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*In vitro* and *in vivo* studies of methadone metabolism and pharmacokinetics

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**Abstract**

Title

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Methadone ((*R,S*)-6-dimethylamino-4, 4-diphenyl-3-heptanone) is a synthetic  $\mu$ -opioid receptor agonist developed in Germany during World War II as a replacement for morphine. Methadone is currently used to aid in opioid cessation and chronic pain control; two ailments that require indefinite drug therapy. While the drug is certainly beneficial to many patients, severe and sometimes fatal adverse events overshadow its usage. In the years between 2000-2009 methadone prescriptions have increased as have the number of unique patients using the medication. Unfortunately trending with this increase in prescription number are the casualties caused by methadone overdose. Currently, an effective prediction and prevention strategy for methadone toxicity remains elusive. This is partially due to high inter-individual variability in the efficacy and toxicity of the drug. Further studies investigating factors that influence variability are necessary to make methadone a safer and more effective medication.

The purpose of this dissertation project is to explore the roles of cytochrome P450 (CYP) enzymes and permeable-glycoprotein (P-gp), an efflux transporter, in the metabolism and disposition of methadone. Previous research focused on CYP3A4, CYP2B6, and P-gp proteins as potential contributors to the overall pharmacokinetic profile of methadone mainly because; 1) methadone is a substrate for each of these proteins and 2) *in vivo* activities of aforementioned proteins are highly variable among individuals. CYP3A4, the most abundant CYP in the liver

and gastrointestinal tract, is the major CYP enzyme responsible for the metabolism and clearance of a large number of xenobiotics. CYP3A has been shown to metabolize methadone *in vitro* but its contribution to *in vivo* clearance has been questioned. CYP2B6 has long been considered a minor player among the CYP super-family, although recent evidence suggest that its role is underestimated due to high substrate overlap and similar regulation pathways with CYP3A4. Interest in CYP2B6 has increased in recent years because of its highly polymorphic nature and several allelic variants have been shown to be clinically relevant in efavirenz therapy. P-gp, an ATP-catalyzed efflux transporter is also highly polymorphic and capable of effluxing numerous compounds of various structures and therapeutic classes. P-gp is expressed in the gastrointestinal tract, kidneys, and along several blood-tissue barriers. Mutations in *ABCB1*, the gene that encodes for P-gp, is thought to lead to variable expression and activity of the protein. Changes in P-gp activity could, therefore, influence methadone drug absorption. This project encompasses *in vitro* and *in vivo* investigations to determine the relative contribution of each protein to methadone metabolism and clearance. A better understanding of factors that underlie methadone's variability will be crucial to improve clinical safety and better predict adverse drug effects.

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I would like to dedicate this to:

My father, Quy Q. Dinh

My mother, Van T. Dinh

My two brothers, Vince N. Dinh and Andrew N. Dinh

Thank you and love you.

## **Chapter 1: Role of cytochrome P450 enzymes and transporters in inter-individual variability in methadone drug response.**

### **I. Introduction**

Methadone ((*R,S*)-6-dimethylamino-4, 4-diphenyl-3-heptanone) is a synthetic  $\mu$ -opioid receptor agonist developed in Germany during World War II as a synthetic replacement for morphine. It was introduced to the United States in 1947 by Eli Lilly and Company and to date remains one of the “essential drugs for health list” compiled by the World Health Organization (WHO, 2013; Scott et al., 1948). Methadone is used clinically to treat opioid addiction and control chronic pain; two ailments that require indefinite drug therapy. While the drug has been advantageous to many patients, severe and sometimes fatal, adverse events are reported, including problems associated with over-dosing. This is evident from the mortality data of methadone, summarized by the Substance Abuse and Mental Health Services Administration (SAMHSA). Between the years 2000-2009, methadone prescriptions have increased as have the number of unique patients using the medication. Paralleling the increase in the number of patients taking methadone are the casualties attributed to the drug (SAMHSA, 2010).

Currently, there is no reliable method to predict which individuals will be susceptible to the adverse side effects. Understanding the source of variability and toxicity of methadone is important because the drug is critical in two disease states that have wide prevalence – opioid cessation and chronic pain control. Therefore, the main focus of this project, will be on proteins that are central in methadone clearance, as toxicity appears to be dose-dependent (Scott et al., 1948). Ultimately, there is potential to develop an effective screen for patients who are at highest risk of methadone toxicity, while ensuring patients who need the medication receive it.

The introduction is divided into the following sections: 1) the importance of methadone in clinical use; 2) the extent of methadone variability, in both the disposition and drug action; and 3) brief description of the three proteins that are the focus of this dissertation project, and the current understanding of how each may contribute to methadone pharmacokinetics. A summary and scope of the research will conclude this chapter.

## **II. Clinical Uses of Methadone**

*Methadone Maintenance Therapy:* The epidemic of heroin use surfaced in the United States as early as 1960s. This prompted medical researchers and clinicians to develop strategies to counteract “The Heroin Plague,” as it was dubbed by Newsweek in 1971 (Peck and Beckett, 1976). Drs. Vincent Dole and Marie Nyswander established the groundwork for methadone maintenance programs. They theorized that heroin drug craving was due to physiological and metabolic changes caused by continual drug use. The changes appeared to be irreversible even when drug was removed from addicted persons for a substantial length of time. Heroin, (diacetylmorphine), is a pro-drug that is biotransformed *in vivo* to the active form, morphine, a  $\mu$ -opioid receptor agonist. Binding to this receptor often causes a “high,” or a state of euphoria. It was hypothesized that using other opioids to control cravings may be a mechanism to aid addicts in heroin cessation. This hypothesis was based on knowledge that opioids exhibit cross-tolerance, meaning that when an individual is tolerant of one opioid, they will also likely be tolerant to other similar drugs (Dole et al., 1966; Dole, 1971; Dole and Nyswander, 1966).

In pilot studies, morphine was initially used to treat cravings. However, escalating doses of morphine were required to prevent cravings and withdrawal symptoms. The high doses result in toxic side effects, such as respiratory and central nervous system (CNS) depression (Peck and

Beckett, 1976). Methadone, remarkably, can be used to prevent cravings at a constant dose (Dole and Nyswander, 1966; Dole, 1971). The chemical structures of heroin, morphine, and methadone are depicted in Figure 1.1. Subsequent studies reported that patients maintained on methadone were rehabilitated into society and were able to lead more productive lives (DuPont and Katon, 1971). When methadone was used long term in maintenance treatment programs, criminal activity and patient mortality decreased. Additionally, indirect benefits of methadone maintenance treatment programs include a decrease in transmission rates of blood-borne diseases, such as HIV, and a lower financial burden on medical institutions and healthcare plans (Mattick et al., 2009; McCarty et al., 2010). The United Nations World Drug Report estimates that 16.5 million people worldwide (approximately 0.4% of the world's population aged 15-64) use opioids (UNODC, 2013).

*Chronic Pain Control:* Methadone is also indicated for the control of chronic pain, particularly within the cancer patient population. Chronic pain is a prevalent concern in modern medicine and adequate treatment is challenging. The symptom is defined as, “pain that lasts beyond the term of an injury or painful stimulus,” or “pain without apparent biological value continuing beyond the normal tissue healing time” (Haroutiunian et al., 2012). While opioids are recommended in this setting, long-term use results in hyperalgesia, or the increased sensitivity to pain, and tolerance to opioids. Patients with chronic pain enter an unproductive cycle of tolerance marked by an increase need for medications, followed by an increase in pain perception. This paradoxical side effect of opioids is mediated through activation of the glutaminergic N-methyl-D-aspartate (NMDA) receptor (Haroutiunian et al., 2012).

Methadone pharmacology is unique among opioids because it is also an NMDA receptor antagonist. When methadone is included in opioid rotation, patients experience less nociceptive

pain (Andrews et al., 2009; Leppert, 2009; Salpeter et al., 2013). Methadone switching in patients with pain that is refractory to morphine is reported to be successful (Mercadante et al., 2012). Because of this, the number of methadone prescriptions has increased steadily over the last decade. Of these new prescriptions, chronic pain control is the most common indication (SAMHSA, 2010). Over 4 million prescriptions were written specifically for methadone in the United States alone in 2009 (SAMHSA, 2010). The estimated global prevalence of chronic pain with a neuropathic component varies between 0.9% - 17.9% (van Hecke et al., 2014).

### **III. Methadone Variability**

*Methadone Pharmacodynamics and Pharmacokinetics:* Attaining a balance between efficacy and toxicity in methadone treatment is complicated. Despite being clinically available for nearly 67 years, methadone is still a difficult drug to dose and use effectively. This is partially due to the high inter-individual variability in drug response, which leads to complications in terms of dosing and therapeutic optimization. When given orally, the duration of action of methadone has been reported to be between 2-10 hours with the time to peak concentration between 2-4 hours. Methadone's elimination following a single oral dose, displays bi-exponential kinetics with an initial phase half-life ( $t_{1/2}$ ) between 12-24 hours, and a secondary phase with a  $t_{1/2}$  of 55 hours (Verebely et al., 1975; Dale et al., 2002; Dale et al., 2004). The oral bioavailability of methadone is also variable. The oral bioavailability is approximately 70-90%, however, it can range from 44% to 99% (Meresaar et al., 1981; Gourlay et al., 1986; Dale et al., 2004). Once absorbed systemically, the drug is highly bound to  $\alpha_1$ -acid glycoprotein (AGP), an acute phase blood protein that is induced in response to stress. Levels of AGP can increase between two- to five-fold with disease and infection (Ceciliani and Pocacqua, 2007). Because only the free fraction of drug can reach the target site, the varying amount of AGP, depending on disease state affects

variability of methadone efficacy (Romach et al., 1981; Behan et al., 2013; Herve et al., 1996). There is extensive empirical evidence for methadone variability as measured by half-lives, bioavailability, and free fraction. The discrepancy between time of pharmacodynamic effect and pharmacokinetic profile has caused the Food and Drug Administration (FDA) to issue a public advisory (FDA, 2013) The concern is that the duration of action suggests the need for multiple daily dosing for efficacy. However, multiple dosing will lead to drug accumulation because of the prolonged  $t_{1/2}$ . This can lead to toxic side effects of methadone, such as respiratory depression, neurological depression, cardiotoxicity and arrhythmias, all of which are dose-dependent (Scott et al., 1948).

*Methadone Stereochemistry:* To complicate matters further, methadone contains a chiral center and is administered as the racemate. The therapeutic activity to treat drug craving and control pain resides almost exclusively in the *R*-enantiomer with a binding affinity to the  $\mu$ -opioid receptor 10-times better than the *S*-enantiomer (Kristensen et al., 1995). The same study also demonstrated that an equi-molar concentration of *R*-methadone, as expected, had twice the binding affinity for  $\mu_1$  and  $\mu_2$  receptors when compared to the racemate (Kristensen et al., 1995). Scott et. al (1948) studied the difference in analgesic activity of methadone enantiomers by administering escalating doses of either *R*- or *S*- methadone and measuring pain tolerance in three healthy volunteers. Doses were increased until equal analgesic effect was achieved between the enantiomers. At a similar dose, the analgesic effect of *R*-methadone was approximately 50-times more potent than *S*-methadone. Interestingly, the toxicological profile was different for the enantiomers. *R*-methadone caused lightheadedness, sleepiness, and decreased cognition that lasted 9-14 hours. In contrast, *S*-methadone generated feelings of either well-being or lethargy which lasted 4-5 hours (Cahen et al., 1948; Scott et al., 1948). Of concern are recent reports that

the *S*-enantiomer has been implicated in the toxic side effects of methadone, such as QT-prolongation and arrhythmias due to more potent inhibition of the hERG channel compared to the *R*-enantiomer (Eap et al., 2007; Ansermot et al., 2010). *In vivo*, methadone displays variable stereoselective pharmacokinetics, with the *R*-enantiomer typically circulating at higher concentrations. However, the ratio of *R*- to-*S*- methadone in plasma varies as evident in a study of twenty-two methadone maintenance treatment patients. In this investigation the plasma *R*- to-*S*- methadone ratio varied from 0.63 – 2.4 (Eap et al., 1996). The combination of stereoselective pharmacokinetics, efficacy, and toxicity make methadone a difficult drug to dose properly.

*Methadone Metabolism Complications:* *R,S*-methadone is metabolized to the major inactive metabolite, (*R,S*)-2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (*R,S*-EDDP) (Sullivan and Due, 1973; Pohland et al., 1971). Hydroxylation occurs on one of the two methyl groups on the amine. The resulting carbinolamine collapses to form *N*-desmethylnmethadone and formaldehyde. The lone pair of electrons on the nitrogen of *N*-desmethylnmethadone attacks the carbonyl group followed by a loss of water to form a five membered ring. EDDP is a mixture of (*E/Z*)-isomers as a double bond forms exo to the ring (Figure 1.2). EDDP can undergo further oxidation, however, to a lesser extent than methadone, to form (*R,S*)-2-ethyl-5-methyl-3,3-diphenylpyrroline (*R,S*-EMDP). This secondary metabolite is also inactive. EMDP is formed by oxidation of the remaining N-methyl group of EDDP. Methadone and its two major oxidative metabolites are shown in Figure 1.2 (Sullivan and Due, 1973; Pohland et al., 1971). The chiral center of methadone is unaffected during metabolism and the two enantiomers do not interconvert (hence, *R*-methadone → *R*-EDDP → *R*-EMDP and the same is true for *S*-methadone). Other minor oxidative metabolites, such as phenols have also been reported, however the EDDP isomers are

mostly frequently studied as markers for methadone oxidation due to their higher abundance in plasma and urine. (Sullivan and Due, 1973).

A number of cytochrome P450 enzymes, discussed below, are capable of catalyzing the oxidation of methadone. *In vitro* data suggest that methadone both induces and inhibits its own metabolism (Tolson et al., 2009; Amunugama et al., 2012). Incubation of primary human hepatocytes with various concentrations of methadone resulted in an increase in mRNA and protein levels of CYP2B6, CYP3A4, UGT1A1, and P-gp (listed in decreasing order) (Tolson et al., 2009). Both isomers of methadone were ligands for nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR). This activation of nuclear receptors and induction of proteins appeared to be concentration-dependent (Tolson et al., 2009). Studies involving recombinantly expressed CYP2B6 demonstrated that racemic methadone inactivated CYP2B6 in a time-dependent, concentration-dependent, and NADPH-dependent manner (Amunugama et al., 2012). Methadone  $K_i$  and  $k_{inact}$  for CYP2B6 are 10  $\mu\text{M}$  and  $0.027 \text{ min}^{-1}$ , respectively. The mechanism of inactivation appears to be through heme destruction (Amunugama et al., 2012). In addition, enantiomeric metabolic interactions can occur with methadone and result in mutual inhibition (Totah et al., 2007). Metabolism is a critical pathway for elimination and clearance of methadone and a source of variability in clearance is the concentration of methadone. Thus, both induction and inhibition could have clinical implications. Induction of metabolism could cause the concentration of active drug to be lower than the therapeutic target. Inhibition of metabolism could result in higher concentrations of active drug and potentially lead to over dosing.

#### **IV. Proteins of Interest—Cytochrome P450 Enzymes**

*Cytochrome P450 Enzymes:* CYP enzymes are a super-family of monooxygenases that catalyze the oxidation of both exogenous and endogenous compounds (de Montellano, 2005). There are a total of 57 functional P450 enzymes in humans. The P450 isozymes are further divided into families and subfamilies based on amino acid sequence homology. Families 1-3 are typically thought to oxidize mainly exogenous compounds and are capable of metabolizing a number of substrates with varying structures. CYP families 1-3 can also exhibit substrate redundancy, where a single compound can be a substrate for more than one CYP. The majority of drug metabolizing isozymes are expressed in the endoplasmic reticulum of hepatocytes. These enzymes are also expressed to a lesser extent along the gastro-intestinal tract, lung, kidney and other organs. In general approximately 70-80% of drugs in clinical use are oxidized by CYP enzymes (Zanger and Schwab, 2013).

The P450 oxidative cycle, along with a description of each step in the cycle is provided in Figure 1.3. The oxidation of exogenous substrates typically results in a more hydrophilic metabolite that is more readily removed from the body either through conjugation (glucuronidation, sulfation, etc.) or filtered by the kidneys for renal excretion. In certain cases, a compound is administered as a pro-drug, and activation by P450s is necessary for therapeutic effect. Both drug activity and also toxicity can be highly dependent on P450 activity. Therefore understanding P450 metabolism is critical to maximizing positive therapeutic outcomes and decreasing toxicity, particularly in drugs with narrow therapeutic indices. In addition, studying polymorphisms that affect P450 activity is also important to better predict therapeutic outcomes.

Currently, there is *in vitro* and *in vivo* data to suggest that there are a number of P450 enzymes capable of metabolizing methadone to the inactive metabolite EDDP. CYP3A4 and CYP2B6, however, appear to be the main isozymes involved *in vivo* and are discussed in further detail below. CYP2D6 was originally thought to contribute to methadone metabolism because it appeared that methadone maintenance patients who were categorized ultra-rapid metabolizers were among the least to be satisfied with treatment. The authors attributed this to a more rapid clearance of methadone in this sub-population (Perez de los Cobos et al., 2007). However, *in vitro* studies do not support this clinical observation (Gerber et al., 2004).

*CYP3A4*: The *CYP3A4* gene in humans is located on chromosome 7 q22.1 (Inoue et al., 1992) and contains thirteen exons and 12 introns (Hashimoto et al., 1993). The members of the CYP3A sub-family—CYP3A4 in particular—are the two major drug-metabolizing isozymes of the P450 super-family. Expression of CYP3A is higher than any other P450 enzyme in adult liver accounting for approximately 14-24% of total P450 enzymes (Shimada et al., 1994; Lin et al., 2002). Additionally, CYP3A is also expressed along the gastrointestinal tract, which, in addition to the liver, is a major site of Phase I metabolism (Paine et al., 1996). The CYP3A4 displays broad substrate specificity and plays a key role in the metabolism of 50% of drugs *in vivo* (Klein and Zanger, 2013). For our purposes, CYP3A4, but not CYP3A5, has been shown to appreciably metabolize methadone (Chang et al., 2011). Therefore, only CYP3A4 will be discussed and investigated further in this dissertation.

*CYP3A4 Variability*: The variability of CYP3A4 enzymatic activity can be as high as ~100 fold (Klein and Zanger, 2013) in the population. The enzyme is subject to many drug-drug interactions through inhibition (by other substrates) or induction via PXR or CAR ligands (Lamba et al., 2005). CYP3A4 activity can also be affected by sex, age, and disease state. It

appears that CYP3A4 activity is higher in women than men, most likely through hormonal signaling and induction of the enzyme (Wolbold et al., 2003). Age and disease states, particularly those that involve inflammation can cause down regulation of CYP3A4 activity (Aitken et al., 2006). Interestingly, to date, there is no clear association between *CYP3A4* genotype and phenotype, a phenomenon that has been called, the “missing heritability” problem (Klein and Zanger, 2013). Despite the lack of a link between *CYP3A4* genotype and phenotype, analyzing variability between individuals is important for ascertaining the role of this enzyme in drug metabolism.

*CYP3A4 and methadone metabolism:* There is experimental evidence, both *in vitro* and *in vivo*, that implicates CYP3A4 in the clearance of methadone. Thus, the high interindividual variability in CYP3A4 activity might explain the differences in pharmacokinetics and pharmacodynamics of the drug. A screen of various CYP Supersomes® at equal protein concentration with 1 and 10 µg/mL methadone provided evidence that CYP2B6, CYP2C19, and CYP3A4 are the main isoforms involved in methadone metabolism (CYP2B6 > CYP2C19 ≥ CYP3A4)(Gerber et al., 2004). Interestingly, CYP3A4, unlike CYP2B6 and CYP2C19, did not metabolize methadone stereoselectively, which is in contrast to *in vivo* evidence mentioned previously in methadone stereochemistry (Gerber et al., 2004). More recent studies evaluated methadone metabolism in CYP2B6, CYP2C19, and CYP3A4 Supersomes®, determined kinetic parameters for each enzyme and scaled activity to protein concentration in the liver. The authors concluded that CYP3A4, having the highest expression in the liver, contributed the most to methadone metabolism. However, the stereoselective profile of metabolite formation was associated with CYP2B6 activity. CYP2C19 activity did not appear to contribute significantly to methadone

metabolism when scaled to protein expression levels in the liver (Totah et al., 2007; Totah et al., 2008).

*In vivo* data regarding CYP3A4 activity and methadone metabolism has been conflicting. Kapur et al. conducted an extensive literature review of drug-drug interactions with methadone and found that in the majority of cases, changes in methadone were a result of inhibition or induction of CYP3A4 (Kapur et al., 2011). In contrast, studies in which chemical inhibitors of CYP3A4 were co-administered with methadone in healthy volunteers resulted in no significant changes to drug exposure (Kharasch et al., 2004; Kharasch et al., 2008; Kharasch et al., 2009). In a study involving methadone maintenance therapy patients, CYP3A4 activity, as phenotyped by 1'-hydroxymidazolam formation, correlated positively with methadone dosage requirements. Patients with higher CYP3A4 activity required higher doses than those with low CYP3A4 activity, most likely due to increased clearance of the drug (Shinderman et al., 2003). Overall, the contribution of CYP3A4 to methadone metabolism is still ambiguous. However, given the relatively high protein expression level, high inter-individual variability and the ability to metabolize methadone *in vitro*, CYP3A4 is still a viable candidate and an important enzyme to consider when elucidating mechanisms of variability in methadone clearance.

*CYP2B6*: The *CYP2B6* gene in humans is located in the *CYP2* cluster on chromosome 19 q13.2 (Hoffman et al., 1995) and is composed of nine exons and eight introns. *CYP2B6* is capable of metabolizing ~8-10% of drugs currently on the market (i.e. greater than 60) (Mo et al., 2009). These drugs differ in structure and size, and represent a variety of drug classes. The enzyme is expressed mainly in the liver, with smaller amounts in kidney, skin, brain, and lung (Gervot et al., 1999; Code et al., 1997). In the liver, *CYP2B6* accounts for approximately 6% of total P450 enzymes, however this value varies greatly. It has been shown by a number of investigators that

hepatic CYP2B6 expression and mRNA levels exhibit 20-278 fold difference between individuals. This is probably due to the nature of CYP2B6 being highly inducible and extensively polymorphic.

There are over 500 SNPs and over 30 allelic variants reported for CYP2B6 (cyp.allele.ki). Regulation of CYP2B6 expression is through CAR and PXR. Induction through CAR can occur through dephosphorylation of the nuclear receptor in the cytosol and translocation into the nucleus where it forms a heterodimer with retinoid X receptor (RXR $\alpha$ ). This hetero-complex binds to the nuclear receptor binding sites and activates the phenobarbital-responsive enhancer module (PBREM). PXR also induces *CYP2B6* through binding to PBREM. Unlike CAR, PXR is permanently located the nucleus and a translocation step is not necessary. Rifampin induces *CYP2B6* through PXR (Faucette et al., 2006; Faucette et al., 2007). Glucocorticoid receptor (GR) is capable of enhancing CYP2B6 transcription in the presence of either CAR or PXR (Mo et al., 2009; Faucette et al., 2006; Faucette et al., 2007). The extensive network for CYP2B6 induction could partially explain the variability observed with CYP2B6 activity.

*CYP2B6 Variability:* CYP2B6 is among the most polymorphic members of the P450 family. As noted previously, there are over 500 reported SNPs and nearly 40 of these SNPs result in non-synonymous changes. Figure 1.4 illustrates the most common CYP2B6 SNPs and the exon in which the base change occurs (Mo et al., 2009). To date, there are 38 reported allelic variants and the list is still growing. These variants can result in either increased or decreased expression or activity of the protein. The most frequent SNPs are studies in this project. These include *CYP2B6\*4* (785 A>G; K262R; rs2279343), *CYP2B6\*5* (1459 C>T; R487C; rs3211371), *CYP2B6\*6* (785 A>G, 516 G>T; K262R, Q172H; rs2279343, rs3745274), *CYP2B6\*7* (785

A>G, 516 G>T, 1459 C>T; K262R, Q172H, R487C; rs2279373, rs3211371, rs3745274) (dbSNP). The frequencies of these mutations vary between ethnic groups. For example, Hiratsuka et al. compared a population of 530 Japanese subjects with 470 Caucasian subjects and demonstrated statistically significant differences in allelic frequencies of the aforementioned SNPs. The observed differences in frequency reported by Hiratsuka et al. are as follows: *CYP2B6\*4* – 9.3% (Japanese) vs. 4.0% (Caucasian); *CYP2B6\*5* – 1.1% (Japanese) vs. 10.9% (Caucasian); *CYP2B6\*6* – 16.4% (Japanese) vs. 25.6% (Caucasian); and *CYP2B6\*7* – 0% (Japanese) vs. 3% (Caucasian) (Hiratsuka et al., 2002).

The influence of CYP2B6 polymorphisms on drug metabolism are most apparent with efavirenz and nevirapine, two drugs used to control the replication of human immunodeficiency virus (HIV) in infected individuals. CYP2B6 metabolizes efavirenz to 8'-hydroxyefavirenz. Subsequent oxidation of this metabolite by CYP2B6 forms 8', 14'-dihydroxyefavirenz (Ward et al., 2003). Carriers of the *CYP2B6\*6* allele experienced increased central nervous system (CNS) toxicity following efavirenz administration. Plasma concentrations of efavirenz were also higher in patients with the 516 G>T mutation (mutation present in allelic variants *CYP2B6\*6* and *CYP2B6\*7*) (King and Aberg, 2008). For some patients, the side effects were so severe that discontinuation of the drug was necessary (King and Aberg, 2008). Genotype effects were noted in patients who had 516 G>T single nucleotide polymorphism (present in *CYP2B6\*6* and *CYP2B6\*7* haplotypes, among others) with nevirapine as well. Patients who were either heterozygous or homozygous for 516 G>T had higher trough concentrations of nevirapine and higher systemic drug exposure (Mahungu et al., 2009; Uttayamakul et al., 2010; Vardhanabhuti et al., 2013).

The clinical effect of CYP2B6 polymorphisms varies depending on the substrate investigated. More often than not, the effect is not as straightforward as the cases presented above. For example cyclophosphamide, a nitrogen mustard-alkylating agent, is used in many chemotherapeutic regimens for the treatment of different cancers. The drug is administered as a pro-drug. Upon activation by oxidative metabolism, the metabolite cross-links DNA and prevents cell replication. The major enzymes involved in cyclophosphamide activation are CYP3A4 and CYP2B6. Genotype effects on cyclophosphamide oxidation are contradictory. Nakajima et al reported that patients carrying the *CYP2B6*\*6 allelic variant had greater cyclophosphamide clearance, suggesting increased activity of the enzyme *in vivo* (Nakajima et al., 2007). This work was followed up by Ariyoshi et al who evaluated the metabolism of cyclophosphamide in recombinant enzymes and found increased activity with CYP2B6.6 protein (Ariyoshi et al., 2011). In contrast, Raccor et al reported no appreciable genotype effect in recombinant enzymes and in a single donor human liver microsome bank (Raccor et al., 2012). Thus, effects of CYP2B6 genotype appear to substrate-dependent.

*CYP2B6 and methadone metabolism:* As described above, the effect of CYP2B6 polymorphisms on xenobiotics metabolism is both varied and substrate dependent. Investigating how these genetic mutations effect methadone metabolism may provide insight into the variability observed with methadone. *In vitro* studies published by a number of researchers established that CYP2B6 has the highest specific activity for methadone metabolism. In addition, CYP2B6 also stereoselectively metabolizes the *S*-enantiomer over the *R*-enantiomer which agrees with *in vivo* observations of methadone pharmacokinetics. These studies were performed in Supersomes<sup>®</sup> and human liver microsomes from single donors. In addition, *R*- and *S*- enantiomers through

competitive inhibition within the active site of CYP2B6 (Gerber et al., 2004; Totah et al., 2007; Totah et al., 2008).

*In vivo* studies in which healthy subjects were co-administered chemical inhibitors or inducers of CYP2B6 in combination with methadone resulted in marked changes in the drug exposure, providing further evidence that CYP2B6 could potentially be the major *in vivo* isoform involved in methadone clearance (Kharasch and Stubbert, 2013; Totah et al., 2008). Efavirenz, as noted above, is a substrate of CYP2B6 and there is evidence that concomitant use of efavirenz and methadone results in a drug-drug interaction. The effect of efavirenz was most consistent with hepatic induction of CYP2B6 and greater clearance of methadone (Kharasch et al., 2012). There is also data linking certain CYP2B6 genotypes to increased drug toxicity. Bunten et al suggested that the *CYP2B6\*6* allele, along with mutations in the opioid receptor, influence methadone toxicity. A post-mortem analysis of individuals who suffered a methadone fatality found that individuals carrying the *CYP2B6\*6* allele had statistically significant differences in methadone concentrations in blood, with approximately twice as high of concentration compared to individuals carrying *CYP2B6\*1* or *CYP2B6\*4* alleles. In addition, there was a statistically significant trend for higher frequency of *CYP2B6\*6* carriers in the post-mortem population (Bunten et al., 2010; Bunten et al., 2011). Given the evidence presented above, it is important to investigate the role of CYP2B6, and the associated allelic variants, to better understand methadone clearance and toxicity.

## **V. Proteins of Interest –ATP-binding cassette Efflux Transporters**

*ATP-binding cassette (ABC) Proteins:* The ATP binding cassette (ABC-ATPase) super-family of proteins encompasses two families: cytosolic non-transporters and transmembrane

transporters/channels (Jones and George, 2004). Both families are critical for homeostasis and cell integrity. The cytosolic proteins play a role in the maintenance and repair of DNA while the transmembrane proteins are important in osmotic homeostasis, nutrient uptake, and resistance to foreign insult (Jones and George, 2004). ABC efflux transporters are proteins that are ubiquitous throughout the three domains of life, archaea, bacteria, eukaryote (Ames et al., 1990; Higgins, 1992). The ABC genes encode for both transporters and channels which transport molecules across cellular membranes. Genetic polymorphisms in members of the ABC transporters are associated with diseases such as cystic fibrosis, Stargardt disease, age-related macular degeneration, etc. (Gottesman and Ambudkar, 2001). In eukaryotes, the ABC genes are efflux transporters and play a critical role in drug resistance and clearance of xenobiotics (Jones and George, 2004). Polymorphisms in drug-effluxing transporters could also have effects on protein expression and result in both variable bioavailability and clearance (Wang and Sadee, 2006; Dean et al., 2001).

*P-gp*: There are seven subfamilies of the ABC transporter family (named A- G) (Dean et al., 2001). For this project, the focus will be on P-glycoprotein (P-gp) and its role in efflux of methadone. P-gp is encoded by *ABCB1* and is located on chromosome 7.q21.12. The gene contains twenty-nine exons and twenty-eight introns (Bodor et al., 2005). The protein is expressed along the apical surface of the gastrointestinal tract (with an inverse relation with CYP3A4 expression), kidneys, and various blood-tissue barriers. P-gp is capable of effluxing both endogenous and exogenous substrates. P-gp substrate specificity overlaps with that of CYP3A4, and hence, CYP2B6 (Kim et al., 1999; Mo et al., 2009; Watanabe et al., 2013). Since it is expressed both in organs of elimination as well as along drug target sites (i.e. CNS), P-gp activity can effect drug action. An example was described by Ho et. al where P-gp, which was

co-expressed with BCRP1 (breast cancer resistant protein 1), was associated with chemotherapy resistance in patients with acute myeloid leukemia (Ho et al., 2008).

*P-gp Variability:* *ABCB1* is highly polymorphic and the most frequently studied polymorphism is the synonymous 3435 C>T (rs1045642). This mutation is in strong linkage disequilibrium with another synonymous SNP, 1263 C>T (rs1128503) (Crettol et al., 2008). While both SNPs do not affect the amino acid sequence, it has been suggested that the presence of both mutations cause mRNA structural deficiencies resulting in unstable mRNA and lower protein expression (Wang and Sadee, 2006). Lower P-gp expression results in more of the drug entering systemic circulation. The other common SNP investigated is 2677 G>T/A (rs2032585) (Crettol et al., 2008), which results in a change from an alanine to a threonine or serine. Individuals with 2677 GG were reported to have lower protein expression. Unfortunately thus far, the studies of genotype effects on P-gp expression have been largely inconclusive. As such, the clinical impact of these polymorphisms is difficult to ascertain as well (Leschziner et al., 2007; Owen et al., 2005).

*P-gp and methadone efflux:* Methadone has been shown to be a substrate of P-gp *in vitro*, and the protein appears to favor effluxing the *S*-enantiomer (Crettol et al., 2007). This finding is consistent with the stereoselective pharmacokinetic profile of methadone. In a study evaluating 60 non-opioid dependent subjects and 60 opioid dependent subjects, carriers of the two *ABCB1* genetic variants (rs1045642 and rs2032585) required lower methadone doses compared to wild-type. The genetic variants have been associated with lower protein expression, resulting in greater drug bioavailability (Coller et al., 2006). It has also been suggested that there are gene-gene interactions that occur between the *ABCB1* and *OPRM1*, the gene that encodes for the  $\mu$ -opioid receptor. Genetic variants in both genes (but not independently) influenced concentrations

of methadone in plasma (Barratt et al., 2012). P-gp activity in relation to methadone efficacy is particularly complex because not only does the protein affect how much drug enters systemic circulation, it also influences entrance of the drug through the blood-brain-barrier to reach the  $\mu$ -opioid receptors. Because of this, P-gp is also an important protein to consider when identifying targets of methadone variability.

## **VI. Summary and Scope of Research**

Metabolism and disposition of methadone is complex and unpredictable. Previous research has focused on the individual contribution of CYP3A4, CYP2B6, or P-gp to methadone variability, but rarely simultaneously. The hypotheses for this dissertation project are the following:

1. CYP3A4, CYP2B6, and P-gp activities contribute to variable methadone absorption and metabolism.
2. *CYP2B6* and *ABCB1* polymorphisms explain variability in enzymatic activity and hence absorption and metabolism of methadone.

These hypotheses will be addressed with both *in vivo* and *in vitro* studies. This dissertation project includes a pilot human subject study in healthy volunteers that characterizes each individual's CYP3A4, CYP2B6, and P-gp activity as a first step with specific probe substrates. The activity of these enzymes will then be correlated with methadone and EDDP clearance and exposure (Chapter 2). The second part will focus on the metabolism of methadone *in vitro* using hepatocytes, a bank of single-donor human liver microsomes (HLMs), and recombinantly expressed CYP2B6 wild type and common variants (Chapter 3). The HLM study will be conducted to determine the relative contribution of CYP3A4 and CYP2B6 to methadone

metabolism. Each single donor HLM used in the study has been fully characterized with regards to CYP3A4 and CYP2B6 protein content, and CYP2B6 genotype. A selected number of HLM samples will be phenotyped for CYP3A4 and CYP2B6 activity using specific probe substrates. The oxidation of methadone to EDDP will also be determined in the same liver samples and correlated to CYP3A4 and CYP2B6 protein content and activity. Finally, to determine if CYP2B6 common variants affect methadone metabolism, CYP2B6.1, CYP2B6.4, CYP2B6.5, and CYP2B6.6 enzymes will be recombinantly expressed and reacted with methadone at therapeutic concentrations. In Chapter 4, the results of both *in vivo* and *in vitro* investigations will be summarized and future studies will be proposed.

## VII. Figures

**Figure 1.1** -- Chemical structures of heroin, morphine, and methadone.

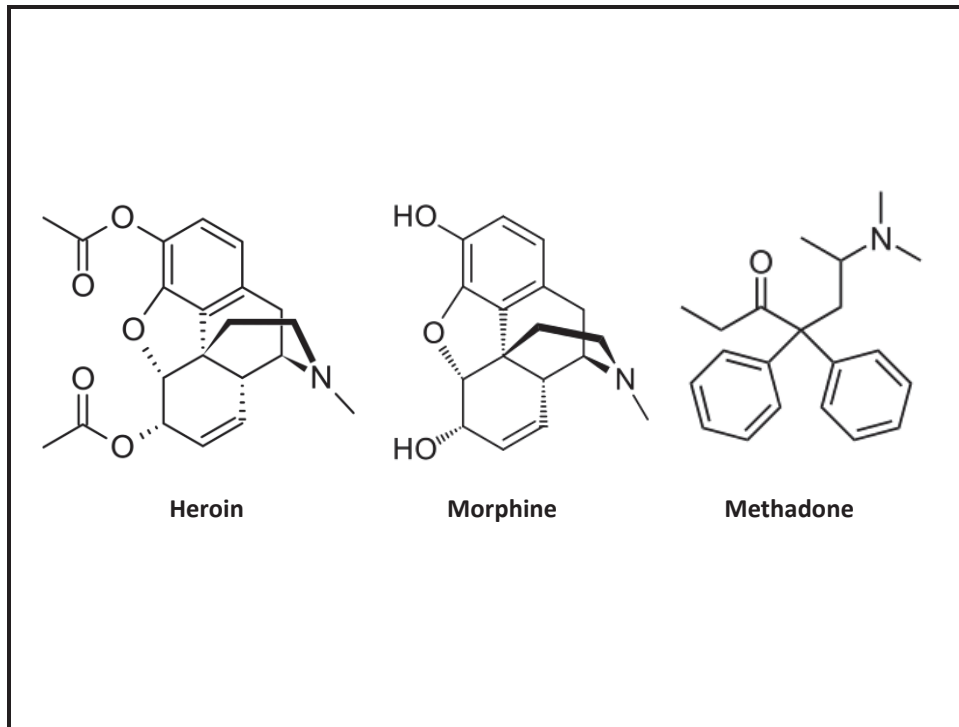
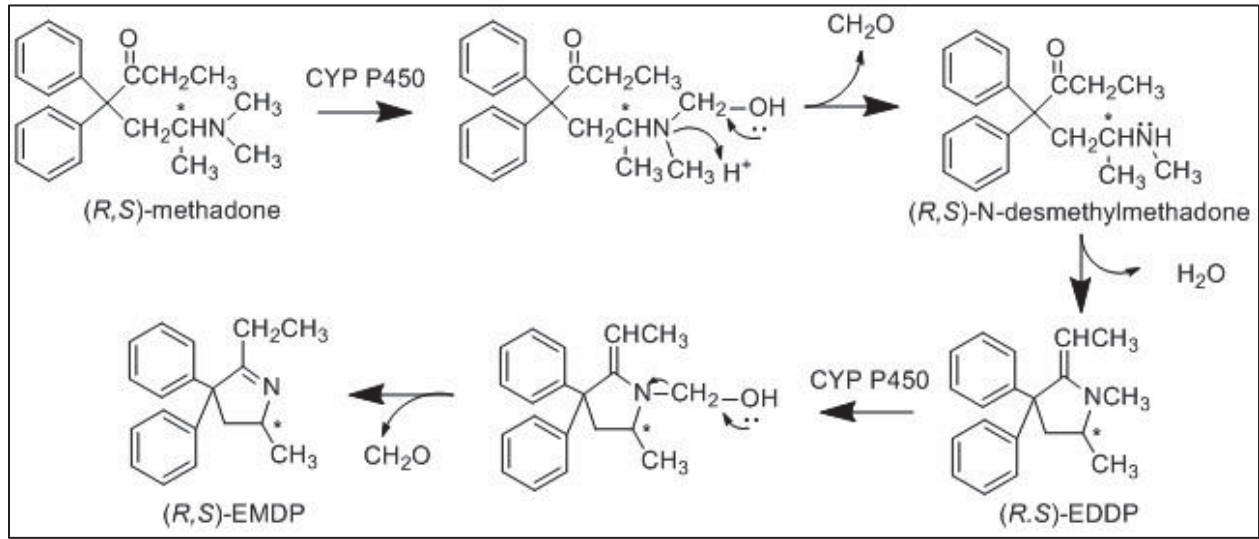
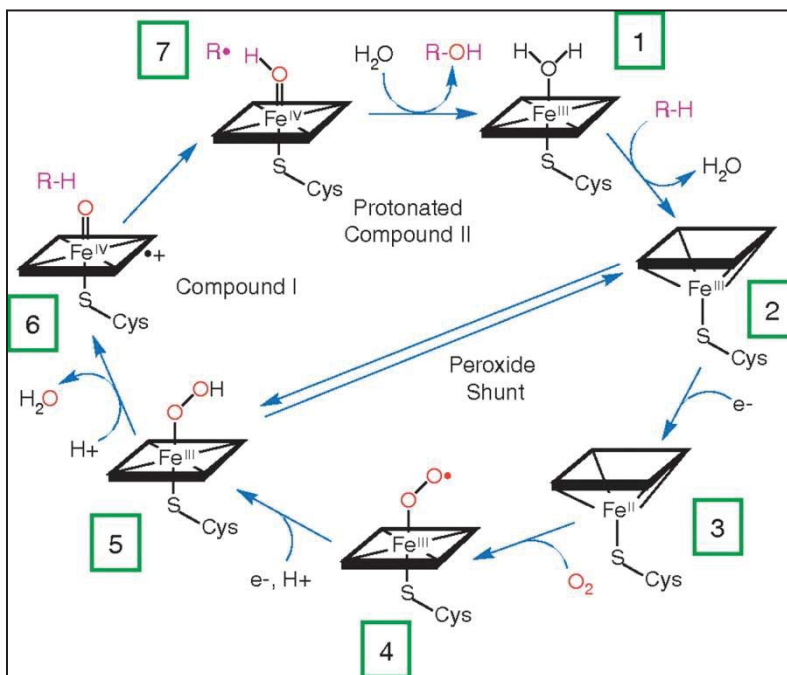


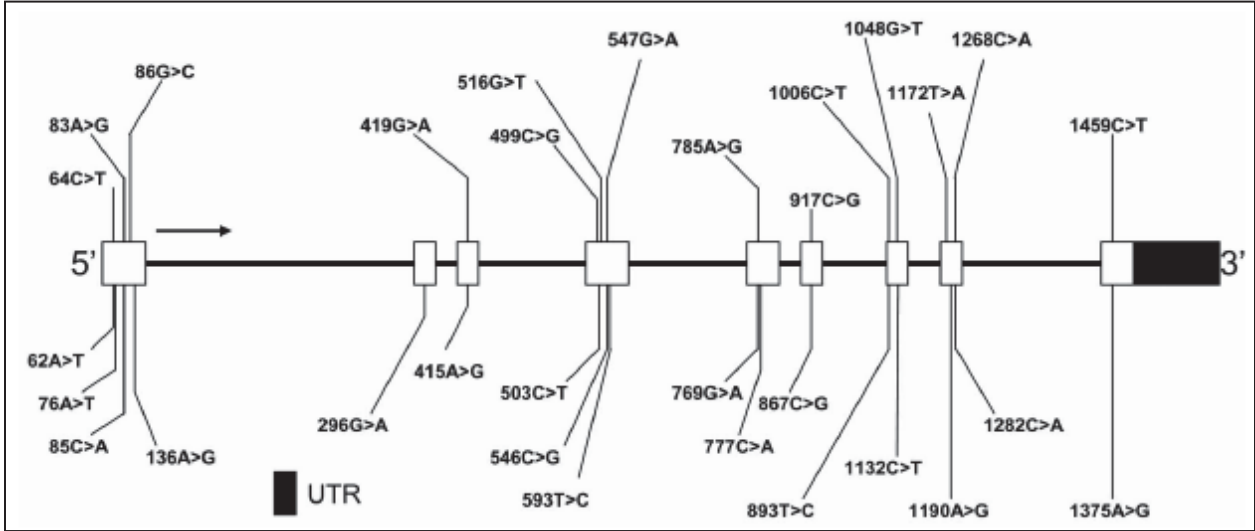
Figure 1.2 -- Methadone metabolism by P450 enzymes.



**Figure 1.3 -- P450 Oxidation Cycle.** (Krest et al., 2013). The first step of the P450 cycle is binding of the substrate replacing water in the 6<sup>th</sup> ligand position and shifting the iron spin state in the heme from a low-spin to high spin (steps 1 and 2). One electron is introduced by cytochrome P450 reductase (CPR) followed by oxygen binding to reduced iron (steps 3 and 4). Subsequent reduction, protonation and loss of water results in Compound I (steps 4, 5 and 6), which oxidizes the substrate of interest and returns enzyme to resting state (step 7).



**Figure 1.4** -- CYP2B6 gene and associated SNPs. Exonic non-synonymous single nucleotide polymorphisms of CYP2B6 (Mo et al., 2009).



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## Chapter 2: *In vivo* contribution of CYP3A4, P-gp, and CYP2B6 to the metabolism and absorption of orally administered methadone

### I. Introduction

Methadone is a  $\mu$ -opioid agonist used in the rehabilitation of opioid and heroin addicts in methadone maintenance treatment (MMT) programs (Peck and Beckett, 1976) and in the treatment of chronic pain (Leppert, 2009). Though the drug was first synthesized in the 1930s as a replacement for morphine, *in vivo* pharmacokinetics of this drug are still not well understood. Methadone displays high inter-individual variability, with 20-100 fold variability in its clearance (Plummer et al., 1988; Inturrisi et al., 1990). The reported time to achieve maximal concentration ranges from 2-4 hours and the duration of action is between 2-10 hours. The elimination of methadone is biphasic, with an initial half-life between 12-24 hours, and terminal half-life approximately 55 hours. The bioavailability ranges between 41%-91%, with a mean of 75% (Dale et al., 2002; Dale et al., 2004). An added complication is that methadone is chiral and administered as a racemic mixture, with *R*-methadone having approximately 10-fold greater affinity for the  $\mu$ -opioid receptor, (Kristensen et al., 1995) making it 50-fold more potent than *S*-methadone (de Vos et al., 1998). *In vivo*, methadone also displays stereoselective pharmacokinetics, with the *R*-enantiomer typically circulating at higher concentrations than *S*-methadone. *R/S*-methadone plasma ratios, when evaluated in a study of methadone maintenance treatment patients, varied from 0.63 – 2.4 (Eap et al., 2000).

Like other opioid drugs, methadone overdose causes severe respiratory and CNS depression that can be fatal. Methadone use has also been associated with life threatening cardiac arrhythmias, a unique toxicity among opioid drugs (Andrews et al., 2009). An incomplete understanding of methadone pharmacokinetics combined with severe adverse effects potentially contributes to the increase in fatal poisonings observed with methadone between the years 1999-

2007 (SAMHS 2010). This increase in fatal poisonings coincides with a surge in number of methadone prescriptions indicated for chronic pain control. While patients undergoing MMT rehabilitation are under rigorous management of their methadone use, the opposite is true for patients diagnosed with chronic pain. This is probably due to 1) many chronic pain patients being treated are in a hospice setting, where relief of pain in the terminal stage of life is the goal 2) pain control is difficult to achieve, 3) the inability of these patients to visit a treatment center daily as MMT patients would. Chronic pain patients are therefore at a higher risk for harm with both less supervision of methadone use and lack of understanding about the drug.

The purpose of this *in vivo* study is to reveal the proteins that contribute to drug disposition processes that determine the pharmacokinetics of methadone and thus contribute to its variability. P-glycoprotein (P-gp) is a transmembrane efflux transporter that is encoded by the multidrug resistance 1 gene, which is also known as ATP-binding cassette B1 gene (*MDR1* or *ABCB1*). Throughout this chapter, *ABCB1* nomenclature will be used. P-gp was first characterized in chemotherapy resistant tumors (Fojo et al., 1987). Since then, it was discovered that P-gp is expressed in a number of tissues, such as the gastro-intestinal tract, liver, kidneys, and at the blood-brain barrier (Fojo et al., 1987). *In vitro* studies show that methadone is effluxed by P-gp with reported stereoselectivity for *S*-methadone (Fojo et al., 1987; Crettol et al., 2007; Eap et al., 2007). The importance of P-gp *in vivo* with regards to oral methadone absorption is still unclear. *ABCB1* is also highly polymorphic which could contribute to inter-individual variability in methadone bioavailability. The most commonly reported SNPs are the: 3435 C>T (a synonymous change, rs1045642) and 2677 G>T/A (A893S, rs2032582). The 3435 C>T change is in linkage disequilibrium with another SNP, 1236 C>T (a synonymous change, rs1128503) (NCBI dbSNP <http://www.ncbi.nlm.nih.gov/projects/SNP/>).

Studies by Collier et al, found that subjects carrying the wild-type 2677G genotype required higher methadone doses compared to those who were either heterozygous or homozygous for the variant SNP 2677 G>T/A (Collier et al., 2006). Meanwhile other studies suggested that P-gp activity and genotype do not influence methadone absorption. However, these results are possibly confounded by the fact that the data gathered on P-gp genotype and methadone were performed in MMT patients who were likely on poly-therapy and/or had other comorbidities (Crettol et al., 2006; Crettol et al., 2008).

Once absorbed, *RS*-methadone undergoes oxidative metabolism by cytochrome P450 (P450) enzymes to form the major inactive metabolites *R*- or *S*- 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (*R*- or *S*-EDDP respectively) (Figure 2.1). EDDP can then be excreted or undergo further oxidation to form EMDP. In most studies, EDDP is the only metabolite studied because it is the most abundant and easily detected in plasma and urine (Sullivan et al., 1972; Sullivan and Due, 1973) (Figure 2.1)

A number of cytochrome P450 enzymes including CYP1A2, 2B6, 2C9, 2C19, 2D6, 3A4/5, 19, etc., are reported to carry out the oxidation of methadone (Eap et al., 2002; Kharasch et al., 2004; Lu et al., 2010). In early studies, CYP3A4 was identified as the key P450 isozyme involved in methadone oxidation because metabolism decreased in presence of CYP3A4 inhibitors (fluconazole, troleandomycin, and quercetin) or increased in presence of inducers (rifampin, phenobarbital, phenytoin, and carbamazepine) (Eap et al., 2002; Kapur et al., 2011; Moody et al., 1997; Moody et al., 2009). However, several of the inhibitors or inducers used were not specific to CYP3A4. Kharasch et al (2004) carried out an *in vivo* study in healthy volunteers evaluating methadone pharmacokinetics while concurrently taking either inhibitors or inducers of CYP3A4. Healthy volunteers were enrolled in a four stage study. Each subject's

hepatic CYP3A4 activity was evaluated with intravenous midazolam. Then each subject was simultaneously given oral stably labeled methadone and intravenous non-labeled methadone after pretreatment with either rifampin (3A4 inducer), troleandomycin (intestinal and hepatic 3A4 inhibitor), grapefruit juice (selective intestinal 3A4 inhibitor), or no treatment. The authors concluded that CYP3A4 activity did not correlate with methadone disposition *in vivo* since no significant difference in methadone exposure was observed with inhibitor and inducer treatments. Instead, their results suggested a more predominant role for other CYPs, such as CYP2B6, and potentially transporters. The *in vitro* work performed by the same group, however, suggested a significant role for both CYP3A4 and CYP2B6 (Kharasch et al., 2004). In contrast to this, Crettol et al conducted a study in 245 MMT patients and evaluated trough levels of methadone and CYP3A4 activity determined using a 30 minute 1-hydroxymidazolam to midazolam concentration ratio from orally administered midazolam as a phenotypic marker. Patients with lower CYP3A4 activity had significantly higher racemic, *R*-methadone, and *S*-methadone trough levels (Crettol et al., 2006).

CYP2B6 has been shown *in vitro* to stereoselectively metabolize methadone (Gerber et al., 2004; Totah et al., 2007; Totah et al., 2008) which is consistent with *in vivo* observations of a stereoselective pharmacokinetic profile. Gerber et al. (2004) demonstrated using a panel of Supersomes<sup>®</sup> that CYP2B6, CYP2C19, and CYP3A4 (in decreasing order) had highest methadone turnover compared to other isozymes tested. Multiple investigators confirmed that CYP3A4 is not stereoselective, CYP2B6 stereoselectively forms more *S*-EDDP while CYP2C19 displays the opposite stereoselectivity and forms more *R*-EDDP (Gerber et al., 2004; Totah et al., 2007). Subsequent *in vitro* studies evaluated Michaelis-Menten parameters of these three enzymes and theoretically scaled the turnover to protein expression levels observed in the liver.

It appears that both CYP3A4 and CYP2B6 play a central role in EDDP formation, however, CYP2B6 was responsible for the stereoselective metabolism of the drug (Totah et al., 2007). Kharasch et al. (2013) also investigated CYP2B6 contribution to methadone metabolism *in vivo*. Healthy subjects were simultaneously given oral stably labeled methadone and intravenous unlabeled methadone following pre-treatment with ticlopidine (CYP2B6 inhibitor). The investigators noted a 20% increase in *R*-methadone and 60% increase for *S*-methadone area-under-the-curve (AUC), a measure of drug exposure (Kharasch and Stubbert, 2013).

CYP2B6 is also of interest in methadone metabolism because it is one of the most polymorphic CYP enzymes, with over 100 reported SNPs, and nearly 40 allelic variants (Ingelman-Sundberg M, accessed April 2014). The number of reported allelic variants of *CYP2B6* continues to grow. It is probable that genetic variation may help explain inter-individual differences with methadone use. The most commonly reported allelic variants are: *CYP2B6*\*4 (785 A>G; K262R; rs2279343), *CYP2B6*\*5 (1459 C>T; R487C; rs3211371), *CYP2B6*\*6 (a combination of \*4 and \*9; and *CYP2B6*\*7 (785 A>G, 516 G>T; K262R, Q172H; rs2279343, rs3745274). As presented in Chapter 1, CYP2B6 genotype effects appear to be substrate dependent. Bunten et al. reported that *OPRM1*, the gene that encodes for the  $\mu$ -opioid receptor, and *CYP2B6* genetic variation lead to increased susceptibility to methadone toxicity and death (Bunten et al., 2010; Bunten et al., 2011). *CYP2B6* allelic variants, therefore, have the potential to be pharmacogenetic risk factors for methadone.

Altogether, studies investigating methadone metabolism and pharmacokinetics, both *in vitro* and *in vivo*, have resulted in divergent conclusions. It is still very much a question as to what extent, CYP3A4 or CYP2B6, are involved in methadone metabolism and elimination. Furthermore, oral administration is the most convenient dosage form for many patients, where

the complexity of absorption and the role of P-gp become important. The goal of this study is to simultaneously investigate the contribution of CYP3A4, CYP2B6, and P-gp to methadone pharmacokinetics in healthy subjects. In this study, midazolam, bupropion and digoxen were used as *in vivo* specific activity probe substrates. These are standard probe drugs for CYP3A4, CYP2B6, and P-gp activity (Hesse et al., 2004; Larsen et al., 2007; Thummel et al., 1994b; Thummel et al., 1994a). If activity of the target enzyme or transporter is determined quantitatively, within each individual with a specific phenotyping probe, confounding factors from previous qualitative studies may be eliminated. Each subject is also screened for common genetic variants of *CYP2B6* and *ABCB1* to determine genetic effects on methadone pharmacokinetics. Probe activity for each individual's CYP3A4, CYP2B6, and P-gp are correlated with *R,S*-methadone clearance, *R,S*-EDDP AUC to *R,S*-methadone AUC ratio, and *R,S*-EDDP AUC. This would establish an association between target enzyme/transporter activities and methadone bioavailability and clearances within the study population, and possibly allow an assessment of their quantitative role in methadone pharmacokinetics. In addition, it is possible that one or more of the proteins (CYP3A4, CYP2B6, or P-gp) in combination determine methadone pharmacokinetics *in vivo*. Therefore, forwards and reverse linear regression analysis is used to describe multiple protein involvement in methadone pharmacokinetics.

## II. Materials and Methods

### *Materials*

Racemic Methadone, racemic d<sub>9</sub>-methadone, racemic EDDP, racemic d<sub>3</sub>-EDDP, midazolam, d<sub>4</sub>-

midazolam, 1'-hydroxymidazolam, d<sub>4</sub>-1'-hydroxymidazolam standards were purchased from Cerilliant (Round Rock, TX). Racemic bupropion, digoxin, and digitoxin standards were purchased from Sigma-Aldrich (St. Louis, MO). Racemic hydroxybupropion-d<sub>6</sub> standard was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Acetonitrile, acetic acid, ammonium acetate, ammonium formate, ammonium hydroxide, formic acid, methanol, and m-tert-butyl-ether (MTBE) were purchased from Life Science –Fisher Scientific (Pittsburgh, PA). Expired plasma was obtained the Puget Sound Blood Center (Seattle, WA). Blank urine was obtained from healthy volunteers.

### *Clinical Protocol*

The human subject study was approved by the University of Washington Institutional Review Board. All subjects provided written consent to participate in the study. Study visits were carried out at the University of Washington Medical Center—Clinical Research Center. To be eligible for the study, subjects were: 1) between 18-40 years of age, 2) within 25% of expected BMI, and 3) healthy, with no evidence of cardiac, renal, or hepatic disease. Exclusion criteria were: 1) regular prescription drug use, with the exception of birth control, and 2) smoker. The study was divided into four visits. All subjects meeting the criteria outlined above were evaluated at a prescreen visit (Visit 1), where the study physician assessed eligibility by establishing a thorough medical history. An EKG was also recorded at the same visit and forwarded to the study cardiologist to ensure absence of underlying cardiac disease. Subjects entering each drug administration phase of the study were instructed to fast beginning midnight prior to each study day, and were asked to refrain from ingesting caffeine or grapefruit for a minimum of 24 hours prior to all subsequent visits. In Visit2, subjects were given 2 mg of oral midazolam, followed by 0.5 mg of oral digoxin one hour later to evaluate CYP3A4 and P-gp activity, respectively.

Venous blood draws were performed at 0, 0.25, 0.5, 1, 2, 3, 4, 6, and 8 hours. The following day, subjects were administered 150 mg of oral racemic bupropion (Visit 3) to evaluate CYP2B6 activity. Blood draws were performed at 0, 0.25, 1, 2, 4, 6, 8, 12, 24, 48, and 72 hours. A two week wash out period was needed following bupropion administration due to the drug's long elimination half-life. At the last visit (Visit 4), 10 mg of oral racemic methadone was administered. Blood draws were performed at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, 24, 48, and 72 hours. Urine was collected for the first 24 hours and dark-adapted pupilometry for the first 12 hours of this visit using a Colvard Oasis Pupillometer (Glendora, CA). Pupil constriction was measured at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 10, and 12 hours. Finally, a small aliquot of the subject's blood was collected during Visit 4 for DNA isolation. Nausea or vomiting experienced by subjects during Visit 4 was treated with ondansetron (4 mg IV or 8 mg po). The study design is depicted in (Figure 2.2).

### *Analytical Methods*

**Midazolam Sample Processing:** Midazolam plasma extraction was performed using liquid-liquid extraction (LLE). Plasma (100  $\mu$ L) was treated with an equal volume of cold acetonitrile containing 0.75 ng d<sub>4</sub>-midazolam and 0.75 ng d<sub>4</sub>-hydroxymidazolam as internal standards (7.5 ng/mL final plasma concentrations). Plasma samples were then centrifuged at room temperature at 16,300 x g on a Spectrafuge 24D microcentrifuge (Labnet International Inc., Edison, NJ) for 30 minutes. Supernatant was collected in a clean microcentrifuge tube and stored at -20°C until further analysis. Calibration standards were prepared using expired plasma, with concentrations of midazolam and hydroxymidazolam ranging from 0 – 25 ng/mL. Quality control samples, at concentrations of 1 and 20 ng/mL, were used to ensure validity of the calibration curves.

**Digoxin Sample Processing:** Digoxin plasma extraction was performed by LLE with digitoxin (1ng) added to 500  $\mu$ L of plasma as an internal standard (2 ng/mL final plasma concentration). Plasma was then treated with concentrated ammonium hydroxide (100  $\mu$ L) followed by 5 mL of methyl-tert-butyl ether (MTBE). The resulting mixture was then placed on a horizontal shaker for 10 minutes and later centrifuged at 4°C at 3488 x g on a Sorvall Legend XTR tabletop centrifuge (ThermoScientific Inc., Waltham, MA) for 10 minutes. To allow ease of removal of organic layer, the samples were frozen overnight at -20°C. The following day, the organic layer was poured into a clean glass test tube and evaporated under N<sub>2</sub> at 25°C. The sample was then reconstituted with 100  $\mu$ L of LC-MS/MS mobile phase at initial run conditions (see below) and vortexed. The vortexed samples were centrifuged at room temperature at 16,300 x g on a Spectrafuge 24D microcentrifuge for 30 minutes. The supernatant was removed and placed in a clean microcentrifuge tube and stored at -20 °C until further analysis. Calibration standards were prepared using expired plasma with digoxin concentrations ranging from 0 – 2 ng/mL. Quality control samples with concentrations of 0.15 and 1.75 ng/mL were used to ensure validity of the calibration curve.

**Bupropion Sample Processing:** Bupropion extraction from plasma was performed by LLE. Plasma (250  $\mu$ L) was treated with cold acetonitrile (500  $\mu$ L) containing 200 ng racemic d<sub>9</sub>-bupropion (400 ng/mL final plasma concentration) and 125 ng racemic d<sub>6</sub>-hydroxybupropion (250 ng/mL final plasma concentration). Deuterated bupropion internal standard was added in excess to ensure an adequate signal during LC-MS/MS analysis. Plasma samples were centrifuged at room temperature at 16,300 x g on a Spectrafuge 24D microcentrifuge for 30 minutes. Supernatant was collected in a clean microcentrifuge tube and stored at -20 °C until analysis. Calibration standards were prepared using expired plasma. Bupropion calibrations

standards ranged from 0– 50 ng/mL (0 – 100 racemic bupropion), and hydroxybupropion standards ranged from 0 – 500 ng/mL (0 – 1000 ng/mL racemic hydroxybupropion). Quality control samples were prepared separately with concentrations 2.5 and 25 ng/mL for bupropion (5 and 50 ng/mL racemic bupropion) and 10 and 100 ng/mL for hydroxybupropion (20 and 200 ng/mL racemic hydroxybupropion) to ensure validity of calibration curves.

**Methadone Sample Processing:** Methadone plasma and urine extractions were performed by LLE. Plasma or urine 250  $\mu$ L was treated with an equal volume of cold acetonitrile containing 12 ng racemic d<sub>9</sub>-methadone (48 ng/mL final plasma concentration) and 2.4 ng racemic d<sub>3</sub>-EDDP (9.6 ng/mL final plasma concentration) for plasma and 12.5 ng racemic d<sub>9</sub>-methadone and 12.5 ng racemic d<sub>3</sub>-EDDP for urine (50 ng/mL final urine concentrations). Plasma and urine samples were centrifuged at room temperature at 16,300 x g on a Spectrafuge 24D microcentrifuge for 30 minutes. The organic layer was collected into a clean microcentrifuge tube and stored in a -20°C freezer until further analysis. Calibration standards were prepared using either expired plasma or donated urine. Methadone calibration standards ranged from 0– 100 ng/mL for plasma (0 – 200 ng/mL racemic methadone) and 0– 500 ng/mL for urine (0 – 1000 ng/mL racemic methadone). EDDP calibration standards ranged from 0– 25 ng/mL for plasma (0 – 50 ng/mL racemic EDDP) and 0– 500 ng/mL for urine (0 – 1000 ng/mL racemic methadone). Quality control concentrations were 3.75 and 37.5 ng/mL for methadone (7.5 and 75 ng/mL racemic methadone) and 2 and 10 ng/mL for EDDP (4 and 20 ng/mL racemic EDDP) in plasma. In urine, quality control concentration for methadone and EDDP were 5, 50, and 125 ng/mL (10, 100, and 250 ng/mL racemic methadone and EDDP). In addition to studying plasma methadone and EDDP levels, an exploratory metabolite identification study was performed in both extracted plasma and urine.

### *Mass Spectrometry Sample Analysis*

**Midazolam:** Midazolam samples were processed on an Agilent Zorbax reverse-phase XDB-C8 (2.1 x 50 mm, 50  $\mu$ M) column attached to a Phenomenex XDB-C8 guard column (4 x 2.0 mm). This was connected to Shimadzu SCL – 10A VP pump (Shimadzu Specific Instruments, Columbia, MD) in tandem with a Micromass UK Limited triple quadrupole mass spectrometer operated in electrospray positive ionization mode (Waters Corporation, Milford, MA). The injection volume was 5  $\mu$ L. The aqueous solvent was 0.1% acetic acid in water (solvent A) and the organic solvent was 0.1% acetic acid in methanol (solvent B). Flow rate was 0.3 mL/min. All changes in solvent percentage were linear with time. The gradient for solvent B was as follows: 45% for 0.5 min; increased to 60% at 2 min which was maintained until 2.5 mins; increased to 90% by 3 mins and this was maintained until 4 mins; by 5 min, the gradient returned to initial starting conditions. The mass fragmentation transitions monitored and the corresponding cone voltage (CV) and collision energy (CE) were: midazolam (326.00 > 291.20, CV = 40.0 V, CE = 27.0 V), d<sub>4</sub>-midazolam (330.00 > 295.20, CV = 40.0 V, CE = 27.0 V), 1'-hydroxymidazolam (342.00 > 324.20, CV = 40.0 V, CE = 22.0 V), and d<sub>4</sub>-hydroxymidazolam (346.00 > 328.20, CV = 40.0 V, CE = 22.0 V). Elution times for midazolam and 1'-hydroxymidazolam were 1.7 min and 2.6 min, respectively. A representative chromatogram is shown in Figure 2.4.

**Digoxin:** Digoxin samples were processed on an Agilent Zorbax XDB-C8 (2.1 x 50 mm, 50  $\mu$ M) column attached to a Phenomenex XDB-C8 guard column (4 x 2.0 mm). This was connected to an Agilent Technologies 1290 Infinity (Agilent Technologies, Santa Clara, CA) in tandem with an Applied Biosystems API4000 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA). The injection volume was 15  $\mu$ L. The aqueous solvent used was 10 mM ammonium formate in Optima water, pH = 3.4 (solvent A) and the organic solvent used was 10 mM

ammonium formate in Optima methanol (solvent B). All changes in solvent percentage occurred linearly. Flow rate was 0.3 mL/min. The gradient used for solvent B is as follows: 30% 1 min; to 37% at 2.5 min; increase to 68% at 3 min and held until 5 min; a final ramp of solvent B to 100% at 5.5 min and this was held until 7.5 min to clean the column. At 8 min, solvent conditions were returned to initial conditions and the column was allowed to re-equilibrate for 2 min. The mass fragmentation transitions monitored and the corresponding declustering potential (DP) and collision energy (CE) were: digoxin (798.4 > 651.5, DP = 11.0, CE = 21.0) and digitoxin (782.4 > 635.5, DP = 46.0, CE = 17.0). Elution times for digoxin and digitoxin were 3.4 min and 4.4 min, respectively. A representative chromatogram is shown in Figure 2.5.

**Bupropion:** Chiral separation of bupropion samples was achieved using a reverse phase chiral-AGP column (100 x 2 mm, 5  $\mu$ M) attached to a chiral-AGP guard cartridge (10 x 2mm, 5  $\mu$ m) (ChromTech, Apple Valley, MO). This was connected to an Acquity Waters UPLC in tandem with a Micromass Premier XE triple quadrupole mass spectrometer (Waters Corporation, Milford, MA). The injection volume was 5  $\mu$ L. The aqueous solvent was 20 mM ammonium formate pH 5.7 (solvent A) and the organic solvent was methanol (solvent B). All changes in solvent B percentage occurred linearly. Flow rate was 0.22 mL/min. The gradient for solvent B used was as follows: 10% 0.5 min; increase to 30% at 5 min and hold until 6 min; increase to 50% at 10 min and held until 15 min. At 16 min, solvent conditions were returned to initial conditions and the column was allowed to re-equilibrate for 4 min. The mass fragmentation transitions monitored and the corresponding cone voltage (CV) and collision energy (CE) were: bupropion (240.1 > 184.1, CV = 20.0, CE = 15.0), *d*<sub>9</sub>-bupropion (249.1 > 184.1, CV = 20.0, CE = 15.0), hydroxybupropion (256.1 > 238.1, CV = 20.0, CE = 15.0), and *d*<sub>6</sub>-hydroxybupropion (262.1 > 244.1, CV = 20.0, CE = 15.0). Elution times for *R*-bupropion, *S*-bupropion, *R,R*-

hydroxybupropion, and *S,S*-hydroxybupropion were 6.8 min, 8.0 min, 11.8 min, and 7.0 min, respectively. A representative chromatogram for bupropion and hydroxybupropion is presented in Figure 2.6.

**Methadone:** Chiral separation of methadone samples was achieved using a reverse phase chiral-AGP column (100 x 2 mm, 5  $\mu$ M) attached to a chiral-AGP guard cartridge (10 x 2mm, 5  $\mu$ m) (ChromTech, Apple Valley, MO). This was connected to an Acquity Waters UPLC in tandem with a Micromass Premier XE triple quadrupole mass spectrometer. The injection volume was 5  $\mu$ L. The aqueous solvent was 20 mM ammonium formate pH 5.7 (solvent A) and the organic solvent was methanol (solvent B). All changes in solvent percentage occurred linearly. Flow rate was 0.22 mL/min. The gradient for solvent B was as follows: 5% for 2 min; increase to 10% at 4 min and held until 7 min; increased to 30% at 9 min and this was held until 10 min; solvent B was then increased to 40% at 11 min and this was held until 14 min. At 16 min, solvent conditions were returned to initial conditions. The column was allowed to re-equilibrate for 4 min. The mass fragmentation transitions monitored and the corresponding cone voltage (CV) and collision energy (CE) were: methadone (310.1 > 268.2, CV = 30.0, CE = 15.0), *d*<sub>9</sub>-methadone (319.1 > 268.2, CV = 30.0, CE = 15.0), EDDP (278.2 > 234.2, CV = 45.0, CE = 30.0), and *d*<sub>3</sub>-EDDP (281.0 > 234.2, CV = 45.0, CE = 30.0). Elution times for *R*-methadone, *S*-methadone, *R*-EDDP, and *S*-EDDP were 14.25 min, 15.2 min, 13.6 min, and 15.0 min, respectively. A representative chromatogram is shown in Figure 2.7.

**Methadone Metabolite Identification Method:** Extracted plasma (pre-dose and 12 hour) and urine (pre-dose and 0-12 hour) samples from each subject were analyzed on a Waters Synapt G2-Si-QTOF UPLC-MS/MS in order to identify all major metabolites of methadone. The injection volume was 2  $\mu$ L. The aqueous solvent was 20 mM ammonium formate pH 5.7 (solvent A) and

the organic solvent was methanol (solvent B). The ionization mode was ESI in positive W mode. The reference file used was ESI sodium formate in positive mode with a mass-to-charge range between 100 and 1000. The lock mass reference used was leucin enkephalin which has a mass to charge ratio of 566.88915. All changes in the gradient occurred linearly. Flow rate was 0.3 mL/min for the entirety of the run. The gradient conditions used are as follows: initial conditions at the beginning of the gradient was 10% solvent B for 1 min; the percentage of solvent B was increased to 98% at 8 min and this was held until 10 min; the system was returned to initial conditions at 10.10 min and this was held until 13 min in order to re-equilibrate the column.

**Genotyping of subjects:** Whole blood (7 mL) was collected at Visit 4 in a PAXgene Blood DNA Tube containing proprietary storage buffer and stored at 4°C until extraction. Genomic DNA was extracted from whole blood using a PreAnalytiX Whole Blood Extraction Kit. Once extracted, all DNA was stored at -20 °C until genotype analysis. DNA concentration was determined using a NanoDrop 2000 UV-Vis Spectrophotometer (ThermoScientific, Wilmington, DE). All subjects' DNA A260/A280 ratio was determined to be greater than 1.7 and of good quality for genotyping assays. Available TaqMan Primers were used to determine *CYP2B6*\*5 (rs3211371), *CYP2B6*\*6 (\*9 rs3745274, which is in linkage disequilibrium with \*4 SNP and results in the \*6 haplotype), and *ABCB1* 3435 C>T (rs1045642) genotype. Final subject DNA was diluted to 1 ng/uL in DNA grade water. Each reaction contained 4 ng of genomic DNA, 7.25 µL DNAase-free water, 12.5 µL 2X TaqMan Universal PCR Mix, and 1.25 µL of SNP-specific Drug Metabolizing Genotyping Assay Mix. All samples were then analyzed with a StepOnePlus PCR and software (Applied Biosystems – Life Technologies, Grand Island, NY). *CYP2B6*\*4 (rs2279343) and *ABCB1* 2677 C>T (rs2032582) genotype was determined by PCR amplification in the region of the SNP and subsequent DNA sequencing. PCR reactions were performed

with 25 ng of genomic DNA, 200 nM of *CYP2B6*\*4 primers or 250 nmol of *ABCB1* 2677 C>T primers, and Herculase II Fusion DNA Polymerase (Agilent Technologies, Santa Clara, CA). The primers used are listed in Table 2.1. The PCR cycling conditions are as follows: 1) 95 °C, 2) 95 °C x 20 seconds, 3) 60 °C x 20 seconds, 4) 72 °C x 60 seconds, 5) repeat steps 2-4 30 times, 6) 72 °C x 3 minutes, and 7) hold at 4 °C. PCR purification was performed using PureLink PCR Purification Kit (Life Technologies, Invitrogen, Grand Island, NY).

### *Data Analysis*

Noncompartmental pharmacokinetic parameters were determined by inputting plasma drug concentration-time data into Phoenix 64 WinNonLin 6.3 (Pharsight – Certara L.P, St. Louis, MO). Mean, standard deviation, median, and range for these parameters are reported in Table 2.3 - Table 2.13. Figure 2.8 -- Figure 2.11 display the average and standard deviation of AUCs of probe substrates, corresponding metabolites where appropriate, and methadone and EDDP. Estimated parameters were used to calculate apparent oral clearance and determine corollary relationships. Midazolam apparent oral clearance ( $Cl_{app} = Cl/F = \text{Dose}/\text{AUC}_{parent}$ ) was used as the *in vivo* phenotypic marker of CYP3A4 activity (Thummel et al., 1994b; Thummel et al., 1994a). Digoxin plasma AUC of the first five hours post drug administration was used to assess P-gp activity in the gastrointestinal tract (Larsen et al., 2007).

To probe *in vivo* CYP2B6 activity, the commonly used probe drug bupropion was administered (Hesse et al., 2004). However, the choice to use bupropion is problematic for the following reasons: 1) bupropion elimination by CYP2B6 is relatively a minor pathway compared to carbonyl reductase and 11 $\beta$ -hydroxysteroid dehydrogenase 1 biotransformation (Meyer et al., 2013), 2) bupropion is administered as a racemic mixture and it is well known that the enantiomers interconvert at neutral pH due to the labile proton that is alpha to the carbonyl

moiety 3) the clearance of *R,R*-hydroxybupropion is elimination-rate limited and circulates at concentration much higher than the parent, while *S,S*-hydroxybupropion clearance is formation rate limited. 4) *in vitro*, CYP2B6 favors the formation of *S,S*- over *R,R*- hydroxybupropion. For these reasons the ratio of *S,S*-hydroxybupropion AUC to *S*-bupropion AUC, total hydroxybupropion AUC, as well as *S,S*-hydroxybupropion AUC were evaluated as possible *in vivo* phenotypic markers of CYP2B6 activity. *S,S*-hydroxybupropion AUC to *S*-bupropion AUC ratio and total hydroxybupropion AUC have been previously used by other investigators to study CYP2B6 activity *in vivo* (Coles and Kharasch, 2007; Benowitz et al., 2013). *S,S*-hydroxybupropion AUC was added to the analysis because CYP2B6 stereoselectively metabolizes *S*-bupropion over *R*-bupropion and the *S,S*-hydroxybupropion metabolite may be a more sensitive probe of CYP2B6 activity.

The following parameters were used to describe methadone disposition *in vivo*: *R*-, *S*-, and total methadone apparent clearance ( $Cl_{app} = Cl/F = \text{Dose}/\text{AUC}_{\text{parent}}$ ), *R*-, *S*-, and total EDDP AUC to methadone AUC ratio, and *R*-,*S*- and total EDDP AUC. Measures of methadone disposition were first correlated with markers of CYP3A4, CYP2B6, and P-gp activity to determine the contribution of each individual enzyme or transporter to methadone absorption and metabolism. Significant association between individual proteins and different outcome variables was analyzed using a Spearman's rank correlation coefficient, rho. A rho value of 1 (or -1) suggests that all variation in the outcome variable studied could be described by the independent variable tested. The positive or negative sign for the Spearman rho indicates either a positive or negative correlation, respectively. Association calculations were performed using GraphPad Prism 5.02 (La Jolla, CA). P-values obtained for association studies were adjusted for multiple

comparisons using the Bonferonni correction. An adjusted p-value of 0.05 was considered significant.

Forward and reverse, step-wise, linear multivariate regression analyses were then performed. These analyses were performed to determine if an additive, multi-enzyme model would result in a better description of either methadone clearance or metabolite exposure. For these analyses, midazolam clearance was used as a phenotypic marker of CYP3A4 activity, digoxin 5-hr AUC was used as a phenotypic marker of P-gp, and *S,S*-hydroxybupropion AUC was used as a measure of *in vivo* CYP2B6 activity because this variable was associated with several outcome variables. The following is the linear model and description of constants used in the analyses. In models where only one or two proteins are considered, the estimate of the slope of the variables not considered is set to zero.

**Model used:**  $Y_X = \beta_0 + \beta_1x_1 + \beta_2x_2 + \beta_3x_3$

- $Y_1$  = Estimate Total methadone apparent oral clearance
- $Y_2$  = Estimate *R*-methadone apparent oral clearance
- $Y_3$  = Estimate *S*-methadone apparent oral clearance
- $Y_4$  = Estimate total EDDP elimination clearance
- $Y_5$  = Estimate *R*-EDDP elimination clearance
- $Y_6$  = Estimate *S*-EDDP elimination clearance
- $\beta_0$  = Y-intercept
- $\beta_1$  = Estimate CYP3A4 slope
- $\beta_2$  = Estimate P-gp slope
- $\beta_3$  = Estimate CYP2B6 slope

The reasoning for modeling both methadone clearance and EDDP AUC was to determine; 1) CYP3A4, CYP2B6, and P-gp additive contribution to total and stereoselective methadone absorption and metabolism of parent drug, and 2) CYP3A4 and CYP2B6 additive contribution to total and *R*-, *S*- or total EDDP AUC. Multiple variable forwards and reverse linear regression tests were performed using R 3.0.1 (The R Foundation for Statistical Computing, Institute for Statistics and Mathematics, Wirtschaftsuniversitet, Vienna, Austria). The effects of genotype were assessed by using a Kruskal-Wallis statistical test using GraphPad Prism 5.02. Subjects of a particular *CYP2B6* and *ABCB1* genotype were stratified and the mean *R*-, *S*- and total methadone clearance, and *R*-,*S*- and total EDDP AUC were compared between genotype groups. Post-hoc analysis was done to compare differences between wild-type and individual allelic variants when the results from the Kruskal-Wallis statistical test suggested clear differences between genotype.

#### *Metabolite identification*

Metabolite identification in patient plasma and urine following methadone administration was performed to determine if EDDP was the main metabolite of methadone oxidation in plasma and urine and to explore the possibility of other pathways of metabolism. Pre-dose samples were used to account for background signal resulting from the matrix analyzed, either plasma or urine. The 12 hour plasma sample and the 0-12 hour urine sample were studied because both methadone and EDDP analytes were readily detectable in these samples by Micromass Premier XE triple quadrupole mass spectrometer. The structure of methadone and exact mass was inputted into Metabolyx XS software. Phase I and II metabolism and associated differences in *m/z* were probed. Correct chemical formula, a mass within 5 ppm of chemical formula, and lack of presence in pre-dose samples were the criteria for identification of a specific metabolite.

### III. Results

#### *Subject Demographics and Variability in Methadone Pharmacodynamics*

Nineteen healthy volunteers were enrolled in the study. The sample population consisted of 13 males and 6 females that were representative of the multi-cultural population of King County, Washington. Details on the cohort demographics are provided in Table 2.2. There were notable differences in pupil miosis, a pharmacodynamic marker of opioid activity, between subjects after administration of methadone. The average change in pupil diameter was 2.2 mm with a standard deviation of 0.8 mm. Average absolute values and standard deviation of pupil diameter change in the nineteen subjects was plotted versus time of measurement (Figure 2.3). Pupil measurement times corresponded with blood sampling. The time to maximal change in pupil diameter varied between subjects from 2-10 hours. This indicates a five-fold difference in time to maximal therapeutic action. Only 25% of subjects' pupil diameter returned to baseline at the final (12 hour) pupil measurement. Interestingly, the severity of nausea and vomiting experienced between subjects was also variable. Some individuals only noted mild dizziness, while others required pharmaceutical intervention (ondansetron 8 mg PO or 4mg IV) to treat adverse side effects. It was determined that the sample of subjects within the study displayed high inter-individual variability in methadone pharmacodynamic effects and served as a representative and diverse population for pharmacokinetic analysis.

#### *Subject Variability in Methadone Pharmacokinetics*

All parameters calculated to describe methadone disposition were variable, typically with the *S*-enantiomer parameters exhibiting greater variability than *R*-enantiomer parameters. The average *R*-methadone and *S*-methadone clearance in the subject population was 14.3 L/hr and

11.8 L/hr, respectively. The values for *R*-methadone clearance varied four-fold, with values ranging from 7.51 – 29.8 L/hr. *S*-methadone clearance appeared to be more variable, with approximately six-fold difference between subjects (4.81 – 27.5 L/hr). The average total methadone clearance was 12.6 L/hr with five fold variation ranging from 5.9 – 28.6 L/hr. The average *R*-EDDP to *R*-methadone AUC ratio was 0.09, with values ranging from 0.05 – 0.16, approximately a three-fold in difference. The *S*-EDDP to *S*-methadone AUC ratio varied more in the subject population, with an average of 0.14 and value ranging from 0.06 – 0.27, approximately a five-fold difference. The total EDDP to methadone AUC ratio average in the subject population was 0.12, and varied approximately four fold with values ranging from 0.06 – 0.22. The average *R*-EDDP and *S*-EDDP AUC values were 34.2 and 64.5 ng/mL\*hr, respectively. Both parameters varied approximately four fold with *R*-EDDP AUC ranging from 16.8 – 73.7 ng/mL\*hr and *S*-EDDP AUC ranging from 34.9 – 145.8 ng/mL\*hr. Total EDDP AUC average was 98.8 ng/mL\*hr and values for this parameter ranged between 51.8 – 219.5 ng/mL\*hr.

Metabolism of methadone was stereoselective in the subject population. Enantiomer percentages were calculated by taking the ratio of either *R*- or *S*-enantiomer to the sum of *R*- and *S*- enantiomers. The average and standard deviation of these percentages within the subject population were plotted versus time. On average, over the course of 72 hours, the percentage of *S*-methadone relative *R*-methadone decreased. Interestingly, *S*-EDDP percentage relative to *R*-EDDP did not change, and *S*-EDDP was always present at greater concentration than *R*-EDDP (Figure 2.12). The average *R/S*-methadone AUC ratio among subjects was 0.81 and ranged from 0.57 – 1.81. The average *R/S*-EDDP AUC ratio in the subject population was 0.53 and ranged from 0.41 – 0.73.

### *Phenotypic Marker Determination of CYP3A4, P-gp, and CYP2B6 Activity*

Midazolam apparent oral clearance is used as the phenotypic marker of CYP3A4 activity. With the exception of two individuals, CYP3A4 activity did not differ greatly between subjects in this population studied. The average midazolam AUC in the subject studied was 32.1 ng/mL\*hr, with a range of 13.4 – 86.2 ng/mL\*hr. Digoxin five hour AUC is used as a probe of P-gp activity. Theoretically, determination of the absorption rate constant,  $K_{abs}$ , of digoxin is the most accurate measure of P-gp activity in the gastrointestinal tract. A surrogate marker of intestinal P-gp activity that has been used with success was introduced by Larsen et al (2007) is a four hour AUC of digoxin. The four hour point was used because it represented the systemic exposure of digoxin during the absorption phase (Larsen et al., 2007). In this investigation, five hour digoxin AUC was used because this data point was closest to four hours and included the entirety of the absorption phase. Digoxin five hour AUC varied approximately four-fold between subjects. The average digoxin AUC was 4.7 ng/mL\*hr and the range was 1.7 – 6.9 ng/mL\* hr within the subject population.

Bupropion hydroxylation was used as an *in vivo* marker for CYP2B6 activity. The ideal measurement of enzyme activity would have been hydroxybupropion formation clearance ( $Cl_f$ ). However, to calculate a formation clearance, both the metabolite of interest and parent must be administered separately. This is because the requisite estimate for fraction metabolized ( $f_m$ ) can only be obtained from knowledge of the elimination clearance of the primary metabolite of interest (Houston, 1981). Direct study with synthetic metabolite is not feasible and the metabolite to parent drug ratio AUC ( $AUC_m/AUC_p$ ) is used as an indirect index of  $Cl_f$ . Unfortunately, the use of this AUC ratio is confounded by the complex disposition of hydroxybupropion and bupropion. Because of this,  $AUC_m$ , both total and *S,S*-hydroxybupropion, were also evaluated as

a potential alternative, phenotypic marker of *in vivo* CYP2B6 activity. Despite these limitations, bupropion is still considered the *in vivo* probe of choice to study CYP2B6 activity especially in studies where CYP2B6 activity is augmented by inhibitors or inducers. The mean *S,S*-hydroxybupropion to *S*-bupropion AUC ratio was 3.85 and this proportion varied six-fold between subjects (ratio range: 1.5 – 9.0). The mean total hydroxybupropion AUC was 10662.7 ng/mL and this parameter varied seven-fold in the subject population (total hydroxybupropion range: 4103.3 – 29242.5 ng/mL\*hr). The mean *S,S*-hydroxybupropion AUC was 559.6 ng/mL\*hr and this parameter varied ten-fold between subjects (*S,S*-hydroxybupropion AUC range 183.3 – 1791 ng/mL).

As previously mentioned, bupropion is primarily metabolized by carbonyl reductases and 11 $\beta$ -hydroxysteroid dehydrogenase to form enantiomers of erythro- and threo-hydrobupropion (Meyer et al., 2013). The effect of these other pathways on bupropion metabolism in confounding the use of metabolite-to-parent drug AUC ratio as a surrogate of formation clearance is more readily apparent when it is illustrated in the diagram detailing drug absorption and metabolism (Figure 2.13). In the Figure 2.13, drug is administered orally ( $D_{po}$ ) and a certain fraction of drug is absorbed from gastrointestinal tract and enters the liver through the hepatic portal vein and is termed the amount absorbed ( $A_{abs}$ ). A fraction of  $A_{abs}$  is metabolized and both parent drug and metabolite then enter systemic circulation where further elimination of the two species can occur. The amount of parent drug and metabolite in the body system is represented in the figure as ARE,  $ARE_{mi}$ , and,  $ARE_{mj}$  where ARE is amount remaining to be excreted of parent and metabolites i and j, respectively. The amounts eliminated are represented by  $A_{el}$ ,  $A_{el}(mi)$ , and  $A_{el}(mj)$ , for parent and metabolites i and j, respectively (Weiss, 1988).

For illustrative purposes, a comparison between an ideal probe (one in which a single metabolite is formed by one enzyme (high  $fm$ )) and hydroxybupropion formation clearance will be made. In an ideal scenario, in Figure 2.13,  $ARE_{mi}$ , relative to  $A_{abs}$  and  $ARE$  is an accurate measure of formation clearance because  $A_{abs}$  and  $ARE$  are both not being biotransformed by any other mechanism in systemic circulation. In other words, the pathway of  $A_{abs}$  and  $ARE$  to  $ARE_{mj}$  are not significant and can be removed from consideration in the case of the ideal probe. The ratio of  $AUC_m/AUC_p$  reflects fraction of parent drug that is absorbed and converted to metabolite  $m_i$ . However, for bupropion, which has multiple, stereoselective, and polymorphic pathways of metabolism, the AUC of metabolite alone may be a more accurate indicator of CYP2B6 activity. The value of  $AUC_m$  is a direct indicator of the amount of metabolite  $i$  absorbed and converted by CYP2B6 to metabolite  $m_i$ . In the case of bupropion, the other pathways of metabolism confound the value of  $AUC_p$  and therefore the calculation of  $AUC_m/AUC_p$ . While previous investigators have used metabolite-to-parent AUC ratio with success with bupropion and methadone, typically those studies were designed as paired studies. An individual subject was administered either an inhibitor or inducer of CYP2B6 and measured changes in metabolite-to-parent AUC ratio. These studies are usually more powerful as each subject serves as their own control (Kharasch et al., 2008; Kharasch and Stubbert, 2013) Given that the present study is one involving basal enzymatic activity and association with methadone clearance, the power of a paired study is lost. For correlation and regression analyses, the metabolite to parent AUC ratio and metabolite AUC for total and *S,S*-hydroxybupropion were used to describe methadone pharmacokinetics as thoroughly as possible.

### *Association Studies*

#### *CYP3A4*

Midazolam parent clearance, the phenotypic marker of CYP3A4 activity, was analyzed for correlation with stereoselective and non-stereoselective methadone clearance, EDDP formation clearance, and EDDP AUC. There was a statistically significant correlation between CYP3A4 and *R*-methadone apparent oral clearance (Figure 2.14). No significant correlation was observed between CYP3A4 activity and either *S*-methadone, total methadone oral clearance, EDDP to methadone AUC ratio, or EDDP AUC. (Figure 2.14, Figure 2.15, and Figure 2.16 depict the association analyses). P-values obtained for association studies were adjusted using the Bonferonni correction, and subsequently, CYP3A4 activity was not significantly associated with any parameters of methadone disposition. Table 2.14 summarizes CYP3A4 association studies.

#### *Pg-p*

Digoxin 5-hour AUC, the phenotypic marker for P-gp, was studied for correlation with stereoselective and non-stereoselective methadone clearance and stereoselective and non-stereoselective methadone AUC four hours post drug administration. P-gp activity did not correlate with any of the outcome variables studied. (Figure 2.17 and Figure 2.18 depict the association analyses). Table 2.15 summarizes P-gp association studies.

#### *CYP2B6*

CYP2B6 activity, represented as either *S,S*-hydroxybupropion to *S*-bupropion area ratio, total hydroxybupropion metabolite AUC, or *S,S*-hydroxybupropion metabolite AUC, was investigated for correlation with stereoselective and non-stereoselective methadone clearance, EDDP to methadone metabolite ratio, and EDDP AUC. There was a statistically significant correlation between CYP2B6 activity, using *S,S*-hydroxybupropion AUC as the phenotypic

marker, with *R*-methadone clearance and *R*-EDDP, *S*-EDDP, and total EDDP AUC.

Interestingly, there were no significant associations when CYP2B6 activity was described as *S,S*-hydroxybupropion to *S*-bupropion AUC ratio or total hydroxybupropion metabolite AUC. *S*-methadone, total methadone clearance, or formation clearance of *R*-, *S*-, or total EDDP could not be described by any CYP2B6 activity parameter. (Figure 2.19 - Figure 2.27 depict the association analyses). P-values obtained for association studies were adjusted using the Bonferonni correction, and subsequently, CYP2B6 activity, when described with *S,S*-hydroxybupropion AUC was only associated with *S*-EDDP AUC. Table 2.16 summarizes CYP2B6 association studies.

#### *Multiple Linear Regression Analyses*

Multiple variable, linear, step-wise ordinary least square regression analysis of CYP3A4, P-gp, and CYP2B6 activity were studied with either *R*-, *S*-, or total methadone apparent oral clearance or *R*-, *S*-, or total EDDP AUC. For these analyses, only stereoselective and total methadone parent clearance and EDDP exposure were used as outcome variables in multiple linear regression analyses. EDDP-to-methadone AUC ratio was excluded from this analysis because it could not be described by any of the independent variables. The values for estimated protein contribution, their corresponding p-value, along with a global F-statistic and model p-value are reported in and Table 2.18. Interestingly, none of the models tested appeared to be statistically significant, which is in contrast to the results of the association studies. Upon further inspection of the results from simple linear regression analyses, it was determined that one subject was a clear outlier and was skewing the linearity of the associations between CYP2B6 activity and total and stereoselective EDDP AUC. There were no outliers that affected the remaining results from the association analyses. The reason why the statistical test used in the association studies

between *S,S*-hydroxybupropion AUC and stereoselective and total EDDP AUC resulted in significant results was because the Spearman rho reports on monotonal relationships between predictor and outcome variable. Monotoal relationships include, but are not limited to, linear relationships. Further evaluation of the data correlating CYP2B6 activity with total and stereoselective EDDP AUC confirmed a monotonic, non-linear relationship. Multiple linear analyses were then performed again, excluding the outlying subject. These results are presented in Table 2.19 and Table 2.20. While many of these models were found to be statistically significant, CYP2B6 activity as represented by *S,S*-hydroxybupropion AUC alone remained the best predictor of methadone metabolism.

#### *Genotype Association Analyses*

*ABCB1* and *CYP2B6* genotype were determined for each subject to assess if genotype affected absorption and/or metabolism. Stereoselective methadone clearance and methadone four hour AUC were studied as outcome variables for *ABCB1* genotype. Stereoselective methadone parent clearance, EDDP-to-methadone AUC ratio, and EDDP AUC were studied as outcome variables for *CYP2B6* genotype. A Kruskal-Wallis one-way ANOVA was used to compare *ABCB1* and *CYP2B6* genotype. Post-test analysis (unpaired t-test) was performed when there were statistically significant results from one-way ANOVA analysis. These plots are depicted in Figure 2.28 and Figure 2.29. *ABCB1* and *CYP2B6* genotype do not have a significant effect on total or stereoselective methadone clearance. However, when analyzing EDDP AUC directly, subjects with *CYP2B6* \*1/\*6 genotype had statistically significant lower metabolite AUC values compared to subjects carrying the wild type allele. Interestingly, subjects carrying the homozygous *CYP2B6* \*6/\*6 (n = 2) and *CYP2B6* \*1/\*5 did not appear to be statistically different. However, the low number of subjects carrying these genotypes limits the interpretation of this

result. Genotype analysis was also performed with probe substrates to evaluate probe sensitivity. There was not a discernible genotype effect with any of the phenotypic markers studied for P-gp or CYP2B6 activity (Figure 2.31 and Figure 2.32).

#### *Metabolite Identification Studies*

Analysis of patient plasma and urine samples determined that EDDP was the major metabolite identified in all subjects. EMDP was not identified in any of the samples. Phase II metabolism was not observed in these samples.

#### **IV. Discussion**

This study attempted to address previous discrepancies in methadone metabolism and absorption by simultaneously evaluating CYP3A4, P-gp, and CYP2B6 in nineteen healthy volunteers of various ethnic backgrounds. The population studied proved to be varied in terms of side effects experienced as well as the pupil diameter constriction, a pharmacodynamic marker of methadone action. Methadone pharmacokinetic parameters within the subject population were also variable. Association analyses were used to identify statistically significant associations between proteins of interest and stereoselective and total methadone clearance, EDDP-to-metabolite ratio, and EDDP AUC.

Initially, it appeared that CYP3A4 activity and CYP2B6 activity (when described as *S,S*-hydroxybupropion AUC) was associated with *R*-methadone clearance. CYP2B6 activity (when described as *S,S*-hydroxybupropion AUC) also correlated with total and stereoselective EDDP AUC *in vivo*. CYP3A4 activity was not associated with *R*- or *S*-EDDP or total AUC. P-gp

activity was not associated with differences in stereoselective or total methadone clearance or methadone 4 hour AUC. Based on the results obtained from the preliminary association analyses, it can be inferred that: 1) CYP3A4 and CYP2B6 activity accounted for ~ 28% and 25%, respectively, of the variation seen in *R*-methadone apparent oral clearance and 2) CYP2B6 activity accounted for 23%, 38%, and 36% of the variation seen in *R*-EDDP, *S*-EDDP, and total EDDP AUC, respectively. However, following a Bonferonni correction, the only association of statistical significance is between *S,S*-hydroxybupropion AUC and *S*-EDDP AUC. A Bonferonni correction is the most stringent method for p-value adjustment. This was chosen because the use of *S,S*-hydroxybupropion AUC to characterize *in vivo* CYP2B6 phenotypic activity has not been previously reported and removal of false-positive relationships was critical.

As mentioned above, an interesting and novel aspect of this analysis is the use of the metabolite AUC (also referred to as exposure) as a phenotypic marker of CYP2B6 activity. Usually the metabolite to parent AUC ratio is the enzyme index activity that is typically used. However, bupropion poses a difficult scenario where the parent drug is metabolized by multiple pathways and the minor pathway is the one of primary interest. In addition, the baseline activity of CYP2B6 is measured in this study, not the activity affected by specific inducers or inhibitors as is typically done (Kharasch et al., 2004; Kharasch and Stubbert, 2013; Kharasch et al., 2009). In this situation, the metabolite AUC appears empirically to be a better indication of basal enzymatic activity because the AUC of parent drug is confounded by all bupropion elimination pathways. A ratio of metabolite to parent drug provides information on the fraction of systemic parent drug that forms the metabolite of interest and therefore gives direct information about the enzyme activity. The use of metabolite AUC is a measure with more variability, especially when considering variable pathways of drug absorption and distribution. However, the use of the *S,S*-

hydroxybupropion AUC in this study allowed a strategy to index subjects based on their CYP2B6 basal activity and to compare inter-subject variability.

An additional measure of association was performed between CYP3A4 and CYP2B6 activity to further test use of metabolite AUC as a measure of enzymatic activity. CYP3A4 and CYP2B6 are co-regulated *in vivo* by CAR and PXR nuclear receptors and the phenotype indices corresponding to enzyme activity should correlate in a positive relationship. Interestingly, there is no correlation between *S,S*-hydroxybupropion-to-*S*-bupropion AUC ratio with midazolam apparent clearance (Figure 2.33). However, there are significant negative associations between total hydroxybupropion AUC and *S,S*-hydroxybupropion AUC with midazolam apparent clearance (Figure 2.33). These results do not corroborate with published data (Faucette et al., 2006; Faucette et al., 2007) and can be the result of the number of subjects studied.

*R*-, *S*- and total methadone clearance is not associated with any of the proteins studied. Stereoselective and total methadone clearance were studied as outcome variables in forwards and reverse regression analysis because initial studies prior to p-value correction suggest that CYP3A4 together with CYP2B6 activity is weakly associated with *R*-methadone clearance. However, multiple variable analyses did not produce a statistically significant model. Stereoselective and total EDDP-to-methadone AUC ratios is not associated with either CYP3A4 or CYP2B6 activity, in contrast to previous studies evaluating these enzymes and methadone (Kharasch and Stubbert, 2013; Kharasch et al., 2004; Iribarne et al., 1998; Foster et al., 2000) using specific inhibitors and inducers to accentuate either CYP3A4 or CYP2B6 activity.

Given that methadone metabolism *in vivo* is both complicated and contradictory, EDDP AUC was then also used as one of the outcome variables studied in both association analyses and

forwards and reverse regression analyses. Total and stereoselective EDDP AUC could potentially allow understanding of methadone metabolism by CYP3A4 and CYP2B6 even if methadone elimination pathways were multi-faceted. Methadone pharmacokinetics, like bupropion, is complex and use of metabolite AUC appeared to be useful in analyzing extent of metabolism between individuals. Association analyses implied that the only significant relationship is between CYP2B6 activity (measured as *S,S*-hydroxybupropion AUC) and *S*-EDDP AUC. However, in the multiple variable regression analyses, CYP2B6 activity produced the most significant models for *R*-, *S*- and total EDDP AUC. This discrepancy may have resulted due to the method of p-value adjustment being too stringent and the number of association studies performed.

Two scenarios could potentially account for the discrepancies observed with methadone clearance and EDDP-to-methadone AUC ratio. The first explanation is that methadone, like bupropion, undergoes several routes of elimination. It is possible that these other routes are stereoselective in nature as well which accounts for the differences in enzyme contribution of parent elimination. Preliminary metabolite identification studies were performed in each subject's plasma and urine to explore this possibility. These studies showed that EDDP is the main metabolite formed *in vivo* from methadone in the population studied. However, because only ~20% of the dose was recovered in urine, parallel pathways of metabolism cannot be ruled out. The second possible cause of the discrepancy may be due to the observation that the metabolite appears to undergo enterohepatic recycling. One of the underlying assumptions of noncompartmental analysis is linearity of the pharmacokinetic system. Because only EDDP, and not parent, appears to be undergoing enterohepatic recycling (Figure 2.30), the proportional relationship between metabolite and parent amount *in vivo* no longer exists. Enterohepatic

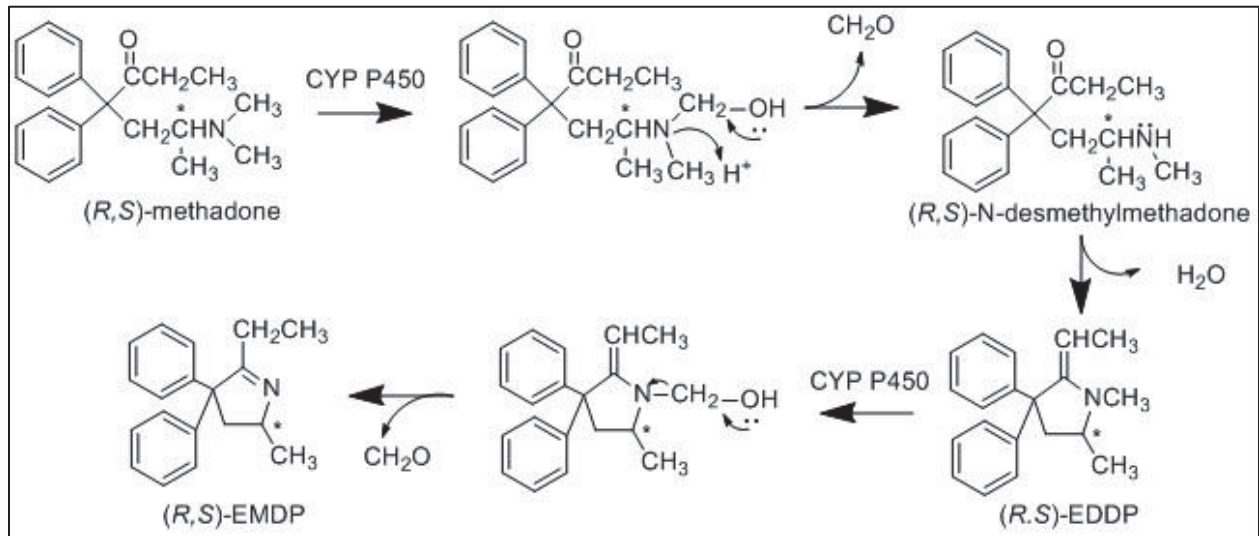
recycling was evaluated on an individual subject basis, not just an average, and there is evidence that several subjects exhibited a second peak in plasma *R*- and *S*- EDDP AUC (data not shown). This affects estimation of formation clearance as a ratio of metabolite to parent AUC, however, EDDP AUC is not affected by this deviation from linearity.

*ABCB1* and *CYP2B6* genotype effects were assessed in methadone absorption and metabolism (Figure 2.28 and Figure 2.29). P-gp and *CYP2B6* genotype were not associated with any changes in methadone absorption or clearance. *CYP2B6* genotype, however, did appear to influence EDDP AUC, as individuals carrying the *\*1/\*6* allelic variant had statistically significant lower EDDP AUC compared to wild-type. There was a trend for lower EDDP AUC in individuals genotyped as *\*1/\*5* and *\*6/\*6*. However, these differences were not statistically significant, most likely due to low subject numbers ( $n = 2$  in both groups). *CYP2B6* has the strongest association with EDDP AUC compared to other proteins studied. Polymorphisms in this gene could affect EDDP AUC and *CYP2B6* genotype could play a role in explaining high inter-individual variability in methadone pharmacokinetics. One limitation to this assertion is the fact that neither digoxin nor bupropion phenotype indices exhibited genotype differences (Figure 2.31 and Figure 2.32). This is probably due to number of subjects studied was too low to detect small differences in patient populations. In the case of digoxin, the percent difference expected between individuals of different *ABCB1* genotype may be low, and thus, the study was not adequately powered to see this difference. For bupropion, the complications of the drug itself may make it difficult to detect differences in *CYP2B6* genotype in a subject size of 19. In a study of 42 subjects (double the number in this study) an association was observed between bupropion and carriers of *CYP2B6\*6* allelic variant (Benowitz et al., 2013).

In summary, this work provided evidence that methadone metabolism and formation of EDDP appears to be through CYP2B6 oxidative metabolism. Therefore, studying EDDP AUC still offers information regarding therapeutic activity of methadone. Our data suggests that CYP2B6 activity and genotype are important in influencing methadone deactivation and should be studied when considering dosing of methadone. However, due to the complicated nature of methadone, it is evident that factors other than metabolism and absorption should be considered when studying methadone variability such as distribution, protein binding and enterohepatic recirculation of EDDP.

## V. Figures and Tables

Figure 2.1 -- Methadone metabolism by P450 enzymes.



**Figure 2.2** -- Human subject study design.

Prescreen (Visit 1)	Visit 2	Visit 3	2 week washout period	Visit 4
1) Health history interview and physical by Dr. Gregory Terman	1) Midazolam 2 mg po. Subjects received digoxin 0.5 mg po 1 hr after midazolam	1) Bupropion 150 mg po		1) Methadone 10 mg po
2) EKG with analysis by Dr. Kris Patton	2) Blood draws 0-8 hours	2) Blood draws 0-72 hours		2) Blood draws 0-72 hours
				3) Total urine collection for first 24 hours
				4) Dark-adapted pupilometry for first 12 hours
				5) Blood collection for DNA analysis

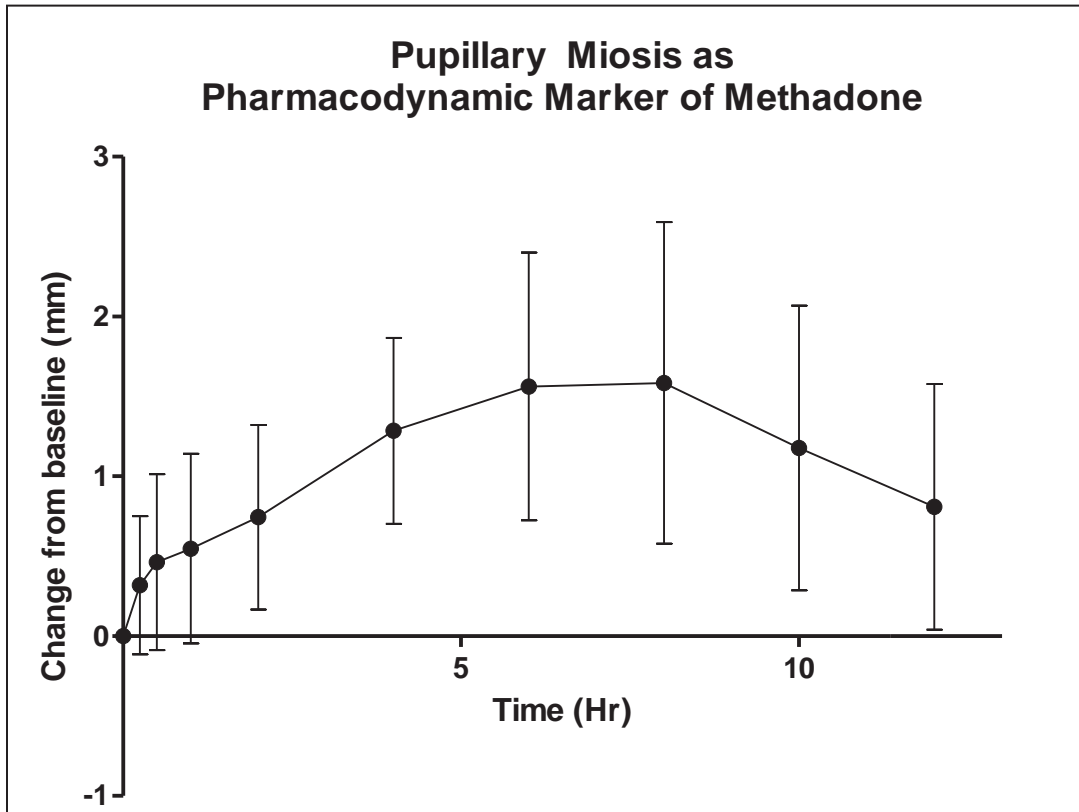
**Table 2.1** – Primers used for sequence amplification and genotyping *CYP2B6\*4* and *ABCB1 2677*

CYP2B6*4 Forward Primer (CYP2B6 -5F) (Lang et al., 2001)	5'-GACAGAAGGATGAGGGAGGAA-3'
CYP2B6*4 Reverse Primer (CYP2B6 -5R) (Lang et al., 2001)	5'-CTCCCTCTGTCTTTCATTCTGT-3'
ABCB1 2677 C>T Forward Primer (Asano et al., 2003)	5'-CAGGCTTGCTGTAATTACCC-3'
ABCB1 2677 C>T Reverse Primer (Asano et al., 2003)	5'-TAGTTTGACTCACCTTCCCA-3'

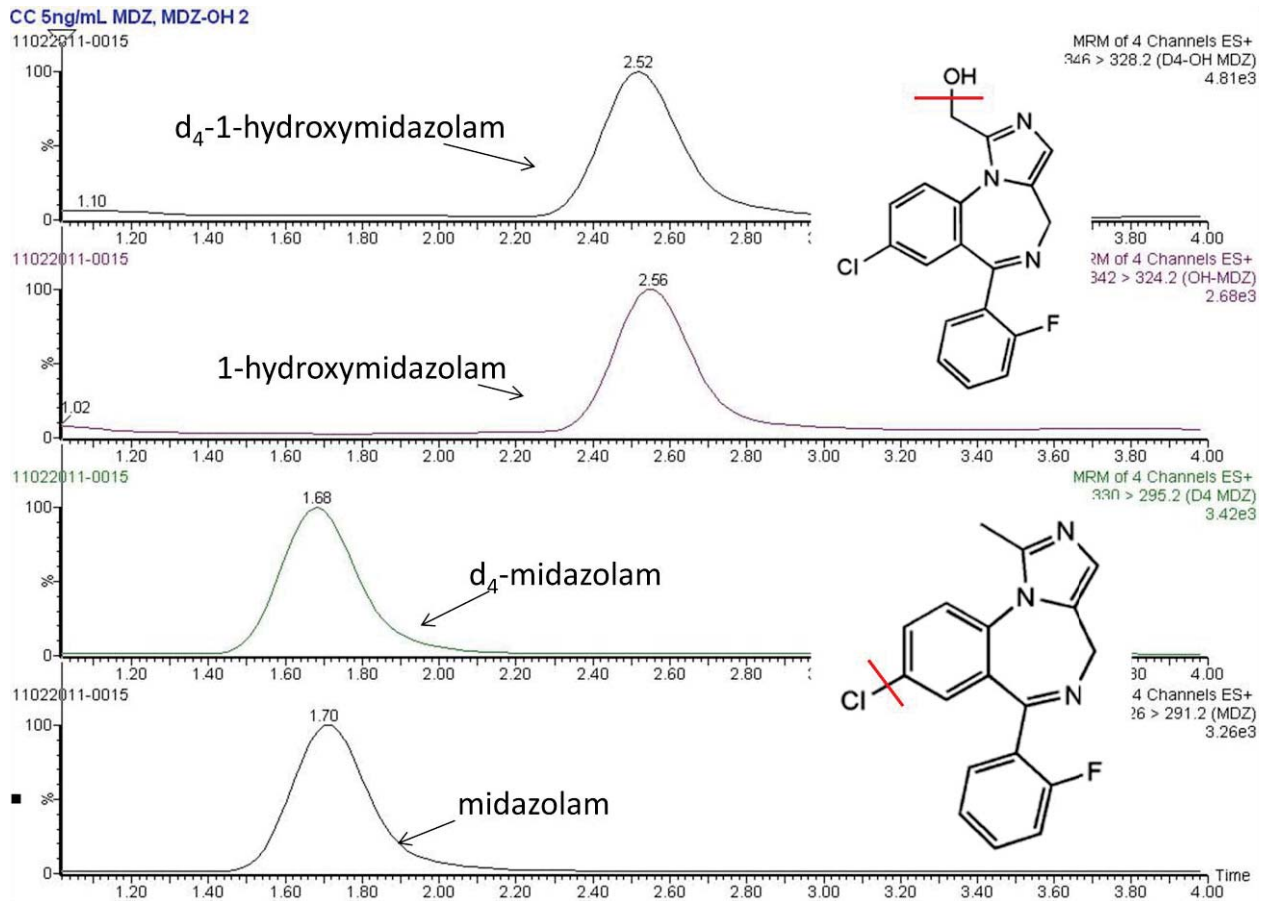
**Table 2.2 -- Human Subject Demographics.**

<b>Subject Number</b>	<b>M/F</b>	<b>Age (yrs)</b>	<b>Ethnicity</b>
MP1	M	28	Caucasian
MP2	F	22	Asian-American
MP3	M	33	Asian-American
MP4	M	25	African-American
MP5	F	30	Caucasian
MP6	F	32	Caucasian
MP7	F	37	Caucasian
MP8	M	25	Caucasian
MP9	M	28	Asian-American
MP11	M	32	Caucasian
MP12	M	40	Caucasian
MP13	M	23	Asian-American
MP14	M	35	Asian-American
MP16	F	30	Caucasian
MP19	M	21	Caucasian
MP21	F	22	African-American
MP22	M	32	African-American
MP24	M	22	Asian-American
MP27	M	39	Caucasian
<b>Summary</b>	13 Males, 6 Females	Average age = 29.2 yrs	10 Caucasian, 6 Asian-Americans, 3 African-Americans

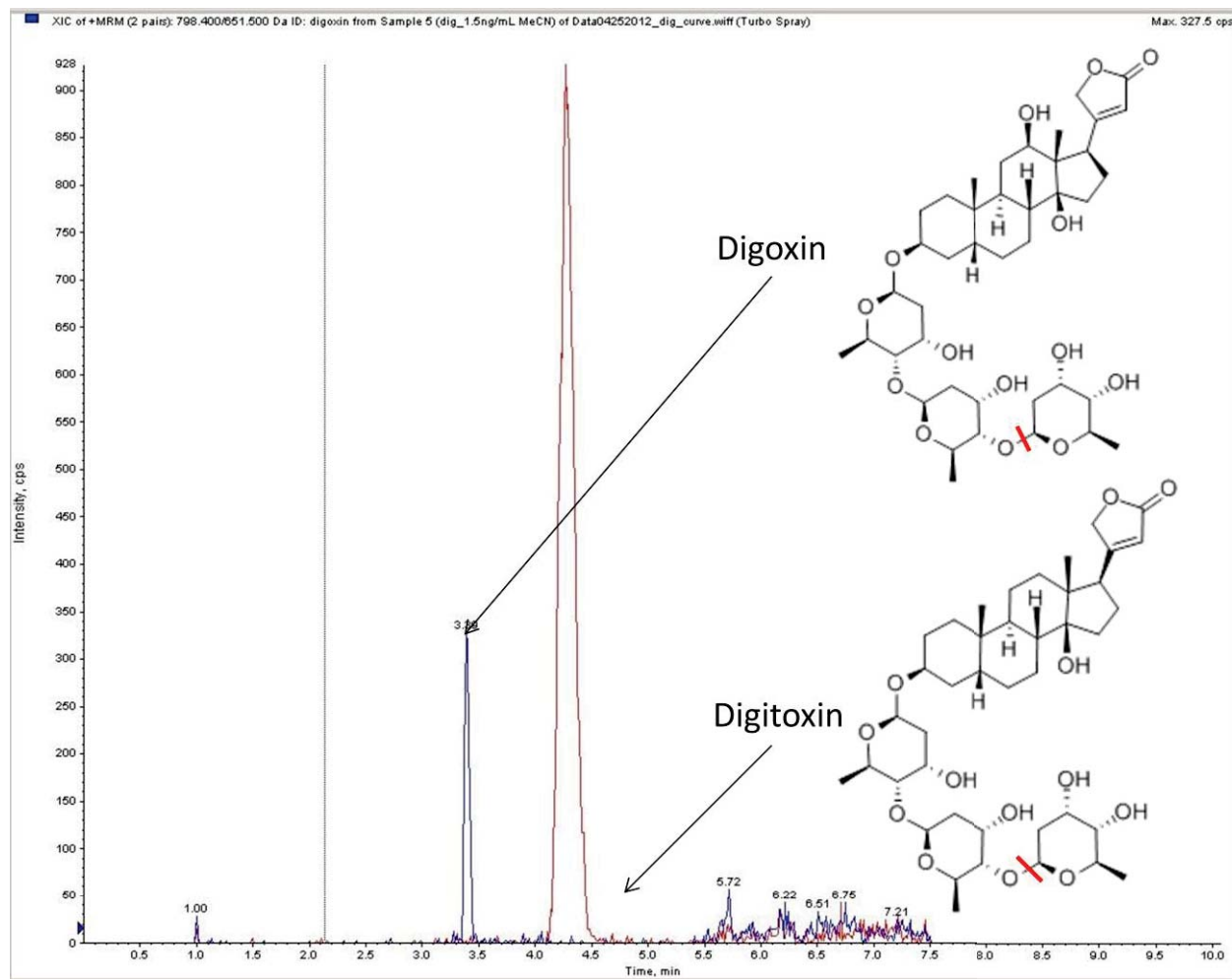
**Figure 2.3** – Pupil constriction over time following methadone administration. Pupil miosis was a measure of methadone pharmacodynamics. Points are the average  $\pm$  standard deviation (n = 19).



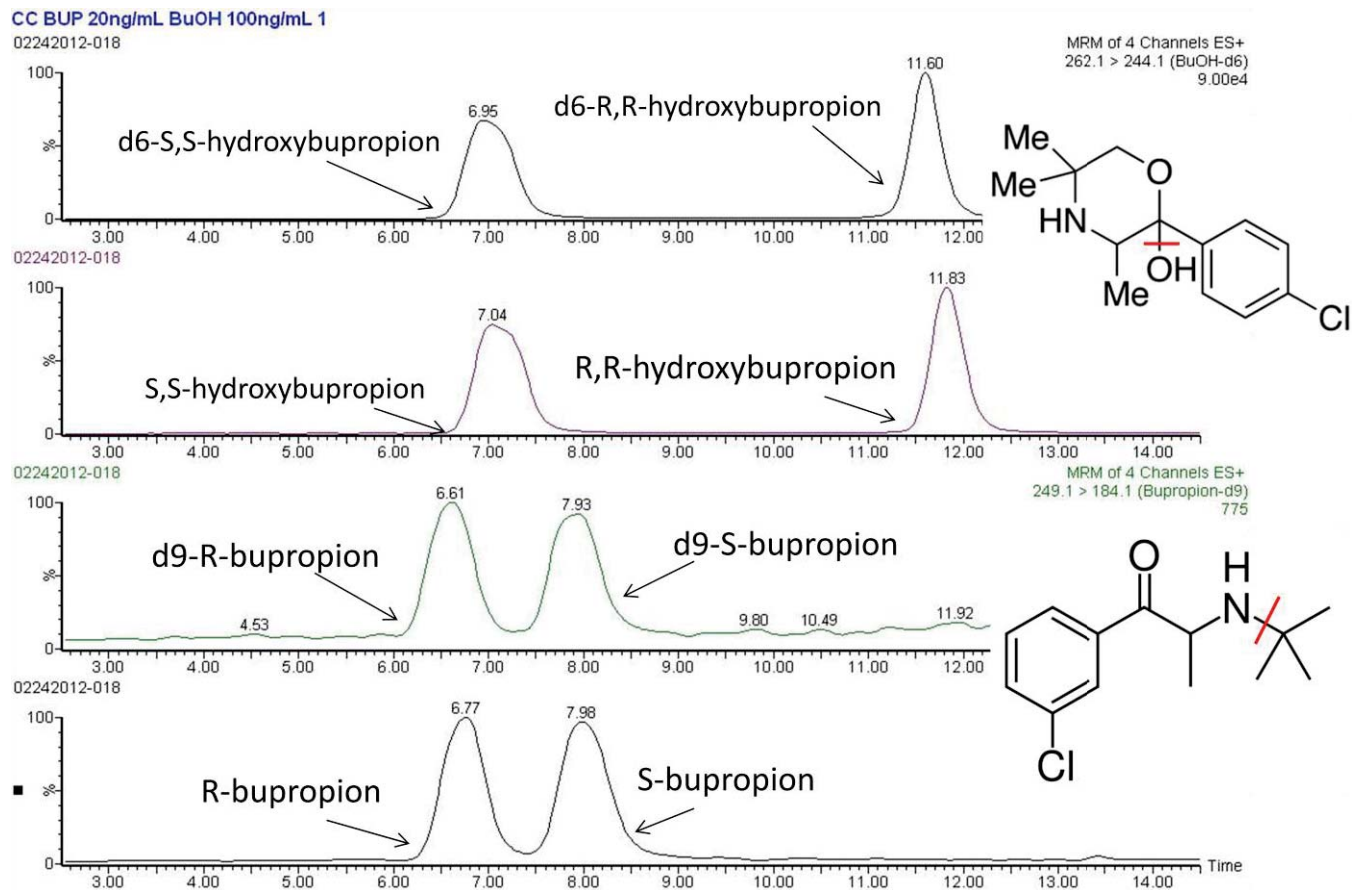
**Figure 2.4** -- Chromatogram of midazolam, 1'-hydroxymidazolam, and deuterated standards. Red lines indicate the where fragmentation on the molecule occurs.



**Figure 2.5** – Chromatogram of digoxin and digitoxin. Red lines indicate where fragmentation occurred on the molecule.



**Figure 2.6** – Chromatogram of bupropion, hydroxybupropion, and deuterated standards. Red lines indicate where fragmentation occurred on the molecule.



**Figure 2.7** -- Chromatogram of methadone, EDDP, and deuterated standards. Red lines indicate where fragmentation occurred on the molecule.

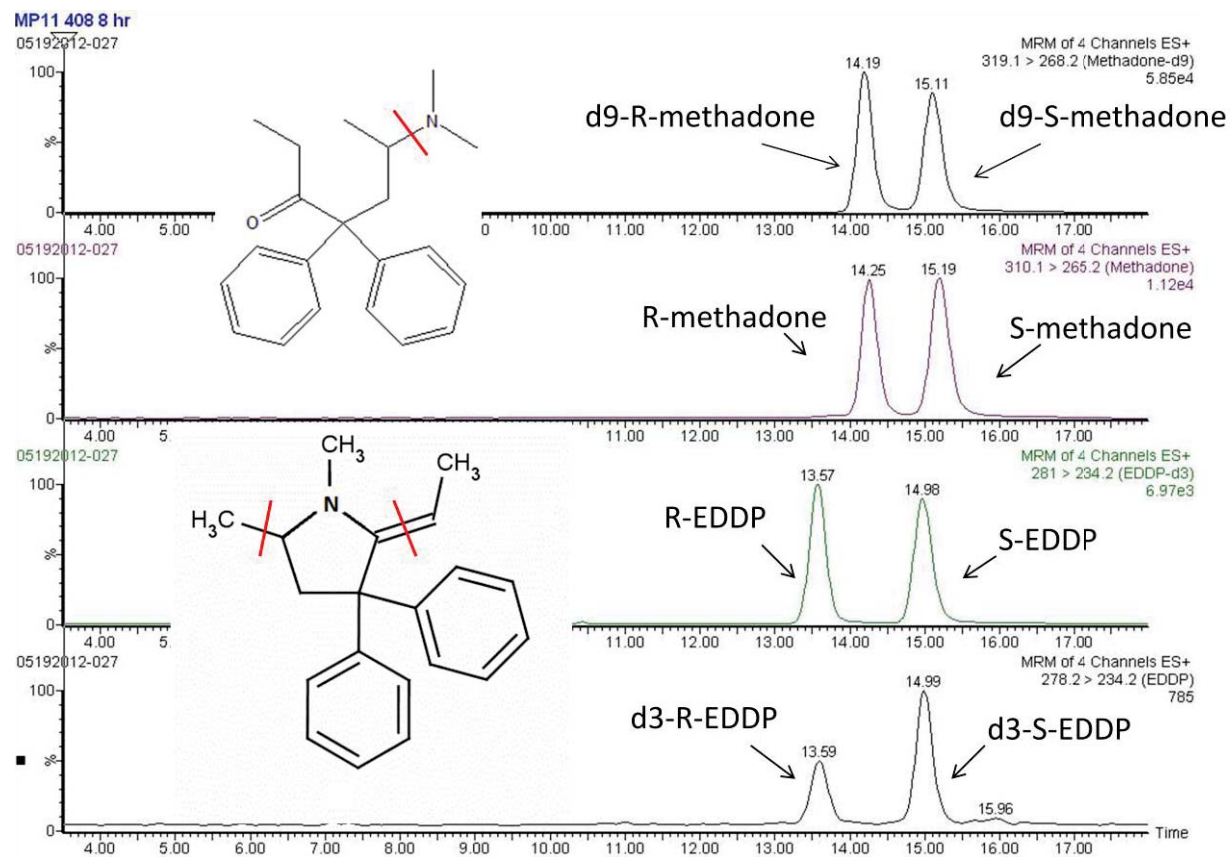


Figure 2.8 – AUC summary for midazolam and 1'-hydroxymidazolam (n = 19).

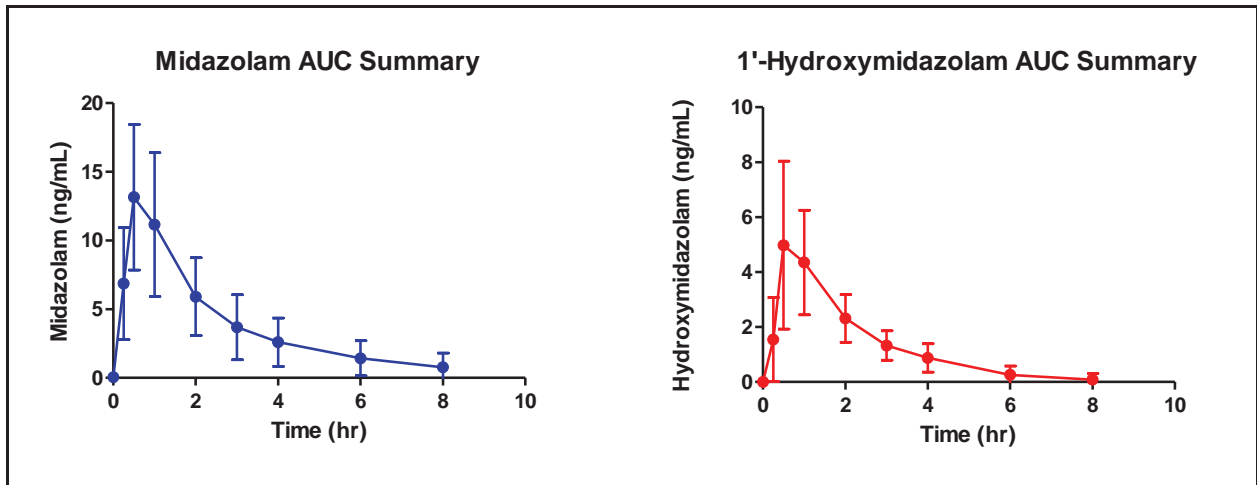


Figure 2.9 -- AUC summary for digoxin (n = 19).

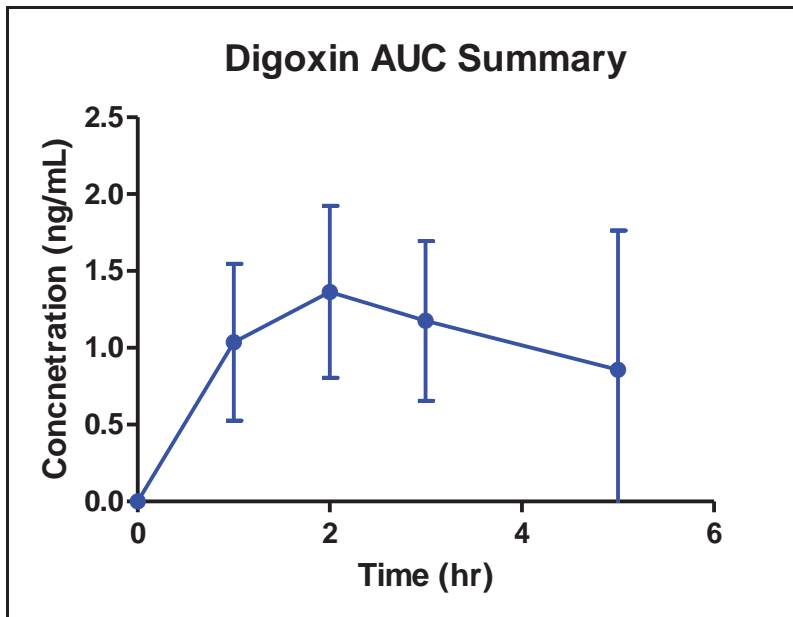


Figure 2.10 – AUC summary for bupropion and hydroxybupropion (n = 19).

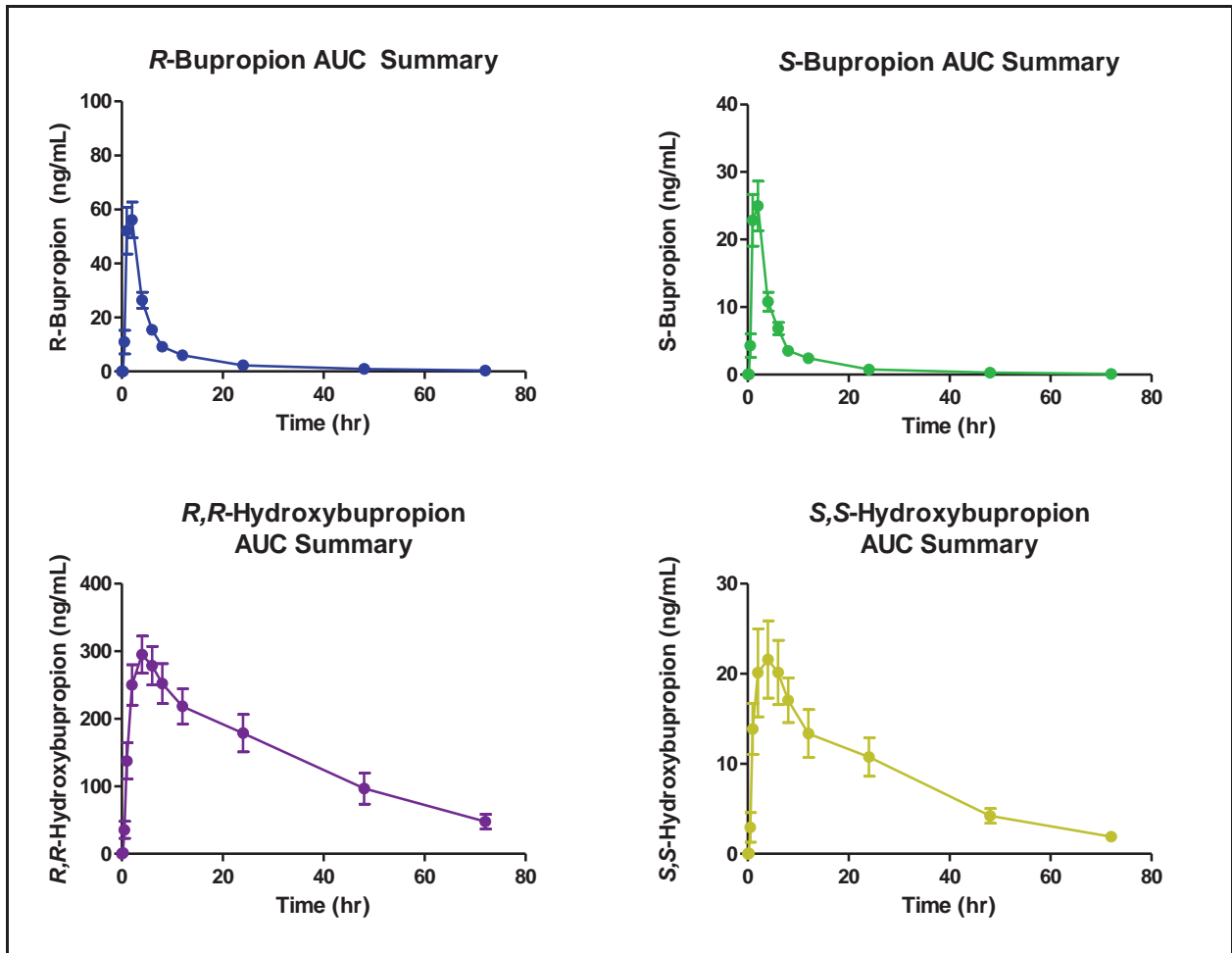
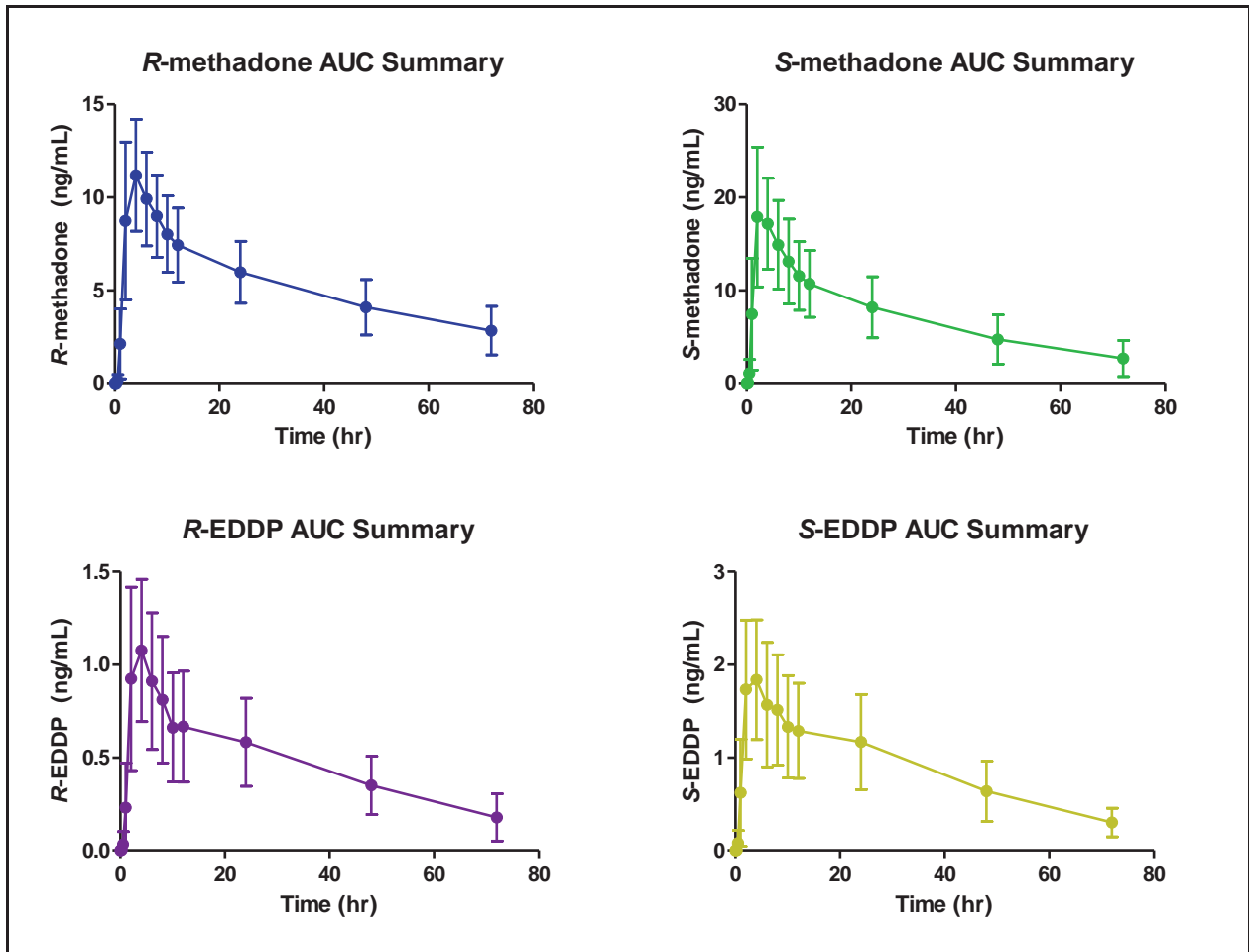
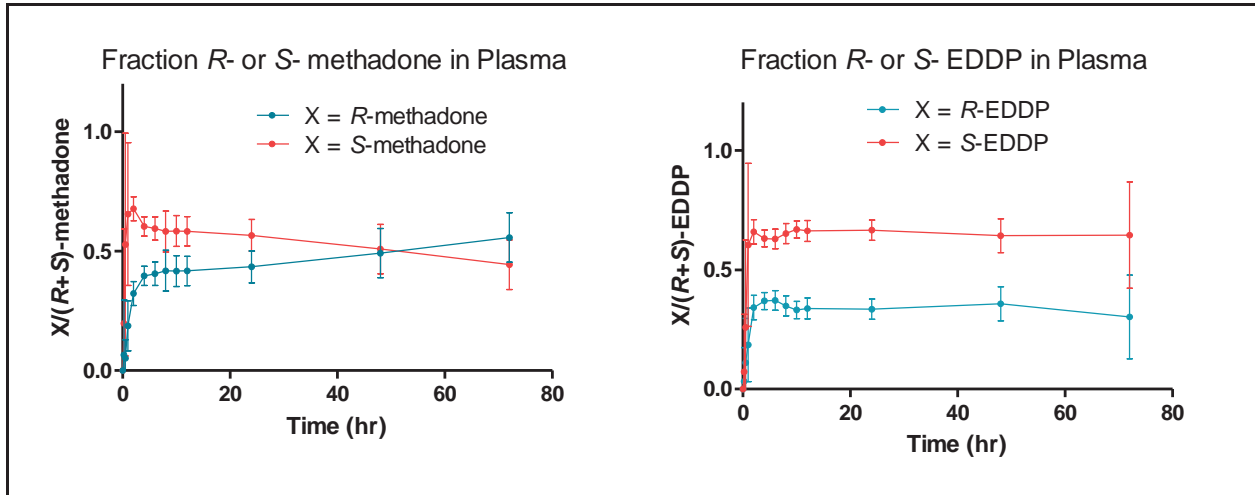


Figure 2.11 -- AUC summary for methadone EDDP (n = 19).



**Figure 2.12** – Summary of fraction *R*- and *S*- methadone and EDDP versus time (n = 19).



**Table 2.3** – Pharmacokinetic parameters for midazolam.

Midazolam Pharmacokinetic Parameters				
	Mean	SD	Median	Range
AUC 0 - 8hr (ng/mL * hr)	32.2	15.2	27.3	13.3 - 86.2
AUC 0 - ∞ (ng/mL * hr)	35.8	19.6	31.2	13.6 - 107.4
Cmax (ng/mL)	14.8	5.7	14.3	6.7 - 29.1
Tmax (hr)	0.7	0.4	0.5	0.5 - 2
Vd/F (L)	194.0	59.6	204.2	89.9 - 315.8
Cl/F (L/hr)	66.2	26.0	64.0	18.6 - 146.7
Half-life (hr)	2.2	0.6	2.1	1.3 - 3.3

**Table 2.4** – Pharmacokinetic parameters for 1'-hydroxymidazolam.

1'-Hydroxymidazolam Pharmacokinetic Parameters				
	Mean	SD	Median	Range
AUC 0 - 8hr (ng/mL * hr)	11.1	4.1	9.7	6.1 - 9.7
AUC 0 - $\infty$ (ng/mL * hr)	12.2	4.4	11.0	6.2 - 20.5
Cmax (ng/mL)	5.9	2.5	5.0	2.8 - 11.4
Tmax (hr)	0.8	0.4	0.5	0.5 - 2.0
Half-life (hr)	1.7	0.9	1.4	0.86 - 5.0

**Table 2.5** – Pharmacokinetic parameters for digoxin.

Digoxin Pharmacokinetic Parameters				
	Mean	SD	Median	Range
AUC 0 - 5hr (ng/mL * hr)	4.7	1.4	4.5	1.7 - 7.0
Cmax (ng/mL)	1.5	0.5	1.5	0.6 - 2.8
Tmax (hr)	1.8	0.7	2.0	1.0 - 3.0

**Table 2.6** – Pharmacokinetic parameters for *R*-bupropion.

<i>R</i> -bupropion Pharmacokinetic Parameters				
	Mean	SD	Median	Range
AUC 0 - 72hr (ng/mL * hr)	374.5	153.4	340.8	144.5 - 617.5
AUC 0 - $\infty$ (ng/mL * hr)	383.9	160.6	356.1	147.1 - 793.1
Cmax (ng/mL)	74.5	36.4	64.8	36.2 - 191.0
Tmax (hr)	1.3	0.5	1.0	0.5 - 2.0
Vd/F (L)	3581.9	1726.0	3429.6	1453 - 8895
Cl/F (L/hr)	229.5	100.9	210.6	94.5 - 509.8
Half-life (hr)	25.0	8.1	25.5	12.2 - 42.7

**Table 2.7** – Pharmacokinetic parameters for *R,R*-hydroxybupropion.

<i>R,R</i> -hydroxybupropion Pharmacokinetic Parameters				
	Mean	SD	Median	Range
AUC 0 - 72hr (ng/mL * hr)	10103.1	5342.9	9470.3	3843.9 - 28295.7
AUC 0 - ∞ (ng/mL * hr)	12148.7	7582.5	11913.1	3976.6 - 38584
Cmax (ng/mL)	300.6	98.8	291.5	169.3-563.7
Tmax (hr)	4.3	1.2	4.0	2.0 - 6.0
Half-life (hr)	25.0	8.1	25.6	12.2 - 42.7

**Table 2.8** – Pharmacokinetic parameters for *S*-bupropion.

<i>S</i> -bupropion Pharmacokinetic Parameters				
	Mean	SD	Median	Range
AUC 0 - 72hr (ng/mL * hr)	158.9	74.3	142.5	60.4 - 360.8
AUC 0 - ∞ (ng/mL * hr)	167.8	79.3	143.7	67.5 - 370.6
Cmax (ng/mL)	35.4	19.9	25.67	17.9 - 98.6
Tmax (hr)	1.4	0.6	1.0	0.5 - 2.0
Vd/F (L)	10419.2	7765.1	7950.3	3603.2 - 38070.4
Cl/F (L/hr)	542.9	239.8	521.8	202.4 - 1111.0
Half-life (hr)	13.7	6.1	14.4	4.2 - 23.8

**Table 2.9** – Pharmacokinetic parameters for *S,S*-hydroxybupropion.

<i>S,S</i> -hydroxybupropion Pharmacokinetic Parameters				
	Mean	SD	Median	Range
AUC 0 - 72hr (ng/mL * hr)	559.7	364.4	474.2	183.3 - 1790.8
AUC 0 - ∞ (ng/mL * hr)	631.9	408.2	538.4	209.3 - 1917
Cmax (ng/mL)	23.4	14.6	20.8	9.4- 78.1
Tmax (hr)	2.5	1.9	2.0	1.0 - 6.0
Half-life (hr)	20.9	6.8	18.7	12.5 - 37.0

**Table 2.10** – Pharmacokinetic parameters for *R*-methadone.

<i>R</i> -methadone Pharmacokinetic Parameters				
	Mean	SD	Median	Range
AUC 0 - 72hr (ng/mL * hr)	382.9	111.4	371.5	167.9 - 666.1
AUC 0 - ∞ (ng/mL * hr)	588.1	240.6	554.4	190.7 - 1203.8
Cmax (ng/mL)	11.6	3.1	10.7	6.3 - 17.2
Tmax (hr)	4	1.3	4.0	2.0 - 6.0
Vd/F (L)	569.3	137.0	563.3	359.0 - 878.0
Cl/F (L/hr)	10.2	5.2	9.0	4.2 - 26.2
Half-life (hr)	44.5	17.2	39.3	21.5 - 78.7

**Table 2.11** – Pharmacokinetic parameters for *R*-EDDP.

<i>R</i> -EDDP Pharmacokinetic Parameters				
	Mean	SD	Median	Range
AUC 0 - 72hr (ng/mL * hr)	34.2	13.7	29.2	16.7 - 73.6
AUC 0 - ∞ (ng/mL * hr)	60.7	47.9	47.5	17.3 - 230.2
Cmax (ng/mL)	1.2	0.4	1.0	0.7 - 2.4
Tmax (hr)	3.7	1.7	4.0	2.0 - 8.0
Half-life (hr)	66.0	104.9	35.2	12.2 - 477.3

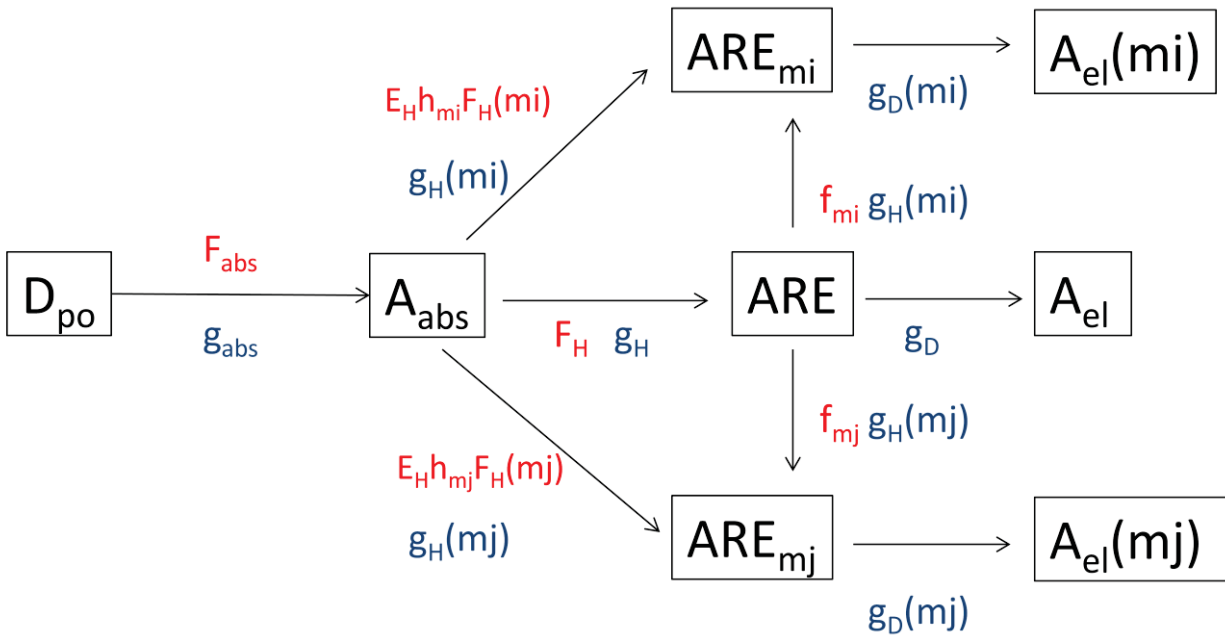
**Table 2.12** – Pharmacokinetic parameters for *S*-methadone.

<i>S</i> -methadone Pharmacokinetic Parameters				
	Mean	SD	Median	Range
AUC 0 - 72hr (ng/mL * hr)	513.5	207.2	530.4	181.9 - 1039.2
AUC 0 - ∞ (ng/mL * hr)	654.1	340.3	619.1	188.5 - 1631.3
Cmax (ng/mL)	19.1	6.3	18.5	10.1 - 31.8
Tmax (hr)	2.8	1.2	2.0	2.0 - 6.0
Vd/F (L)	363.6	147.4	351.3	217.6 - 856.5
Cl/F (L/hr)	10.0	6.0	8.1	3.1 - 26.5
Half-life (hr)	29.5	11.6	27.1	13.6 - 51.7

**Table 2.13** – Pharmacokinetic parameters for *S*-EDDP.

<i>S</i> -EDDP Pharmacokinetic Parameters				
	Mean	SD	Median	Range
AUC 0 - 72hr (ng/mL * hr)	64.6	25.8	59.3	34.8 - 145.8
AUC 0 - ∞ (ng/mL * hr)	83.5	31.6	82.5	38.9 - 161.1
Cmax (ng/mL)	2.0	0.7	2.0	0.99 - 3.7
Tmax (hr)	3.8	2.3	4.0	1.0 - 10.0
Half-life (hr)	33.9	17.9	25.5	16.3 - 90.1

**Figure 2.13** – A general scheme of metabolite kinetics. Modified from (Weiss, 1988).



$D_{po}$  – Dose of drug administered orally

$A_{abs}$  – Cumulative amount of drug absorbed

$A_{el}$  – Cumulative amount of eliminated

ARE – Amount remaining to be excreted

$F_{abs}$  – Availability of drug from absorption

$F_H$  – Fraction of drug after first pass through liver ( $1-E_H$ )

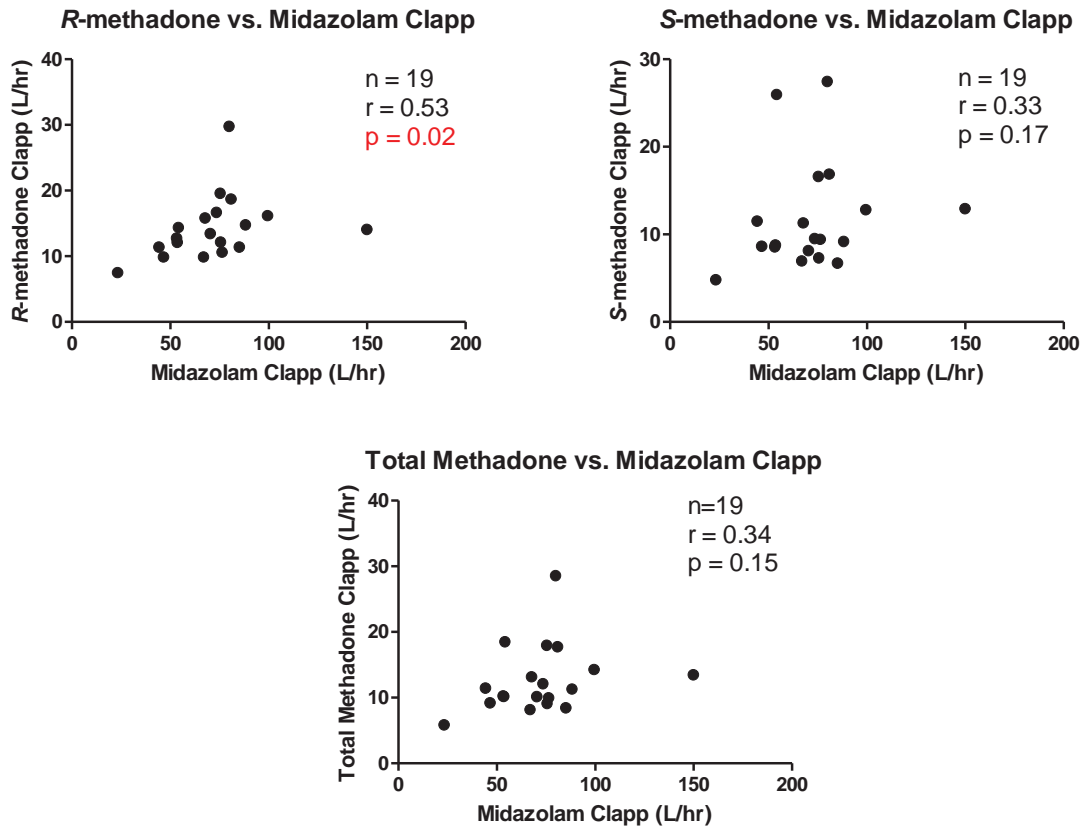
$E_H$  – Hepatic extraction ratio

$f_{mi}$  – Fraction of drug metabolized to metabolite mi

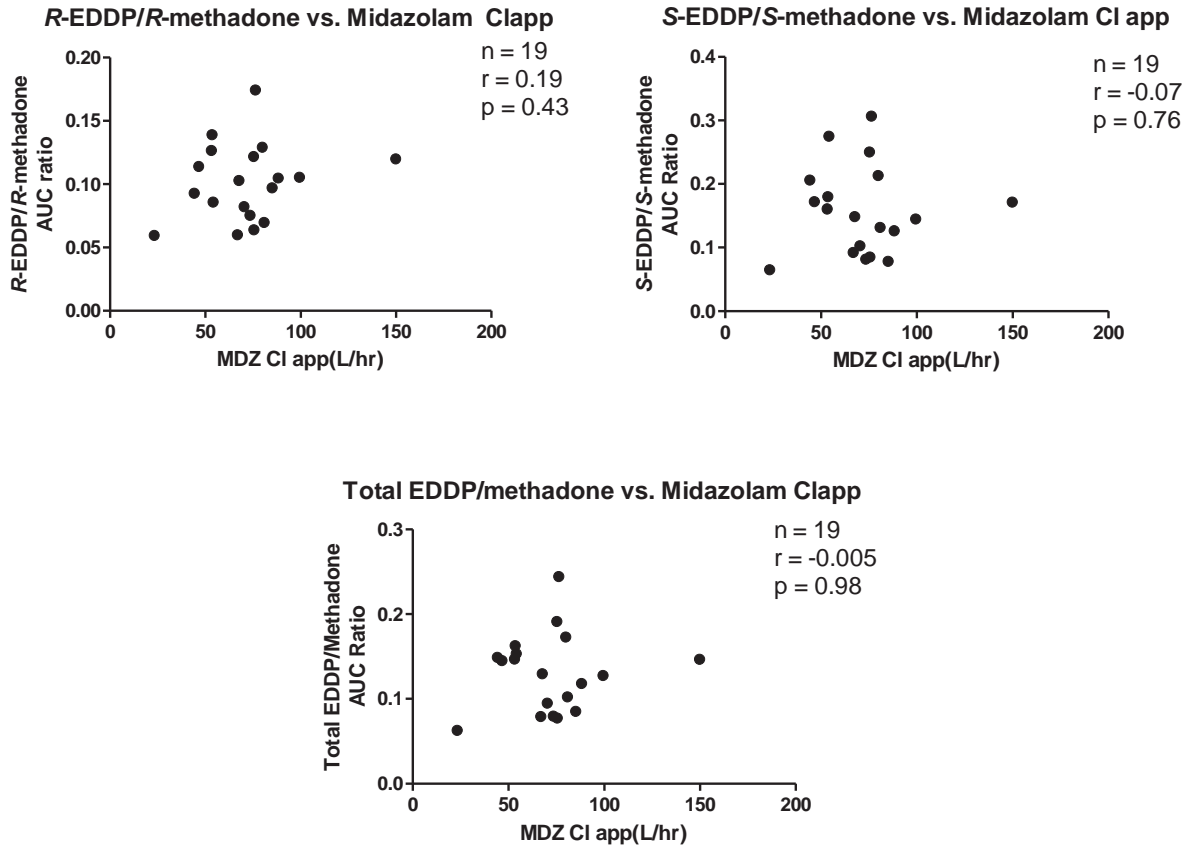
$f_{mj}$  – Fraction of drug metabolized to metabolite mj

$g$  – density function describing cumulative residence time as a function of time

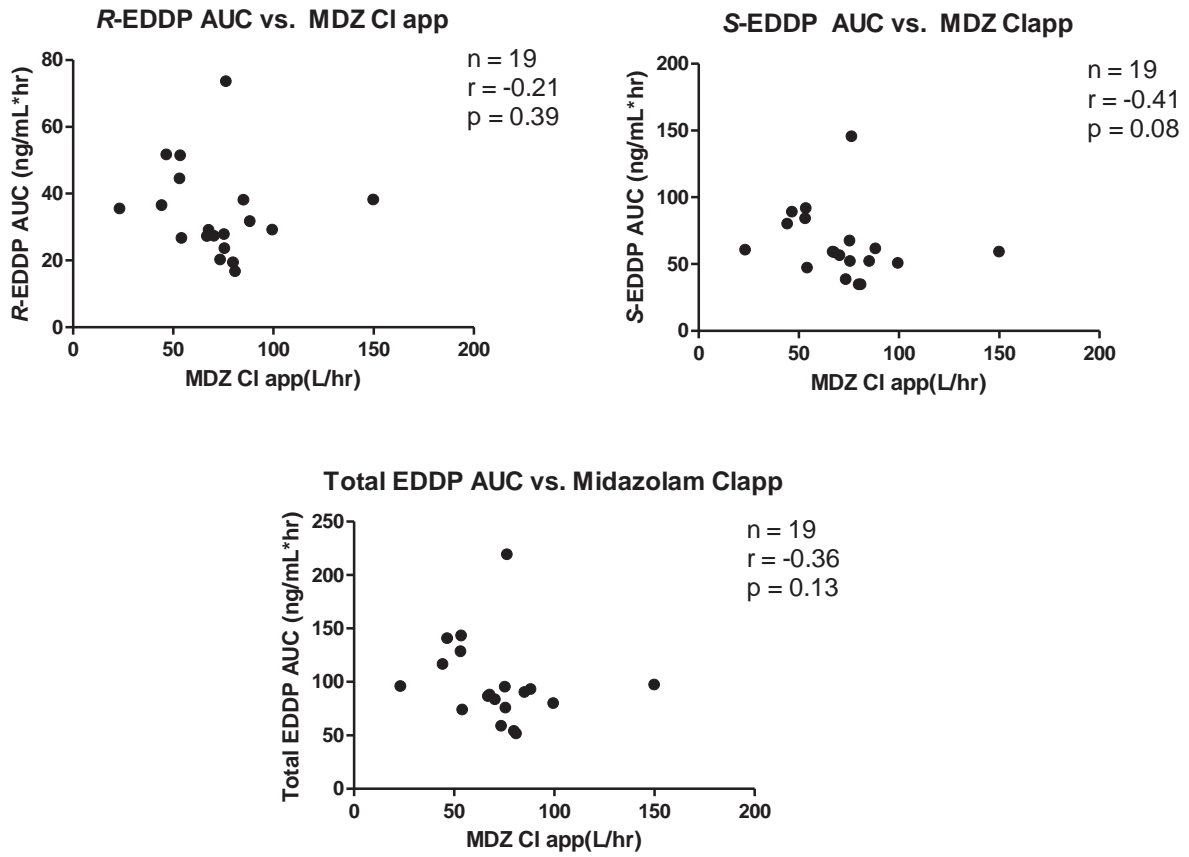
**Figure 2.14** -- CYP3A4 activity (midazolam apparent clearance) correlated with *R*-, *S*- and total methadone clearance.



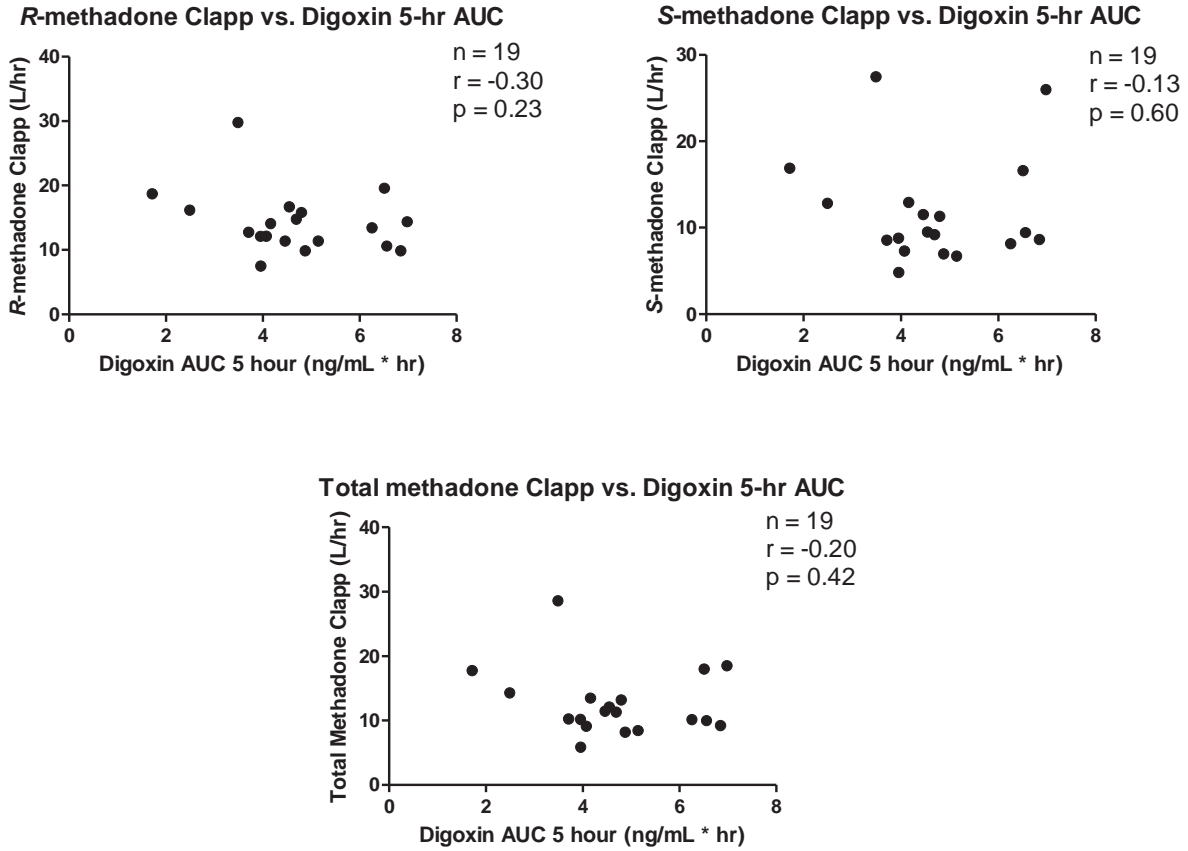
**Figure 2.15** -- CYP3A4 activity (midazolam apparent clearance) correlated with *R*-, *S*-, and total EDDP to methadone AUC ratio.



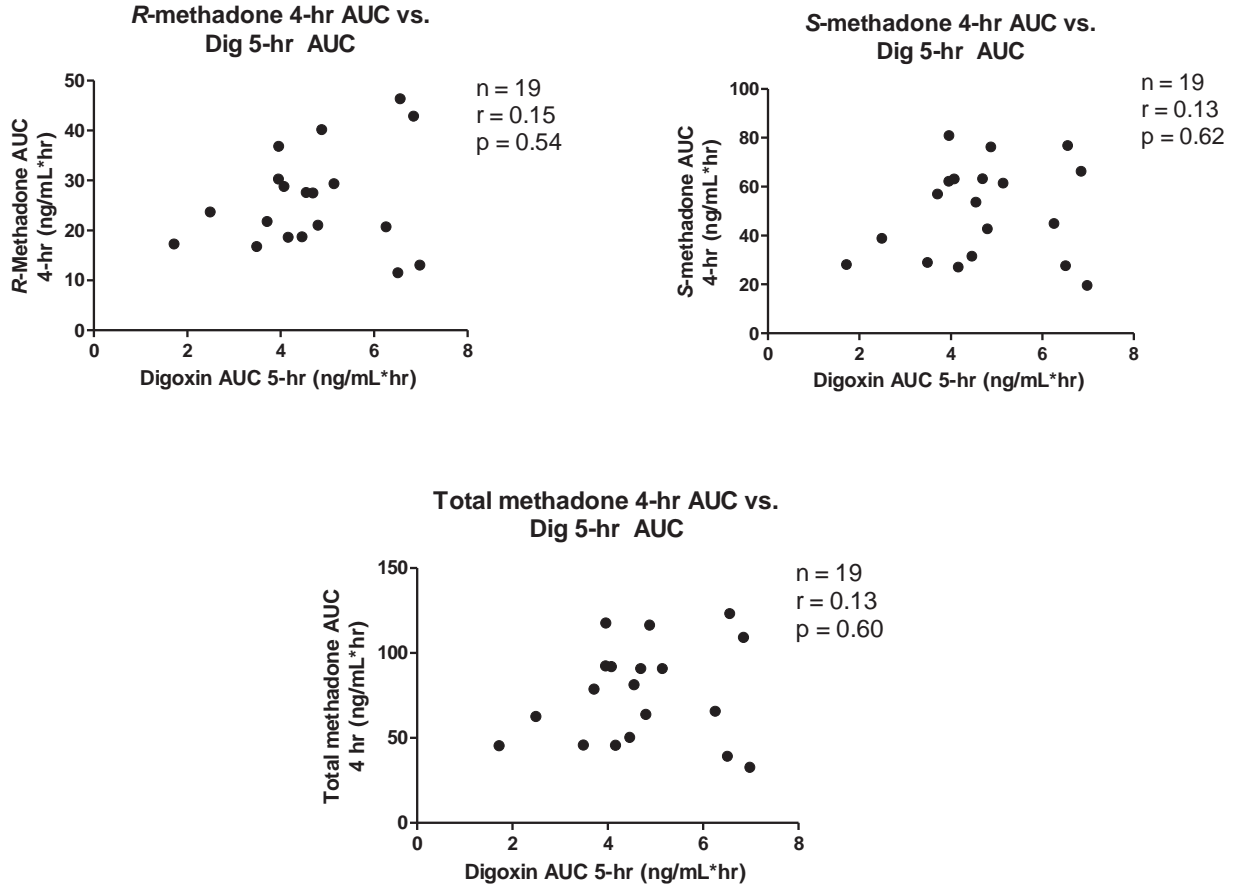
**Figure 2.16** -- CYP3A4 activity (midazolam apparent clearance) correlated with *R*-, *S*-, and total AUC.



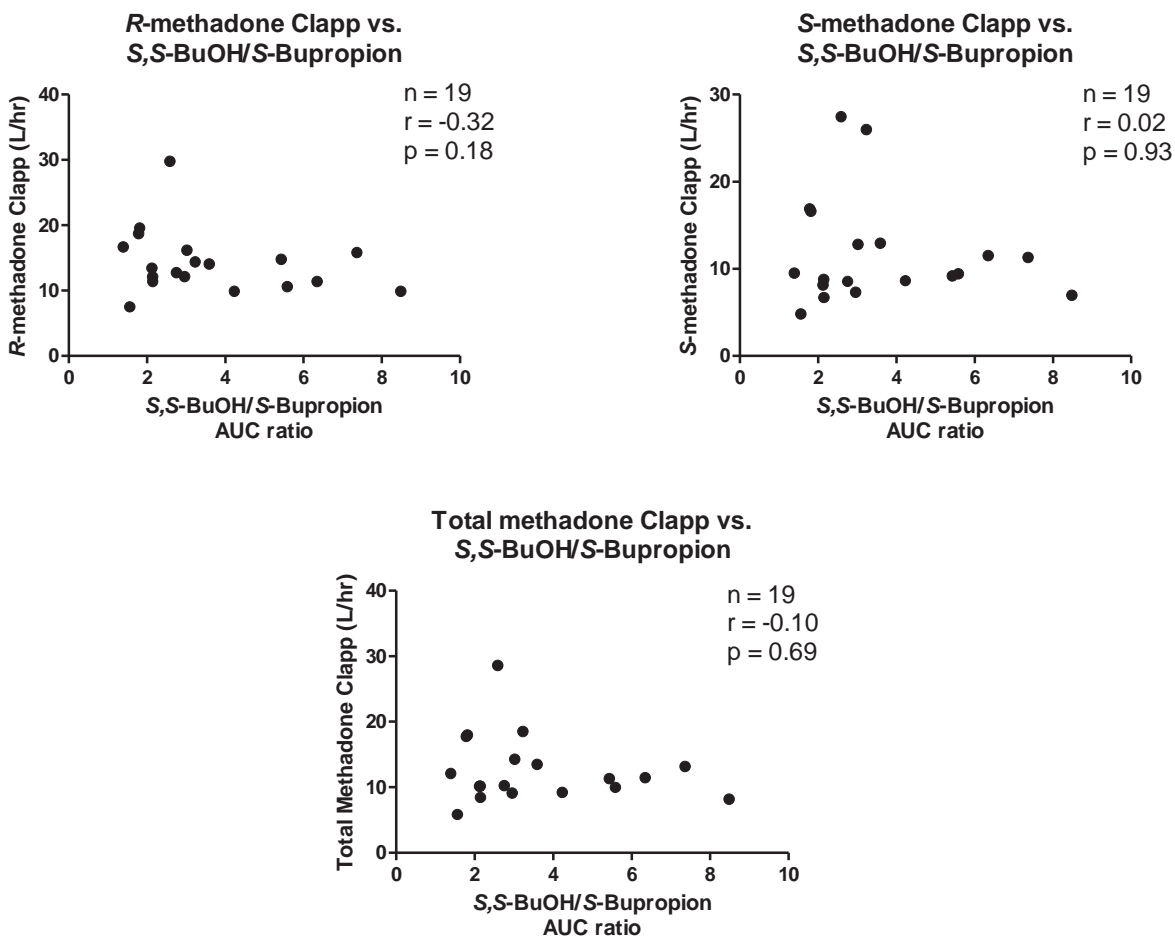
**Figure 2.17** – P-gp activity (digoxin 5-hr AUC) correlated with *R*-, *S*-, and total methadone clearance.



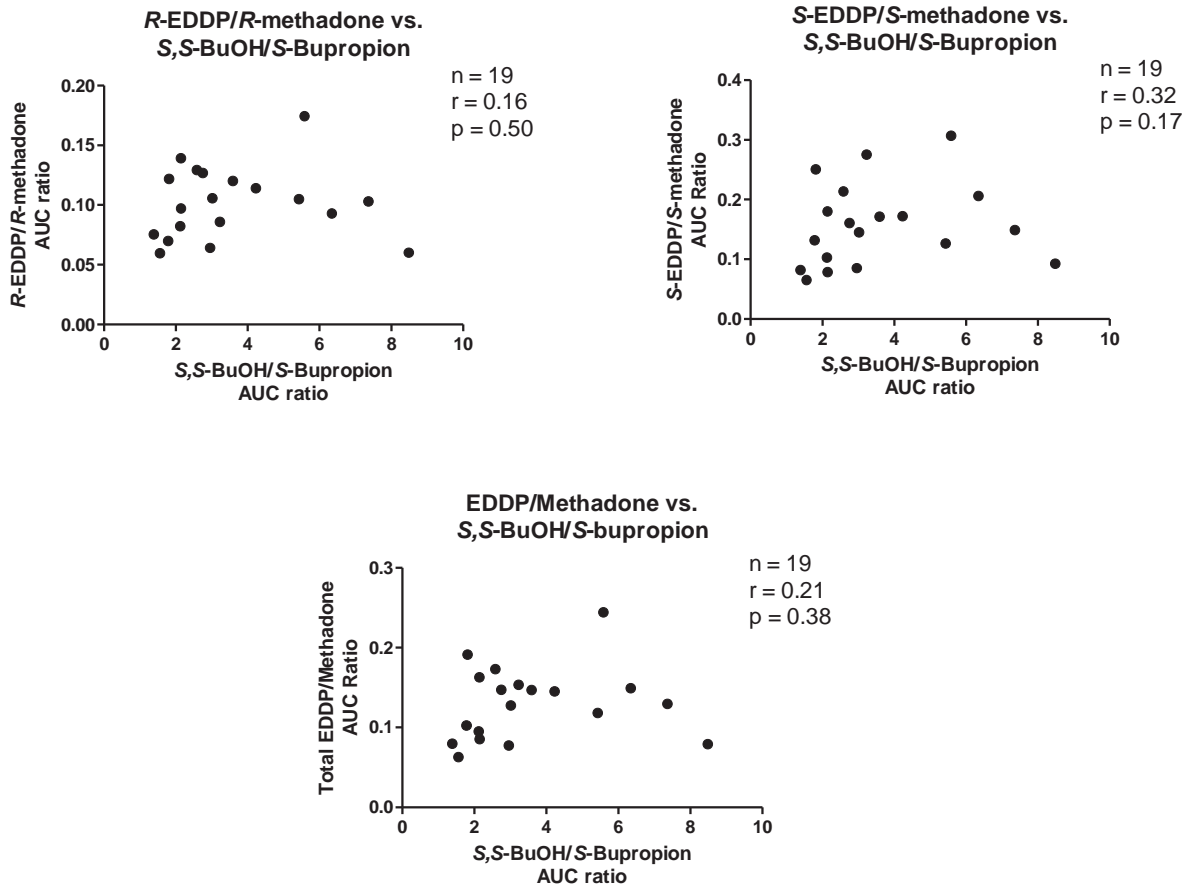
**Figure 2.18** – P-gp activity (digoxin 5-hr AUC) correlated with *R*-, *S*-, and total methadone 4-hr AUC.



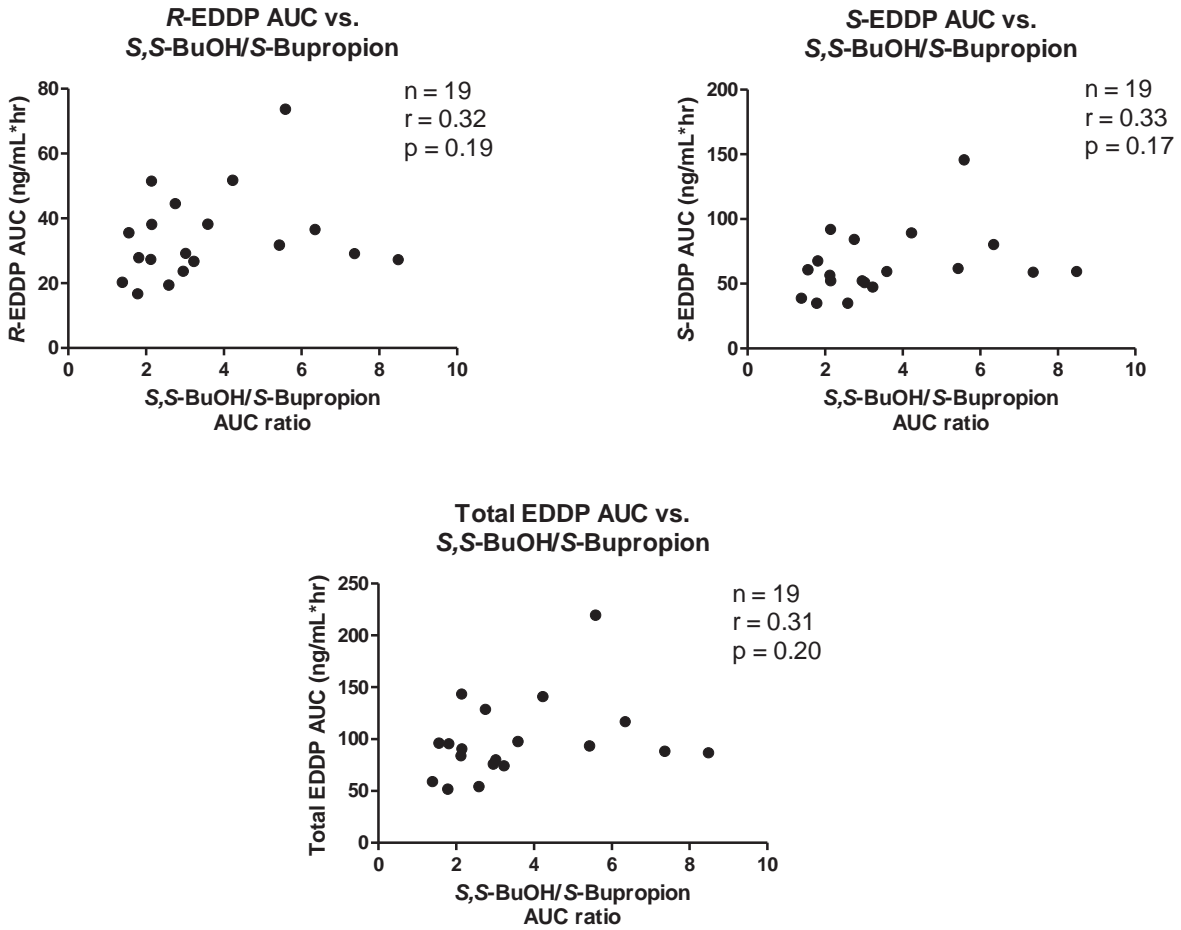
**Figure 2.19--** *S,S*-hydroxybupropion to *S*-bupropion AUC ratio (a possible phenotypic marker of CYP2B6 activity) correlated with *R*-, *S*-, and total methadone clearance.



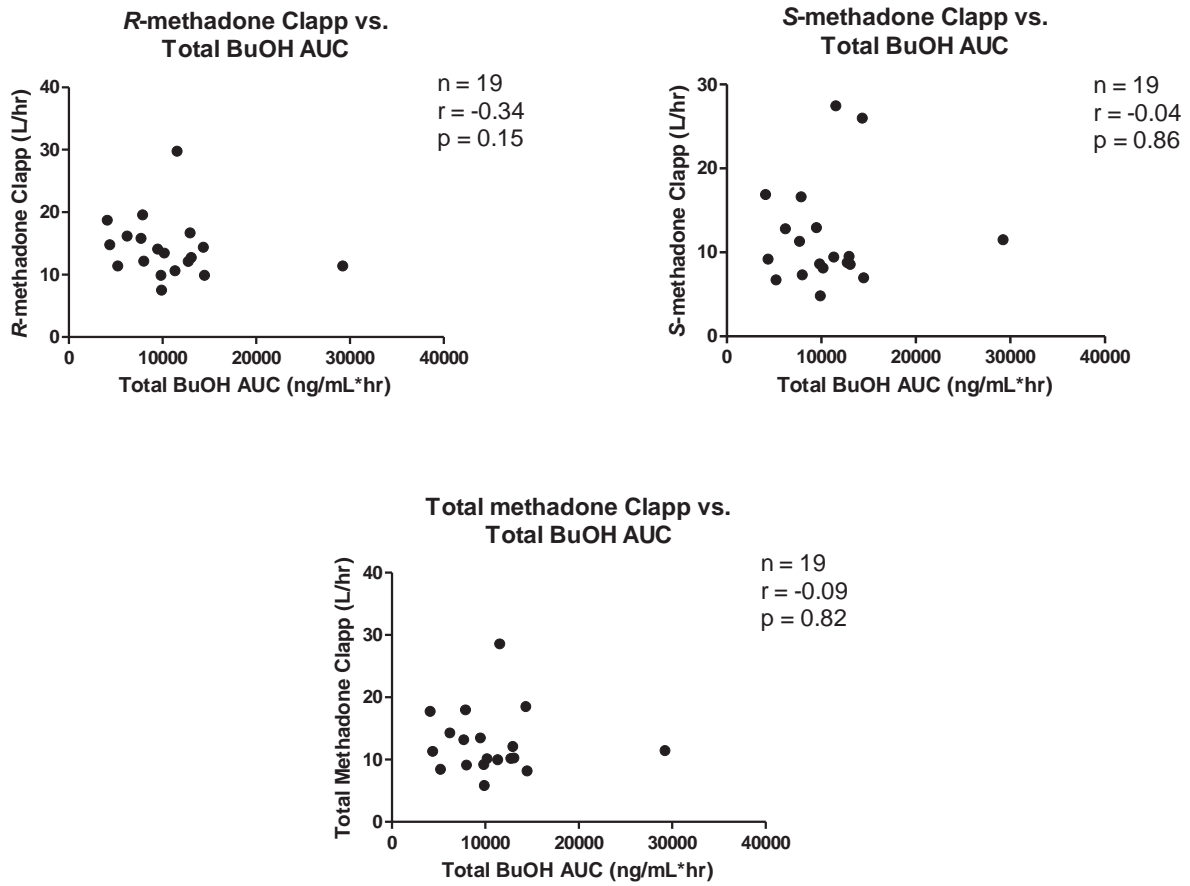
**Figure 2.20** -- *S,S*-hydroxybupropion to *S*- bupropion AUC ratio ratio (a possible phenotypic marker of CYP2B6 activity) correlated with *R*-, *S*-, and total EDDP to methadone AUC ratio.



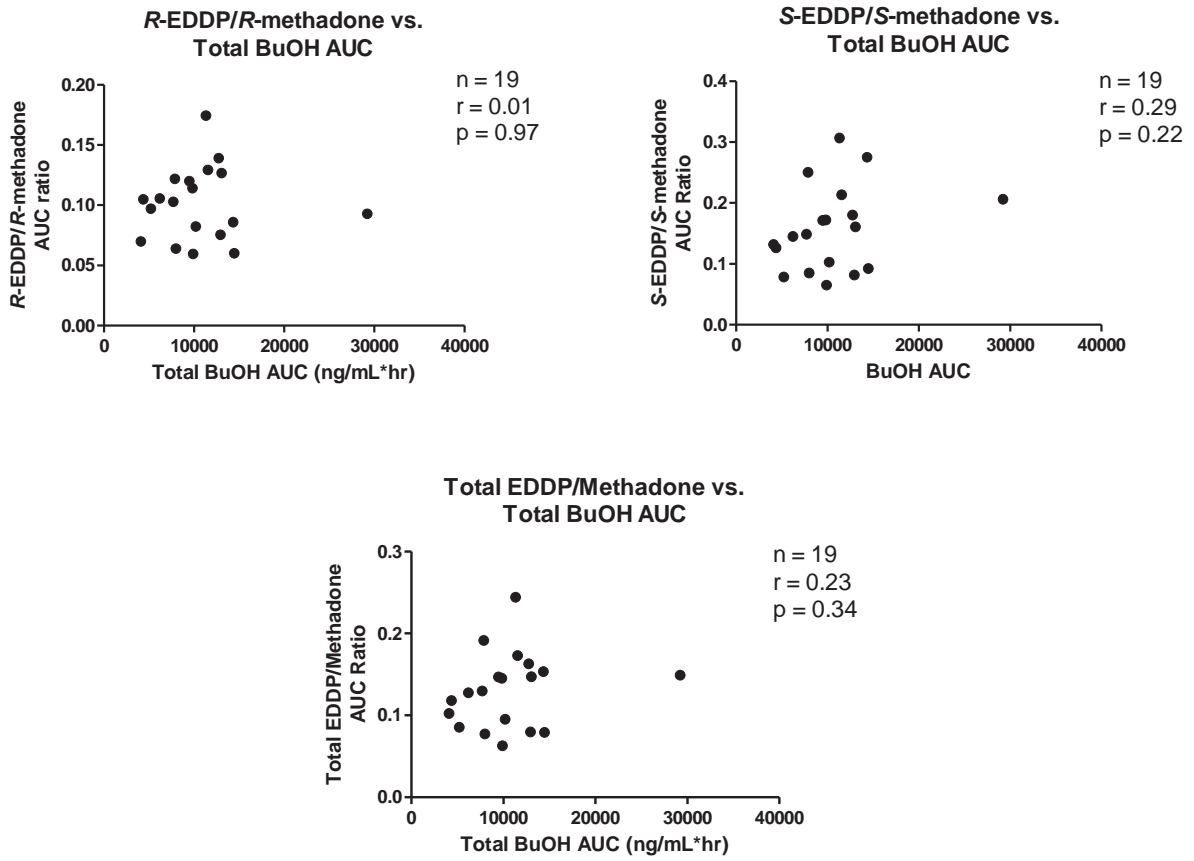
**Figure 2.21** -- *S,S*-hydroxybupropion to *S*-bupropion AUC ratio (a possible phenotypic marker of CYP2B6 activity) correlated with *R*-, *S*-, and total EDDP AUC.



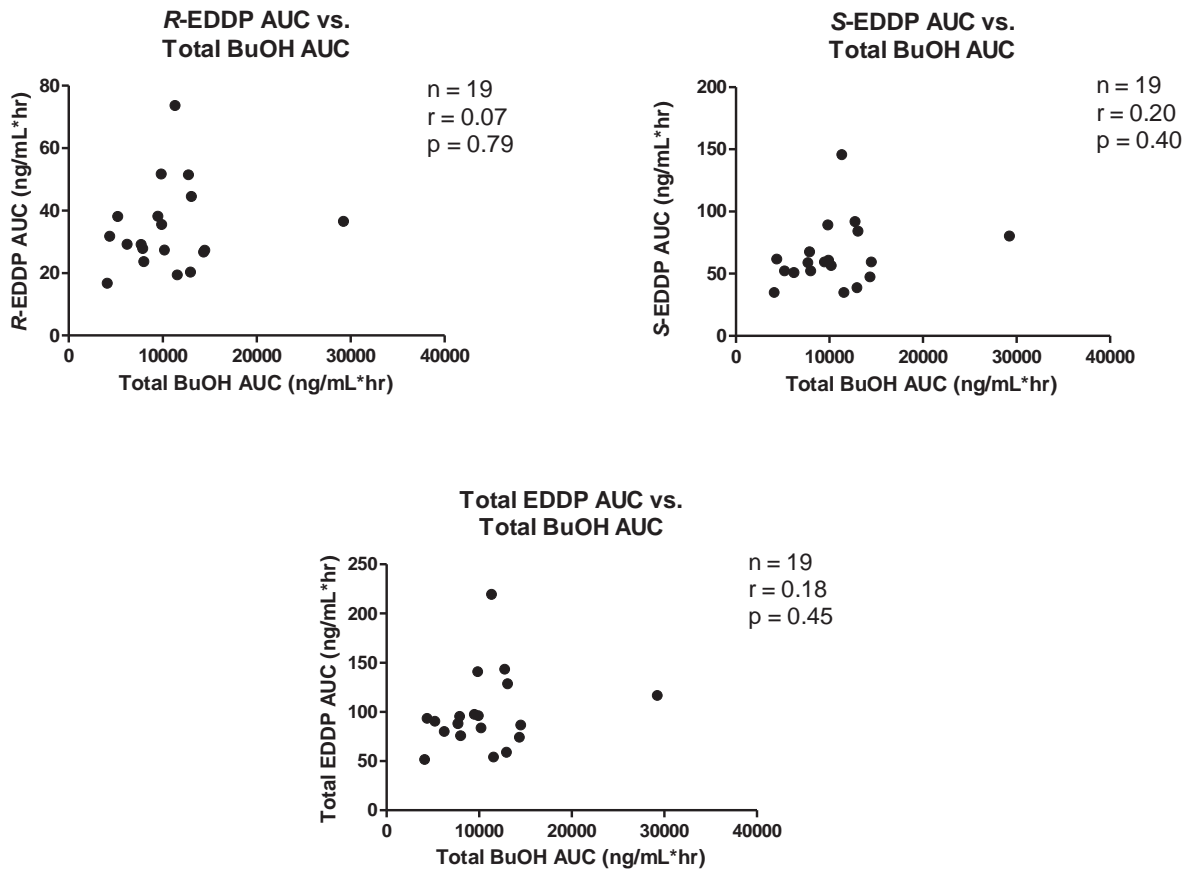
**Figure 2.22** -- Hydroxybupropion AUC (a possible phenotypic marker of CYP2B6 activity) correlated with *R*-, *S*-, and total methadone clearance.



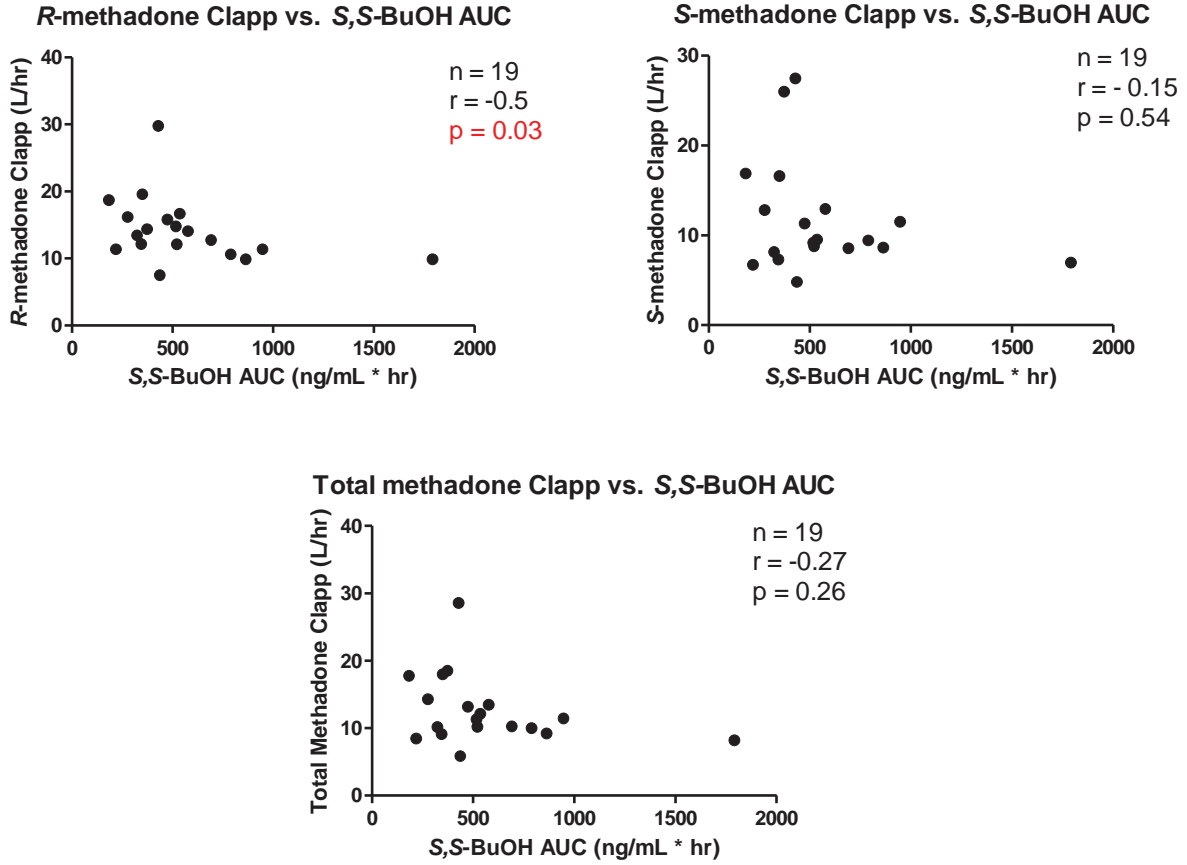
**Figure 2.23** -- Hydroxybupropion AUC (a possible phenotypic marker of CYP2B6 activity) correlated with *R*-, *S*-, and total EDDP to methadone AUC ratio.



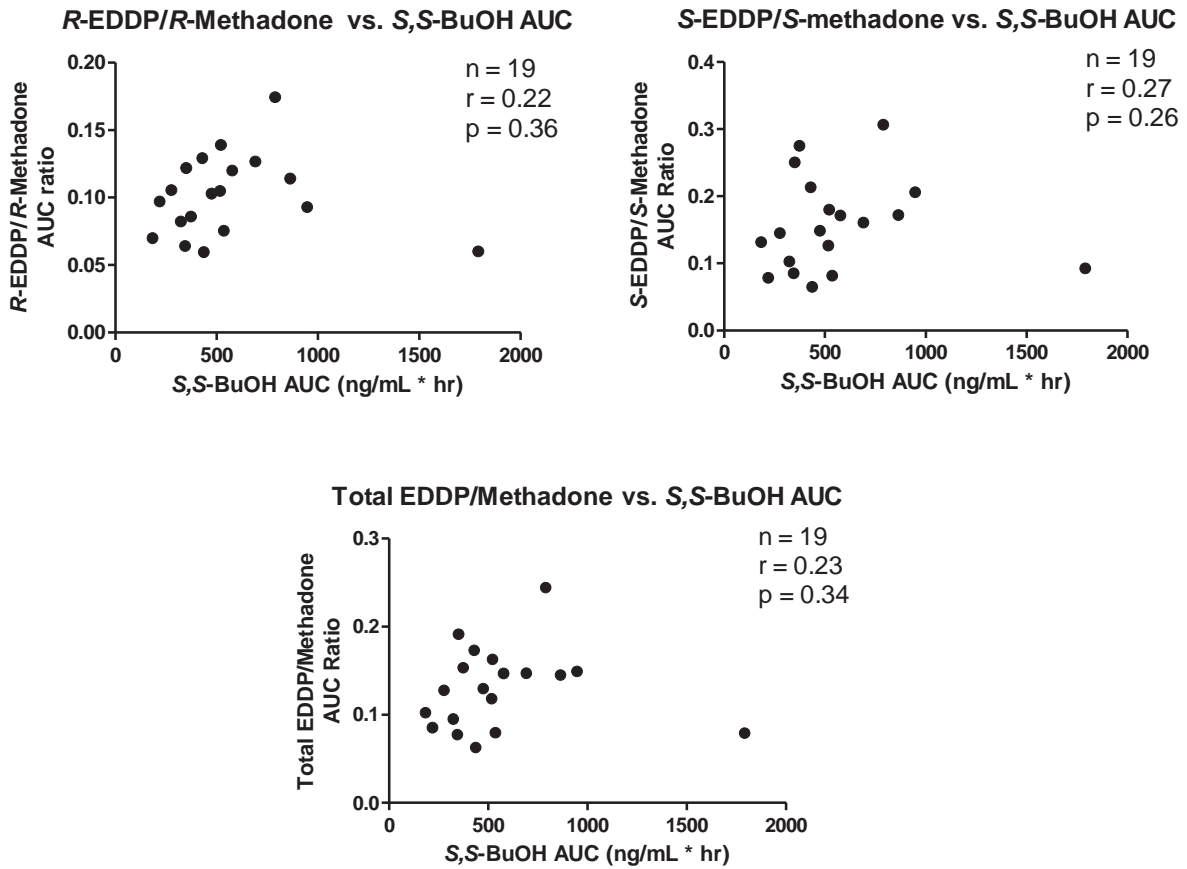
**Figure 2.24** -- Hydroxybupropion AUC (a possible phenotypic marker of CYP2B6 activity) correlated with *R*-, *S*-, and total EDDP AUC.



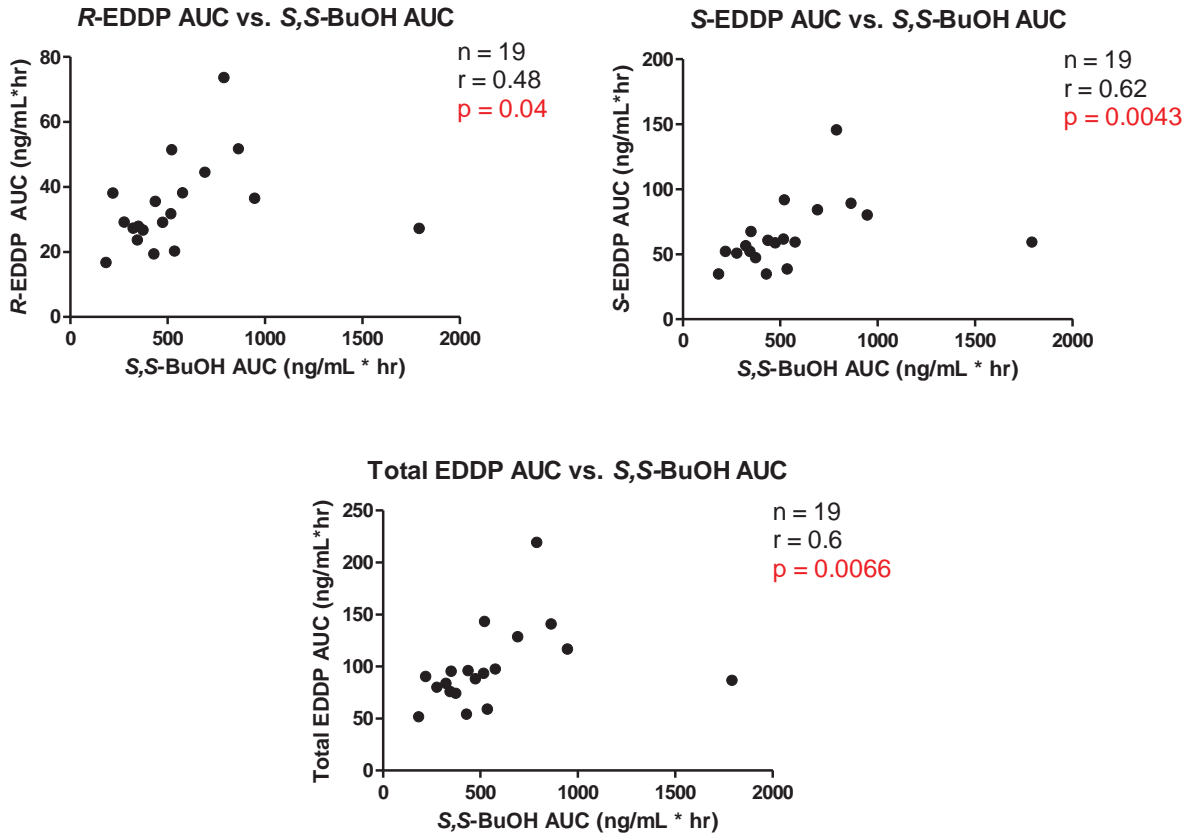
**Figure 2.25** -- *S,S*-hydroxybupropion AUC (a possible phenotypic marker of CYP2B6 activity) correlated with *R*-, *S*-, and total methadone clearance.



**Figure 2.26** -- *S,S*-hydroxybupropion AUC (a possible phenotypic marker of CYP2B6 activity) correlated with *R*-, *S*-, and total EDDP to methadone AUC ratio.



**Figure 2.27** -- *S,S*-hydroxybupropion AUC (a possible phenotypic marker of CYP2B6 activity) correlated with *R*-, *S*-, and total EDDP AUC.



**Table 2.14** -- Summary of correlation analyses with CYP3A4. Reported are the Spearman coefficient and the corresponding p-value. All p-values were corrected with a Bonferonni adjustment.

CYP3A4 Correlation studies, n = 19				
Independent variable	Outcome variable	Spearman Coefficient-rho	p-value	Adjusted p-value
Midazolam Clapp	<i>R</i> -methadone Clapp	0.53	0.02	0.18
Midazolam Clapp	<i>S</i> -methadone Clapp	0.33	0.17	1.53
Midazolam Clapp	Total methadone Clapp	0.34	0.15	1.35
Midazolam Clapp	<i>R</i> -EDDP/ <i>R</i> -methadone	0.19	0.43	3.87
Midazolam Clapp	<i>S</i> -EDDP/ <i>S</i> -methadone	-0.07	0.76	6.84
Midazolam Clapp	Total EDDP/methadone	-0.005	0.98	8.82
Midazolam Clapp	<i>R</i> -EDDP AUC	-0.21	0.39	3.51
Midazolam Clapp	<i>S</i> -EDDP AUC	-0.41	0.08	0.72
Midazolam Clapp	Total EDDP AUC	-0.36	0.13	1.17

**Table 2.15** -- Summary of correlation analyses with P-gp. Reported are the Spearman coefficient and the corresponding p-value. All p-values were corrected with a Bonferonni adjustment.

P-gp Activity Correlation studies, n = 19				
Independent variable	Outcome variable	Spearman Coefficient-rho	p-value	Adjusted p-value
Digoxin 5 hr AUC	<i>R</i> -methadone Clapp	-0.29	0.23	1.38
Digoxin 5 hr AUC	<i>S</i> -methadone Clapp	-0.13	0.6	3.6
Digoxin 5 hr AUC	Total methadone Clapp	-0.19	0.42	2.52
Digoxin 5 hr AUC	<i>R</i> -methadone 4 hr AUC	0.15	0.54	3.24
Digoxin 5 hr AUC	<i>S</i> -methadone 4 hr AUC	0.13	0.62	3.72
Digoxin 5 hr AUC	Total methadone 4 hr AUC	0.13	0.6	3.6

**Table 2.16** -- Summary of correlation analyses with CYP2B6. Reported are the Spearman coefficient and the corresponding p-value. All p-values were corrected with a Bonferonni adjustment.

A. CYP2B6 activity described as *S,S*-hydroxybupropion (*S,S*-BuOH)/*S*-Bupropion AUC ratio.

CYP2B6 Activity ( <i>S,S</i> -BuOH/ <i>S</i> -Bupropion) Correlation studies, n = 19				
Independent variable	Outcome variable	Spearman Coefficient- rho	p-value	Adjusted p-value
<i>S,S</i> -BuOH/ <i>S</i> -Bupropion	<i>R</i> -methadone Clapp	-0.32	0.18	1.62
<i>S,S</i> -BuOH/ <i>S</i> -Bupropion	<i>S</i> -methadone Clapp	0.02	0.93	8.37
<i>S,S</i> -BuOH/ <i>S</i> -Bupropion	Total methadone Clapp	-0.1	0.69	6.21
<i>S,S</i> -BuOH/ <i>S</i> -Bupropion	<i>R</i> -EDDP/ <i>R</i> -methadone	0.14	0.58	5.22
<i>S,S</i> -BuOH/ <i>S</i> -Bupropion	<i>S</i> -EDDP/ <i>S</i> -methadone	0.4	0.09	0.81
<i>S,S</i> -BuOH/ <i>S</i> -Bupropion	Total EDDP/methadone	0.16	0.5	4.5
<i>S,S</i> -BuOH/ <i>S</i> -Bupropion	<i>R</i> -EDDP AUC	0.32	0.19	1.71
<i>S,S</i> -BuOH/ <i>S</i> -Bupropion	<i>S</i> -EDDP AUC	0.33	0.17	1.53
<i>S,S</i> -BuOH/ <i>S</i> -Bupropion	Total EDDP AUC	0.31	0.2	1.8

B. CYP2B6 activity described as total hydroxybupropion (BuOH).

CYP2B6 Activity (Total BuOH) Correlation studies, n = 19				
Independent variable	Outcome variable	Spearman Coefficient- rho	p-value	Adjusted p-value
Total BuOH AUC	<i>R</i> -methadone Clapp	-0.34	0.15	1.35
Total BuOH AUC	<i>S</i> -methadone Clapp	-0.04	0.86	7.74
Total BuOH AUC	Total methadone Clapp	-0.09	0.72	6.48
Total BuOH AUC	<i>R</i> -EDDP/ <i>R</i> -methadone	0.01	0.97	8.73
Total BuOH AUC	<i>S</i> -EDDP/ <i>S</i> -methadone	0.29	0.22	1.98
Total BuOH AUC	Total EDDP/methadone	0.23	0.34	3.06
Total BuOH AUC	<i>R</i> -EDDP AUC	0.07	0.79	7.11
Total BuOH AUC	<i>S</i> -EDDP AUC	0.2	0.4	3.6
Total BuOH AUC	Total EDDP AUC	0.18	0.45	4.05

C. CYP2B6 activity described as *S,S*-hydroxybupropion (*S,S*-BuOH)

CYP2B6 Activity ( <i>S,S</i> -BuOH) Correlation studies, n = 19				
Independent variable	Outcome variable	Spearman Coefficient- rho	p-value	Adjusted p-value
<i>S,S</i> -BuOH AUC	<i>R</i> -methadone Clapp	-0.5	0.03	0.27
<i>S,S</i> -BuOH AUC	<i>S</i> -methadone Clapp	-0.15	0.54	4.86
<i>S,S</i> -BuOH AUC	Total methadone Clapp	-0.27	0.26	2.34
<i>S,S</i> -BuOH AUC	<i>R</i> -EDDP/ <i>R</i> -methadone	0.22	0.36	3.24
<i>S,S</i> -BuOH AUC	<i>S</i> -EDDP/ <i>S</i> -methadone	0.27	0.26	2.34
<i>S,S</i> -BuOH AUC	Total EDDP/methadone	0.23	0.34	3.06
<i>S,S</i> -BuOH AUC	<i>R</i> -EDDP AUC	0.48	0.04	0.36
<i>S,S</i> -BuOH AUC	<i>S</i> -EDDP AUC	0.62	0.0043	0.0387
<i>S,S</i> -BuOH AUC	Total EDDP AUC	0.6	0.0066	0.0594

**Table 2.17** -- Multivariate linear regression analysis with methadone clearance as the outcome variable (n = 19).

Model	Total Methadone Cl	R-methadone Cl	S-methadone Cl
CYP3A4	$\beta_0 = 8.8$ p = 0.023; $\beta_1 = 0.054$ p = 0.26; F = 1.35 p = 0.26	$\beta_0 = 9.75$ p = 0.008; $\beta_1 = 0.063$ p = 0.16; F = 2.2 p = 0.16	$\beta_0 = 8.9$ p = 0.052; $\beta_1 = 0.04$ p = 0.5; F = 0.5 p = 0.5
P-gp	$\beta_0 = 9.3$ p = 0.007; $\beta_2 = 0.03$ p = 0.30; F = 1.42 p = 0.25	$\beta_0 = 10.6$ p = 0.0015; $\beta_2 = 0.03$ p = 0.18; F = 1.98 p = 0.177	$\beta_0 = 9.36$ p = 0.02; $\beta_2 = 0.02$ p = 0.49; F = 0.5 p = 0.5
CYP2B6	$\beta_0 = 15.18$ p = 2.08 e-06; $\beta_3 = -0.0045$ p = 0.181; F = 1.95 p = 0.181	$\beta_0 = 17.07$ p = 1.44 e-7; $\beta_3 = -0.005$ p = 0.115; F = 2.76 p = 0.115	$\beta_0 = 14.37$ p = 3.68 e-5; $\beta_3 = -0.0047$ p = 0.252; F = 1.41 p = 0.252
CYP3A4 + P-gp	$\beta_0 = 6.6$ p = 0.13; $\beta_1 = 0.04$ p = 0.36; $\beta_2 = 0.02$ p = 0.35; F = 1.15 p = 0.34	$\beta_0 = 7.45$ p = 0.066; $\beta_1 = 0.053$ p = 0.236; $\beta_2 = 0.025$ p = 0.266; F = 1.78 p = 0.2	$\beta_0 = 7.4$ p = 0.17; $\beta_1 = 0.03$ p = 0.58; $\beta_2 = 0.017$ p = 0.58; F = 0.4 p = 0.7
CYP3A4 + CYP2B6	$\beta_0 = 11.7$ p = 0.015; $\beta_1 = 0.04$ p = 0.37; $\beta_3 = -0.004$ p = 0.25; F = 1.4 p = 0.28	$\beta_0 = 13.0$ p = 0.004; $\beta_1 = 0.051$ p = 0.24; $\beta_3 = -0.004$ p = 0.17; F = 2.2 p = 0.15	$\beta_0 = 12.1$ p = 0.03; $\beta_1 = 0.03$ p = 0.63; $\beta_3 = -0.004$ p = 0.31; F = 0.80 p = 0.47
CYP2B6 + P-gp	$\beta_0 = 12.3$ p = 0.009; $\beta_2 = 0.02$ p = 0.42; $\beta_3 = -0.004$ p = 0.30; F = 1.3 p = 0.30	$\beta_0 = 13.9$ p = 0.002; $\beta_2 = 0.022$ p = 0.34; $\beta_3 = -0.004$ p = 0.22; F = 1.9 p = 0.19	$\beta_0 = 12.7$ p = 0.022; $\beta_2 = 0.011$ p = 0.71; $\beta_3 = -0.004$ p = 0.34; F = 0.74 p = 0.49
CYP3A4 + P-gp + CYP2B6	$\beta_0 = 9.7$ p = 0.09; $\beta_1 = 0.04$ p = 0.43; $\beta_2 = 0.017$ p = 0.51; $\beta_3 = -0.003$ p = 0.36; F = 1.05 p = 0.40	$\beta_0 = 10.8$ p = 0.039; $\beta_1 = 0.05$ p = 0.30; $\beta_2 = 0.02$ p = 0.43; $\beta_3 = -0.004$ p = 0.28; F = 1.64 p = 0.22	$\beta_0 = 11.0$ p = 0.11; $\beta_1 = 0.03$ p = 0.67; $\beta_2 = 0.009$ p = 0.77; $\beta_3 = -0.004$ p = 0.40; F = 0.53 p = 0.67

**Table 2.18** -- Multivariate linear regression analysis with EDDP AUC as the outcome variable (n=19).

Model	Total EDDP AUC	R-EDDP AUC	S-EDDP AUC
CYP3A4	$\beta_0 = 118.9$ p = 0.0004; $\beta_1 = -0.28$ p = 0.44; F = 0.62 p = 0.44	$\beta_0 = 39.3$ p = 0.0008; $\beta_1 = -0.07$ p = 0.58; F = 0.32 p = 0.58	$\beta_0 = 79.6$ p = 0.0003; $\beta_1 = -0.21$ p = 0.38; F = 0.80 p = 0.38
P-gp	$\beta_0 = 134.7$ p = 1.04 e-5; $\beta_2 = -0.3$ p = 0.09; F = 3.2 p = 0.09	$\beta_0 = 46.4$ p = 1.44 e-5; $\beta_2 = -0.10$ p = 0.106; F = 2.91 p = 0.11	$\beta_0 = 88.3$ p = 1.14 e-5; $\beta_2 = -0.2$ p = 0.09; F = 3.2 p = 0.09
CYP2B6	$\beta_0 = 79.5$ p = 0.0001; $\beta_3 = 0.03$ p = 0.18; F = 2.0 p = 0.18	$\beta_0 = 28.7$ p = 0.0001; $\beta_3 = 0.01$ p = 0.28; F = 1.23 p = 0.28	$\beta_0 = 50.8$ p = 0.0001; $\beta_3 = 0.02$ p = 0.14; F = 2.36 p = 0.14
CYP3A4 + P-gp	$\beta_0 = 144.7$ p = 0.0002; $\beta_1 = -0.17$ p = 0.63; $\beta_2 = -0.28$ p = 0.13; F = 1.63 p = 0.23	$\beta_0 = 48.3$ p = 0.0004; $\beta_1 = -0.03$ p = 0.80; $\beta_2 = -0.1$ p = 0.14; F = 1.4 p = 0.27	$\beta_0 = 96.5$ p = 0.0002; $\beta_1 = -0.14$ p = 0.56; $\beta_2 = -0.18$ p = 0.13; F = 1.71 p = 0.21
CYP3A4 + CYP2B6	$\beta_0 = 95.0$ p = 0.011; $\beta_1 = -0.20$ p = 0.59; $\beta_3 = 0.03$ p = 0.23; F = 1.1 p = 0.36	$\beta_0 = 32.4$ p = 0.015; $\beta_1 = -0.05$ p = 0.72; $\beta_3 = 0.009$ p = 0.34; F = 0.65 p = 0.54	$\beta_0 = 62.6$ p = 0.01; $\beta_1 = -0.15$ p = 0.53; $\beta_3 = 0.02$ p = 0.19; F = 1.34 p = 0.29
CYP2B6 + P-gp	$\beta_0 = 115.3$ p = 0.0014; $\beta_2 = -0.25$ p = 0.18; $\beta_3 = 0.02$ p = 0.35; F = 2.04 p = 0.16	$\beta_0 = 41.5$ p = 0.001; $\beta_2 = -0.09$ p = 0.18; $\beta_3 = 0.006$ p = 0.51; F = 1.63 p = 0.23	$\beta_0 = 73.8$ p = 0.002; $\beta_2 = -0.16$ p = 0.19; $\beta_3 = 0.018$ p = 0.29; F = 2.2 p = 0.14
CYP3A4 + P-gp + CYP2B6	$\beta_0 = 123.8$ p = 0.006; $\beta_1 = -0.13$ p = 0.73; $\beta_2 = -0.24$ p = 0.21; $\beta_3 = 0.022$ p = 0.40; F = 1.3 p = 0.30	$\beta_0 = 42.9$ p = 0.008; $\beta_1 = -0.02$ p = 0.87; $\beta_2 = -0.09$ p = 0.21; $\beta_3 = 0.005$ p = 0.55; F = 1.03 p = 0.41	$\beta_0 = 80.9$ p = 0.006; $\beta_1 = -0.10$ p = 0.66; $\beta_2 = -0.15$ p = 0.22; $\beta_3 = 0.02$ p = 0.34; F = 1.46 p = 0.26

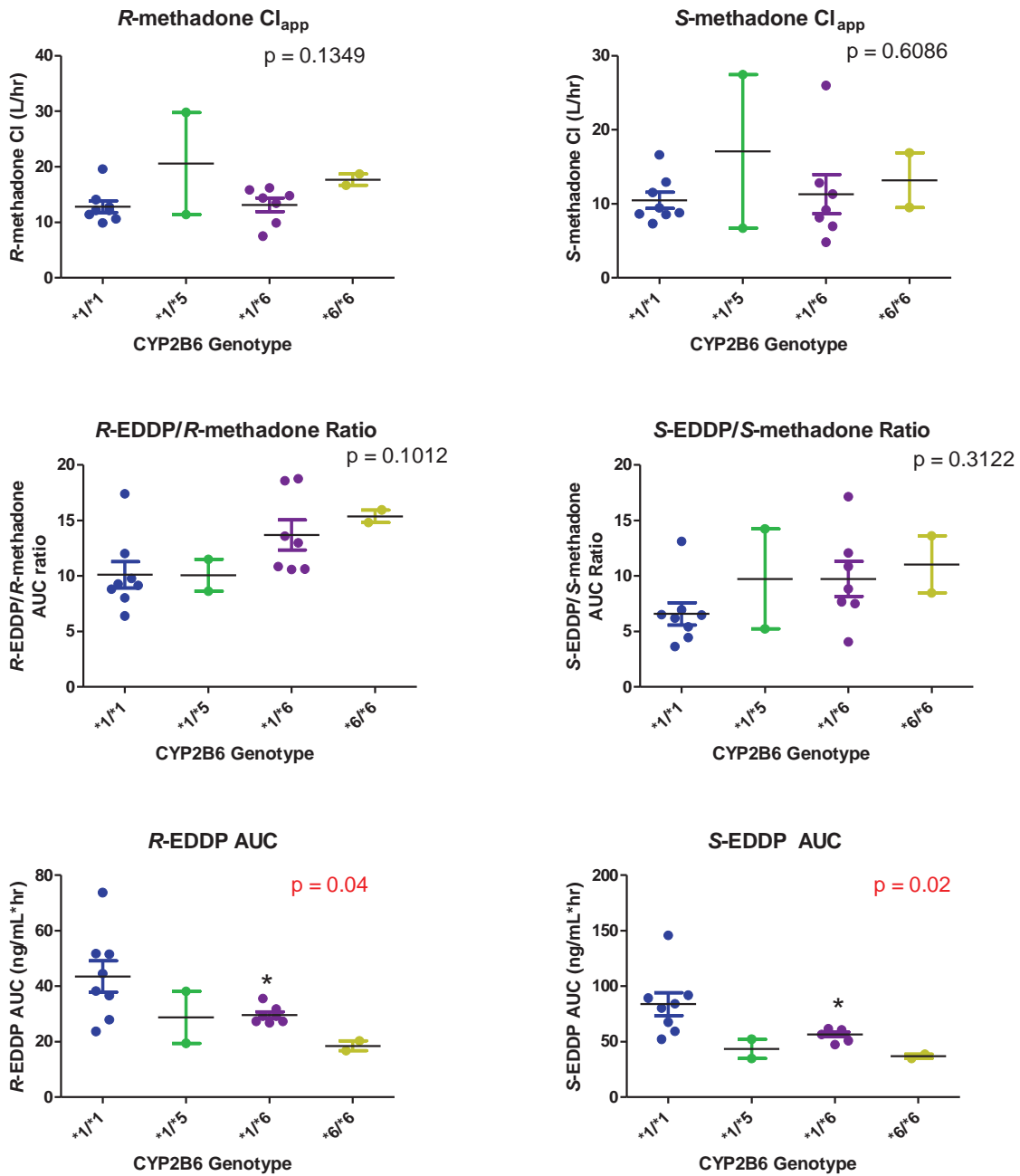
**Table 2.19** -- Multivariate linear regression analysis with methadone clearance as the outcome variable (n = 18).

Model	Total Methadone CI	R-methadone CI	S-methadone CI
CYP3A4	$\beta_0 = 9.12, p = 0.02; \beta_1 = 0.05, p = 0.28; F = 1.24, p = 0.28$	$\beta_0 = 10.1, p = 0.007; \beta_1 = 0.06, p = 0.17; F = 2.04, p = 0.17$	$\beta_0 = 9.3, p = 0.05; \beta_1 = 0.04, p = 0.52, F = 0.44, p = 0.52$
P-gp	$\beta_0 = 9.7, p = 0.007; \beta_2 = 0.03, p = 0.28; F = 1.23, p = 0.28$	$\beta_0 = 11.0, p = 0.0015; \beta_2 = 0.03, p = 0.2; F = 1.8, p = 0.2$	$\beta_0 = 9.8, p = 0.02; \beta_2 = 0.02, p = 0.53, F = 0.41, p = 0.53$
CYP2B6	$\beta_0 = 16.1, p = 9.5e-05; \beta_3 = -0.006, p = 0.28; F = 1.2, p = 0.28$	$\beta_0 = 18.4, p = 7.7e-06; \beta_3 = -0.007, p = 0.16; F = 2.2, p = 0.16$	$\beta_0 = 15.1, p = 9.9e-4; \beta_3 = -0.006, p = 0.39, F = 0.78, p = 0.39$
CYP3A4 + P-gp	$\beta_0 = 7.1, p = 0.12; \beta_1 = 0.04, p = 0.38; \beta_2 = 0.02, p = 0.38, F = 1.02, p = 0.38$	$\beta_0 = 7.9, p = 0.06; \beta_1 = 0.05, p = 0.25; \beta_2 = 0.02, p = 0.3, F = 1.6, p = 0.23$	$\beta_0 = 7.9, p = 0.15; \beta_1 = 0.03, p = 0.60; \beta_2 = 0.02, p = 0.62; F = 0.34, p = 0.72$
CYP3A4 + CYP2B6	$\beta_0 = 12.4, p = 0.035; \beta_1 = 0.04, p = 0.42; \beta_3 = -0.005, p = 0.42; F = 0.95, p = 0.41$	$\beta_0 = 14.2, p = 0.01; \beta_1 = 0.05, p = 0.3; \beta_3 = -0.006, p = 0.27; F = 1.7, p = 0.22$	$\beta_0 = 12.8, p = 0.071, \beta_1 = 0.03, p = 0.67, \beta_3 = -0.005, p = 0.5, F = 0.46, p = 0.64$
CYP2B6 + P-gp	$\beta_0 = 12.9, p = 0.03; \beta_2 = 0.02, p = 0.49; \beta_3 = -0.005, p = 0.49; F = 0.85, p = 0.45$	$\beta_0 = 15.1, p = 0.008; \beta_2 = 0.02, p = 0.44; \beta_3 = -0.005, p = 0.33, F = 1.4, p = 0.28$	$\beta_0 = 13.4, p = 0.06; \beta_2 = 0.01, p = 0.76; \beta_3 = -0.005, p = 0.52, F = 0.41, p = 0.67$
CYP3A4 + P-gp + CYP2B6	$\beta_0 = 9.9, p = 0.17; \beta_1 = 0.04, p = 0.46; \beta_2 = 0.02, p = 0.55; \beta_3 = -0.003, p = 0.61; F = 0.74, p = 0.55$	$\beta_0 = 11.6, p = 0.08; \beta_1 = 0.04, p = 0.34; \beta_2 = 0.02, p = 0.50; \beta_3 = -0.005, p = 0.45; F = 1.25, p = 0.33$	$\beta_0 = 11.4, p = 0.2; \beta_1 = 0.02, p = 0.70; \beta_2 = 0.009, p = 0.8; \beta_3 = -0.004, p = 0.82$

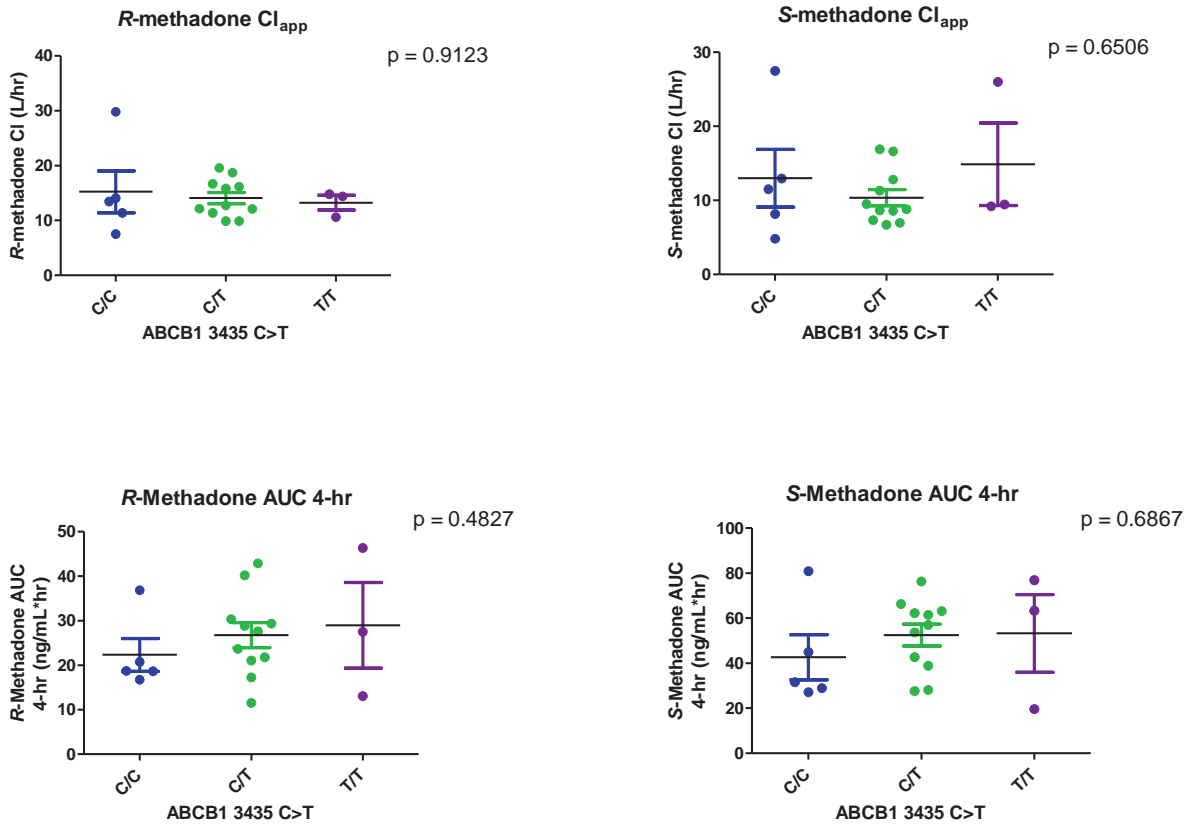
**Table 2.20** -- Multivariate linear regression analysis with EDDP AUC as the outcome variable (n = 18).

Model	Total EDDP AUC (Y4)	R-EDDP AUC (Y5)	S-EDDP AUC (Y6)
CYP3A4	$\beta_0 = 120.1$ , $p = 5.7e-4$ ; $\beta_1 = -0.29$ , $p = 0.44$ ; $F = 0.61$ , $p = 0.45$	$\beta_0 = 40.0$ , $p = 9.5e-4$ ; $\beta_1 = -0.074$ , $p = 0.57$ ; $F = 0.33$ , $p = 0.57$	$\beta_0 = 80.2$ , $p = 5.0e-4$ ; $\beta_1 = -0.21$ , $p = 0.40$ ; $F = 0.77$ , $p = 0.39$
P-gp	$\beta_0 = 136.5$ , $p = 1.8e-5$ ; $\beta_2 = -0.31$ , $p = 0.10$ ; $F = 3.14$ , $p = 0.1$	$\beta_0 = 47.2$ , $p = 2.12e-5$ ; $\beta_2 = -0.10$ , $p = 0.104$ ; $F = 2.98$ , $p = 0.10$	$\beta_0 = 89.2$ , $p = 2.1e-5$ ; $\beta_2 = -0.2$ , $p = 0.098$ ; $F = 3.09$ , $p = 0.098$
CYP2B6	$\beta_0 = 38.4$ , $p = 0.05$ ; $\beta_3 = 0.12$ , $p = 0.0024$ ; $F = 12.96$ , $p = 0.002$	$\beta_0 = 14.5$ , $p = 0.05$ ; $\beta_3 = 0.041$ , $p = 0.0051$ ; $F = 10.52$ , $p = 0.0051$	$\beta_0 = 23.9$ , $p = 0.065$ ; $\beta_3 = 0.08$ , $p = 0.002$ ; $F = 13.6$ , $p = 0.002$
CYP3A4 + P-gp	$\beta_0 = 146.9$ , $p = 3.12e-4$ ; $\beta_1 = -0.17$ , $p = 0.63$ ; $\beta_2 = -0.30$ , $p = 0.13$ ; $F = 1.62$ , $p = 0.23$	$\beta_0 = 49.3$ , $p = 4.9e-4$ ; $\beta_1 = -0.035$ , $p = 0.79$ ; $\beta_2 = -0.101$ , $p = 0.13$ ; $F = 1.44$ , $p = 0.27$	$\beta_0 = 97.5$ , $p = 2.9e-4$ ; $\beta_1 = -0.14$ , $p = 0.56$ ; $\beta_2 = -0.19$ , $p = 0.14$ ; $F = 1.66$ , $p = 0.22$
CYP3A4 + CYP2B6	$\beta_0 = 40.12$ , $p = 0.24$ ; $\beta_1 = -0.02$ , $p = 0.95$ ; $\beta_3 = 0.12$ , $p = 0.004$ ; $F = 6.08$ , $p = 0.01$	$\beta_0 = 13.13$ , $p = 0.29$ ; $\beta_1 = 0.015$ , $p = 0.89$ ; $\beta_3 = 0.04$ , $p = 0.008$ ; $F = 4.95$ , $p = 0.02$	$\beta_0 = 27.0$ , $p = 0.22$ ; $\beta_1 = -0.03$ , $p = 0.86$ ; $\beta_3 = 0.08$ , $p = 0.004$ ; $F = 6.4$ , $p = 0.0098$
CYP2B6 + P-gp	$\beta_0 = 59.0$ , $p = 0.09$ ; $\beta_2 = -0.13$ , $p = 0.45$ ; $\beta_3 = 0.11$ , $p = 0.01$ ; $F = 6.62$ , $p = 0.009$	$\beta_0 = 22.04$ , $p = 0.081$ ; $\beta_2 = -0.04$ , $p = 0.45$ ; $\beta_3 = 0.03$ , $p = 0.02$ ; $F = 5.4$ , $p = 0.02$	$\beta_0 = 36.9$ , $p = 0.10$ ; $\beta_2 = -0.076$ , $p = 0.47$ ; $\beta_3 = 0.076$ , $p = 0.0087$ ; $F = 6.88$ , $p = 0.0076$
CYP3A4 + P-gp + CYP2B6	$\beta_0 = 58.7$ , $p = 0.2$ ; $\beta_1 = 0.004$ , $p = 0.99$ ; $\beta_2 = -0.12$ , $p = 0.5$ ; $\beta_3 = 0.11$ , $p = 0.02$ ; $F = 4.1$ , $p = 0.03$	$\beta_0 = 20.14$ , $p = 0.20$ ; $\beta_1 = 0.024$ , $p = 0.83$ ; $\beta_2 = -0.05$ , $p = 0.45$ ; $\beta_3 = 0.04$ , $p = 0.025$ ; $F = 3.41$ , $p = 0.05$	$\beta_0 = 38.5$ , $p = 0.18$ ; $\beta_1 = -0.02$ , $p = 0.92$ ; $\beta_2 = -0.075$ , $p = 0.49$ ; $\beta_3 = 0.075$ , $p = 0.013$ ; $F = 4.29$ , $p = 0.024$

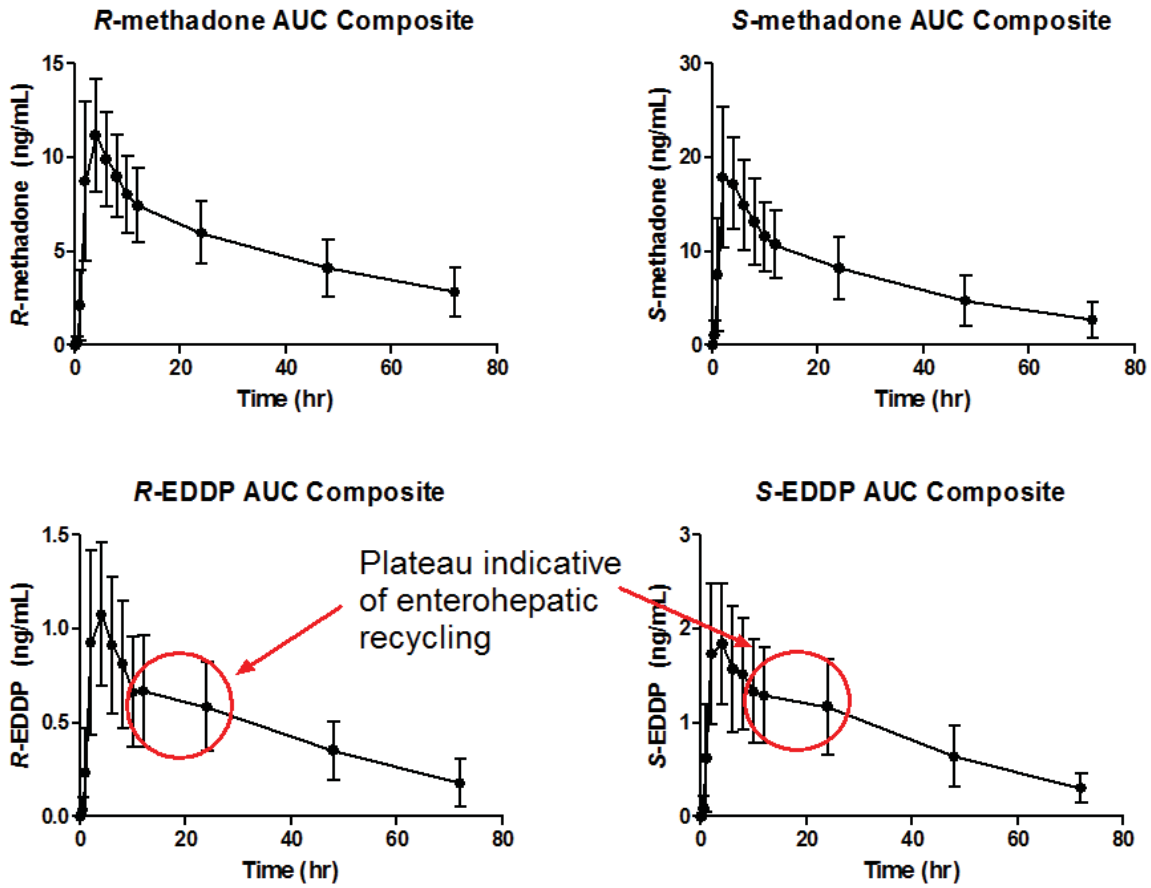
**Figure 2.28** -- *CYP2B6* genotype effect on methadone clearance, EDDP-to-methadone AUC ratio, and EDDP AUC.



**Figure 2.29** -- *ABCB1* genotype effect on methadone clearance and 4-hr AUC.



**Figure 2.30** -- Average AUC of *R*-methadone, *S*-methadone, *R*-EDDP, and *S*-EDDP. Circled in red is the apparent plateau *R*-EDDP and *S*-EDDP.



**Figure 2.31** -- *CYP2B6* genotype effect on *S,S*-hydroxybupropion-to-*S*-bupropion AUC ratio, *S,S*-hydroxybupropion AUC, and total hydroxybupropion AUC. There appeared to be an outlier in *S,S*-hydroxybupropion-to-*S*-bupropion AUC ratio and *S,S*-hydroxybupropion AUC analysis, and this individual was removed and the data was re-analyzed.

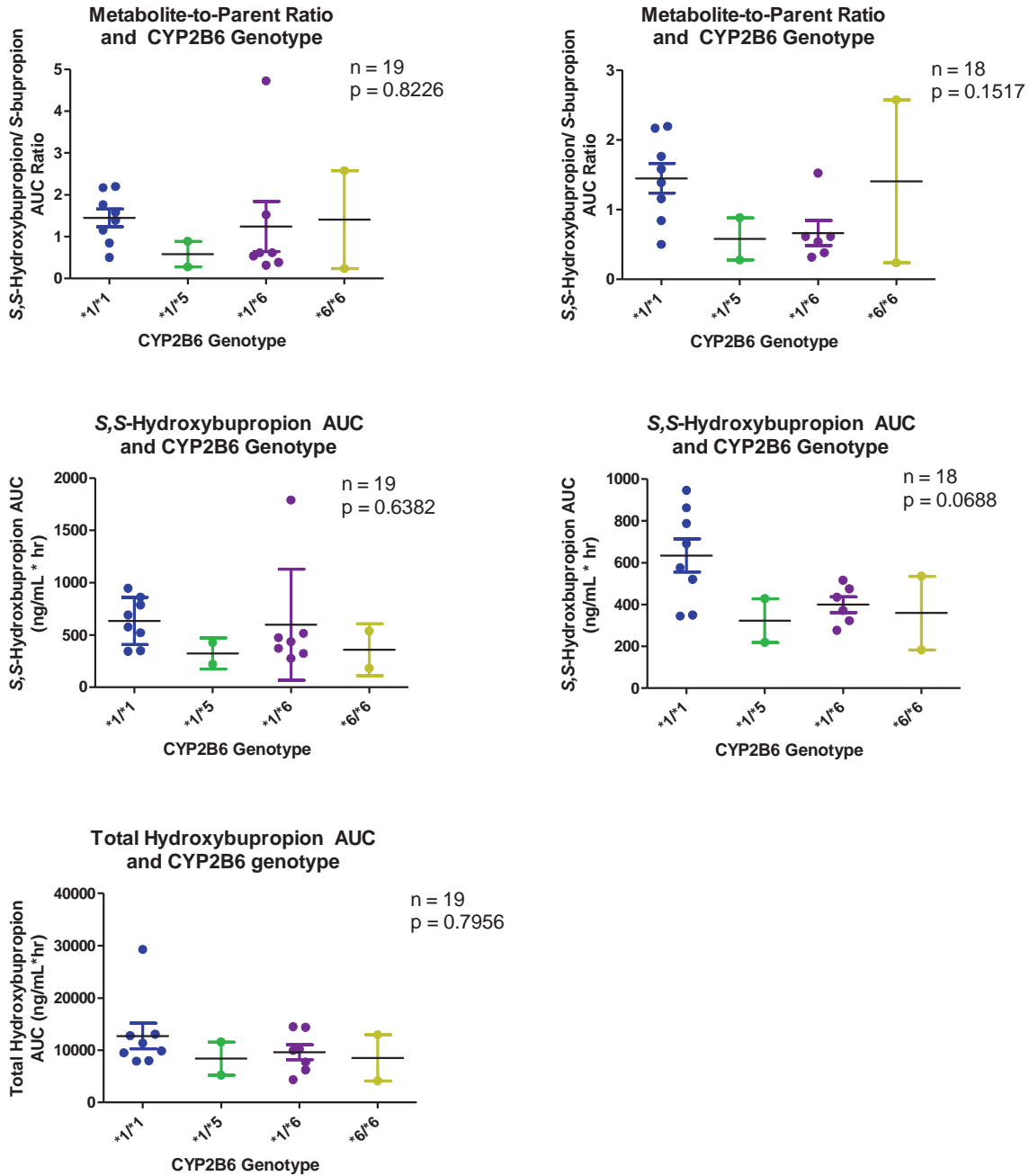
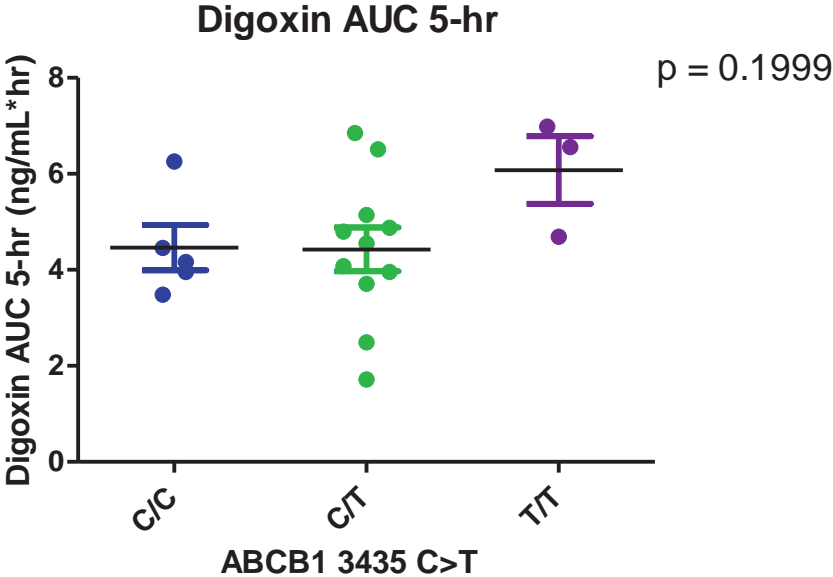
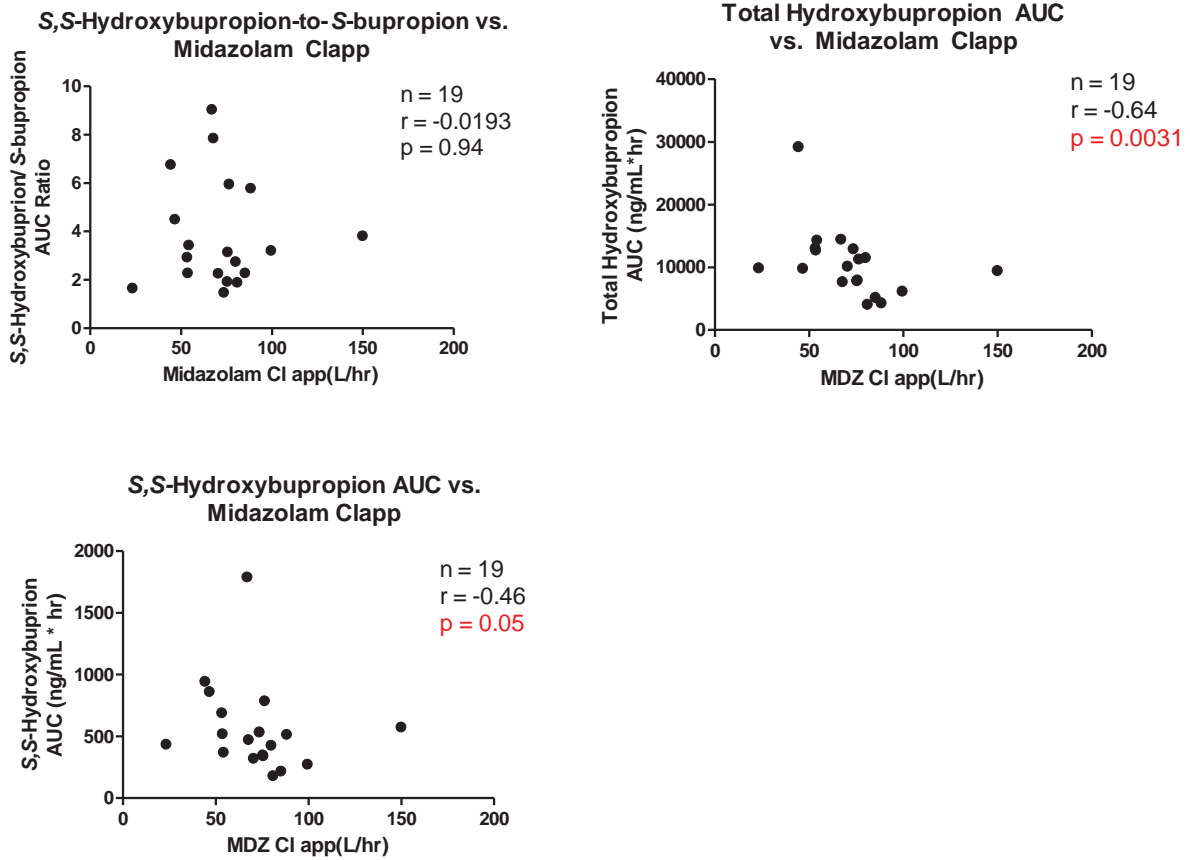


Figure 2.32 -- ABCB1 genotype and digoxin 5-hr AUC.



**Figure 2.33** -- CYP3A4 and CYP2B6 activity association analyses. CYP2B6 activity is presented as *S,S*-hydroxybupropion-to-*S*-bupropion AUC ratio, total hydroxybupropion AUC, and *S,S*-hydroxybupropion AUC.



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### **Chapter 3: *In vitro* studies on contribution of CYP3A4 and CYP2B6 to methadone metabolism**

#### **I. Introduction**

Methadone is the cornerstone of opioid maintenance treatment programs to rehabilitate heroin and opioid addicts. The drug is also widely used for the control of chronic pain. However, effective and safe use of methadone is plagued by high inter-individual variability stemming from unpredictable pharmacodynamics and pharmacokinetics. Metabolism of methadone occurs primarily through oxidation of the parent molecule by cytochrome P450 enzymes to form 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium (EDDP), an inactive metabolite. EDDP can be further metabolized to 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP) (Figure 3.1). A number of CYP enzymes have been implicated in oxidative metabolism of methadone. Subsequent oxidation of EDDP is presumably also performed by cytochrome P450 enzymes. Several studies conducted by Sullivan et. al in the 1970s suggest that EDDP is the major metabolite of methadone and thus, has been the focus of analysis when studying methadone clearance (Sullivan and Blake, 1972; Sullivan and Due, 1973; Sullivan et al., 1972). However, it is possible that EDDP formation is not the only route of metabolism for methadone and thus the variable disposition of methadone may be due to the unstudied and competing pathways of metabolism (Figure 3.1)

Although there is substantial evidence as to the role of cytochrome P450 enzymes in methadone metabolism, there is still debate surrounding which isoform contributes the most to *in vivo* methadone clearance. CYP3A4, CYP2B6, CYP2D6, CYP2C19, CYP2C9, and CYP1A2 have been implicated in methadone metabolism (Moody et al., 1997; Perez de los Cobos et al., 2007; Totah et al., 2008; Eap et al., 2001; Kapur et al., 2011). Gerber et al performed a screen

with methadone at 3.23  $\mu\text{M}$  (1  $\mu\text{g}/\text{mL}$ ) and 32.3  $\mu\text{M}$  (10  $\mu\text{g}/\text{mL}$ ) in CYP3A4, CYP2B6, CYP2D6, CYP2C19, CYP2C9, CYP1A2, and CYP2E1 Supersomes<sup>®</sup> to determine which isoforms had the highest activity. From these studies, it was determined that CYP3A4, CYP2B6, and CYP2C19 are the major isoforms capable of forming EDDP from methadone. It was also determined that CYP2B6 and CYP2C19 metabolize methadone stereoselectively. CYP2B6 metabolism results in higher formation of *S*-EDDP, while CYP2C19 metabolism exhibits higher *R*-EDDP formation (Gerber et al., 2004). Subsequent studies evaluating both enzymatic activity and protein content of CYP3A4, CYP2B6, and CYP2C19 concluded that CYP3A4 metabolizes methadone to the greatest extent, albeit non-stereoselectively. In addition, CYP2B6 is also the major enzyme involved in methadone stereoselective disposition (Totah et al., 2007; Totah et al., 2008). CYP2B6 is also a highly polymorphic enzyme and genetic variants of CYP2B6 could explain some of the inter-individual variability observed with methadone use.

This *in vitro* investigation aims to, first; probe alternative and subsequent routes of methadone metabolism in human hepatocytes treated with racemic methadone and EDDP. Evaluating other metabolites of methadone would lead to a better understanding of methadone metabolism *in vivo*, and possible alternative clearance pathways to investigate. Secondly, this study aims to evaluate the relative contribution of CYP3A4 and CYP2B6 to methadone metabolism in a panel of human liver microsomes (HLMs) that are phenotyped for CYP3A4 and CYP2B6 protein content and activity, and genotyped for common CYP2B6 variants. Lastly, this investigation aims to study the effects of CYP2B6 genetic variants on methadone metabolism in recombinantly expressed CYP2B6 wild type and the most common CYP2B6 variants at physiologically relevant concentrations of methadone. Results obtained in this chapter will be compared with *in vivo* data presented in Chapter 2 and summarized in Chapter 4

## II. Materials and Methods

### *Materials*

Racemic methadone, racemic EDDP, racemic d<sub>9</sub>-methadone and racemic d<sub>3</sub>-EDDP were purchased from Cerilliant (Round Rock, TX). Racemic bupropion was purchased from Sigma-Aldrich (St. Louis, MO). Racemic d<sub>6</sub>-hydroxybupropion was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). L- $\alpha$ -Dilauryl-*sn*-glycero-3-phosphocholine and Avanti Mini-Extruder were from Avanti Polar Lipids (Alabaster, AL). Emulgen 911 was a gift from KAO Chemicals (Tokyo, Japan). Isopropyl- $\beta$ -D-1-thiogalactopyranoside was from Gold Biotechnologies (St. Louis, MO). The pGRO7 plasmid was from Takara Bio Inc. (Otsu, Shiga, Japan, through Thermo Fisher Scientific, Waltham, MA). The PCR-Blunt II-TOPO vector was from Invitrogen (Carlsbad, CA). Acetonitrile, acetic acid, ammonium formate, ammonium hydroxide, formic acid, and methanol were purchased from Life Science—Fischer Scientific (Pittsburgh, PA). Single-donor primary human hepatocytes, Cryopreserved Hepatocyte Recovery Medium (CHRM®), Williams E medium containing Hepatocyte Plating Supplement Pack, and serum free Williams E. Medium containing Hepatocyte Maintenance Supplement Pack were a kind gift from Life Technologies (Carlsbad, CA). All other materials were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification.

### *Methods*

#### *Primary Human Hepatocyte treatment with methadone and EDDP*

Cryopreserved plateable human hepatocytes were obtained from three donors (two females and one male). Hepatocyte donor CYP activity was characterized and reported by supplier.

Hepatocytes were thawed and plated according to the supplier's protocol. Cells were thawed at

37 °C and diluted into 50 mL of warmed Cryopreserved Hepatocyte Recovery Medium (CHRM<sup>®</sup>) (Life Technologies). The cell suspension was centrifuged at 100 x g for 10 minutes. The cell pellet was resuspended in Williams E medium containing Hepatocyte Plating Supplement Pack. Cells were plated in 96-well Geltrex coated plate at approximately 0.5-0.7 x 10<sup>5</sup> cells per well. Cells were allowed to adhere for 6 hours before the media was replaced with Williams E. Medium containing Hepatocyte Maintenance Supplement Pack (serum-free). The cells were maintained for an additional 48 hours and were treated with maintenance media containing either methadone (10 µM) or EDDP (1 µM) for 24 hours. The reaction was quenched with an equal volume of acetonitrile containing d<sub>9</sub>-methadone and d<sub>3</sub>-EDDP (final concentration for both 0.1 µM). Each well was scraped with a pipette tip and the sample was transferred to a clean microcentrifuge tube. Cell debris was pelleted by centrifugation at 16,300 x g on a Spectrafuge 24D microcentrifuge (Labnet International Inc., Edison, NJ) and the supernatant was stored at -20 °C until analysis.

#### *Human Liver Microsome Screen*

**Bupropion:** Single-donor human liver microsomes from the University of Washington School of Pharmacy liver bank, with predetermined CYP2B6 content and genotype, were incubated with bupropion to determine CYP2B6 activity. Microsomes (final concentration 0.1 mg/mL) were pre-incubated with 75 µM racemic bupropion for 5 minutes. The reaction was initiated with an NADPH regeneration system (final concentration 100 mM Kpi, 50 mM MgCl<sub>2</sub>, 100 mM glucose-6-phosphate, 10 mM NADP, 10 units/mL glucose-6-phosphate dehydrogenase). The reaction was quenched after 20 minutes with an equal volume of cold acetonitrile containing racemic d<sub>6</sub>-hydroxybupropion (0.125 ng/uL). The samples were centrifuged at 4 °C at 2670 x g

for 10 minutes on a Sorvall Legend XTR table-top centrifuge (Thermo Fisher Scientific Inc., Waltham, MA). Samples were stored at -20 °C until analysis.

**Methadone:** The same liver microsomes used to phenotype for CYP2B6 activity were incubated with methadone. Microsomes (final concentration 0.1 mg/mL) were pre-incubated with 40 μM racemic methadone for 5 minutes. The reaction was initiated with NADPH (final concentration 1 mM). The reaction was quenched after 20 minutes with an equal volume of cold acetonitrile containing racemic d<sub>3</sub>-EDDP (0.15 ng/μL) as an internal standard. The samples were centrifuged at 4 °C at 2670 x g for 10 minutes on a Sorvall Legend XTR table-top centrifuge and stored at -20 °C until analysis.

#### *Cloning, Expression, and Purification of CYP2B6 Wild-type protein and Variants:*

**Cloning:** Human CYP2B6 cDNA was obtained from OriGene (Rockville, MD) and designed primers (Table 3.7) were used to make a modified and truncated *CYP2B6* gene. The following modifications were introduced to the gene to increase solubility and aid purification of the gene product: 1) the first nineteen amino acid residues were removed from the N-terminus, 2) residues 20-29 were replaced with MAKKTSSGK sequence, and 3) a six residue histidine tag was attached to the C-terminus to simplify purification. The CYP2B6 cDNA was then inserted into a pCWori vector to make a pCW2B6.1 plasmid. This plasmid was subsequently used to create the *CYP2B6* variant plasmids pCW2B6.4, pCW2B6.5, and pCW2B6.6. Mutagenesis was accomplished using a Stratagene QuikChange kit (Stratagene, La Jolla, CA) and designed primers (Table 3.7). pCW2B6.1, pCW2B6.4, pCW2B6.5, and pCW2B6.6 were sequenced at the University of Washington Biochemistry Sequencing Facility to ensure that intended base changes were successfully introduced and that no extraneous mutations occurred following PCR.

Plasmids were transformed into competent cells containing GroES/EL chaperone proteins.

Glycerol stocks were made for each plasmid and stored at -80 °C until expression. (Raccor et al., 2012)

**Protein expression:** The wild-type and variant CYP2B6 starter culture consisted of the following: 5 mL of Luria Broth (LB) chloramphenicol (final concentration 40 µg/mL), ampicillin (final concentration 100 µg/mL), and approximately 5 µL of frozen *CYP2B6* wild-type (or variant) glycerol stock. The starter culture was placed in a shaker at 37 °C for approximately 12-16 hours. The following day, Terrific Broth (TB) (Appendix) was prepared. 500 mL of TB was required for every 5 mL starter culture. Typical expression batches were 6 L in scale, with twelve 500 mL expression flasks. Chloramphenicol (final concentration 40 µg/mL) and ampicillin (final concentration 100 µg/mL), thiamine (final concentration 1 mM), and trace elements (Appendix) were added to each 500 mL expression flask. Lastly, 5 mL of starter culture was added to each expression flask and allowed to shake for one hour at 37 °C and 225 rotations per minute in the dark. After one hour, 5 mL of arabinose (0.4 g/mL) was added to each 500 mL expression flask to induce chaperone proteins. Three hours after addition of arabinose, heme formation and P450 enzyme induction were initiated with the addition of d-aminolevulinic acid (d-ALA) (final concentration 1 mM) and isopropyl β-D-1-thiogalactopyranoside (IPTG) (final concentration 1.5 mM), respectively. The cultures were allowed to shake for an additional 48 hours at 28 °C at 150 rpm on a New Brunswick Innova 44 incubated shaker (Eppendorf Corporate, Hauppauge, NY) the dark. Cultures were combined and centrifuged. The supernatant was discarded. Cell pellets were covered with a small amount of resuspension buffer (Appendix) and stored at -80 °C until purification (Raccor et al., 2012). (See Appendix for all solutions and buffers used in expression of CYP2B6)

**Protein Purification:** Protein purification was accomplished first by resuspending and homogenizing cell pellets in resuspension buffer. The homogenized pellet was processed through a Micro DeBee homogenizer (BEE Internation, South Easton, MA) to lyse the cells and centrifuged to remove cell debris. The supernatant was then loaded onto a nickel affinity column. Several buffers were prepared to load and purify protein: resuspension/equilibration buffer, wash buffer, super-wash buffer, and elution buffer. Following elution, the protein was dialyzed three times against phosphate buffer to remove imidazole. (See Appendix for all buffers used for purification via nickel column) Protein was further purified using a hydroxyapatite column to remove Emulgen. (See Appendix for all buffers used for purification via hydroxyapatite column). Accurate mass of all expressed enzymes was verified on a Micromass Synapt-TOF mass spectrometer (Waters, Milford, MA). Expressed enzymes were stored at -80 °C until use (Raccor et al., 2012).

*Kinetic Experiments using recombinant enzymes and bupropion:*

Protein and time linearity experiments and negative controls (deprived of substrate, enzyme, or NADPH) were first performed with wild-type CYP2B6 to ensure first order reaction conditions. CYP2B6 enzyme was reconstituted in 1,2-dilauryl-sn-glycero-3-phosphocholine (DLPC) (final concentration 62.5  $\mu$ M) with cytochrome P450 reductase (CPR) and cytochrome b<sub>5</sub> (cyt-b<sub>5</sub>) (molar ratio cyt-b<sub>5</sub>:CPR:P450 1:2:1). Formation kinetics were obtained by mixing 10 pmol CYP2B6, 20 pmol CPR, and 10 pmol cyt-b<sub>5</sub> with racemic bupropion concentrations ranging from 20 – 2000  $\mu$ M (single enantiomer concentration 10 – 1000  $\mu$ M) at 37 °C. Each reaction was performed in duplicate. The final concentration of organic solvent in each reaction was 1%. Previous studies determined that enzyme activity was not compromised at 1% v/v organic. Following a five minute pre-incubation time, the reaction was initiated with NADPH (final

concentration 1 mM). The duration of the incubation was 10 minutes, after which the reaction was quenched with an equal volume of cold acetonitrile containing racemic d<sub>6</sub>-hydroxybupropion (0.25 ng/μL) as an internal standard. The samples were centrifuged at 4 °C at 2670 x g for 10 minutes on a Sorvall Legend XTR table-top centrifuge and stored at -20 °C until analysis.

*Screen of CYP2B6 wild-type and variants at clinically relevant concentrations of methadone:*

**Methadone:** Methadone incubations were performed with expressed CYP2B6 wild-type and variants. A NADPH negative control was included for each reaction concentration. CYP2B6 enzyme was reconstituted in DLPC (final concentration 62.5 μM) with cytochrome CPR and cyt-b<sub>5</sub> (molar ratio cyt-b<sub>5</sub>:CPR:P450 1:2:1). Rates of EDDP formation were obtained by mixing 10 pmol CYP2B6, 20 pmol CPR, and 10 pmol cyt-b<sub>5</sub> with racemic methadone concentrations of 10 μM and 20 μM (single enantiomer concentration of 5 μM and 10 μM) at 37°C. Each reaction was performed in duplicate. The final concentration of organic solvent in each reaction was 1%. Previous studies determined that there was no compromise in enzyme activity at 1% v/v organic. Following a five minute pre-incubation, the reaction was initiated with NADPH (final concentration 1 mM). The duration of the incubation was 20 minutes, after which the reaction was quenched with an equal volume of cold acetonitrile containing racemic d<sub>3</sub>-EDDP (0.15 ng/μL) as an internal standard. Samples were centrifuged at 4 °C at 2670 x g for 10 minutes on a Sorvall Legend XTR table-top centrifuge and stored at -20 °C until analysis by mass spectrometry.

*Mass Spectrometry Sample Analysis*

**Methadone Metabolite Identification using an UPLC-MS/MS Method:** Metabolite identification in hepatocytes was performed on an Acquity UPLC using a BEH C18 column (2.1

x 100 mm, 1.7  $\mu$ M) connected to a Waters Synapt G2-Si-QTOF UPLC-MS/MS (Waters Corporation, Milford, MA). The injection volume was 2  $\mu$ L. The aqueous solvent used was 20 mM ammonium formate pH 5.7 (solvent A) and the organic solvent was methanol (solvent B). The ionization mode was electrospray ionization (ESI) positive in W mode. The reference file used was ESI sodium formate in positive mode with a mass-to-charge range between 100 and 1000. The lock mass reference used was leucin enkephalin which has a mass to charge ratio of 566.88915. All changes in the gradient occurred linearly. Flow rate was 0.3 mL/min. The gradient conditions used were as follows: initial conditions at the beginning of the gradient were 10% solvent B for 1 min; solvent B was increased to 98% at 8 min and this was held until 10 min; the system returned to initial conditions at 10.10 min and this was held until 13 min in order to re-equilibrate the column.

**Bupropion Achiral LC-MS Method:** Hydroxybupropion quantitation in HLM incubations was accomplished by using a Phenomenex Synergi Fusion reverse phase column (150 mm x 2.0 mm, particle size 4  $\mu$ m, pore size 80 angstroms) connected to an Agilent HPLC/MS series 1100 SL. The aqueous mobile phase was 10 mM ammonium acetate, pH 4 (solvent A) and the organic mobile phase used was acetonitrile (solvent B). All changes in liquid chromatography gradient occurred linearly. The liquid chromatography gradient used is as follows: initial conditions at the beginning of the gradient were 20% solvent B with total flow rate of 0.2 mL/min; the percentage of B was increased to 40% at 5 min with a total flow rate of 0.2 mL/min; at 7 min, the percentage of B was increased to 50% with a total flow rate of 0.3 mL/min and this was maintained until 9 min; at 10 min, the gradient returned to initial starting conditions. An additional 3 minutes at initial conditions allowed for re-equilibration of the column. The ionization mode was atmospheric pressure ionization electrospray (API-ES) in positive mode. The following mass

ions and corresponding fragmentor voltage were monitored: bupropion ( $m/z$  240.2, fragmentor = 140 V), hydroxybupropion ( $m/z$  256.2, fragmentor = 40 V), and  $d_6$ -hydroxybupropion ( $m/z$  262.2, fragmentor = 140 V). The elution times for bupropion and hydroxybupropion (and deuterated standard) were 8.15 min and 6.84 min, respectively.

**Bupropion Chiral LC-MS/MS Separation Method:** Chiral separation and quantitation of hydroxybupropion diastereomers for recombinant CYP2B6 experiments was achieved by applying samples onto a reverse phase chiral-AGP column (100 x 2 mm, 5  $\mu$ M) attached to a chiral-AGP guard cartridge (10 x 2 mm, 5  $\mu$ m) (ChromTech, Apple Valley, MO). This was connected to an Acquity Waters UPLC in tandem with a Micromass Premier XE triple quadrupole mass spectrometer. The injection volume was 5  $\mu$ L. The aqueous solvent used was 20 mM ammonium formate pH 5.7 (solvent A) and the organic solvent used was methanol (solvent B). All changes in the gradient occurred linearly. Flow rate was 0.22 mL/min for the entirety of the run. The liquid chromatography gradient used is as follows: initial conditions at the beginning of the run was 10% B for 0.5 min; the percentage of solvent B increased to 70% at 1 min and held until 4 min; the percentage of B increased to 50% by 4.1 min and held until 7.5 min; the system returned to initial conditions at 8 min; and an additional 5 min at initial conditions allowed the column to re-equilibrate. The ionization mode was ESI in positive mode. The mass fragmentations monitored and the corresponding cone voltage (CV) and collision energy (CE) were as follows: bupropion (240.1 > 184.1, CV = 20.0, CE = 15.0), hydroxybupropion (256.1 > 238.1, CV = 20.0, CE = 15.0), and  $d_6$ -hydroxybupropion (262.1 > 244.1, CV = 20.0, CE = 15.0). Elution times for *R,R*-hydroxybupropion, and *S,S*-hydroxybupropion (and their corresponding deuterated internal standards) were 8.4 min, and 6.3 min, respectively.

**Methadone Chiral LC-MS/MS Method:** Chiral separation and quantitation of EDDP enantiomers for recombinant CYP2B6 experiments and HLM screen was achieved by applying samples to reverse phase chiral-AGP column (100 x 2 mm, 5  $\mu$ M) attached to a chiral-AGP guard cartridge (10 x 2mm, 5  $\mu$ m). This was connected to an Acquity Waters UPLC in tandem with a Micromass Premier XE triple quadrupole mass spectrometer (Waters Corporation, Milford, MA). The injection volume was 5  $\mu$ L. The aqueous solvent used was 20 mM ammonium formate pH 5.7 (solvent A) and the organic solvent used was methanol (solvent B). All changes in the gradient occurred linearly. Flow rate was 0.22 mL/min for the entirety of the run. The gradient conditions used are as follows: initial conditions at the beginning of the gradient were 5% solvent B for 2 min; the percentage of B increased to 10% at 4 min and this was held until 7 min; the percentage of B was increased to 50% at 10 min and was held until 13 min; the system returned to initial conditions at 13.5 min; with an additional 2.5 min at initial conditions added to re-equilibrate the column. The mass fragmentations monitored and the corresponding cone voltage (CV) and collision energy (CE) were: methadone (310.1 > 268.2, CV = 30.0, CE = 15.0), EDDP (278.2 > 234.2, CV = 45.0, CE = 30.0), and d<sub>3</sub>-EDDP (281.0 > 234.2, CV = 45.0, CE = 30.0). Elution times for *R*-EDDP, and *S*-EDDP (and their corresponding deuterated internal standards) were 13.8 min, and 14.4 min, respectively.

**Analysis of Accurate Mass for CYP2B6 protein:** Mass of CYP2B6 wild-type and all allelic variants were analyzed by applying samples to a POROS R2 column (2.1 x 250 mm) attached to a chiral-AGP guard cartridge (10 x 2 mm, 5  $\mu$ m) (Applied Biosystems by Life Technologies, Grand Island, NY). This was connected to a Waters Synapt G2-Si-QTOF UPLC-MS/MS (Waters Corporation, Milford, MA). The aqueous mobile phase used was 0.05% v/v trifluoroacetic acid (TFA) in water (solvent A) and 0.05% TFA in acetonitrile (solvent B). The ionization mode was

ESI positive in V mode. All changes in the gradient occurred linearly. Flow rate was 1 mL/min for the entirety of the run. The liquid chromatography gradient used is as follows: initial conditions at the beginning of the gradient were 20% solvent B and held until 3 min; the percentage of B was increased to 60% at 23 min. (Cheesman et al., 2003). Spectra were deconvoluted and an exact mass was determined.

### *Data Analysis*

**Hepatocyte Metabolite Identification Studies:** All hepatocyte data was analyzed with Metabolynx XS Software (Waters Corporation, Milford, MA). The structure of methadone and was inputted into Metabolynx XS software. Phase I and II metabolism and associated differences in mass-to-charge ratio were probed. Correct chemical formula and a mass within 5 ppm of predicted value were criteria for identification of a metabolite.

**Correlation Studies in HLMs:** The correlation of CYP3A4 and CYP2B6 and methadone metabolism was determined using GraphPad Prism version 5.02 (GraphPad Software Inc., San Diego, CA). Formation of *R*- and *S*-EDDP and the ratio of *R*- to *S*- EDDP in individual HLMs were plotted against CYP3A4 and CYP2B6 content and activity. CYP3A4 activity was previously determined by quantitating 1'-OH-midazolam formation in individual HLMs (Lin et al., 2002). Analogously, CYP2B6 activity was assessed by determining total hydroxybupropion formation. The associations between methadone metabolism and CYP3A4 and CYP2B6 content or activity were determined by using Spearman rank order correlation coefficient statistical test. *R*- and *S*- EDDP formation were normalized to CYP2B6 content to assess the effect of *CYP2B6* genotype on metabolite formation. A Kruskal-Wallis statistical test was used to determine if there were statistically significant differences in activity between variant CYP2B6 and wild-type.

A Kruskal-Wallis test was chosen because a Gaussian distribution in protein content and activity could not be assumed. Because basal CYP3A4 and CYP2B6 expression may be under the control of the same nuclear receptors (Faucette et al., 2006; Faucette et al., 2007), association of protein content and activity between CYP3A4 and CYP2B6 was assessed as well. A statistically significant correlation could present a confounding factor when analyzing HLM data. For this reason, a ratio of and *R*- to *S*- EDDP was also studied as an outcome variable to discern CYP2B6 contribution to methadone stereospecific metabolism.

**Kinetic parameter estimation:** Analysis of kinetic data obtained from studies with bupropion and recombinant enzyme were performed using GraphPad Prism version 5.02 (GraphPad Software Inc., San Diego, CA). Metabolite formation rate was plotted against substrate concentration and analyzed using a Michaelis-Menten and substrate inhibition model. From the fitted model, values of  $K_m$  and  $V_{max}$  were obtained. Intrinsic clearance was then calculated as  $V_{max}/K_m$ .

**Differences in EDDP formation rates by CYP2B6 variants:** Analysis of methadone incubations in recombinantly expressed enzymes was performed using GraphPad Prism version 5.02. Rates of *R*- and *S*- EDDP formation rates after incubation at 5  $\mu$ M and 10  $\mu$ M were plotted. A one-way ANOVA statistical test was used to determine whether there were differences in methadone turnover between wild-type CYP2B6 and genetic variants.

### III. Results and Discussion

#### Hepatocyte Metabolite Identification:

Primary hepatocytes from single donor livers were used to characterize metabolites of methadone formed following a 24-hour incubation. Each of these livers' CYP2B6 activity, as

characterized by bupropion hydroxylation, was previously determined by Life Technologies (Carlsbad, CA). All hepatocytes had relatively high basal CYP2B6 activity. All samples were treated with clinically relevant, albeit higher, concentrations of methadone and EDDP, 10  $\mu$ M and 1  $\mu$ M, respectively. Higher concentrations were chosen to increase chances of detecting metabolites. Three biological replicates of methadone and EDDP treatment were performed for each donor. Analysis of samples treated with methadone showed that EDDP is the major metabolite formed, with no EMDP detected (a representative scan and elemental composition report are shown in Figure 3.2 and Table 3.1). Additional metabolites characterized by Sullivan et. al (1973) (Figure 3.1) in methadone maintenance treatment patients were also queried in these samples, however, none were detected (Sullivan and Due, 1973). Interestingly, in samples treated with EDDP, the secondary metabolite EMDP was also not detected. Aromatic hydroxylation of EDDP, another potential metabolite of EDDP was also not detected. (A representative scan and elemental composition report after treatment with EDDP are shown in Figure 3.3 and Table 3.2).

### **Correlation Studies in HLMs:**

There was a statistically significant correlation between CYP3A4 and CYP2B6 protein content and activity (see Figure 3.4). This agrees with previous data showing co-regulation of the expression of the two enzymes (Faucette et al., 2006; Faucette et al., 2007). As CYP3A4 and CYP2B6 expression appear to be co-regulated, it becomes difficult to distinguish the contribution of individual CYP isoforms to methadone metabolism in HLMs.

CYP3A4 protein content and activity correlates with *R*- and *S*- methadone metabolism (Figure 3.5 and Figure 3.6). By extrapolating from correlation data, CYP3A4 protein content

accounts for 55% of the variability observed with *R*- and *S*- EDDP formation. By examining the activity plots, CYP3A4 activity accounts for 52% of the variability in *R*-EDDP formation and 48% of the variability observed with *S*-EDDP formation. CYP2B6 protein content and activity also correlated significantly with *R*-and *S*-EDDP formation (Figure 3.8 and Figure 3.9). CYP2B6 protein content accounts for 27% and 33% of the variability observed in *R*-EDDP and *S*-EDDP formation, respectively. CYP2B6 activity describes 33% and 38% of the variability detected in *R*- and *S*- EDDP formation, respectively.

Previous data in Supersomes<sup>®</sup> and recombinant enzymes provided evidence of a stereoselective bias for *S*-methadone over *R*-methadone with CYP2B6 metabolism (Gerber et al., 2004; Totah et al., 2007). Therefore, a Spearman rank correlation coefficient was calculated between the *R/S*-EDDP ratio (to determine stereoselective bias) with varying protein content and activity for CYP3A4 and CYP2B6. CYP3A4 protein content and activity was not associated with any stereoselective bias (Figure 3.7). This is consistent with results obtained in Supersomes<sup>®</sup> and recombinant enzyme (Gerber et al., 2004; Moody et al., 1997; Totah et al., 2008). Conversely, CYP2B6 protein content and activity has a statistically significant association with *R/S*-EDDP ratio (Figure 3.10). The Spearman coefficient for protein content and activity was negative, which indicates that CYP2B6 content and activity is inversely correlated with *R/S*-EDDP formation ratio. Extrapolation of correlation results reveal that CYP2B6 protein content accounts for 49% of the variability in stereoselective metabolism and CYP2B6 activity accounts for 43% of the variability observed in *R/S*-EDDP ratio. In summary, the data from the HLM screen suggests that CYP3A4 accounts for the majority of methadone oxidative metabolism, but CYP2B6 impacts the stereoselective profile of EDDP formation in human liver microsomes. Table 3.3 and Table 3.4 summarize the correlation studies with CYP3A4 and CYP2B6.

### **Effect of *CYP2B6* genotype on methadone metabolism:**

*R*- and *S*- EDDP formation was normalized to CYP2B6 content in HLMs (n=37) and stratified by genotype to determine pharmacogenetic effects on methadone metabolism (Figure 3.11). A Kruskal-Wallis statistical test was used to determine differences with genotype. There are no significant differences in either *R*- or *S*- EDDP formation with CYP2B6 genotype. This could also be partially due to the low number of livers with the following genotypes *CYP2B6*\*1/\*4 (n = 1), *CYP2B6*\*1/\*5 (n = 2), *CYP2B6*\*1/\*7 (n = 1), and *CYP2B6*\*6/\*6 (n = 1). However, even when comparing livers carrying the \*1/\*1 (n = 19) with \*1/\*6 (n = 13) genotype, no notable difference in EDDP formation was detected. An analysis of *R/S*-EDDP ratio compared between livers of carrying different genotypes was also performed with a Kruskal-Wallis statistical test (Figure 3.12). CYP2B6 genotype also does not appear to influence EDDP stereoselective formation.

In this study, protein content and overall CYP2B6 activity, more so than genotype, influenced stereoselective EDDP formation to the greatest extent. This is in contrast to previous data performed in CYP2B6.1 and CYP2B6.6 Supersomes<sup>®</sup>, in which there was a clear genotype effect. CYP2B6.6 Supersomes<sup>®</sup> had three to four times lower activity compared to CYP2B6.1 when studying methadone at clinically relevant concentrations (0.25 μM – 2 μM) (Gadel et al., 2013). It possible that the use of a higher concentration of methadone (40 μM) in this study, combined with the contribution of CYP3A4 in HLMs obscured CYP2B6 pharmacogenetic

effects on methadone metabolism. In addition, the liver donors may have been taking drugs that are inducers of CYP2B6, which could also mask the effect of CYP2B6 genotype.

### **Kinetic Experiments:**

All CYP2B6 enzymes studied had higher rates of formation of *S,S*-hydroxybupropion than *R,R*-hydroxybupropion (Figure 3.13). This result is somewhat confounded given the fact that the bupropion enantiomers readily interconvert in solution, yet *R,R*-hydroxybupropion can only be formed from *R*-bupropion (and the same is true of *S,S*-hydroxybupropion). The rapid racemization in solution makes enantiomeric interactions difficult to discern. A comparison analysis between Michaelis-Menten and substrate inhibition models was performed and the kinetic parameters are reported in Table 3.5 and Table 3.6. Goodness of fit was determined using the sum of squares F-test. *R*-bupropion metabolism is best described by a Michaelis-Menten model, while *S*-bupropion metabolism is best described by a substrate inhibition model. The exception to this was *R*-bupropion metabolism by CYP2B6.6, in which the fit was ambiguous and the model of best fit could not be determined. Overall, wild-type CYP2B6 had higher intrinsic clearance of both bupropion enantiomers compared to CYP2B6 variants.

### **Methadone metabolism with Recombinant CYP2B6 enzymes:**

*R*-EDDP and *S*-EDDP formation rates were compared at clinically relevant concentrations of methadone in CYP2B6 wild-type and variants (Figure 3.14). At these concentrations of methadone, there does not appear to be any differences in metabolism between any of the CYP2B6 enzymes studied. Of note, all CYP2B6 enzymes showed clear

stereoselectivity for *S*-methadone over *R*-methadone. These data agree with conclusions drawn from studies performed in HLMs.

#### IV. Summary

Treatment of hepatocytes with methadone confirmed that EDDP is the major metabolite formed from in the liver. Furthermore, EDDP does not appear to undergo significant oxidation to EMDP. This result supports utilizing EDDP AUC and EDDP formation as an indicator of methadone clearance both *in vivo* and *in vitro*, respectively. Sullivan et al first discovered alternative metabolites in MMT patients who were stabilized on methadone (Sullivan and Due, 1973). It is likely that chronic use of methadone results in a different metabolite profile by inducing certain clearance pathways. More studies in hepatocytes, either increasing incubation time or concentrations of methadone and EDDP, are warranted to investigate whether the other metabolites detected by Sullivan et. al (Sullivan and Due, 1973) are formed in the liver.

The screen of single-donor HLMs provided insight to the relative contribution of CYP3A4 and CYP2B6 to EDDP formation. CYP3A4 appears to be the main isoform involved in the metabolism of methadone, however, CYP2B6 accounts for the stereoselective metabolism of the drug. This is the first investigation to show this in multiple HLM samples.

The effects of CYP2B6 genotype were difficult to determine because of the low number of livers of certain genotypes and also, the contribution of CYP3A4 confounded the results. Studies involving recombinant wild-type CYP2B6 and genetic variants of CYP2B6 showed that

CYP2B6 content, more so than genotype, influences methadone metabolism. Michaelis-Menten experiments with bupropion, the probe substrate for CYP2B6 activity, and CYP2B6 recombinant enzymes show differences in intrinsic clearance between wild-type CYP2B6 and variants.

However, in a screen with methadone at clinically relevant concentrations, there is little variation in EDDP formation noted between wild-type and variant recombinant CYP2B6 enzyme. Because CYP2B6 content appears to be critical to methadone clearance, pathways and mechanisms of CYP2B6 induction and inhibition are important to investigate moving forward.

## V. Figures and Tables

**Figure 3.1** -- Methadone metabolites and possible pathways of metabolism. Exact mass and chemical formulas are included. Reproduced from Sullivan and Due ((Sullivan and Due, 1973).

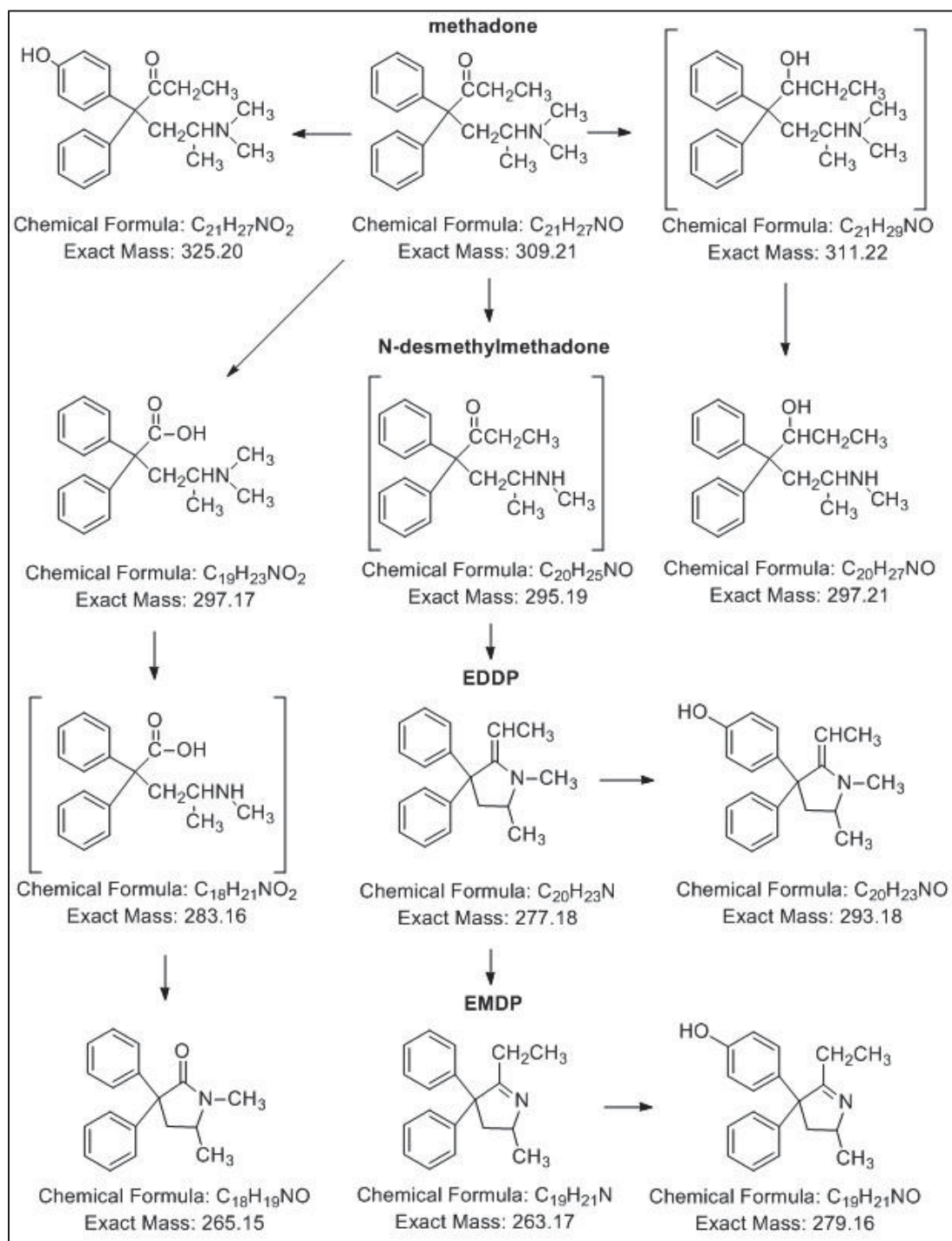
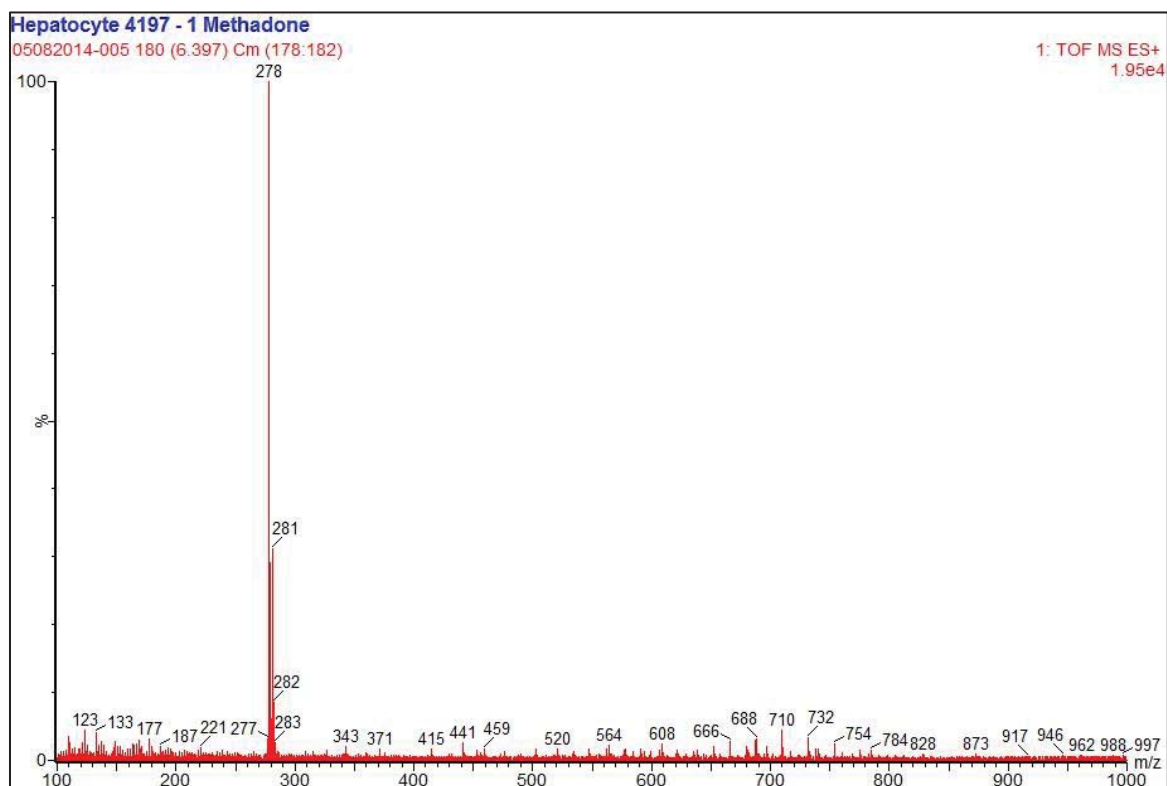


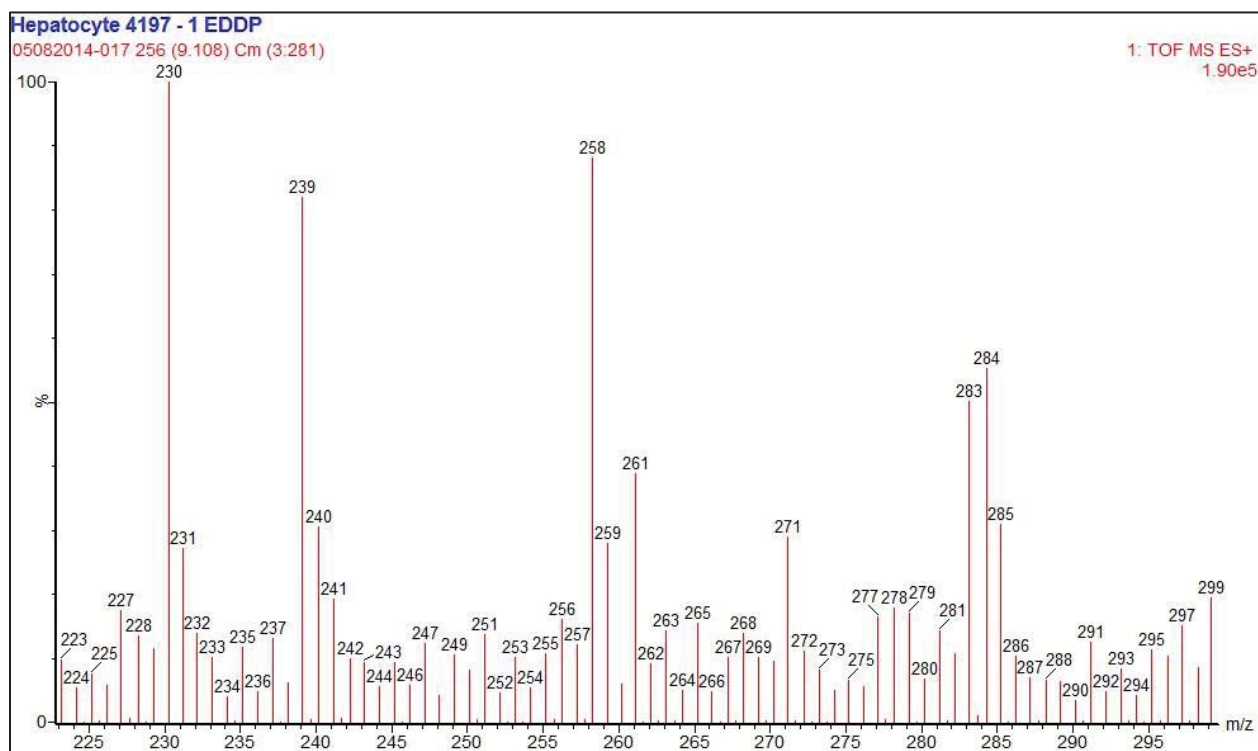
Figure 3.2 -- Representative scan of hepatocytes treated with methadone (10  $\mu$ M) for 24 hours.



**Table 3.1** -- Elemental composition report from scan shown in Figure 3.2. Elemental composition and exact mass of EDDP were predicted accurately. Accurate mass and correction prediction of chemical formula of EDDP is highlighted in red.

Elemental Composition Report Liver 4197								
Single Mass Analysis								
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0								
Element prediction: Off								
Number of isotope peaks used for i-FIT = 3								
Monoisotopic Mass, Odd and Even Electron Ions								
128 formula(e) evaluated with 5 results within limits (up to 50 best isotopic matches for each mass)								
Elements Used:								
C: 0-30 H: 0-100 N: 0-5 O: 0-5								
Minimum: -1.5								
Maximum: 5 10 50								
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
278.1918	278.1909	0.9	3.2	9.5	38.5	0.7	49.63	C <sub>20</sub> H <sub>24</sub> N
	278.1882	3.6	12.9	5	39.4	1.539	21.46	C <sub>17</sub> H <sub>26</sub> O <sub>3</sub>
	278.1869	4.9	17.6	5.5	39.8	1.947	14.28	C <sub>15</sub> H <sub>24</sub> N <sub>3</sub> O <sub>2</sub>
	278.1967	-4.9	-17.6	0.5	40.3	2.473	8.44	C <sub>13</sub> H <sub>28</sub> NO <sub>5</sub>
	278.1954	-3.6	-12.9	1	40.6	2.781	6.2	C <sub>11</sub> H <sub>26</sub> N <sub>4</sub> O <sub>4</sub>

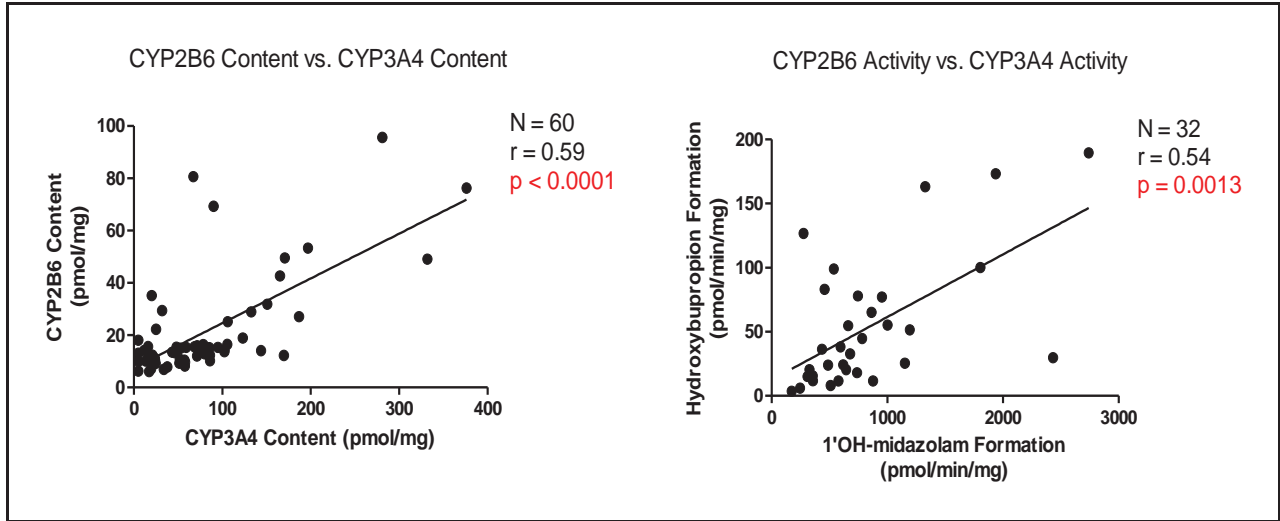
**Figure 3.3** -- Representative scan of hepatocytes treated with EDDP (1  $\mu$ M) for 24 hours.



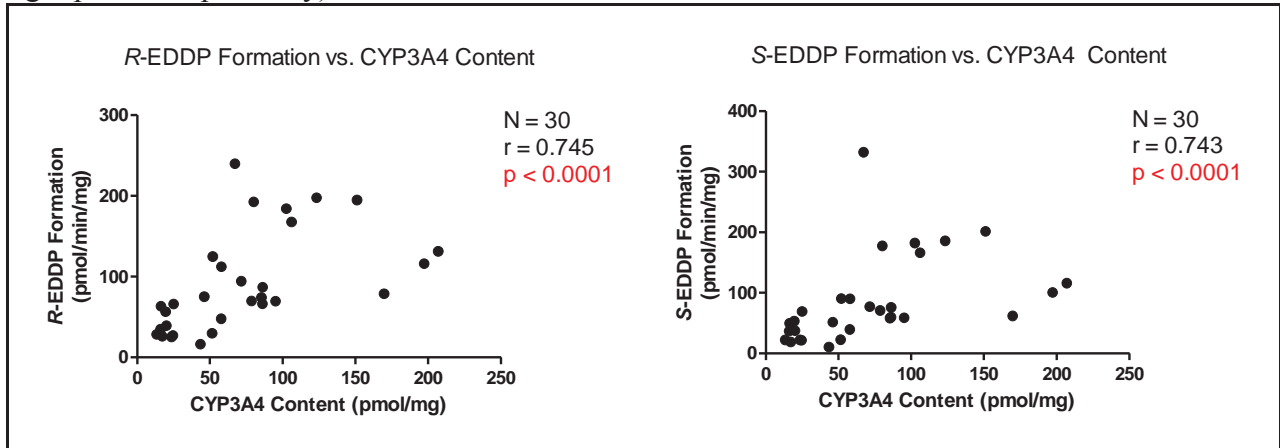
**Table 3.2** -- Elemental Composition Report from scan shown in Figure 3.3. EMDP mass error was larger than threshold (> 5ppm) and incorrect prediction of EMDP chemical formula are highlighted in red.

Elemental Composition Report								
Single Mass Analysis								
Tolerance = 5.0 mDa / DBE: min = -1.5, max = 50.0								
Element prediction: Off								
Number of isotope peaks used for i-FIT = 3								
Monoisotopic Mass, Odd and Even Electron Ions								
122 formula(e) evaluated with 4 results within limits (up to 50 best isotopic matches for each mass)								
Elements Used:								
C: 0-30 H: 0-100 N: 0-5 O: 0-5								
Minimum:				-1.5				
Maximum:				5 10 50				
Mass	Calc. Mass	mDa	PPM	DBE	i-FIT	Norm	Conf(%)	Formula
264.1824	264.1798	2.6	9.8	1	48.5	1.346	26.02	C <sub>10</sub> H <sub>24</sub> N <sub>4</sub> O <sub>4</sub>
	264.1824	0	0	5.5	48.5	1.38	25.16	C <sub>13</sub> H <sub>22</sub> N <sub>5</sub> O
	264.1811	1.3	4.9	0.5	48.5	1.383	25.07	C <sub>12</sub> H <sub>26</sub> NO <sub>5</sub>
	264.1838	-1.4	-5.3	5	48.6	1.438	23.75	C <sub>15</sub> H <sub>24</sub> N <sub>2</sub> O <sub>2</sub>

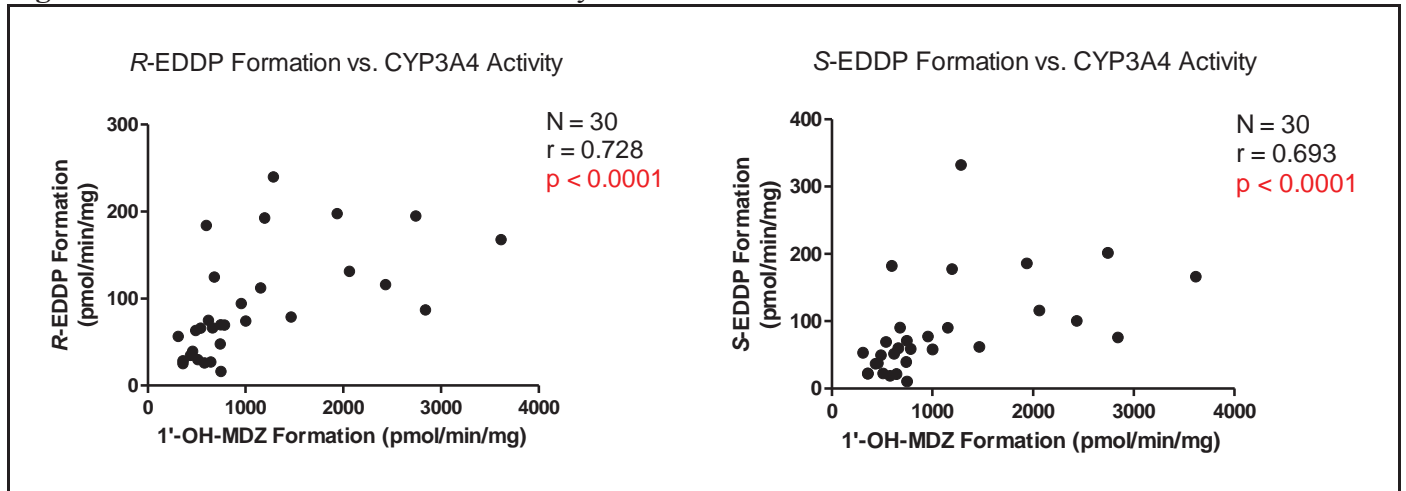
**Figure 3.4** -- Correlation of protein content (left) and activity (right) between CYP3A4 and CYP2B6.



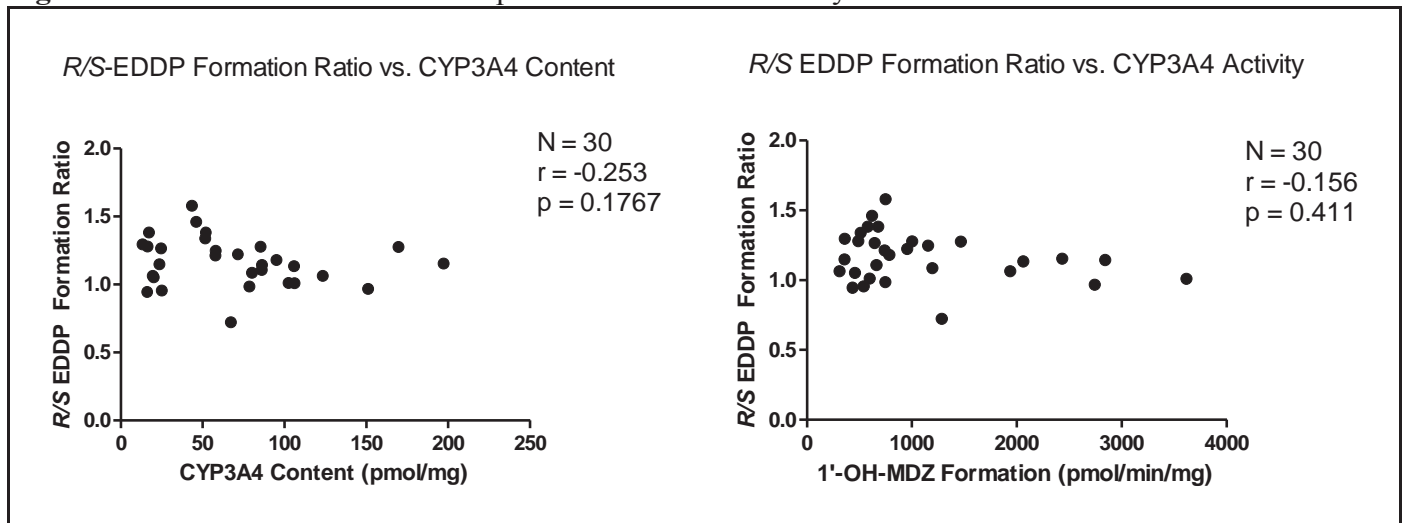
**Figure 3.5** -- Correlation of CYP3A4 protein content with *R*- and *S*- EDDP formation (left and right panels respectively).



**Figure 3.6** -- Correlation of CYP3A4 activity with *R*- and *S*- EDDP formation.



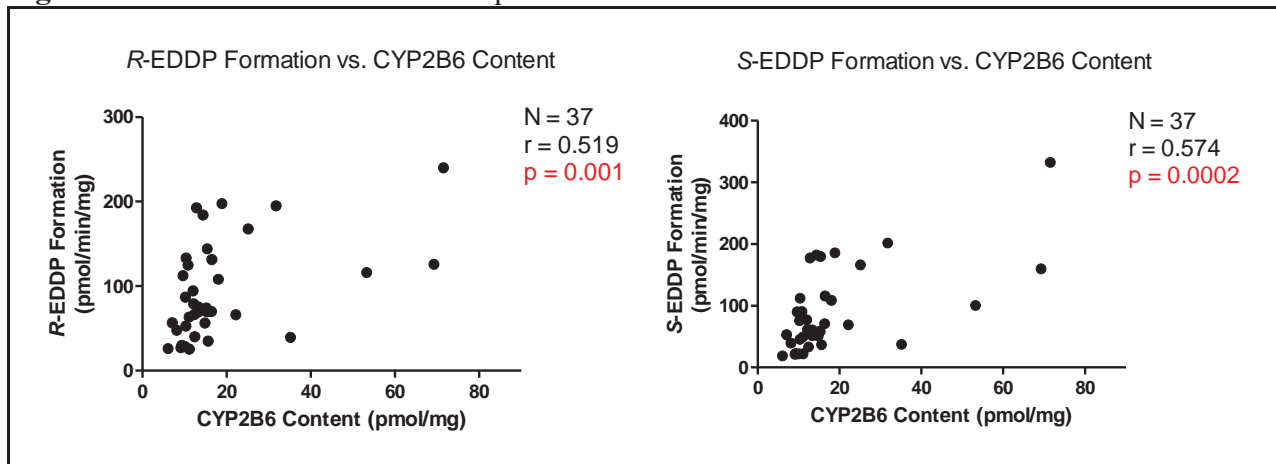
**Figure 3.7** -- Correlation of CYP3A4 protein content and activity with *R/S*- EDDP formation.



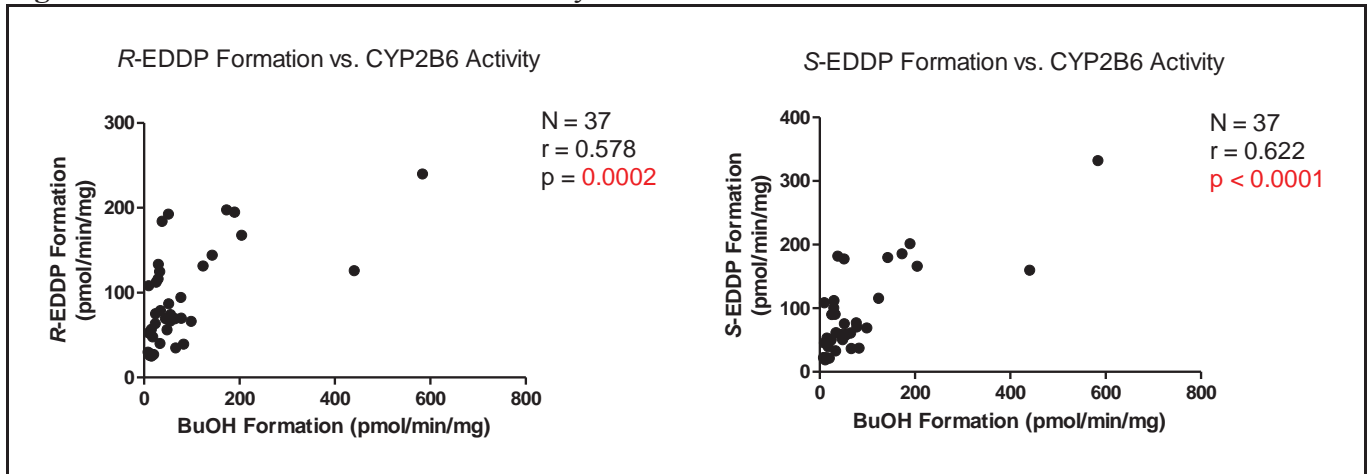
**Table 3.3** -- Summary of correlation studies between CYP3A4 and EDDP formation.

CYP3A4 Correlation Studies, n = 30				
Independent Variable	Outcome Variable	Spearman's coefficient		
		rho	p-value	
CYP3A4 Content	R-EDDP Formation	0.745	<0.0001	
CYP3A4 Content	S-EDDP Formation	0.743	<0.0001	
CYP3A4 Content	R/S Formation Ratio	-0.253	0.1767	
CYP3A4 Activity	R-EDDP Formation	0.728	<0.0001	
CYP3A4 Activity	S-EDDP Formation	0.693	<0.0001	
CYP3A4 Activity	R/S Formation Ratio	-0.156	0.411	

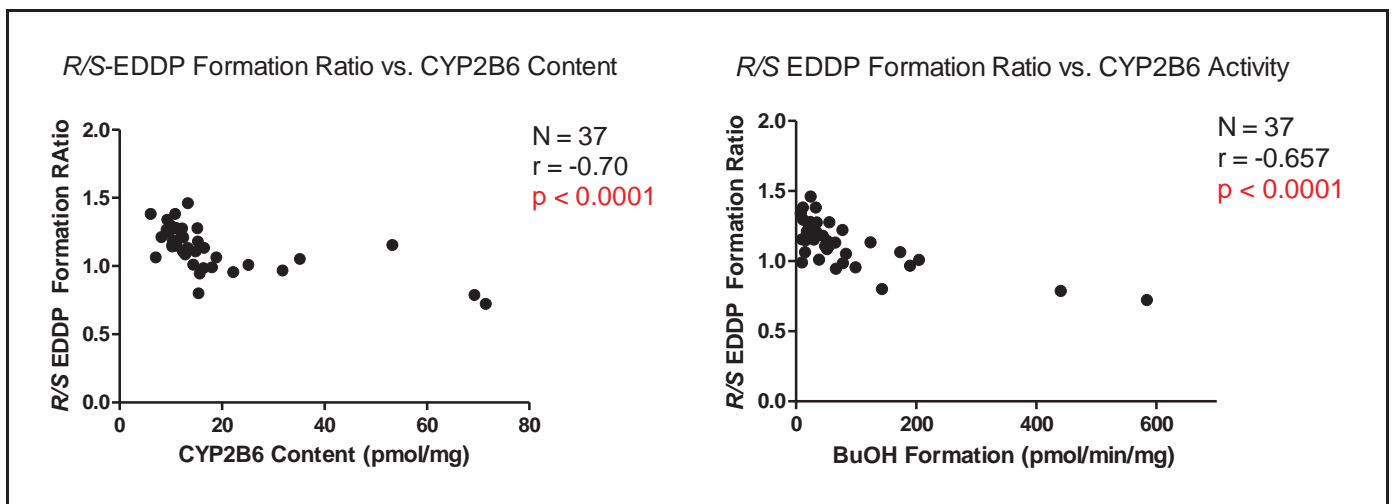
**Figure 3.8** -- Correlation of CYP2B6 protein content with R- and S- EDDP formation.



**Figure 3.9** -- Correlation of CYP2B6 activity with *R*- and *S*- EDDP formation.



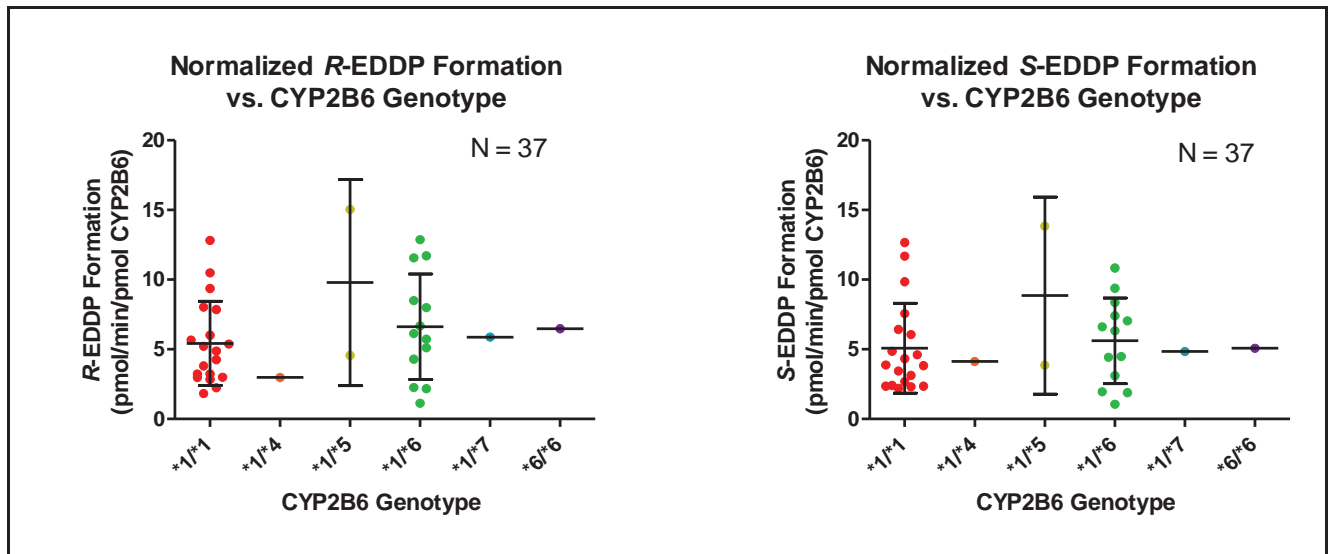
**Figure 3.10** -- Correlation of CYP2B6 protein content and activity with ratio of *R/S*- EDDP formation.



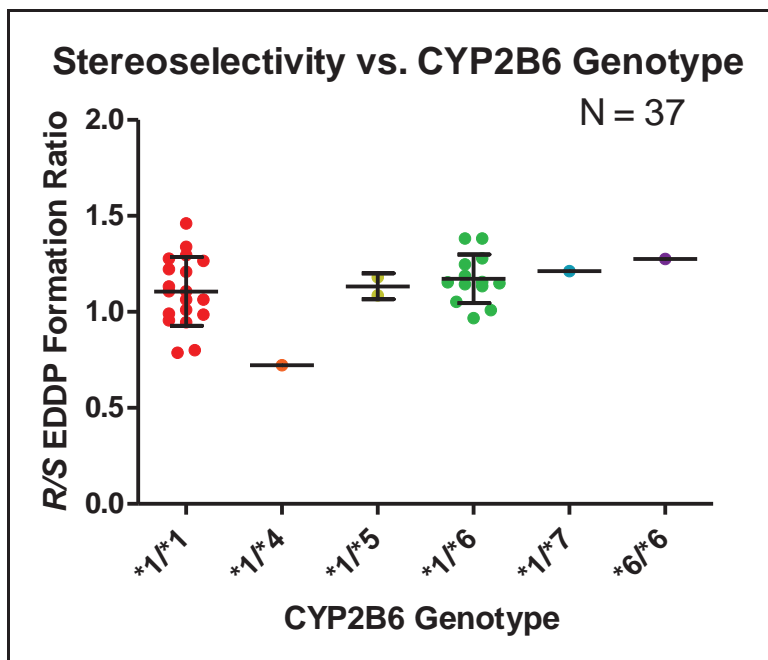
**Table 3.4** -- Summary of correlation studies between CYP2B6 and EDDP formation.

CYP2B6 Correlation Studies, n = 37		Spearman's coefficient	
Independent Variable	Outcome Variable	rho	p-value
CYP2B6 Content	R-EDDP Formation	0.519	0.001
CYP2B6 Content	S-EDDP Formation	0.574	0.0002
CYP2B6 Content	R/S Formation Ratio	-0.7	<0.0001
CYP2B6 Activity	R-EDDP Formation	0.578	0.0002
CYP2B6 Activity	S-EDDP Formation	0.622	<0.0001
CYP2B6 Activity	R/S Formation Ratio	-0.657	<0.0001

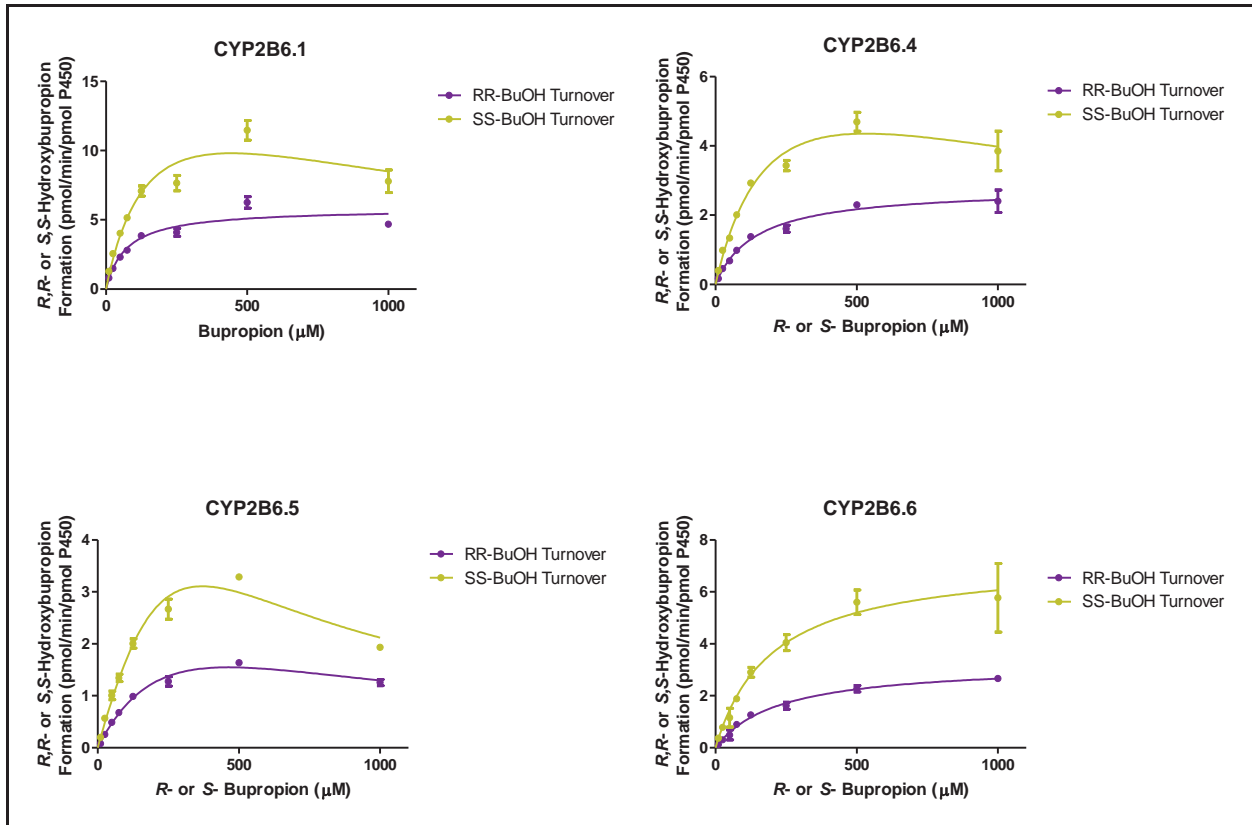
**Figure 3.11** -- R- and S- EDDP Formation normalized to CYP2B6 content to assess for genotype effects. There were no significant differences in rates of formation between livers of different CYP2B6 genotype.



**Figure 3.12** -- *R/S*-EDDP ratio formation analyzed between livers of different CYP2B6 genotype. There are no significant differences in stereoselectivity among livers with different CYP2B6 genotype.



**Figure 3.13** -- Formation of hydroxybupropion versus concentration of bupropion plots in CYP2B6 wild-type and all variants.



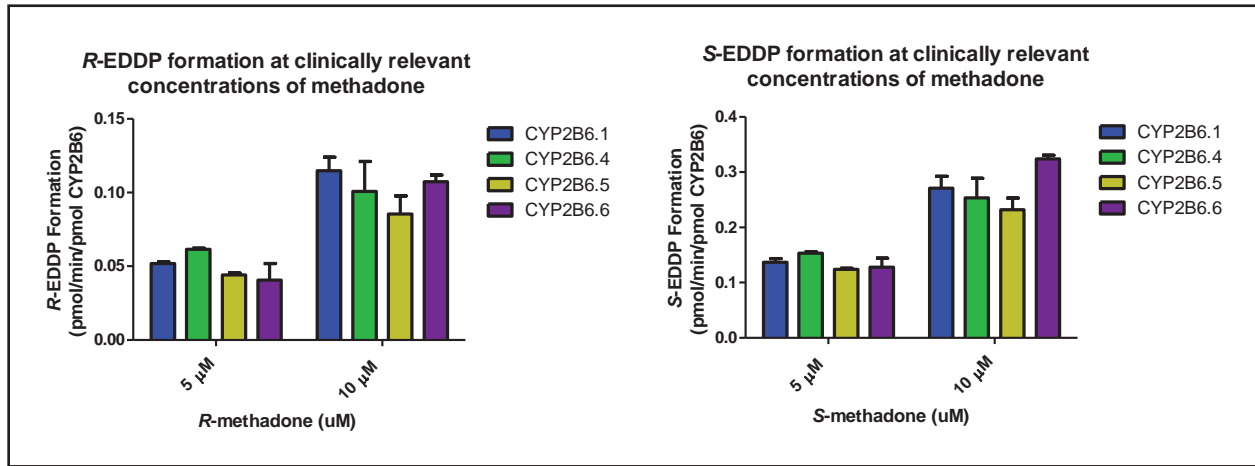
**Table 3.5 -- Michaelis-Menten parameters fit to CYP2B6 wild-type and variant metabolism of bupropion. Goodness of fit was determined using an extra sum of squares F-test.**

Michaelis-Menten Model. Bolded text indicates model (Michaelis-Menten or substrate inhibition) of better fit.						
Enzyme	Substrate	K <sub>m</sub> (μM)	K <sub>m</sub> std error	k <sub>cat</sub> (pmol/min/pmol P450)	k <sub>cat</sub> std error	Intrinsic Clearance (nL/min/pmol P450)
CYP2B6.1	R-Bupropion	<b>72.34</b>	<b>15.9</b>	<b>5.82</b>	<b>0.37</b>	<b>80.51</b>
	S-Bupropion	69.08	18.22	10.3	0.79	149.39
CYP2B6.4	R-Bupropion	<b>142.5</b>	<b>16.85</b>	<b>2.79</b>	<b>0.11</b>	<b>19.56</b>
	S-Bupropion	98.14	18.66	4.84	0.29	49.27
CYP2B6.5	R-Bupropion	99.26	21.31	1.66	0.11	16.76
	S-Bupropion	75.8	26.2	2.98	0.31	39.37
CYP2B6.6	R-Bupropion	223.9	21.65	3.26	0.12	14.54
	S-Bupropion	<b>200.4</b>	<b>36.26</b>	<b>7.28</b>	<b>0.50</b>	<b>36.33</b>

**Table 3.6 -- Substrate inhibition model parameters fit to CYP2B6 wild-type and variant metabolism of bupropion. Goodness of fit was determined using an extra sum of squares F-test.**

Substrate Inhibition Model. Bolded text indicates model (Michaelis-Menten or substrate inhibition) of better fit.								
Enzyme	Substrate	K <sub>m</sub> (μM)	K <sub>m</sub> std error	k <sub>cat</sub> (pmol/min/pmol P450)	k <sub>cat</sub> std error	K <sub>i</sub> (μM)	K <sub>i</sub> std error	Intrinsic Clearance (nL/min/pmol P450)
CYP2B6*1	R-Bupropion	131.5	49.41	8.021	1.68	2133	1602	61.00
	S-Bupropion	<b>178.3</b>	<b>79.2</b>	<b>17.67</b>	<b>4.90</b>	<b>1108</b>	<b>733</b>	<b>99.10</b>
CYP2B6*4	R-Bupropion	147.4	35.91	2.843	0.37	45444	289969	19.29
	S-Bupropion	<b>226</b>	<b>73.89</b>	<b>8.051</b>	<b>1.71</b>	<b>1255</b>	<b>651.8</b>	<b>35.62</b>
CYP2B6*5	R-Bupropion	<b>387.6</b>	<b>111.2</b>	<b>4.137</b>	<b>0.89</b>	<b>553.1</b>	<b>197</b>	<b>10.67</b>
	S-Bupropion	<b>1261</b>	<b>1339</b>	<b>24.31</b>	<b>23.56</b>	<b>108.4</b>	<b>119.9</b>	<b>19.28</b>
CYP2B6*6	R-Bupropion	223.9	22.47	3.255	0.1269	ambiguous	ambiguous	14.54
	S-Bupropion	366.2	172.5	10.98	3.608	1897	1786	29.98

**Figure 3.14** -- Rates of formation for EDDP at clinically relevant concentrations of methadone. There was no statistically significant difference between rates of turnover and CYP2B6 genotype.



**Table 3.7** -- Primers used for CYP2B6 truncation and modification (Raccor et al., 2012).

Primer Name	Sequence (5' to 3')
2B6-truncation forward	CCATCGATCATATGGCTAAGAAGACTAGTAGTAAAGGAAA ACT CCCACCAGGGCCCCGCCCTCTG
2B6-truncation reverse	AGTCGACCGGGGGAGGAAGCGGATCTGGTATGTT
Internal Sall forward	GAAAAATCGCCATGGTGGACCCATT
<i>CYP2B6</i> *4 forward	CCCAGCGCCCCCA <b>G</b> GGACCTCATCGAC
<i>CYP2B6</i> *5 forward	GACCCACCTTCCTCTTCCA <b>T</b> TCCATTACCGC
<i>CYP2B6</i> *6 forward	CAACATACCAGATC <b>T</b> GCTTCCTGCCCCGC

Base changes are highlighted in red

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## Chapter 4: General conclusions and future directions

### I. General Conclusions

This dissertation project aimed to elucidate the contribution of CYP3A4, CYP2B6, and P-gp in the variability observed in methadone pharmacokinetics. In addition, *CYP2B6* and *ABCB1* genotype impact on methadone pharmacokinetic parameters were also explored. In particular, the present investigation focused on relative contribution of CYP3A4 and CYP2B6 to methadone metabolism and how protein content, activity, and allelic variants (of CYP2B6) affected EDDP formation rates. Both *in vivo* and *in vitro* studies were conducted to achieve these goals.

This project included the first *in vivo* study to simultaneously characterize CYP3A4, CYP2B6, and P-gp activities in healthy individuals and ascertain their association with methadone absorption and clearance. In order to characterize CYP2B6 activity, the *in vivo* study also introduced using the AUC of *S,S*-hydroxybupropion as a phenotypic marker of CYP2B6 activity. Parent drug clearance or metabolite formation clearance, estimated as metabolite-to-parent AUC ratio are typically used as indices for protein activity, but the use of bupropion as a specific *in vivo* probe for CYP2B6 presented a unique challenge as is discussed in detail in Chapter 2. The unconventional and novel use of *S,S*-hydroxybupropion AUC may be applicable to other substrates whose clearance is governed by several variable and competing pathways and when the basal level of enzyme activity (without inclusion of inducers or inhibitors) is important.

Data from the *in vivo* study revealed that CYP3A4 and P-gp were not associated with stereoselective or total methadone clearance, estimated formation clearance of EDDP (calculated EDDP-to-methadone AUC ratio), or EDDP AUC *in vivo*. Activities from both proteins do not

seem to play a significant role in the variation observed in the methadone pharmacokinetic parameters calculated.

*In vivo* CYP2B6 activity (when characterized as the AUC of *S,S*-hydroxybupropion) is significantly associated with AUC of *S*- EDDP. This data agrees with results presented in Chapter 3 and previously published *in vitro* data (Totah et al., 2007; Totah et al., 2008). Multiple linear regression analyses also support a more significant role for CYP2B6 in the metabolism of methadone *in vivo* compared to CYP3A4 and P-gp. *CYP2B6* and *ABCB1* variants were also assessed to determine trends in absorption and metabolism *in vivo*. There is a trend for lower *R*- and *S*- EDDP AUC in CYP2B6 allelic variants compared to wild-type individuals. However, the number of subjects studied is too low to draw strong conclusions and larger studies are needed to establish the influence of CYP2B6 genotype on *R*- and *S*- EDDP AUC, and more importantly, methadone metabolism. *ABCB1* genotype did not affect methadone absorption or overall clearance.

The *in vitro* investigations involved studying methadone metabolism in primary human hepatocytes, a human liver microsome (HLM) bank, and recombinantly expressed CYP2B6 wild type and variant enzymes. Studies in human hepatocytes confirm that EDDP is the major oxidative metabolite formed in the liver. The HLM screen was insightful in determining the relative contribution of CYP3A4 and CYP2B6 to methadone metabolism. In contrast to the *in vivo* studies, CYP3A4 protein content and activity accounts for approximately 50% of the variability observed with *R*- and *S*- EDDP formation. CYP2B6 *in vivo* and *in vitro* data are in agreement. CYP2B6 protein content and activity in HLMs, although statistically significant, only accounts for 25%-30% of the variability observed in *R*- and *S*- EDDP formation respectively.

CYP2B6 genotype in HLMs does not appear to influence EDDP formation, in contrast to *in vivo* data.

One possible reason for the discrepancies between *in vivo* and *in vitro* studies may be the methadone concentration (40  $\mu$ M) used in these studies. Typical *in vivo* concentrations of methadone are approximately 2  $\mu$ M (Gadel et al., 2013). The choice to screen HLMs at methadone concentration of 40  $\mu$ M was because this concentration is the reported methadone  $K_m$  for CYP2B6, where genotype effects would be more apparent at this concentration (Totah et al., 2008). CYP2B6 genotype does not appear to influence rate of formation of EDDP, however the presence of CYP3A4 in HLMs may be masking the effect of CYP2B6 genotype. Another reason for this discrepancy may be due to the nature of the human liver bank. Cytochrome P450 enzyme expression in this system are typically changed, which could cause genotype effects to be dampened. In addition, CYP2B6 is a highly polymorphic enzyme and only the most common SNPs are screened. It is possible that individual livers are incorrectly genotyped because the entire *CYP2B6* gene has not been sequenced. Whole gene sequencing is beyond the scope of this research project, and budget of this project therefore we limited the *CYP2B6* genotyping to the most common variants.

Activity studies in recombinant CYP2B6 variants are inconclusive; however, all enzymes studied have markedly higher affinity for *S*-methadone over *R*-methadone. Interestingly, CYP2B6.1 had much higher rates of formation for hydroxybupropion compared to allelic variants. This would suggest that catalytic activities of these enzymes are different. However, the rates of EDDP formation for all CYP2B6 enzymes studied are approximately equal at clinically relevant concentrations of methadone highlighting the substrate specific effect of *CYP2B6* genotype.

## II. Future Directions

Further studies are needed to establish the use of *S,S*-hydroxybupropion AUC as an *in vivo* phenotypic marker for basal CYP2B6 activity. Validation would require using *S,S*-hydroxybupropion AUC to describe clearance of other substrates reported to be partly metabolized by CYP2B6, such as cyclophosphamide or efavirenz. Future studies characterizing variable methadone metabolism *in vivo* include studying more individuals carrying various *CYP2B6* polymorphisms and determining how methadone clearance is affected. The results obtained indicate a trend for lower EDDP AUC in individuals carrying certain polymorphisms of *CYP2B6* especially *CYP2B6\*6*. However, a larger sample size is needed to confirm this observation. The *in vivo* study also provides evidence that absorption and metabolism of methadone only partially explain its variability. Studying processes of methadone distribution or characterizing how methadone metabolism changes in MMT or chronic pain patients would be helpful to understanding the variable pharmacokinetics of the drug.

Additional *in vitro* studies in hepatocytes are needed to characterize CYP3A4 and CYP2B6 metabolism of methadone. Hepatocytes represent a more realistic model to perform metabolism studies. It would be ideal to conduct studies in hepatocytes with certain CYP2B6 genotypes to fully characterize the pharmacogenetic effect on methadone metabolism. However, to date, commercial hepatocytes genotyped for CYP2B6 variants are not available. Our studies establish a role for CYP2B6 in the *in vivo* and *in vitro* variability of methadone metabolism but further studies are needed to determine other causes for the variable pharmacokinetics and toxicity of methadone.

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## Appendix

Recipes for over-expression of CYP2B6 in *E. Coli* cells.

*Terrific Broth (TB)*: Mix 6 L of deionized water, 330 g of dry TB media mix, and 24 mL of glycerol. Aliquot the 6 L into 500 mL portions using 12 expression flasks. Autoclave the media no earlier than the day before seeding (the day the starter cultures are inoculated).

*Luria Broth (LB)*: Mix 500 mL of deionized water, 5 g tryptone, 2.5 g yeast extract, 5 g NaCl, and 7.5 g agar (if making plates).

*Trace Elements*: Combine 2.7 g  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.2 g  $\text{ZnCl}_2 \cdot 4\text{H}_2\text{O}$ , 0.2 g  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.2 g  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.2 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.1 g  $\text{CuCl}_2$ , 0.05 g  $\text{H}_3\text{BO}_3$ , and 10 mL of concentrated HCl. Deionized water was added to bring the volume up to 100 mL. Trace elements were autoclaved prior to use in expression.

Buffers used for recombinant enzyme purification on nickel column

*Resuspension/Equilibration Buffer*:

<b>Total Volume</b>	<b>100 mL</b>	<b>500 mL</b>	<b>1 L</b>
<b>1 M KPi pH=7.4</b> (0.1M final conc.)	10 mL	50 mL	100 mL
<b>Glycerol</b> (20% final conc.)	20 mL	100 mL	200 mL
<b>10 % Emulgen</b> (1% final conc.)	10 mL	50 mL	100 mL
<b>Protease Inhibitor</b> *Add right before use ** 1:1000 Dilution	100 $\mu\text{L}$	500 $\mu\text{L}$	1 mL

*Wash Buffer:*

<b>Total Volume</b>	<b>100 mL</b>	<b>500 mL</b>	<b>1 L</b>
<b>1 M KPi pH=7.4</b> (0.1M final conc.)	10 mL	50 mL	100 mL
<b>Glycerol</b> (20% final conc.)	20 mL	100 mL	200 mL
<b>NaCl</b> (300 mM final conc.)	1.75 g	8.77 g	17.52 g
<b>Glycine</b> (100 mM final conc.)	750 mg	3.75 g	7.5 g
<b>1M Imidazole OR</b> <b>Imidazole (Solid)</b> (5 mM final conc.)	500 µL <b>OR</b> 34 mg	2.5 mL <b>OR</b> 170 mg	5 mL <b>OR</b> 340 mg
<b>Cholate</b> (0.05% final conc.)	50 mg	250 mg	500 mg
<b>Protease Inhibitor</b> *Add right before use ** 1:1000 Dilution	100 µl	500 µL	1 mL

*Super Wash Buffer:*

<b>Total Volume</b>	<b>100 mL</b>	<b>500 mL</b>	<b>1 L</b>
<b>1 M KPi pH=7.4</b> (0.1M final conc.)	10 mL	50 mL	100 mL
<b>Glycerol</b> (20% final conc.)	20 mL	100 mL	200 mL
<b>NaCl</b> (300 mM final conc.)	1.75 g	8.77 g	17.52 g
<b>Glycine</b> (100 mM final conc.)	750 mg	3.75 g	7.5 g
<b>1M Imidazole OR</b> <b>Imidazole (Solid)</b> (40 mM final conc.)	4 mL <b>OR</b> 272 mg	20 mL <b>OR</b> 1.36 g	40 mL <b>OR</b> 2.72 g
<b>Cholate</b> (0.05% final conc.)	50 mg	250 mg	500 mg
<b>Protease Inhibitor</b> *Add right before use ** 1:1000 Dilution	100 µl	500 µL	1 mL

*Elution Buffer:*

<b>Total Volume</b>	<b>100 mL</b>	<b>500 mL</b>	<b>1 L</b>
<b>1 M KPi pH=7.4</b> (0.1M final conc.)	10 mL	50 mL	100 mL
<b>Glycerol</b> (20% final conc.)	20 mL	100 mL	200 mL
<b>Imidazole (Solid)</b> (500 mM final conc.)	3.404 g	17.02 g	34.04 g
<b>Cholate</b> (0.05% final conc.)	50 mg	250 mg	500 mg

*Dialysis Buffer:*

<b>Total Volume</b>	<b>1 L</b>	<b>2 L</b>	<b>4 L</b>
<b>1 M KPi pH =7.4</b> (0.1M final conc.)	100 mL	200 mL	400 mL
<b>Glycerol</b> (20% final conc.)	200 mL	400 mL	800 mL

**Scheme 3.17** – Buffers used to purify recombinant enzyme on hydroxyapatite column

*Dialysis/Loading Buffer:*

<b>Total Volume</b>	<b>1 L</b>	<b>2 L</b>	<b>4 L</b>
<b>1 M KPi pH =7.4</b> (10 mM final conc.)	10 mL	20 mL	40 mL
<b>Glycerol</b> (20% final conc.)	200 mL	400 mL	800 mL
<b>NaCl</b> (300 mM final conc.)	17.5 g	35 g	70 g
<b>Cholate</b> (0.2% final conc.)	2 g	4 g	8 g

*Wash Buffer:*

<b>Total Volume</b>	<b>1 L</b>	<b>2 L</b>	<b>4 L</b>
<b>1 M KPi pH =7.4</b> (20 mM final conc.)	25 mL	50 mL	100 mL
<b>Glycerol</b> (20% final conc.)	200 mL	400 mL	800 mL
<b>NaCl</b> (100 mM final conc.)	5.8 g	11.6 g	23.2 g
<b>Cholate</b> (0.5% final conc.)	5 g	10 g	20 g

*Elution Buffer:*

1:1 HA Wash Buffer : 1 M KPi pH 7.

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- Protein expression and purification of CYP2B6 and four variants (CYP2B6.4, CYP2B6.5, CYP2B6.6, and CYP2B6.7).
- Determined and compared Michaelis-Menten kinetics of methadone and bupropion with CYP2B6 wild-type and all variants expressed.
- Phenotyping reaction assays studying CYP isozymes contribution to cyclophosphamide metabolism.
- Study coordinator and researcher for human subject trial assessing role of CYP2B6, CYP3A4, and P-gp in methadone metabolism.
- Designed and optimized plasma and urine extraction protocols and developed LC-MS/MS methods to process samples collected from human subject study.
- Performed pharmacokinetic analysis of methadone absorption, metabolism, and excretion in healthy human subjects.
- Design and performed *in vitro* experiments screening CYP2B6 activity with bupropion and methadone in University of Washington School of Pharmacy Human Liver Microsome bank. The data was analyzed for correlation.
- *In vitro* experiments involving CYP2J2 inhibition by methadone and its main metabolite EDDP in Supersomes and primary human cardiomyocytes.
- Characterized Michaelis-Menten pharmacokinetics of bupropion deuterated at various sites of metabolism by wild-type CYP2B6 and a common variant, CYP2B6.6.
- Familiarity with a variety of statistical and modeling packages: GraphPad Prism, STATA, R, and Pharsight Phoenix.

**Pharmacy Intern**, July 2005 — June 2009

Harborview Medical Center, Seattle, Washington

- Assisted in filling prescriptions, verifying dosing, answering phones, and counseling patients in both inpatient and outpatient settings.
- Performed monthly narcotic audits and clinic inspections.
- Intern at Pioneer Square Clinic with focus on monitoring drug therapy and education of patients and who were homeless, indigent, and afflicted with drug addiction.
- Managed dosing of patients with renal failure.
- Managed dosing of patients undergoing anticoagulation therapy.

**Undergraduate Research Experience**, April 2003 — June 2005

**University of Washington Chemistry Department**, Seattle, Washington

- Helped design and carried out experiments in development of anti-malarial drug.
- Polypeptide synthesis, purification, and spectroscopic analyses (specifically UV analysis, HPLC, NMR, and mass spectroscopy).
- Presented findings at Undergraduate Research Symposium and Honors Research Colloquium.

**PUBLICATIONS AND PRESENTATIONS**

**Peer-Reviewed Publications**

Raccor, BS, Claessens AJ, **Dinh JC**, Park JR, Hawkins DS, Thomas SS, Makar KW, McCune JS, Totah RA. Potential contribution of cytochrome P450 2B6 to hepatic 4-hydroxycyclophosphamide formation in vitro and in vivo. *Drug Metab Dispos.* **40**, 54-63 (2012).

**Book Chapter**

Raccor BS, Teitelbaum AM, **Dinh JC**, Totah RA. “Principles of Drug Metabolism 3: Enzymes and Tissues” in Comprehensive Medicinal Chemistry III. Ed. Testa B and van de Waterbeemd H. Elsevier, Oxford, United Kingdom. *Chapter Under Review*.

**Oral and Poster Presentations**

2013 Cytochrome P450 Meeting:

**Dinh J**, Racor B, Uttamsingh V, Harbeson S, Totah R. “CYP2B6 Metabolism of Bupropion and Deuterated Analogues.”

2013 University of Washington Corporate Advisory Board Meeting Talk: “*In Vitro* and *In Vivo* Studies of Methadone Metabolism: Role of CYP2B6 and CYP3A4.”

2012 University of Washington Corporate Advisory Board Meeting:

**Dinh J**, Raccor B, Terman G, Patton K, Totah R. “*In vivo* and *In vitro* Contributions of CYP3A4, CYP2B6, and P-gp to Methadone Pharmacokinetics.”

2011 Gordon Research Conference on Drug Metabolism:

**Dinh J**, Raccor B, Kaspera R, Masse C, Uttamsingh V, Harbeson S, Totah R. “CYP2B6 Metabolism of Bupropion and Deuterated Analogues.”

**FELLOWSHIPS RECEIVED**

- NIH TL-1 Multidisciplinary Predoctoral Training Grant (2012).
- Elmer M. Plein Endowed Research Fund (2007).

- NIH T-32 Multidisciplinary Predoctoral Training Grant (2007).
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## **PROFESSIONAL AFFILIATIONS**

### **UAW 4121, 2007—present**

#### **University of Washington, Seattle, Washington**

- Trustee (2009—2012). Audited local chapter's finances twice a year. Part of the union's board members that worked to preserve the quality of higher education despite budget cuts to Washington state.
- Steward (2012—present). Represented health sciences during union meetings.
- Board member that participated in negotiating the 2010, 2011, and 2012 labor contract for all graduate students (includes both teaching assistants and research assistants).
- Volunteered for I1098 campaign for a state income tax for the state of Washington.
- Went to Olympia (Washington state capital) several times to speak to senators and legislators about preserving funding for higher education.
- Phone-banked for various campaigns: Immigration Reform, Marriage Equality, Tax Reform, Washington State budget cuts to higher education, etc.

### **Graduate Professional Student Senate (GPSS) Senator, 2010—2012**

#### **University of Washington, Seattle, Washington**

- Attended monthly meetings to develop graduate student agenda on issues such as the Washington State budget, health insurance for graduate students, and childcare, etc.

### **Associate Students of University of Washington (ASUW) Senator, 2010—2011**

#### **University of Washington, Seattle, Washington**

- Attended weekly meetings to develop student agenda and represent the graduate student voice at the University of Washington.

### **Unified Professional Pharmacy Organizations of Washington (UPPOW), 2005 – 2009**

#### **University of Washington School of Pharmacy, Seattle, Washington**

- Attended APhA conference in San Francisco (2006), Atlanta (2007), and Mid-year Regional Meeting in Spokane (2006).
- Volunteered at Hypertension Booth (2007).
- Volunteered at Health Fairs (2006, 2007).
- Volunteered on Legislation Day (2006).
- Volunteered on Diabetes Committee (2006).

### **Lambda Kappa Sigma (LKS), 2005 — 2009**

#### **University of Washington School of Pharmacy, Seattle, Washington**

- Co-president (2006-2007).
- Webmaster (2007-2008) – built webpage for LKS.
- Organized several community volunteer projects at Hearthstone Retirement Community, fundraising for breast cancer, and organized food drives for Northwest Harvest.

### **Phi Lambda Sigma, Pharmacy Leadership Society, 2007 — 2009**

#### **University of Washington School of Pharmacy, Seattle, Washington**

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## AWARDS RECEIVED

- Muriel C. Vincent Endowment (2008).
  - Leadership Center Research Fund Recipient (2008).
  - 5<sup>th</sup> place in UW School of Pharmacy counseling competition (2006).
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## OTHER RELEVANT EXPERIENCE

**Volunteer**, April 2014

**Paws-On-Science**, Seattle, Washington

- Volunteered at “Mini-Pharmacy” booth with other graduate students and faculty from the University of Washington School of Pharmacy.
- Taught public about the importance of taking medications correctly by performing an experiment with calcium carbonate, vinegar, and cabbage juice.

**Teaching Assistant**, January 2010 — June 2010

**University of Washington School of Pharmacy**, Seattle, Washington

- Teaching assistant for two core classes in medicinal chemistry for pharmacy students.
- Wrote homework sets and keys.
- Participated in grading of all exams.

**Technical Assistant**, April 2002 — June 2005

**University of Washington Graduate School**, Seattle, Washington

- Updated computers to ensure security and safety.
- In charge of updating and securing National Association of Graduate Admissions Professionals (NAGAP) website.
- Helped to make sure that the Graduate School webpage for application and admissions was maintained and accurate.

**Special Olympics Volunteer Coach**, June 2001 – June 2003

**Lynnwood Recreation Center**, Lynnwood, Washington

- Swim coach for younger brother and his teammates.
- Assisted with swim team at state meet for Special Olympics.

**Volunteer**, September 2001 — September 2002

**University of Washington Medical Center**, Seattle, Washington

- Transported patients between services, delivered specimens to laboratories, ran errands for patients when needed.
- Helped answer questions from hospital visitors.

**Lifeguard and Swim Instructor**, June 2001 — December 2001

**Lynnwood Recreation Center**, Lynnwood, Washington

- Taught swim lessons and substituted as coach for swim team.
- Worked to ensure safety and education of patrons as lifeguard.