Chapter 6
Summary & Conclusion
6.1 Summary

The thesis is summarized as follows:

- Chapter 1 gives a crisp introduction to bioanalysis, pharmacokinetics and conduct of bioequivalence study. The compound of interest, EZM is introduced in detail giving a start to the thesis. Chapter also explains the aims and objectives of study.

- Chapter 2, describes literature review & methodology and includes an exhaustive review on the systematic development of liquid chromatographic methods. It includes an exhaustive review on the methods available for the estimation of EZM alone or in combination, from biological samples.

- Chapter 3: Determination of EZM by HPLC
  - Section I describes development and validation of a HPLC method for the analysis of total EZM. A liquid-liquid extraction technique is employed to extract total EZM from human plasma.
  - Section II describes the development of a qualitative method for the determination of EZM-G. The solid phase extraction technique is used to extract EZM-G from human plasma.

- Chapter 4: Determination of EZM by LC-MS/MS
  - Section I describes development and validation of a LC-MS/MS method for the determination of total EZM. The liquid-liquid extraction used in chapter 3 section I, was used for the extraction of total EZM from human plasma, with minor modifications to suit the LC-MS/MS requirements.
  - Section II describes development and validation of a LC-MS/MS method for the determination of unconjugated EZM. A simple liquid-liquid extraction was employed for the extraction of unconjugated EZM from human plasma.

- Chapter 5 illustrates the application of the above mentioned methods in the pharmacokinetic analysis of unconjugated, conjugated and total EZM in 12
healthy human subjects under fasting and fed state. The concentrations achieved underwent pharmacokinetic and statistical analysis.

- Chapter 6 summarizes the entire thesis and concludes it based on the findings of the thesis.

### 6.2 Conclusion

An analytical methodology is an important aspect of bioequivalence study. The drug concentrations are measured from biological samples through bioanalytical method. Development of selective, precise and accurate analytical methods, are essential to rightfully analyze the drug concentration at each time point to obtain quality pharmacokinetic data, which in turn will decide the interchangeability of the innovator and multisource drug product. The Bioanalytical methods are validated to ensure the acceptability of the performance and reliability of analytical results.

For many drugs, glucuronidation is the major metabolic pathway of detoxification, which changes the compounds to a less toxic, more water soluble form that can be rapidly excreted from the body. Direct conjugation with glucuronic acid generally terminates the pharmacological activity of the drug. However, in few cases phase II metabolites have been found to be pharmacologically active for example, the phenolic glucuronide conjugate of EZM, which constitutes approximately 80-90% of total drug in plasma. EZM-G is found to be more potent than the parent molecule in inhibiting cholesterol absorption. For bioequivalence studies when metabolite contributes meaningfully to the efficacy, the parent drug and metabolites are to be measured (King 2009). In accordance with FDA guidance, unconjugated EZM and total EZM (unconjugated + conjugated EZM) are to be measured for the bioavailability/bioequivalence studies. However, the concentrations of unconjugated EZM in plasma being very low and also EZM-G being the main contributing factor for the efficacy of the drug, following aims and objectives were outlined for the study.

The research work was divided into following steps:

- Development of the liquid chromatographic methods for the estimation of analytes under study.
- Validation of the developed method as per the regulatory guidelines.
• Application of the methods for the determination of pharmacokinetic parameters to study the comparative bioavailability of the two investigational product of EZM.

The application of LC-MS/MS in clinical laboratories is well documented and forms the first method of choice for bioanalysis. Despite of higher specificity and better sensitivity, the initial setup of instrument is costly and requires dedicated highly trained personnel for using and maintaining the instruments. Thus, third chapter of the thesis is dedicated to develop a HPLC method for the determination of total EZM from human plasma samples.

The extraction procedure was optimized to ensure complete hydrolysis of EZM-G to product EZM and was estimated as total EZM with the use of symmetry shield C18 (250 × 4.6mm, 5µ) column and a mobile phase consisting of acetonitrile: 1mM ammonium acetate in the proportion of 60:40 (v/v). Enzyme glucuronidase was used to cleave glucuronic acid moiety of EZM-G to form EZM. The rate of enzyme reactions is generally different for different substrates in different biological matrices. Thus a reaction pattern for a particular enzyme source and a substrate, in a particular biological matrix has to be established. Thus enzyme reaction parameters like enzyme concentration, effective substrate concentration, pH, temperature and time were studied. The enzymatic cleavage process gained maximum ezetimibe levels after incubation of glucuronide with 1457 units of enzyme per mL of plasma for 6 hours at 50 - 55°C. An interesting observation was that the activity of the enzyme persisted in the pH range of 4 – 6, while the recommended pH range for β-glucuronidase enzyme from *Helix Pomatia* source is 4 – 5. The effective substrate concentration was found to be between 25-9500 ng/mL indicating that the enzyme is effective in hydrolyzing very broad range of substrate concentration. The optimized extraction procedure was found to convert EZM-G to EZM efficiently and the total EZM was estimated within the acceptable limits of precision and accuracy. The enzyme used for the analysis was reduced significantly as compared to the reported methods (Bae et al., 2012; Li et al., 2006; Oliveira et al., 2006), thereby reducing the cost of analysis. The limit of quantification obtained was 30 ng/mL and the method was found to be linear in the concentration range of 30 – 500 ng/mL. The recovery of the method was 79.12% with a precision of 6.64%. The developed HPLC method was applied for the estimation of
total EZM in 6 healthy human subjects under fasting conditions. In order to quantitate lower concentration levels of total EZM generally encountered in real samples, 50 ng/mL EZM standard was spiked to actual concentrations of the subject samples so as to lift the concentrations in the concentration range of 30 – 500 ng/mL. Post quantitation, concentrations obtained, underwent subtraction of 39.5 ng/mL, accounting for the recovery of the method and final concentrations were subjected to pharmacokinetics and statistical analysis.

In the section II, a HPLC method was developed for the determination of EZM-G from human plasma. Due to highly polar nature of metabolite, developing a method proved to be a real challenge. The optimized chromatographic conditions to detect EZM-G consisted of Kromasil C18 (250 × 4.6mm, 5µ) column with 0.05M HCOOH: acetonitrile in the proportion 60:40 (v/v) as mobile phase. Various extraction procedures were employed for the extraction of EZM-G from plasma. The protein precipitation technique resulted in a crude extract, thereby making the identification of analyte peak difficult. Although various solvents and solvent combinations were tried, liquid-liquid extractions lead to poor recovery of the analyte. Protein precipitation and liquid-liquid extraction served as precursors for the development of solid phase extraction procedure. A solid phase extraction method was developed for extracting the phenolic glucuronide from human plasma. The developed method was found to give good plasma profile and response area, but the precision obtained for the response area of the analyte peak was found to be beyond the acceptance limits. Nonetheless, the method developed allowed qualitative estimation of EZM-G. The chapter could serve as a forerunner to other scientist wishing to quantitate EZM-G from human plasma either separately or simultaneously with the unconjugated EZM.

In chapter 4, section I, a LC-MS/MS method was developed and validated for the quantitative determination of total EZM from human plasma. The purpose of developing LC-MS/MS method for estimation of total EZM, was to check and compare, validity of HPLC results obtained, after the method is applied for the pharmacokinetic analysis of total EZM in healthy human subjects. During method development it was observed that, as the concentration of enzyme added to the reaction mixture, exceeds beyond certain concentration, the recovery obtained for
total EZM decreases. This could be possibly due to excess of enzyme leading to incomplete deconjugation (Gomes et al., 2009). LC-MS/MS method was found to be sensitive at 1.03 ng/mL, with a precision of 2.15% and accuracy of 97.09%. The calibration curve was linear in the concentration range of 1.02 to 303.0 ng/mL. No significant matrix effect or carryover was observed. The validated method was applied for the evaluation of comparative bioavailability of two investigational product of EZM, under fasting and fed state. When the developed method is compared with reported methods (Bae et al., 2012; Li et al., 2006; Oliveira et al., 2006), the current method was found to be economical, with comparable accuracy, sensitivity and precision.

The purpose of chapter 4, section II was to develop and validate a method for the quantitative determination of unconjugated EZM, so as to establish equilibrium between conjugated and total EZM. Unconjugated EZM was chromatographed on ACE C18 (50 × 4.6mm, 5µ) column with methanol: 2 mM ammonium acetate (75:25, v/v) as mobile phase. A simple liquid-liquid extraction procedure was employed for the extraction of unconjugated EZM from human plasma samples. The LLOQ was set at 51.0 pg/mL and calibration curve ranged from 51.0 to 20188.9 pg/mL. The method developed was simple, fast, precise and accurate as indicative from the validation results. Application of the method was demonstrated for studying pharmacokinetics of unconjugated EZM.

A bioequivalence study was designed and conducted to test the bioavailability of two test products of EZM. Since, USFDA has recommended two way crossover study for EZM (Guidance for Ezetimibe, USFDA, 2008), the study was an open label, balanced, analyst blind, randomized, two-treatment, two-period, two-sequence, single dose crossover bioequivalence study on 12 healthy, adult, human subjects under fasting and fed conditions. The findings of this chapter are as follows:

- **Total EZM by HPLC:** The plasma peak concentrations achieved was 47.86 ± 10.69 ng/mL, at a T_max of 1.93 ± 1.21 hours; which is in the agreement of the reported values elsewhere (Bae et al., 2012; Li et al 2006; Oliveira et al., 2006). The AUC₀₋₄ and AUC₀₋∞ achieved were 580.49 ± 117.36 ng.hrs/mL and 647.56 ± 161.28 ng.hrs/mL, respectively. Although, a limited sample size of six was used, the values obtained for pharmacokinetic parameters did not
differ the reported findings. The T_{1/2} obtained was 18.34 ± 4.51 hours; identical to the reported value of 22 hours (Zhu et al., 2000), indicating that the elimination rate was estimated accurately. Thus an alternative method has been developed for the estimation of total EZM using HPLC and can be routinely used for the pharmacokinetic study.

- **Total EZM by LC-MS/MS:** 90% confidence interval for the log-transformed pharmacokinetic parameters for C_{max}, AUC_{0-t} and AUC_{0-∞} was within 80 to 125%. The mean C_{max} under fasting state for A and B formulation was 96.65 ± 38.91 ng/mL and 87.37 ± 52.27 ng/mL; while in fed state it was 137.64 ± 50.08 ng/mL and 139.82 ± 76.92 ng/mL, respectively. The mean AUC_{0-t} under fasting state for A and B formulation was 1079.14 ± 603.42 ng.hr/mL and 873.89 ± 486.53 ng.hr/mL; while in fed state it was 882.94 ± 386.52 ng.hr/mL and 875.61 ± 354.35 ng.hr/mL, respectively. 90% CI in the fasting state was 97.09 to 122.67% for C_{max}, 104.52 to 115.55% for AUC_{0-t} and 106.89 to 117.48% for AUC_{0-∞}; while in the fed state, 90% CI was 92.58 to 112.24% for C_{max}, 93.24 to 105.68% for AUC_{0-t} and 91.95 to 105.12% for AUC_{0-∞}. The food increases the rate of absorption for total EZM.

- **Unconjugated EZM by LC-MS/MS:** The mean C_{max} under fasting state for A and B formulation was 4507.50 ± 1741.11 pg/mL and 4348.12 ± 2394.75 pg/mL; while in fed state it was 5608.85 ± 2831.84 pg/mL and 6007.58 ± 3957.83 pg/mL, respectively. The mean AUC_{0-t} under fasting state for A and B formulation was 97184.79 ± 35572.18 pg.hr/mL and 83719.26 ± 40676.75 pg.hr/mL; while in fed state it was 79582.56 ± 44748.42 pg.hr/mL and 75823.09 ± 34369.44 pg.hr/mL, respectively. 90% CI in the fasting state was 95.61 to 114.94% for C_{max}, 102.32 to 119.90% for AUC_{0-t} and 102.29 to 118.32% for AUC_{0-∞}; while in the fed state, 90% CI was 88.37 to 111.16% for C_{max}, 92.14 to 109.99% for AUC_{0-t} and 92.29 to 110.18% for AUC_{0-∞}. Thus, food has no effect on the pharmacokinetics of unconjugated EZM.

- **Conjugated EZM-G:** The plasma concentrations of EZM-G were obtained by subtracting unconjugated EZM concentrations from total EZM concentrations at individual time points. After the pharmacokinetic analysis, the mean C_{max} under fasting state for A and B formulation was 93.50 ± 37.60ng/mL and
85.08 ± 51.13 ng/mL; while in fed state it was 130.74 ± 52.13 ng/mL and 133.98 ± 75.01 ng/mL, respectively. The mean AUC\textsubscript{0-t} under fasting state for A and B formulation was 985.27 ± 567.59 ng.hr/mL and 786.17 ± 442.57 ng.hr/mL; while in fed state it was 806.03 ± 358.95 ng.hr/mL and 803.45 ± 343.02 ng.hr/mL, respectively. 90% CI in the fasting state was 96.95 to 122.16% for C\textsubscript{max}, 105.09 to 115.78% for AUC\textsubscript{0-t} and 106.92 to 117.48% for AUC\textsubscript{0-∞}; while in the fed state, 90% CI was 90.65 to 110.57% for C\textsubscript{max}, 93.23 to 105.45% for AUC\textsubscript{0-t} and 93.36 to 105.93% for AUC\textsubscript{0-∞}. Food increases the rate of absorption for conjugated EZM.

- The intra-subject variability observed for fasting and fed state, was found to be less than 30% for total, unconjugated and conjugated EZM, indicating that the drug is not a highly variable drug (WHO, 2001).

- Since the subjects used for HPLC analysis are different from those used in the LC-MS/MS analysis, the pharmacokinetic parameters obtained by both the methods differ from each other. Yet the pharmacokinetic parameters obtained by HPLC matched with those reported in the literature (Bae et al., 2012; Li et al., 2006; Oliviera et al., 2006). Thus, applicability of the HPLC method is checked and found to be suitable for the determination of total EZM from human plasma samples.

- The statistical results for test and reference formulation shows that test and reference formulation were comparable. None of the subjects under study showed any clinically significant adverse event during or after the study. The efficacy and safety of the test product is equivalent to that of the innovator product.

In future, if the direct measurement of EZM-G along with the unconjugated EZM is made possible, without the need to deconjugate the glucuronide, it will benefit the analyst with fast and thus economical bioanalysis of EZM.

Thus the work presented in this thesis has demonstrated

- Equivalence between two methods (HPLC and LC-MS/MS) for measurement of total EZM
- Applicability of HPLC and LC-MS/MS method for measurement of total EZM.

- Applicability of LC-MS/MS method for measurement of unconjugated EZM

- Estimation of conjugated EZM from results of total and unconjugated EZM

- Pharmacokinetic evaluation of the subject concentration data obtained for measurement of unconjugated EZM, total EZM and conjugated EZM.

Based on the pharmacokinetic evaluation and the subject concentration data in both fasting and fed studies, it can be concluded that the measurement of total EZM will be the best parameter for determining the bioequivalence of the two products. This is mainly because the results have shown that the food has effect on the rate of absorption of total EZM. But the same is not being observed for unconjugated EZM. Thus the variability of two formulations can be better determined, by measuring the total EZM levels to evaluate the bioequivalence.