Summary as per the previously proposed objectives of the thesis is as follows:


**Summary of Findings:**

Liver microsomes of male and female rat, rabbit and monkey were prepared through differential centrifugation technique. BCA (Bicinchoninic acid) method was used for the estimation of protein content of prepared microsomal fraction and their activity was checked through CYP3A4 specific standard substrate testosterone in terms of its %turnover. All prepared and purchased microsomes were found to have optimum activity to conduct metabolic stability studies. Before starting metabolic stability studies, a selective, sensitive, specific, accurate and precise HPLC-UV method was developed and validated in *in-vitro* reaction milieu. In addition preliminary studies as solubility and stability of S002-333 and isomers were conducted in potassium phosphate buffer pH-7.4. Compounds were soluble and stable at set *in-vitro* reaction conditions. In results, there was significant difference found between the male and female genders with respect to phase-I metabolic degradation of S002-333 and isomers S004-1032 and S007-1558. Therefore, proper consideration should be given to gender based safety and efficacy studies in future clinical trials. Based upon the *in-vitro*
intrinsic clearance values obtained for male genders of each species, most appropriate animal species was predicted as surrogate to humans in order to carry out further preclinical studies. Rabbit was found to be common surrogate species for all the three lead candidates. Moreover, the hepatic clearance values for male new zeland white (NZW) rabbits were calculated from scaled *in-vitro* intrinsic clearance values by using well stirred, parallel tube and dispersion models. The predicted *in-vivo* hepatic clearance values $\text{CL}_H(\text{pred})$, obtained were compared using two tailed unpaired t-test, with the observed hepatic clearance, $\text{CL}_H(\text{obs})$,obtained after single i.v injection of S002-333 to male NZW rabbits. There was no significant difference, found among the values. Similarly, for humans predicted hepatic clearance values were calculated based upon scaled *in-vitro* intrinsic clearance values of male human liver microsomes for all the three lead candidates. Moreover, the results suggest that for S002-333 and isomers, any one of the model can be used for the extrapolation of *in-vitro* intrinsic clearance to *in-vivo* hepatic clearance. This showed appropriateness of the method for prediction of *in-vivo* pharmacokinetic parameters from *in-vitro* experiments as well as categorize S002-333 and isomers as low clearance compounds in rabbits as well as in humans.
Objective: 2. In-vitro and in-vivo pharmacokinetic studies of S002-333.

Summary of Findings: The aim to perform these studies was to predict pharmacokinetic behavior of the NCE from the time it is administered orally and reach in the systemic circulation through various in-vitro and in-vivo experiments. In-vitro studies included simulated gastric and intestinal fluid stability studies (SGF/SIF), parallel artificial membrane permeability assay (PAMPA) and plasma protein binding studies while i.v, oral pharmacokinetics and bioavailability studies were in-vivo studies conducted in male rabbits. In SGF/SIF studies S002-333 was found to be unstable in gastric and intestinal fluids while both the enantiomers S004-1032 and S007-1558 separately were stable. In PAMPA permeability assay, permeability of the test compounds through gastrointestinal tract was conducted at two different pH (pH 4.0 and pH 7.0). The scientific logic was to cover the entire pH range of intestine. All the three lead candidates showed low permeability characteristic at both pH when compared with high and low permeability markers except S007-1558 at pH-4 where it was showing slightly high permeability, which means it has high gastric permeability and low intestinal permeability. US-FDA Guidance, concerning in-vivo bioavailability and bioequivalence studies for immediate release solid oral dosage forms states importance of these studies in defining BCS classification system. Therefore, determining drug substance permeability, the degree of drug
degradation in the gastrointestinal fluid prior to intestinal membrane permeation should also be considered. Documenting that drug loss from the gastrointestinal tract is due to intestinal membrane permeation, rather than a degradation process, assists in establishing the drug’s permeability classification and thus bioavailability of a new molecule. Further, plasma protein binding studies were performed through equilibrium dialysis method. All three lead candidates were found to be greater than 90% bound to plasma proteins. This information will be helpful in explaining bioavailability and distribution characteristics of the molecules. In addition, the effect of protein binding is more influential on drugs, which are highly protein bound as a slight change in protein binding characteristics of these drugs may drastically increase the free concentration in systemic circulation. Thus a clear understanding of plasma protein binding is essential for safe and proper use of drug. A bioanalytical method was developed and partially validated in terms of specificity, recovery, matrix effect, dilution integrity, linearity, inter and intraday accuracy and precision, in rabbit plasma to study pharmacokinetics, bioavailability and dose proportionality of S002-333 in rabbit plasma. Validation results showed that there is no significant matrix effect on analytes and selected IS. This method utilizes a short run time of 4.0 min for each sample analysis. Due to good sensitivity (LLOQ-2.02 ng/mL) of the assay, it offers a suitable platform for the determination of S002-333 in pre-
clinical studies. The volume of distribution ($V_d$) of S002-333 was found to be significantly higher than the total blood volume and the total water volume indicating higher tissue distribution. The CL of S002-333 was found to be 3.17 L/h/kg after i.v. administration which is smaller than mean hepatic blood flow, indicating that S002-333 is slowly metabolized by the liver and had low clearance property. The oral bioavailability of S002-333 was found to be 14.26% at 20 mg dose. This may be due to low permeability, low aqueous solubility, extensive first-pass metabolism and biliary elimination. Dose proportionality studies indicate non linear pharmacokinetics of S002-333 in rabbits. Evaluation of the pharmacokinetics of S002-333 will be useful in assessing concentration-effect relationships for the potential therapeutic applications of S002-333.

**Objective:** 3. *In-vitro* Cytochrome P450 phenotyping and metabolite profiling of S002-333 and isomers

**Summary of Findings:** The study has highlighted basic metabolic properties of S002-333 and its enantiomers with respect to involvement of various enzymes (CYP phenotyping), oxidative metabolites formed (metabolite identification) and the associated enzyme kinetics during phase-I metabolism. In CYP phenotyping studies, CYP2C19 was found to be the major contributor for S002-333 and S007-1558 while CYP3A4 showed greater involvement in S004-1032
Summary

metabolism. Apart from that CYP2C9, 2D6 and 2B6 were also involved in the metabolism of S002-333 and isomers. Four major phase-I metabolites of S002-333; M-1 and M-3 (oxidative), M-2 (O-demethylated) and M-4 (dehydrogenated) were characterized in human liver microsomes. These metabolites (sum of M-1 through M-4) constituted 11.2, 11.3 and 21.5% of the parent in comparison to the net phase-I metabolism of 29.9, 31.4 and 38.3% of S002-333, S004-1032 and S007-1558 respectively. Moreover P450 involvement in metabolite formation was studied using recombinant CYP2B6, 2C9, 2C19, 2D6 and 3A4 as well as P450 specific chemical inhibitors. Results showed that CYP3A4 was found to be responsible for M-1 and M-4 formation whereas M-2 was formed from multiple enzymes, CYP2B6, 2C9, 2C19 and 2D6 except CYP3A4. Metabolite M-3 was formed through all tested P450s except for S002-1558 where CYP2C9 was not found to be involved in M-3 formation.

However, microsomes does not fully represent phase-I machinery but are the best tool to describe the metabolic fate of the molecules in preclinical phase. The knowledge that CYP2C9, 2C19 and 3A4 play a significant role in the metabolism of all three lead candidates, will give an insight related to interaction potential with concomitantly administered drugs which are modulators or substrates of these CYP450s. The in-vitro data and in-vivo animal studies to address these potential interactions generate confidence in pursuing further
preclinical safety and toxicological evaluations in the drug development path and also constitute an important set of preclinical pharmacokinetic and metabolic data necessary for approval of IND application by regulatory authorities for clinical trials in human beings. Similarly, consideration to genetic polymorphism need to be given since major allelic variations related to CYP2C9 and 2C19 have been reported, which result into poor metabolizers with a potentially undesirable frequency of adverse events. Metabolites M-1, M-2 and M-3 follow classical hyperbolic michaelis-menten kinetics while M-4 undergoes auto activation. Enzyme kinetic parameters predicted from these models confer clearance characteristics of these metabolites. Further, testing of identified metabolites on suitable animal species will predict their role in undulating effectiveness or adverse effects. These studies also help in making conclusions for subsequent studies like inhibition and induction of the metabolizing enzymes.