CYTOCHROME P450 2C8 REACTION PHENOTYPING: SUBSTRATE SELECTION AND INHIBITION PROFILE

By

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To the sweet memories of my little heroes, Jayesh and Anay.

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β-NADP:	β-nicotinamide adenine dinucleotide phosphate
ADR:	Adverse drug reaction
AQ:	Amodiaquine
$AUC_{0-\infty}$:	Area under the plasma concentration time curve
CYP:	Cytochrome P450
DEAQ:	*N*-desethylamodiaquine
DME:	Drug metabolizing enzyme
DMSO:	Dimethyl sulfoxide
ESI:	Electrospray ionization
FDA:	Food and Drug Administration
FWHM:	Full width at half maximum
HILIC:	Hydrophilic interaction chromatography
HPLC:	High pressure liquid chromatography
IC_{50}:	Concentration of the inhibitor that reduces the maximum initial velocity to 50%
LC/MS/MS:	Liquid chromatography tandem mass spectrometry
LOQ:	Limit of quantification
M-IV	Hydroxypioglitazone
MRM:	Multiple reaction monitoring
NCE:	New chemical entity
NIDDM:	Non insulin dependent diabetes mellitus
PHLM:	Pooled human liver microsomes
PIO:	Pioglitazone
PPAR:	Peroxisome proliferator activated receptor

QC:	Quality control
RSD:	Relative standard deviation
SRM:	Single reaction monitoring
$T_{1/2}$	Terminal elimination half-life
UV:	Ultraviolet
WHO:	World Health Organization

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About 20% of the adverse drug reactions are related to drug-drug interactions caused by inhibition or induction of a drug metabolizing enzyme determining the elimination of a drug by another concomitantly administered drug. These drug-drug interactions could be predicted by identifying the major drug metabolizing enzymes responsible for the metabolism of a new chemical entity.

The reaction phenotyping of Cytochrome P450 2C8 (CYP2C8) did not gain enough attention due to the lack of an enzyme specific probe substrate and inhibitor. Paclitaxel is used as a conventional probe substrate of CYP2C8 *in vitro*. Recently, amodiaquine (AQ) is reported to be a high affinity and turnover probe substrate of CYP2C8. However, it is not ideal for clinical drug metabolism studies due to its toxicity and very long elimination half-life of its CYP2C8 specific metabolite, N-desethylamodiaquine (DEAQ). Pioglitazone (PIO) hydroxylation to form M-IV is also a CYP2C8 specific reaction.

We recognized a need for development of a LC/MS/MS based analytical method for determination of DEAQ and M-IV. The present work involves the development and validation of analytical methods for quantification of these two CYP2C8 specific metabolites. Further, we

successfully applied these methods to *in vitro* drug metabolism studies using pooled human liver microsomes. The potential of M-IV formation as a CYP2C8 specific probe reaction was evaluated by studying the inhibition of montelukast, ketoconazole, terfenadine, β-estradiol and midazolam and comparing their IC_{50} values to those obtained by using other CYP2C8 specific probe reactions.

CHAPTER 1
INTRODUCTION

Cytochrome P450 Reaction Phenotyping

Drug-drug interactions pose a significant health concern causing approximately 20% of adverse drug reactions (ADRs) (Backmann et al., 2003). Clearly, the probability of ADRs associated with drug-drug interactions increases as the number of concomitantly administered drugs increase (Bachmann et al., 2003). Inhibition of one or more drug metabolizing enzymes (DMEs) could result in an increased exposure of the parent drug and therefore, an increased chance of observing drug related toxicity. On the other hand, induction of a DME might result in subtherapeutic response leading to failure of drug therapy (Venkatakrishnan et al., 2003; Frye, 2004). In order to avoid any unforeseen interactions in the clinic, the Food and Drug Administration (FDA) requires pharmaceutical companies to identify drug metabolizing enzymes involved in the elimination of a new chemical entity (NCE) as well as the effect of its coadministration on the pharmacokinetics of marker substrates of DMEs that are responsible for its elimination (http://www.fda.gov/cder/guidance). Common experimental methods to identify the drug metabolizing enzymes responsible for the metabolism of the NCE are as follows:

(1) Chemical inhibitors: In order to determine the contribution of a particular DME, the metabolism of the NCE is studied in presence of an enzyme selective chemical inhibitor. In this case, selectivity could be defined by two factors, the mechanism of inhibition and the relative affinity of both the inhibitor and the test substrate for the enzyme. The limiting factor in the use of chemical inhibitors for *in vitro* studies has historically been the lack of adequate selectivity of inhibition among cytochrome P450 (CYP) enzymes.

(2) Expressed CYP enzymes: The ability of a panel of expressed CYP enzymes to metabolize a specific NCE reduces reaction phenotyping to the simplest system of only one enzyme and a

substrate. Although these studies give important qualitative information, the importance of each CYP enzyme in presence of other enzymatic pathways is difficult to quantify thus providing incomplete information about overall metabolic fate of the NCE.

(3) Antibodies: The use of anti-CYP antibodies as biological inhibitors of enzyme activity allows the direct assessment of the role of specific CYP to the metabolism of the NCE in an enzyme mixture like pooled human liver microsomes (pHLM). A primary limitation of anti-CYP antibodies for reaction phenotyping is their cross-reactivity with related CYPs. Additionally, the inhibition of reactions known to be highly specific to a particular CYP isoform is rarely 100% and a small fraction of the enzyme retains its function.

(4) Correlation analysis: It involves the comparison of the inter-liver variability in the rate of formation of a specific drug metabolite with the measured activities towards CYP marker substrates. This approach warrants prior establishment of enzyme kinetic parameters, a thorough investigation of the correlation between the different CYP enzymes and the use of considerable number of liver samples with wide range of enzyme activities (Wienkers and Stevens, 2003).

The potential of a NCE to inhibit the metabolism of currently available drugs could be determined by studying the metabolism of an enzyme specific probe substrate in the presence of the NCE. The identification of substrates that are selectively metabolized *in vitro* by specific CYPs allows for the investigation of the inhibition by the NCE. The results from *in vitro* inhibition studies, yielding IC_{50} or K_i values allows for the prediction of the potential for the NCE to inhibit specific CYPs *in vivo*. Information thus obtained is used by the clinician to design *in vivo* interaction studies that are relevant to the NCE (Bachmann et al., 2003).

The Centrality of Cytochrome P450 Enzymes in Drug Metabolism

Cytochrome P450 constitutes a superfamily of drug metabolizing enzymes that contributes to the Phase I metabolism of approximately three-forths of all drugs (Wilkinson, 2001). From the

drug metabolism point of view, the most important CYPs in humans are CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4/5. The CYP2C subfamily consists of four members namely, CYP2C8, CYP2C9, CYP2C18 and CYP2C19 and is the second most abundant CYP subfamily after CYP3A, representing about 20% of the total hepatic P450 (Montellano, Handbook of Drug Metabolism, 1999). Collectively, the CYP2C subfamily is responsible for the metabolism of about 20% of clinically prescribed drugs. Clinical importance of CYP2C9 and CYP2C19 is well known and therefore, these two isoforms are extensively studied and always included in the battery of enzymes used in reaction phenotyping studies during the development of a new chemical entity. However, until recently, CYP2C8 was often neglected due to the limited knowledge of its importance as well as the lack of CYP2C8 specific probe substrate and inhibitor.

Cytochrome P450 2C8

CYP2C8 is a major human hepatic P450, constituting about 7% of total microsomal CYP content in the liver. It is responsible of at least 5% of drugs cleared by Phase I metabolism (Rendic and Di Carlo, 1997). Although it is primarily expressed in the liver, CYP2C8 protein is also found in kidney, intestine, adrenal glands, mammary glands, ovary, heart, aorta as well as in breast cancer tumors (Klose et al., 1999; Thum and Borlak, 2000; Nishimura et al., 2003; Knupfer et al., 2004). Ferguson et al. found that the pregnane X receptor , constitutive androstane receptor and glucocorticoid receptor are involved in the regulation of *CYP2C8* gene expression (Ferguson et al., 2005).

In humans, the *CYP2C8* gene is located on chromosome 10q24, spanning 31 kilobases and consisting of nine exons. Single nucleotide polymorphisms have been identified in exons 3, 5 and 8 (Klose et al., 1999). The most common varient alleles are *CYP2C8*2* and *CYP2C8*3*. The protein product CYP2C8.2 contains Ile269Phe substitution and is expressed in African American

population with an allle frequency of 18%. *CYP2C8*3* has two amino acid susbstitutions, Arg139Lys and Lys399Arg. It is most commonly found in Caucasians with an allele frequency of 13% and rarely expressed in African Americans (2%) (Bahadur et al., 2002). Interestingly, *CYP2C8*3* is in incomplete genetic equilibrium with *CYP2C9*2*.

CYP2C8 plays an important role in the metabolism of various drugs including paclitaxel (Dai et al., 2001; Bahadur et al., 2002), amodiaquine (AQ) (Li et al., 2002), troglitazone (Yamazaki et al., 1999), rosiglitazone (Baldwin et al., 1999), pioglitazone (PIO) (Deng et al., 2005), repaglinide (Deng et al., 2005), cerivastatin (Backman et al., 2002), amiodarone (Soyama et al., 2002) and verapamil (Busse et al., 1995). It also contributes to the metabolism of various endogenous substances like *all-trans* retinoic acid (McSorley and Daly, 2000) and arachidonic acid (Dai et al., 2001). Common substrates and inhibitors used in *in vitro* drug metabolism experiments are listed in Table 1-1.

Table 1-1.Substrates and inhibitors of CYP2C8 for in vitro experiments.

Substrates	Inhibitors
Paclitaxel	Gemfibrozil
Torsemide	Trimethoprim
Cerivastatin	Montelukast
Repaglinide	Quercetin
Amodiaquine	Thiazolidinediones
Pioglitazone	

Paclitaxel: A Conventional Probe of CYP2C8 Activity

Paclitaxel, an antineoplastic agent, was originally isolated from the stem bark of the western yew, *Taxus brevifolia*. It is a potent inhibitor of cell replication, blocking the cells in the late G2 mitotic phase of the cell cycle, presumably by stabilizing the microtubule cytoskeleton. As a therapeutic antineoplastic agent, paclitaxel is used in patients with breast, lung, esophageal, head and neck, and advanced platinum-refractory ovarian carcinomas (Walle et al., 1995). In

humans, paclitaxel is primarily metabolized by CYP2C8 to 6-α-hydroxypaclitaxel and to lesser extent by CYP3A4 to form 3'-p-hydroxypaclitaxel. Further, both metabolites are hydroxylated to lead to dihydroxypaclitaxel. Paclitaxel and its metabolites are secreted in the bile and feces. The ratio of 6-α-hydroxypaclitaxel to 3'-p-hydroxypaclitaxel is 6:1 in bile (Cresteil et al., 1994). Similarly, in pHLM, the concentration of 6-α-hydroxypaclitaxel was about two-fold that of 3'-p-hydroxypaclitaxel suggesting the major role of CYP2C8 in the elimination of paclitaxel (Taniguchi et al., 2005). Therefore, formation of 6-α-hydroxypaclitaxel is widely used as a marker substrate of CYP2C8 activity in reaction phenotyping studies. Dai et al. found that CYP2C8.2 exhibits two-fold higher K_m for paclitaxel 6-hydroxylation (31 μM) compared to that of CYP2C8.1 (15 μM) whereas CYP2C8.3 had a turnover number of 15% that of CYP2C8.1 for paclitaxel 6-α-hydroxylation (Dai et al., 2001). Other research groups indicated a moderate decrease (31 to 84 %) in the enzyme activity by presence of *CYP2C8*3* allele (Bahadur et al., 2002; Soyama et al., 2002).

Amodiaquine: A High Affinity and Turnover Probe Substrate of CYP2C8

Amodiaquine , a 4-aminoquinolone antimalarial drug, is clinically effective against certain chloroquine resistant strains of *Plasmodium falciparum*. However, it is no longer recommended for prophylactic antimalarial therapy due to the high risk of agranulocytosis and hepatitis caused by the reactive quinine-imine metabolite. Although the global use of AQ has declined due to the intrinsic toxicity, it is still being used as a first-line drug in the treatment of uncomplicated falciparum malaria, especially in African countries. In 2005, World Health Organization (WHO) recommended the use of AQ in combination with artemisinin-based antimalarial therapy due to higher clinical efficacy of the combination, revitalizing the interest in the use of AQ in treating malaria.

In vivo pharmacokinetic studies have shown that the primary route of systemic elimination in humans is via the extensive first-pass metabolism to *N*-desethylamodiaquine (DEAQ). Li et al. characterized the metabolism of AQ in various expressed CYP enzymes and pHLM. They found that AQ is almost exclusively metabolized by CYP2C8 to produce DEAQ (Li et al., 2002). Therefore, AQ clearance and its metabolism to DEAQ could be used as a measure of CYP2C8 enzyme activity for *in vitro* reaction phenotyping studies. Recently, Walsky et al. examined 209 frequently prescribed drugs and related xenobiotics for inhibition of CYP2C8 using amodiaquine N-desethylation as a CYP2C8 specific marker reaction. Forty-eight compounds exhibiting greater than 50% inhibition were further evaluated for determination of IC_{50} using expressed CYP2C8. In pHLM, the leukotriene receptor antagonist, montelukast was found to be the most potent inhibitor of CYP2C8 with an IC_{50} of 19 nM (Walsky et al., 2005). In a recent report, Parikh et al. found that the presence of *CYP2C8*2* allele increased the K_m of AQ metabolism by three-fold and decreased intrinsic clearance by six-fold. They also reported a marked decrease in the AQ metabolism with the presence of *CYP2C8*3* allele (Parikh et al., 2007).

Various analytical methods are described for determination of DEAQ (Trenholme et al., 1974; Pussard et al., 1985; Mount et al., 1986; Pussard et al., 1987; Winstanley et al., 1987; Laurent et al., 1993; Li et al., 2002; Minzi et al., 2003; Dua et al., 2004; Gitau et al., 2004; Walsky and Obach, 2004; Bell et al., 2007; Dixit et al., 2007; O'Donnell et al., 2007). Earlier methods have long run times with high mobile phase flow rates and lack the sensitivity required for *in vitro* drug metabolism assays. Most of the recent methods use highly aqueous mobile phases in order to improve the retention of the polar DEAQ on the column, which is not ideal for detection by mass spectrometry.

Pioglitazone as Probe Substrate of CYP2C8

Pioglitazone, a thiazolidinedione, is used in the treatment of non-insulin dependent diabetes mellitus (NIDDM) as monotherapy or in combination with other hypoglycemic agents (e.g., sulfonylureas, metformin and insulin). Like other thiazolidinediones (e.g., troglitazone and rosiglitazone), PIO mediates its hypoglycemic effects by activation of peroxisome proliferator activated receptor (PPAR) γ, thereby enhancing the sensitivity of insulin responsive tissues rather than increasing release of insulin from islet β-cells. In clinical studies, PIO is shown to produce antihyperglycemic effects by increasing insulin stimulated glucose uptake in peripheral tissues as well as the ability of insulin to suppress endogenous glucose production in the liver. It was also shown to decrease plasma levels of insulin, thus reducing the risk of hypoglycemia. From the toxicity point of view, PIO has a much better safely profile as compared to troglitazone and rosiglitazone. Additionally, PIO is found to exert hypolipidemic effects by reducing the serum concentrations of free fatty acids (Mizushige et al., 2002).

After oral administration (30 mg once daily), PIO undergoes minimal first pass metabolism in the gut and is almost completely absorbed with an absolute bioavailability of 83%. Although it is distributed to peripheral tissues, plasma concentrations are always higher than tissues concentrations indicating a small volume of distribution due to high plasma protein binding (>97%). In humans, PIO undergoes extensive metabolism in the liver to form various hydroxylated and oxidized metabolites (M-I to M-VII). Oxidative cleavage of aliphatic C-O bond leads to formation of M-I. M-II and hydroxypioglitazone (M-IV) are formed by the hydroxylation of aliphatic methylene group whereas terminal ethyl group is hydroxylated to give M-VII and is oxidized to form M-V. Oxidation of the hydroxyl group in M-IV to a ketone generates M-III (Yki-Jarvinen, 2004).

In vitro studies in pHLM have shown that PIO undergoes extensive metabolism in the liver primarily by CYP2C8, with minor contribution from CYP3A4. The metabolites M-II, M-III and M-IV are pharmacologically active possessing about 40-60% anti-hyperglycemic potency as compared to PIO. In humans, M-III and M-IV are found to be the major metabolites with considerably longer terminal half lives ($t_{1/2}$, 26-28 hours) than that of PIO (mean $t_{½}$ 5.8 hours), presumably contributing to the extended pharmacological activity allowing once-daily administration of PIO. In another study, a potent and highly selective CYP2C8 inhibitor, montelukast (1 μM), significantly inhibited depletion of PIO (IC_{50} = 0.51 μM) and more strongly inhibited formation of M-IV (IC_{50} = 0.18 μM), clearly indicating the major role of CYP2C8 in the formation of M-IV (Jaakkola et al., 2006). In a clinical drug interaction study, gemfibrozil alone raised the mean area under the plasma concentration-time curve ($AUC_{0-\infty}$) of PIO 3.2-fold and prolonged its $t_{1/2}$ from 8.3 to 22.7 hours. Additionally, it also decreased the AUC_{0-48} of M-III and M-IV by 42% and 45%, respectively. However, itraconazole, a potent CYP3A4 inhibitor, did not have any significant effect on the pharmacokinetics of PIO or either of its metabolites suggesting a minor role of CYP3A4 in the formation of its major plasma metabolite M-IV (Jaakkola et al., 2005). In a recent report, Tornio et al., found that trimethoprim, a known inhibitor of CYP2C8 increased the $AUC_{0-\infty}$ of PIO by 42% and reduced the apparent formation rate of M-IV by 27%, validating the major contribution of CYP2C8 in the formation of M-IV (Tornio et al., 2007). From the above mentioned *in vitro* and *in vivo* drug interaction studies it is evident that M-IV is almost exclusively formed by CYP2C8. Therefore, formation of M-IV could be employed as a marker reaction for the quantification of CYP2C8 activity for *in vitro* reaction phenotyping studies as well as clinical drug interaction studies. The proposed structures of PIO metabolites in pHLM are depicted in Figure 1-1. Tornio et al. also investigated the effect

of carrying the *CYP2C8*3* allele on the pharmacokinetics of PIO. The weight-adjusted $AUC_{0-\infty}$ of PIO was 34% lower in the subjects with the *CYP2C8*3/*3* genotype and 26% lower in case of subjects carrying *CYP2C8*1/*3* genotype (Tornio et al., 2007).

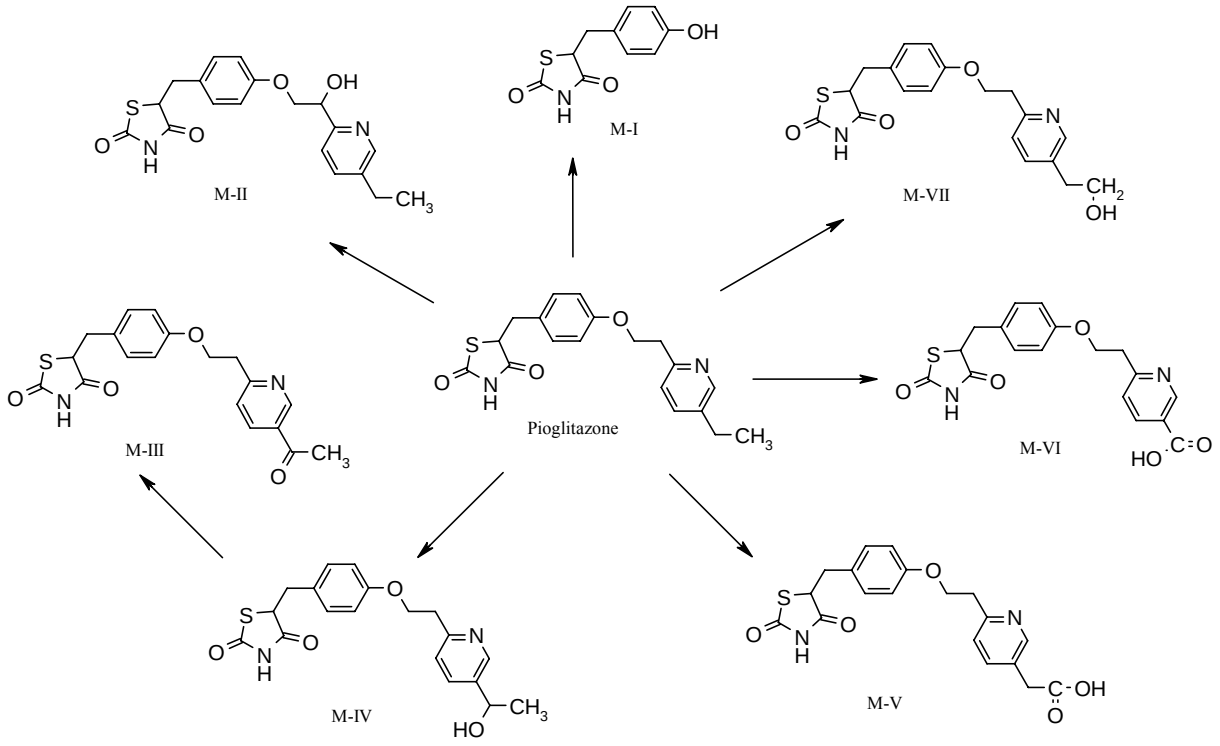

Figure 1-1.Proposed structures of pioglitazone metabolites in pHLM.

Several analytical methods are available for the quantification of M-IV in various biological fluids (e.g., plasma and urine) (Kiyota et al., 1997; Lin et al., 2003; Deng et al., 2005; Jaakkola et al., 2005; Tornio et al., 2007) and subcellular fractions (e.g., recombinant CYP enzymes and pHLM) (Shen et al., 2003; Baughman et al., 2005; Jaakkola et al., 2006; Tornio et al., 2007). Most of these methods involve slow mobile phase gradients with long run times as they were developed for characterization of all of the metabolites. Many of the recent methods

are either not sensitive enough for the detection of metabolites or assign arbitrary units to M-IV due to the lack of commercially available metabolite standard at the time of development.

The Scope of Present Work

Considering the limitations of the current analytical methods for determination of DEAQ and potential application of DEAQ formation as a CYP2C8 specific reaction for *in vitro* reaction phenotyping studies, there is a need for development of a simple, sensitive and robust mass spectrometric method that could be easily applied to drug metabolism studies. Therefore, the current work involves development and validation of a liquid chromatography tandem mass spectrometry (LC/MS/MS) based method for determination of DEAQ and its application to drug metabolism studies using pHLM.

Despite the number of drug interaction studies involving PIO, until recently, very little was known about the affinity of PIO towards CYP2C8. Therefore, one of the aims of the present work is to characterize the formation of M-IV with respect to Michaelis-Menten kinetics in pHLM. In order to facilitate the detection of M-IV, we developed and validated a LC/MS/MS based method that can be applied to drug metabolism studies. Additionally, the focus of the current work was to evaluate the potential of PIO as a CYP2C8 specific substrate by comparing the IC_{50} values of montelukast, ketoconazole, β-estradiol, midazolam and terfenadine with those obtained using AQ.

CHAPTER 2
DETERMINATION OF N-DESETHYLAMODIAQUINE BY LC/MS/MS: APPLICATION TO IN VITRO DRUG METABOLISM STUDIES

Introduction

Amodiaquine, a 4-aminoquinolone antimalarial drug, is clinically effective against certain chloroquine resistant strains of *Plasmodium falciparum*. Although the global use of AQ has declined due to the the high risk of agranulocytosis and hepatitis caused by the reactive quinine-imine metabolite (Jewell et al., 1995), it is still being used as a first-line drug in the treatment of uncomplicated falciparum malaria, especially in African countries (Basco et al., 2002; Cavaco et al., 2005; Hombhanje et al., 2005; Rower et al., 2005). After oral administration, AQ undergoes rapid and extensive metabolism in the liver to form the pharmacologically active metabolite DEAQ, which is primarily responsible for the antimalarial effects (Winstanley et al., 1987). In humans, desethylation of AQ is the major pathway of elimination with other minor metabolites being 2-hydroxyl DEAQ and N-bisdesethylAQ (Pussard et al., 1985a; Pussard et al., 1987; Laurent et al., 1993; Jewell et al., 1995). Studies in pHLM and recombinant enzymes show that AQ desethylation is almost exclusively catalyzed by Cytochrome P450 2C8 (Li et al., 2002). Therefore, AQ is used as an enzyme-selective probe substrate to quantify CYP2C8 enzyme activity *in vitro* (Li et al., 2002).

Several analytical methods are available for quantification of DEAQ in various biological fluids (e.g., blood, plasma and urine) and subcellular fractions (e.g., pHLM) (Trenholme et al., 1974; Pussard et al., 1985b; Mount et al., 1986; Pussard et al., 1987; Winstanley et al., 1987; Laurent et al., 1993; Li et al., 2002; Minzi et al., 2003; Dua et al., 2004; Gitau et al., 2004; Walsky and Obach, 2004; Bell et al., 2007). Early reverse phase chromatographic methods suffered from poor retention of AQ and DEAQ, long run times and high mobile phase flow rates (Pussard et al., 1985b; Gitau et al., 2004). Analytical methods based on UV detection did not

permit the accuracy and sensitivity required for the quantification of the analytes due to endogenous interferences from the biological matrices as a result of poor baseline resolution between AQ and its metabolites. Additionally, all of these methods involved tedious multiple extraction steps and large volumes of organic solvents (Pussard et al., 1987; Winstanley et al., 1987; Laurent et al., 1993; Minzi et al., 2003; Dua et al., 2004; Gitau et al., 2004; Bell et al., 2007). Higher sensitivity was achieved by Trenholme and coworkers through conversion of AQ to a fluorescent product by refluxing it with borate buffer. Although this normal phase chromatographic method improved sensitivity and retention, it was found to be non-specific because the concentration of AQ was confounded by its metabolites (Trenholme et al., 1974). Mount and coworkers developed the most sensitive method for assaying DEAQ in human blood and urine (LOQ 1 ng/ml) by employing electrochemical detection (Mount et al., 1986). However, it involved lengthy extraction steps and consumed high amounts of organic solvents making it unsuitable for analysis of large number of samples. Considering the prospects of the use of amodiaquine as a CYP2C8 probe substrate in drug metabolism studies, high throughput LC/MS/MS based methods were developed for analysis of DEAQ (Li et al., 2002; Walsky and Obach, 2004; Turpeinen et al., 2005; Walsky et al., 2005; Dixit et al., 2007; O'Donnell et al., 2007). All of these methods use simple processing methods and are sensitive enough for determination of DEAQ concentration in *in vitro* assays as well as clinical studies. However, DEAQ was separated on reverse phase columns resulting in the use of highly aqueous mobile phase gradients to prolong retention of DEAQ, which is not ideal for mass spectrometric detection. Additionally, they involved separation of DEAQ by gradient elution with long run times. Thus, a simple, sensitive and robust mass spectrometric method that could be easily applied to drug metabolism studies is needed.

The purpose of the present work was to develop a LC/MS/MS method using hydrophilic interaction chromatography (HILIC) that involved minimal sample preparation. HILIC chromatography yielded excellent separation of AQ from DEAQ by prolonging DEAQ retention time while using high proportions of organic solvent in the mobile phase. The method was used to determine enzyme kinetic parameters for DEAQ formation in pHLM.

Experimental

Chemicals and Reagents

Amodiaquine, β-nicotinamide adenine dinucleotide phosphate (β–NADP), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, magnesium chloride and ammonium acetate were purchased from Sigma (St. Louis, MO, USA). The DEAQ metabolite standard and deuterated internal standard, DEAQ-d3, were obtained from BD-Gentest (San Jose, CA, USA). Potassium phosphate, sodium citrate and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Glucose 6-phosphate dehydrogenase solution was prepared by dissolving lyophilized enzyme in 5 mM sodium citrate to produce 40 U/ml solution and was stored at -20°C until use. All chemicals used in the study were of analytical grade. HPLC grade acetonitrile was obtained from EMD Chemicals (Gibbstown, NJ, USA). Deionized water was prepared by using a Barnstead Nanopure Diamond UV Ultrapure Water System (Dubuque, IA, USA). Pooled HLM were purchased from BD Biosciences (San Jose, CA, USA).

Preparation of DEAQ Standards and Quality Control Samples

Two sets of stock solutions were prepared in acetonitrile and water (50:50 v/v) at concentrations of 0.1 and 0.5 mM. One set of stock solutions was used to spike standards and the other set was used to spike quality control (QC) samples. Standards were prepared by spiking phosphate buffer (50 mM, pH 7.4) at seven concentrations ranging from 10 nM to 1500 nM. For validation, QC samples were prepared by spiking phosphate buffer (50 mM, pH 7.4) at three

concentration levels (50, 500 and 1200 nM). The standards and QC samples were stored at -20°C until analysis. The internal standard solution was prepared by dissolving DEAQ-d3 in acetonitrile to produce a final concentration of 200 nM and stored at 4 °C.

Sample Preparation

The internal standard solution in acetonitrile (200 nM, 1400 µl) was added to DEAQ standard or QC sample (250 µl). After shaking for 2 minutes on a vortex shaker, samples were centrifuged at 20,817 *g* for 8 minutes. An aliquot of clear supernatant was transferred to a 96 well plate and 10 µl was injected on the column. All samples were protected from light exposure during processing in order to avoid photodecomposition (Motten et al., 1999).

LC/MS/MS Conditions

The LC system was comprised of a ThermoFinnigan Surveyor HPLC autosampler and ThermoFinnigan Surveyor MS quaternary pump. Chromatographic separation was achieved on a BETASIL Silica-100 (50 x 2.1 mm, 5µ, ThermoElectron Corporation) analytical column. Isocratic elution was performed at a flow rate of 220 µl/min for 4.7 minutes using a mobile phase consisting of 5 mM ammonium acetate and 0.1% (v/v) formic acid in water and 5 mM ammonium acetate and 0.1% (v/v) formic acid in acetonitrile (15:85 v/v). The autosampler was maintained at 10°C and 10 µl of sample was injected on the column. The mobile phase flow was diverted from the mass spectrometer to waste for the first 1.5 minutes of run time to remove nonvolatile salts. After each injection, the needle was washed and flushed with 1000 µl of solution containing: acetonitrile:2-propanol:water (35:35:30 v/v) and 0.1% (v/v) formic acid.

The mass spectrometer was a TSQ Quantum Discovery triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) source. The mass spectrometer was calibrated with a solution of polytyrosine-1, 3, 6 per manufacturer's instructions. The operating

conditions were optimized by infusing DEAQ in the mobile phase in order to maximize the detector signal. ESI source was operated in positive mode and was set orthogonal to the ion transfer capillary tube.

For quantification, the TSQ quantum was operated in multiple reaction monitoring (MRM) mode and the precursor-product ion pair was 328 → 283 *m/z* for DEAQ and 331 → 283 *m/z* for DEAQ-d3. The acquisition parameters were: spray voltage 4.0 kV, source CID -10 V, heated capillary temperature 325°C and capillary offset 35V. Nitrogen was used as a sheath and auxiliary gas set to 35 and 10 (arbitrary units), respectively. The argon collision gas pressure was set to 1.5 mTorr. The collision energy was 24 eV for the analyte as well as the internal standard. The peak full width at half maximum (FWHM) was set at 0.2 Th and 0.7 Th for Q1 and Q3, respectively. Scan width was fixed to 0.1 Th for both SRM channels and scan time was set to 250 ms.

Calibration curves were constructed by linear regression of the peak area ratio of analyte to that of the internal standard (Y-axis) and the nominal standard concentration (X-axis) with a weighting factor of $1/y^2$. Concentrations of QCs and incubation samples were calculated by using the regression equation of the calibration curve. Chromatographic peaks were quantified by using Xcalibur™ software (version 1.4) and the peak area ratio of DEAQ to DEAQ-d3 was plotted against the nominal DEAQ concentrations.

Validation

The newly developed analytical method was validated with respect to selectivity, carry over, linearity, precision, accuracy and autosampler stability.

For selectivity, samples of blank incubation matrix were analyzed to check the lack of interference in the quantification of DEAQ. Carry-over was evaluated by placing vials of blank mobile phase at several locations in the analysis set.

Standards at all concentrations were analyzed in duplicate except the LOQ, which was run in triplicate. To assess linearity, the maximum allowable deviation of the back calculated concentration was set at 15% for all standards and at 20% for the LOQ.

The accuracy and precision of the assay was determined by the analysis of QC samples of DEAQ at concentrations of 50.0, 500.0 and 1200.0 nM. Six of each QC sample were analyzed on the same day to determine intra-day precision and accuracy, and on three different occasions to assess inter-day precision and accuracy.

Reanalysis of standards and QC samples tested the stability of the analyses. The stability of the samples in the autosampler was tested after the samples were left in the autosampler for up to 36 hours by reanalyzing the standards and QC samples. Stability was defined as less than 10% deviation in concentration from that on the day samples were processed.

Incubation Conditions

Preliminary experiments were conducted to optimize the microsomal protein concentration (0.01-0.2 mg/ml) and incubation time (5-20 minutes) in order to assure the linearity of DEAQ formation . Amodiaquine and pHLM (0.1 mg/ml) were mixed with phosphate buffer (50 mM, pH 7.4) and warmed at 37°C for five minutes. Incubations were commenced by addition of the NADPH regenerating system, which consisted of $MgCl_2$ (assay concentration, 3.3 mM), NADP+ (1.25 mM), glucose 6-phosphate (3.3 mM) and glucose 6-phosphate dehydrogenase (0.32 U/ml) in 5 mM sodium citrate solution. Final incubation volume was 250 µl. After incubating for 10 minutes at 37°C, the reaction was terminated by addition of 1400 µl of ice-cold acetonitrile containing DEAQ-d3 (0.28 nmol). Samples were processed as described above.

Enzyme kinetic parameters were obtained by performing incubations at nine different concentrations of AQ ranging from 0.5 µM to 80 µM. AQ was dissolved in acetonitrile and water (50:50 v/v, final acetonitrile concentration of 0.4% v/v). Microsomes were stored at -80°C

and thawed immediately before use. Polypropylene microcentrifuge tubes were used to store AQ stocks as well as to conduct the microsomal incubations. All incubations were performed in duplicate and were protected from light to avoid photodecomposition of AQ and the metabolite.

Data Analysis

Enzyme kinetic parameters were obtained by nonlinear regression using GraphPad Prism (San Diego, CA, USA). Data were typically fit to the following Michaelis-Menten equation:

$$V = \frac{V_{max} \times S}{K_m + S} \qquad \text{Equation 2-1}$$

in which V is the initial velocity, V_{max} is the maximal velocity, S is the substrate concentration and K_m is the substrate concentration at half-maximal velocity.

Results and Discussion

Method Development

In order to improve the retention of DEAQ and to avoid the use of highly aqueous mobile phases, we selected BETASIL Silica-100 (50 x 2.1 mm, 5 μ) column, which separates analytes based on the principles of HILIC. It elutes analytes by passing a hydrophobic or mostly organic mobile phase across a neutral hydrophilic stationary phase causing solutes to elute in order of increasing hydrophilicity resulting in better separation of highly polar compounds. Alternative to reverse phase chromatography, HILIC worked best for separation of DEAQ (retention time, 2.9 min) from AQ (retention time, 1.2 min) while still allowing use of 85% acetonitrile in the mobile phase.

Ammonium acetate buffer was used to volatilize the mobile phase and aid ionization of DEAQ. Addition of 0.1% (v/v) formic acid in the mobile phase also enhanced ionization and improved the peak shape of DEAQ. ESI was chosen as the mode of ionization because it gave high signal intensity for DEAQ. A full scan mass spectrum of DEAQ was obtained in the

positive and negative mode. The most abundant parent ion of DEAQ (328 *m/z*) was obtained in the negative mode, which was selected for SRM scanning. Further, the fragmentation pattern of the precursor ion was obtained and a highly specific ion pair (328 → 283 *m/z*) was selected based on the intensities of three most abundant product ions. Thus, for quantification purpose, TSQ quantum was operated in MRM mode and the precursor-product ion pair of 328 → 283 *m/z* and 331 → 283 *m/z* was followed for DEAQ and DEAQ-d3, respectively. In order to minimize the sample preparation time, a one step protein precipitation method was utilized by addition of a solution of internal standard in acetonitrile followed by a short mixing and centrifugation step. Considering the simplicity of sample processing, the present method could potentially be applied to a high throughput drug metabolism assay.

Method Validation

Validation of the assay method was conducted according to the FDA guidelines with respect to selectivity, carry over, linearity, precision, accuracy and autosampler stability (http://www.fda.gov/cder/guidance). For validation purposes, QC samples at low, medium and high concentrations were prepared independently and six of each QC sample was analyzed on three occasions.

Selectivity, carry over and matrix effect

To determine the selectivity of the method, blank microsomal incubation samples were used to investigate the potential interferences due to the endogenous compounds in the matrix. A clear baseline was observed without any significant interference at the retention times of DEAQ and DEAQ-d3. Representative chromatograms of (A) blank buffer and buffer spiked with DEAQ at LOQ (10 nM) and (B) blank incubation sample and a sample after 10 minute incubation of AQ at 0.5 μM are depicted in Figure 2-1. No carry over was observed in any of the blank samples. The potential of ion suppression or enhancement due to the matrix components was evaluated for

DEAQ and DEAQ-d3. No significant matrix effect was observed in microsomal incubations, as the use of a deuterated internal standard compensated for any variation in matrix effect.

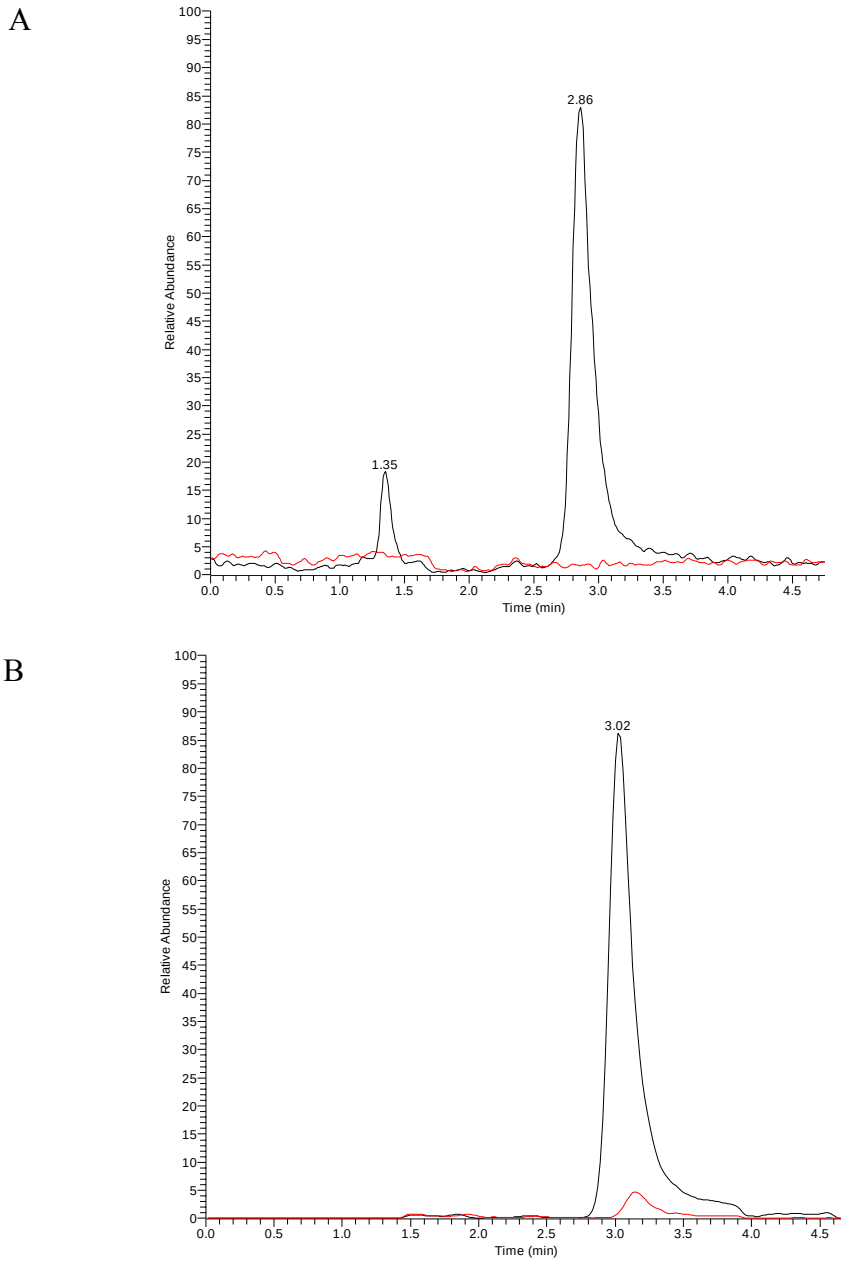

Figure 2-1. Representative chromatograms of (A) blank incubation buffer (red) and buffer spiked with DEAQ at LOQ (black) and (B) incubation sample: blank (red) and after 10 min incubation of AQ at 0.5 μM (black) (m/z 328 → 283, overlay offset = 0%).

Linearity

Calibration curve demonstrated good linearity for the entire concentration range with a mean correlation coefficient (R^2) of 0.9969 ± 0.0012.

Precision and accuracy

Precision was represented as the relative standard deviation (%RSD) whereas accuracy was calculated as the percent deviation (% bias) from the respective nominal concentration. The maximum acceptable limit for precision and accuracy was set at 15%. The intra-day and inter-day precision and accuracy were within 7.9% and 4.3%, respectively, for all standards and QC samples (Table 2-1). Thus, the present method was found to be highly reproducible and demonstrated a high degree of accuracy.

Table 2-1.Intraday (n=6) and Interday (n=18) precision (%RSD) and accuracy (% deviation) for analysis of DEAQ in 50 mM phosphate buffer, pH 7.4.

Concentration (nM)			
Nominal	Back calculated (mean ± SD)	% RSD	% Deviation
Intraday			
50.00	51.70 ± 0.7	1.4	3.4
500.0	492.8 ± 9.1	1.8	-1.4
1200	1181 ± 28	2.4	-1.5
Interday			
50.00	51.20 ± 3.6	7.0	2.5
500.0	487.9 ± 13	2.6	-2.4
1200	1209 ± 83	6.9	0.8

Autosampler stability

As the autosampler was maintained at 10°C during the run, stability of analytes at 10°C was determined by reanalyzing the same standards and QC samples after 36 hours. Both DEAQ and DEAQ-d3 were found to be stable at 10°C for at least 36 hours.

Metabolism of AQ in Pooled Human Liver Microsomes

AQ was incubated with pHLM at nine different concentrations (0.5 -80 μM). The linearity of AQ metabolism with respect to the microsomal protein content was studied at five protein concentrations (0.01-0.2 mg/ml). In order to avoid non-specific protein binding, the lowest protein concentration that produced quantifiable metabolite (0.1 mg/ml) was selected. Formation of DEAQ was linear up to 20 minutes. Considering the photosensitivity of AQ, samples were protected from light. Following the incubation of AQ in pHLM, DEAQ was detected by using the validated method. The rate of formation of DEAQ was measured as the index of CYP2C8 enzyme activity (Li et al., 2002; Walsky and Obach, 2004; Turpeinen et al., 2005; Walsky et al., 2005; Dixit et al., 2007; O'Donnell et al., 2007). When modeled by using GraphPad Prism (version 4), the formation of DEAQ exhibited typical Michaelis-Menten kinetics (Figure 2-2) with the maximal rate of formation of DEAQ (V_{max}) of 2060 ± 94 pmol/min/mg protein and the concentration of AQ at half maximal velocity (K_m) was 5.78 ± 0.94. In a recent study, Li et al., characterized the disappearance of AQ as well as the formation of DEAQ in various expressed CYP enzymes and pHLM. They reported AQ to be a high affinity and turnover probe substrate of CYP2C8. Our results are in agreement with the findings of the study conducted by Li et al recommending the use of DEAQ formation as a CYP2C8 specific probe reaction (Table 2-2) (Li et al., 2002; Li et al., 2003; Walsky and Obach, 2004). Thus, the present method was successfully applied to *in vitro* drug metabolism studies.

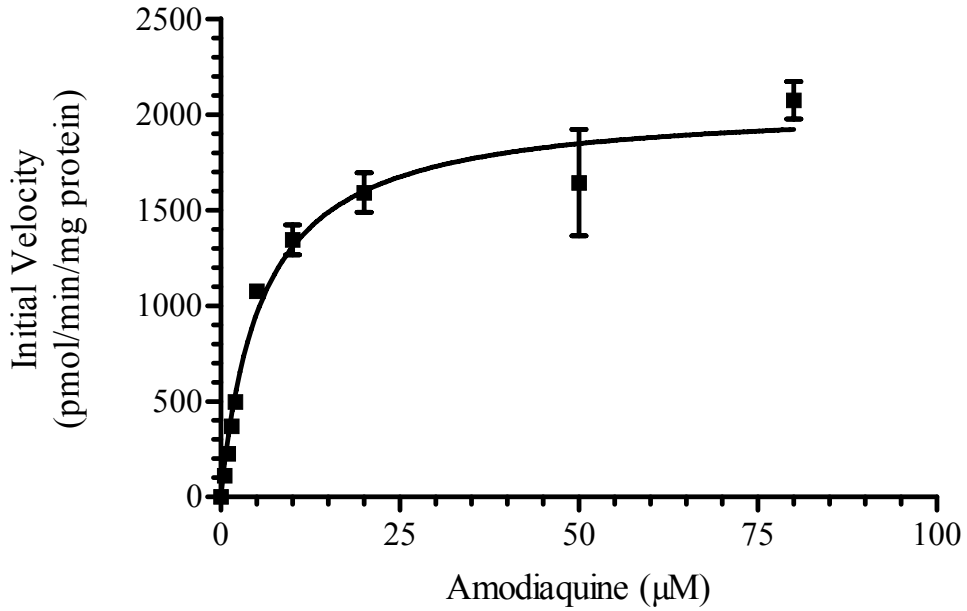

Figure 2-2. Plot of initial velocity versus amodiaquine concentration for the formation of desethylamodiaquine in pHLM (n=2).

Table 2-2. Enzyme kinetic parameters of AQ in pHLM.

	Km ± SE (μM)	Vmax (pmol/min/mg protein)
In house	5.8 ± 0.94	2060 ± 94
Walsky and Obach, 2004	1.9 ± 0.06	1480 ± 20
Li et al., 2003	3.4	1696
Li et al., 2002	2.4	1462

Conclusion

Application of HILIC technique to separate DEAQ resulted in a simple and robust LC/MS/MS based method. The method was validated with respect to selectivity, carry over, matrix effects, linearity, precision, accuracy and autosampler stability. Enzyme kinetic

parameters obtained by incubating AQ with pHLM in presence of NADPH regenerating system were in accordance with the available literature. Therefore, the present method could be applied for future CYP2C8 drug metabolism studies.

CHAPTER 3
DETERMINATION OF HYDROXYPIOGLITAZONE (M-IV) BY LC/MS/MS: APPLICATION TO IN VITRO DRUG METABOLISM STUDIES

Introduction

Cytochrome P450 2C8 plays an important role in the metabolism of various drugs including paclitaxel (Dai et al., 2001; Bahadur et al., 2002), amodiaquine (Li et al., 2002), troglitazone (Yamazaki et al., 1999), rosiglitazone (Baldwin et al., 1999), pioglitazone (Deng et al., 2005), repaglinide (Bidstrup et al., 2003; Kajosaari et al., 2005), cerivastatin (Backman et al., 2002), amiodarone (Soyama et al., 2002), verapamil (Busse et al., 1995), and endogenous substances like *all-trans* retinoic acid (McSorley and Daly, 2000) and arachidonic acid (Dai et al., 2001). Therefore, it is important to know whether a new drug candidate is a substrate or an inhibitor of CYP2C8. Considering the contribution of CYP2C8 in the metabolism of various drugs and the impact of inhibition of CYP2C8 on the pharmacokinetics of its substrates, it has been added to the panel of CYP enzymes for reaction phenotyping studies. Until recently, there was a lack of information about CYP2C8 specific reactions that could be used to quantify the activity of this enzyme.

In vivo pharmacokinetic studies as well as *in vitro* experiments with expressed CYP2C8 and pHLM have shown that AQ is almost exclusively metabolized to DEAQ by CYP2C8. Therefore, amodiaquine desethylation is used as a CYP2C8 specific marker reaction to quantify CYP2C8 activity *in vitro* (Li et al., 2002). However, AQ cannot be used as in *in vivo* probe of CYP2C8 as it was withdrawn from the US market due to its intrinsic toxicity. Thus, there is a need to identify a CYP2C8 specific probe substrate that could be used for *in vitro* as well as *in vivo* reaction phenotyping studies.

Pioglitazone, a thiazolidinedione antidiabetic agent, is widely used in the treatment of non-insulin dependent diabetes mellitus either as monotherapy or in combination with other

hypoglycemic agents (e.g., metformin, sulfonylurea or insulin). Like other Peroxisome Proliferator Activated Receptor γ (PPARγ) agonists, pioglitazone mediates its antidiabetic effects by increasing insulin stimulated glucose uptake in peripheral tissues as well as the ability of insulin to suppress endogenous glucose production in the liver (Yki-Jarvinen, 2004). *In vitro* studies in human liver microsomes have shown that PIO (Figure 3-1) undergoes extensive metabolism in the liver primarily by CYP2C8 and CYP3A4 to five primary metabolites (M-I, M-II, M-IV, M-VI and M-VII). Further, M-IV (Figure 3-1) is oxidized to a ketone to form M-III, whereas oxidation of M-V leads to formation of M-VI (Shen et al., 2003; Baughman et al., 2005; Jaakkola et al., 2006). In humans, M-III and M-IV are found to be the major metabolites with about 40-60% hypoglycemic potency thus, contributing significantly to the pharmacological activity of PIO.

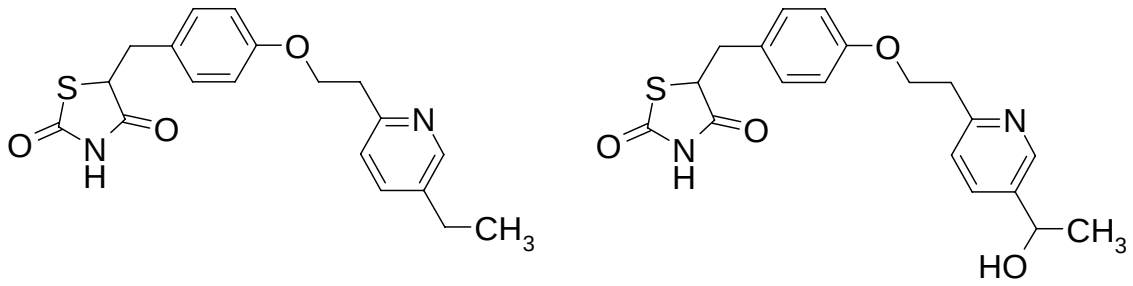

Figure 3-1.Chemical structures of (A) pioglitazone and (B) hydroxypioglitazone (M-IV)

Studies with various recombinant CYP isoforms as well as pHLM have shown that montelukast, a potent and highly selective CYP2C8 inhibitor, significantly inhibited depletion of PIO (IC_{50} = 0.51 μM) and more strongly inhibited formation of M-IV (IC_{50} = 0.18 μM) indicating the major role of CYP2C8 in the formation of M-IV (Jaakkola et al., 2006). In human pharmacokinetics study, gemfibrozil alone raised the mean area under the plasma concentration-time curve ($AUC_{0-\infty}$) of PIO 3.2-fold and prolonged its elimination half-life ($t_{1/2}$) from 8.3 to 22.7

hours. It also decreased the AUC_{0-48} of M-III and M-IV by 42% and 45%, respectively. The effect of gemfibrozil on the pharmacokinetics of PIO and its metabolites due to CYP2C8 inhibition could be attributed to the acylglucoronide of gemfibrozil. In the same study, a potent CYP3A4 inhibitor itraconazole did not have any significant effect on the pharmacokinetics of PIO or either of its metabolites indicating a minor role of CYP3A4 in the metabolism of PIO and formation of M-IV (Jaakkola et al., 2005). In a recent study, trimethoprim, a known inhibitor of CYP2C8, was found to increase $AUC_{0-\infty}$ of PIO by 42% and reduced the apparent formation rate of M-IV by 27% validating the major role of CYP2C8 in the formation of M-IV (Tornio et al., 2007). From above mentioned *in vitro* and *in vivo* drug interaction studies it is evident that CYP2C8 plays a major role in the formation of M-IV. Therefore, formation of M-IV could be employed as a marker reaction for quantification of CYP2C8 activity in reaction phenotyping studies.

Several analytical methods are available for the quantification of M-IV in various biological fluids (e.g., plasma and urine) (Kiyota et al., 1997; Lin et al., 2003; Deng et al., 2005; Jaakkola et al., 2005; Tornio et al., 2007) and subcellular fractions (e.g., recombinant CYP enzymes and liver microsomes) (Shen et al., 2003; Baughman et al., 2005; Jaakkola et al., 2006; Tornio et al., 2007). Most of the LC/MS/MS based methods were developed with the purpose of identification and characterization of various metabolites of PIO and therefore, involve slow mobile phase gradients with long run times. Many of these methods assigned arbitrary units to M-IV due to the lack of metabolite standards at the time of development. More recent LC/MS/MS based methods used in drug interaction studies in humans involve sample processing by solid phase extraction and suffer from the disadvantage of very long run times. Analytical methods developed by Lin et al and Deng et al separate PIO and its metabolites by isocratic

elution and quantify M-IV based on metabolite standards (Lin et al., 2003; Deng et al., 2005). Although both of these methods could be successfully employed for quantification of M-IV (LOQ of M-IV 0.5 and 1.1 ng/ml, respectively) in pharmacokinetic studies in humans, they are not sensitive enough to be used for *in vitro* enzyme assays and inhibition studies. Thus, the purpose of this study was to develop a rapid, sensitive and robust method for determination of M-IV that could be easily applied to *in vitro* drug metabolism studies.

Experimental

Chemicals and Reagents

β-Nicotinamide adenine dinucleotide phosphate (β–NADP), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, magnesium chloride and ammonium acetate were procured from Sigma (St. Louis, MO, USA). Pioglitazone hydrochloride, M-IV metabolite standard and the stable labeled internal standard, M-IV-d4, were obtained from Torronto Research Chemicals Inc. (North York, Ontario, Canada). Glucose 6-phosphate dehydrogenase solution was prepared by dissolving lyophilized enzyme in 5 mM sodium citrate to produce 40 U/ml solution and was stored at -20°C until use. Potassium phosphate was purchased from Fisher Scientific (Fair Lawn, NJ, USA) and formic acid was obtained from Mallinckrodt baker Inc. (Phillipsburg, NJ, USA). HPLC grade acetonitrile and methanol were purchased from EMD Chemicals (Gibbstown, NJ, USA). All other chemicals used in the study were of analytical grade. Deionized water was obtained from a Barnstead Nanopure Diamond UV Ultrapure Water System (Dubuque, IA, USA). Pooled HLM were obtained from *In Vitro* technologies (Baltimore, MD, USA).

Preparation of M-IV Standards and Quality Control samples

Two sets of stock solutions were prepared in methanol at concentrations of 0.1 and 1.0 μg/ml. One set of stock solutions was used to spike standards and the other set was used to spike QC samples. Standards were prepared by spiking phosphate buffer (50 mM, pH 7.4) at seven

concentrations ranging from 0.1 to 20 ng/ml. For validation, QC samples were prepared by spiking phosphate buffer (50 mM, pH 7.4) at three concentration levels (spiked at 0.5, 2.0 and 10 ng/ml). The standards and QC samples were stored at -20°C until analysis. The internal standard solution was prepared by dissolving M-IV-d4 in methanol to produce a final concentration of 12 ng/ml and was stored at -20°C.

Sample Preparation

Acetonitrile (750 µl) was added to 250 µl of standard solution, QC or incubation sample followed by the addition of the internal standard solution in methanol (50 µl; 12 ng/ml). Samples were processed by liquid-liquid extraction using dichloromethane (2 ml) as the extraction solvent. After shaking for 10 minutes on a horizontal shaker at low speed, samples were centrifuged at 3200 *g* for 10 minutes. The top aqueous layer was aspirated and the organic layer was transferred to glass tubes and dried under a stream of nitrogen at 50°C. The obtained residue was redissolved in methanol: water (50:50, 150 µl) and an aliquot was transferred to autosampler vials.

LC/MS/MS Conditions

The LC system was comprised of a ThermoFinnigan Surveyor HPLC autosampler and ThermoFinnigan Surveyor MS quaternary pump. Chromatographic separation was achieved on a SYNERGI MAX-RP 80A (150 x 2.00 mm, 4µ, Phenomenex) analytical column. Gradient elution was performed at a flow rate of 220 µl/min using the following mobile phase system: A = 5 mM ammonium acetate and 0.1 % (v/v) formic acid and B = 5 mM ammonium acetate and 0.1 % (v/v) formic acid in methanol. The column was started at 40:60 of A:B and at 0.5 min, the mobile phase composition was changed to 90% of B over 0.5 minutes and held for 1.2 min (2.2 min total) before returning to the starting conditions, which was held for 2.3 minutes (total run time 5.0 minutes). The autosampler was maintained at 10°C and 20 µl of sample was injected on

the column. The mobile phase flow was diverted from the mass spectrometer to waste for the first 1.0 min of run time to remove nonvolatile salts. After each injection, the needle was washed and flushed with 500 µl of solution containing: acetonitrile:2-propanol:water (35:35:30 v/v) and 0.1% (v/v) formic acid.

The mass spectrometer was a TSQ Quantum Discovery triple quadrupole mass spectrometer equipped with an ESI source. It was calibrated with a solution of polytyrosine-1, 3, 6 per manufacturer's instructions. The operating conditions were optimized by infusing M-IV in the mobile phase in order to maximize the detector signal. ESI source was operated in the positive mode and was set orthogonal to the ion transfer capillary tube.

For quantification, the TSQ quantum was operated in the MRM mode and the precursor-product ion pair was 373 → 150 *m/z* for M-IV and 377 → 154 *m/z* for M-IV-d4. The acquisition parameters were: spray voltage 4.0 kV, source CID -3 V, heated capillary temperature 325°C and capillary offset 35 V. Nitrogen was used as a sheath and auxiliary gas set to 35 and 10 (arbitrary units), respectively. The argon collision gas pressure was set to 1.5 mTorr. The collision energy was 35 eV for the analyte as well as the internal standard. The peak FWHM was set at 0.2 Th and 0.7 Th for Q1 and Q3, respectively. Scan width was fixed to 1.0 Th for both SRM channels and scan time was set to 250 ms.

Chromatographic peaks were quantified using Xcalibur™ software (version 1.4) and calibration curves were constructed by linear regression of the peak area ratio of M-IV to that of M-IV-d4 (Y-axis) and the nominal standard concentration (X-axis) with a weighting factor of $1/y^2$. The concentration in QCs and incubation samples was calculated by using the regression equation of the calibration curve.

Validation

The newly developed analytical method was validated with respect to selectivity, carry over, linearity, precision, accuracy and autosampler stability. For selectivity, samples of blank incubation matrix were analyzed to check for lack of interference at the retention time of M-IV and M-IV-d4. Carry-over was evaluated by placing vials of methanol at several locations in the analysis set.

Calibration curves were constructed by plotting the ratio of the peak area of M-IV to that of M-IV-d4 against the nominal M-IV concentration. Standards at all concentrations were analyzed in duplicate except the LOQ, which was run in triplicate. To assess linearity, the maximum allowable deviation of the back calculated concentration was set at 15% for all standards and at 20% for LOQ.

The accuracy and precision of the assay was determined by the analysis of QC samples of M-IV at concentrations of 0.5, 2.0 and 10 ng/ml. Twelve of each QC sample were analyzed on the same day to determine intra-day precision and accuracy, and six of each QC sample on two different occasions to assess inter-day precision and accuracy.

The stability of the samples in the autosampler was tested after the samples were left in the autosampler for up to 48 hours by reanalyzing the standards and QC samples. Stability was defined as less than 10% deviation in concentration from that on the day samples were processed.

Incubation Conditions

Preliminary experiments were conducted to optimize the microsomal protein concentration (0.05-0.4 mg/ml) and incubation time (5-20 minutes) in order to assure the linearity of M-IV formation. Pioglitazone and pHLM (0.05 mg/ml) were mixed with phosphate buffer (50 mM, pH 7.4) and warmed at 37°C for five minutes. Incubations were commenced by addition of the

NADPH regenerating system, which consisted of $MgCl_2$ (assay concentration, 3.3 mM), NADP+ (1.25 mM), glucose 6-phosphate (3.3 mM) and glucose 6-phosphate dehydrogenase (0.32 U/ml) in 5 mM sodium citrate solution. Final incubation volume was 250 µl. After incubating for 10 minutes at 37°C, the reaction was terminated by addition of 750 µl of ice-cold acetonitrile containing internal standard and samples were processed as described above.

Enzyme kinetic parameters were obtained by performing incubations at nine different concentrations of PIO ranging from 0.25 µM to 25 µM. Pioglitazone was dissolved in acetonitrile and methanol (90:10 v/v, final organic solvent concentration of 0.8% v/v corresponding to final methanol content 0.08%). Microsomes were stored at -80°C and thawed immediately before use. Polypropylene microcentrifuge tubes were used to store PIO stocks and microsomal incubations were conducted in polypropylene tubes (4 ml) to minimize any non-specific binding. All incubations were performed in duplicate.

Results and Discussion

Method Development

Pioglitazone is primarily metabolized by CYP2C8 and CYP3A4 to four hydroxylated (M-II, M-IV, M-VII and M-VIII) and various other metabolites. Baughman et al have identified M-II, M-IV, M-VII and M-VIII in freshly isolated human hepatocytes and in pHLM (Baughman et al., 2005). Shen et al. (Shen et al., 2003) also reported formation of M-VII along with M-IV in various preclinical species and pHLM. In the LC/MS/MS based method described by Shen et al., M-VII elutes very close (retention time 38.3 min) to M-IV (40.2 min) in a total of 90 min run time (Shen et al., 2003). Although these metabolites differ in the position of hydroxylation they have similar fragmentation pattern and therefore, possess the same transition pair of Q1 and Q3 (373 → 150 *m/z*). Therefore, it was important to separate M-IV from M-VII on the analytical column. With the purpose of shortening the run time while achieving good baseline resolution

between M-IV and M-VII, we selected SYNERGI MAX-RP 80A (150 x 2.00 mm, 4 μ, Phenomenex) column. We were able to achieve baseline resolution keeping the run time relatively short (5 minutes). M-IV eluted at 2.7 minutes on the column.

A mobile phase consisting of ammonium acetate buffer was used to aid ionization of M-IV. Addition of 0.1% (v/v) formic acid in the mobile phase also enhanced ionization and improved the peak shape of M-IV. A full scan mass spectrum of M-IV was obtained in the positive and negative mode. The most abundant parent ion of M-IV (373 *m/z*) was obtained in the positive mode and the specific ion pair (373 → 150 *m/z*) was selected based on the intensities of three most abundant product ions. Thus, for quantification purpose, the TSQ quantum was operated in MRM mode and the precursor-product ion pair of 373 → 150 *m/z* and 377 → 154 *m/z* was followed for M-IV and M-IV-d4, respectively. In order to improve sensitivity of the method, M-IV was extracted by a liquid-liquid extraction method. Various solvents including acetonitrile, methyl tert butyl ether, dichloromethane, ethyl acetate and their combinations were used as extraction solvents. The highest and most consistent recovery was with dichloromethane (lower limit of quantification, LOQ 0.1 ng/ml), which was selected as the extraction solvent for future experiments. Considering the high sensitivity, simplicity of sample processing and short run time, the present method could potentially be applied to drug metabolism assays in future.

Method Validation

Validation of the assay method was conducted according to the FDA guidelines with respect to selectivity, carry over, linearity, precision, accuracy and autosampler stability (http://www.fda.gov/cder/guidance).

Selectivity, carry over and matrix effect

To determine the selectivity of the method, blank microsomal incubation samples were used to investigate the potential interferences due to the endogenous compounds in the matrix.

The present method demonstrated high degree of selectivity by means of MRM mode. A clear baseline was observed without any significant interference at the retention times of M-IV and M-IV-d4. The ratio of signal to noise obtained from an extracted standard at LOQ (0.1 ng/ml) was at least 50 for M-IV. Representative chromatograms of (A) blank buffer and buffer spiked with M-IV at the low QC (0.5 ng/ml) and (B) blank incubation sample and a sample after 10 minute incubation of PIO at 10 µM are depicted in Figure 3-2. No carry over was observed in any of the blank samples.

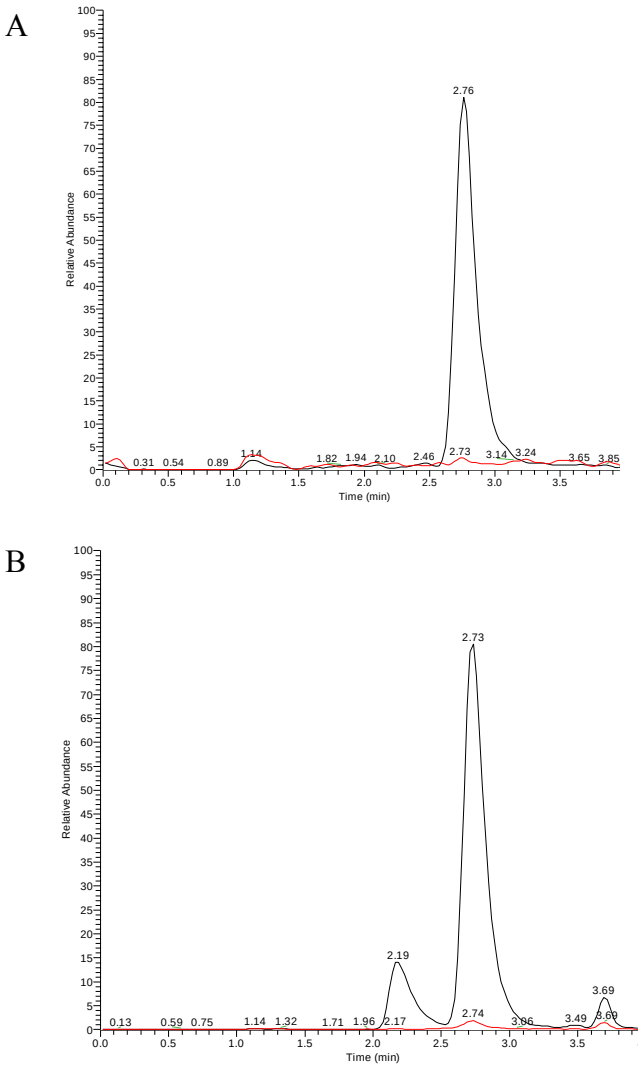

Figure 3-2. Representative chromatograms of (A) blank buffer (red) and buffer spiked with OH-PIO at low QC (black) and (B) incubation sample: blank (red) and after 10 min incubation of PIO at 10µM (black) (*m/z* 373 → 150, overlay offset = 0%).

Linearity

Calibration curves were linear in the concentration range of 0.1 - 20 ng/ml using a linear regression equation with a weighting factor of $1/y^2$. The mean correlation coefficient (R^2) of the calibration curves was 0.9967.

Precision and accuracy

Precision was represented as the relative standard deviation (%RSD) whereas accuracy was calculated as the percent deviation (% bias) from the respective nominal concentration. The maximum acceptable limit for precision and accuracy was set at 15%. The intra-day and inter-day precision and accuracy were within 4.0% and 10%, respectively, for all standards and QC samples (Table 3-1). Thus, the present method was found to be highly reproducible and demonstrated a high degree of accuracy.

Table 3-1.Intraday (n=6) and Interday (n=18) precision (%RSD) and accuracy (% deviation) for analysis of M-IV in 50 mM phosphate buffer, pH 7.4.

Concentration (ng/ml)			
Nominal	Back calculated (mean ± SD)	% RSD	% Deviation
Intraday			
0.50	0.48 ± 0.02	1.4	3.4
2.00	2.10 ± 0.05	1.8	-1.4
10	9.0 ± 0.4	2.4	-1.5
Interday			
0.50	0.48 ± 0.02	3.6	-4.5
2.00	2.07± 0.06	3.0	3.7
10.0	9.2 ± 0.4	3.9	-7.7

Autosampler stability

The processed stability of analytes was determined by reanalyzing the same standards and QC samples after 48 hours in the autosampler maintained at 10^{o}C. Both M-IV and M-IV-d4 were found to be stable at 10^{o}C for at least 48 hours.

Formation of M-IV in Pooled Human Liver Microsomes

PIO was incubated with pHLM at nine different concentrations (0.25-25 μM). The linearity of M-IV formation with respect to the microsomal protein concentration was studied at five protein concentrations (0.05-0.4 mg/ml). In order to avoid non-specific protein binding, the lowest protein concentration that produced quantifiable metabolite (0.05 mg/ml) was selected. Formation of M-IV was linear up to 20 minutes and was dependent on the presence of NADPH regenerating system. Following the incubation of PIO in pHLM, the concentration of M-IV was determined by using the newly validated method. The rate of formation of M-IV was measured as an index of CYP2C8 enzyme activity and data were analyzed by nonlinear regression using GraphPad Prism (version 4). The formation of M-IV exhibited typical Michaelis-Menten kinetics (Figure 3-3) with the maximal rate of formation of M-IV (V_{max}) of 150.3 ± 13.9 pmol/min/mg protein and the concentration of PIO at half maximal velocity (K_m) was 8.71 ± 1.9 μM, which are in agreement with the present literature (Table 3-2) (Tornio et al., 2007). Thus, the present method was successfully applied to *in vitro* drug metabolism studies.

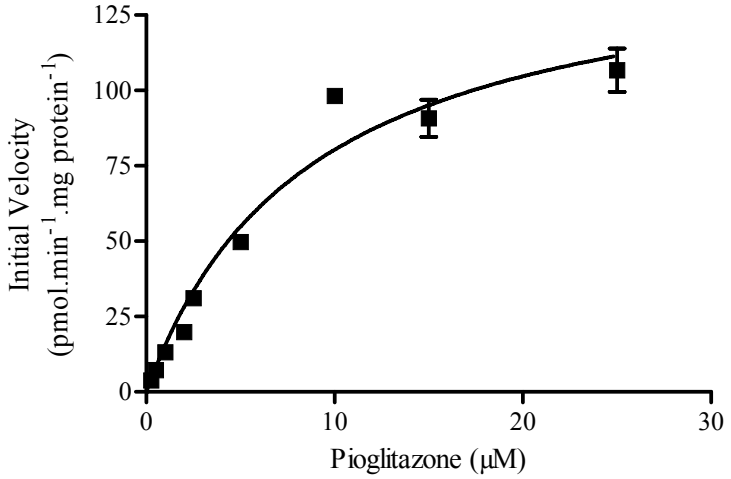

Figure 3-3. Plot of initial velocity versus pioglitazone concentration for the formation of hydroxypioglitazone (M-IV) in pHLM (n=2).

Table 3-2.Enzyme kinetic parameters of M-IV formation in pHLM.

	Km ± SE (μM)	Vmax (pmol.min^{-1}.mg protein^{-1})
In house	8.71 ± 1.90	150.3 ± 13.9
Tornio et al., 2007	9.8	640

Conclusion

A LC/MS/MS based method for determination of M-IV, the CYP2C8 specific metabolite of PIO was developed and validated. The method is sensitive (LOQ 0.1 ng/ml) and robust with a very short run time (5 minutes). It was validated with respect to selectivity, carry over, matrix effects, linearity, precision, accuracy and autosampler stability. Enzyme kinetic parameters obtained by incubating PIO with pHLM in presence of NADPH regenerating system were in accordance with the available literature. Therefore, the present method could be applied for future *in vitro* reaction phenotyping studies of CYP2C8.

CHAPTER 4
DETERMINATION OF IC_{50} IN POOLED HUMAN LIVER MICROSOMES USING M-IV FORMATION AS A CYP2C8 SPECIFIC REACTION

Introduction

It has become increasingly clear that the inhibition of CYP enzymes is often the key mechanism underlying drug-drug interactions leading to an ADR. In many cases, the drug interaction occurs via alterations in the activity of CYP enzymes. In other words, one drug (the perpetrator) alters the activity of an enzyme that is responsible for the metabolism of another drug (the victim or object), thus affecting its metabolic clearance. If the second drug has a wide therapeutic window, then the interaction might be clinically insignificant. However, if the victim drug has a narrow margin of safety, then the inhibition of its metabolism might result in drug related toxicity. On the other hand, induction of metabolism of the victim drug by the perpetrator might cause subtherapeutic effects leading to failure of drug therapy.

CYP2C8 plays an important role in the metabolism of various drugs including paclitaxel (Dai et al., 2001; Bahadur et al., 2002), amodiaquine (Li et al., 2002), troglitazone (Yamazaki et al., 1999), rosiglitazone (Baldwin et al., 1999), pioglitazone (Deng et al., 2005), repaglinide (Bidstrup et al., 2003; Niemi et al., 2003b), cerivastatin (Backman et al., 2002), amiodarone (Soyama et al., 2002) and verapamil (Busse et al., 1995). Reports of CYP2C8 inhibition by gemfibrozil have shown an increase in rosiglitazone $AUC_{0-\infty}$ from 1.8 to 2.8-fold (Niemi et al., 2003a), repaglinide $AUC_{0-\infty}$ from 5.5-15-fold (Niemi et al., 2003b), and cerivastatin $AUC_{0-\infty}$ from 1.3-20-fold (Backman et al., 2002). Cerivastatin was withdrawn from the market after about 500 ADRs, half of which involved coadministration of the CYP2C8 inhibitor gemfibrozil. In a recent study, Walsky et al. examined 209 frequently prescribed drugs and related xenobiotics for CYP2C8 inhibition using AQ as a CYP2C8 specific probe. In order to evaluate the potential of PIO as a CYP2C8 marker substrate, we studied the inhibition of CYP2C8 by five

drugs that were the part of the comprehensive study by Walsky et al. and other smaller studies using different CYP2C8 substrates (Walsky et al., 2005).

Experimental

Chemicals and Reagents

Ketoconazole, terfenadine, β-estradiol, midazolam, β–NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, magnesium chloride and ammonium acetate were procured from Sigma (St. Louis, MO, USA). Pioglitazone hydrochloride, M-IV metabolite standard and the deuterated internal standard, M-IV-d4, were obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Montelukast was purchased from LKT Labs (St. Paul, MN, USA). Pooled HLM were obtained from *In vitro* Technologies (Baltimore, MD, USA). Glucose 6-phosphate dehydrogenase solution was prepared by dissolving lyophilized enzyme in 5 mM sodium citrate to produce 40 U/ml solution and was stored at -20°C until use. Potassium phosphate was purchased from Fisher Scientific (Fair Lawn, NJ, USA) and formic acid was obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ, USA). HPLC grade acetonitrile and methanol were purchased from EMD Chemicals (Gibbstown, NJ, USA). All other chemicals used in the study were of analytical grade. Deionized water prepared by using a Barnstead Nanopure Diamond UV Ultrapure Water System (Dubuque, IA, USA) was used throughout the study.

Incubation Conditions

Preliminary experiments were conducted to optimize the microsomal protein concentration and incubation time in order to assure the linearity of M-IV formation (Chapter 3). The mixture containing pioglitazone (assay concentration, 7.5 μM) and pHLM (0.05 mg/ml) in phosphate buffer (50 mM, pH 7.4) was mixed with the inhibitor solution (in methanol:acetonitrile, 10:90) at various concentrations and warmed at 37°C for five minutes. The final incubation concentration

range that was studied for various inhibitors was as follows: ketoconazole (0.1-100 μM), terfenadine (0.1-100 μM), β-estradiol (0.1-600 μM), midazolam (0.1-600 μM), and montelukast (0.1-1000 nM). Equivalent amount of solvent was added to the incubation containing no inhibitor. Incubations were commenced by addition of the NADPH regenerating system, which consisted of $MgCl_2$ (assay concentration, 3.3 mM), NADP+ (1.25 mM), glucose 6-phosphate (3.3 mM) and glucose 6-phosphate dehydrogenase (0.32 U/ml) in 5 mM sodium citrate solution. Final incubation volume was 250 μl and the content of total organic solvent was kept to the minimum (1.2%). Control incubations were conducted without addition of NADPH regenerating system to check any interference at the metabolite retention time. After incubating for 10 minutes at 37°C, the reaction was terminated by addition of 750 μl of ice-cold acetonitrile followed by the addition of internal standard solution in methanol (50 μl, 12 ng/ml). Samples were processed as described in Chapter 3. Microsomes were stored at -80°C and thawed immediately before use. All incubations were performed in duplicate.

Analysis of Hydroxypioglitazone (M-IV)

Concentrations of M-IV were determined as described in Chapter 3. Briefly, the chromatographic separation was achieved on a SYNERGI MAX-RP 80A (150 x 2.00 mm, 4 μ, Phenomenex) analytical column by gradient elution. The mass spectrometer was a TSQ Quantum Discovery triple quadrupole mass spectrometer equipped with ESI source. The ESI source was operated in the positive mode and was set orthogonal to the ion transfer capillary tube. For quantification, TSQ quantum was operated in MRM mode and the precursor-product ion pair was 373 → 150 m/z for M-IV and 377 → 154 m/z for M-IV-d4. The acquisition parameters were optimized to maximize the signal to noise ratio.

Data Analysis

Enzyme kinetic and inhibition data were obtained by nonlinear regression using GraphPad Prism (San Diego, CA, USA). Data for IC_{50} determinations were typically fit to the following equation:

$$\%\,\text{Control activity} = 100 - \frac{I_{max} \times I}{IC_{50} + I} \qquad \text{Equation 4-1}$$

in which I is the inhibitor concentration, Imax is the initial velocity in the presence of inhibitor, IC_{50} is the inhibitor concentration that reduces initial velocity by 50%.

Results and Discussion

Five drugs namely, montelukast, ketoconazole, β-estradiol, midazolam and terfenadine were examined for their potential to inhibit the formation of the CYP2C8 specific metabolite (M-IV) in pHLM. Chemical structures of these inhibitors are shown in Figure 4-1.

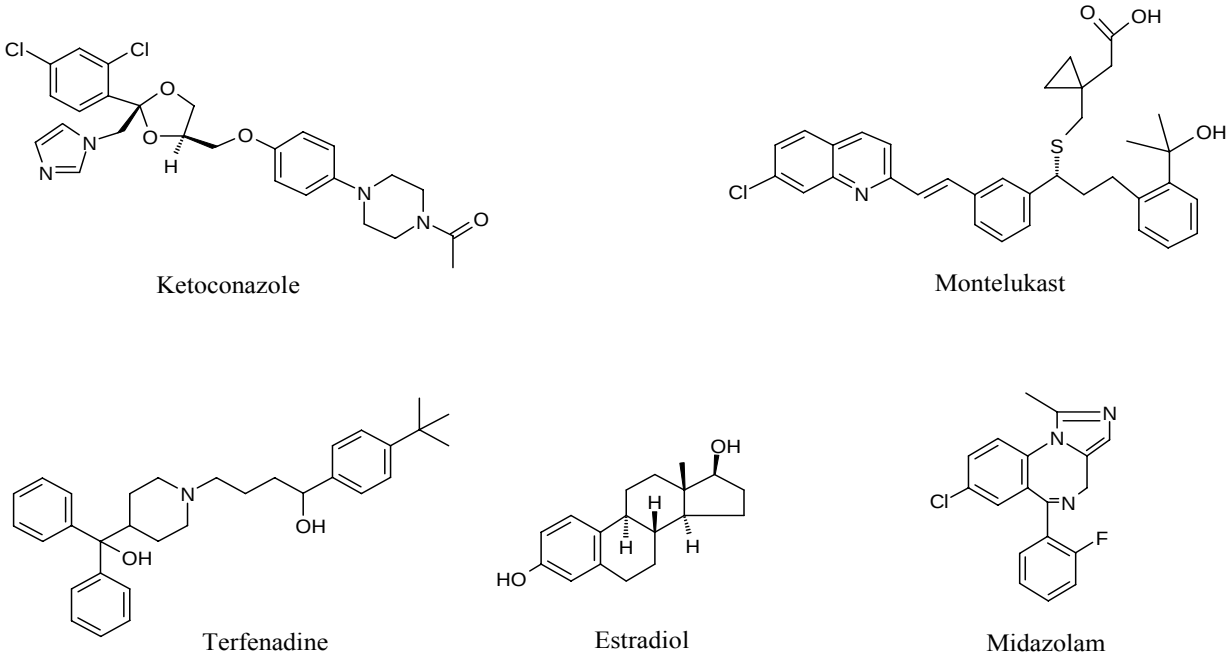

Figure 4-1. Structures of the compounds tested for inhibition of *in vitro* CYP2C8 activity.

The concentration of inhibitor that reduces the initial velocity by 50% (IC_{50}) was determined by conducting the enzyme assay in the presence of seven different inhibitor concentrations. The results of the present study demonstrate that all the test inhibitors inhibited the activity of CYP2C8 in a concentration dependent manner when studied as a function of formation of M-IV from PIO. IC_{50} plots of pioglitazone hydroxylase inhibition are shown in Figure 4-2.

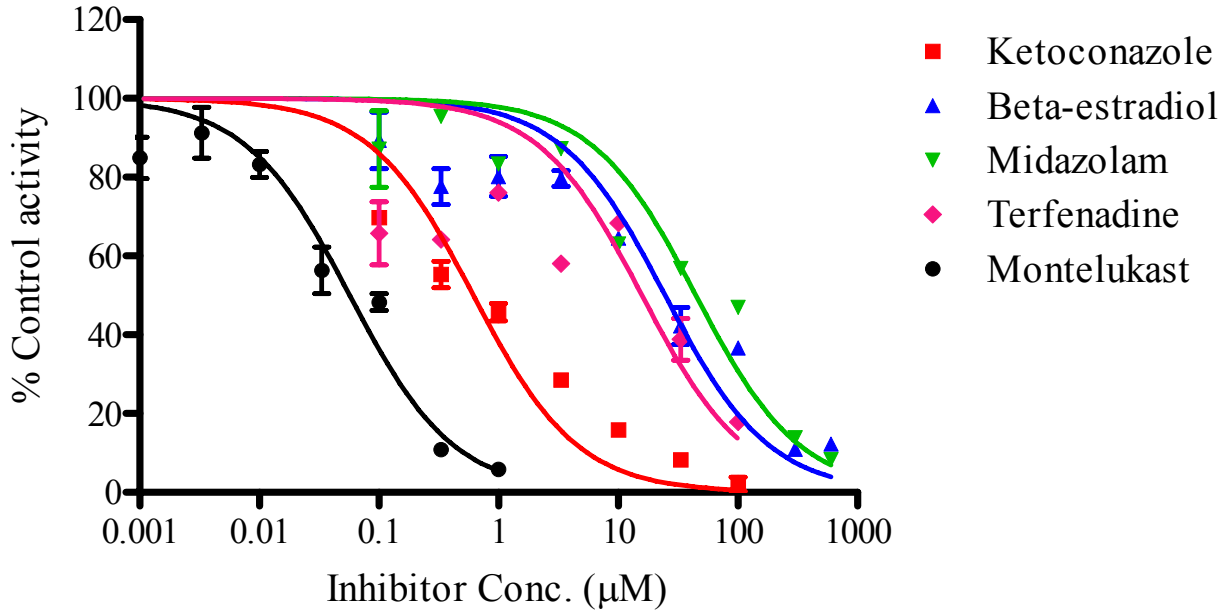

Figure 4-2. IC_{50} plots of pioglitazone hydroxylase inhibition in pHLM by montelukast (circles), ketoconazole (squares), terfenadine (diamonds), β-estradiol (triangles) and midazolam (reverse triangles). Data points reflect the average for incubations run in duplicate ± SE.

Montelukast was the most potent inhibitor of formation of M-IV with an IC_{50} of 57.16 ± 12.12 nM (R^2 = 0.8896) in pHLM. Walsky et al. examined the inhibition of CYP2C8 activity by 209 drugs and related compounds in expressed CYP2C8 as a function of AQ desethylation. They also reported montelukast to be the most potent inhibitor of CYP2C8 activity with an IC_{50} of

19.6 nM in pHLM (Walsky et al., 2005). Our results are in accordance with another study by Jaakkola et al. that examined the CYP2C8 inhibition potential of montelukast using pioglitazone as a substrate (IC_{50} = 180 nM) (Jaakkola et al., 2006).

The antifungal agent ketoconazole was the next potent inhibitor of CYP2C8 activity with an IC_{50} of 0.618 ± 0.12 μM (R^2 = 0.9039). In the present study, we found that terfenadine, a H1 receptor antagonist, inhibited the activity of CYP2C8 with an IC_{50} of 15.79 ± 7.32 μM (R^2 = 0.4247). Ketoconazole and terfenadine are known inhibitors of CYP3A4. Ketoconazole also inhibited 6-α-hydroxylation of paclitaxel (Desai et al, 1998) and tolyl methylhydroxylation of torsemide (Ong et al., 2000), both of which are CYP2C8 specific reactions, where as terfenadine was shown to inhibit AQ desethylation (IC_{50} 11.5 ± 5.1 μM) (Walsky et al., 2005) as well as tolyl methylhydroxylation of torsemide in expressed CYP2C8 (Ong et al., 2000). Inhibition of CYP2C8 by ketoconazole and terfenadine could be explained by the fact that both enzymes, CYP3A4 and CYP2C8, have a large active site and many CYP2C8 substrates are also metabolized by CYP3A4. Walsky et al. reported a moderate inhibition of the metabolism of AQ by β-estradiol in expressed CYP2C8 (21.5 ± 5.8) (Walsky et al., 2005). In the current study, we proved the moderate inhibitory potential of β-estradiol to inhibit CYP2C8 activity measured as the formation rate of M-IV. Midazolam, a benzodiazepine drug, moderately inhibited CYP2C8 mediated hydroxylation of PIO (IC_{50} = 44.60 ± 7.1). It also inhibited the formation of DEAQ (Walsky et al., 2005) as well as formation of tolyl methylhydroxy torsemide in expressed CYP2C8 (Ong et al., 2000). Midazolam is used as a maker substrate of CYP3A4, once again indicating the overlap between the substrate and inhibitor profiles of CYP2C8 and 3A4.

Conclusion

We examined the potential of montekulast, ketoconazole, terfenadine, β-estradiol and midazolam to inhibit the formation of M-IV which is CYP2C8 specific metabolite of PIO using

pHLM. All test inhibitors inhibited the M-IV formation in the concentration dependent manner and the inhibitory potentials of each of them are comparable to that studied with other CYP2C8 substrates. Therefore, formation of M-IV could potentially be used as a CYP2C8 specific probe reaction.

CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

Drug-drug interactions due to induction or inhibition of DMEs significantly add to the ADRs. In order to avoid any unforeseen interactions in the clinic, the FDA requires pharmaceutical companies to identify the major drug metabolizing enzymes involved in the elimination of a NCE as well as the effect of its coadministration on the pharmacokinetics of marker substrates of DMEs that are responsible for its elimination. Common experimental methods used to characterize the metabolism of a NCE and predict its drug-drug interactions involve the use of enzyme specific chemical inhibitors, expressed enzymes, antibodies and correlation analysis.

The polymorphic CYP2C8 is involved in the metabolism of paclitaxel, amodiaquine, troglitazone, rosiglitazone, pioglitazone, repaglinide, cerivastatin, amiodarone and verapamil. Conventionally, paclitaxel has been used as a CYP2C8 specific probe substrate for *in vitro* reaction phenotying studies. Recently, AQ was found to be a high turnover and affinity substrate of CYP2C8 and is used for *in vitro* drug metabolism studies however, it can not be administered to humans due to its toxocity. Therefore, identification of a CYP2C8 specific substrate that can be used for *in vitro* as we as *in vivo* drug interaction studies is needed. Hydroxylation of PIO to form M-IV is primarily medicated by CYP2C8, therefore, formation of M-IV could be used as a CYP2C8 specific probe reaction.

We developed a simple and robust LC/MS/MS based method using HILIC chromatography to separate DEAQ. The method was validated with respect to selectivity, carry over, matrix effects, linearity, precision, accuracy and autosampler stability. Enzyme kinetic parameters obtained by incubating AQ with pooled HLM in presence of NADPH regenerating

system were in accordance with the available literature. Therefore, the present method could be applied for future CYP2C8 drug metabolism studies.

A LC/MS/MS based method for determination of M-IV, the CYP2C8 specific metabolite of PIO was developed and validated. The method is sensitive (LOQ 0.1 ng/ml) and robust with a very short run time (5 minutes). It was validated with respect to selectivity, carry over, matrix effects, linearity, precision, accuracy and autosampler stability. Enzyme kinetic parameters obtained by incubating PIO with pooled HLM in presence of NADPH regenerating system were in accordance with the available literature. Therefore, the present method could be applied for future in vitro reaction phenotyping studies of CYP2C8.

We examined the potential of montekulast, ketoconazole, terfenadine, estradiol and midazolam to inhibit the formation of M-IV which is CYP2C8 specific metabolite of PIO using pooled human liver microsomes. All test inhibitors inhibited the M-IV formation in the concentration dependent manner and the inhibitory potentials of each of them are comparable to that studied with other CYP2C8 substrates. Therefore, formation of M-IV could potentially be used as a CYP2C8 specific probe reaction.

Although, the presence of *CYP2C8*3* allele reduced the intrinsic clearance of AQ in human liver microsomes, its effect of AQ pharmacokinetics is unknown. In future, it will be interesting to study the effect of *CYP2C8*3/*3* genotype on the disposition of AQ. On the other hand, *CYP2C8*3* allele reduced the $AUC_{0-\infty}$ of PIO. However, its effect on PIO metabolism has not been studied at enzymatic level. Further, we would like to study the effect of *CYP2C8*3* allele on M-IV formation in genotyped human liver microsomes.

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BIOGRAPHICAL SKETCH

Prajakta Dravid was born in Maharashtra, India. In 2000, she received her B.S. in Pharmacy from University of Pune, India. During her bachelors, she developed an interest in research and development and followed her interest by getting M.S. degree in Pharmaceutics from the National Institute of Pharmaceutical Education and Research (NIPER), India. Chasing her interest in the area of pharmacokinetics and drug metabolism, Prajakta joined the Bioanalysis, Drug Metabolism and Pharmacokinetics Department at Dr. Reddy's Laboratories in Hyderabad, India. At Dr. Reddy's she gained extensive experience in the field of bioanalysis and preclinical pharmacokinetics. After two years of industrial experience, she joined the PhD program at the Department of Pharmaceutics, University of Florida in the August of 2004. Under the supervision of Dr. Reginald Frye, she looked at the reaction phenotyping aspects of Cytochrome P450 2C8 with a focus on substrate selection and inhibition profile. She received M.S. in Pharmacy from University of Florida in December 2007 and pursuing her PhD in the same program.