Neonatal abstinence syndrome: comparing the hepatic metabolism of addictive medicines *in vitro* in the fetus, neonate and adult female

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Abstract

Neonatal abstinence syndrome (NAS) is defined as neonatal withdrawal symptoms due to antepartum maternal substance abuse. Substances that are most likely to cause NAS are opioids, alcohol and central nervous system (CNS) stimulants or depressants, such as cocaine and benzodiazepines, respectively. Opioid addiction in adults as well as neonates is treated with methadone or buprenorphine. In severe maternal addiction and neonatal withdrawal cases, morphine is administered as an additive to methadone or buprenorphine. Mothers who are on methadone or buprenorphine treatment are often found to have plasma traces of alprazolam, a common benzodiazepine prescribed for the treatment of anxiety. However, the *in vitro* metabolism of these drugs, either alone or when taken together, has never been well established in fetuses or neonates. Here, we analyze the activity of methadone, buprenorphine, morphine, and alprazolam in fetal, neonate and adult female livers. Since the cytochrome P450 3A enzymes (CYP3A4 and CYPA5) are common and major routes of metabolism for these drugs, the resulting metabolite of each can be measured to assess the activity. This was done by *in vitro* incubations with human liver microsomes (HLM) from adult females (20-75 years old) and neonates (male and female, 13 days – 11 months old). The metabolites were identified by high-performance liquid chromatography (HPLC). We found there was no significant difference in alprazolam, buprenorphine or methadone metabolite formation rate between adults and neonates. Morphine showed inhibition of alprazolam metabolite formation at high concentrations in both female adult and neonatal liver microsomes. We also saw high variability between the individual microsomes and performed a multiple linear regression analysis to determine if age, CYP content, and

alcohol or cigarette use could be responsible for this variability. From these analyses, CYP content was a significant variable for predicting metabolite formation in all groups except neonates in 4-hydroxyalprazolam (4-OHALP) and 2-ethylidene-1,5-dimethyl-3,3-dipehnylpyrrolidine (EDDP) production. Additionally, norbuprenorphine (NBUP) and EDDP formation rate was partially related to alcohol use in adult females. From these results, we can conclude: 1) the metabolite formation rate differences of 4-OHALP, BUP, and EDDP between adult females and neonates are not predictors of neonatal abstinence syndrome onset, but *in vitro* studies with fetal microsomes still need to be done and 2) the interaction between morphine and alprazolam occurs at a concentration too high to be of pharmacokinetic importance, but there may be an underlying pharmacodynamic mechanism leading to additive effects.

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List of Abbreviations

ALP	Alprazolam
α-OHALP	alpha-hydroxyalprazolam
4-OHALP	4-hydroxyalprazolam
B	Black
B3G	Buprenorphine-3-glucuronide
BUP	Buprenorphine
C	Caucasian
CNS	Central Nervous System
CYP	Cytochrome P450
DDI	Drug-drug interaction
EDDP	2-ethylidene-1,5-dimethyl-3,3-dipehnylpyrrolidine
EMDP	2-ethyl-5-methyl-3,3-diphenylpyrroline
F	Female
H	Hispanic
HLM	Human Liver Microsomes
HPLC	High performance liquid chromatography
IC50	Concentration of inhibitory substance reducing activity to 50% of maximum
IS	Internal standard
K_m	Substrate concentration when V_{max} is 50%
M	Male
MET	Methadone
MOR	Morphine
μOR	μ- opioid receptor
N3G	Norbuprenorphine-3-glucuronide
NADP	β-Nicotinamide adenine dinucleotide phosphate sodium salt
NAS	Neonatal abstinence syndrome
NBUP	Norbuprenorphine
NMDAR	N-methyl-D-aspartate receptor
P-gp	P-glycoprotein
RAU	Relative absorbance units
$t_{1/2}$	Time required for drug concentration or amount to fall by 50%
UGT	UDP-glucuronosyl transferases
V_{max}	Maximum velocity of metabolite formation
VWD	Variable wavelength detector

Chapter 1. Introduction

Neonatal abstinence syndrome (NAS) has been an issue for many years, more recently increasing in number of incidences due to the rising opioid epidemic (Brandt and Finnegan, 2017). NAS is due to maternal use of addictive substances, most commonly with opioids, but can also be from abuse of benzodiazepines, and CNS stimulants (McQueen and Murphy-Oikonen, 2016). These substances pass through the placental membrane and into the fetus where they travel through the blood-brain barrier into the CNS (Burke and Beckwith, 2017). Withdrawal symptoms affect metabolism, gastrointestinal tract manifestations, and overall development, including the central nervous system and respiratory system (McQueen and Murphy-Oikonen, 2016). NAS has been a known issue for many years, however it has been increasing in number of incidences over the past 15 years with a ratio of NAS births to healthy births being 5.8:1000 in 2012, and continuing to rise (Brandt and Finnegan, 2017). Treatment and prevention of NAS starts with the mother. To date, there are only two approved treatments for substance addiction, methadone (MET) and buprenorphine (BUP), with morphine (MOR) being administered as a supplement in severe addiction cases (Mozurkewich and Rayburn, 2014). Both methadone and buprenorphine act on the μ-opioid receptor as a full and partial agonist, respectively (Dinis-Oliveira, 2016; Silva and Rubinstein, 2016). Methadone is the more accepted form of treatment and has been used for the treatment of opioid addiction since 1965 (Dinis-Oliveira, 2016). Buprenorphine is new in comparison, but has consistently shown a more favorable safety profile in neonates and mothers compared to methadone (Lemon et al., 2018). Commonly found in mothers on buprenorphine or methadone treatment are plasma traces of anti-anxiety

medication, like alprazolam (ALP), and other addictive substances like oxycodone (Fields et al., 2015). There have been studies to show an increase in hospitalizations and deaths on those who are taking methadone or buprenorphine and alprazolam at the same time (Fields et al., 2015; McCance-Katz et al., 2010).

1.1. Cytochrome P450 enzyme involvement

Cytochrome P450 enzymes (CYPs) are heavily abundant in the liver and are responsible the majority of phase I drug metabolism (Jia and Liu, 2007). They are also found within the epithelial cells of the gastrointestinal tract, and may contribute to the amount of drug absorbed into the body, thus the drug's bioavailability (Jia and Liu, 2007). They are a superfamily comprised of more than 50 isoforms, with only a fraction of those contributing to the metabolism of 70-80% of drugs used clinically (Zanger and Schwab, 2013). They are responsible for oxidation of a drug, thus transforming the parent drug into its metabolite (Ogu and Maxa, 2000). Some of the principal CYPs involved in biotransformation, and also the most expressed in the liver, are CYP3A, 2C9, 2D6, 1A2, 2B6, 2C19, and 2E1, with CYP3A responsible for the biotransformation of over 50% of all drugs (Jia and Liu, 2007; Zanger and Schwab, 2013). With the ability of CYPs, especially CYP3A, to metabolize such a large fraction of substrates, comes the inevitability of drug-drug interactions (DDI) (Ogu and Maxa, 2000). CYPs can be affected by genetic polymorphisms, and can also be induced or inhibited, altering the metabolism and effect of the substrate (Zanger and Schwab, 2013). A well-known example of this are the highly profiled CYP2D6 alleles, which can produce a phenotype of either normal, poor, or extensive metabolism (Zanger and Schwab, 2013). The severity

of a DDI is related to the therapeutic drug of interest, the form of drug that is active, and the main metabolizing CYP (Ogu and Maxa, 2000; Zanger and Schwab, 2013). As much as neonatal drug pharmacology differs from adults, fetal drug exposure, main contributing CYPs and pathway of metabolism differ vastly compared to adults (Fanni et al., 2014; Lewis et al., 2015; Sun et al., 2006).

In neonates, not only are adverse drug reactions more likely to occur compared to adults, but body weight between each individual can vary up to 10-fold, allowing large variability in the pharmacokinetics and pharmacodynamics of a drug (Fanni et al., 2014; Tayman et al., 2011). There are additionally many developmental changes that occur within the first month after birth that can alter a drug's action. Gastric pH and emptying time, bacterial flora, plasma protein, fluid compartment sizes, and maturation of absorptive surfaces like the gastrointestinal lining, muscles, and skin, can all contribute to changes in drug absorption, distribution, and elimination (Tayman et al., 2011). Additionally, CYPs are variable in comparison to adults. Neonatal CYP3A4 reaches adult levels at one month, but has only 30-40% of its full activity, taking around 6 months to reach adult activity. This has obvious implications given its role in metabolizing most of the clinical drugs available (Fanni et al., 2014; Tayman et al., 2011). Similarly, fetal liver CYP3A4 levels are found to be very low or completely absent (Lacroix et al., 1997). Instead, another isoform, CYP3A7, is highly expressed in fetal liver and is found to disappear as CYP3A4 appears in neonates (Lacroix et al., 1997; Tayman et al., 2011). The two isoforms have greater than 90% similarities in gene sequence encoding each enzyme, yet have variable affinities for substrates and formation of metabolites (Lacroix

et al., 1997; Vyhlidal et al., 2016). Other commonly found adult enzymes (CYP2D6, CYP2C9, CYP2C19 and CYP1A2) also have varied expression and activity, being low or absent in fetal livers, and highly reduced in neonates (Tayman et al., 2011). CYPs are just one factor in the complexity of fetal metabolism. Also taken into account are transport proteins, which may alter drug exposure, and the placental membrane, of which all drugs need to cross to have an effect on the fetus (Lewis et al., 2015; Smit et al., 1999).

1.2. Methadone

Methadone (MET), 6-dimethylamino-4,4-diphenyl-3-heptane, is a synthetic opioid approved by the FDA as an analgesic and antitussive in 1947 and was first seen to have clinical importance for opioid detoxification in 1965 (Richard A. Rettig, 1995). In 1972, MET was approved for clinical use in the United States and as of 1997, has become widely prescribed with new clinical guidelines (Greenblatt, 2014; Richards-Waugh et al., 2014). As of 2013, MET was one of the most prescribed opioids, with 3.9 million prescriptions in the year (Greenblatt, 2018). Now, it is the main form of treatment for opioid dependence and is also used extensively in pediatric medicine for anesthesia, critical care and oncology (Störmer et al., 2001; Ward et al., 2014). It possesses complex
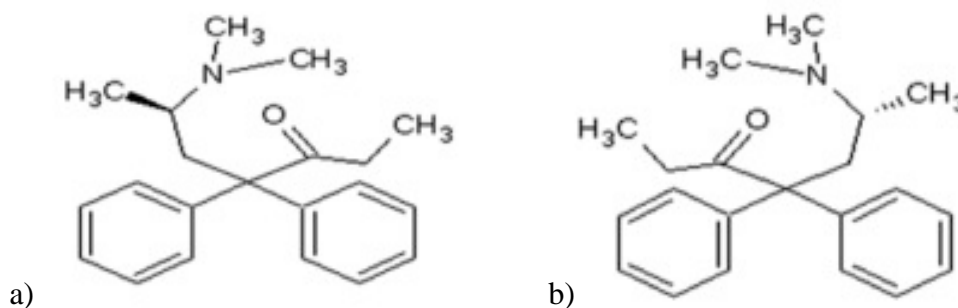
Figure 1.1. Racemic methadone structure: a) *R*-(-)-methadone, b) *S*-(+)-methadone

chemical properties and can act as a full agonist on multiple receptors. Since it is a

racemic drug, with *R*- and *S*- enantiomers (Fig 1.1), there is variability in how it is

metabolized between individuals and thus, a large variability in the resulting therapeutic

response (Dinis-Oliveira, 2016).

MET has affinity for the μ-opioid receptor (μOR) and the N-methyl-D-aspartate receptor

(NMDAR) (Ward et al., 2014). The enantiomers of MET have different receptor

affinities and therapeutic effects. *R*-(-)-MET has 10 times greater affinity for the μOR,

contributes up to 50 times the analgesic effect, and prevents opioid withdrawal

symptoms, while *S*-(+)-methadone may be responsible for toxicity, is much less potent

for pain relief, and is ineffective for opioid treatment (Foster et al., 1999; Lewis et al.,

2015). MET is metabolized by *N*-demethylation in the liver through a phase I metabolic

pathway, mainly by CYP2B6 and CYP3A, but also CYP2D6, CYP2C19/2C9/2C8 and

CYP1A2 (Crettol et al., 2006; Moody et al., 1997). Additionally, CYP2B6 is the

predominant enzyme of MET metabolism to *S*-MET compared to *R*-MET, while

Figure 1.2. Methadone metabolite structures: a) EDDP and b) EMDP *chiral carbon

CYP3A4 has higher selectivity for *R*-MET (Crettol et al., 2006; Greenblatt, 2014; Wang and DeVane, 2003). The extent CYP3A involvement on EDDP formation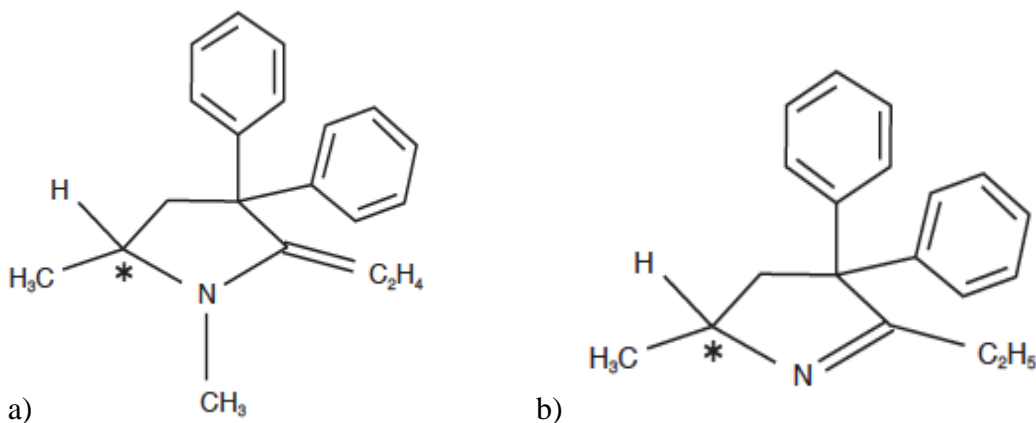 was found to be insignificant when tested *in vivo* with a known CYP3A inhibitor combination, ritonavir/indinavir, confirming the primary role of CYP2B6 (Kharasch et al., 2009; Totah et al., 2008). In early studies to establish the metabolic properties of MET, nine metabolites were identified (Sullivan, 1972). The principal quantifiable metabolites are 2-ethylidene-1,5-dimethyl-3,3-dipehnylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP) (Figure 1.2), both of which are inactive (Foster et al., 1999; Magalhaes et al., 2017). After EDDP formation, it is further *N*-demethylated to EMDP by re-entering the hepatic circulation and undergoing spontaneous cyclization (Foster et al., 1999; Wang and DeVane, 2003). However, EMDP levels are often found to be negligible in humans compared to EDDP and MET (Magalhaes et al., 2017).

When used as an opioid dependency treatment, dosing is started at a fixed oral dose of 20 mg, and given additionally in a dose of 5 to 10 mg every 3 to 6 hours (Mozurkewich and Rayburn, 2014). However, because of CYP variability, and bioavailability ranging from 41-95%, one dose can produce different plasma levels between patients, leading to varied therapeutic effects, and cause unanticipated adverse reactions (Crettol et al., 2006; Ferrari et al., 2004). It is highly lipid soluble and can reach the central compartment rapidly, allowing distribution to tissues including the necessary site of action, the brain (Ferrari et al., 2004). The half-life ($t_{1/2}$) in pregnant women can be as low as 8 hours compared to 22-24 hours in non-pregnant women due to increased hepatic and renal clearance, absorption, and volume expansion (Mozurkewich and Rayburn, 2014). This decrease can

contribute to an increase in opioid cravings, higher dose requirements, more MET

crossing into the fetal bloodstream, and the potential for more severe NAS withdrawal

symptoms (Mozurkewich and Rayburn, 2014).

1.3. Buprenorphine

Buprenorphine (BUP) is a synthetic derivative of the opioid morphine alkaloid thebaine,

with partial agonist activity on the µOR (Picard et al., 2005). Prior to being approved for

opioid treatment, BUP was used for chronic, post-operative, and cancer related pain

management (Ohtani et al., 1995; Robbie, 1979). Its partial agonist activity allows for

potential advantages over MET for opioid dependence treatment with less withdrawal

symptoms and sedation, decreased respiratory depression, and a lower risk of toxicity at

high doses (Welsh and Valadez-Meltzer, 2005). In the course of its discovery as an
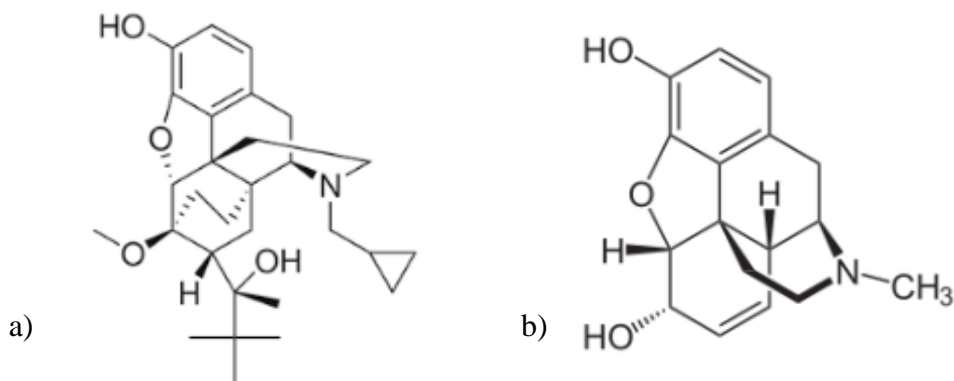
Figure 1.3. Buprenorphine compared to morphine structures. a) Buprenorphine.
b) Morphine

opioid treatment, BUP was evaluated in comparison to morphine due to their cyclic

structural similarities (Figure 1.3). BUP was found to be 25-50 times more potent than

morphine, had ability to block the effects of morphine for over 29 hours, and had lower

dependence liability (Jasinski et al., 1978). BUP is metabolized by phase I metabolism in

the liver, with CYP3A contributing mainly to 80-90% of metabolite formation and CYP2C8 showing activity also (Brown et al., 2011). Other CYPs, CYP3A7 and
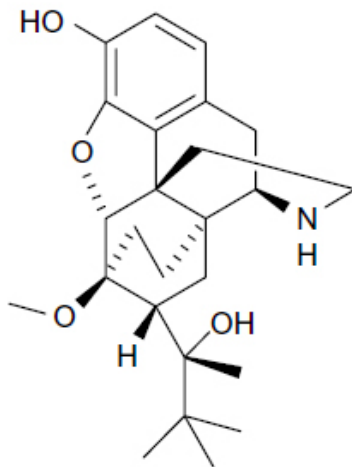
Figure 1.4. Norbuprenorphine structure

CYP2C9/2C18/2C19 contribute to biotransformation of BUP (Picard et al., 2005). The main metabolite formed by N-dealkylation is norbuprenorphine (NBUP) (Figure 1.4), which retains some pharmacological activity (Ohtani et al., 1995). After formation of NBUP, this and the parent compound, undergo phase II metabolic glucuronidation by UDP-glucuronosyl transferases (UGT) (Cone et al., 1984). The glucuronide metabolites, buprenorphine-3-glurcuronide (B3G) and norbuprenorphine-3-glucuronide (N3G), are formed mainly by UGT2B7 and UGT1A1/1A3 (Brown et al., 2011). Buprenorphine's extensive metabolism is exemplified by B3G, N3G, and NBUP plasma levels similar to or exceeding the plasma levels of BUP (Brown et al., 2011). Little clinical information is available on the activity of these glucuronides, however recent animal studies show buprenorphine glucuronides are the first active opioid-3-glucuronides identified, with B3G having affinity for the μOR and nociception receptor leading to mild pain-relief and N3G causing sedation (Brown et al., 2011). The activity of these glucuronides is

comparable with the other known active glucuronide metabolite, morphine-6-glucuronide (Brown et al., 2011).

Due to extensive first-pass metabolism, BUP has a low oral bioavailability (15%) and can only be administered sublingually (Ciraulo et al., 2006). BUP treatment is started at a 2 mg sublingual dose in patients with opioid dependency, a vast reduction from methadone dose (Mozurkewich and Rayburn, 2014). Due to its partial agonistic properties, there is a maximum possible effect even with higher than recommended doses (24 to 32 mg), resulting in a ceiling effect (Brown et al., 2011; Ciraulo et al., 2006; Mozurkewich and Rayburn, 2014). While this leads to an improved safety profile, it can also incompletely prevent cravings, leading to use of higher doses or seeking of other opioids (Mozurkewich and Rayburn, 2014). There is a highly variable rate of elimination, with a $t_{1/2}$ of 3-44 hours when administered sublingually (Lewis et al., 2015). Similar to methadone, blood volume increases during advancing gestation can lead to a potential decreased effect (Mozurkewich and Rayburn, 2014).

1.4. Alprazolam

Alprazolam (ALP), more commonly known throughout the world as Xanax, is a commonly prescribed benzodiazepine of the triazolo-class used for the treatment of generalized anxiety, panic disorder and depression (Venkatakrishnan et al., 1998; von Moltke et al., 1993). Of the 82.6 million anxiolytics prescribed in the US from 2014-2015, ALP accounted for 28.8 million (~35%) of them (Greenblatt et al., 2018). Once absorbed into the bloodstream, ALP crosses the blood-brain barrier and binds to the

gamma amino butyric acid-A (GABA-A) receptor as a positive allosteric modulator, increasing the affinity of GABA to its receptor in the central nervous system (Griffin et al., 2013). It is metabolized almost exclusively by CYP3A. This enzyme contributes solely to the formation of the primary main metabolite, 4-hydroxyalprazolam (4-OHALP), and CYP2C9/2C19 additionally contributing to the primary minor metabolite, α-hydroxyalprazolam (α-OHALP) (Figure 1.5) (Venkatakrishnan et al., 1998). α-OHALP can be further metabolized to α,4-dihydroxyalprazolam, and 4-OHALP can be metabolized to this and a benzophenone (von Moltke et al., 1993). While the primary metabolites are active, they have lower GABA-A receptor affinity compared to the parent drug and have plasma concentrations of less than 10% that of ALP (Greenblatt and Wright, 1993; von Moltke et al., 1993). ALP is also the only form to cross into the brain, thus clinical activity and pharmacologic effects are attributable to the intact parent drug (Arendt et al., 1987; Greenblatt et al., 1993). It is short acting, with a $t_{1/2}$ of 6-27 hours, and has a high affinity for the GABA-A receptor (Griffin et al., 2013).
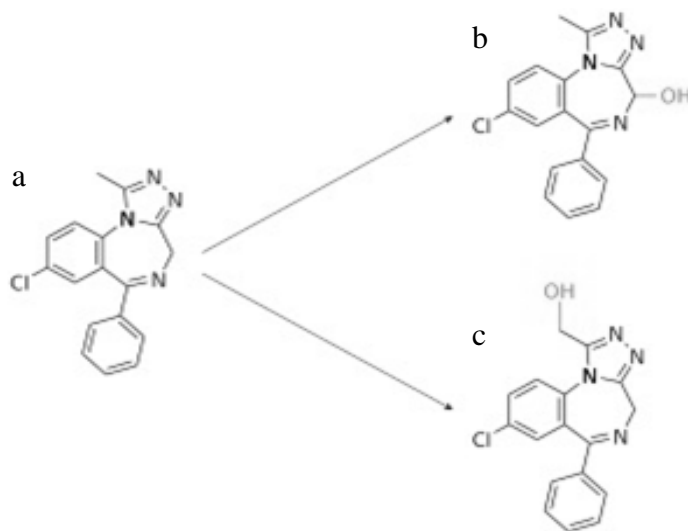

Figure 1.5. Structure of Alprazolam and its metabolites. a) ALP. b) 4-OHALP. c) α-OHALP

1.4.1. Opioid-Benzodiazepine Drug-drug Interactions

Patients who have opioid addiction on MET and BUP treatment often have concurrent illnesses that require treatment with additional medications (McCance-Katz et al., 2010). The use of a benzodiazepine in combination with opioid use increased from 9 to 17% from 2001-2013, an 80% relative increase (Sun et al., 2017). With the prescription use of benzodiazepines, alprazolam particularly, being high, there has been an increased incidence of ALP present in plasma in regular opioid users, and in opioid overdoses (Fields et al., 2015). The pharmacokinetic interactions that are inevitably occurring between opioid treatment medications and alprazolam are likely due to CYP interactions, mainly CYP3A4 (McCance-Katz et al., 2010). Studies have shown the similarities in activity of CYP3A4 and CYP3A5, however when it comes to inhibition and DDIs, CYP3A4 is more significant (Patki et al., 2003).

In vitro metabolism with human liver microsomes (HLM) showed midazolam inhibiting the metabolism of buprenorphine, however this was not seen in every case (Jones et al., 2012). When methadone and alprazolam are taken together, methadone plasma levels remain the same as if methadone were being ingested alone. However, side effects of methadone were much more evident, some cases resulting in fatalities (McCance-Katz et al., 2010). Studies have shown that a common explanation for the severity of this drug combination is the μOR and GABA-A receptor localization in medulla respiratory centers leading to respiratory depression (Jones et al., 2012). The pharmacokinetic interaction of benzodiazepines and opioids would lead to higher opioid levels, resulting in inhibition of the respiratory centers through saturation of the μOR (Jones et al., 2012).

Use of these drugs has an obvious potential impact on pregnant women, yet the exact impact and severity on the fetus is unknown.

1.5. Aims of the study

The purpose of this study was to first, identify the main metabolites of the two approved and used opioid addiction treatments (buprenorphine and methadone) and of alprazolam, commonly abused in patients on buprenorphine or methadone by HPLC in adult female, neonatal and fetal HLM. Secondly, to evaluate the metabolite formation rates between these groups for potential differences, which could be used as a predictor of neonatal abstinence syndrome. Thirdly, to identify and analyze morphine, commonly administered simultaneously with methadone or buprenorphine, as a potential inhibitor of alprazolam metabolism *in vitro* and whether this would be a pharmacokinetic interaction of clinical significance.

Chapter 2. Materials and Methods

HPLC methods were modified from previous publications to identify the parent, metabolite, and internal standard peaks for each substrate by using pure compounds. Preliminary incubations to determine the optimal substrate concentration, HLM concentration, and incubation time using an established CYP protocol were performed for each substrate. After determining optimal conditions, incubations were performed in 26 individual adult female HLM and 7 individual neonatal HLM. Each individual HLM was performed in duplicate to measure inter-assay variability. This was done for BUP, ALP and MET separately. Metabolite formation peak heights were normalized to the internal standard peak heights and averaged to determine the metabolite formation rate in RAU. The formation rates in both groups were separately averaged and compared for significance using an independent t-test. Variability of metabolite formation rate was analyzed by multiple linear regression using CYP content, age, and alcohol or cigarette use as potential determinants of metabolite formation. Morphine was analyzed as a potential inhibitor of CYP3A4 using concentrations of 0-1500 μM morphine and 1250 μM ALP as the substrate.

2.1. Chemicals and Reagents

Phosphate buffer solution, magnesium chloride, ammonium bicarbonate, potassium hydroxide, β-Nicotinamide adenine dinucleotide phosphate sodium salt (NADP), DL-isocitric acid trisodium salt hydrate, isocitric dehydrogenase, morphine sulfate salt pentahydrate, morphine-3-glucuronide, morphine-6-glucuronide, racemic methadone hydrochloride, and nitrazepam were purchased from Sigma-Aldrich. Buprenorphine,

norbuprenorphine, and EDDP were purchased from Cerilliant. Potassium phosphate

monobasic, hydrochloric acid, and HPLC grade acetonitrile and methanol were purchased

from Fisher Scientific. Alprazolam, α-OH alprazolam, and 4-OH alprazolam was gifted

by the Upjohn Company (Kalamazoo, MI), which is now a subsidiary of Pfizer. Stock

solutions of drugs were dissolved in either methanol or water, based on solubility data,

and stored in -20° or 4° C, respectively.

2.2. Liver Microsomes

HLM liver numbers 11-34 were purchased from Xenotech, LLC (Kansas City, KS) at a

concentration of 20 mg/mL. HLM numbers 1-10 were prepared from donor liver tissue as

described previously (von Moltke et al., 1993). Liver tissue, thawed or fresh, was kept at

4°C during preparation. Tissue was homogenized in 50 mM Tris buffer containing 0.1 M

potassium chloride and adjusted to pH 7.4 with hydrochloric acid at 25° C. Homogenates

were then spun in a temperature controlled centrifuge, 2-4° C at 10,000 g for 20 minutes.

The temperature control of the centrifuge is crucial to ensure enzyme activity is at a

minimum and will not degrade. Supernatants from this spin were spun again by

ultracentrifuge at 105,000 g for 60 minutes at 2-4°C. The resulting microsomal pellet was

suspended in 0.1 M potassium phosphate butter with 20% glycerol and stored at -80° C

until use. The HLM demographics can be found in Tables 2.1, 2.2, and 2.3.

Table 2.1. Fetal HLM

Lab ID	Liver ID	Sex	Age	Race	Alcohol	Smoker
35	242	M	20 weeks	ND	N/A	N/A
36	244	M	19 weeks	ND	N/A	N/A
37	235	M	21 weeks	ND	N/A	N/A
38	238	F	22 weeks	ND	N/A	N/A

Table 2.2. Adult HLM

Lab ID	Liver ID	Age	Sex	Race	Alcohol	Smoker
1	9408	35	F	C	no	N/A
2	9411	26	F	B	social	N/A
3	9503	46	F	B	no	N/A
4	9511	45	F	H	no	N/A
5	SH/5H	65	F	C	no	N/A
6	776	65	F	C	no	N/A
7	858	75	F	C	no	N/A
8	OD38344	66	F	C	N/A	N/A
9	103951	52	F	C	heavy	N/A
10	45976	64	F	C	N/A	N/A
11	H0158	36	F	B	yes	yes
12	H0177	45	F	C	no	yes
13	H0227	55	F	H	yes	yes
14	H0266	72	F	B	no	no
15	H0267	27	F	C	yes	no
16	H0288	30	F	B	yes	no
17	H0295	54	F	B	no	no
18	H0324	53	F	B	no	yes
19	H0028	20	F	C	no	no
20	H0090	47	F	C	no	yes
21	H0115	48	F	C	no	no
22	H0170	28	F	C	no	no
23	H0313	49	F	C	yes	yes
24	H0327	52	F	C	yes	yes
25	H0290	44	F	B	yes	no
26	H0355	71	F	C	no	no
27	H0285	49	F	C	N/A	N/A
28	866	21	M	C	N/A	N/A
29	Pooled	N/A	N/A	N/A	N/A	N/A

Table 2.3. Neonatal HLM

Lab ID	Liver ID	Sex	Age	Race	Alcohol	Smoker
28	H0238	M	3 mo	B	N/A	N/A
29	H0268	M	4 mo	C	N/A	N/A
30	H0354	F	13 days	C	N/A	N/A
31	H0395	M	5.5 mo	C	N/A	N/A

32	H0671	M	3 mo	H	N/A	N/A
33	H0825	M	11 mo	C	N/A	N/A
34	H0845	M	1 mo	C	N/A	N/A

2.3. Incubations

HLM were thawed on ice and all sample preparation was carried out on ice until the final

incubation was performed at 37°C. Cofactors NADP and isocitric acid were dissolved in

1.9 mL of 50 mM potassium phosphate buffer (pH= 7.4), 1.0 mL of 100 mM potassium

phosphate buffer (pH=7.4), 1.0 mL of 50 mM magnesium chloride, and 100 μL of

isocitric acid dehydrogenase for a total of 4 mL cofactor mix. The final incubation

volume was 250 μL for all samples. The final concentration of NADP and isocitric acid

was 0.5 mM and 5.2 mM, respectively. Linearity tests were done with protein (0-1.0

μg/μL), substrate (0-1500 μM ALP; 0-150 μM BUP; 0-1500 uM MET) and incubation

time (up to 60 minutes) to determine optimal conditions. Final HLM concentrations were

made to 0.25 μg/μL for ALP, 0.20 μg/μL for BUP, and 0.20 μg/μL for MET by dilution

with 50 mM potassium phosphate buffer (pH=7.4). Final substrate concentrations and

incubation times were; 1250 μM ALP 20 minutes, 35 μM BUP 25 minutes, and 50 μM

MET 15 minutes.

Each substrate (ALP, BUP and MET) was dried down separately in incubation tubes

from their respective stock solutions until all liquid was evaporated and only powdered

drug remained. Each individual HLM incubation was performed in duplicate, with two

incubation tubes of substrate per HLM. Variability within the duplicates did not exceed

15%. Each duplicate incubation set had its own master mix tube, in which 100 μL of

cofactor mix and 150 μL of diluted HLM was combined for each sample. This was then vortex mixed, and 250 μL was added into each incubation tube. This was done for each individual HLM. The incubation tubes were then placed in a 37°C water bath with shaking, to represent physiological temperature and movement. The tubes were removed after 20 minutes for ALP, 25 minutes for BUP, and 15 minutes for MET. The reaction was stopped by the addition of 100 μL of stop solution, consisting of ice cold acetonitrile and an internal standard (IS) (4.3 μM nitrazepam for ALP, 3.1 μM morphine for BUP and 5 μM midazolam for MET per sample) and placing the samples on ice. Acetonitrile is used as the stopping solvent to produce protein precipitation, and the ice reduces temperature so that enzyme activity is stopped. Samples stayed on ice for 2-3 minutes, then were spun down by centrifugation for at least 10 minutes at 14,000 rpm. After centrifugation, the supernatant was removed, the pellet left undisturbed, and transferred into HPLC vials for injection. Control studies were performed, leaving out either cofactor, substrate, or HLM.

2.4. Alprazolam, Buprenorphine, and Methadone HPLC Method

All methods were run on an Agilent Technologies 1200 series HPLC system, coupled with a quaternary pump, a degasser, sample auto-injector, and variable wavelength detector (VWD) with a deuterium lamp. The mobile phase for all substrates were filtered through a Millipore vacuum filtration apparatus with a 0.2 μm, 47 mm pre-sterilized nylon-66 membrane disc filter. Mobile phases were made by first dissolving necessary salts in distilled water, filtered, organic phase added, and allowed to stir for at least 20 minutes before fixing the pH with hydrochloric acid, phosphoric acid (methadone) or

potassium hydroxide. Methods were developed from previous publications, with modifications (Chang-Liang Liao, 2008; Gerber et al., 2004; von Moltke et al., 1993).

For ALP, the mobile phase was an isocratic solution of 50 mM potassium phosphate buffer with acetonitrile at a ratio of 65:35. The mobile phase was fixed to pH 6.0 and was delivered through the system at a flow rate of 1.2 mL/min. A Waters Nova-Pak C18 4 μm, 3.9 x 150 mm, silica packed column was used for separation and the VWD was set to 214 nm. Injection volume was 10 μL and run time was 7 minutes for each sample.

For BUP, an isocratic mobile phase of 63 mM ammonium bicarbonate with acetonitrile at a ratio of 30:70 was delivered through the system at a flow rate of 0.8 ml/min. The pH was fixed to 8.8. An Agilent ZORBAX Eclipse XDB-C18, 5 μm, 4.6 x 150 mm column was used. This column was used for its ability to withstand a broad pH range. The VWD was set to 220 nm, injection volume was 10 μL and run time was 21 minutes for each sample.

For MET, the mobile phase was an isocratic solution of distilled water and acetonitrile (65:35) with 1% trimethylamine and fixed to pH 2.8 with phosphoric acid. It was delivered through the system at a flow rate of 1.0 mL/min. The Agilent ZORBAX Eclipse XDB-C18, 5 μm, 4.6 x 150 mm column was used with detection was set to 210 nm. Injection volume was 15 μL and run time was 7 minutes for each sample.

2.5. IC50 Determination

Inhibitory studies were performed to determine if morphine (MOR) had an effect on the formation of the alprazolam metabolite, 4-OHALP, and whether any inhibitory effect was time-dependent. First, an initial IC50 determination was performed. Varied concentrations of morphine (0 – 1500 μM) were added to 1250 μM ALP and vacuum dried. Control samples did not have morphine added. Ketoconazole (0.075 μM) was added to ALP as a positive control for IC50 determination, based on previous publications (Greenblatt et al., 2011). The assay was performed following the conditions for ALP.

To compare the inhibitory potential between pre- and no pre-incubation, two sets of experiments were done simultaneously. For the no pre-incubation set, incubations were performed as described above. For the pre-incubation set, ALP and morphine were dried down separately. The set containing morphine was incubated with HLM and cofactor mix for 20 minutes at 37 °C with shaking. It was then transferred into the vials with dried ALP and returned to the incubator for another 20 minutes. The rest of the assay was performed following the conditions for ALP.

The purpose of evaluating pre- and no pre-incubation conditions is to determine if the inhibition by morphine is time-dependent. Time-dependent (mechanism-based) inhibitors bind slowly to the enzyme and cause a slowed onset of inhibition. There are cases where this mechanism-dependent inhibition can be reversible or irreversible. Irreversible inhibitors have slow dissociation constants from the enzyme, independent of substrate

presence, leading to slower recovery of enzymatic activity and prolonged inhibition (Strelow et al., 2004). There are also inhibitors that form a covalent bond with the active site of the inhibitor, destroying enzymatic activity. In many cases, this covalent bond is reversible and enzyme activity can return (Silverman, 1995). Ultimately, if morphine is a time-dependent inhibitor of CYP3A, we would see an increase in inhibition evident as reduced formation of the alprazolam metabolite, in the pre-incubation condition compared to the no pre-incubation condition.

2.6. Metabolite Quantification, Equations and Statistics

Agilent ChemStation was the software program coupled with the HPLC and used for instrument control, data acquisition, data processing, data analysis and reporting. The metabolite of quantification for each substrate, 4-OHALP for ALP and NBUP for BUP, were integrated by setting the area of integration to the baseline of either side of the peak. The same was done for the internal standard. The samples were then quantified, calculating the area and height of the metabolite and internal standard previously integrated. After report output, metabolite formation was determined by the ratio of metabolite peak height divided by internal standard peak height. Intra- and inter-variability between samples was expressed as a percent of variation and calculated using the following equation (1):

$$C_v = \frac{|C_1 - C_2|}{\frac{C_1 + C_2}{2}} * 100$$

C_v was less than 15% for all data sets.

For IC50 calculations with fixed substrate (1250 μM ALP) and variable inhibitor concentrations (0-1500 μM morphine), reaction velocities with the addition of inhibitor were expressed as a percentage ratio compared to reaction velocities with no inhibitor (R_v). The relationship of the inhibitor concentration to R_v was determined by nonlinear regression using GraphPad Prism 7.0 and the following equation (2):

$$R_v = 100\left(1 - \frac{E_{max} * C^b}{C^b + IC^b}\right)$$

E_{max} represents the maximum inhibition; C, inhibitor concentration; IC, inhibitor concentration when $R_v = 50\%$ of $(100-E_{max})$; and b, an exponent. The E_{max}, IC and b values given after data input into GraphPad Prism were used to calculate the actual IC_{50} using the following equation (3):

$$IC50 = \frac{IC}{(2E_{max} - 1)^{1/b}}$$

Profiling of metabolite formation for BUP and MET with variable substrate concentrations (0-150 μM and 0-1500 μM, respectively) and no inhibitor, was performed on GraphPad Prism using the Michaelis-Menten equation (4):

$$V = \frac{V_{max}[S]}{[S] + K_m}$$

V_{max} represents the maximum velocity of metabolite formation; S is the varying substrate concentration; and K_m, the substrate concentration when V_{max} is 50%. For ALP, kinetics followed a sigmoidal curve, needing to be evaluated by the Hill equation (5):

$$V = \frac{V_{max}[S]^\alpha}{S_{50}{}^\alpha + [S]^\alpha}$$

S_{50} represents the substrate concentration resulting in 50% of V_{max}; α represents the Hill coefficient. This is a two-substrate model that when substrate concentration (S) equals S_{50} there is a mixed population of one molecule (ES) or two molecules (ESS) bound to the enzyme. When $S < S_{50}$, ES predominates over ESS (Stresser et al., 2000).

Two-tailed independent t-tests were executed on Microsoft Excel to test for significance. Any data presented as significant has a p-value of 0.05 or lower. Multiple linear regression analysis to determine the relation between metabolite formation and age, CYP content, alcohol or cigarette use was performed on Sigma Plot 13.0. XY plot for metabolite formation versus CYP content was also generated on Sigma Plot 13.0.

Chapter 3. Results

Results include metabolite formation differences between ALP, BUP and MET in adult
female and neonate livers *in vitro* and inhibitory data when ALP is used as a substrate
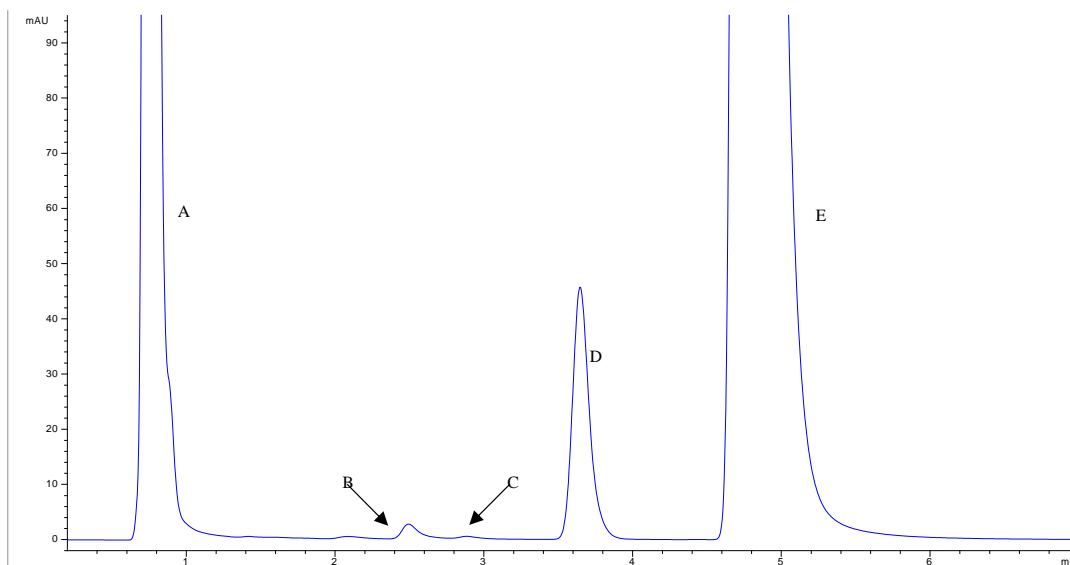and morphine as an inhibitor. Metabolite, substrate, and IS chromatogram peaks for ALP

Figure 3.1. ALP incubation chromatogram. Incubation with HLM pooled, peaks
represented: A) cofactor and HLM; B) 4-OHALP; C) α-OHALP; D) IS; E)
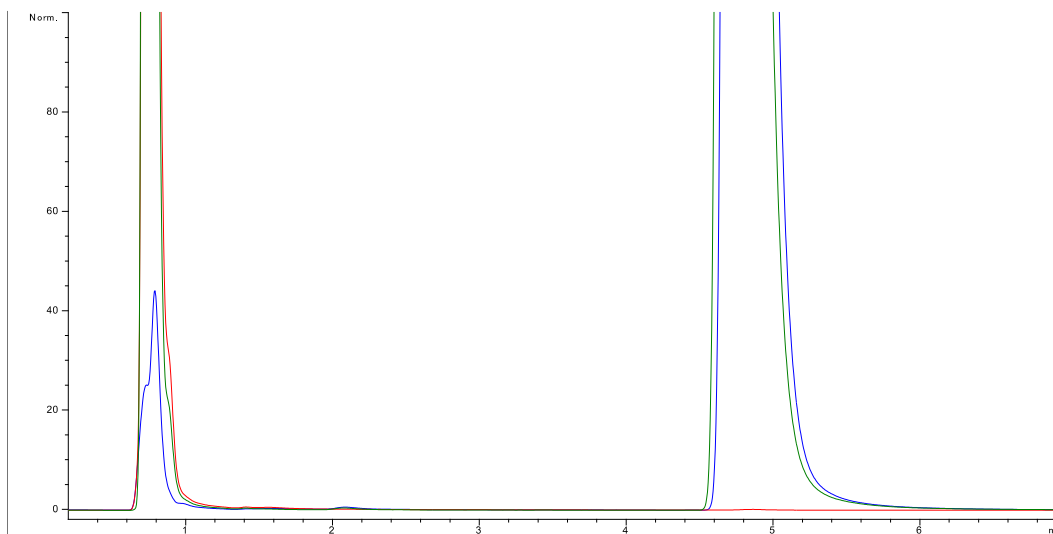ALP. Y axis is RAU and X axis is time.

Figure 3.2. ALP control chromatograms overlaid; Red=HLM + cofactors;
Green=cofactors + ALP; Blue= HLM + ALP.

Figure 3.1 and 3.2), BUP (Figure 3.3 and 3.4) and MET (Figure 3.5. and 3.6). Incubations were confirmed with pure compound standards and control incubations.
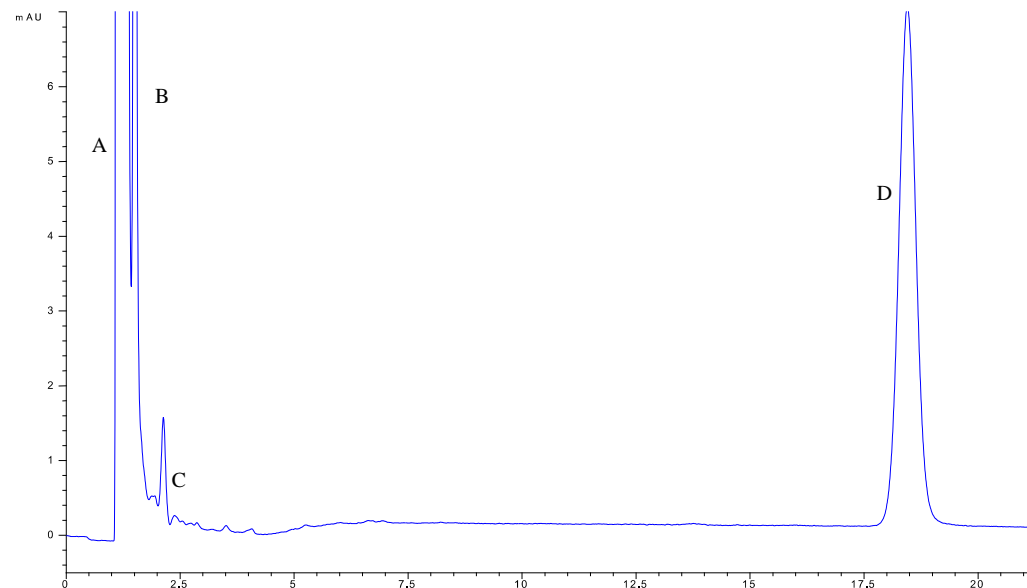
Figure 3.3. BUP incubation chromatogram. Incubation with HLM0266, peaks represented: A) cofactor and HLM; B) IS; C) NBUP; D) BUP. Y axis is RAU and X axis is time.

Figure 3.4. BUP control chromatograms overlaid; Red=HLM + BUP; Green=cofactors + BUP; Blue= HLM + CF.
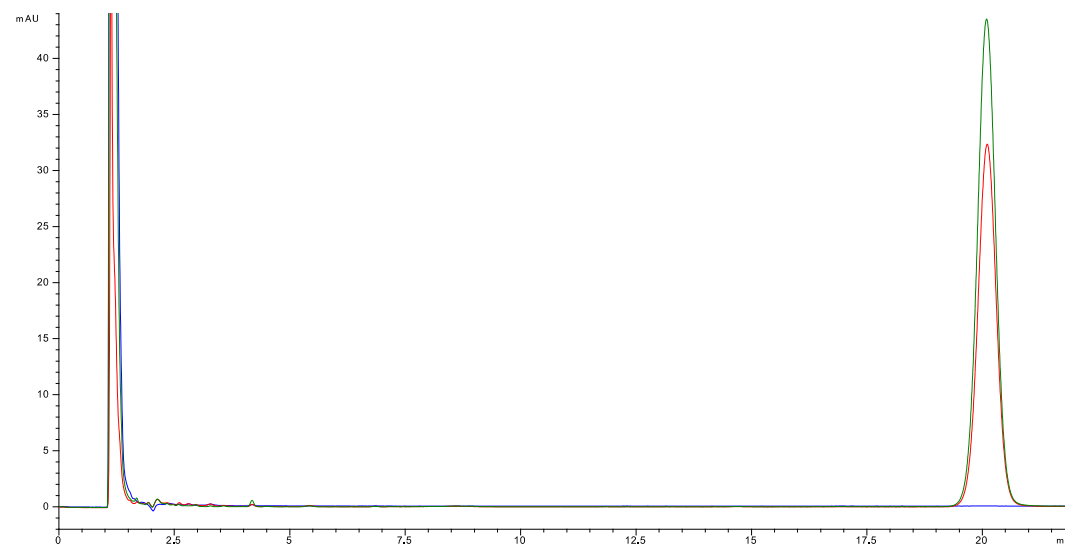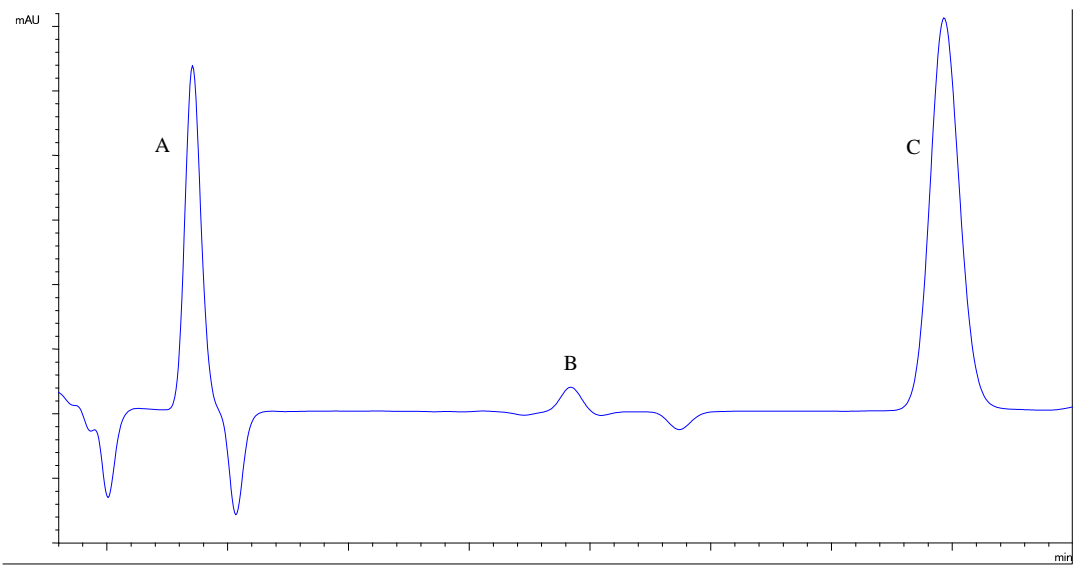
Figure 3.5. MET incubation chromatogram. Incubation with HLM 866, peaks represented: A) IS; B) EDDP; C) Methadone. Y axis is RAU and X axis is time.
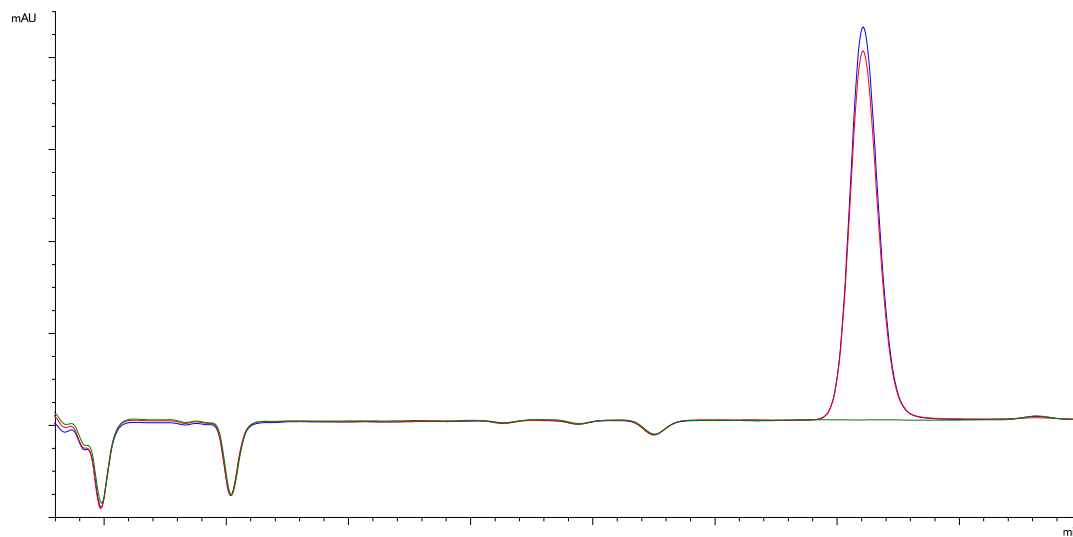

Figure 3.6. MET control chromatograms overlaid; Red=MET + HLM; Green=HLM+CF ; Blue= MET + HLM.

3.1. Determination of incubation conditions

Based on previous literature and methods for ALP metabolism, 1000µM was used as a starting concentration to determine microsomal concentration (Figure 3.7). Incubation time was confirmed from previous studies (Greenblatt et al., 1993). Based on the results, we determined a HLM concentration 0.25 µg/µL would produce a sufficient metabolite formation rate. After determining this, we performed a concentration assay to determine the optimal ALP concentration for each subsequent study (Figure 3.8). ALP showed kinetics similar to previous studies, showing sigmoidal kinetics. This required data to be fit to the Hill equation (5). Differences in V_{max} are due to variability within each HLM, most likely CYP3A activity and content. The α-value of greater than 1 shows a positive correlation between the binding of one molecule of ALP and subsequent molecules.
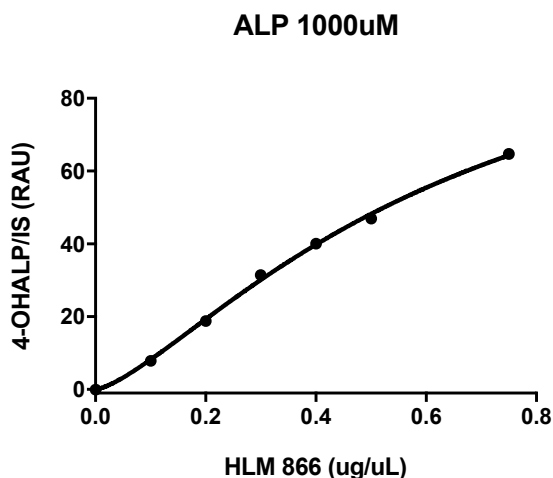
ALP 1000uM

Figure 3.7. Relation of HLM protein concentration to metabolite formation rate. Representative of HLM 866 using fixed ALP concentration (1000µM) and varied HLM concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.75 µg/µL). Each data point was performed in duplicate, normalized to IS (nitrazepam) and averaged. Hill equation used for analysis.
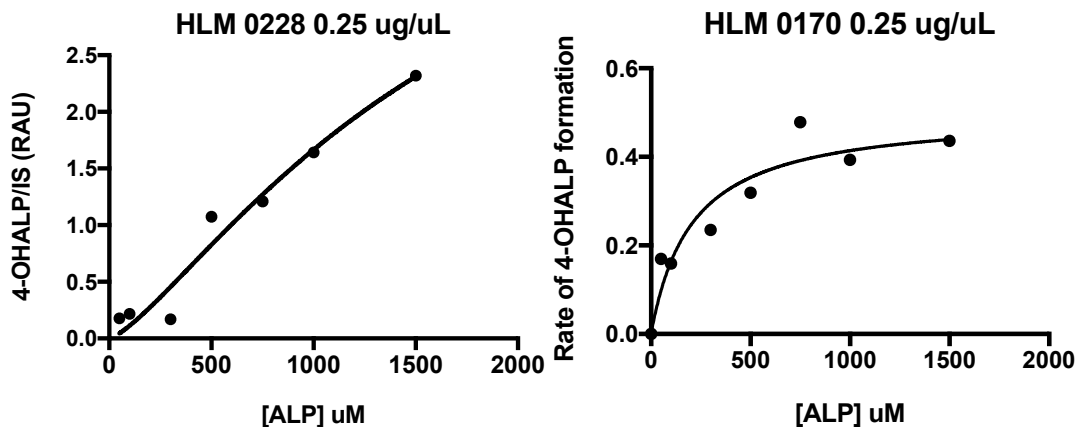
Figure 3.8. ALP concentration determination. Fixed concentration of HLM. A) HLM 0228; V_{max}=5.038, K_m=1698, α=1.333, r^2= 0.96. B) HLM 0170; V_{max}=0.499, K_m=207.1, α=1.0, r^2=0.91. Each data point was performed in duplicate, normalized to IS (nitrazepam) and averaged.

Taking into account the results from ALP HLM concentration determination and previous literature, a similar HLM concentration (0.20 μg/μL) was used to determine initial BUP concentration and incubation time (Figure 3.9). Since CYP3A is responsible for 80-90% of BUP biotransformation, we expected optimal NBUP formation rates at a similar incubation time as ALP. From this data, we used a 25 minute incubation time and a concentration of 35 μM BUP.

We then determined initial HLM concentration, substrate concentration, and incubation time for MET (Figure 3.10). Both incubation time and HLM concentration relative to a fixed concentration of MET (50 μM) were linear. Metabolite formation rate with 0.20 μg/μL HLM and a 15 minute incubation was satisfactory to perform the following experiments.

27

A)

10 min incubation

20 min incubation

30 min incubation

40 min incubation

60 min incubation

B)

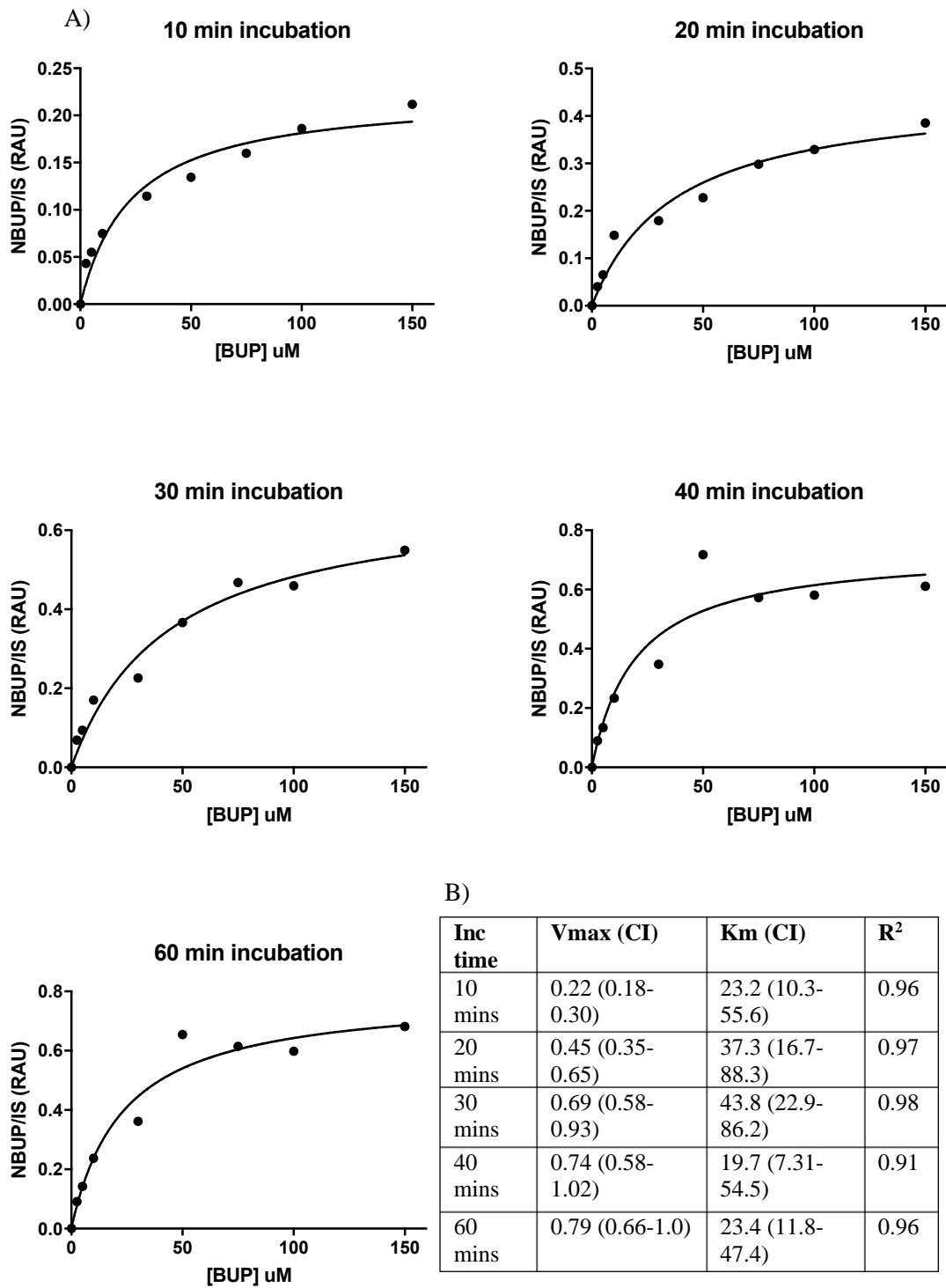| Inc time	Vmax (CI)	Km (CI)	R^2
10 mins	0.22 (0.18-0.30)	23.2 (10.3-55.6)	0.96
20 mins	0.45 (0.35-0.65)	37.3 (16.7-88.3)	0.97
30 mins	0.69 (0.58-0.93)	43.8 (22.9-86.2)	0.98
40 mins	0.74 (0.58-1.02)	19.7 (7.31-54.5)	0.91
60 mins	0.79 (0.66-1.0)	23.4 (11.8-47.4)	0.96

Figure 3.9. BUP concentration and incubation time curves. A) Incubations for all time points were performed with HLM pooled in duplicate, normalized to IS and averaged. B) Michaelis-Menten parameter values, confidence interval (CI) set to 95%.

Using these conditions, the MET kinetics were analyzed using increasing concentrations (0-1500 μM). Similar to previous literature, MET followed hyperbolic kinetics and was fit to the Michaelis-Menten equation (Figure 3.11). Incubations with adult female and neonatal HLM were carried out with 50 μM MET, 0.20 μg/μL HLM and a 15 minute incubation.

A)
Methadone 50 uM with HLM 866

B) **Methadone 50 uM with HLM 866 0.20 ug/uL**

Y = 0.3092*X + 0

Y = 0.003983*X + 0
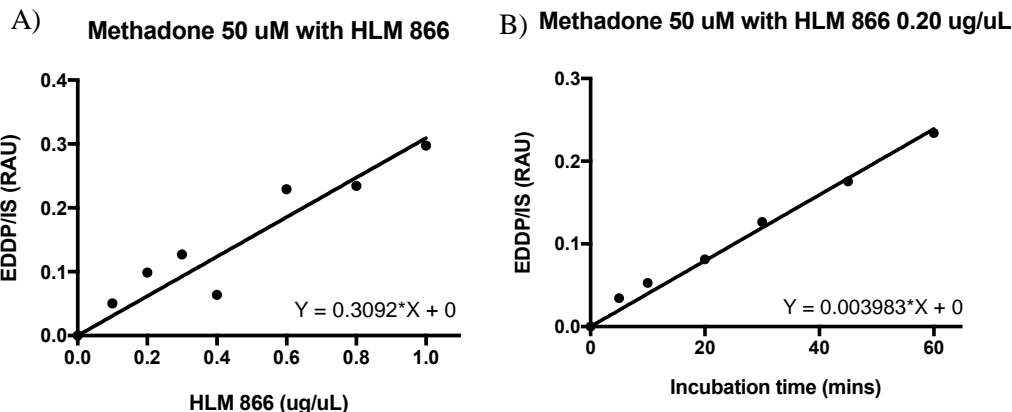
Figure 3.10. Methadone initial incubation conditions with HLM 866. A) Using fixed concentration of 50 μM MET with different HLM concentrations (0, 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0 μg/μL). B) Using fixed concentration of 50 μM MET and 0.20 μg/μL with varied incubation times (up to 60 minutes). Each data point was performed in duplicate, normalized to the IS (midazolam) and averaged.

EDDP formation rate with HLM 866
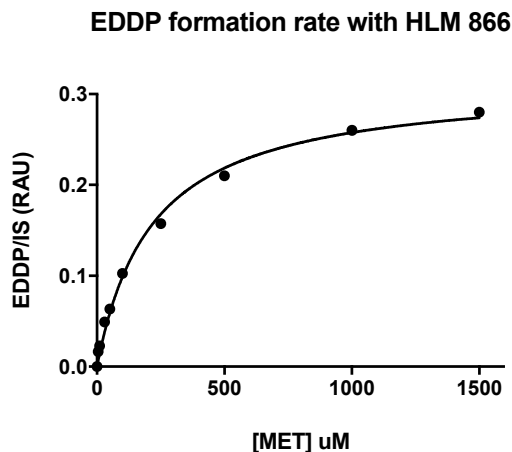
Figure 3.11 Methadone concentration determination. MET concentrations (0, 5, 10, 30, 50, 100, 250, 500, 1000, 1500 μM) with 0.20 μg/μL HLM 866 with 15 minute incubation. V_{max}=0.31; K_m= 217.7; R^2= 0.99. Each data point was performed in duplicate, normalized to the IS and averaged.

3.2. Metabolite formation

The specific concentrations of HLM and substrate described above for ALP, BUP, and MET individually, were applied to each individual adult female HLM and neonatal HLM to determine metabolite formation rate.

When under identical incubation conditions described in the methods section for 4-OHALP, NBUP and EDDP, the metabolite formation of each was quantified for adult female and neonate liver microsomes *in vitro*. We showed that at a fixed concentration of

A)

Female Adult 4-OHALP Formation

B)

Neonatal 4-OHALP Formation

Figure 3.12. 4-OHALP formation as a measurement of RAU for individual HLMs. A) Female adult HLM, n=26 and B) neonate HLM, n=7. All samples were performed in duplicate, normalized to the IS (nitrazepam) and averaged to get an individual value. Dotted line represents the average value. ALP=1250 µM, nitrazepam=3.1 µM.

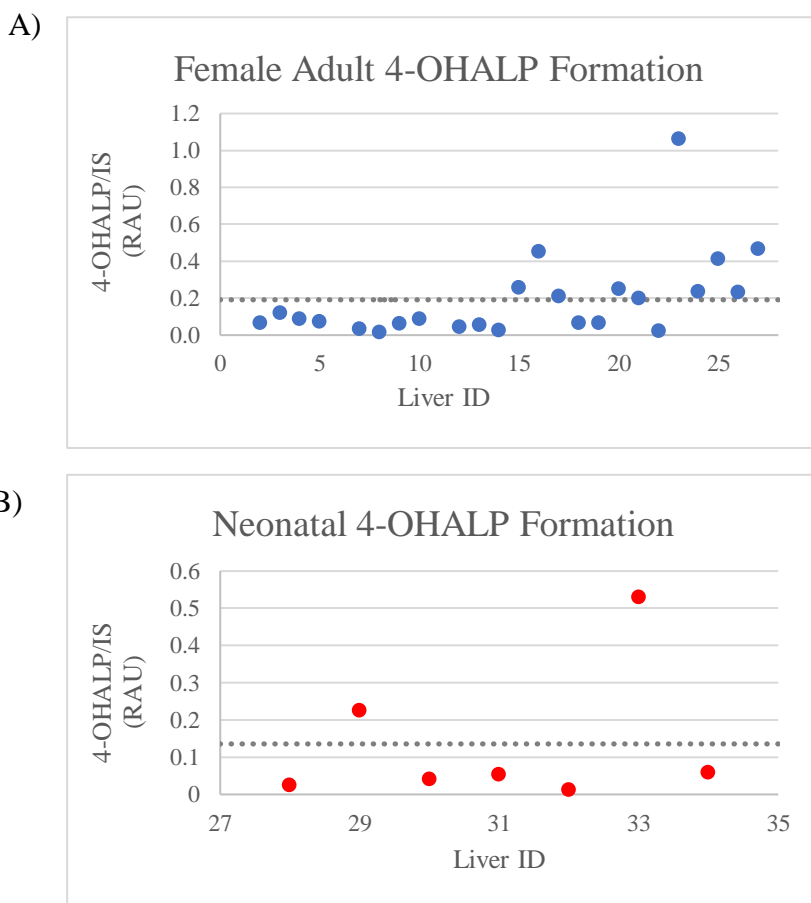ALP, formation of 4-OHALP in adult female HLM had a maximum of 1.063 RAU and minimum of .021 RAU. The maximum value however is over 2.2 times that of the second highest, 0.465 RAU, which is more relevant based on these findings (Figure 3.12). The arithmetic mean of 4-OHALP/IS from female adult HLM was 0.191 RAU and geometric mean was 0.110. Maximum 4-OHALP formation from neonate HLM was 0.530 RAU and minimum was 0.013 RAU with a total average of 0.136 RAU and geometric mean of 0.066 (Figure 3.12). The range of neonate metabolite formation fell within that of adult female suggesting no difference. This was confirmed by an independent t-test p-value of 0.564.

Using identical incubation conditions for each liver, NBUP metabolite formation was quantified and normalized to the IS, morphine (Figure 3.13). For female adult HLM, maximum formation of NBUP was 0.583 RAU with a minimum of 0.032 RAU. The arithmetic mean was 0.154 and geometric mean was 0.121. For neonatal HLM, maximum NBUP formation was 0.247 RAU and minimum was 0.051 RAU, with an average of 0.117 and geometric mean of 0.099. Similar to ALP metabolite formation, the range of NBUP produced by neonatal HLM was within the range of female adult HLM. A paired t-test confirmed this with a p-value of 0.460.

Figure 3.13. NBUP formation as a measurement of RAU for individual HLMs. A) Female adult HLM, n=26 and B) neonate HLM, n=7. All samples were performed in duplicate, normalized to the IS (morphine) and averaged to get an individual value. Dotted line represents the average value. BUP=35 μM, morphine=4.3 μM per sample.

Similarly, with incubations conditions as described above, EDDP formation rate was quantified from its parent compound, MET. EDDP metabolite height was normalized to the IS, midazolam (Figure 3.14). For neonatal HLM, maximum formation rate of EDDP was 0.080 RAU with a minimum of 0.051 RAU. The average of the seven HLMs was 0.024 and the geometric mean was 0.017 RAU. Similar to 4-OHALP and NBUP formation, liver ID #29 and #33 produced EDDP at a higher rate. This is probably due to higher CYP content compared to the other neonatal livers. Adult female EDDP formation

was similar to neonatal, with a maximum formation rate of 0.122 RAU, minimum of 0.003 RAU. The average formation rate of the 23 HLMs was 0.024 RAU and geometric mean was 0.014 RAU. Again, no difference in EDDP formation between adult female and neonates was seen, with a p-value of 0.969. Interestingly, liver ID #8 produced no EDDP, suggesting little to no activity of CYP2B6.
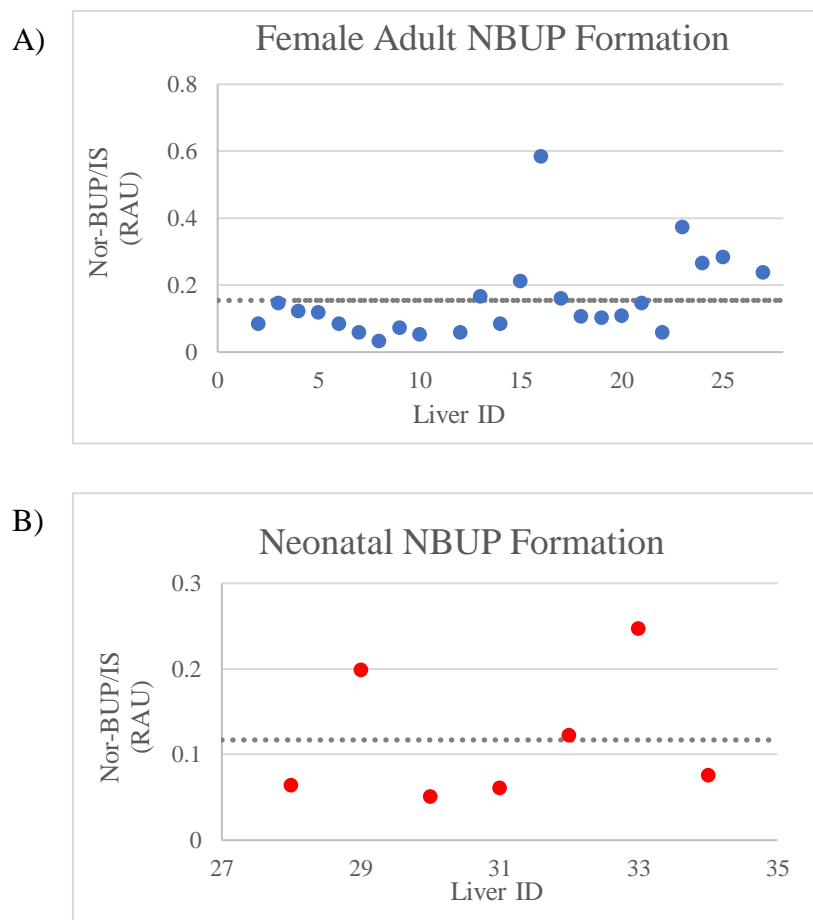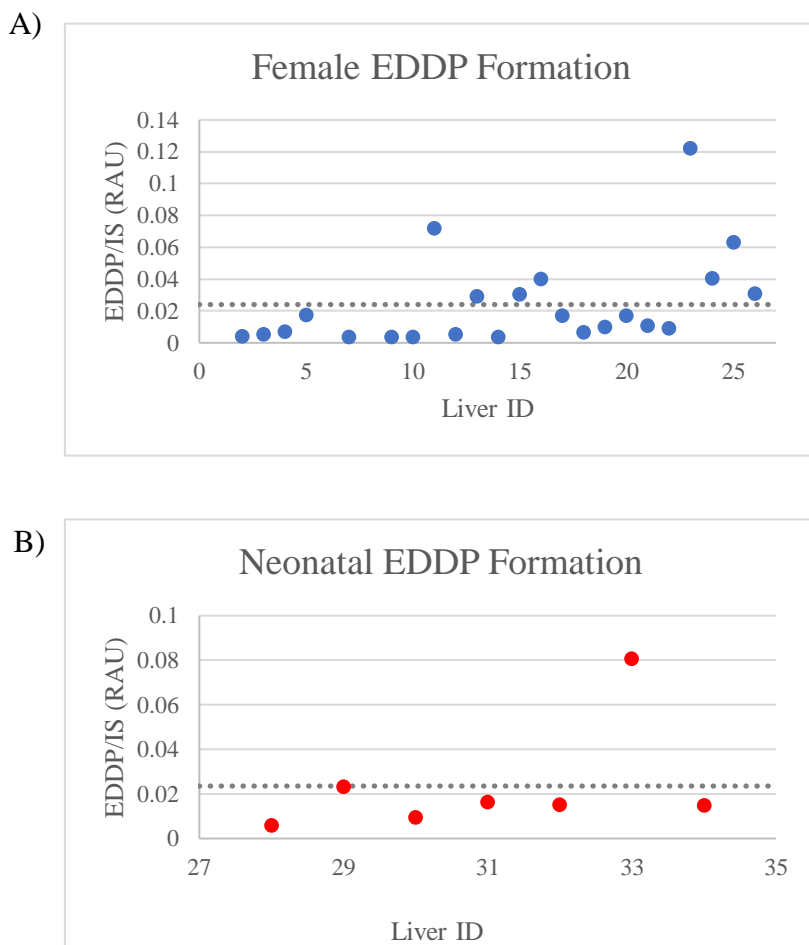

Figure 3.14. EDDP formation as a measurement of RAU for individual HLMs. A) Female adult HLM, n=24 and B) neonate HLM, n=7. All samples were performed in duplicate, normalized to the IS (methadone) and averaged to get an individual value. Dotted line represents the average value. MET= 50 μM, midazolam=5 μM per sample.

3.3. Variability of metabolite formation

Even with a fixed concentration of HLM, figures 3.12, 3.13, and 3.14 show great variability in metabolite formation, which coincides with previous literature on individual variability in CYP activity. With the profiled HLMs, we looked at age, CYP content, cigarette and alcohol use as factors that could contribute to differences in metabolite formation. Since race cannot be set to a quantifiable variable, it was excluded from this analysis. Multiple linear regression analysis was performed for the HLM's that had most variability, liver #'s 12-35, for AP, BUP and MET (Table 3.1, 3.2 and 3.3). Separate analyses were performed for adult human HLM and neonate HLM. The p-values for each variable in 4-OHALP formation (Table 3.1B and 3.1D) are shown under their respective tables. The analysis yeilded an $R^2=0.64$ for adult female 4-OHALP formation and $R^2=0.951$ for neonatal 4-OHALP formation.

A)

Liver #	4-OHALP/IS (RAU)	Age (yrs)	CYP content (nmol/mg of protein)	Alcohol	Smoker
27	0.465	49	0.255	N/A	N/A
12	0.043	45	0.455	no	yes
13	0.055	55	0.225	yes	yes
14	0.025	72	0.229	no	no
15	0.257	27	0.453	yes	no
16	0.453	30	0.727	yes	no
17	0.209	54	0.356	no	no
18	0.065	53	0.453	no	yes
19	0.067	20	0.327	no	no
20	0.248	47	0.288	no	yes
21	0.200	48	0.222	no	no
22	0.021	28	0.236	no	no
23	1.064	49	0.872	yes	yes
24	0.236	52	0.587	yes	yes
25	0.411	44	0.444	yes	no
26	0.233	71	0.604	no	no

B)

	Age (yrs)	CYP content	Alcohol	Smoker
P-value	0.91	0.012	0.369	0.865

C)

Liver #	4-OHALP/IS (RAU)	Age (days)	CYP content (nmol/mg of protein)
28	0.026	93	0.26
29	0.226	124	0.483
30	0.041	13	N/A
31	0.055	170.5	N/A
32	0.013	93	0.352
33	0.530	341	0.486
34	0.059	31	0.344

D)

	Age (days)	CYP content
P-value	0.076	0.233

Table 3.1. 4-OHALP multiple linear regression analysis. A) Female adult HLM; B) generated p-values; C) neonatal HLM; D) generated p-values. 4-OHALP/IS (RAU) was set as the dependent (Y) variable and age, CYP content (nmol/mg of protein), alcohol and smoker set as each individual X variables.

From these results, CYP content shows to be the most significant in predicting 4-OHALP

metabolite formation in adults, but not neonates. Plots showing this relationship for

neonatal and adult female HLM are below (Figure 3.14).
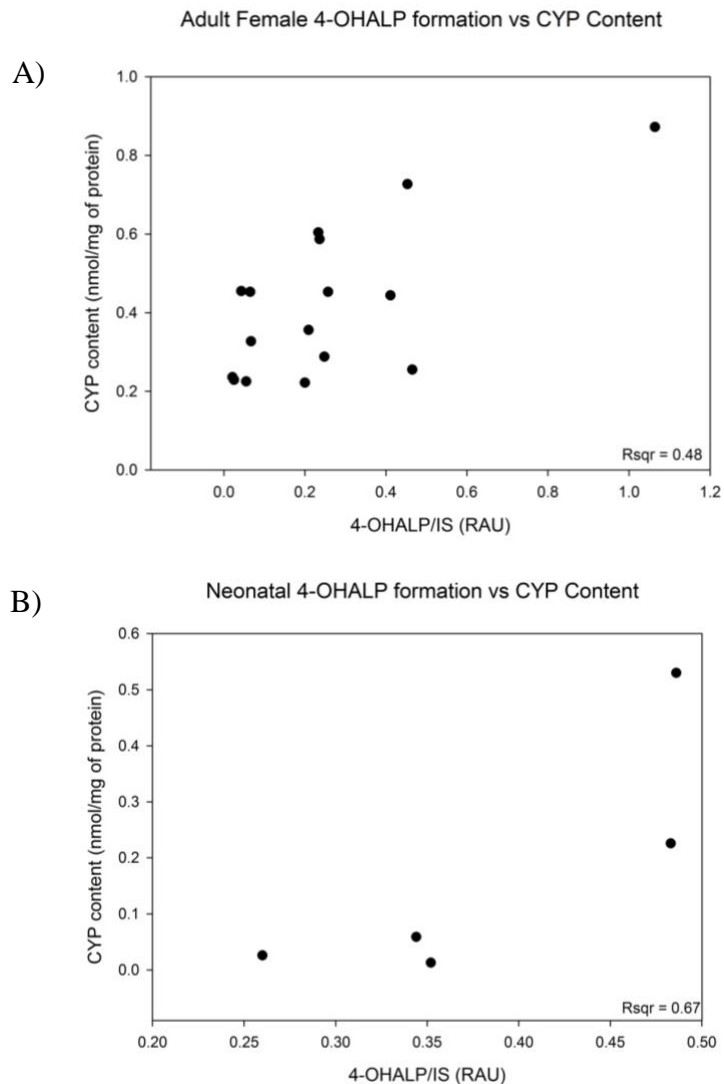
Figure 3.15. Relationship of 4-OHALP formation rate to CYP content. A) adult female HLM with an r^2=0.48; B) neonatal HLM with an r^2=0.67.

The same analyses were done with NBUP formation in adult female and neonatal HLM (Table 3.2.). The p-values for each variable in NBUP formation (Table 3.2B and 3.2D) are shown under their respective tables. The analysis yeilded an R^2=0.82 for adult female NBUP formation and R^2=0.98 for neonatal NBUP formation.

A)

Liver #	NBUP/IS (RAU)	Age (yrs)	CYP content (nmol/mg of protein)	Alcohol	Smoker
27	0.237	49	0.255	N/A	N/A
12	0.059	45	0.455	no	yes
13	0.166	55	0.225	yes	yes
14	0.083	72	0.229	no	no
15	0.211	27	0.453	yes	no
16	0.583	30	0.727	yes	no
17	0.159	54	0.356	no	no
18	0.106	53	0.453	no	yes
19	0.102	20	0.327	no	no
20	0.108	47	0.288	no	yes
21	0.146	48	0.222	no	no
22	0.058	28	0.236	no	no
23	0.372	49	0.872	yes	yes
24	0.266	52	0.587	yes	yes
25	0.283	44	0.444	yes	no

B)

	Age (yrs)	CYP content	Alcohol	Smoker
P-value	0.692	0.009	0.043	0.072

C)

Liver #	NBUP/IS (RAU)	Age (days)	CYP content (nmol/mg of protein)
28	0.064	93	0.26
29	0.199	124	0.483
30	0.051	13	N/A
31	0.061	170.5	N/A
32	0.123	93	0.352
33	0.247	341	0.486
34	0.076	31	0.344

D)

	Age (days)	CYP content
P-value	0.083	0.03

Table 3.2. NBUP multiple linear regression analysis. A) Female adult HLM; B) generated p-values; C) neonatal HLM; D) generated p-values. NBUP/IS (RAU) was set as the dependent (Y) variable and age, CYP content (nmol/mg of protein), alcohol and smoker set as each individual X variables.

With NBUP formation, we saw CYP content was the most significant variable in predicting metabolite formation with both adult female and neonatal HLM. Plots showing this relationship are below (Figure 3.16).

A)

B)

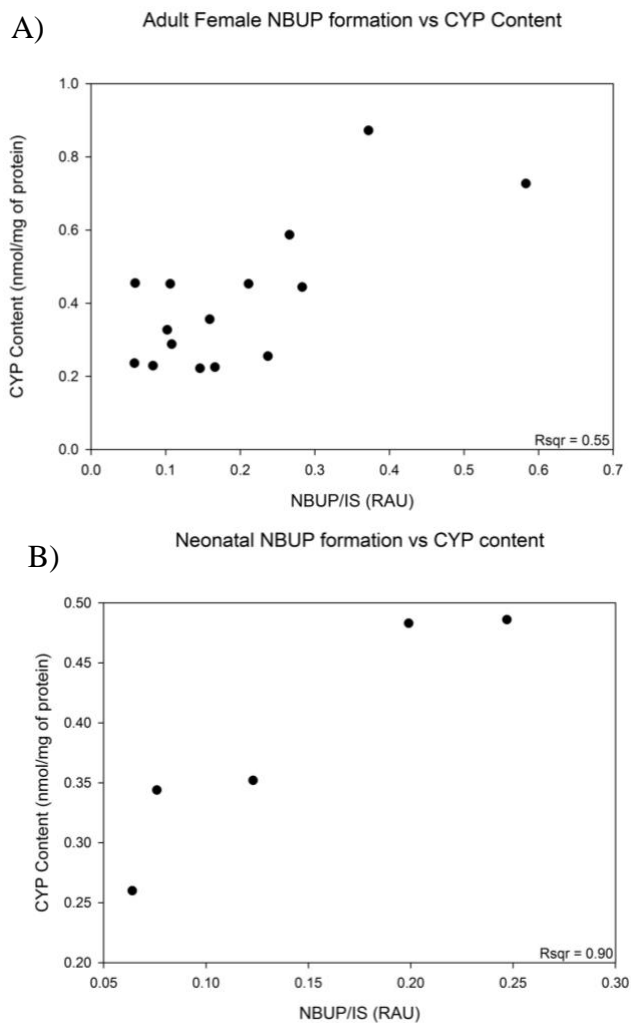Figure 3.16. Relationship of NBUP formation rate to CYP content. A) adult female HLM with an r^2=0.55; B) neonatal HLM with an r^2=0.90.

Multiple linear regression analysis was repeated again for variability in EDDP formation rate (Table 3.3). Adult female EDDP formation resulted in an R^2 =0.704 and neonatal with R^2=0.933. CYP content was not a significant predictor of metabolite formation rate

in neonates but was significant in adult female HLM (p=0.034). Similar to BUP results, alcohol use in adult females showed to be a significant predictor of EDDP formation (Table 3.3B).

A)

Liver #	EDDP/IS (RAU)	Age (yrs)	CYP content (nmol/mg of protein)	Alcohol	Smoker
11	0.072	36	0.475	yes	yes
12	0.005	45	0.455	no	yes
13	0.029	55	0.225	yes	yes
14	0.004	72	0.229	no	no
15	0.031	27	0.453	yes	no
16	0.040	30	0.727	yes	no
17	0.017	54	0.356	no	no
18	0.007	53	0.453	no	yes
19	0.010	20	0.327	no	no
20	0.017	47	0.288	no	yes
21	0.011	48	0.222	no	no
22	0.009	28	0.236	no	no
23	0.122	49	0.872	yes	yes
24	0.041	52	0.587	yes	yes
25	0.063	44	0.444	yes	no
26	0.031	71	0.604	no	no

B)

	Age (yrs)	CYP content	Alcohol	Smoker
P-value	0.733	0.034	0.034	0.707

C)

Liver #	EDDP/IS (RAU)	Age (days)	CYP content (nmol/mg of protein)
28	0.006	93	0.26
29	0.023	124	0.483
30	0.009	13	N/A
31	0.016	170.5	N/A
32	0.015	93	0.352
33	0.08	341	0.486
34	0.015	31	0.344

D)	Age (days)	CYP content
P-value	0.074	0.542

Table 3.3. EDDP multiple linear regression analysis. A) Female adult HLM; B) generated p-values; C) neonatal HLM; D) generated p-values. EDDP/IS (RAU) was set as the dependent (Y) variable and age, CYP content (nmol/mg of protein), alcohol and smoker set as each individual X variables.

CYP content versus EDDP formation rate was plotted for both adult female and neonates to visualize this relationship (Figure 3.17).
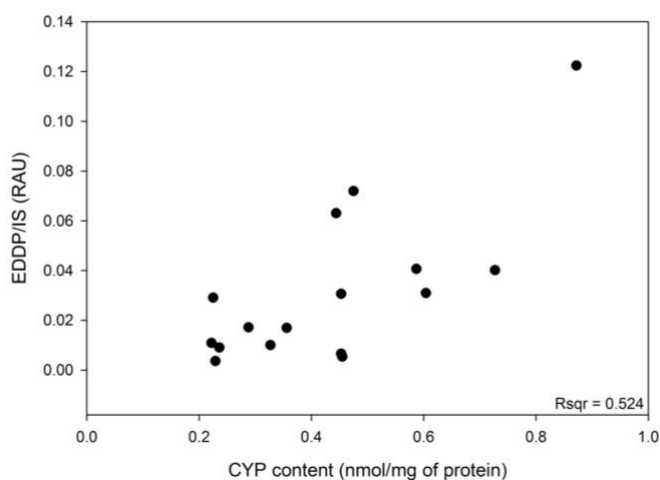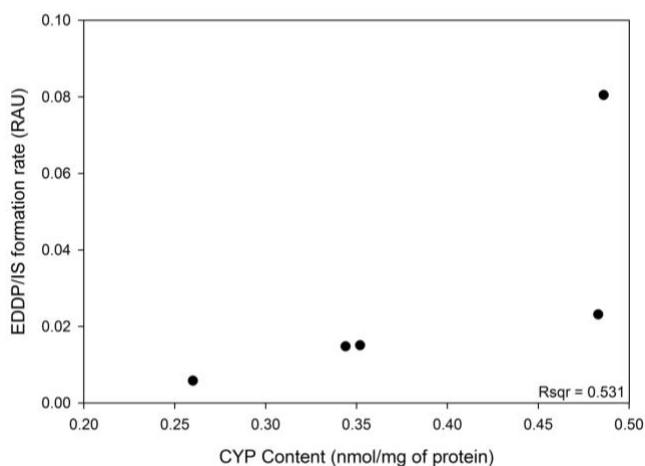

Figure 3.17. Relationship of EDDP formation rate to CYP content. A) adult female HLM with an r^2=0.52; B) neonatal HLM with an r^2=0.53.

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3.4. 4-OHALP inhibition by Morphine

Based on recent literature of benzodiazepines and opioids being taken together and the potential effects, we investigated possible inhibition of alprazolam metabolism by morphine. We evaluated time-dependent inhibition to determine if the inhibition, if any at all, would depend on pre-incubation of HLM with the inhibitor. We found that without pre-incubation, there was no inhibition of 4-OHALP. However, with a 20 minute pre-incubation of HLM and morphine prior to the addition of ALP, there was a clear inhibition of 4-OHALP at high morphine concentrations (Figure 3.18). After seeing inhibition by analyzing female adult HLM 0290, we looked at neonatal HLM 0285 and saw a similar response. For HLM 0290, the IC50 of 1635 μM translates to about 466.5 μg/mL and for HLM 0825, 1193 μM is equivalent to about 340.4 μg/mL. These concentrations are greater than 100x the plasma concentration of an average 30 mg controlled-release dose of morphine, 2254-2596 ng/mL. This inhibition is therefore not likely to be clinically relevant.
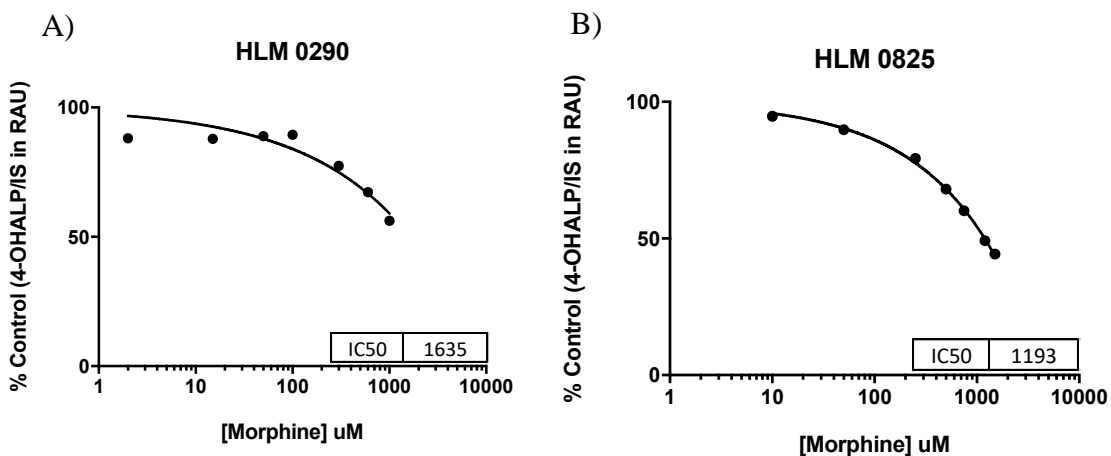

Figure 3.18. ALP inhibition by morphine. A) Adult female HLM 0290 and B) neonatal HLM 0825 using incubation conditions described in methods. IC50 recorded in μM.

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Chapter 4. Discussion

The purpose of this study was to evaluate the *in vitro* metabolite formation of opioid treatment medication, and drugs commonly co-administered by treatment patients, in adult female, neonate, and fetal livers. By doing this we evaluate the possible role of differences in metabolic phenotype as predictors of neonatal abstinence syndrome. To do this, we first confirmed the metabolite formation and retention times by using HPLC for alprazolam, buprenorphine and methadone. When looking at *in vitro* metabolite formation of these drugs, only female liver microsomes were used since NAS is the result of maternal opioid drug and treatment medication use. We saw no significant difference in 4-OHALP metabolite formation rates between adult female and neonates, agreeing with previous studies that ALP metabolism is not dependent on age when studied in mice (Charpentier et al., 1997). However clinical use has a reduction in clearance of alprazolam in the elderly (Greenblatt and Wright, 1993). Neonatal CYP3A4 expression varies greatly, requiring up to 6 months to reach the activity similar to adults (Tayman et al., 2011). This could contribute to the variability in metabolite formation seen.

NBUP formation for neonates was also within the range of female adults. CYP3A4 is responsible for 80-90% of BUP metabolism, so this similarity is to be expected. There is no data on age affecting BUP metabolism, but many studies have shown a more favorable NAS outcome when buprenorphine was administered maternally compared to methadone. EDDP formation also showed no difference in the neonatal and adult female groups. Interestingly, the average rate of formation was almost identical, differing only

by 0.001 RAU. Recent studies show that during gestation, CYP2B6 has little activity, and increases drastically after birth into adulthood, similar to CYP3A4 (Sadler et al., 2016).

 In vitro data regarding fetal exposure to alprazolam, buprenorphine and methadone is lacking. Fetal livers are known to have minimal levels of CYP3A4, a required enzyme for metabolism of buprenorphine and alprazolam, and contributing less to methadone metabolism (Lacroix et al., 1997). Similarly, CYP2B6 expression and activity is low in fetal livers, suggesting difference in methadone metabolism in the fetus compared to adult and neonate (Sadler et al., 2016). By evaluating these drugs in fetal livers, the mechanism of how these drugs are biotransformed can be understood.

In many cases of opioid treatment, patients who take buprenorphine, methadone, or morphine for severe cases, often have other diseases that require the prescription of other drugs, commonly alprazolam (Sun et al., 2017). To evaluate this relationship, we looked at the inhibition of alprazolam by morphine. Inhibition of alprazolam metabolism would lead to higher plasma concentrations of the parent drug, and the potential for greater toxicity. We found that morphine inhibits alprazolam metabolism in a time-dependent manner (mechanism-based inhibition) in that a decrease in 4-OHALP formation was observed with pre-incubation of HLM, cofactors and morphine before addition of ALP. However, the IC50 of morphine in both adult females and neonates was much higher than the plasma concentration of morphine from usual clinical doses. While these findings suggest there is no pharmacokinetic interaction between morphine and ALP, there may be a pharmacodynamic interaction due to additive sedation.

Fetal exposure to drugs is an extremely complex system that cannot be solely based on *in vitro* data. There are many variables that could contribute to drug metabolism *in utero*, such as the drug permeability to the placental membrane, differences in enzyme expression and activity levels, and transporter proteins like P-glycoprotein (P-gp). Additionally, gestational age of the fetus affects the expression and activity of CYPs and important transport proteins (Sadler et al., 2016). In a transgenic mouse model expressing CYP3A4, there was an increase in hepatic CYP3A4 with increasing gestational age (Zhang et al., 2008). Other findings show an increase in a variety of metabolic enzymes during mouse development, also suggesting a mechanistic relevance to enzyme development in humans (Shuster et al., 2013). This finding, if translatable to humans, would mean an increase in exposure to drug metabolites in the developing fetus.

Future experiments could look at umbilical cord blood levels of 4-OHALP, NBUP, EDDP and P-gp levels in fetal liver and the substrate affinity for P-gp. By looking at umbilical cord blood, we can determine the amount of parent drug and metabolite crossing into the fetus. This will give us a better understanding of fetal drug exposure. Also, umbilical cord tissue from patients on opioid treatment can be cultured to examine activity and expression, if any, of CYP and P-gp. Inhibitory studies with buprenorphine and alprazolam, and methadone and alprazolam, can also be done to determine the extent of drug-drug interaction, and whether it is clinically important (McCance-Katz et al., 2010).

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