrigeration, as soon as possible to separate plasma. All post-dose plasma samples were divided into 2 aliquots and transferred to suitably labeled tubes and re-checked to ensure transfer of plasma to the correct tube. The plasma samples were stored below – 15°C, pending transfer to the analytical facility for assay. All samples were collected and processed under low light conditions.

3.1.14 Safety: Clinical safety parameters

3.1.14.1 Vitals signs recording

Vital signs of sitting blood pressure, radial pulse rate and oral temperature were measured and recorded on the day of admission, pre-dose (within 2.0 hours) and post-dose at 4, 24 and 48 hours (within ±2.0 hours) in each period.

3.1.14.2 Clinical Examination

A brief clinical examination of the subjects was performed on the day of admission and prior to discharge in each period.

3.1.14.3 Laboratory Evaluations for Safety

Laboratory parameters of hematology and biochemistry [as mentioned in section 3.1.5.1] were repeated at the end of the study. Any laboratory parameter(s) outside ‘Acceptable Limits’ (Annexure II) were termed as laboratory abnormality and followed
up until the results were normal / clinically not significant or till the subjects were lost to follow-up.

3.1.15 Adverse event monitoring, recording and reporting
The subjects were under medical surveillance until 48 hours post-dose during each period. A medically qualified designate was on call for the remaining period of study. Subjects were monitored throughout the study period for adverse events. Subjects were informed to bring to the notice of the nurse or the doctor any adverse event that may occur. Subjects were specifically asked about any adverse events on the day of admission, at pre-dose [within 2.0 hour prior to dosing] and approximately 4, 12, 24, 36 and 48 hours post-dose [within ± 2.0 hours of the scheduled time] in each period. Management of adverse events was done by a physician.

Adverse events were reported and followed up at regular intervals until resolution of adverse event or determination that no further medical intervention is deemed necessary or when the adverse event was otherwise explained or the subject was lost to follow-up.

3.1.16 Discharge
All subjects were discharged 48 hours after administration of the study drug during each period.

<table>
<thead>
<tr>
<th>Study Phase</th>
<th>Screening</th>
<th>Study Days in Period I and II</th>
<th>End of Study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity</td>
<td>Admissions Day 0</td>
<td>Day 1</td>
</tr>
<tr>
<td>Written Informed Consent (in period I only)</td>
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<td>√</td>
<td></td>
</tr>
<tr>
<td>Screening procedures</td>
<td></td>
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<tr>
<td>Randomization</td>
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<tr>
<td>Clinical examination</td>
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<tr>
<td>Vital Signs Recording</td>
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<tr>
<td>Hematology</td>
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<tr>
<td>Biochemistry</td>
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</table>
### Materials and Methods

#### Study Days in Period I and II

<table>
<thead>
<tr>
<th>Study Phase</th>
<th>Screening</th>
<th>Study Days in Period I and II</th>
<th>End of Study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Within 28 days prior to admission.</td>
<td>admission Day 0</td>
<td>Day 1</td>
</tr>
<tr>
<td>Activity</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serology &amp; urinalysis</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Urine drug screen</td>
<td>√</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breath alcohol test</td>
<td>√</td>
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<tr>
<td>Criteria check (in period I only)</td>
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<tr>
<td>Check-in Procedures</td>
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<tr>
<td>Admission</td>
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<tr>
<td>Study Medication Administration (dosing will be suitably staggered)</td>
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<tr>
<td>PK Blood Sampling</td>
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<tr>
<td>- Pre-dose</td>
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<td></td>
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</tr>
<tr>
<td>- Post-dose</td>
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</tr>
<tr>
<td>Confinement in Study Unit</td>
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<tr>
<td>Discharge</td>
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</table>

### 3.1.17 Ethical Considerations

#### 3.1.17.1 Basic principles

This research was carried out in accordance with the Principles of Good Clinical practices defined in the Ethical Guidelines for Biomedical Research on Human Participants issued by Indian Council of Medical Research (ICMR), New Delhi, Good Clinical Practices guidelines for Clinical Research in India issued by Central Drugs Standard Control Organization, Ministry of Health and Family Welfare, Schedule Y, Drug & Cosmetics acts (amended 2005), the ICH E6 'Guidance for 'Guidance on Good Clinical Practice' and the principles enunciated in the Declaration of Helsinki (59th WMA General Assembly, Seoul, October 2008).
3.1.17.2 Informed Consent

‘Informed consent form’ was reviewed and approved by the Institutional board. Informed consent was obtained from all the subjects for the purpose of inclusion into the study. The purpose of the study, the procedures to be carried out and the potential hazards that may be encountered during the conduct of the study were described to the subjects in non-technical terms before the subjects were admitted. ‘Medical Query Resolution’ was a part of the Informed consent procedure where Medical Queries of the volunteers as regards the study drug were satisfactorily resolved by a Research Physician. Subjects were required to understand and sign the ‘consent form’ summarizing the discussion prior to admission for the study in Period I. Subjects were enrolled after they provided formal written informed consent after attending an oral presentation and after reading the Informed consent form (ICF) [Annexure III].

3.1.17.3 Drop-out/ Withdrawal of Subjects from Study

Subjects were informed that they are free to dropout from the study at any time without stating any reason. The investigator had the right to withdraw a subject from the study for any of the following reasons:

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

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

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

iv. The subject was positive for breath alcohol test/urinary screen testing of drugs of abuse (opiates or cannabinoids) on the day of admission of any period or consumed alcohol during the period from 48 hrs prior to admission till last sample collection in each period.
v. If the subject required concomitant medications which could affect pharmacokinetic comparison of the study medication. The decision to withdraw/ not withdraw the subject was to be based on the following:

a. The pharmacology and pharmacokinetics of the non-study medication.

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

c. The time of administration of the non-study medication.

It was required that details of reasons for withdrawal, if any, of subjects be recorded and reported and a complete follow-up for any withdrawn subject was to be obtained.

3.1.17.4 Volunteer Compensation

The subjects were adequately compensated on account of their participation in the study as per the guidelines issued by the Institutional Review Board.

3.1.17.5 Medical Treatment for Injury

In case of research related injury, first aid was available at study site and treatment of adverse reactions requiring hospitalization was to be undertaken at a nearby hospital.

3.1.18 Study documentation

All data generated during the conduct of the study were directly entered or transcribed in the raw data recording forms or study related forms, log books etc. The computer-generated chromatograms were also treated as raw data.

3.1.19 Confidentiality of data

The data identifying each study subject by name was kept confidential and was accessible to the study personnel, Quality Assurance Auditor during audits and if necessary, to the Institutional Review Board and various regulatory agencies.
3.2 BIO-ANALYTICAL PHASE

3.2.1 Bio-Analytical Method Development
A High Performance Liquid Chromatography Tandem Mass Spectrometric Method for the determination of Levofloxacin in Human K₃EDTA Plasma using Levofloxacin-d8 as an Internal Standard was developed and validated at the Bioanalytical site. Different Mobile phase compositions were tried and optimum resolution, run time and better peak shape was obtained with a mobile phase composition of Buffer-1: Acetonitrile (50:50, v/v) as mobile phase. Columns of different manufacturers, dimensions, particles size, pH range and carbon load were tried and Hypurity Advance (150 × 4.6 mm, 5 µ) column were selected to achieve better and reproducible results. Sample preparation process was performed by Solid-phase technique extraction using cartridges [Bond Elut Plexa, PCX, 30 mg/1cc]. The processed samples were chromatographed on Hypurity Advance (150 × 4.6 mm, 5 µ) column using Buffer-1: Acetonitrile (50:50, v/v) as mobile phase. Levofloxacin and Levofloxacin-d8 (ISTD) were detected by LC/MS/MS detection. Signals from the detector were captured by computer and processed using Analyst software.

3.2.2 Instrumentation and Reagents

**Instrumentation** HPLC from Cohesive Technologies, MS from Sciex (MS-15) and Analyst software Version 1.4.1 for data processing were used.

**Reagents**

1. Acetonitrile (HPLC Grade)
2. Ammonium formate (Analytical Grade)
3. Chloroform (Analytical Grade)
4. Formic Acid (Analytical Grade)
5. Levofloxacin (Working Standard)
6. Levofloxacin-d8 (Working Standard)
7. Methanol (HPLC-Grade)
8. Ortho Phosphoric Acid (Analytical Grade)
9. Solid phase extraction Cartridges (Bond Elut PLEXA - 30mg/1cc)
10. Water (HPLC-Grade)

3.2.3 A Summary of the Chromatographic and Mass Spectrometric Conditions are as follows:

Column : Hypurity Advance (150 × 4.6mm, 5 µ)
Mobile Phase : Buffer-1: Acetonitrile (50:50, v/v)
Flow Rate : 0.600 mL/min
Column Oven Temperature : 45°C ± 1.0°C
Sample Cooler Temperature : 10°C ± 1.0°C
Injection Volume : 10 µL
Retention Time : Levofloxacin: 1.3 to 2.3 minute
               Levofloxacin-d8: 1.3 to 2.3 minute
Ion source : Turbo Ion Spray in positive Ion mode
Rinsing solution-1 : Buffer-1: Acetonitrile (50:50,v/v)
Rinsing solution-2 : Chloroform:Methanol:Acetonitrile (20:30:50, v/v/+0.1% Formic Acid; v/v)
Total Run Time : 3.5 Minutes
API - 3200  
(MS-15)

Detection:

<table>
<thead>
<tr>
<th></th>
<th>Q1 Mass (amu)</th>
<th>Q3 Mass (amu)</th>
<th>Dwell Time (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levofloxacin</td>
<td>362.30</td>
<td>261.30</td>
<td>200.00</td>
</tr>
<tr>
<td>Levofloxacin-d8</td>
<td>370.30</td>
<td>265.30</td>
<td>200.00</td>
</tr>
</tbody>
</table>

Integration Parameters:

<table>
<thead>
<tr>
<th>Integration Parameter</th>
<th>Levofloxacin</th>
<th>Levofloxacin-d8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bunching factor</td>
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<td>1</td>
</tr>
<tr>
<td>Noise threshold</td>
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<td>1</td>
</tr>
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<td>Area threshold</td>
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</tr>
<tr>
<td>Number of smooth</td>
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</tr>
<tr>
<td>Separation width</td>
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<td>Separation Height</td>
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<td>1</td>
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<tr>
<td>RT window</td>
<td>Maximum 40.0 Sec</td>
<td>Maximum 40.0 Sec</td>
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</tbody>
</table>

Compound Parameters:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Levofloxacin</th>
<th>Levofloxacin-d8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declustering Potential (DP)</td>
<td>50.0 V</td>
<td>50.0 V</td>
</tr>
<tr>
<td>Entrance Potential (EP)</td>
<td>10.0 V</td>
<td>10.0 V</td>
</tr>
<tr>
<td>Collision Energy (CE)</td>
<td>40.0 V</td>
<td>40.0 V</td>
</tr>
<tr>
<td>Collision cell Exit Potential (CXP)</td>
<td>5.0 V</td>
<td>5.0 V</td>
</tr>
<tr>
<td>Cell Entrance Potential (CEP)</td>
<td>15.0 V</td>
<td>18.0 V</td>
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Source/Gas Parameters:

<table>
<thead>
<tr>
<th>GS1</th>
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</tr>
</thead>
<tbody>
<tr>
<td>GS2</td>
<td>50°</td>
</tr>
<tr>
<td>CAD</td>
<td>4°</td>
</tr>
<tr>
<td>CUR</td>
<td>40°</td>
</tr>
<tr>
<td>IS</td>
<td>5500 V</td>
</tr>
<tr>
<td>TEM</td>
<td>650°C</td>
</tr>
<tr>
<td>ihe</td>
<td>ON</td>
</tr>
</tbody>
</table>

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

Resolution Q1: Unit
Resolution Q3: Unit

3.2.4 SOURCE AND TYPE OF BIOLOGICAL MATRIX

Human Plasma lots were used. Human Haemolysed Plasma and Human Lipemic Plasma were also used. K$_3$EDTA was used as anticoagulant for human plasma as well as Human Haemolysed Plasma and Human Lipemic Plasma.

3.2.5 Sample preparation

1. Plasma samples were withdrawn as per requirement of method validation experiments from the freezer room.
2. These samples were allowed to thaw at room temperature and vortexed.
3. The samples were centrifuged at 4000 rpm for a minimum of 5 minutes.
4. 100 μL aliquot of each plasma sample was pipetted out into appropriately labeled polypropylene tubes.
5. 50 μL of internal standard stock dilution (approximately 400.0 ng/mL of Levofloxacin-d8) was added into these tubes (except standard blank).

6. 250 μL of Solution-1 was added to each polypropylene tube and the samples were vortexed to ensure complete mixing of contents.

7. The samples were centrifuged at 4000 rpm for a minimum of 5 minutes.

8. The cartridges [Bond Elut Plexa, PCX, 30 mg/1cc] were conditioned using 0.5 mL of methanol followed by 0.5 mL of HPLC-grade water by running the centrifuge at 4000 rpm for a minimum of 1 min after each addition.

9. The samples were loaded onto the cartridges and the centrifuge was run for a minimum of 1 minute at 4000 rpm.

10. The cartridges were washed with 1 mL of HPLC-grade water followed by 1 mL of solution-2 by running the centrifuge at 4000 rpm for a minimum of 1 minute

11. The samples were eluted into appropriately labeled polypropylene tubes with 1 mL of acetonitrile by running centrifuge at 4000 rpm for a minimum of 1 min.

12. The eluted samples were dried under stream of nitrogen at 50°C ± 2°C at about 20 psi.

13. The dried residue was reconstituted with 1000 μL of mobile phase.

14. The samples were transferred into glass vials for analysis.

Note:

1. All the samples were processed under low light condition.

2. Temperature of centrifuge was set between 2-10°C and maintained up to an ambient temperature condition, not exceeding 25°C.

3. During the centrifugation step, in case of any interruption or incomplete filtration from the cartridge, the same step was repeated by centrifuging the samples again at specified rpm for specified time.
3.2.6 Preparation of Solutions

**Buffer-1 (2mM Ammonium formate)**

0.126 ±0.006g of Ammonium formate was weighed and transferred into a reagent bottle and 1000 mL HPLC grade water was added to it. 3.0 ml of Formic Acid was added to it. It was mixed well and sonicated in an ultrasonic bath. This solution was stored at room temperature and used within 5 days from the date of its preparation.

**Mobile Phase (Buffer-1: Acetonitrile:: 50:50; v/v)**

500 mL of Buffer-1 was transferred into a reagent bottle. 500 mL of acetonitrile was added to it. It was mixed well and sonicated in an ultrasonic bath. This solution was stored at room temperature and used within 5 days from the date of its preparation.

**Solution-1 (5% Orthophosphoric Acid in Water; v/v)**

5 mL of orthophosphoric acid was transferred into a 100 mL volumetric flask. Volume was made up with HPLC-grade water. It was mixed well. This solution was stored at room temperature and used within 5 days from the date of its preparation.

**Solution-2 (5% methanol in HPLC-grade water; v/v)**

5 mL of methanol was transferred into a 100 mL volumetric flask. The volume was made up with HPLC grade water. It was mixed well. This solution was stored at room temperature and used within 5 days from the date of its preparation.

**Diluent-1 (HPLC-grade water:Methanol:: 50:50; v/v)**

500 mL of HPLC-grade water was transferred into a reagent bottle. 500 mL of Methanol was added to it. It was mixed well. This solution was stored at room temperature and used within 5 days from the date of its preparation.

**Rinsing Solution-1(Buffer-1: Acetonitrile::50:50,v/v)**

500 mL of Buffer-1 was transferred into a reagent bottle. 500 mL of acetonitrile was added to it. It was mixed well and sonicated in an ultrasonic bath. This solution was stored at room temperature and used within 5 days from the date of its preparation.
Rinsing Solution-2 (Chloroform:Methanol:Acetonitrile::20:30:50, v/v+0.1% Formic Acid; v/v)
100 mL of Chloroform was transferred into a reagent bottle. 150 mL of Methanol was added to it. 250 mL of Acetonitrile was added to it. 500 µL of Formic Acid was added to it. It was mixed well and sonicated in an ultrasonic bath. This solution was stored at room temperature and used within 5 days from the date of its preparation.

Rinsing Solution-3 (HPLC-grade water: Methanol::50:50; v/v)
500 mL of HPLC-grade water was transferred into a reagent bottle. 500 mL of Methanol was added to it. It was mixed well. This solution was stored at room temperature and used within 5 days from the date of its preparation. Note: During the method validation rinsing solution-1 and 2 were used with CTC Autosampler.

3.2.7 Preparation of Standard Stock Solutions

Levofloxacin Standard Stock Solution
Levofloxacin working standard was weighed accurately and transferred to a volumetric flask. It was dissolved in methanol and the volume was made up with the same to prepare a solution of approximately 5 mg/mL. The final concentration of Levofloxacin was corrected accounting for its potency, molecular weight and the actual amount weighed. It was stored in refrigerator between 1-10°C in a glass container, protected from light and used within 13 days from the date of its preparation. Stock solution and all further dilutions from the above stock solution were prepared under low light condition. All further dilutions from the above stock solution were prepared in polypropylene container and kept under low light condition.

Levofloxacin-d8 Internal Standard Stock Solution
Levofloxacin -d8 working standard was weighed accurately and dissolved in methanol to prepare a solution of approximately 1 mg/mL. The final concentration of Levofloxacin -d8 was corrected accounting for its potency, molecular weight and the actual amount weighed. It was stored in refrigerator between 1-10°C in a glass
container, protected from light and used within 13 days from the date of its preparation. Stock solution and all further dilutions from the above stock solution were prepared under low light condition. All further dilutions from the above stock solution were prepared in polypropylene container and kept under low light condition.

3.3 BIO-ANALYTICAL METHOD VALIDATION

The analytical method for the determination of levofloxacin in human K₃EDTA plasma using levofloxacin-d₈ as internal standard was validated for various Validation Parameters.

Validation Parameters

This validation was performed to evaluate the method in terms of selectivity, Selectivity of analyte in presence of concomitant medication (Acetaminophen, Diclofenac, Amoxicillin and Clavulanic acid), sensitivity, linearity of response, carry-over effect in human plasma, precision and accuracy in human plasma, recovery, stability, re-injection reproducibility, dilution integrity, matrix effect, matrix factor, ruggedness, extended precision and accuracy and long term stability.

The sensitivity, linearity, precision and accuracy evaluations were performed on three batches of spiked samples and all of them are reported. Each precision and accuracy batch consisted of one complete calibration curve (comprising of one blank plasma, one blank plasma with internal standard and eight different non-zero concentrations in which LOQ and ULOQ were in duplicate) and six replicates of quality control samples at LOQ, Low, Middle and High levels.
3.3.1 REFERENCE/WORKING STANDARDS

<table>
<thead>
<tr>
<th>Name of Standard</th>
<th>Batch No./Lot No.</th>
<th>Purity</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levofloxacin</td>
<td>1425507</td>
<td>99.4% (HPLC)</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Levofloxacin-d8</td>
<td>4-SBK-128-3</td>
<td>98.9% (HPLC)</td>
<td>TRC Canada</td>
</tr>
<tr>
<td>Diclofenac Sodium salt</td>
<td>075K1896</td>
<td>99% by TLC</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Clavulanate Lithium</td>
<td>J0G109</td>
<td>95.7% w/w on as is basis as Clavulanic acid</td>
<td>USP</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>K0H332</td>
<td>86.4% w/w on as is basis</td>
<td>USP</td>
</tr>
<tr>
<td>Acetaminophen (Paracetamol)</td>
<td>CS-PT-465</td>
<td>99.99% by HPLC as is basis</td>
<td>Clearsynth labs, Mumbai</td>
</tr>
</tbody>
</table>

3.3.2 STANDARDIZATION AND CALCULATION

The chromatographic data were acquired and processed using computer based Analyst software Version 1.4.1. The best-fit curves using weighted (1/Concentration²) linear least square regression analysis were obtained by peak area ratio of Levofloxacin to Levofloxacin-d8 (ISTD). The concentrations of Levofloxacin in plasma samples were calculated using linear regression parameters of the corresponding calibration curve. Weighting factor 1/Concentration² was selected after weighting factor evaluation using three precision and accuracy batches (with weighting: None, 1/Concentration and 1/Concentration²).

Refer: Table MV 3 for weighting factor evaluation of Levofloxacin.
### 3.3.3 Calibration Curve Standards and Quality Control Samples

<table>
<thead>
<tr>
<th>Type of Standards</th>
<th>Nominal Concentration (ng/mL)</th>
<th>Storage Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration curve standards in pooled normal human plasma</td>
<td>30.8 (in duplicate), 85.6, 237.9, 660.8, 1835.5, 5098.5, 10197.1, 13073.2 (in duplicate)</td>
<td>Below -15°C in freezer room in polypropylene container</td>
</tr>
<tr>
<td>Quality control samples in pooled normal human plasma</td>
<td>LOQQC – 31.2, LQC – 86.6, MQC – 5095.7, HQC – 10191.5</td>
<td>Below -15°C in freezer room in polypropylene container</td>
</tr>
</tbody>
</table>

Note:

1. Matrix IDs [260111-17, 260111-23, 260111-26 and 260111-28] were pooled (Pooled Plasma ID V501PP/01) for bulk spiking to prepare calibration curve standards and quality control samples in normal human plasma.
2. Matrix ID [240211-07] was used for spiking of quality control samples in Haemolysed plasma and Matrix ID [240211-13] was used for spiking of quality control samples in Lipemic plasma.
3. QC IDs V501/LQC, HQC #127 to 150 were stored in freezer room below -15°C, for long-term stability.
4. Freshly spiked calibration curve standards and quality control samples (in normal, haemolysed and lipemic human plasma) were prepared as per requirements of individual validation parameters.
5. Spiking was done at room temperature under low light condition.
Validation Procedure and Acceptance Criteria

3.3.4 Selectivity and S/N Ratio

Selectivity

It is the ability of the bioanalytical method to measure and differentiate the analytes in the presence of components that may be expected to be present. These could include metabolites, impurities, degradants or matrix components. For selectivity, analyses of blank samples of the biological matrix, obtained from matrix batches, was done. Each blank sample was tested for interference, and selectivity was ensured at the lower limit of quantification. The specific steps followed for the determination of the same are detailed below:

- Ten different blank matrix batches were screened for interference at the retention time (RT) of all peaks of interest using proposed extraction procedure and chromatographic conditions.

- Eight batches [180211-18, 180211-28, 260111-12, 260111-17, 260111-21, 260111-23, 260111-26, 260111-28] were from normal matrix, one from hemolyzed matrix [240211-07] and other one from lipemic matrix [240211-13].

- Blank matrix batches were processed and injected (without addition of internal standard).

- 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.

- Appropriate analyte dilution was spiked in pooled plasma to obtain concentration approximately equivalent to LOQ of the method.

- Six aliquots of the spiked LOQ samples were processed and injected (with addition of internal standard).
- 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:

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

\[
\text{% Interference for internal standard} = \frac{\text{Peak area response at RT of internal standard in blank matrix}}{\text{Mean peak area response of the internal standard in extracted LOQ samples}} \times 100
\]

**The Acceptance Criteria followed for Selectivity were:**

1) 80% of screened normal matrix batches along with both hemolyzed and lipemic matrix batches should meet the following acceptance criteria.

- Response of interfering peaks at the retention time of analyte in blank matrix must be \( \leq \) 20% of the mean peak area response of the analyte in LOQ samples.

- Response of interfering peaks at the retention time of internal standard in blank matrix must be \( \leq \) 5% of the mean peak area response of the internal standard in LOQ samples.

2) % C.V. should be \( \leq \) 20% for both analyte area and internal standard area in the LOQ samples.
Note: If LOQ sample is rejected due to analytical reason (e.g. Lost during processing/analysis, Bad chromatography etc.), mean of at least five accepted LOQ samples should be available for calculating % interference at RT of all peak of interest in the blank samples.

**S/N Ratio**

- Signal to Noise (S/N) ratio was determined for all accepted normal blank matrix batches and LOQ samples of selectivity exercise was calculated.
- Mean of S/N ratio for accepted normal blank matrix samples was calculated.
- Ratio of (S/N ratio of each LOQ sample) and mean S/N ratio of normal blank matrix samples was calculated.

**Acceptance Criteria followed for S/N ratio were:**

- Ratio of (S/N ratio of each LOQ sample) and mean S/N ratio of normal blank matrix samples should be \( \geq 5 \) for all LOQ samples.

\[
\frac{\text{S/N of LOQ sample}}{\text{Mean of S/N of Normal Blanks}} \geq 5
\]

**Selectivity of analyte in presence of concomitant medication**

- LOQ solution of analyte with internal standard was prepared at a concentration approximately equivalent to validated LOQ concentration assuming actual recovery.

- Stock solution of 1mg/mL was prepared for the following drugs (in the solvents specified as below):

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Solvent for Stock Solution</th>
<th>Stability Duration (Days)</th>
<th>Storage condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>HPLC Grade Methanol</td>
<td>14</td>
<td>Refrigerator between 1-10°C, protected from light</td>
</tr>
</tbody>
</table>
From the above stock, stock dilution of each drug was prepared at a concentration of approximately 1.0 µg/mL using solution used for injecting analyte in the mass spectrometer.

Stock dilution of each drug was injected in duplicate into the LC/MS/MS system along with six injections of LOQ solution using chromatographic conditions of analyte.

The interference at the RT of an analyte was evaluated by comparing the response in each injection of drug against the mean peak area response of the analyte in LOQ samples.

The interference at the RT of the internal standard was evaluated by comparing the response in each injection of drug against the mean peak area response of the internal standard in LOQ samples.

Acceptance Criteria followed for Selectivity of Analyte in presence of concomitant medication were:

- 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.
• If a LOQ sample is rejected due to an analytical reason (e.g. Lost during analysis, Bad chromatography etc.), mean of at least five accepted LOQ samples should be available for calculating % interference at RT of all peak of interest in the blank samples.

3.3.5 Sensitivity

The lowest standard was accepted as the limit of quantification (LOQ) of the method if:

• Between batch precision (%C.V.) at the LOQQC was ≤ 20%. (Taken from PA batches)
• Between batch accuracy (% nominal) at the LOQQC was between 80-120%. (Taken from PA batches)
• S/N ratio of LOQ samples should be at least 5 times of mean S/N ratio of blank matrix samples (taken from Selectivity and S/N ratio exercise).

3.3.6 Linearity

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

• Three calibration curves were used of accepted PA batches to establish linearity.
• Linear regression algorithm was selected.
• Appropriate weighting factor was statistically determined using the following formula:

\[
\left| \frac{\sum \% \text{ dev}}{\sum (\% \text{ dev})^2} \right|
\]

where % dev represents the percent deviation for each non-zero calibrant,

i.e.: (% Nominal – 100) or \( \frac{\text{Back Calculated concentration} - \text{Nominal concentration}}{\text{Nominal concentration}} \times 100 \)
• **Weighting factors were chosen by the following procedure for all three-calibration curves.**

  ➢ Weighting factor-None was chosen and the calibration standard concentrations and % Nominal for all three-calibration curves were calculated.

  ➢ % dev for each non-zero calibrant were calculated for CC curve I and sum the results to get Σ% dev (X1).

  ➢ ( % dev )^2 for each non-zero calibrant were calculated for CC curve I and sum the results to get Σ ( % dev )^2 (Y1).

  ➢ √Y1 was calculated and |X1| and √Y1 were summed to get Z1 value.

  ➢ Similarly Z2 and Z3 value were calculated for CC curve II and III.

  ➢ Z1, Z2 and Z3 values were summed to get Z value for the linear regression algorithm without any weighting.

  \[
  Z = Z_1 + Z_2 + Z_3
  \]

  ➢ Z value was also calculated using weighting 1/x and 1/x^2 weighting factor.

  ➢ Lowest Z value was indicative of the weighting factor used.

  ➢ Mean concentrations, S.D., % C.V. and % nominal were determined for all calibrants of the three calibration curves using the chosen weighting factor.

Note: Acceptability of each PA batch was checked as per section 3.3.7 using 1/x^2 weighting factor for selecting weighting factor.

3.3.7 **Precision and Accuracy**

The precision of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. The precision of the assay was measured by the percent coefficient of variation over concentration range of LOQQC, I.QC, MQC and HQC quality control samples of levofloxacin.
The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. The deviation of the mean from the true value serves as the measure of accuracy. The accuracy of the assay was defined as the absolute value of the calculated mean values of the quality control samples to their respective nominal values, expressed as percentage.

The specific steps followed for determination of precision and accuracy are detailed below:

Levosofloxac in Standard Stock Solution and Levosofloxac in-d8 Internal Standard Stock Solution were prepared as mention below:

Levosofloxac in Standard Stock Solution:

1. Levosofloxac in working standard was weighed accurately and transferred to a volumetric flask. It was dissolved in methanol and the volume was made up with the same to prepare a solution of approximately 5 mg/mL.
2. The final concentration of Levosofloxac in was corrected accounting for its potency, molecular weight and the actual amount weighed. It was stored in refrigerator between 1-10°C in a glass container,

Levosofloxac in-d8 Internal Standard Stock Solution

1. Levosofloxac in -d8 working standard was weighed accurately and dissolved in methanol to prepare a solution of approximately 1 mg/mL.
2. The final concentration of Levosofloxac in-d8 was corrected accounting for its potency, molecular weight and the actual amount weighed. It was stored in refrigerator between 1-10°C in a glass container

CC standards and QC samples were prepared following appropriate steps
Each validation batch consisted of the following samples in the following order:

- Reference solution [analyte and internal standard]
- Matrix blank
- Matrix blank with internal standard
- Spiked calibration standards (One set of eight non-zero concentrations in which LOQ and ULOQ were in duplicate)
- LOQ QC
- Low QC
- Middle QC
- High QC

Note:

- Six replicates of each QC set (containing one LOQQC, LQC, MQC and HQC) were processed and analyzed.
- LOQ of the PA batch was equivalent to LOQ of the selectivity (preferably within 2%).
- Processed and analyzed three (3) PA batches, two PA batches on same day and third batch on another day.
- Calibration curve were prepared using all accepted CC standards including duplicate LOQ and ULOQ standard.
- QC concentrations were calculated and mean concentration, S.D., % C.V. and % nominal values were determined.
- Both within and between batch precision and accuracy were assessed.
- Both Intraday (using two PA batches run on same day) and Interday (using all three PA Batches) precision and accuracy were assessed.
% C.V. = \frac{\text{Standard Deviation}}{\text{Mean Calculated Concentration}} \times 100

% Nominal = \frac{\text{Mean Calculated Concentration}}{\text{Nominal Concentration}} \times 100

The Acceptance Criteria followed for Calibration Curve were:

1) Blank and Blank + Internal standard should be free of significant interference at the retention times of the analyte and internal standard.
   - Interference should be considered significant if the peak area response at the retention time of the analyte in the Blank, Blank + Internal standard is > 20% of peak area response of each accepted LOQ standard.
   
   Note: If one of the LOQ samples is rejected, interference should be compared with accepted LOQ sample.

   - Interference should be considered significant if the peak area response at the retention time of the internal standard in the blank is > 5% of the mean internal standard response of the standards used in the calculation of calibration curve.

2) If blank or Blank + Internal standard sample is rejected due to any analytical reason [(e.g. Lost during processing/analysis, Bad chromatography, Internal standard variation Chromatographic acceptance criteria and verification of chromatograms) etc.], the analytical batch should be rejected.
Internal standard variation shall be considered in the following cases:

- If the peak area response of the ISTD in a subject sample has a response which is less than 40% or greater than 180% of the mean peak area response of ISTD in the accepted non-zero standards used to calculate the calibration function, then the sample shall be identified for repeat analysis.

- Furthermore Quality control samples showing the above mentioned ISTD variation shall also be rejected.

- In case ISTD variation is suspected in any calibration standard then that standard shall be confirmed for ISTD variation if peak area response of ISTD in the calibration standard is less than 40% or more than 180% of the mean peak area response of ISTD for rest of calibration standards. This calibration standard shall be rejected and not included to calculate the mean peak area response of ISTD used for evaluating ISTD variation in rest of the batch.

3) At least 75% of non-zero standards should meet the following criteria, including at least one LOQ and ULOQ standard.

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

4) Calibration curve should be prepared using all accepted CC standards including duplicate LOQ and ULOQ standard. Rejected calibration standard should not be used for preparing calibration curve.

CC standard were to be rejected due to following reasons.

- CC standard not meeting the acceptance criteria as defined in point No. 3

- Due to any analytical reason [e.g. Lost during processing/analysis, Bad chromatography, Internal standard variation. Etc]

5) Linear coefficient of correlation should be ≥ 0.98
Acceptance Criteria followed for QC samples were:

1) At least 67% of total QC samples including at least 50% at each concentration should meet following acceptance criteria.
   - LQC, MQC and HQC samples should be within ±15% of their respective nominal values and LOQQC samples should be within ±20% of their respective nominal values.

2) Precision
   The % C.V. at low, middle and high concentration level should be ≤ 15% and at LOQ QC level should be ≤ 20%.

3) Accuracy
   The mean concentrations should be within ± 15% of the nominal concentrations at low, middle and high QC concentrations and should not deviate by more than ± 20% at the LOQ QC concentration.

4) In case a QC sample is rejected due to an analytical reason (e.g. Internal standard variation, Lost during processing / analysis, Bad chromatography etc.), at least five QC samples at each concentration should be available to calculate precision and accuracy.

3.3.8 EXTENDED PRECISION AND ACCURACY BATCH
Fourty five sets of low, middle and high QC samples were processed and analyzed against a single calibration curve.

Acceptance Criteria followed for Calibration Curve

For Acceptance criteria for Calibration Curve refer section 3.3.7

Acceptance Criteria followed for QC samples were:

At least 67% of the LQC, MQC and HQC samples including at least 50% at each concentration should be within ±15% of their respective nominal values.
Acceptance Criteria followed for Precision and Accuracy were:

**Precision**

% C.V. should be \( \leq 15\% \) for low, middle and high QC concentrations.

**Accuracy**

The mean back calculated concentrations for low, middle and high QC concentrations should be within \( \pm 15\% \) of their nominal concentrations.

3.3.9 **Recovery**

It is the extraction efficiency of an analytical process, reported as a percentage of the known amount of analyte carried through the sample extraction and processing steps of the method. The recovery of an analyte in an assay is the detector response obtained from an amount of the analyte added to and extracted from the biological matrix, compared to the detector response obtained for the true concentration of the pure authentic standard. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and of the internal standard should be consistent, precise, and reproducible. Recovery experiments were performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery.

**Recovery of Levofloxacin**

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

The specific steps followed were:

- Dilution of analyte, at approximate concentrations representing 100% extraction of QC samples at low, middle and high concentrations were prepared. These served as non-extracted samples.

- Diluent used for preparation of the non-extracted samples was similar in constitution as that of final extracted sample to be injected.

- Six (6) replicates of QC samples at each low, middle and high concentration were extracted (i.e., internal standard was added during the sample pre-treatment procedure). These samples served as the extracted samples.

- Six (6) replicates of the “non-extracted” samples and 6 replicates of the “extracted samples”, at each low, middle and high QC concentration were injected.

- The peak area responses of extracted and non-extracted samples at each low, middle and high QC level were tabulated and the mean response, S.D., % C.V. and % Recovery were calculated.

- The percent recovery at each QC level was calculated as follows:

\[
\text{% Recovery} = \frac{\text{Mean Peak Area Response of Extracted Samples}}{\text{Mean Peak Area Response of Non Extracted Samples} \times \text{C.F.}} \times 100
\]

\[
\text{C.F.} = \frac{\text{Concentration of extracted sample}}{\text{Concentration of non extracted sample}}
\]
Acceptance Criteria

The recovery was considered acceptable if

- C.V. was < 20% for % recovery between low, middle and high QC concentrations.
- Mean % recovery between low, middle and high QC concentrations was < 115%.

Internal Standard (ISTD)

Recovery of Levofloxacin-d8 (ISTD)

The percentage recovery of Levofloxacin-d8 (ISTD) was determined by comparing the mean peak area response of Levofloxacin-d8 (ISTD) in extracted quality control samples (MQC) against the mean peak area response of unextracted internal standards solution prepared at concentration representing 100% extraction of internal standard.

The specific steps followed are detailed below:

- Internal standard dilution at approximate concentration representing 100% extraction of the internal standard used for the method was prepared. This sample served as the non-extracted sample.
- Six (6) replicates of the “non extracted” sample were injected from the same vial.
- Peak area response for the extracted internal standard samples was taken from the experiment used to assess the recovery of analyte at MQC concentration. These samples served as extracted samples.
- The peak area responses of the ‘extracted’ and ‘non-extracted’ internal standard samples were tabulated. And the mean response, S.D., %C.V. and % Recovery were calculated and reported.
Acceptance Criteria

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

3.3.10 Ruggedness

One batch of precision and accuracy was processed and analyzed using different solutions (prepared by analyst performing ruggedness) and different column (same type) by a different analyst employing the same instrument.

Acceptance Criteria

As described in section 3.3.7 for Precision and Accuracy batch.

3.3.11 Carry over effect in matrix in human plasma

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

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

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

Carryover effect for analyte and internal standard was calculated as per following formula.
Response of interfering peak at RT of analyte in second blank sample - Response of interfering peak at RT of analyte in first blank sample

\[ \text{Carry over effect for analyte} = \frac{\text{Mean peak area response of the analyte in processed LOQ samples}}{\times 100} \]

Response of interfering peak at RT of internal standard in second blank sample - Response of interfering peak at RT of internal standard in first blank sample

\[ \text{Carry over effect for internal standard} = \frac{\text{Mean peak area response of the internal standard in processed LOQ samples}}{\times 100} \]

Acceptance Criteria

- The response of interfering peak at RT of analyte in first blank sample should be \( \leq 20\% \) of the mean peak area response of the analyte in processed LOQ sample.
- The response of interfering peak at RT of internal standard in first blank sample should be \( \leq 5\% \) of the mean peak area response of the internal standard in processed LOQ sample.
- Carryover effect should be \( \leq 20\% \) for analyte and \( \leq 5\% \) for internal standard.

3.3.12 Matrix Effect

The direct or indirect alteration or interference in response due to the presence of unintended analytes (for analysis) or other interfering substances in the sample. Matrix effect was performed by freshly spiking LOQQC and HQC samples into each of six lots of accepted blank matrices including one hemolyzed and one lipemic matrix. Two aliquots of LOQQC and HQC samples from each batch of blank matrix were taken and internal standard was added and processed. The values of these QCs were back calculated against freshly spiked calibration curve.
The specific steps followed are detailed below:

- LOQQC and HQC samples were spiked into six different batches of accepted blank matrix including one lipemic and one hemolyzed matrix.

- Blank matrix batch were considered acceptable if response of interfering peaks in the blank matrix at the retention time of analyte was $\leq 20\%$ of the mean peak area response of analyte in the extracted LOQ samples and response of interfering peaks in the blank matrix at the retention time of internal standard was $\leq 5\%$ of the mean peak area response of the internal standard in the extracted LOQ samples.

- 2 aliquots of LOQQC and HQC samples were taken from each batch of blank matrix, internal standard was added and processed. These samples served as ‘matrix effect samples’.

- Also, freshly spiked calibration standards, six LOQQC samples and six HQC samples were prepared in pooled plasma and processed.

- LOQQC samples were prepared at an approximate concentration of the LOQ standard (not to exceed more than 2 % of the LOQ and not less than LOQ).

- CC standards and QC samples were injected.

- The values of QC samples were back calculated against calibration curve and mean concentration, S.D., % C.V. and % nominal values were determined for matrix effect samples at LOQQC and HQC level, freshly spiked LOQQC samples and freshly spiked HQC samples.

\[
\text{Standard Deviation} \quad \frac{\% \text{ C.V.}}{\text{Mean Calculated Concentration}} \times 100
\]

\[
\text{Mean Calculated Concentration} \times \frac{\% \text{ Nominal}}{\text{Nominal Concentration}} \times 100
\]
Acceptance Criteria

Acceptance Criteria for Calibration Curve

➢ For Acceptance criteria for Calibration Curve refer section 3.3.7.

Acceptance Criteria for freshly prepared LOQQC and HQC samples

➢ For Acceptance criteria for LOQQC and HQC samples refer section 3.3.7

Acceptance Criteria for Matrix Effect Samples

The matrix effect is nullified if

• The mean concentration is within ± 20% of the nominal concentrations for matrix effect samples at LOQQC level and within ± 15 % of the nominal concentrations for matrix effect samples at HQC level.

• % C.V. should be ≤ 20% for matrix effect samples at LOQQC level and ≤ 15% for matrix effect samples at HQC level.

• At least 67% of total QC samples including at least 50% at each concentration should meet following acceptance criteria for each type of matrix.

  - HQC samples should be within ±15% of their respective nominal values and LOQQC samples should be within ±20% of their respective nominal values.

3.3.13 Matrix Factor

It is a quantitative measure of the matrix effects due to suppression or enhancement of ionization in mass spectrometric detection.

Matrix factor was calculated by the following steps:

• Reference mixtures of internal standard and analyte at approximate concentrations representing 100% extraction of internal standard and analyte at low, middle and high QC concentrations were prepared. These served as ‘matrix factor reference samples’.
Six aliquots from each of six different batches of accepted blank matrix including one lipemic and one hemolyzed matrix were taken and processed without addition of Internal Standard.

Two aliquots of each blank matrix were reconstituted with matrix factor reference samples at low QC concentrations, two aliquots of each blank matrix were reconstituted with matrix factor reference samples at middle QC concentrations and two aliquots of each blank matrix were reconstituted with matrix factor reference samples at high QC concentrations were reconstituted.

These samples served as ‘matrix samples reconstituted with matrix factor reference samples’.

These 36 samples along with 4 replicates of each reference mixture of internal standard and analyte at low, middle and high QC level (Total 48 samples) were injected.

Peak area ratio of each was tabulated. Also mean peak area ratio at low, middle and high QC level for each blank matrix was calculated.

Matrix factor (MF) was calculated at low, middle and high QC level for each blank matrix.

Mean peak area ratio of ‘matrix samples reconstituted with matrix factor reference samples at the LQC/MQC/HQC level at each blank matrix

Mean peak area ratio of matrix factor reference samples at the LQC/MQC/HQC level

Mean, S.D. and % C.V. of matrix factor was calculated at Low, Middle and High QC level.

Mean, S.D. and % C.V. was calculated for mean matrix factor calculated at Low, Middle and High QC level.
Acceptance Criteria followed for matrix factor were:

- The % C.V. of matrix factor at low, middle and high QC level should be ≤ 15%.
- The % C.V. of matrix factor between low, middle and high QC level should be ≤ 15%.
- In case a QC sample is rejected due to an analytical reason (e.g. Lost during processing / analysis, Bad chromatography etc.), at least one QC sample should be available for calculating matrix factor at low, middle and high QC level for each matrix batch.

3.3.14 Re-injection Reproducibility

Re-injection reproducibility was evaluated by re-injecting the low, middle and high QC samples of the already injected PA Batch. The re-injected QC samples were quantitated against the calibration curve standards analysed with Precision and Accuracy Batch.

The specific steps followed are detailed below:

- PA batch (Precision-Accuracy batch) analyzed and meeting the acceptance criteria was used for establishing re-injection reproducibility.
- All QC samples of the PA batch were re-injected.
- The re-injected QC concentrations were calculated using CC curve of PA batch and the mean concentration, S.D., %C.V. and % nominal concentrations were determined at all QC levels.
- % Difference for each QC concentration was calculated.

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

\[
\% \text{ Difference} = \frac{\text{Absolute (Original Value} - \text{Reinjected Value)}}{\text{Original Value}} \times 100
\]
Acceptance Criteria followed for Re-injection Reproducibility were:

- All re-injected QC samples must meet the acceptance criteria specified in section 3.3.7
- % Difference of 80% of all QCs re-injected should be within 15%.

### 3.3.15 Dilution Integrity

Dilution integrity sample was prepared by spiking Levofoxacin stock dilution into blank plasma sample to get 160-180% of ULOQ concentration. Six replicates of dilution integrity sample were diluted by a factor of 2 and Six replicates were diluted by a factor of 4, using similar blank matrix. These diluted samples were processed and analysed against freshly spiked calibration curve.

The specific steps followed are detailed below:

- Dilution integrity sample at a concentration between 160-180% of ULOQ was prepared.
- Appropriate volume of the spiked dilution integrity sample was taken and six replicates were diluted by a factor of 2 times and six replicates by a factor of 4 times, using similar blank matrix.
- Dilution integrity samples were processed and analyzed along with freshly spiked calibration standards and four replicates of freshly spiked low and high concentrations QC samples (comparison samples).
- The concentrations for dilution integrity samples and freshly spiked QC samples were calculated and the mean concentration, S.D., % C.V. and % nominal values were determined for both two times and four times diluted dilution integrity samples and comparison QC samples at low and high QC levels.

Acceptance Criteria for calibration curve:

- For Acceptance criteria for Calibration Curve refer section 3.3.7
Acceptance Criteria for comparison QC samples:

- At least 67% of the QC samples including at least 50% at each concentration should be within ±15% of their respective nominal values.

- % C.V. should be ≤ 15% and the mean concentrations should be within ± 15% of the nominal concentrations at both low and high QC concentrations.

- In case a QC sample is rejected due to an analytical reason (e.g. Internal standard variation, Lost during processing/analysis, Bad chromatography etc.), at least three should be available for accuracy and precision.

Acceptance criteria for dilution integrity samples:

- The mean concentrations should be within ± 15% of the nominal concentrations for two times and four times diluted dilution integrity samples.

- Precision should be ≤ 15% for two times and four times diluted dilution integrity samples.

- Accuracy of at least 50% QCs must be within ±15% of the nominal concentration for each dilution factor (two times and four times).

- In case a dilution integrity sample is rejected due to an analytical reason (e.g. Internal standard variation, Lost during processing/analysis, Bad chromatography etc.), at least five samples should be available to determine precision and mean accuracy.

3.3.16. Stability

3.3.16.1 Analyte and Internal standard stock stability

Stock solution stability of Levofloxacin and Levofloxacin-d8

The stability of stock solution of levofloxacin and levofloxacin–d8 was tested after a particular storage period when stored under refrigeration at 1-10°C temperature. Six replicates of each were run along with six replicates of freshly prepared stock solutions, the latter being considered as comparison stocks. Percentage [%] stability of stock
solution [stability samples] was calculated by comparing mean peak area ratios with that of comparison stock [comparison samples].

- To determine stock solution stability, analyte [Levofloxacin] and internal standard [Levofloxacin-d8] stock solutions were prepared (stability stocks) and aliquots of the same were kept under refrigeration at 1-10°C temperature. These served as stability stock solutions.

- Following a particular storage period, fresh stock solutions of the analyte and internal standard were prepared. These served as comparison stock solutions.

- Appropriate dilutions in mobile phase were prepared from stability and comparison stock solutions of analyte and internal standard.

- Appropriate ISTD dilution were prepared and added in both stability and comparison dilutions of analyte.

- Appropriate analyte dilution were prepared and added in both stability and comparison dilutions of internal standard.

- Six (6) replicates from the vial of the stability stock dilution and 6 replicates from the vial of comparison stock dilution were injected.

- The peak area ratio obtained from the stability and comparison stock dilutions were tabulated. Mean ratio, S.D., %C.V. and % Stability were calculated.

- Peak area ratio of analyte and internal standard was used for stock solution stability of analyte and peak area ratio of internal standard and analyte was used for stock solution stability of internal standard.

- % Stability was calculated as follows:

\[
\% \text{ Stability} = \frac{\text{Mean Peak Area Ratio of Stability Samples}}{\text{Mean Peak Area Ratio of Comparison Samples} \times \text{C.F.}} \times 100
\]
Concentration of Stability Samples
C.F. = \frac{\text{Concentration of Stability Samples}}{\text{Concentration of Comparison Samples}}

Acceptance criteria
The stability should be considered acceptable if % stability is within the range of 90-110%.

3.3.16.2 Short-term stability for Analyte dilution, Internal standard dilution and their mixture (Reference Solution)

Short-term stability of Levofloxacin and Levofloxacin-d8 (ISTD)
Short-term stability for Levofloxacin and Levofloxacin-d8 (ISTD) was determined. The appropriate dilutions of stability stock for Levofloxacin and Levofloxacin-d8 (ISTD) were prepared; these dilutions were stored at specified conditions for a specified period. These were analyzed along with freshly prepared dilutions prepared from the same respective standard stock solutions (comparison stock dilutions), which was used for preparing the stability dilutions.

Short-term stability was determined by comparing the mean peak area response of analyte [Levofloxacin] or internal standard [Levofloxacin-d8] in stability dilutions against mean peak area response of analyte [Levofloxacin] or internal standard [Levofloxacin-d8] in comparison dilutions.

Short-term stability of reference mixture dilution solution
Short-term stability of reference mixture dilution solution containing Levofloxacin and Levofloxacin-d8 (ISTD) was determined. The stability reference dilutions stored under specified conditions for short term duration were analyzed with freshly prepared reference solution dilutions.
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Short-term stability was determined by comparing the mean peak area ratio of analyte [Levofloxacin] / Internal standard [Levofloxacin-d8] in stability dilutions against mean peak area ratio of analyte [Levofloxacin] / Internal standard [Levofloxacin-d8] in comparison dilutions.

The specific steps followed are detailed below:

- Appropriate analyte [Levofloxacin], internal standard [Levofloxacin-d8] and reference mixture dilutions [containing Levofloxacin and Levofloxacin-d8 (ISTD)] from the respective standard stock solutions were prepared. These served as the stability dilutions.

- These dilutions were stored at room temperature under low light condition for a period of 7.78 hours for Analyte & Internal standard and a period of 7.8 hours for the Reference mixture.

- Following the stability period, fresh dilutions of the analyte, internal standard and reference mixture were prepared from the same respective standard stock solutions from which the stability dilutions were made. These served as the comparison dilutions.

- Analyte and internal standard dilution for short-term stability were prepared in the solvent/solution used for dilution of standard solution of analyte and internal standard, respectively.

- Reference mixture dilution for short-term stability was prepared in the solvent/solution used for daily preparation of reference mixture.

- Final analyte and internal standard stability and comparison dilutions were prepared in mobile phase.

- Appropriate ISTD dilution was prepared and added in both stability and comparison dilutions of analyte.

- Appropriate analyte dilution was prepared and added in both stability and comparison dilutions of internal standard.
Six (6) replicates of the stability dilutions from the same vial and 6 replicates of the comparison dilutions from the same vial were injected.

The Short term stability duration was calculated as the difference between the time at which comparison dilutions were prepared less the time at which stability dilutions were prepared (in hours).

Peak area ratio obtained from the stability and comparison stock dilutions were tabulated.

Mean ratio, S.D., %C.V. and % Stability were calculated.

% Stability was calculated as follows:

\[
\frac{\text{Mean Peak Area Ratio of Stability Samples}}{\text{Mean Peak Area Ratio of Comparison Samples}} = \frac{\text{Mean Stability for Analyte or Internal Standard or Reference Mixture}}{}
\]

The acceptance criterion followed was:

The stability should be considered acceptable if % stability is within the range of 90-110%.

3.3.16.3 Bench top stability (Short-term temperature stability)

3.3.16.3.1 Bench top stability in human plasma

The bench-top stability evaluation involved analysis of four replicates of low and high quality control samples of Levofloxacin (stability samples) [prepared by spiking of human plasma] which were stored unprocessed. These were compared with four replicates of freshly spiked low and high quality control samples of Levofloxacin (comparison samples) prepared in human plasma against calibration curve prepared with freshly spiked calibration standards.
The percentage stability was determined by comparing the mean concentration of Levofloxacin in stability quality control samples against the mean concentration of Levofloxacin in comparison quality control samples.

The specific steps followed are detailed below:

- Four (4) replicates of QC samples at low and high concentrations were removed from the freezer room and stored unprocessed for a period of 6.27 hours at room temperature under low light conditions. These served as stability samples.
- Freshly spiked calibration standards and four replicates of freshly spiked low and high concentrations QC samples were prepared just before processing. (Comparison samples).
- The stability QC samples were processed after the intended storage duration along with the freshly spiked calibration standards and freshly spiked quality control samples (Comparison samples).
- The CC standards and QC samples were injected.
- The bench top stability duration was calculated as the difference between the processing start time of QC samples and the time samples were removed from the freezer room (in hours).
- QC concentrations were calculated and the mean concentration, S.D., %C.V. and % nominal concentrations were determined for both the stability and comparison QC samples at low and high QC levels.

- % Stability was calculated at both low and high QC concentrations.

\[
\text{% Stability} = \left( \frac{\text{Mean concentration of stability samples}}{\text{Mean concentration of comparison samples \times C.F.}} \right) \times 100
\]

\[
\text{C.F.} = \frac{\text{Concentration of Stability Samples}}{\text{Concentration of Comparison Samples}}
\]
Acceptance Criteria followed for calibration curve:

- For Acceptance criteria for Calibration Curve refer section 3.3.7.

Acceptance Criteria for comparison QC samples

- At least 67% of the QC samples including at least 50% at each concentration should be within ±15% of their respective nominal values.
- % C.V. should be ≤ 15 % and the mean concentrations should be within ± 15% of the nominal concentrations at both low and high QC concentrations.

Acceptance Criteria for stability QC samples

- % Stability should be within the range of 85-115% for both low and high QC concentrations.
- % C.V. should be ≤ 15 % for both low and high QC concentrations.
- In case a QC sample is rejected due to an analytical reason (e.g. Internal standard variation, Lost during processing/analysis, Bad chromatography etc.), at least three stability and three comparison QC samples should be available to determine % Stability.

3.3.16.3.2 Bench top stability during extraction

The bench-top stability evaluation during sample extraction involved analysis of four replicates of low and high quality control samples of Levofloxacin, which were removed from the freezer room and kept on the bench during processing (stability samples) at room temperature under low light condition.

- 1.53 hours after pretreatment
- 1.55 hours before drying step
- 1.57 hours after drying step
- 1.97 hours after reconstitution
Following the stability duration, the stability quality control samples were analyzed with four replicates of freshly spiked low and high quality control samples of Levofloxacin (comparison samples) against freshly spiked calibration curve.

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

The specific steps followed are detailed below:

- Four (4) replicates of QC samples at low and high concentrations were removed from the freezer room, and thawed at room temperature.

- These samples were stored at room temperature under low light conditions for approximately 1.5 hr. at the processing steps at which study samples were anticipated to be kept on the bench.

- After completion of processing, these samples were placed in the auto-sampler (stability samples) and the time of placement of the samples in the auto-sampler was recorded.

- Freshly spiked calibration standards and 4 replicates of freshly spiked low and high concentrations QC samples were prepared and processed (Comparison samples).

- After completion of processing, these samples were placed in the auto-sampler and the time of placement of the samples in the auto-sampler was recorded.

- CC standards along with stability and comparison QC samples were injected.

- The bench top stability duration during extraction was calculated as the time taken from start of processing of stability QC samples to their transfer into the auto sampler.

- QC concentrations were calculated and the mean concentration, standard deviation, %C.V., and % nominal concentrations were determined for both the stability and comparison QC samples at low and high QC levels.
% Stability was calculated by using following formula at both low and high QC concentrations.

\[
\text{% Stability} = \frac{\text{Mean concentration of stability samples}}{\text{Mean concentration of comparison samples} \times \text{C.F.}} \times 100
\]

\[
\text{C.F.} = \frac{\text{Concentration of Stability Samples}}{\text{Concentration of Comparison Samples}}
\]

**Acceptance Criteria for calibration curve**

- For Acceptance criteria for Calibration Curve refer section 3.3.7

**Acceptance Criteria followed for comparison QC samples were:**

- At least 67% of the QC samples including at least 50% at each concentration should be within ±15% of their respective nominal values.
- % C.V. should be ≤15 % and the mean concentrations should be within ± 15% of the nominal concentrations at both low and high QC concentrations.

**Acceptance Criteria followed for stability QC samples were:**

- % Stability should be within the range of 85-115% for both low and high QC concentrations.
- % C.V. should be ≤ 15 % for both low and high QC concentrations.
- In case a QC sample is rejected due to an analytical reason (e.g. Internal standard variation, Lost during processing/analysis, Bad chromatography etc.), at least three stability and three comparison QC samples should be available to determine % Stability.

### 3.3.16.4 Freeze-thaw stability

**Freeze-thaw stability in human plasma**

The stability of spiked plasma samples was determined after three freeze-thaw cycles. Four replicates of low and high quality control samples of Levofloxacin (stability
samples) stored in polypropylene containers were analysed after third freeze-thaw cycle with four replicates of freshly spiked low and high quality control samples of Levofloxacin (comparison samples) against freshly spiked calibration curve. The percentage stability was determined by comparing the mean concentration of Levofloxacin in stability quality control samples against the mean concentration of Levofloxacin in comparison quality control samples.

The specific steps followed are detailed below:

- Freeze and thaw stability was assessed in matrix by assaying 4 replicates of QC samples at low & high concentrations previously frozen and thawed over multiple cycles.
- Freeze and thaw cycle consisted of first freezing for at least 24 hours at below -15 °C followed by unassisted thawing at room temperature under low light conditions.
- When completely thawed, samples were refrozen for a minimum of 12 hours under the same conditions.
- Freeze-thaw cycle was repeated two more times and the stability was then evaluated for the third freeze thaw cycle (Stability samples)
- Freshly spiked calibration standards were prepared.
- Four (4) replicates of freshly spiked low and high concentrations QC samples (Comparison samples) were prepared.
- The comparison and stability QC samples were processed along with the freshly spiked calibration standards.
- CC standards and QC samples were injected.
- QC concentrations were calculated and the mean concentration, S.D., %C.V. and % nominal concentrations were determined of both the stability and comparison QC samples at low and high QC levels.
- % Stability was calculated between stability and comparison samples at both low and high QC concentrations.
\[
\% \text{ Stability} = \frac{\text{Mean concentration of stability samples}}{\text{Mean concentration of comparison samples} \times \text{C.F.}} \times 100
\]

\[
\text{C.F.} = \frac{\text{Concentration of Stability Samples}}{\text{Concentration of Comparison Samples}}
\]

Acceptance Criteria for calibration curve

- For Acceptance criteria for Calibration Curve refer section 3.3.7

Acceptance Criteria for comparison QC samples

- At least 67% of the QC samples including at least 50% at each concentration should be within ±15% of their respective nominal values.

- % C.V. should be ≤ 15 % and the mean concentrations should be within ± 15% of the nominal concentrations at both low and high QC concentrations.

Acceptance Criteria for stability QC samples

- % Stability should be within the range of 85-115% for both low and high QC concentrations.

- % C.V. should be ≤ 15 % for both low and high QC concentrations.

- In case a QC sample is rejected due to an analytical reason (e.g. Internal standard variation, Lost during processing/analysis, Bad chromatography etc.), at least three stability and three comparison QC samples should be available to determine % Stability.

3.3.16.5 Auto sampler stability (In injector stability / Post processing stability)

In-injector stability (post processing stability) of Analyte Levofloxacin -

- In-injector stability evaluation involved analysis of processed four replicates of low and high quality control samples of levofloxacin (stability samples) stored in auto injector for 73.58 hours (at 10°C ± 1.0°C) with four replicates of freshly spiked low and high
quality control samples of levofloxacin (comparison samples) against freshly spiked calibration curve.

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

The specific steps followed are detailed below:

- Four (4) replicates of QC samples at low and high concentrations were processed and placed in the auto-sampler.

- The time of placement of the QC samples in the auto-sampler was recorded.

- Following the stability period of 73.58 hours (anticipated run time for the analytical batch during subject analysis) at 10°C ± 1.0°C, 4 replicates of freshly spiked low and high concentrations QC samples (Comparison samples) were prepared along with the freshly spiked calibration standards.

- Comparison QC samples were processed along with the freshly spiked calibration standards just prior to analysis.

- Comparison QC samples and freshly spiked CC standards along with stability QC samples were injected.

- The in-injector stability duration was calculated as the time of injection of the last QC sample less the time of their placement in the auto-sampler.

- % Stability was calculated at both low and high QC concentrations.

For Analyte

- QC concentrations were calculated and the mean concentration, S.D., %C.V. and % nominal concentrations of both the stability and comparison QC samples were determined at low and high QC levels.
% Stability for analyte = \( \frac{\text{Mean concentration of stability samples}}{\text{Mean concentration of comparison samples \times C.F.}} \times 100 \)

C.F. = \( \frac{\text{Concentration of Stability Samples}}{\text{Concentration of Comparison Samples}} \)

Acceptance Criteria for calibration curve

- For Acceptance criteria for Calibration Curve refer section 3.3.7.

Acceptance Criteria followed for comparison QC samples were:

- At least 67% of the QC samples including at least 50% at each concentration should be within ±15% of their respective nominal values.
- % C.V. should be ≤ 15% and the mean concentrations should be within ±15% of the nominal concentrations at both low and high QC concentrations.

Acceptance Criteria followed for stability QC samples were:

- % Stability should be within the range of 85-115% for both low and high QC concentrations.
- % C.V. should be ≤15% for both low and high QC concentrations.
- In case a QC sample is rejected due to an analytical reason (e.g. Internal standard variation, Lost during processing/analysis, Bad chromatography etc.), at least three stability and three comparison QC samples should be available to determine % Stability.
Auto sampler stability for Internal standard

In-injector stability (post processing stability) of Levofloxacin-d8 (ISTD) -

In-injector stability of replicate quality control samples was determined. Four replicates of low and high quality control samples stored in auto injector for 73.58 hours (at 10°C ± 1.0°C) after processing (stability samples) in glass vials were analyzed and the peak area ratio for Levofloxacin-d8 (ISTD) to Levofloxacin was calculated for stability samples and comparison samples (four replicates of freshly spiked low and high quality control samples).

The percentage stability was determined by comparing the mean peak area ratio of Levofloxacin-d8 (ISTD)/ Levofloxacin in stability quality control samples against the mean peak area ratio of Levofloxacin-d8 (ISTD)/ Levofloxacin in comparison quality control samples.

- Peak area ratio of Internal standard / Analyte, obtained from both the stability and comparison QC samples at low and high QC levels were tabulated.
- Mean peak area ratio, S.D., %C.V. and % stability were calculated.
- % Stability was calculated as follows:

\[
\frac{\text{% Stability for ISTD}}{\text{Mean Peak Area Ratio of Stability Samples}} = \frac{\text{Mean Peak Area Ratio of Comparison Samples} \times \text{C.F.}}{\text{Mean Peak Area Ratio of Comparison Samples}} \times 100
\]

\[
\text{C.F.} = \frac{\text{Concentration of ISTD in Stability Samples}}{\text{Concentration of Analyte in Comparison Samples}} \times \frac{\text{Concentration of ISTD in Comparison Samples}}{\text{Concentration of Analyte in Stability Samples}}
\]
Acceptance Criteria followed for comparison QC samples were:

- % C.V. should be ≤ 15 % at both low and high QC concentrations.

Acceptance Criteria followed for stability QC samples were:

- % Stability should be within the range of 85-115% for both low and high QC level.
- % C.V. should be ≤ 15 % for both low and high QC level.
- In case a QC sample is rejected due to an analytical reason (e.g. Internal standard variation, Lost during processing/analysis, Bad chromatography etc.), at least three stability and three comparison QC samples should be available to determine % Stability.

3.3.16.6 Sample collection process stability (blood stability testing)

- Sample collection process stability was performed at the temperature or condition used during collection of blood samples in the clinic.
- Analyte was spiked at low and high QC concentrations in appropriate volume of blood, to get at least 4 aliquots of plasma sample volume required.
- These samples were stored unprocessed under low light conditions at room temperature, for a period of at least 2 hours (2.10 hours). These samples served as stability samples.
- The samples were shaken gently without vortex.
- After storage period, analyte at low and high QC concentration was spiked in same volume of blood as for stability samples. These samples served as comparison samples.
- Stability and comparison samples were centrifuged together at 4±2°C and 4000 rpm for at least 15 minutes to separate plasma from blood.
- 4 aliquots of low and high QC concentration samples from stability and comparison samples were taken, processed and injected.
The stability duration was calculated as the difference between the times of spiking of comparison samples less the time of spiking of stability samples.

The peak area ratio of stability and comparison samples was tabulated.

Mean, S.D. and %C.V for stability and comparison samples was calculated at low and high QC concentration.

% Stability was calculated at low and high QC concentrations as follows:

\[
\% \text{ Stability} = \frac{\text{Mean peak area ratio of stability samples}}{\text{Mean peak area ratio of comparison samples}} \times 100
\]

Acceptance Criteria followed for Sample collection process stability:

- Stability should be within the range of 85 to 115% for both low and high concentrations.
- % C.V. should be \( \leq 15 \% \).
- In case a sample is rejected due to an analytical reason (e.g. lost during processing/analysis, bad chromatography etc.), at least three stability and three comparison samples should be used to determine % stability.

3.3.16.7 Long-term stability 1

The bioanalytical method was validated for long-term stability (45 days). For details of Analytical Method, Reagents, Standard Stock Solutions, Preparation of Solutions, Sample Preparation, Integration Parameters and Chromatographic Conditions refer Section 3.2

HPLC from Shimadzu Technologies, MS from Sciex (MS-14) and Analyst software Version 1.4.2 for data processing were used in the experiment.
Following parameters optimized during the prior experiments on levofloxacin were used during the experiment [MS-14]:

**API – 3200 (MS-14)**

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<thead>
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<th>Detection</th>
<th>Q1 Mass (amu)</th>
<th>Q3 Mass (amu)</th>
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<td>261.10</td>
</tr>
<tr>
<td>Levofloxacin-d8</td>
<td>370.10</td>
<td>265.10</td>
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</table>

**Compound Parameters:**

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<thead>
<tr>
<th>Parameter</th>
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<th>Levofloxacin-d8</th>
</tr>
</thead>
<tbody>
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<td>50 V</td>
</tr>
<tr>
<td>Entrance Potential (EP)</td>
<td>10 V</td>
<td>10 V</td>
</tr>
<tr>
<td>Collision Energy (CE)</td>
<td>40.0 V</td>
<td>40.0 V</td>
</tr>
<tr>
<td>Collision cell Exit Potential (CXP)</td>
<td>2.0 V</td>
<td>2 V</td>
</tr>
<tr>
<td>Cell Entrance Potential (CEP)</td>
<td>23 V</td>
<td>23 V</td>
</tr>
</tbody>
</table>

**Source/Gas Parameters:**

- GS1: 55°
- GS2: 50°
- CAD: 6°
- CUR: 40°
- IS: 2500 V
- TEM: 600°C

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

- Ion Source: ESI
- Polarity: Positive
- Resolution Q1: Unit
- Resolution Q3: Unit
Source and Type of Biological Matrix:

Pooled plasma (ID: V501PP/01) was used for preparation of freshly spiked calibration curve standards and comparison quality control samples of Long-term stability exercise. Pooled plasma (ID: V501PP/01) was prepared from screened and acceptable matrix lots during method validation.

Standardization and calculation:

The chromatographic data were acquired and processed using computer based Analyst software Version 1.4.2. The best-fit curves using weighted (1/Concentration\(^2\)) linear least square regression analysis were obtained by peak area ratio of Levofloxacin to Levofloxacin-d8 (ISTD). The concentrations of Levofloxacin in plasma samples were calculated using linear regression parameters of the corresponding calibration curve.

Reference/Working Standards

Levofloxacin and Levofloxacin-d8 as mentioned in section 3.3.1 were used.

Calibration Curve Standards and Quality Control Samples

Freshly spiked Calibration curve standards and comparison quality control samples were prepared for long term stability exercise.

Stability quality control samples with QC IDs V501/LQC, HQC # 127 to 130, stored in Freezer Room below \(-15^\circ\text{C}\) were used for Long-term stability exercise.

Validation Procedure and Acceptance Criteria for long-term stability

- Following an appropriate storage period, four replicates of the stored low and high concentration QC samples were removed from the freezer room and allowed to thaw.

- Freshly spiked calibration standards and four replicates of freshly spiked low and high concentrations QC samples (Comparison samples) were prepared.
• Comparison and stability QC samples were processed along with freshly spiked calibration standards.

• CC standards and QC samples were injected.

• QC concentrations were calculated and the mean concentration, S.D., %C.V. and % nominal concentrations were determined for both the stability and comparison QC samples at low and high QC levels.

• % Stability between stability and comparison samples was calculated at both low and high QC concentrations.

• The long-term stability duration was calculated as the date of processing of QC samples less the date of storage of the stability QC samples (in days).

\[
\% \text{ Stability} = \left( \frac{\text{Mean concentration of stability samples}}{\text{Mean concentration of comparison samples} \times \text{C.F.}} \right) \times 100
\]

\[
\text{C.F.} = \frac{\text{Concentration of Stability Samples}}{\text{Concentration of Comparison Samples}}
\]

Acceptance Criteria for Calibration Curve

➢ For Acceptance criteria for Calibration Curve refer section 3.3.7.

Acceptance Criteria followed for comparison and stability QC samples were:

➢ At least 67% of total QC samples including at least 50% at each concentration should be within ±15% of their respective nominal values

➢ % C.V. should be ≤ 15% and the mean concentrations should be within ±15% of the nominal concentrations at both low and High QC concentrations.
Acceptance Criteria followed for stability QC samples were:

- % stability should be within the range of 85-115 % for both low and high QC concentrations.
- % C.V. should be ≤ 15% for both low and high QC concentrations.
- In case a QC sample is rejected due to an analytical reason (e.g. Internal standard variation, Lost during processing / analysis, Bad chromatography etc.), at least three stability and three comparison QC samples should be available to determine % stability.

3.3.16.8 Long-term stability 2

Long term stability exercise 2 was conducted to validate the bioanalytical method for estimation of Levofloxacin in human plasma for Precision and Accuracy batch and long-term stability for 123 days.

Thus the Validation Parameter(s) were

a. Precision and Accuracy Batch
b. Long term Stability-2 of Levofloxacin

For details of Analytical Method, Reagents, Standard Stock Solutions, Preparation of Solutions, Sample Preparation, Integration Parameters and Chromatographic Conditions refer Section 3.2. HPLC from Cohesive Technologies, MS from Applied Biosystems (MS-15) and Analyst software Version 1.4.2 for data processing were used in the experiment.
Following parameters were optimized on MS-15:

**API 3200 (MS-15)**

**Detection:**

<table>
<thead>
<tr>
<th></th>
<th>Q1 Mass (amu)</th>
<th>Q3 Mass (amu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Levofloxacin</td>
<td>362.0</td>
<td>261.1</td>
</tr>
<tr>
<td>Levofloxacin-d8</td>
<td>370.1</td>
<td>265.2</td>
</tr>
</tbody>
</table>

**Compound Parameters:**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Levofloxacin</th>
<th>Levofloxacin-d8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declustering Potential (DP)</td>
<td>45 V</td>
<td>45 V</td>
</tr>
<tr>
<td>Entrance Potential (EP)</td>
<td>10 V</td>
<td>10 V</td>
</tr>
<tr>
<td>Collision Energy (CE)</td>
<td>40 V</td>
<td>40 V</td>
</tr>
<tr>
<td>Collision cell Exit Potential (CXp)</td>
<td>5 V</td>
<td>5 V</td>
</tr>
<tr>
<td>Cell Entrance Potential (CEP)</td>
<td>15 V</td>
<td>15 V</td>
</tr>
</tbody>
</table>

**Source/Gas Parameters:**

GS1: 50º  
GS2: 50º  
CAD: 4º  
CUR: 40º  
IS: 5500 V  
TEM: 650ºC  

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

Ion Source: ESI  
Polarity: Positive  
Resolution Q1: Unit  
Resolution Q3: Unit
Source and Type of Biological Matrix:

Pooled plasma (ID: V501PP/01) was used for preparation of freshly spiked calibration curve standards and comparison quality control samples of Long-term stability exercises. Pooled plasma (ID: V501PP/01) was prepared from screened and acceptable matrix lots during method validation.

Standardization and calculation:

The chromatographic data were acquired and processed using computer based Analyst software Version 1.4.2. The best-fit curves using weighted \((1/\text{Concentration}^2)\) linear least square regression analysis were obtained by peak area ratio of Levofloxacin to Levofloxacin-d8 (ISTD). The concentrations of Levofloxacin in plasma samples were calculated using linear regression parameters of the corresponding calibration curve.

Reference/Working Standards

Levofloxacin and Levofloxacin-d8 as mentioned in section 3.3.1 were used

Calibration Curve Standards and Quality Control Samples

1. Calibration curve standards and quality control samples prepared and stored in freezer room below -15°C, were used for Precision and Accuracy batch

2. Freshly spiked calibration curve standards and comparison quality control samples were prepared for long-term stability exercise.

Stability quality control samples of Levofloxacin with QC IDs V501/LQC, HQC # 131 to 134 stored in freezer room below -15°C, were used for Long-term stability exercise.

Validation Procedure and Acceptance Criteria for Precision and Accuracy

- CC standards and QC samples were prepared as mentioned in section 3.3.7.

- The validation batch consisted of the following samples in the following order:
  - Reference solution [analyte and internal standard]
- Matrix blank
- Matrix blank with internal standard
- Spiked calibration standards (One set of eight non-zero concentrations in which LOQ and ULOQ were in duplicate)
- LOQ QC
- Low QC
- Middle QC
- High QC

Note: Six replicates of each QC set (containing one LOQQC, LQC, MQC and HQC) were processed and analyzed.

- Calibration curve using all accepted CC standards including duplicate LOQ and ULOQ standard were prepared.

- QC concentrations were calculated and mean concentration, S.D., % C.V. and % nominal values were determined.

\[
\text{Standard Deviation} = \frac{\% \text{ C.V.}}{\text{Mean Calculated Concentration}} \times 100
\]

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

Acceptance Criteria for Calibration Curve

➢ For Acceptance criteria for Calibration Curve refer section 3.3.7.

Acceptance Criteria for QC samples

➢ For Acceptance criteria for QC samples refer section 3.3.7.
Long term stability

- Following an appropriate storage period, four replicates of the stored low and high concentration QC samples were removed from the freezer room and allowed to thaw.

- Freshly spiked calibration standards and four replicates of freshly spiked low and high concentrations QC samples (Comparison samples) were prepared.

- Comparison and stability QC samples were processed along with freshly spiked calibration standards.

- CC standards and QC samples were injected.

- QC concentrations were calculated and the mean concentration, S.D., %C.V. and % nominal concentrations were determined for both the stability and comparison QC samples at low and high QC levels.

- % Stability between stability and comparison samples was calculated at both low and high QC concentrations.

- The long-term stability duration was calculated as the date of processing of QC samples less the date of storage of the stability QC samples (in days).

\[
\text{% Stability} = \frac{\text{Mean concentration of stability samples}}{\text{Mean concentration of comparison samples} \times \text{C.F.}} \times 100
\]

Concentration of Stability Samples

\[
\text{C.F.} = \frac{\text{Concentration of Stability Samples}}{\text{Concentration of Comparison Samples}}
\]
Acceptance Criteria for Calibration Curve

➢ For Acceptance criteria for Calibration Curve refer section 3.3.7.

Acceptance Criteria for comparison and stability QC samples

Acceptance Criteria followed for comparison QC samples were:

➢ At least 67% of the QC samples including at least 50% at each concentration should be within ±15% of their respective nominal values.

➢ % C.V. should be ≤ 15 % and the mean concentrations should be within ± 15% of the nominal concentrations at both low and high QC concentrations.

Acceptance Criteria followed for stability QC samples were:

➢ % Stability should be within the range of 85-115% for both low and high QC concentrations.

➢ % C.V. should be ≤ 15 % for both low and high QC concentrations.

➢ In case a QC sample is rejected due to an analytical reason (e.g. Internal standard variation, Lost during processing/analysis, Bad chromatography etc.), at least three stability and three comparison QC samples should be available to determine % Stability.
3.4 ANALYSIS OF CLINICAL STUDY SAMPLES

3.4.1 Sample Collection and Storage

During clinical conduct of the study, a total of 1176 blood samples were collected, in periods 1 and 2, for the estimation of levofloxacin in plasma. In each period, after each timepoint, the blood samples were centrifuged at a speed of 4000 RPM for duration of 15 minutes, at temperature of 4 ± 2°C under refrigeration, as soon as possible to separate plasma. All post-dose plasma samples were divided into 2 aliquots and transferred to suitably labeled tubes and re-checked to ensure transfer of plasma to the correct tube. The plasma were stored below –15°C, pending transfer to the analytical facility for assay. All samples were collected and processed under low light conditions. Aliquot 1 of plasma samples of period I and period II were packed with dry ice and transferred to the analytical facility and subsequently aliquot 2 of plasma samples of period I and period II were also packed with dry ice and transferred to the analytical facility, in a second shipment, for analysis. Analysis was performed on 1176 plasma samples for Levofloxacin from 28 subjects who completed both periods of the study. Dosing Compliance was verified by measuring plasma Levofloxacin, during analysis.

3.4.2 Assay Method Description

The following assay method was followed for analysis of samples:

- Plasma samples were withdrawn as per requirement of method validation experiments from the freezer room.
- These samples were allowed to thaw at room temperature and vortexed.
- The samples were centrifuged at 4000 rpm for a minimum of 5 minutes.
- 100 μL aliquot of each plasma sample was pipetted out into appropriately labeled polypropylene tubes.
- 50 μL of internal standard stock dilution (approximately 400.0 ng/mL of Levofloxacin d8) was added into these tubes (except standard blank).
250 µL of Solution-1 was added to each polypropylene tube and the samples were vortexed to ensure complete mixing of contents.

- The samples were centrifuged at 4000 rpm for a minimum of 5 minutes.
- The cartridges [Bond Elut Plexa, PCX, 30 mg/1cc] were conditioned using 0.5 mL of methanol followed by 0.5 mL of HPLC-grade water by running the centrifuge at 4000 rpm for a minimum of 1 min after each addition.
- The samples were loaded onto the cartridges and the centrifuge was run for a minimum of 1 minute at 4000 rpm.
- The cartridges were washed with 1 mL of HPLC-grade water followed by 1 mL of solution-2 by running the centrifuge at 4000 rpm for a minimum of 1 minute.
- The samples were eluted into appropriately labeled polypropylene tubes with 1 mL of acetonitrile by running centrifuge at 4000 rpm for a minimum of 1 min.
- The eluted samples were dried under stream of nitrogen at 50°C ± 2°C at about 20 psi.
- The dried residue was reconstituted with 1000 µL of mobile phase.
- The samples were transferred into glass vials for analysis.

3.4.3 Sample analysis

The clinical study samples were analyzed using a validated LC/MS/MS method for the determination of Levofoxacin in K$_2$EDTA human plasma.

3.4.4 Batch Acceptance Criteria

All the batches were evaluated rigorously and considered for the repeat analysis if failed with respect to any of the criteria like Quality control samples acceptance criteria (Refer Details included in section 3.3.7), Standard Calibration curve acceptance criteria and the Blank and blank plus internal standard acceptance criteria (as detailed below)

1. Batch acceptance required that one Blank and one Blank+ISTD be free of significant interference at the retention times of the analyte and internal standard.
Interference was deemed significant if the peak area response at the retention time of the analyte in the blank, blank + ISTD is > 20% of the peak area response of each accepted LOQ standard.

Interference was deemed significant if the peak area response at the retention time of the internal standard in the blank is > 5% of the mean ISTD response of the standards used in the calculation of calibration curve.

2. If Blank or Blank + ISTD Sample was rejected due to an analytical reason [eg. lost due to processing / analysis, bad chromatography, Internal standard variation etc.] the analytical batch was rejected.

3. Calibration curve was prepared using all acceptable CC standards including duplicate LOQ and ULOQ standard.

4. At least 75% of non-zero standards met the below criteria, including one LOQ and ULOQ standard: Accuracy of the LOQ in the standard curve was within ± 20% of the nominal value and within ±15% for other concentrations.

5. Linear coefficient of correlation ≥ 0.98.

3.4.5 Protocol deviations

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

The concentration data obtained from all 28 subjects who completed both periods of study was entered in WinNonlin version 5.0.1 Pharmacokinetic software for further processing and Non-Compartmental Analysis. The following pharmacokinetic parameters were calculated for Levofloxacin in plasma:

**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-∞</sub>:** The area under the plasma concentration versus time curve, from time zero to infinity. AUC<sub>0-∞</sub> was 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 

\[ \frac{(AUC_{0-\infty} - AUC_{0-t})}{AUC_{0-\infty}} \times 100 \]

**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).
T1/2: The apparent first-order terminal elimination half-life was calculated as 0.693/Kel.

3.6 STATISTICAL ANALYSIS
Statistical analyses were performed on plasma Levofloxacin using the SAS system for Windows, release 9.1.3 or above. The analysis included data from all 28 subjects who completed the study.

3.6.1 Summary Statistics
Arithmetic means, standard deviations and coefficients of variation were calculated for the parameters listed in section 3.5. Additionally, geometric means and percentage coefficient of variation of geometric means were calculated for AUC_{0-t}, AUC_{0-\infty}, and C_{max}.

3.6.2 Analysis of Variance (ANOVA)
The log-transformed pharmacokinetic parameters C_{max}, AUC_{0-t} and AUC_{0-\infty} were analyzed using a mixed effects ANOVA model using Type III sum of squares with the main effects of sequence, period and treatment as fixed effects and subjects nested within sequence as random effect. The sequence effect was tested at the 10% level of significance using the subjects nested within sequence mean square as the error term and treatment and period effects were tested at the 5% level of significance against the residual error (mean square error) from the ANOVA model as the error term.

3.6.3 90% Confidence Intervals and Ratio Analysis
The least-squares means (LSM) were calculated for the log-transformed pharmacokinetic parameters C_{max}, AUC_{0-t} and AUC_{0-\infty}. 90% confidence intervals for the ratios of Test (T) and Reference (R) product averages (least squares means) derived from the analysis of log transformed pharmacokinetic parameters C_{max}, AUC_{0-t} and AUC_{0-\infty} were calculated for Levofloxacin. The comparison of interest is T vs R, so
the ratios were of the form: - Test/Reference. Ratios of means were calculated using the LSM for log-transformed $C_{\text{max}}$, $\text{AUC}_{0-t}$ and $\text{AUC}_{0-\infty}$. Ratios of means were expressed as a percentage of the LSM for the reference formulations. $T_{\text{max}}$ was analyzed as an individual difference (Test-Reference) building a 90% confidence interval, using a non parametric test.

For Levofloxacin:- The 90% confidence intervals for the ratio of test (T) and reference (R) product averages (least square means) for pharmacokinetic parameters $C_{\text{max}}$, $\text{AUC}_{0-t}$ and $\text{AUC}_{0-\infty}$ should be between 80% and 125% for the log-transformed data.