CHAPTER 3

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3.0 Materials and Methods

3.1 In-vitro Dissolution Rate Studies

The dissolution of the tablets was performed according to United States Pharmacopoeia (USP29) specifications Test number 1 and 2 with the Apparatus 1 using Hanson SR8-Plus (Hanson Research-USA) Dissolution Test System. Mean concentration at each time point is represented by six tablets of each formulation.

Test I:
Medium: Dilute hydrochloric acid (7 in 1000); Volume-800 ml
Apparatus I: 100 rpm
Times: 15, 45, 90, 120 and 150 minutes.
Procedure: The dissolution medium was maintained at 37° C. At each time, 5 ml of the solution was withdrawn under test, and passed through a filter having a 35- μm or finer porosity. Using the filtrate as the assay preparation suitably diluted with Medium and using Medium to prepare the Standard Solution, the amount of Lithium carbonate dissolved was determined by employing a flame photometer.

Test II:
Medium: Water; 900 ml
Times: 0.5, 1, 2, 3, 5 and 7 hours
Apparatus and Procedure: As in Test I.

Procedure for evaluation:
Dilute hydrochloric acid (1 in 200) — 5 ml of hydrochloric acid was diluted with water to obtain 1000 ml of solution.

Standard preparation and Assay preparation was diluted quantitatively with Dilute hydrochloric acid (1 in 200), to yield solutions of suitable concentrations acceptable to the linear or working range of the instrument.
Standard preparation: transfer to a 100 ml volumetric flask about 30 mg of USP lithium carbonate RS, accurately weighed. Add about 20 ml of water and 0.5 ml of hydrochloric acid, shake until dissolved, dilute with water to volume, and mix to obtain a solution having a known concentration of about 300 μg of USP Lithium Carbonate RS per ml.

Flame photometer was adjusted with dilute hydrochloric acid and was calibrated with four known concentrations of 30 μg/ml, 20 μg/ml, 10 μg/ml and blank. Then unknown samples were aspirated to estimate the concentration.

3.2 In-vivo Bioequivalence Studies

These are described under 4 sub-heads.

1. Clinical study methodology
2. Analytical methodology
3. Analysis of clinical study samples
4. Pharmacokinetic and Statistical analysis

3.2.1 Clinical study methodology

3.2.1.1 Objective

To compare the bioavailability and steady-state levels of three brands of lithium carbonate sustained release tablets in healthy, adult male human subjects.

3.2.1.2 Products Evaluated

Reference (R) Lithosun SR tablets containing lithium carbonate 400 mg, of Sun Pharmaceutical Industries Ltd., Mumbai, Maharashtra, India.
Batch number PC007, Mfg Date: October 2004

Test (A) Lalithium XR tablets containing lithium carbonate 400 mg, of Manas Pharma Mfg. Ahmedabad, Gujarat, India.
Batch number GK41486, Mfg Date: November 2004
Test (B) Licab XL tablets containing lithium carbonate 400 mg, of Torrent Labs (P) Ltd. Ahmedabad, Gujarat.
Batch number 7044008, Mig Date: October 2004

3.2.1.3 Study Design
The study was conducted as an open label, balanced, randomized, three periods, multiple dose and steady-state, crossover bioequivalence study comparing three marketed brands of lithium carbonate.

3.2.1.4 Number of Subjects
Enough healthy adult male human subjects were screened to allow dosing of 15 subjects in the study. Subsequent dropouts were not replaced. Data is presented on all completed subjects.

3.2.1.5 Selection of Subjects
Adequate number of subjects was selected randomly from the volunteer bank of Clinical Pharmacology Unit and the subjects underwent a standardized screening procedure along with an additional screening for Thyroid Function Test.

> Screening Assessments
Medical histories and demographic data, including name, sex, age, body weight (kg), height (cm) and tobacco use were recorded. Each subject underwent physical examination and the laboratory tests of hematologic, hepatic and renal functions as listed below.
### Laboratory Tests

<table>
<thead>
<tr>
<th>HEMATOLOGY</th>
<th>URINALYSIS</th>
<th>ADDITIONAL TESTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>PHYSICAL EXAMINATION</td>
<td>• HIV I &amp; II</td>
</tr>
<tr>
<td>• Total Leukocyte Count</td>
<td>Colour</td>
<td>• HBsAg</td>
</tr>
<tr>
<td>• Differential Leukocyte Count</td>
<td>Appearance</td>
<td>• HCV</td>
</tr>
<tr>
<td>• Platelet count</td>
<td>• PH</td>
<td>• VDRL</td>
</tr>
<tr>
<td></td>
<td>• Specific Gravity</td>
<td>URINE DRUG SCREEN</td>
</tr>
<tr>
<td>BIO-CHEMISTRY</td>
<td>• Protein</td>
<td>• Cannabinoids</td>
</tr>
<tr>
<td>• BUN</td>
<td>Glucose</td>
<td>Opioids</td>
</tr>
<tr>
<td>• Creatinine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Total Bilirubin</td>
<td>MICROSCOPIC EXAMINATION</td>
<td></td>
</tr>
<tr>
<td>• Alkaline Phosphatase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• AST</td>
<td>• RBC</td>
<td></td>
</tr>
<tr>
<td>• ALT</td>
<td>• WBC</td>
<td></td>
</tr>
<tr>
<td>• Glucose</td>
<td>• E. cells</td>
<td></td>
</tr>
<tr>
<td>• Cholesterol</td>
<td>• Crystals</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Casts</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Others</td>
<td></td>
</tr>
</tbody>
</table>
Only medically healthy subjects, with clinically normal laboratory profiles, were selected if they met following inclusion and exclusion criteria. The thyroid function test was done to assess the baseline value of thyroid functions, and from those subjects who were in normal range, fifteen healthy male human subjects were selected.

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

3.2.1.7 **Exclusion Criteria**
- History of any allergy or hypersensitivity to lithium carbonate or related compounds.
- History of vomiting and diarrhoea in last 15 days.
- History of drug induced rash, anaphylaxis.
- History of seizures, psychiatric illness, tremors, polyuria, slurred speech, giddiness and vertigo.
- Any evidence of severe debilitation and low body sodium levels including, for example, dehydrated subjects, those on low sodium diet, or those with Addison's disease (Characteristics of the disease are —chronic worsening fatigue, muscle weakness, loss of appetite, weight loss).
- Any evidence of organ dysfunction or any clinically significant deviation from the normal, in physical or clinical determinations.
- History of serious gastrointestinal, hepatic, renal, cardiovascular, pulmonary, neurological or haematological disease, diabetes or glaucoma.
• Presence of disease markers of HIV 1 and 2, Hepatitis B and C viruses or syphilis infection. Presence of values which are significantly different from normal reference ranges and/or judged clinically significant for haemoglobin, total white blood cells count, differential WBC count or platelet count.

• Positive for urinary screen testing of drugs of abuse (opiates or cannabinoids).

• Presence of values, which are significantly different from normal reference ranges and/or judged clinically significant for serum creatinine, blood urea nitrogen, serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), serum alkaline phosphatase, serum bilirubin, plasma glucose or serum cholesterol.

• Clinically abnormal chemical and microscopic examination of urine defined as presence of RBC, WBC (>4/HPF), glucose (positive) or protein (positive).

• Clinically abnormal ECG or Chest X-ray.

• Difficulty with donating blood.

• Regular smokers who smoke more than 10 cigarettes daily or have difficulty abstaining from smoking for the duration of each study period.

• History of drug dependence or excessive alcohol intake on a habitual basis of more than 2 units of alcoholic beverages per day (1 unit equivalent to half pint of beer or 1 glass of wine or 1 measure of spirit) or have difficulty in abstaining for the duration of each study period.

• Use of any enzyme modifying drugs within 30 days prior to Day 1 of this study.

• Participation in any clinical trial within 12 weeks preceding Day 1 of this study.

• Subjects who, through completion of this study, would have donated and/or lost more than 350 ml of blood in the past 3 months.

3.2.1.8 Treatments

Lithium carbonate SR 400 mg tablet was administered twice daily for five days and on the morning of day 6 during each period. On the first day, the sample profile was
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taken for 12 hours after morning doses. On the 6th day, the sample profile was taken after morning dose till 24 hours.

Reference (R):
Lithosun SR tablet containing lithium carbonate 400 mg, of Sun Pharmaceutical Industries Ltd., Mumbai, Maharashtra was administered orally with 240 ml of drinking water in morning and evening on days 1, 2, 3, 4 and 5. On the 6th day, only the morning dose was administered.

Test (A):
Lalithium XR tablet containing lithium carbonate 400 mg, of Manas Pharma Mfg. Ahmedabad, Gujarat, was administered orally with 240 ml of drinking water in morning and evening on days 1, 2, 3, 4 and 5. On the 6th day, only the morning dose was administered.

Test (B):
Licab XL tablet containing lithium carbonate 400 mg, of Torrent Labs (P) Ltd. Ahmedabad, Gujarat, India was administered orally with 240 ml of drinking water in morning and evening on days 1, 2, 3, 4 and 5. On the 6th day, only the morning dose will be administered.

3.2.1.9 Washout Period
A washout period of seven days was enforced between dosing of each period.

3.2.1.10 Assignment to Treatment Sequences
The order of receiving study treatments for each subject during the 3 periods of the study was determined according to the SAS-generated balanced randomization schedule.
3.2.1.11 Assessment of Compliance

Compliance was assessed by conducting a thorough examination of the oral cavity by trained study personnel, after the morning dose on each day of the period. The evening compliance was based on the collection of the empty containers and verbal questioning. The final confirmation of compliance was done by the measurement of serum lithium carbonate during the analytical phase of the study.

3.2.1.12 Fasting/Meals

Post admission to Ranbaxy CPU on day 1 and day 6 of each period, all subjects were required to fast overnight for at least 10 hours before the morning dose and for 4 hours post-dose. All subjects received standard meals - lunch, snacks and dinner 4, 9 and 12.5 hours, respectively, after morning drug administration. During housing, all meal plans were identical for all 3 periods. In case meals and blood sample collection coincide, samples were collected before meals are provided. Drinking water was not allowed from 1 hour before dosing until 2 hours post-dose. Thereafter, it was allowed at all times.

3.2.1.13 Sampling Schedule

A total of 29 blood samples (3 ml each) per period were collected from each subject in vacutainers through an indwelling cannula placed in forearm vein. The blood samples were collected on day 1 at predose and at 1, 2, 3, 3.5, 4, 4.5, 5, 6, 8, 10, 12 hours post dose after the first dose. Predose sample (trough) was collected on days 2, 3, 4, and 5 before the morning dose. The blood samples were again collected on day 6 at predose and at 1, 2, 3, 3.5, 4, 4.5, 5, 6, 9, 12, 18 and 24 hours post dose. The total volume of blood drawn including 16 mL for screening, 24 mL for safety analysis at the end of periods, and 43.5 mL 'discarded' blood prior to venous cannula collections, was not exceeded 345 mL.

A total of eighty seven 3-mL blood samples were collected during the course of the study through indwelling cannulae placed in forearm veins. Predose samples (trough) were collected on days 2, 3, 4, and 5 before the morning dose. The pre-
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dose blood samples were collected within a period of 1 hour before dosing on days 1-6. The post-dose samples on day 1 and 6 were collected generally within 2 minutes of the scheduled time. The actual end-point time of collection of each blood sample (to the nearest minute) was recorded.

Intravenous indwelling cannula was kept in situ as long as possible when multiple samples were collected. The cannula was maintained patent by injection of 1 mL of 5 IU/mL of heparin in normal saline solution. At each time point, the blood samples were collected after discarding the first 0.5 mL of heparinised blood and heparin solution from the tubing.

After collection, the blood samples were kept for 10 minutes to complete coagulation. Blood samples were centrifuged under refrigeration to separate serum. All serum samples were transferred to suitable labeled tubes and rechecked to ensure transfer of serum to the correct tube. The serum samples were then stored at -20°C or lower, pending transfer to the analytical facility for assay.

The blood sampling and sample separation were done under low light.

3.2.1.14 Dosing, Admission and Stay

Since this was a single dose and steady-state study, the study subjects were admitted and housed in the Clinical Pharmacology Unit on day 1 and again on day 6, after twice a day dosing. On day 1, the subjects were admitted at least 12 hours before dose administration. The subjects were discharged after administration of the study medication at evening. Subjects were administered morning doses in Clinical Pharmacology Unit and the evening dose was provided to them to have at home to be taken at 9:00 PM along with the directions for use. On day 5, the subjects were admitted before evening dose administration and were discharged 24 hours after administration of the study medication on day 6, if the subjects do not suffer from any adverse drug event. In case of an adverse event, the subjects were monitored until the event subsided.
Period I
Period I of the study was conducted between dates 22 December 2005 and 29 December 2005.

Period II:
Period II of the study was conducted between dates 5 January 2005 and 12 January 2005.

Period III:
Period III of the study was conducted between dates 19 January 2005 and 26 January 2005.

During each study period, the following procedure was carried out: All the subjects reported to Ranbaxy Clinical Pharmacology Unit (CPU) at least 12 hours before dose administration on day 1. After sampling for 12 hours as per schedule for day 1, evening dose was administered. Subjects were discharged after having dinner. Subjects reported to Ranbaxy Clinical Pharmacology Unit (CPU) on days 2, 3, 4 and 5 of each period between 7:30 – 8:00 AM. Vitals were recorded and the pre-dose samples were collected at 8:30 AM. At 9:00 AM. The morning dose was administered to the subjects and the evening dose was provided to them to have at home to be taken at 9:00 PM along with the directions for use. On day 5 of the dosing, all subjects reported to the Ranbaxy CPU at 1600 hours. Vitals for the subjects were recorded at 6:00 PM. Evening dose was administered at 9:00 PM followed by dinner.

On day 6, pre-dose samples were collected at 8:30 AM and the last dose of the study medication was administered sequentially starting at 9:00 AM to the subjects, followed by the sampling as per the schedule mentioned in the section 3.1.11.

Discharge was done on day 7 between 9:15 AM till 9:45 AM, provided the subjects had no adverse events, in which case, subjects were discharged only after the event subsided. A safety sample was also collected before the discharge.
3.2.1.15 Restrictions

- Medications
  All subjects were instructed not to take any other medications including OTC during the 2 weeks period prior to the onset of the study. The medication was advised only in case of medical emergencies.

- Diet
  All subjects abstained from any xanthine containing food or beverages or alcoholic products for 48 hours prior to dosing and throughout the sampling schedule during each period.

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

3.2.1.16 Safety

- Clinical Safety Measurements
  Vital signs of oral temperature, sitting blood pressure and radial pulse were measured during subject admission, prior to each dosing and 2, 6 and 24 hours after administration of study drug and at ambulatory visits in each study period. Vital signs to be measured prior to administration of the dose were taken within 1 hour of the scheduled dosing time. At all other times, vital signs were taken within 30 minutes of the scheduled times.

  Brief clinical examination of the subject was conducted by a qualified medical designate on duty after subject admission, prior to dosing of study drug and before discharge.
Laboratory tests (haemoglobin, serum creatinine, blood urea nitrogen, serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), serum alkaline phosphatase (ALP) and serum bilirubin were repeated at discharge day of all subjects. Any elevations in laboratory value were to be followed up till they subsided.

➤ Adverse Events
All the subjects were monitored throughout the study period for adverse events. Subjects were specifically asked about any adverse events on each ambulatory visit (days 2-5), after admission (day 1 and 6), before administration of the dose (day 1 and 6) and approximately every four hours thereafter till discharged in each period.

3.2.1.17 Ethical Considerations
➤ Basic Principles
This research was carried out in accordance with the basic principles defined in US 21CFR Part 320, ICH E6 (May 1996) 'Guidance for Good Clinical Practice' and the principles enunciated in the Declaration of Helsinki (Edinburgh, October 2000).

➤ Institutional Review Board
This protocol and the corresponding informed consent form (ICF) used to obtain informed consent of study subjects were reviewed and approved by the Jamia Hamdard Institutional Review Board and the study subjects were not dosed until the Board approved the protocol and the ICF, as submitted or with modifications.

The version 2 of the protocol and the ICF for this study were reviewed and approved by the Jamia Hamdard Institutional Review Board on 30th November 2005.

➤ Informed Consent
The purpose of the study, procedures to be carried out, potential hazards and rights of the subjects were described to the subjects in non-technical terms before the subjects were admitted to the Ranbaxy CPU for Period I. All the subjects provided
formal written consent after attending an oral presentation and after thoroughly reading the version 2 of the Informed Consent Form.

3.2.1.18 Drop-out / Withdrawal of Subjects from Study
Subjects were informed that they are free to drop-out from the study at any time without stating any reason. The decision of withdrawal of a subject from the study was considered for any of the following reasons:

- The subject suffers from significant intercurrent illness or undergoes surgery during the course of the study.
- The subject experiences adverse event and withdrawal is in the best interest of the subjects.
- The subject fails to comply with the requirements of the protocol. This would include pre-study directions regarding alcohol and drug use, fasting or if the subject is uncooperative during the study.

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

3.2.1.19 Volunteer Compensation
The subjects were adequately compensated on account of their participation in the study. In case of drop-out/withdrawal of a subject before completion of the study, the guidelines issued by the Jamia Hamdard Institutional Review Board were final and binding on both Ranbaxy Research Laboratories and the study subjects.

3.2.1.20 Study Documentation
All data generated during the conduct of the study was directly entered in the raw data recording forms except the analytical data of clinical laboratory of the clinical Pharmacology Unit, which was transcribed into the study related forms and the raw data retained by the laboratory for their records. All raw data and transcribed data
forms were completed by the study personnel assisting in the study and were checked wherever applicable for completeness and logistics by the investigator.

3.2.2 Analytical methodology
A flame photometric method as per Flame Photometer Instrument Manual for the determination of lithium in human serum was used. The method was validated before starting analysis of the samples.

3.2.2.1 Instrumentation
Flame Photometer 129 (Systronics) is a microprocessor-based unit designed for Medical applications. The microprocessor provides automation in operation, measurements and end - result presentations. The unit can do the estimation of Sodium, Potassium, Lithium and Calcium in a single aspiration of a sample.

System Configuration
The system consists of:
1. Flame Photometer 129, the main unit.
2. FPM Compressor 126.

3.2.2.2 Principle of Operation
The principle of operation of a flame photometer is simple. The fluid under analysis is sprayed as a fine mist into a non- luminous flame which becomes colored according to the characteristic emission of elements (Li: 671 nm, Na: 589, K: 768, Ca: 622). The flame is monitored by a photodetector which views the flame through a selected narrow band optical filter that only passes the wavelengths centered around the characteristic emission of the selected element. The output of the photodetector is fed to and electronic module which provides digital readout of the concentration of the selected element(s). Before analyzing the unknown fluid sample, the system is standardized with solutions of known concentrations of elements of interest.
In a measurement set-up, compressed air from a compressor is supplied to an atomizer. Due to a draught of air at the tip of the atomizer, the sample solution is sucked in and enters in a mixing chamber as a fine atomized jet. Liquid Petroleum Gas (LPG) – the household cooking gas from a suitable source is also injected in the mixing chamber at a controlled pressure. The mixture of gas and atomized sample is passed on to a burner and is ignited. The emitted light from the flame is collected by optical lenses and passed on to a photodetector through a selected filter. An electronic unit processes the output of the detector and the results are appropriately displayed.

Table 7: Specifications of the Flame Photometer

<table>
<thead>
<tr>
<th>Specification</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range of operation (lithium)</td>
<td>0-2 mEq/l ; 1:10 dilution</td>
</tr>
<tr>
<td>Maximum concentration of aspirated solutions</td>
<td>0.2 mEq/l</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>± 2% FS ± 2 digit</td>
</tr>
<tr>
<td>Curve fitting accuracy</td>
<td>± 2% FS</td>
</tr>
<tr>
<td>Filter</td>
<td>Li (671), 10 nm Bandwidth (typical)</td>
</tr>
<tr>
<td>Minimum sample</td>
<td>Approx. 3 ml</td>
</tr>
<tr>
<td>Averaging time</td>
<td>2-15 seconds, selectable</td>
</tr>
<tr>
<td>Aspiration time</td>
<td>5 Second + Avg. time per element + 4 Sec</td>
</tr>
<tr>
<td>Operation air pressure</td>
<td>0.45 kg/cm² (typical), regulated</td>
</tr>
<tr>
<td>Fuel gas</td>
<td>LPG (Liquid Petroleum Gas), regulated</td>
</tr>
</tbody>
</table>
3.2.2.3 Reagents

  i. Lithium Carbonate working standard
  ii. Hydrochloric acid (AR) grade
  iii. Sodium Chloride (AR grade)
  iv. Potassium Chloride (AR grade)
  v. Milli-Q water

Preparation of reagents

  0.1N Hydrochloric Acid

About 820 µL of AR grade HCl was taken in a 100 mL of volumetric flask and to it was added 25 mL of HPLC grade water and was mixed well. The volume was made up with the same.

Preparation of Stock Standard Solution (1 mEq/l)

Approximately 18.473 mg of Lithium Carbonate was dissolved in 50 ml of HPLC grade water in a 500 mL of volumetric flask. And to it 5 ml 0.1 N HCl was added. This was then dissolved and the volume was made up to 500 mL with HPLC grade water. Thereafter a batch number was provided to it.

Preparation of Stock Standard Blank Solution

0.1865 gms of Potassium chloride and 4.09 gms of sodium chloride were dissolved in 500 ml of HPLC grade water.

3.2.2.4 Procedures

Preparation of Working Standard solution

Stock Standard Blank and Stock Standard solutions were taken as per the table given below and diluted to 50 ml with HPLC grade water and properly labeled.

<table>
<thead>
<tr>
<th>Stock Standard (1mEq/l) µL</th>
<th>Stock Blank ml</th>
<th>Volume made upto ml</th>
<th>Final concentration mEq/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.0</td>
<td>50.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2.5</td>
<td>5.0</td>
<td>50.0</td>
<td>0.05</td>
</tr>
<tr>
<td>5.0</td>
<td>5.0</td>
<td>50.0</td>
<td>0.1</td>
</tr>
<tr>
<td>10.0</td>
<td>5.0</td>
<td>50.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>
Preparation of Working Standard solution in Serum instead of Stock Blank

It was prepared as the solutions above. The only difference was that serum was used in place of stock blank and labeled properly. Discarded Serum of the screened volunteers was collected from Laboratory of Ranbaxy Clinical Pharmacology Unit. Serum was used in place of stock blank in order to see the difference in both the calibration curves (one with stock blank and other with serum) as the drug to be estimated was present in biological matrix. The biological matrix has many other constituents that may have their own effects.

<table>
<thead>
<tr>
<th>Stock Standard (mEq/l)</th>
<th>Serum Volume (ml)</th>
<th>Volume made up to (ml)</th>
<th>Final concentration (mEq/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.0</td>
<td>50.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2.5</td>
<td>5.0</td>
<td>50.0</td>
<td>0.05</td>
</tr>
<tr>
<td>5.0</td>
<td>5.0</td>
<td>50.0</td>
<td>0.1</td>
</tr>
<tr>
<td>10.0</td>
<td>5.0</td>
<td>50.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

CC (calibration curve standard) standards

A standard stock solution of lithium carbonate of concentration approximately 1.0 mEq/L was prepared by dissolving lithium carbonate in HPLC grade water with 0.1 N HCl and making up the volume with HPLC grade water. A batch number was provided. The solution was then stored in the refrigerator between 2-10°C, protected from light. This stock solution was to be consumed within 7 days of its preparation.

Preparation of lithium carbonate calibration curve standards (CC)

A calibration curve standards of lithium carbonate, consisting of seven different concentrations in the range from 0.1 - 1.0 mEq/l, was also prepared in serum using HPLC grade water as presented in the following table.
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<table>
<thead>
<tr>
<th>Stock Standard (1mEq/l)</th>
<th>Serum</th>
<th>HPLC grade water</th>
<th>Final volume</th>
<th>Final concentration in spiked plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>µl</td>
<td>µl</td>
<td>ml</td>
<td>ml</td>
<td>mEq/l</td>
</tr>
<tr>
<td>50</td>
<td>500</td>
<td>4.45</td>
<td>5</td>
<td>0.01</td>
</tr>
<tr>
<td>100</td>
<td>500</td>
<td>4.4</td>
<td>5</td>
<td>0.02</td>
</tr>
<tr>
<td>200</td>
<td>500</td>
<td>4.3</td>
<td>5</td>
<td>0.04</td>
</tr>
<tr>
<td>300</td>
<td>500</td>
<td>4.2</td>
<td>5</td>
<td>0.06</td>
</tr>
<tr>
<td>400</td>
<td>500</td>
<td>4.1</td>
<td>5</td>
<td>0.08</td>
</tr>
<tr>
<td>500</td>
<td>500</td>
<td>4.0</td>
<td>5</td>
<td>0.1</td>
</tr>
<tr>
<td>600</td>
<td>500</td>
<td>3.9</td>
<td>5</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Transferred appropriate volume of the above-described stock standard into 10 mL beaker. 500 µl of serum was added and then final volume was made up with HPLC grade water to 5 ml. Each above described stock dilution was adequately labeled with date of preparation. After aspiration of the CC sample, the unit auto cancels the dilution factor i.e. 10 and showed the end results as shown directly.

➢ **Preparation of Quality control samples**

Quality control samples of lithium carbonate were also prepared in serum using HPLC grade water as presented in the following table.

<table>
<thead>
<tr>
<th>Stock Standard (1mEq/l)</th>
<th>Serum</th>
<th>HPLC grade water</th>
<th>Final volume</th>
<th>Final concentration in spiked plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>µl</td>
<td>µl</td>
<td>ml</td>
<td>ml</td>
<td>mEq/l</td>
</tr>
<tr>
<td>100</td>
<td>500</td>
<td>4.4</td>
<td>5</td>
<td>0.02 (LQC)</td>
</tr>
<tr>
<td>300</td>
<td>500</td>
<td>4.2</td>
<td>5</td>
<td>0.06 (MQC)</td>
</tr>
<tr>
<td>500</td>
<td>500</td>
<td>4.0</td>
<td>5</td>
<td>0.1 (HQC)</td>
</tr>
</tbody>
</table>

Transferred appropriate volume of the above-described stock standard into 10 mL beakers. 500 µl of serum was added and then final volume was made up with HPLC grade water to 5 ml to achieve the following QC samples and labeled them as LQC, MQC, and HQC respectively as described in the table below. After aspiration of the
QC sample, the unit auto cancels the dilution factor i.e. 10 and showed the end results as shown directly.

This was used for precision and accuracy during the method validation only.

Sample preparation
Calibration curve standards and QC samples were prepared fresh on the day of analysis. Subject's serum samples were withdrawn from cold room and allowed to thaw at the room temperature. The thawed samples were vortexed to ensure complete mixing of the contents. A 500 µL of each sample was aliquoted in a glass cuvette. Thereafter, 4.5 mL of distilled water was added to all samples and vortexed to ensure proper mixing.

After testing system suitability, CC samples were aspirated. Then subject samples were aspirated having in between QC samples. The system was standardized after every 15–20 samples. Distilled water was aspirated after every sample instead of blank. All the samples were protected from light during the whole procedure.

3.2.2.5 Validation Parameters
The validation of this procedure was performed in order to evaluate the method in terms of linearity, precision, accuracy, sensitivity and stability (US Pharmacopoeia 2000).

The linearity, precision and accuracy evaluations were performed on three batches of spiked serum samples. Each batch of spiked serum samples included one complete calibration curve (consisting of seven different concentrations in the range from low (0.1 mEq/l) to High (1.0 mEq/l)).
3.2.3 Clinical study sample analysis

3.2.3.1 Objective
The objective of the study was to analyze the clinical study samples using a validated flame photometric method for the determination of lithium carbonate.

3.2.3.2 Dates of the sample collection
The serum samples for period I, II and III were collected between 22\textsuperscript{nd} - 29\textsuperscript{th} December 2005; 5\textsuperscript{th} - 12\textsuperscript{th} January 2006 and 19\textsuperscript{th} - 26\textsuperscript{th} January 2006 respectively.

3.2.3.3 Total number of samples
As per the protocol, a total number of 1305 samples should have been collected from 15 subjects over in three periods. However, two subjects dropped out during the study and two subjects were withdrawn following adverse reactions. One trough sample of subject number 5 could not be collected on day 3 in period I, as subject did not come. Therefore, a total of 956 samples were collected during the whole study. One analytical batch consisted of all the samples of one subject (29) in one period along with the calibration standards (7) and quality control samples (4 LQC, 4 MQC and 4 HQC).

3.2.3.4 Storage Sites
The samples were stored at \(-30 \pm 4^\circ\text{C}\) in a Haereus deep freezer at the clinical facility.

3.2.4 Pharmacokinetic and statistical analysis

3.2.4.1 Pharmacokinetic analysis
The following pharmacokinetic parameters will be calculated for lithium carbonate using WinNonlin-Node from Pharsight. The calculation for steady state will be based upon one dosing interval i.e.0-12 hours; however, sampling time were collected up to 24 hours is for information basis only.
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Single dose PK Parameters-

AUC_{0→t}  The area under the plasma concentration versus time curve, from time zero to the last measurable concentration (12 hours), as calculated by the linear trapezoidal method.

AUC_{0→∞}  The area under the plasma concentration versus time curve, from time zero to infinity. AUC_{0→∞} is calculated as the sum of AUC_{0→t} plus the ratio of the last measurable plasma concentration to the elimination rate constant.

\frac{AUC_{0→t}}{AUC_{0→∞}}  The ratio of AUC_{0→t} to AUC_{0→∞}.

C_{max}  Maximum measured plasma concentration over the time span specified.

T_{max}  Time of the maximum measured plasma concentration. If the maximum value occurs at more than 1 time point, T_{max} is defined as the first time point with this value.

K_{el}  Apparent first-order terminal elimination rate constant calculated from a semi-log plot of the plasma concentration versus time curve. The parameter will be calculated by linear least-square regression analysis using the maximum number of points in the terminal log-linear phase (e.g. three or more non-zero plasma concentrations).

T_{1/2}  The apparent first-order terminal elimination half-life will be calculated as \frac{0.693}{K_{el}}.

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CHAPTER 3

MATERIALS AND METHODS

- Steady-state PK Parameters-

\( \text{AUC}_{\text{ss,0-t}} \) The area under the plasma concentration versus time curve, from time zero to 12 hours, as calculated by the linear trapezoidal method.

\( \text{Css}_{\text{min}} \) Minimum measured plasma concentration over the time span specified at steady state.

\( \text{Css}_{\text{max}} \) Maximum measured plasma concentration over the time span specified at steady state.

\( \text{Css}_{\text{avg}} \) The average plasma concentration at steady-state.

\( \text{PTF} \) Peak-to-trough fluctuations.

\( \text{Tss}_{\text{max}} \) Time of maximum measured plasma at steady-state. If the maximum value occurs at more than one time point, \( \text{Tss}_{\text{max}} \) is defined as the first time point with this value.

No value of \( K_{\text{el}}, \text{AUC}_{\text{ss,0-t}}, \) or \( T_{1/2} \) will be reported for cases that do not exhibit a terminal log-linear phase in the concentration versus time profile.
3.2.2 Statistical analysis

Statistical analyses were performed on serum lithium carbonate using the SAS system for Windows, release 8.2 (SAS Institute Inc., USA). The analyses included data from all subjects except subject number 1, 6, 12 and 14 as these subjects did not complete the study. Subject number 1 and 6 were withdrawn from the study due to adverse effects, while subject number 12 and 14 dropped out from the study.

Students paired t-test was used to determine significance of results between different treatments. A p < 0.05 signifies statistically significant result.

Summary Statistics

The summary statistics (for the relevant pharmacokinetic parameters) was reported for both test and reference products: The arithmetic means, the geometric mean, standard deviations and the coefficient of variation for the original (untransformed) data; and the arithmetic means, standard deviation and the coefficient of variation for the log (natural) transformed data. For the relevant pharmacokinetic parameters, the ratios of the test and reference product averages were also reported.

Ratios of mean (in percentage) were calculated using the LSM for both untransformed and log-transformed $C_{ss_{max}}$, $AUC_{ss_{0-12}}$, $C_{ss_{min}}$, $C_{ss_{avg}}$ and % fluctuations at steady-state and $C_{max}$, $AUC_{0-12}$ and $AUC_{0-\infty}$ of single dose. The geometric mean values were reported for log-transformed parameters. Ratios of means were expressed as a percentage of the LSM for the respective treatment comparisons.

The confidence interval (90%) as recommended by US FDA, Canadian Regulatory Agency and DCGI (India) were constructed for all the treatment comparisons for both untransformed and log-transformed data.
The intra subject variability was reported for the pharmacokinetic parameters $C_{SS_{\text{max}}}$, $\text{AUC}_{SS_{0-12}}$, $C_{SS_{\text{min}}}$, $C_{SS_{\text{avg}}}$ and % fluctuation at steady-state and $C_{\text{max}}$, $\text{AUC}_{0-12}$ and $\text{AUC}_{0-\infty}$ of single dose for log transformed data.

Analysis of variance was performed and a P value < 0.05 was considered statistically significant at 95% level of significance and $\alpha$-0.05.

The pharmacokinetic parameter $T_{\text{max}}$ was statistically evaluated using a non-parametric test i.e., the Wilcoxon's Signed Rank Test at $\alpha$ = 0.05 level of significance.