Chapter 9
Summary
&
Conclusion
Chapter 9: Summary and Conclusion

The overall study was initiated in order to investigate the formation kinetics of the metabolites from parent drug in vitro and to predict the specific enzymes involved in its metabolic pathway and possible metabolism based Drug/Drug and Drug/Food interactions as well as to develop a cocktail probe substrate assay system for inhibition screening of multiple CYP isoforms by NME in vitro using human liver microsomes. All these methods and their results are summarized in the following section.

- **In vitro oxidative biotransformation of GLM as a model substrate for CYP450** conclusively demonstrates the use of a $3^3$ factorial design in the optimization of initial velocity conditions affecting turnover of GLM. P450 reaction phenotyping is defined as a set of experiments that aim to define which human cytochrome P450 enzyme(s) is involved in a given metabolic transformation. Such data are useful in the prediction of pharmacokinetic drug-drug interactions and interpatient variability in drug exposure. Any prolonged incubation in a closed in vitro system such as liver microsomes can cause formation of secondary metabolites from the primary metabolites of a drug. Inactivation or denaturation of enzymes can become significant over time in the in vitro systems. Thus it is of critical importance that initial velocity conditions are defined.

- This study examines the effects of the main control factors and attempts to enhance the turnover rate of GLM’s oxidative biotransformation by optimizing these factors using full factorial design. The derived reduced polynomial equation, contour plot and response surface plot aid in predicting the values of selected independent variables. Contour plots obtained by applying a computerized optimization process suggested a level of 30 minute incubation time ($X_2$) and 0.5mg/ml protein ($X_3$) as an ideal condition. At this level the turnover rate ($\%Y$) was found to be ranging from 18.91% to 19.91%. Thus the rate of GLM disappearance was linear at the chosen concentrations of substrate using the assay conditions and detection system. However, a decrease in the level of incubation time and protein concentration below the selected level, typically yield nonlinear initial velocities of enzyme activity. Once the optimal conditions (30 min incubation time, 0.5mg/ml HLM) were obtained, the substrate concentration...
of metabolite formation was examined. The $K_m$ ($28.9 \pm (0.559 \pm 0.017 \ \mu\text{Mole/min/mg protein})$ values were
determined by nonlinear regression of a plot of enzyme activity versus substrate
concentration. The Michaelis constant, $K_m$ accounts for the concentration of
substrate at which half the active sites are filled. Thus, $K_m$ provides a measure of
the substrate concentration required for significant catalysis to occur. $V_{max}$ is the
rate at which substrate will be converted to product once bound to the enzyme. A
substrate concentration around or below the $K_m$ is ideal for determination of
competitive inhibitor activity. The $\text{Cl}_{\text{int}}$ value as predicted after in vitro studies
was found to be 0.019 $\mu\text{l/min/mg}$ suggesting a direct measure of enzyme activity
towards glimepiride. Hence further inhibition studies are needed to confirm the
performance of GLM’s oxidative biotransformation in vitro.

- It was possible to optimize the turnover of the candidate drugs within the limits of
developed assay design such that all subsequent in vitro incubations can be
performed using the condition that ensures linearity with time and HLM
concentration, and less than 20% of the initial substrate is consumed. Thus the
precise information about the effects of each factor on metabolism can be used to
flexibly adjust the system performance. The best estimates of $K_m$ and $V_{max}$ values
were obtained with linear (Michaelis Menten plot) as well as nonlinear
transformation (Lineweaver Burk plot) for the enzymatic assay of GLM under
initial velocity conditions. The low $K_m$ value of GLM (28.9 $\mu\text{Mole}$) as compared
to literature value of tolbutamide (50 $\mu\text{Mole}$) for CYP2C9 suggest that enzyme
has a high affinity for the substrate GLM. Thus GLM can be used as a alternative
probe substrate for CYP2C9 reaction phenotyping of new molecular entities.

- The study “In vitro Evaluation of the Pharmacokinetic Alterations Caused by
SMZ on GLM Hydroxylation” evaluated the inhibitory effects of sulfonamides on
GLM metabolism mediated by CYP2C9. With concentrations ranging from 30 to
1100 $\mu\text{Mole}$, SMZ exhibited a selective inhibitory effect on CYP2C9-mediated
GLM-hydroxylation with an apparent $IC_{50}$ value of 400 $\mu\text{Mole}$ and $K_i$ value of
290 $\mu\text{Mole}$. The pattern of inhibition was found to be competitive as $K_m$ value was
increased (32.26 ± 4.31 \text{µMole}) and \( V_{\text{max}} \) (0.526 ± 0.031) almost remain unaffected as predicted by Michaelis Menten plot and Lineweaver Burk plot. Also the \( K_i \) value obtained by Dixon plot to the left of the ordinate (-290 \text{µMole}) suggests competitive inhibition. \textit{IVIVC} findings suggest that AUC of GLM was increased around or more than 1.5 fold by SMZ. This predicted increase in plasma concentration of GLM is high, suggesting the risk of hypoglycemia when SMZ is coadministered with GLM.

- Caution must be exercised as sulfamethoxazole can potentiate the hypoglycemic effect of glimepiride when given in combination as is predicted by the \textit{in vitro} \textit{IVIVC} study. Hence coadministration of sulfamethoxazole with glimepiride (to avoid hypoglycemic attack) and CYP2C9 substrates with narrow therapeutic ranges such as phenytoin (an antiepileptic) and warfarin (an anticoagulant) should be monitored. The study also demonstrated that GLM and SMZ can be used as a probe substrate and selective inhibitor of CYP2C9 respectively, which can provide a reliable \textit{in vitro} approach for kinetic studies.

- This interaction study predicts that coadministration of sulfamethoxazole with glimepiride and CYP2C9 substrates with narrow therapeutic ranges such as phenytoin (an antiepileptic) and warfarin (an anticoagulant) should be monitored closely as sulfonamides can potentiate the hypoglycemic effect of sulfonylurea agents when given in combination.

- The study \textit{“In vitro assessment of PIJ and POJ on CYP2C9 mediated GLM metabolism in vitro”} investigated that pineapple as well as pomegranate juice affected the CYP2C9 activity \textit{in vitro} which suggests the possible interaction of juices with substrates of CYP2C9 in humans. At concentrations 0.5\% v/v the percentage inhibition was 61.26\% and at 1.5\% v/v it was 22.42\% for pineapple juice. Similarly for pomegranate juice, at concentrations 0.5\% v/v the percentage inhibition was 77.05\% and at 1.5\% v/v it was 53.98\%. Pineapple juice was found to be a potent inhibitor of human CYP2C9 as compared to pomegranate juice. In human liver microsomes, the mean 50\% inhibitory concentrations (IC\(_{50}\)) for PIJ and POJ versus CYP (glimepiride hydroxylation) were 1.50 ± 0.233 \text{µl} and 4.25 ±
Chapter 9: Summary and Conclusion

Thus, POJ does not significantly alter metabolism of GLM to PIJ which suggests its beneficial effects in subjects with type 2 diabetes. From the comparative study or results of Km and Vmax for GLM alone (27.98 ± 2.77 µM, 0.564 ± 0.015 µM/min/mg protein), Km, Vmax and IC₅₀ for GLM in presence of PIJ (47.50 ± 10.99 µM, 0.492 ± 0.038 µM/min/mg protein, 1.50 ± 0.23 µl (0.75% v/v) ) Km, Vmax and GLM in presence of POJ (34.00 ± 4.96 µM, 0.50 ± 0.021 µM/min/mg protein, 4.25 ± 0.53µl (2.12% v/v)), it was observed that PIJ exerts significant competitive inhibitory effect than POJ on GLM metabolism.

Our results supports the surprising finding by Aviram that the sugars contained in POM juice although similar in content to those found in other fruit juices did not worsen diabetes disease parameters in patients but in fact reduced the risk for atherosclerosis. This is because in most juices, sugars are present in free and harmful forms but in POJ juice however the sugars are attached to unique antioxidants, which make these sugars protective against diabetes and atherosclerosis.

This study demonstrated that the metabolism of GLM was altered by PIJ and POJ. Addition of 10 µl (5% v/v) of pineapple juice resulted in almost complete inhibition. Amongst the fruits evaluated, PIJ showed strong inhibition towards CYP2C9 activity while POJ appears to make minor contributions to the oxidative metabolism of GLIM. One of the ways to control diabetes mellitus is through the diet and it is here that pomegranate juice can play a part. The low inhibitory potential of pomegranate towards GLM in vitro metabolism suggests beneficial effects in subjects with type 2 diabetes. Pomegranate juice may be considered as a healthy fruit juice and awaits additional clinical research to further strengthen for its unique antidiabetic effect.

Pomegranate juice may be considered as a healthy fruit juice and awaits additional clinical research to further strengthen for its unique antidiabetic effect. Although our in vitro evidence in favor of using pomegranate juice for diabetics is very promising, extensive studies are required to fully understand its possible contribution to human health before recommending its regular consumption. In addition, the effects of fruit juices on pharmacokinetics of drugs in vitro may not
be consistent with those in humans. Therefore further investigations in humans are necessary to elaborate our findings.

- The *in vitro* drug fruit interaction study predicts that pineapple juice is more inhibitory in nature as compared to pomegranate juice. Hence coadministration of juices should be closely monitored in diabetic patients.

- The study “Simultaneous method development of cocktail substrate assay system for EFV (CYP2B6), DIC (CYP2C9), CHZ (CYPE1), ATV (CYP3A4)” describes HPLC-PDA method development and its validation in the presence of HLM. The probes CHZ/ATV/DIC/EFV to be used in this cocktail approach were chosen based on their CYP specificity, availability and recommendations in regulatory guidance. The probe substrates selected were EFV (CYP2B6), DIC (CYP2C9), CHZ (CYPE1), ATV (CYP3A4). All the substrates were soluble in common solvent methanol and stable during the analysis i.e., no additional interacting peaks of probe substrate were observed in chromatograms after cocktail incubation. All the peaks of probe substrate 4.08 min for CHZ, 9.76 min for ATV, 10.89 min for DIC, 15.58 min for EFV and their respective metabolite M₁ at 2.58 min for CHZ, M₂ at 4.8 min for DIC, M₃ at 7.9 min for ATV, M₄ at 8.6 min for EFV were well resolved. Glimepiride (40 mcg/ml) was selected as the internal standard of choice as it was stable during the analysis, readily available, was well resolved from CHZ, ATV, DIC, EFV, its peak shape was good (tailing factor at 230 nm 1.25, tailing factor at 247 nm 1.20), and its elution time (12.58 min) was shorter than that of last eluting analyte peak, EFV (15.58 min) saving run time per sample. The method showed a linear calibration curve with correlation coefficients greater than 0.999 for the analytes in the investigated concentration range and absolute recoveries of all analytes were >90%. The acceptable intraday and interday precision were <15% relative standard deviation from nominal values. The lower limit of detection (LLOD) was 1.10 μM for CHZ, 0.92 μM for ATV, 0.88 μM for DIC, and 0.54 μM for EFA, respectively. The lower limit of quantitation (LLOQ) was 1.98 μM for CHZ, 1.85 μM for ATV, 1.28 μM for DIC, and 1.25 μM for EFA, respectively. The enzyme reactions for assessment of CYP2B6, CYP2C9, CYP2E1 and CYP3A4 activities were linear.
Chapter 9: Summary and Conclusion

The developed isocratic LC/UV method has been shown to provide sufficient sensitivity and linear concentration range for the analysis of probe substrate and its metabolites with good resolution from in vitro individual incubations as well as cocktail incubations. Overall, the simultaneous development of cocktail substrate assay system for efavirenz (CYP2B6), diclofenac (CYP2C9), chlorzoxazone (CYPE1), atorvastatin (CYP3A4) is simple, uses conventional instrumentation and provides a scope to analyse all cytochrome P450 combination sets continuously in a single run.

The developed method can be used to improve throughput and cost-effectiveness in preclinical drug studies. Hence these in vitro findings can be extrapolated to carry out P450 probe substrate inhibition assays to determine whether an NME inhibits a particular P450 enzyme activity.

The study “Evaluation of cocktail substrate assay system for inhibition screening of CYP2B6, CYP2C9, CYP2E1 & CYP3A4 by MCR-706 and MCR-742,” describes that inhibition reactions were evaluated via two approaches i) individual dosing of a substrate (CHZ, ATV, DIC, EFV) and of an inhibitor (KET, FLX, CLP, MCR-706, MCR-742). ii) cassette dosing of substrates (CHZ,ATV,DIC,EFV) combined with individual dosing of inhibitor (KET,FLX,CLP,MCR-706,MCR-742).The method was validated by incubating known CYP inhibitors (clopidogrel, CYP2B6; fluoxetine, CYP2C9 and ketoconazole, CYP3A4; with the individual substrate they were known to inhibit (EFV; DIC; and ATV respectively) and with the substrate cocktail. Both the approaches generated similar IC\(_{50}\) values for each CYP isozyme and all measured IC\(_{50}\) values were compared with the literature values. CLP, FLX, KET at 1, 50 and 1 \(\mu\)M caused 48.28, 38.92 and 41.76% inhibition of EFV, DIC, and ATV hydroxylation respectively in individual incubation while in cocktail incubation it showed 49.49, 46.385 and 45.106% inhibition respectively. The IC\(_{50}\) values of
I determined with the individual substrates were in good agreement with the IC50 values of 1.58, 41.8 and 0.90 μM using the substrate cocktail of EFV/DIC/ATV. The IC50 values determined using the individual substrates agreed with the values determined using substrate cocktail. Exception to this agreement with published IC50 values of 0.046, 33, and 0.72 μM is observed for CLP/FLX/KET in this study. This could be due to the use of different substrates or expressed enzyme versus human liver microsomes. The Ki values were also estimated using obtained IC50 values of CLP, FLX, and KET when co incubated with their respective substrate at fixed concentration (at fixed or below its Km values) in individual and cocktail incubation.

- A HPLC-PDA method has been developed for the inhibition screening of the four major human CYP enzymes (CYP2B6, CYP2C9, CYP3A4, CYP2E1) using an in vitro individual substrate and substrate cocktail. The IC50 values of selective CYP inhibitors (ketoconazole, CYP3A4; fluoxetine, CYP2C9; clopidogrel, CYP2B6) and two new molecular entities (MCR-706 and MCR 742) determined using the substrate cocktail were in good agreement with individual substrates. The developed assay offers a reliable and sensitive screening method for the prediction of the P450 inhibitory potential of new molecular entities using individual and cocktail substrate incubation approach.

- The developed method has the potential to be used for the characterization of P450 enzyme activity in human liver microsomal preparations. In addition, a P450 inhibition profile using this screening method can allow a number of new molecular entities to be screened rapidly for P450 inhibitory potential, which can help in selection of potential drug candidates, and can guide the quantitative prediction of clinical drug interactions.