There is no such thing as a failed experiment, only experiments with unexpected outcomes

\textit{Materials and Methods}
4 MATERIALS AND METHODS

4.1 Part A: Method Development for Atorvastatin
In several clinical studies regarding statins in general, including atorvastatin, determination of the statins has been performed using alternative methods, such as enzyme inhibition assays, other than determination of the actual plasma concentrations of the compounds (Asberg et al., 2001; Amsden et al., 2002; Hsyu et al., 2001; Siedlik et al., 1999). Such indirect measurements are relevant when determining the HMG-CoA reductase inhibitory activity of the statin in plasma, but they do not give any further information on metabolites. Information about the actual plasma concentration of both parent compound and metabolites is of interest in Phenotyping and pharmacokinetic studies. Thus, our aim was to develop a chromatographic method for determining the acid forms of atorvastatin, O- and p-hydroxyatorvastatin, in human plasma using SPE for sample preparation.

4.1.1 Chemicals and reagents
Atorvastatin, o-hydroxy atorvastatin and p-hydroxy atorvastatin were purchased from Synfine Chemicals (Canada) Deuterated analogs of atorvastatin were not available, and Pravastatin (Sigma-Aldrich, Norway) was therefore used as internal standard. Pravastatin is also an HMG-CoA reductase inhibitor and concomitant use of both the drugs are not possible and it was therefore unlikely to be used by patients, in subsequent pharmacokinetic studies applying this method. All chemicals used for chromatographic purposes were of analytical grade.
4.1.2 Experimental Conditions:

The HPLC equipment: Alliance HT with integrated system of quarternary pump

Detector: Mass spectrometry a Quattro LC tandem quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an ESI source.

Operating System: Mass Lynx version 4 software.

Chromatographic Column: An Cyno 125 X 4 mm, 5μm column

Mobile Phase: Acetonitrile–methanol–0.1% formic acid in water (50–30–20, v/v).

The flow Rate: 0.5 ml/min.

Auto Sampler Tray Temperature: 10 °C.

Column Oven Temperature: 45 °C.

At the above conditions the total time of analysis in the chromatographic system was 3.5 min. and the retention times of atorvastatin, metabolites and internal standard were at around 2.4 min.

4.1.3 Sample preparation

Preparation of samples from healthy volunteers and plasma samples spiked with aliquots of the analytes was performed by SPE, The plasma samples (0.5 ml) were spiked with 5μg of the internal standard
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pravastatin and 0.5 ml of 2% formic acid in water and was vortexed for 30 seconds. The content was subsequently transferred to 1 ml C18 (100 mg) SPE cartridges (Oasis, Waters Corporations, Milford, Mass, USA) pre-conditioned with 2 ml methanol followed by 1 ml water. The cartridges were washed with 2 ml 2% formic acid in water, and the analytes were eluted with 1 ml 0.1 % formic acid in methanol after the extraction of samples, the extracts were concentrated and evaporated to dryness under a stream of N2 at around 60° C and the residues were reconstituted in 200 μl mobile phase. Resulting solution was vortexed for 30 seconds, prior to transfer to HPLC vials and injection of 25 μl in the HPLC system.

A set of seven calibration standards, a zero, a blank and three sets of quality control samples (QCS) were analyzed with every series of standard curves. For each calibration standard, the peak height ratio of atorvastatin to I.S (Pravastatin) was calculated. A straight-line equation describing the relationship between this ratio and concentration, with a weighting factor of 1/X was arrived at.

4.1.4 Method Validation:

Specificity: Blank plasma samples of six different sources were screened to check for any interference due to endogenous components. Same samples were used for all further analytical development work and QCs.

Linearity: The linearity was demonstrated over the concentration range if the coefficient of variation (r2) of calibration curve is more than 0.98.

Precision:

With in day precision: Three sets of calibrations and six replicates of QCs by level were assayed the same day, the concentration of all the QCs were determined with first calibration curve. The within day
precise was expressed as coefficient of variation (\%CV) of six determinations at each level of QCs and must not exceed 15\% except at lowest level where 20\% is acceptable.

Inter-day Precision: It was performed on three days with one set of calibrators and six replicates of QCs by level per day. The inter-day precision was expressed as coefficient of variation (\%CV) of eighteen determinations at each level of QCs and must not exceed 15\% except at lowest level where 20\% is acceptable.

Accuracy: Inter day and Intra day accuracy was calculated by dividing the concentration measured by concentration spiked and was expressed as percentage.

4.2 Part B: Pharmacokinetic Study:

4.2.1 Subjects and Methods

This study was conducted at Synchron Research Pvt. Ltd, Ahmedabad, according to the principles of the Declaration of Helsinki and according to ICH, GCP. Institutional ethics committee approved the protocol, and the participants gave written informed consent before participation in the study. In total, 44 healthy volunteers were enrolled in this study. All participants were in good health according to medical history and physical examination, electrocardiogram, and clinical laboratory measurements. Participants had no clinically significant respiratory, Cardio vascular, renal, hepatic, gastrointestinal, gallbladder (including gallstones), hematologic, neurologic, psychiatric, endocrine, or other medical disorder that would interfere with participation in the study. The following concomitant drugs were not permitted during this study: (i) other lipid-lowering drugs or preparations (acipimox, niacin, fibrates, bile sequestrants, other statins, soluble fibre preparations like psyllium and Metamucil); (ii) other drugs known to modulate lipid parameters (corticosteroids, isotretinoin); (iii) antioxidant vitamins; (iv)
immunosuppressive drugs; (v) drugs known to be associated with myopathy in combination with HMG-CoA reductase inhibitors, due to competition for metabolic pathways (cyclosporin, macrolide antibiotics, azole antifungals). Subjects were asked not to change their eating habits during the course of the study.

4.2.1.1 Screening
The screening was carried out after taking an initial informed consent from volunteers for study screening (-14 to -1 day) procedure and included the following:

- Demographic data, including name, sex, date of birth, height and weight
- Medical and treatment history including present complaints such as skin diseases, rhinitis, glaucoma and other (if any), relevant past medical history, family history, history of any allergy to food or drug, medication history in the last six months
- Complete physical examination including recording of vital signs (B.P., Pulse, Temperature and Respiration) and systemic examination.
- 12-lead ECG for heart rate, rhythm and specific finding (if any)
- Chest X-ray

4.2.1.2 Clinical Laboratory Tests
- Complete blood count - haemoglobin, hematocrit, total and differential leucocytic count, red blood cell count, and platelet count, ESR, CT, BT and PT.
- Biochemistry - blood glucose, sodium and potassium
- Fasting state lipid profile.
- Hepatic profile - SGOT, SGPT & Bilirubin (total, direct and indirect)
- Renal profile - serum creatinine and urea
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- Urine - physical examinations, chemical examination, microscopic examination and drugs of abuse (benzodiazepines, opioids and amphetamine)
- HIV, HBS Ag and HCVAb

4.2.1.3 Inclusion Criteria
- Healthy males of any race within the age range of 18 to 45 years.
- Willingness to provide written informed consent to participate in the study.
- Body Mass Index (BMI) > 18.0 Kg/m2 and <25.0 Kg/m2.
- Absence of significant disease or clinically significant abnormal laboratory values on the laboratory evaluations, medical history or physical examination during the screening.
- Normal blood lipid profile.
- Have a normal 12-lead ECG or one with abnormally considered to be clinically insignificant.
- Have a normal chest X-ray.
- Comprehension of the nature and purpose of the study and compliance with the requirement of the entire protocol.

4.2.1.4 Exclusion Criteria
- History/evidence of allergy or hypersensitivity to any drugs.
- Any major illness in the past three months or any significant ongoing chronic medical illness.
- Severe renal or liver impairment.
- History of alcoholism (more than two years), moderate drinkers (more than three drinks per day) or having consumed alcohol within 48 hours prior to dosing (one drink is equal to one unit of alcohol (one glass wine, half pint beer, one measure of spirit)).
- High caffeine (more than 5 cups of coffee or tea/day) or tobacco (more than 10 cigarettes/day) consumption.
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- Use of any recreational drug or a history of drug addiction.
- Participation in any clinical trial within the past three months.
- History of difficulty with donating blood or difficulty in accessibility of veins in left or right arm.
- Donation of blood (one unit or 350 ml within three months prior to receiving the first dose of study medication.
- Receipt of any prescription drug therapy within two weeks or over-the-counter (OTC) drugs within one week prior to receiving the first dose of study medication.

4.2.1.5 Withdrawal Criteria
Subjects were withdrawn from the study by the investigator for any of the following reasons during the course of the study:
- If the subject suffers from significant illness.
- If the subject requires unacceptable concomitant medications.
- If the subject has entered the study in violation of the inclusion and the exclusion criteria.
- If the subject is found to be non co-operative.
- If the subject decides to voluntarily withdraw from the study.

4.2.1.6 Protocol of Drug Administration and Blood Sampling
The protocol is shown in Appendix. Participants were randomized to one of the two treatment groups: A and B. An oral dose of one tablet of atorvastatin 40 mg tablets (test (A) or reference (B)) was administered as per the randomization schedule every morning for 14 days for both periods. Fourteen days were enough to reach the steady state concentration (Cilla et al., 1996a). Dose up to 80 mg/day were well tolerated in healthy subjects (Product information Lipitor, 1999). Subjects received the alternate treatment in the subsequent period following crossover with the following possible treatment sequence (B-A or A-B). The subjects visited Synchron facility everyday for dosing, at
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specified time from day 01 to day 13 and from day 43 to day 55. They were educated, requested and expected to maintain the fasting condition during non-in-house phase for at least 10 hours before every dose administration and 4 hour after dose in both periods. On days 01 and 43 the subjects remained in facility four hours after dose for vital monitoring after two hours of dosing. They were provided with standard meals four hours post dose. On day 13th and 55th the subjects were admitted in the Synchron facility in respective periods. On day 14th and day 56th the dosing was done as per everyday schedule (Table -2) after ensuring the 10-hour pre dose and 4 hour post dose fasting. The meals were provided at approximately 4 hours and 12 hours post dose, and snacks at approximately 8 hours post dose of day 14 and day 56. Water was restricted for one hour pre-dose to two hours post-dose; at other times drinking water was permitted ad libitum.

4.2.2 Pharmacokinetic Analysis

Pharmacokinetic parameters of atorvastatin and ortho- and para-hydroxy atorvastatin was calculated as follows:

Tssmax: Maximum time corresponding to Cssmax.

Cssmax: Maximum steady state drug concentration during a dosing interval.

Cssmin: Minimum steady state drug concentration during a dosing interval.

AUCss: Area under the curve during a dosing interval at steady state.

%ptf: Peak through fluctuation
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4.2.3 Statistical analysis

4.2.3.1 Summary Statistics:
The summary statistics (for relevant pharmacokinetic parameters) will be reported for the test and reference formulations. The reported parameters will be the arithmetic means, geometric mean, maximum value, minimum value, standard deviations and the coefficient of variation for untransformed data.

4.2.3.2 Analysis of Variance (ANOVA)
The log-transformed pharmacokinetic parameters (Cssmax and AUCss) will be analysed using an ANOVA model with the main effects of sequence, subject nested within sequence, period and treatment. Each analysis of variance will include calculation of least-square means, adjusted differences between treatment means and the standard error associated with these differences.

4.2.3.3 Intra-subject Variability
The intra-subject variability for each of the pharmacokinetic parameters reflect the residual variability after accounting for the difference between subjects, periods and treatments and will be reported in terms of the overall coefficient of variation (% C.V.), from the ANOVA results using log-transformed data.

4.2.3.4 90% Confidence Intervals
For the pharmacokinetic parameters (Cssmax and AUCss) 90% confidence intervals for the ratios of test and reference formulations average will be calculated using the ANOVA output from the analysis of the log-transformed data.
4.2.3.5  **Bioequivalence criteria**
The 90% confidence interval for the ratio of test and reference formulations will be calculated for the log-transformed pharmacokinetic parameters (C<sub>ssmax</sub> and AUC<sub>ss</sub>).

The 90% confidence interval will form the basis for concluding the equivalence of reference B with Test A. If the point estimate of the geometric mean ratio and the confidence intervals for the entire log transformed pharmacokinetic parameters (C<sub>ssmax</sub> and AUC<sub>ss</sub>) are entirely included in the range of 80-125%, then the treatments will be claimed to be bio-equivalent.

4.2.3.6  **Ratio analysis**
Ratio of test formulation will be compared to the reference formulation for all un-transformed pharmacokinetic parameters (C<sub>ssmax</sub> and AUC<sub>ss</sub>). Ratio of geometric means will be expressed as point estimates of test/reference mean ratio.

4.2.3.7  **T<sub>ssmax</sub>**
T<sub>ss-max</sub> will be analysed according to the Kruskal Wallis non-parametric test.
STUDY FLOW CHART

Screening ICF and Screening

Eligibility Criteria

Subject Selection (n = 44)

Randomization

Check in, ICF and safety Assessments

Pre-dose blood draw and safety Assessments

Dosing for 14 days

Treatment A for 14 days (n=22)  Treatment B for 14 days (n=22)

Period I (n=44)

Blood draw and safety Assessments

Check out and safety Assessments

Washout period of 4 weeks and crossover
Blood draw for lab evaluation on days 16, 28 and 42

Treatment B for 14 days (n=22)  Treatment A for 14 days (n=22)

Period II (n=44)

Post-dose blood draw and safety Assessments

Post-study lab evaluation sample

Checkout and Safety assessment
### Table 2 SCHEMATIC REPRESENTATION OF STUDY ASSESSMENT

<table>
<thead>
<tr>
<th>Assessments</th>
<th>Screening Period (-14 to -1Day)</th>
<th>Study Days</th>
<th>Wash period D_{28} and D_{42}</th>
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<td>Written Consent for screening</td>
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<td>Demographics</td>
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<td>Medical &amp; Treatment History</td>
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<td>Physical Examination - including</td>
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<td><strong>3. Written Informed Consent for dosing/sampling</strong></td>
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<td>Vital signs</td>
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<td>Medical examination: general &amp; systemic</td>
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<td>Adverse events monitoring (Subject Questionnaire)</td>
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<td><strong>5. Pharmacokinetics</strong></td>
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<td>Pre-dose Blood Sampling</td>
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<td>Post-dose Blood Sampling</td>
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<td>Post study sample (end of period II/after drop out/withdrawal)</td>
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4.3 Part C: Genetic Polymorphism of CYP3A4 in Gujarati Population.

4.3.1 Selection of volunteers.
Based upon genetic distances, the people of India have been broadly classified into four main ethnic groups: Caucasoid Aryans, Caucasoid Dravidians, Australoids and Mongoloids. The non-tribal population of India consists mainly of Caucasoid Aryans in North India and Caucasoid Dravidians in South India. Gujarati subjects, a perfect blend of north India and south India were enrolled for the study. The basic protocol of the study was explained to each volunteer. The screening was carried out after taking an initial informed consent from volunteers for study screening procedure and included the following:

- Demographic data, including name, sex, date of birth, height and weight
- Medical and treatment history including present complaints such as skin diseases, rhinitis, glaucoma and other (if any), relevant past medical history, family history, history of any allergy to food or drug, medication history in the last six months
- Complete physical examination including recording of vital signs (B.P., Pulse, Temperature and Respiration) and systemic examination.

4.3.1.1 Clinical Laboratory Tests
Complete blood count - haemoglobin, hematocrit, total and differential leucocytic count, red blood cell count, and platelet count, ESR, CT, BT and PT.
4.3.2 Inclusion Criteria

- Healthy males or females of any race within the age range of 18 to 45 years and resident of Gujarat.
- Willingness to provide written informed consent to participate in the study.
- Body Mass Index (BMI) > 18.0 Kg/m2 and < 25.0 Kg/m2.
- Absence of significant disease or clinically significant abnormal laboratory values on the laboratory evaluations, medical history or physical examination during the screening.
- Comprehension of the nature and purpose of the study and compliance with the requirement of the entire protocol.

4.3.3 Exclusion Criteria

- History/evidence of allergy or hypersensitivity to any drugs.
- Any major illness in the past three months or any significant ongoing chronic medical illness.
- History of alcoholism (more than two years), moderate drinkers (more than three drinks per day) or having consumed alcohol within 48 hours prior to dosing (one drink is equal to one unit of alcohol (one glass wine, half pint beer, one measure of spirit)).
- Pregnant women and Nursing mothers were excluded from study
- High caffeine (more than 5 cups of coffee or tea/day) or tobacco (more than 10 cigarettes/day) consumption.
- Use of any recreational drug or a history of drug addiction.
- Participation in any clinical trial within the past three months.
- History of difficulty with donating blood or difficulty in accessibility of veins in left or right arm.
- Donation of blood (one unit or 350 ml within three months prior to receiving the first dose of study medication.
- Receipt of any prescription drug therapy within two weeks or over-the-counter (OTC) drugs within one week prior to receiving the dose of study medication.
4.3.4 Withdrawal Criteria
Subjects were withdrawn from the study by the investigator for any of the following reasons during the course of the study:

- If the subject suffers from significant illness.
- If the subject requires unacceptable concomitant medications.
- If the subject has entered the study in violation of the inclusion and the exclusion criteria.
- If the subject is found to be non-co-operative.
- If the subject decides to voluntarily withdraw from the study.

Those found fit by these criteria were phenotyped after each subject provided a written and voluntary consent in accordance with the ethical guidelines of the Helsinki convention on research involving human volunteers. The Ethics Committee approved the study.

4.3.5 Phenotyping of subjects
Atorvastatin was used to phenotype subjects with respect to hepatic CYP3A4. For phenotyping, each healthy Gujarati volunteer was given 20 mg atorvastatin (Zivast, FDC, India) orally with 250 ml water after an overnight fasting. No food was allowed throughout the study. Subjects were not taking any concurrent medication and were asked to abstain from drinking tea or coffee 10 h before drug administration and 3 h afterwards. The post-dose samples were collected at 2 hour after drug administration. Seven millilitres of venous blood was drawn into a pre-labelled vacutainers containing K3EDTA. The samples collected were centrifuged to separate plasma, immediately within 30 minutes after receiving the blood samples from all the subjects. The separated plasma samples were then transferred to deep freezer in pre-labelled tubes below -80°C for storage. Plasma samples were analysed for the parent compound atorvastatin, its ortho- and para-hydroxy-
metabolites, by a high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS) assay.

### 4.3.6 Statistical Analysis

Metabolic ratios MRs of atorvastatin/ortho-hydroxy atorvastatin were calculated in a 2-h plasma sample. Analysis of interindividual variations in the metabolism of atorvastatin was expressed by a probit plot and a frequency distribution histogram between log MR on the abscissa and the number of the subjects on the ordinate. The Kolmogorov Smirnov test was also used to test the normality of the metabolic distribution. The level of significance for all statistical tests was $p<0.05$. All values are presented as mean ±SD unless otherwise stated. The Microsoft Excel (Microsoft Corp., Redmond, Wash) was used for statistical test and probit plots were used to determine the bimodal distribution and antimode value of the log MR of atorvastatin/ortho-hydroxy atorvastatin in the Gujarat subjects. Multiple regression was used to calculate the inflection point in the probit plot.
4.4 Part D: Genetic polymorphism of CYP 3A4 in Coronary Artery Disease patients (CAD)

4.4.1 Selection of Patients

Patients of either sex between 18 and 75 years of age with documented Coronary artery Disease either single vessel, double vessel or multiple vessel coronary artery disease confirmed with prior angioplasty were recruited from sterling hospital Ahmedabad. The basic protocol of the study was explained to each Patient. The screening was carried out after taking an initial informed consent from patients for study screening procedure and included the following:

- Demographic data, including name, sex, date of birth, height and weight
- Medical and treatment history including present complaints, relevant past medical history, family history, history of any allergy to food or study drug,
- Complete physical examination including recording of vital signs (B.P., Pulse, Temperature and Respiration) and systemic examination.

4.4.1.1 Inclusion Criteria

- Healthy males or females of any race within the age range of 18 to 75 years and resident of Gujarat.
- Willingness to provide written informed consent to participate in the study.
- Documented Coronary artery Disease single vessel, double vessel or multiple vessel coronary artery disease confirmed with prior angioplasty
- Compliance with the requirement of the entire protocol.
4.4.1.2 Exclusion Criteria

- History/evidence of allergy or hypersensitivity to any drugs.
- Consuming concurrent medicines which were CYP3A4 inhibitors.
- History of alcoholism (more than two years), moderate drinkers (more than three drinks per day) or having consumed alcohol within 48 hours prior to dosing (one drink is equal to one unit of alcohol (one glass wine, half pint beer, one measure of spirit)).
- High caffeine (more than 5 cups of coffee or tea/day) or tobacco (more than 10 cigarettes/day) consumption.
- Use of any recreational drug or a history of drug addiction.
- Participation in any clinical trial within the past three months.
- History of difficulty with donating blood or difficulty in accessibility of veins in left or right arm.
- Donation of blood (one unit or 350 ml) within three months prior to receiving the single dose of study medication.
- Patients were excluded if they had known hepatic or renal dysfunction,
- A serum creatinine of more than 1.5 mg/dL
- Thyrotoxicosis.
- Clinically significant valvular disease, Wolf-Parkinson-White syndrome, a recent acute coronary syndrome,
- New York Heart Association Class III or IV heart failure.
- Terminal malignancy, paroxysmal atrial fibrillation resulting in pulmonary edema or syncope.
- Pregnancy or childbearing potential without adequate contraception, or no written informed consent.

4.4.1.3 Withdrawal Criteria

Subjects were withdrawn from the study by the investigator for any of the following reasons during the course of the study:

- If the subject suffers from significant illness.
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- If the subject requires unacceptable concomitant medications (CYP3A4 Inhibitors).
- If the subject has entered the study in violation of the inclusion and the exclusion criteria.
- If the subject is found to be non co-operative.
- If the subject decides to voluntarily withdraw from the study.

Those found fit by these criteria were phenotyped after each subject provided a written and voluntary consent in accordance with the ethical guidelines of the Helsinki convention on research involving human volunteers. The Ethics Committee approved the study.

4.4.2 Phenotyping of Patients

Atorvastatin was used to phenotype subjects with respect to hepatic CYP3A4. For phenotyping, each CAD patient was given 20 mg atorvastatin (Zivast, FDC, India) orally with 250 ml water at bedtime (nearly 8.00 P.M.). Subjects were not taking any concurrent medication which were known CYP3A4 inhibitors and were asked to abstain from drinking tea or coffee 10 h before drug administration and 3 h afterwards. The post-dose samples were collected at 2 hour after drug administration (10.00 PM). Seven millilitres of venous blood was drawn into a pre-labelled vacutainers containing K3EDTA. The samples collected were centrifuged to separate plasma, immediately within 30 minutes after receiving the blood samples from all the patients. The separated plasma samples were then transferred to deep freezer in pre-labelled tubes below -80°C for storage. Plasma samples were analysed for the parent compound atorvastatin, its ortho- hydroxy-metabolites, by a high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS) assay.
4.4.3 Statistical Analysis

Metabolic ratios MRs of atorvastatin/ortho-hydroxy atorvastatin were calculated in a 2-h plasma sample. Analysis of interindividual variations in the metabolism of atorvastatin was expressed by a probit plot and a frequency distribution histogram between log MR on the abscissa and the number of the patients on the ordinate. The Kolmogorov Smirnov test was also used to test the normality of the metabolic distribution. The level of significance for all statistical tests was p<0.05. All values are presented as mean ±SD unless otherwise stated. The Microsoft Excel (Microsoft Corp., Redmond, Wash) was used for statistical test and probit plots were used to determine the bimodal distribution and antimode value of the log MR of atorvastatin/ortho-hydroxy atorvastatin in the Gujarati subjects. Multiple regression was used to calculate the inflection point in the probit plot.