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Buprenorphine Metabolism in Placentas from Preterm Pregnancies: A Pilot Study

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by
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Buprenorphine Metabolism in Placentas from Preterm Pregnancies: A Pilot Study

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The focus of this investigation is to attempt to identify and characterize the major enzyme responsible for metabolism of buprenorphine to nor-buprenorphine in human preterm placentas and to elucidate whether the development/gestational age of the placenta affects metabolism of buprenorphine. The estimated apparent K_m did not appear to change with gestational age suggesting the affinity of buprenorphine does not change throughout gestation. On the other hand, the estimated V_{max} did appear to increase with gestational age suggesting the activity increases with gestational age. Our attempt to identify the enzyme responsible for metabolism in earlier gestational age placentas revealed that CYP19 remains the major metabolizing enzyme of buprenorphine but suggests additional enzymes may be involved, namely, CYP3A4, 1A1, 2E1, 2D6 and 2C19. Further studies must be conducted for conclusions to be drawn but we hope this report can contribute to the design of future studies on preterm placental metabolism of drugs.

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CHAPTER 1: INTRODUCTION

Buprenorphine (BUP) is an opioid drug first marketed in the 1980s for the treatment of moderate to severe chronic pain or for peri-operative analgesia (Martin, 1982). In 2002, the FDA approved buprenorphine for treatment of opioid addiction in men and non-pregnant women, and as such the drug is now also used for this purpose (Substance Abuse and Mental Health Service Administration, 2007). Prior to the approval of BUP, methadone was the only option for the management of opioid dependence, termed *maintenance therapy*. Methadone remains the only drug approved by the FDA for the treatment of the pregnant opiate addict (Johnson, 2003). Women maintained on methadone lower their risk of complications during pregnancy for both themselves and the fetus. It remains controversial whether the improved neonatal outcome observed is due solely to methadone treatment or the enhanced pre-natal care that accompanies treatment (Suffet et al., 1984; Chang et al., 1992). Recently, BUP entered into clinical trials for treatment of the pregnant opiate addict. The current clinical trials and initial results prompt the need to study hepatic and extrahepatic biotransformation of BUP in the pregnant woman. The placenta is the only protective barrier separating the maternal and fetal circulation. Therefore, the placenta's role in metabolism of BUP is important because of the unknown effects the drug could have on the developing fetus. The focus of this investigation is to attempt to identify and characterize the major enzyme responsible for metabolism of BUP to norBUP in human preterm placentas and to elucidate whether the development/gestational age of the placenta affects metabolism of BUP. Although the human placenta is similar to guinea pigs, rat, and rabbit placenta, it is different enough to favor the study of human placenta over animal models (Syme et al., 2004).

Pharmacodynamics of Opiates

BUP, methadone and heroin all belong to the class of drugs termed *opiates*. The site of action of opiates is a class of receptors named opioid receptors found throughout the body. The classic receptors of this family are considered to be mu, kappa, and delta although some argue for a fourth receptor type, the opioid-like receptor 1 (ORL1) (Evans,

2004; Waldhhoer et al., 2004). In fact, BUP is one ligand that has been found to interact with the ORL1 receptor (Evans, 2004; Waldhhoer et al., 2004). However, ORL1 does not interact with naloxone, an opioid antagonist considered a classic opioid ligand, and this lack of interaction is why some argue against ORL1 receptors inclusion into the opioid receptor family (Evans, 2004).

Most of the potent analgesic drugs used today are mu-opioid receptor agonists or mixed receptor agonists. Mu receptors tend to be associated with the beneficial analgesic and euphoric effects as well as the undesirable effects of opiates including respiratory depression and physical dependence (Martin, 1984). Kappa receptor agonists have been associated with dysphoria and hallucinations and delta agonists have been found to induce seizures in animal models, along with their analgesic effect (Evans, 2004).

Central nervous system effects from the use of opiates include: a slight decrease in body temperature via heat-regulatory mechanisms in the hypothalamus, decreased concentrations of pituitary hormones, miosis, convulsions, a depressed cough reflex, emetic effects, and most importantly, respiratory depression (Martin, 1984). Respiratory depression is a direct effect of opiates on the brainstem centers and depresses all phases of respiration. Decreased responsiveness of the brainstem to carbon dioxide levels is the primary mechanism of respiratory depression (Martin, 1984).

BUP is a semi-synthetic derivative of thebaine, a naturally occurring alkaloid from the opium poppy (Evans, 2004). BUP has unique pharmacological properties as a partial mu-opioid receptor agonist and kappa antagonist (Lutfy et al., 2003). The drug's effects on analgesia and respiratory depression are affected by its pharmacological action as both agonist and antagonist at the mu receptor and afford BUP its unique safety profile. BUP acts as an agonist at the mu-receptor up until a certain dose and then will antagonize any subsequent effects of higher doses. The risk of severe respiratory depression is decreased with BUP due to the "ceiling effect"—with an increasing dose the effect will reach a maximum level and then level off (Davids et al., 2004). The extent of analgesia is also decreased due to the "ceiling effect" (Davids et al., 2004). Thus, the hazardous side effect of increased respiratory depression seen with increased doses of a full mu-opioid receptor agonist is not apparent with increased doses of BUP especially in opiate tolerant patients. The ceiling effect of BUP is reached in a dosing range of 8-32 mg and doses above 32 mg

elicit the same response and only extend the length of time of the maximal response (Davids et al., 2004). The kappa-opioid receptor antagonist property of the drug allows it to block agonist activity through high affinity for the receptor. The kappa receptor, as noted previously, is implicated in the dysphoric effects of opiates. Therefore, BUP could potentially have an antidepressant effect on patients (Davids et al., 2004). In addition to the decreased risk of respiratory depression, BUP's duration of action is much longer than methadone. The long-lasting occupation of mu-receptors by BUP allows dosing typically to be given every other day (possibly every three days) as opposed to daily for methadone (Greenwald et al., 2007).

Pharmacokinetics of Buprenorphine

Opiates are absorbed readily and as with all drugs, the greater lipophilicity, the more readily absorbed. BUP has very low oral bioavailability due to metabolism in the intestine and liver. Therefore, BUP is administered sublingually to circumvent first-pass metabolism (David et al., 2004). In sublingually administered doses, the bioavailability is 60-70%. The peak plasma concentration is reached within 90 minutes and the half life is 4-5 hours (Sporer, 2004). BUP is lipophilic and is highly bound to alpha and beta globulin in the plasma, approximately 95% (Suboxone/Subutex drug label).

BUP undergoes N-dealkylation and conjugation to inactive and active metabolites. The principle enzyme for N-dealkylation of BUP's N-cyclopropylmethyl group to form norBUP is CYP3A4 (Iribarne et al., 1997), the most abundant drug metabolizing P450 enzyme in human liver and intestine. NorBUP and BUP can also undergo further metabolism via glucuronidation (Cone et al., 1984).

Recent reports have implicated involvement of additional enzymes in the metabolism of BUP. Picard et al. reported that in addition to CYP3A4, the principle enzyme, 2C8, 3A5, and 3A7 were also involved in production of norBUP as determined using both human liver microsomes, P450-transfected cells, and LC-MS/MS detection (Picard et al., 2005). In addition to norBUP production, a further oxidative metabolic pathway was found to involve CYP 2C9, 2C18, 2C19, and 3A. The new metabolites were identified as hydroxy-BUP and hydroxy-norBUP. In 2006, Chang et al. identified 5 new phase I metabolites of BUP using human liver microsomes and LC-MS/MS detection.

These new metabolites differed from those described by Picard et al. but involved the same enzymes in formation of the metabolites. Chang et al. confirmed that CYP3A4 was the predominate enzyme involved in N-dealkylation of BUP to norBUP and that CYP2C8, 3A5, and 3A7 were also involved. However, these enzymes were also shown to form newly identified metabolites termed M1, M3, and M5 (Chang et al., 2006). These new discoveries reveal how complex the metabolism of BUP is as compared to the previously mentioned opiates. There is much to be studied and elucidated in order to fully understand this drug.

Cytochrome P450 Enzymes

The cytochrome P450 (CYP) superfamily is a group of heme-containing enzymes found throughout almost every organism (Scott et al., 2005). Over 7000 P450 enzymes, in total from all organisms, have been reported (Guengerich, 2004). The term "P450" was coined because of their absorbance at 450 nm when the reduced proteins formed complexes with carbon monoxide (Omiecinski et al., 1999). In general, P450 enzymes catalyze mono-oxygenation of the substrate following the general stoichiometric reaction:
$RH + O_2 + NADPH + H \rightarrow ROH + H_2O + NADP^+$, where RH represents the endogenous compound or xenobiotic and ROH the product (Coon, 2005).

P450 enzymes are organized into families and subfamilies based on protein sequence similarity. CYPs sharing >40% homology are grouped into the same family, >55% homology are in the same subfamily (Omiecinski et al., 1999). Fifty-seven genes for P450 enzymes have been found in humans (Guengerich, 2004).

The P450 enzymes are broadly divided into two groups: those that metabolize xenobiotics and those that metabolize endogenous compounds (Guengerich, 2004). The major xenobiotic metabolizing enzymes are CYP3A4, 2D6, 2C19, 2C9, 1A1, and 1A2 (Guengerich, 2004). P450 enzymes participate in Phase I metabolism of exogenous and endogenous compounds. They play a role in detoxification through modification of the compound into a more soluble form that can subsequently be conjugated by phase II enzymes for elimination (Scott et al., 2005).

Aromatase, or CYP19, involved in metabolism of endogenous compounds, is found in steroidegenic tissue (Simpson, 2002). Such tissues include but are not limited to the

brain, bone, adipose tissue, ovaries, and placenta. CYP19 in involved in conversion of androgens to estrogens and has recently been targeted as a therapy, through inhibition of the enzyme, for treatment of estrogen-dependent breast cancer (Subramanian et al., 2007).

Effect of Pregnancy on Pharmacokinetics of Drugs

Changes in maternal physiology influence pharmacokinetics of drugs: their absorption, distribution, metabolism, and elimination (Dawes et al., 2001). For example, increased progesterone levels influence absorption of orally administered drugs. Gastric emptying and motility through the small intestine are delayed which affect absorption and bioavailability of drugs (Parry et al., 1970). Specifically, slowing of gastric emptying and motility should increase time of peak plasma concentration while decreasing overall peak plasma concentration (Dawes et al., 2001). Some drugs may be better absorbed and reach higher concentrations than the therapeutic dose while different drugs may show a decrease in concentration (Amon et al., 1984).

During pregnancy, total body water can increase up to 8 liters (Dawes et al., 2001). Distribution of a drug can be influenced by this change, especially with hydrophilic drugs. Overall due to increased plasma volume, decreased albumin, and drug displacement, the total amount of free drug is increased (Ward, 1993, Dawes et al., 2002). However, other parameters of pharmacokinetics, such as distribution, metabolism and excretion, cause the change in free drug to be physiologically insignificant in most cases (Loebstein et al., 1997).

Hepatic enzymes are also affected by the change in hormone levels during pregnancy. Some drug metabolizing enzymes are induced by higher levels of circulating estrogens and progesterone such as CYP3A4, CYP2D6, CYP2C9, and uridine 5' diphosphate glucuronosyltransferase isozymes (Dawes et al., 2001, Myllynen et al., 2007). Conversely, CYP1A2 and CYP2C19 activity is decreased in pregnant women. CYP3A4 has been shown to metabolize 50% of pharmaceutical drugs and the changing hormone levels that occur with pregnancy could drastically affect therapeutic efficacy. BUP is primarily metabolized by CYP3A4 in the liver and intestine. Thus it can be inferred that higher doses of BUP are needed during treatment of the pregnant woman but an obvious trend has not been found (Kahila et al., 2007; Lejune et al., 2006; Johnson et al., 2003).

One confounding possibility is that other enzyme activities are attenuated due to competitive inhibition by progesterone and estrogen (Dawes et al., 2001).

In addition to hepatic metabolism, placental metabolism also plays a role in xenobiotic transformation. The placenta is especially important in terms of drug exposure and the consequent well-being of the fetus because it is the one protective barrier between the maternal circulation and the fetus. BUP is metabolized in the placenta, but has been reported not to occur via CYP3A4 (Deshmukh et al., 2003). CYP19 was reported to be the enzyme responsible for metabolism of BUP in term placenta (Deshmukh et al., 2003).

Elimination by the kidneys is also greatly influenced by pregnancy. Renal blood flow and glomerular filtration increase by 60-80% and 50%, respectively (Dawes et al., 2001). Increase in excretion by the kidneys should influence the bioavailability of those drugs that are unchanged before excretion.

Drug Transfer from the Mother to the Fetus

Organogenesis occurs within the first 2 months of pregnancy (Syme et al., 2004). The embryo becomes a fetus after this time. During the time of organogenesis, the maternal circulation does not perfuse the placenta, thus exchange between fetal and maternal blood is absent. Despite lack of perfusion, drugs can still cross the placenta due to the primary transfer mechanism of passive diffusion (Myllynen et al., 2007). During the early stages of gestation, the embryo/fetus is most susceptible to malformations. The fact that the exchange of blood between maternal and fetal circulation does not occur does not preclude potential harm to the fetus by toxic xenobiotics.

Drug transfer from the mother to the fetus can occur through passive diffusion, facilitated diffusion, and active transport. Passive diffusion does not require energy and is the prominent transplacental mode of transfer for lipophilic, un-ionized drugs of low molecular weight (<500DA) (Syme et al., 2004). Lipophilicity is one of the major determinants in passive diffusion of placental transfer (Myllynen et al., 2007). In addition, lipophilicity can affect the placental function as a drug depot which could have a positive or negative effect on the transfer of drug or metabolite to the fetus (Nanovskaya et al., 2002).

Facilitated diffusion is the predominate process for transport of nutrients to the fetus but few known drugs cross the placenta in this way. Typically, in facilitated diffusion the maternal: fetal drug concentration ratio is 1 (Syme et al., 2004). Finally, much study has been devoted to nutrient transfer across the placenta through active drug transfer (Syme et al., 2004). Based on the studies reviewed by Syme et al. and others, it has been made clear that drugs transported in this way are often structurally similar to endogenous substances (2004). P-glycoprotein, an ABC transporter, carries uncharged compounds with molecular weights between 200-1800Da (Syme et al., 2004). The MRP family of proteins, of which three have been identified in placental tissue, transport unconjugated, amphiphilic anions and conjugated, lipophilic compounds. These proteins could function in a protective role by removing metabolic waste products.

Drug Metabolism in the Placenta

The placenta contains many of the same phase I and phase II drug metabolizing enzymes as the liver although the concentration of the P450 isozymes (phase I enzymes) in the placenta are three times less than in the liver due to the different roles each organ plays (Bourget et al., 1995). The placenta is an organ that develops and changes throughout gestation to accommodate the needs of the growing fetus. As mentioned previously, one function of the placenta is drug metabolism but it is by no means its primary function as is metabolism in the liver. The concentrations of P450 isozymes in the placenta are lower than in the liver, possibly because of its role in protecting the fetus, rather than the entire organism. In addition, Hakkola et al. have shown a wider number of mRNA transcripts for P450 isozymes present in first trimester placentas than those at term (1996a,b). Activities of these enzymes and their abundance are less defined. A plausible explanation for this temporal difference is that during the first trimester of pregnancy (when organogenesis occurs and the fetus is more susceptible to teratogens) more protection in the form of cytochrome P450 metabolizing enzymes is provided (Syme et al., 2004).

There are conflicting reports on which cytochrome P450 enzymes are present in the placenta at different gestational ages, which may depend on whether mRNA, protein level, or activity was measured. The P450 enzymes currently found in the placenta include

CYP19 (Kitawaki et al., 1989), as well as CYPs 1A1, 1A2, and 2E1 (Hakkola at al. 1996; Syme et al., 2004; Myllynen et al., 2007).

Since CYP19 will be discussed in great detail in the subsequent sections, only CYPs 1A1, 1A2, 2E1, and 3A will be considered in this section. CYP1A1 has been found in the placenta throughout term and can be induced by environmental factors such as polycyclic aromatic compounds from cigarette smoke (Hakkola et al., 1998). The amount of CYP1A1 varies significantly between placentas. CYP1A2 mRNA has been found in the first trimester but not at term (Syme et al., 2004). In addition, CYP1A1 has been reported to be much higher in women that smoked during pregnancy (Collier et al., 2002, Hakkola et al., 1998). The mRNA and protein of CYP2E1 have been detected in the first trimester and throughout gestation, although controversy remains on whether it is a functional enzyme in the placenta (Hakkola et al., 1998). Thus, we can conclude that variable and small amounts of CYP2E1 are present in the placenta because of reports that this enzyme is induced by ethanol, similar to CYP1A1 induction by cigarette smoke (Syme et al., 2004, Collier et al., 2002). However, chlorozoxazone, used as a marker substrate to measure activity, yielded negative results (Pasanen et al., 1999, Collier et al., 2002).

The CYP3 family of enzymes has been detected in human placenta, via RT-PCR and western blot; but there have been no reports of marker substrate activity of any of these enzymes to date (Syme et al., 2004, Myllynen et al., 2007).

CYP2D6 has been reported to be present in the first trimester in placentas but no catalytic activity has been observed (Pasanen at al. 1999). Whether it remains and is active during the second trimester has not been reported. CYP2D6 is also an important enzyme in hepatic drug metabolism and has recently been implicated in metabolism of BUP by human liver microsomes (Zhang et al., 2003). In addition, CYP2C enzymes have been detected at the mRNA level in placentas from the first trimester but are absent at term (Syme et al., 2004).

Opiate Use in Pregnancy

Illicit opiate use during pregnancy significantly increases the risk of complications for both maternal and neonatal outcomes (Finnegan, 1991). Complications for the mother include breakthrough bleeding during the third trimester, preterm labor, placental

abruption, and the presence of sexually transmitted disease. Neonates can also experience a myriad of complications due to intermittent opiate use of the mother and their withdrawal from the drug (Beauman, 2005; Dattel, 1990). Intrauterine growth retardation, fetal distress, and microencephaly are of concern for the fetus, while postnatal growth deficiencies, as well as an increased risk of sudden infant death syndrome, become concerns for neonates whose mothers were opiate-addicts while pregnant (Beauman, 2005; Dattel, 1990).

Women who opt to be maintained on methadone for their addiction lower their risk of complications during pregnancy for both themselves and the fetus (Kahila et al., 2007; Lejune et al., 2006; Johnson et al., 2003). Methadone treatment provides a steady state of an opiate in the maternal circulation which lowers the risk of complications that result from drug seeking behavior and withdrawal suffered by the mother, as well as eliminating intermittent withdrawal of the fetus and the adverse effects/risks that go along with withdrawal suffered by the fetus. However, it is unclear whether the association between improved maternal and neonatal outcome is due to methadone therapy itself or due to the enhanced prenatal care that goes along with maintenance therapy (Suffet et al., 1984; Chang et al., 1992). Whether a mother's use of opiates is illicit or therapeutic, her newborn often exhibits *neonatal abstinence syndrome* (NAS) when born.

NAS

Common withdrawal symptoms include: vomiting, poor feeding, continuous sucking, diarrhea, sneezing, yawning, and tachypnea (Ebners et al., 2006). Complications such as dehydration and malnourishment can become an issue when these symptoms appear and are left untreated. The presence and severity of NAS are assessed on a semi-objective scale referred to as the Finnegan scale (Jones et al., 2005). The Finnegan scale rates the presence or absence of each symptom and provides guidelines for pharmaco-management of the neonate. Symptoms usually appear from 12-48 hours after delivery but sometimes do not present until 7-14 days later (Dunlop et al., 2003). Varying dosages of morphine, which has a much shorter duration of action than methadone, are administered to the infant to ease the withdrawal according to the severity of NAS (Ebners et al., 2006). Once symptoms subside, recommendations are to slowly taper off the morphine while

continuing to assess for NAS. The treatment for NAS varies depending on symptoms but neonates eventually recover once the drug clears their system. Non-pharmacological approaches for treatment include: reducing stimuli, cuddling, and use of pacifiers (Dunlop et al., 2003).

NAS and BUP

Thirty to eighty percent of neonates exposed to opiates during gestation require treatment for NAS. For pregnant women maintained on methadone, 60-87% of their neonates require treatment for NAS with 10-30% being admitted to intensive care. Estimates for BUP-related NAS are limited. Compiled, non-controlled data from all studies on pregnant women undergoing BUP-maintenance for opiate addiction reveal 63% of neonates born to these women were reported as suffering from NAS. Of these, 48% required treatment and 10% of these required admission to the neonatal intensive care unit (Johnson et al., 2003).

It is still unclear whether there are long lasting psychological, developmental, and physiological effects resulting from opiate exposure and withdrawal *in utero* as a fetus (Nocon, 2006; Kahila et al., 2007; Fischer et al., 2006; Lejeune et al., 2006). Specifically, one prospective study carried out in France observed that out of 34 BUP treated pregnancies, only 13 exhibited NAS. Of those 13, nine were confounded by poly-drug use (benzodiazepines, opiates, cannabis) (Nocon, 2006). A recent prospective study of pregnant women treated with BUP found that the only significant difference between their neonates and the national registry was a lower birth weight (7.5% versus 4.4%) (Kahila et al., 2007). Although the lower birth weight was significant, there were no more higher incidences of premature birth, cesarean section, or low Apgar score (≤6) than the Finnish national registry. Of the 67 BUP-exposed pregnancies, 91% of the infants required care in the neonatal care unit. 76% were determined to have NAS and 57% required morphine therapy (Kahila et al., 2007). Most disturbingly, a high number of sudden infant deaths occurred as compared to the national registry (2 deaths out of 67 births or a 150 times higher incidence than documented in the national registry). Other comparative and observational studies have shown no statistically significant differences in outcomes of pregnancy and prevalence of NAS between methadone and BUP treatment in pregnant

women (Fischer et al., 2006; Lejeune et al., 2006). Overall, the conclusions that can be made so far are that BUP affords the mother and her fetus the same benefits as other maintenance therapies but *could* also decrease the severity of NAS. More controlled studies must be conducted before making the final decision about this route of treatment versus the classic treatment but the initial trend is positive. A broader understanding of the drug and its pharmacokinetics during pregnancy, both hepatic and placental, will contribute to better treatment options for the mother and her fetus.

Experimental Introduction

An area of interest for Dr. Mahmoud Ahmed's laboratory and the report that provoked much of their investigational study was that the incidence and severity of NAS cannot be correlated with maternal methadone dose and circulating concentration of the drug (Berghella et al. 2003). One would assume that the intensity of the addiction of the newborn to the drug would be related to the concentration of the drug in the maternal circulation: higher concentration of drug should cause more severe addiction and hence more severe withdrawal. The reason why this is not the case is unclear and important to elucidate.

Many additional questions have been provoked by previous reports. For example, the metabolite to parent drug ratio for plasma concentrations in maternal circulation of norBUP: BUP is 6:1 whereas in neonates it is 1:1 (Johnson et al., 2003). These ratios document the need for better understanding of the disposition of BUP during pregnancy. How much of the metabolite is formed? Where does the metabolite go after it is formed? Is more metabolite formed at different points in gestation?

Dr. Ahmed's laboratory has shown using perfused placentas that the rate of transfer of methadone from maternal to fetal circulation is almost three times higher than that of BUP. Less than 5% of the perfused BUP is metabolized to norBUP and BUP concentrates in the placental tissue (Nanovkaya et al., 2002; Nekhaeva et al., 2005). The observed high concentration gradient and subsequent retention of BUP in placental tissue prompted further study of the metabolic biotransformation of BUP in the placenta. Dr. Ahmed's laboratory isolated microsomal fraction from six placentas from full term deliveries to determine the affinity and activity of the P450 isozyme involved in BUP metabolism.

Using untreated microsomes and microsomes incubated with antibodies against major drug-metabolizing P450s, as well as specific inhibitors against these enzymes, the laboratory investigated the major enzyme responsible for metabolism of BUP in term placenta (Deshmukh et al., 2003). Based on the data obtained, they concluded that placental metabolism of opiates is not carried out by CYP3A4 as in the liver. Instead, a different P450 enzyme, CYP19, is involved. This was an unexpected finding because before the pioneering work in Dr. Ahmed's laboratory, CYP19 was thought to participate in metabolism of steroids only, catalyzing the conversion of androgens to estrogens in steroidegenic tissue (Simpson et al., 2002). In addition to BUP, M. Ahmed's laboratory has reported that in term placenta, CYP19 is the major metabolizing enzyme for methadone and levo-alpha-acetylmethadol (LAAM) (Deshmukh et al., 2003, Deshmukh et al., 2004, Nanovskaya et al., 2004).

This project was undertaken to establish how BUP is metabolized in pre-term placentas because there was no available information on placental BUP biotransformation at early gestational ages. The rationale for this project was the following: It has been shown that the concentration of placental microsomal CYP19 increases during gestation by four-fold at term, while estrogen formation (as a of measure aromatase activity) increases only two fold (Kitawaki et al., 1992). Further, it is incorrect to assume that one can extrapolate placental functions at term to those during preterm. There are numerous differences between the periods in hormone production, transfer of nutrients as well as enzyme expression. As mentioned previously, it has been shown that the expression of metabolizing enzymes in the placenta varies during gestation and that a greater expression of CYP isoforms in the placenta can be found during the first trimester as compared with those at term (Hakkola et al1996a,b).

This project sought to address two specific questions. First, does the affinity and activity of the BUP-metabolizing enzyme(s) change with gestational age? Second, is aromatase the major metabolizing enzyme of BUP throughout gestation? Experiments were carried out on individual and pooled placental microsomal preparations from varying gestational ages. Assay conditions were the same as those in the previous work on term placentas (Deshmukh et al., 2003). Preterm placentas were not readily available and are often themselves abnormal since obtained from pregnancies that required early delivery of

the fetus. Since it is still unclear whether these abnormal placentas would yield reliable information, ideally, control studies on tissue from healthy pregnancies are required to unambiguously interpret the data. Another limitation of this project is that appropriate enzyme assay conditions were not re-optimized and were assumed to be the same as in the term placentas (this will be discussed in the Methods and Discussion sections). Nonetheless, I believe that despite the mentioned limitations, this project provides some insight into the metabolism of drugs by placental tissue of differing gestational ages.

CHAPTER 2: MATERIALS AND METHODS

Chemicals

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Acetonitrile was purchased from Fischer Scientific Co. (Pittsburgh, PA). All monoclonal antibodies were purchased from Xenotech LLC (Lenexa, KS). Rabbit antiserum to human placental aromatase was purchased from Hauptman-Woodward Institute (Buffalo, NY) and the specificity of their effect on placental aromatase was previously reported (Kitawaki et al., 1989). BUP, norBUP, and LAAM were a gift from the National Institute on Drug Abuse (NIDA) drug supply unit.

Clinical Material

Placentas from term (≥ 34 weeks of gestation) and preterm (between 17 and 34 weeks of gestation) deliveries were obtained, immediately after delivery, from the John Sealy Hospital labor and delivery ward according to a protocol approved by the Institutional Review Board of the University of Texas Medical Branch, Galveston, TX. The exact gestational ages used for experiments can be found in Table 1 and subsequent figures.

Exclusion criteria included presence of sexually transmitted disease (STD) and pharmacological treatment or diagnosis for any medical condition such as gestational diabetes, STD, and anemia. Microsomal fractions obtained from placentas of the following gestational ages were arbitrarily divided into three groups: late second trimester (17-26 weeks gestation), early third trimester (27-33 weeks gestation), and late third trimester (34-40 weeks gestation). The rationale for having the third group begin with 34 weeks which is not considered "full term" (full term ≥ 37 weeks) was due to the fact that when a woman presents in labor at 34 weeks the physician will not try to prevent the progression of labor. Neonatal outcomes do not vary as significantly after 34 weeks as compared to those delivered before 34 weeks (personal communication, Dr. Mahmoud Ahmed).

Preparation of Placental Microsomes

Villus tissue (8-10 mm depth) was dissected randomly from each placenta, rinsed thoroughly with ice-cold saline, and homogenized in 0.1M potassium phosphate buffer (pH

7.4) using a Tekmar SDT-1810 Tissumizer (Cincinnati, OH; Deshmukh et al., 2003). The crude homogenate was centrifuged for 10 min at 800-1000 g and pellet containing nuclear fraction was discarded. The supernatant was then centrifuged for 10 min at 9000-10,000 g, supernatant removed and centrifuged again for 10 min at 9000-10,000 g and the pellet containing mitochondrial fraction discarded. The supernatant was centrifuged at 100,000 g for 1 hour. The 100,000 g pellet containing the microsomes was re-suspended in 1-2 ml of 0.1 M potassium phosphate buffer (pH 7.4). Aliquots were stored a -80ºC until used. Protein content of microsomal fraction was determined by the Bradford dye-binding Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Briefly, dye reagent was mixed with dH_2O in a 1:4 dilution. Five ml of the mixture was added to 5 µl of microsomal protein and mixed by inversion. Absorbance of the mixture was measured at a wavelength of 595 nm. Amount of protein was determined using bovine serum albumin as the standard.

BUP Metabolism by Placental Microsomes

The activity of placental microsomal fractions in catalyzing the n-dealkylation of BUP to norBUP was determined as previously described with some modifications (Deshmukh et al., 2003). Modifications included increased range of substrate concentration, modification of high performance liquid chromatography (HPLC) conditions, and modification of m/z monitoring in mass spectrometry (MS) for norBUP. In addition, the extraction procedure was not described in Deshmukh et al. (2003), but after communication with the author, directions were given for the proper extraction procedure.

Briefly, the reaction mixture, with a final volume of 1 ml, contained 0.1 M potassium phosphate buffer, pH 7.4; 1 mg microsomal protein; a NADPH-regenerating system prepared immediately before assay (NADP, 0.4 mM; glucose-6-phosphate (G6P), 4 mM; G6P-dehydrogenase, 1 U/ml; and $MgCl_2$, 2 mM); and BUP in a concentration range between 0 and 120 µM. BUP was pre-incubated with the pool of microsomal fractions for five minutes at 37ºC. The reaction was initiated by the addition of the NADPH-regenerating system, carried out at the same temperature for 15 min and terminated by adding 100 µl of 35% trichloroacetic acid (TCA). Additionally, following termination of the reaction, 20 µl of 2 µg/ml LAAM was added as the internal standard and the tubes were

placed on ice. The reaction mixture was centrifuged at 12,000 g for twelve minutes, the supernatant transferred to a new tube and treated with butyl chloride in a 1:1.5 ratio for extraction. The mixture was shaken vigorously for 15 min, the butyl chloride layer separated, evaporated to dryness and re-dissolved in 200 µl of the mobile phase for the high performance liquid chromatography/mass spectrometry (HPLC/MS). In the control reaction TCA was added prior to addition of NADPH to denature the protein.

A minimum of three individual placentas were investigated in each group. In addition, pooled placental preparations were prepared and used. Each pool consisted of eight to ten placental preparations with varying gestational ages within each group.

Identification of NorBUP

Separation and identification of the metabolite(s) formed were performed by HPLC/MS. The HPLC system consisted of a Waters 600E multisolvent delivery system, Waters 2487 dual wavelength absorbance detector, and a Waters 717 autosampler, all of which were controlled by Waters Millennium chromatography manager (Waters, Milford, MA). Chromatographic separation was achieved using a Luna 5µ C18 column (250 mm x 4.6 mm) purchased from Phenomonex (Torrance, CA).

Isocratic elution (0.8 ml/min) was used in place of gradient elution as previously described by Deshmukh et al. (2003). This type of elution gives higher resolution and sharper peaks when compared to using gradient elution previously described. The mobile phase for isocratic elution consisted of acetonitrile and water (25:75 v/v), pH 3.0, adjusted by orthophosphoric acid.

The eluant was monitored at a wavelength of 210 nm as previously reported (Moody et al., 2002). Retention times for norBUP, BUP, and LAAM were ~6 minutes, 8 minutes, and 25 minutes respectively. Eluant corresponding to retention times of norBUP, BUP, and LAAM were further analyzed by MS because of the weak absorbance of these compounds at the concentrations used in the enzyme assay.

Experimental Conditions for the Mass Spectrometer

Eluant from HPLC was applied to a Waters EMD 1000 single-quadrupole mass spectrometer. The spectrometer was equipped with an electrospray ion source operated in

positive-ion mode and maintained at a temperature of 118°C with liquid nitrogen as the nebulizing gas. The ionization temperature was determined through adjusting temperature in a stepwise fashion and measuring intensities of ions under observation. The mass resolution of the instrument is 1DA or 1amu. Mass range of 100-1000 daltons was scanned at a speed of 1 second. Previous work in the laboratory utilized the *m/z* value 454.7 corresponding to the potassium-norBUP adduct (Deshmukh et al., 2003). In the present work we used the *m/z* value 414 corresponding to the peak and mass for norBUP because the peak at this *m/z* value is more prominent than the peak at *m/z* 454.7. Select ion monitoring (SIM) at *m/z* 354 was performed to measure the internal standard, LAAM. LAAM was substituted for the internal standard, terfenadine, used previously (Deshmukh et al., 2003). The choice was made because terfenadine did not give consistent peaks that correlated with the concentration used. Further examination of the solution revealed precipitate which may have been the terfenadine, explaining why there were inconsistent peaks. Despite remaking the solution and using fresh terfenadine, precipitation persisted. LAAM has similar chemical properties to BUP, does not precipitate, and exhibits stable peaks in SIM, as well as during HPLC.

The BUP stock supplied from NIDA was found to be contaminated with norBUP. The contamination was accounted for in each of the experimental assays by addition of BUP to the denatured reaction mixture at the same concentration(s) used for each experiment. The area under the curve (AUC) for the SIM peak (*m/z* 414) from norBUP contamination was then subtracted from the AUC for norBUP formation measured from the corresponding reaction. After the norBUP contamination was accounted for by subtraction of the AUC, the ratio of norBUP: LAAM was determined and used for calculation of the correct norBUP formation.

Chemical Inhibition

The effect of selective inhibitors for different CYP isoforms on the formation of norBUP by microsomal fractions was determined as described previously (Deshmukh et al., 2003). The concentration of the inhibitor was based on previously reported IC_{50}, K_i, or K_m values for the specified P450 isozyme. The reported K_i value for each inhibitor were as follows: 0.008 μM α-napthaflavone, 0.3 μM sulfaphenazole, 0.06 μM quinidine, 50 μM 4-

methylpyrazole, 0.015-0.7 μM ketoconazole, and 10 μM nifedipine (Newton et al., 1995; Bourrie et al., 1996; Pelkonen et al., 1998). Speculation on selection criteria for the concentrations of each chemical inhibitor is that concentrations were selected by increasing the Ki values. This was done presumably to be able to see significant inhibition in cases where the enzyme selected to inhibit was involved. The previously selected concentration of the inhibitors and corresponding CYP isoforms (in parentheses) were as follows: 0.1 μM α-napthaflavone (CYP1A); 10 μM sulfaphenazole (CYP2C); 5 μM quinidine (CYP2D6); 50 μM 4-methylpyrazole (CYP2E1); 2.5 μM ketoconazole (CYP3A); 20 μM nifedipine (CYP3A4) (Newton et al., 1995; Bourrie et al., 1996; Pelkonen et al., 1998). Two known inhibitors for CYP19 were used: 1 μM 4-hydroxyandrostenedione (4OHA); and 1 μM aminoglutethimide (AG) (Stresser et al., 2000). Inhibitors (5 μl) were added from stock solutions in methanol. The assay mixture, 1 ml, consisted of BUP (12 μM, the apparent K_m for term placenta), the inhibitor, the microsomal preparation (1 mg protein/ml). After 5 minutes pre-incubation at 37ºC the reaction was initiated with NADPH regenerating system and allowed to proceed for 30 min. The reaction was terminated by TCA and prepared for analysis as stated previously. Control reaction contained all components except for the inhibitor which was substituted with 5 μl methanol.

For the inhibition studies we used pools consisting of 8-12 microsomal preparations from placentas obtained from 17-26 weeks GA, 27-33 weeks GA, and 34-40 weeks GA.

Antibody Inhibition

Volumes of the monoclonal antibodies to human liver enzymes used in this assay for 0.1 mg pooled placental microsomal protein—and volumes recommended for use in 0.1 mg human liver microsomes for inhibition of 80% of the enzyme specified—are listed in parentheses prior to the enzyme selected to inhibit: (12.5 μl/ 50 μl) CYP1A2, (12.5 μl/ 50 μl) 2B6, (12.5 μl/ 50 μl) 2C9, (2 μl/ 5 μl) 2C19, (2 μl/ 2μl) 2D6, (12.5 μl/ 50 μl) 2E1, and (5 μl/ 10 μl) 3A4/5. Polyclonal rabbit serum to human placental CYP19 was also tested to identify the CYP enzyme responsible for metabolism of BUP. It is assumed that some of the volumes recommended by Xenotech Inc. were decreased for this experimental assay because the concentrations of placental CYP isozymes have been reported to be three times

less than in the liver (Bourget et al., 1995). As previously published by Deshmukh et al. (2003), 0.1 mg protein of placental microsomal protein was incubated with antibody at its concentration causing 80% inhibition of the CYP isoform the antibody was raised against (Xenotech LLC manual). The fifteen minute co-incubation was followed by addition of 80 μM BUP and the NADPH regenerating system. The reaction was allowed to continue for 120 min at 37°C and then terminated. A concentration of 80 μM BUP was used instead of the previously reported 50 μM BUP (Deshmukh et al., 2003). Unlike incubation with term placental microsomes, saturation was not reached at 50 μM in preterm placental microsomes. Therefore, the change was made to ensure that a decrease in norBUP production was not caused by use of un-saturable levels of the substrate but rather that of inhibition of the enzyme.

Data Analysis

All data represent the mean ± standard error, except where noted. Data was fit to the Michaelis-Menten equation: $v = [S \times V_{max}]/[S + K_m]$ where v- velocity, S- concentration of substrate, and V_{max}- maximum velocity (SPSS 11.0 for Windows software). Statistical significance for BUP kinetics was determined using Excel two-tailed t-test and considered significant if the P-value was <0.05. Statistical significance for inhibition data was determined using one-way ANOVA with a Tukey's comparison and considered significant if the *P*-value was <0.05.

CHAPTER 3: RESULTS

Metabolism of BUP by Placental Microsomes

Incubation of placental microsomes with BUP resulted in increased formation of norBUP as compared to control incubation with TCA-denatured proteins. The identity of norBUP was confirmed by the HPLC retention times corresponding to that of the authentic standard and by the *m/z* value (data not shown).

The rate of norBUP formation was dependent on BUP concentration and exhibited a typical Michaelis-Menten saturation kinetics, irrespective of placental gestational age (Fig. 1, Table 1). Estimated apparent K_m values of 11 ± 2 μM, 12 ± 1 μM, and 13 ± 3 μM were obtained for placentas of gestational ages (GA) 17-26 weeks, 27-33 weeks, and ≥34 weeks, respectively. These estimated apparent K_m values suggest that the affinity of BUP for the enzyme(s) does not change with gestation development.

In contrast, the estimated V_{max} values appeared to increase with gestational age and were 2.1 ± 0.3 pmol.mgP^{-1} min^{-1}, 3 ± 0.6 pmol.mgP^{-1} min^{-}, and 3.8 ± 0.6 pmol.mgP^{-1} min^{-1} for 17-26 weeks GA, 27-33 weeks GA, and ≥34 weeks GA, respectively.

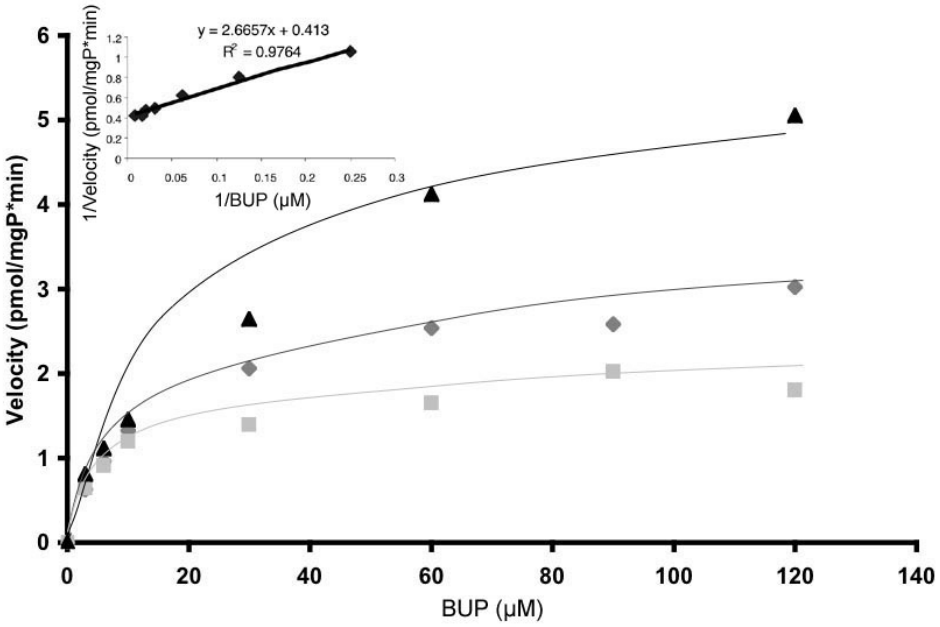

Figure 1. Estimated kinetics of BUP metabolism by microsomal fractions of individual placenta for each gestational age group (black-34-40 weeks, dark grey-27-33 weeks, light grey- 17-26 weeks). Insert shows the Lineweaver Burke plot used to calculate the apparent Km and Vmax for the experiment representing the 17-26 week gestational age group.

Table 1. Estimated apparent Km, and Vmax values for the conversion of BUP to norBUP by placental microsomal fractions of different gestational ages.
[1]

Gestational Period (weeks)	Placentas from Gestational Age (weeks)	K_m (μM)	V_{max} (pmol· mg.protein^{-1}min^{-1})
Late 2nd Trimester (17-26 weeks)	19	7	2.4
	19	7	2.1
	26	13	0.9
	Pool 1a[1]	12	2.6
	Pool 1b[2]	15	2.7
		11 ± 2	**2.1 ± 0.3***
Early 3rd Trimester (27-33 weeks)	27	10	4.4
	31	12	3.6
	32	10	2.1
	Pool 2a[3]	16	1.9
		12 ± 1	**3.0 ± 0.6**
Late 3rd Trimester (≥34 weeks)	34	5	4.2
	35	16	5.0
	35	14	2.2
	36	18	3.7
		13 ± 3	**3.8 ±0.6**

Microsomal poo1 using 8 placental preparations from gestational ages 17-26 weeks.
[2] Microsomal pool using 10 placental preparations from gestational ages 17-26 weeks.
Bold values represent mean ± standard error.
* $p<0.05$ (17-26 weeks versus ≥34 weeks) Statistical analysis was made between each group using a two tailed t-test**.**
[3] Microsomal pool using 10 placental preparations from gestational ages 27-33 weeks.

Inhibition of BUP Metabolism

Chemical Inhibition

Of eight chemical inhibitors tested, six inhibited the formation of norBUP (Fig. 2, Table 2). Aminoglutethimide (AG) and 4-hydroxy-androstenedione (4OHA), inhibitors of CYP19, showed greatest inhibition of norBUP formation (60%) as compared with the control ($p<0.0001$). A lesser inhibition (30-40%) was observed with nifedipine and ketoconazole, inhibitors of CYP3A4 and the CYP3A family, respectively ($p<0.03$, $p<0.04$). Ketoconazole is labeled a chemical inhibitor of the CYP3A family of enzymes because, at the concentration used, it has been shown to inhibit other members of the family in addition

to CYP3A4 (Bourrie et al., 1996). Sulfaphenazole and quinidine, inhibitors of CYP2C and 2D6, respectively, showed no significant inhibition of norBUP formation throughout gestation.

Significant differences in norBUP formation between 17-26 weeks GA and 27-33 weeks GA placental pools compared to 34-40 weeks GA placental pools were observed with α-napthaflavone, a selective inhibitor of CYP1A. The 17-26 weeks GA placental pools showed 34% more inhibition of norBUP formation than in 34-40 weeks GA placental pools ($p<0.04$). The 27-33 weeks GA placental pools showed 23% more inhibition of norBUP formation than the 34-40 weeks GA placental pools ($p<.02$). Four-methlypyrazole exhibited similar inhibition of norBUP formation in 17-26 weeks GA and 27-33 weeks GA.

Taken together, these data suggest CYP19 is the major enzyme responsible for dealkylation of BUP to norBUP in placentas from late 17-26 week GA and 27-33 week GA. These data are in agreement with earlier reports stating that CYP19 is the major enzyme involved in metabolism of BUP in term placenta. In addition to CYP19, CYP1A appears to play a role in N-dealkylation of BUP by placental microsomal fractions of 17-26 weeks GA and 27-33 weeks GA. Although it was not determined significant, 4-methylpyrazole exhibited similar inhibition of norBUP formation in 17-26 weeks GA and 27-33 weeks GA suggesting that CYP2E1 may also play a role in metabolism of BUP. Data for CYP3A(4) with ketoconazole alone were not conclusive because this compound has also been reported to be an inhibitor of CYP19 (Deshmukh et al., 2003; Ayub et al., 1988). Therefore, a second selective inhibitor for CYP3A4, nifedipine, was used in addition to ketoconazole. Similar to ketoconazole, nifedipine showed a modest 30%-40% inhibition throughout gestation ($p<0.03$). Collectively, the data on ketoconazole and nifedipine suggest the involvement of CYP3A4 in metabolism of BUP in preterm placentas.

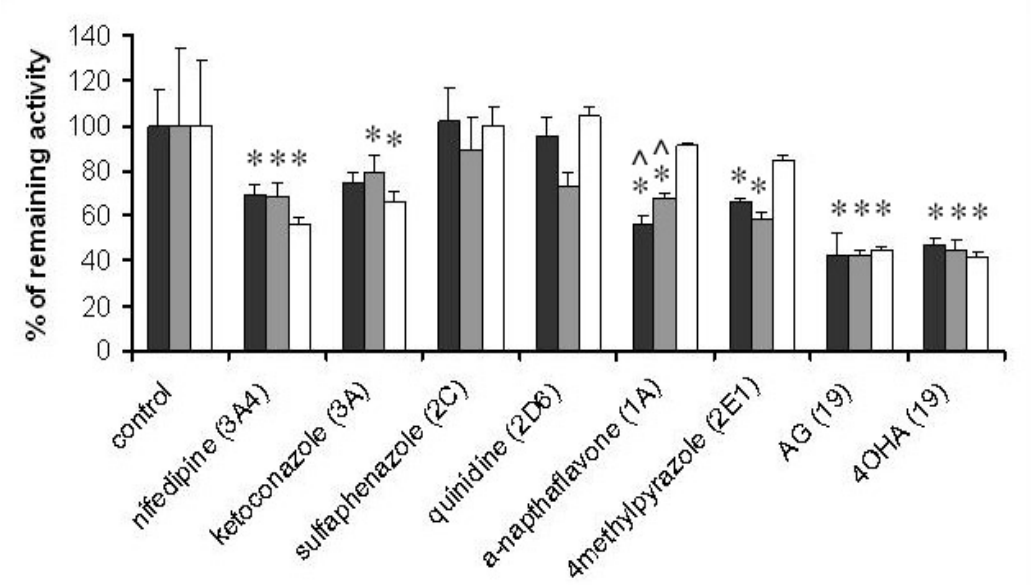

Figure 2. The effect of chemical inhibitors on BUP N-dealkylation by placental microsomes from all gestational age groups (black-17-26 weeks, dark grey-27-33 weeks, light grey- 34-40 weeks).(* versus control $p<0.05$, ^ versus 34-40 weeks $p<0.05$). For each gestational age group, n = 6.

Table 2: Chemical and immuno-inhibition of norBUP formation.

Inhibitor	GA 17-26, %[1]		GA 27-33, %		GA 34-40, %	
	Chemical	*Immuno*	*Chemical*	*Immuno*	*Chemical*	*Immuno*
Control[2]	100 ± 16	100 ± 6	100 ± 34	100 ± 3	100 ± 30	NA
Nifedipine	69 ± 5*	-	69 ± 6*	-	56 ± 3*	-
Ketoconazole	75 ± 4	-	79 ± 8*	-	64 ± 4*	-
3A4 antibody (ab)	-	42 ± 5*	-	36 ± 8*	-	NA
Sulfaphenazole	102 ± 15		89 ± 14	-	100 ± 8	-
2C8 ab	-	104 ± 21	-	122 ± 6	-	NA
2C9 ab	-	100 ± 8	-	85 ± 13	-	NA
2C19 ab	-	43 ± 4*	-	39 ± 2*	-	NA
Quinidine	95 ± 8	-	73 ± 6	-	104 ± 3	-
2D6 ab	-	46 ± 7*	-	41 ± 5*	-	NA
A-napthaflavone	57 ± 3*^	-	68 ± 2*^	-	91 ± 1	-
1A2 ab	-	103 ± 18	-	95 ± 10	-	NA
4-methylpyrazole	66 ± 1*^	-	59 ± 3*^	-	85 ± 2	-
2E1 ab	-	72 ± 3*	-	105 ± 14	-	NA
AG	42 ± 10*	-	42 ± 2*	-	45 ± 1*	-
4OHA	47 ± 3*	-	45 ± 5*	-	42 ± 2*	-
19 ab	-	22 ± 5*	-	28 ± 6*	-	NA
2B6 ab	-	103 ± 17	109 ± 35	-	-	NA

[1] Percent remaining activity compared to control where no inhibitor was present.
[2] The results represent the mean of 6 determinations ± standard error. Pools of 8-10 placental microsomes were used for each experiment as described in the Materials and Methods section.
* Versus control $p<0.05$, ^ versus 34-40 weeks $p<0.05$.
NA- Not available.

Immuno-inhibition

Antibodies to CYP19 caused 80% inhibition of norBUP formation in 17-26 weeks GA pools (Fig. 3) ($p<0.05$). Antibodies to CYP3A4, CYP2C19, and CYP2D6 also produced significant inhibition (60%) of norBUP formation ($p<.0.05$). Antibodies to CYP2E1 produced the lowest inhibition (30%) ($p<0.05$) (Table 2).

The 27-33 weeks GA pool exhibited a similar pattern of antibody inhibition as the 17-26 weeks GA's antibody inhibition results (Fig. 3). Again, CYP19 showed the most significant inhibition with 75%, followed by CYP3A4, CYP2C19 and CYP2D6 with 65%, 60%, and 60% inhibition, respectively ($p<0.05$). No statistically significant inhibition was observed with antibodies against CYP1A2, 2B6, 2C8, and 2C9.

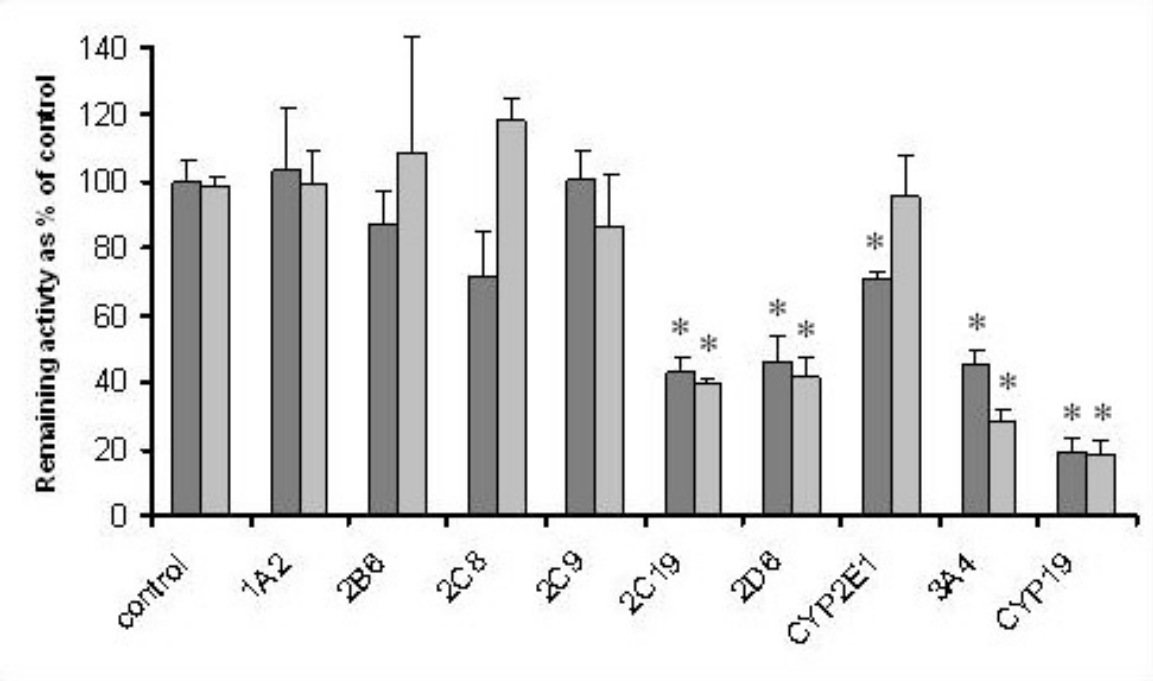

Figure 3: A comparison of immuno-inhibition from 17-26 week gestational age (dark grey) with immuno-inhibition from 27-33 week gestational age. (*$p<0.05$) The pools for each gestational age group included placental preparations from 10 placentas. For each gestational age group , n = 3.

CHAPTER 4: DISCUSSION

Pool of Preterm Placental Microsomes

This project clearly demonstrates that placentas from the second and third trimester metabolize BUP to norBUP as previously shown for term placentas (Deshmukh et al., 2003). Unfortunately, access to placentas earlier than 17 weeks was not available. Therefore, we could not compare our data with the literature data that only reports on enzyme expression from first trimester and term placentas but not from second and early third trimester placentas (Hakkola et al., 1996a,b; Syme et al., 2004). Further investigations using western blot, mRNA detection, and marker substrate activity are necessary to confirm the results of our study for identification of the enzyme(s) responsible for metabolism of BUP.

We were also unable to use the same placental pools throughout the project due to the scarcity of tissue. In most cases, different placentas or pools were used for kinetics, chemical inhibition and immuno-inhibition experiments. Variation between the individual placentas is evident in our results, for example see Table 1. Due to the existing IRB protocol, information on whether the mother smoked or consumed alcohol during pregnancy was not obtained for this study. Since CYP1A1 and 2E1 are known to be induced by cigarette and alcohol consumption, this information would certainly have been helpful in controlling the variability of the results and the overall understanding of drug metabolism by the placenta.

Determination of Estimated Apparent K_m

Estimated apparent K_m values for BUP were 11 ± 2 μM and 12 ± 1 μM, respectively, for placental microsomes obtained from late second and early third trimesters. There was no statistically significant difference between these values or versus the estimated apparent K_m of 13 ± 3 μM for late third trimester placentas. Therefore, the affinity of BUP to the major placental enzyme(s) responsible for its metabolism does not seem to change throughout gestation. As mentioned earlier, this project utilized the same conditions as the previous study of term placentas (Deshmukh et al., 2003). Since re-optimization of the conditions was not carried out for this project, it is not clear whether the estimated apparent K_m values obtained for BUP are reasonable,

precise and do, indeed, remain equivalent throughout gestation. Specifically, proper determination of appropriate incubation time and protein concentration would have helped to clarify these data.

Determination of Estimated V_{max}

The activity of the enzyme, as measured by V_{max}, appeared to increase with gestational age. The estimated V_{max} from late second trimester was 2.1 ± 0.3 pmol.mgP^{-1} min^{-1} and increased with gestational age to 3.8 ± 0.6 pmol.mgP^{-1} min^{-1} during the late third trimester.

Initially, a maximum BUP concentration of 80 μM was used for constructing saturation curves as in the previous report by Deshmukh et al. (2003). In their report, it appeared saturation levels were reached but the saturation levels remained linear when evaluated, by plotting the velocity versus [BUP], in this project. The additional concentration of 120 μM BUP was added in order to reach saturation. The reason behind this discrepancy is unclear but could be due to norBUP contamination in the donated BUP because the actual amount of contamination, as percent, was never investigated. The extent of contamination could be different between batches of BUP, thus leading to a variation in maximal BUP concentration needed for this project as compared to the study of Deshmukh et al. (2003).

CYP19

Identification of the enzyme(s) responsible for metabolism of BUP in placentas from early second trimester to third trimester was carried out by chemical inhibition and immuno-inhibition. Data from both inhibition assays suggest that CYP19 is a major enzyme metabolizing BUP from the gestational age of 17 weeks onward.

CYP19 levels from preterm placentas (17-26 weeks, 27-33 weeks, and 34-40 weeks) were measured previously in the laboratory of Dr. Ahmed using western blot. Although variable within groups, averages from each group did not change significantly with increasing gestational age (Hieronymus et al., 2006). In contrast, Kiwataki et al. (1992) measuring CYP19 expression and activity, using an enzyme-linked immunosorbent assay developed specifically for this study, reported that the CYP19

concentration increased four-fold when comparing placental microsomal preparations from 10 weeks gestation to those from 40 weeks gestation. Kitawaki et al. (1992) further reported that the activity of the enzyme increased only two-fold with gestation as measured by the tritiated water method using [1β-^{3}H, 4-^{14}C] androstenedione as a substrate and the 1β-elimitation mechanism. A major difference between the two reports by the investigators concerns the range of placentas assessed and this variable could account for the discrepant results. Furthermore, it is possible that the increase in activity between gestational ages could be due to increasing concentrations of CYP19 with gestation. Observations from this project are compatible with the CYP19 activity range increase reported by Kitawaki et al. (1992) since these observations also indicate a two-fold increase in activity from late second trimester to late third trimester.

As mentioned previously, the endogenous function of CYP19 is the conversion of androgens to estrogens. Two such conversions are testosterone to 17β-estradiol (E2) and 16α-hydroxytestosterone to estriol (E3) (Zharikova et al., 2006). Previous reports have described a decrease in E3 levels in women maintained on methadone (Facchinetti et al., 1986). Since E3 is a product solely of the placenta, the levels measured are often used as an indicator for the well being of the fetus (Zharikova et al., 2006). Estrogens are very important in development of the fetus and regulation of pregnancy. Estrogens are involved in uteroplacental blood flow, uterine growth, uterine contractility and progesterone production (Zharikova et al., 2006). Zharikova et al. (2006) reported that methadone and BUP, along with their metabolites, act as competitive inhibitors of CYP19 in term placenta. Specifically, the K_i of BUP was 39 μM and 6 μM for E2 and E3 formation, respectively (Zharikova et al., 2006). Reports on BUP plasma concentrations are not available from pregnant women, but reports from a study using male and non-pregnant female volunteers estimate the mean C_{max} value to be 10.4 ng/ml ± 3.5 after administration of an 8 mg sublingual dose of BUP (Compton et al., 2006). BUP has been shown to be a substrate of CYP19 and acted as a competitive inhibitor of CYP19 when endogenous substrates were added and their biotransformation measured. The extent of inhibition of estrogen formation was dependent on the concentration of BUP. BUP appears to be a more potent inhibitor in the conversion of 16α-hydroxytestosterone to E3 possibly because the endogenous substrate has a lower affinity

for the CYP19 enzyme than BUP. Therefore, based on these data, at potential therapeutic concentrations BUP should inhibit CYP19, a key enzyme in the production of important hormone regulators in pregnancy. Based on this inhibitor effect, further investigations of BUP effects on fetal development are clearly warranted.

Additional Enzyme Involvement

In addition to CYP19, new observations from the chemical and immuno-inhibition aspects of this project suggest involvement of CYP3A4 and CYP2E1. However, there was less an agreement between chemical and immuno-inhibition observations for CYP1A, 2D6, and 2C19. In the case of CYP1A, the antibody to CYP1A2 showed no inhibition. Antibodies for CYP1A1, an extra-hepatic, inducible P450 enzyme, were not available. Therefore, the inhibition seen by the selected chemical inhibitor of the CYP1A family, α-napthaflavone, is likely due to its effect on CYP1A1. As mentioned previously, CYP1A1 has been reported to be present and active throughout gestation (Syme et al., 2004; Pasanen, 1999). The discrepancies seen with CYP2D6 and 2C19 could be resolved using a more stringent experimental design which includes the imperative cross-reactivity experiments. It is possible that the antibodies to CYP2D6 and CYP2C19 were cross-reactive with confounding effects on CYP19 that explain the inhibition observed. Specifically, the epitope on the enzyme that the monoclonal antibody is specific for could be present on another enzyme since the P450s are a broad class of enzymes that are similar in structure, composition, and function. Therefore, further studies should be conducted to explain these results. Studies would include extensive characterization of appropriate experimental conditions such as determinations of incubation times and protein concentrations that lie within the linear range for norBUP formation. The current incubation time was selected presumably to allow for maximal norBUP formation since a smaller concentration of microsomal protein was used. The limitation to this parameter is that incubation for 120 minutes at 37 ºC may not approximate steady state conditions. It could be speculated that some enzymes were denatured and were not functional while others continued to be active allowing for the discrepancies observed within the immuno-inhibition data. Additionally, investigation into the specificity of the antibodies supplied would be valuable. Lastly, selection and

determination of suitable antibody concentrations that lie within the higher limit of a linear range of inhibition using typical marker substrates for the enzyme selected would be valuable to test in placental preparations as well as similar concentrations of purified enzymes. This test would not only help determine appropriate antibody concentrations but could also play a role in the investigation of enzyme presence and activity in the placenta.

An additional area of debate about the estimated kinetic data discussed previously arose after conducting the experiments attempting to identify the enzyme(s) responsible for metabolism of BUP. The equation for Michaelis-Menten saturation kinetics is formulated to account for one enzyme and one substrate. After our data suggested that other enzymes could be involved in the metabolism of BUP, the validity of the determination of the apparent K_m and V_{max} values could be argued. Additionally, the determination of these values using the Lineweaver-Burke plot, which is a double reciprocal plot, is not the most rigorous way to calculate the apparent K_m and V_{max} values due to the minimization of the variation between activity data points at a given substrate concentration.

BUP Metabolites

Previous studies in Dr. Ahmed's laboratory utilizing placental perfusion with BUP identified norBUP as the only metabolite formed. No glucuronidated BUP or norBUP was found (Nanovskaya et al., 2002). However, hepatic phase II metabolism studies by Cone et al. (1984) and later confirmed by Chang et al. (2006) have identified glucuronidated BUP and norBUP. Uridine diphosphate glucuronosyltransferase mRNA and protein have been identified in the syncitium throughout gestation although in varying amounts (Syme et al., 2004). As mentioned, it was reported that no glucuronidation of BUP or norBUP was found by the placenta in perfusion studies, but a deeper investigation into this mechanism should be pursued (Nanovskaya et al., 2002). Perhaps the level of glucuronic acid is significantly decreased after removal of the tissue from the mother and through the subsequent preparation of microsomes, so that glucuronidation no longer exists as a mechanism of detoxification in the *in vitro* studies. Therefore, other possible metabolites, especially the newly identified metabolites by

Chang et al. (2006), cannot be ruled out as products of metabolism of BUP by the placenta.

Conclusion

Overall, this project has contributed the groundwork that warrants careful use of preterm placentas for investigations of drug metabolism and the enzymes involved. Recent reports reveal that BUP metabolism, both hepatic and placental, is much more complex than initially thought (Zhang et al., 2003, Picard et al., 2005, Chang et al., 2006, Zharikova et al., 2006). This project indicates that activity of the enzymes metabolizing BUP increases with gestational age. Metabolism of BUP by the placenta could very likely be carried out by more than just CYP19 and could involve different enzymes at different times of gestation. Future research to validate these observations should include an inquiry into CYP isozyme presence and activity at different gestational ages, cross-reactivity of antibodies to CYP19 using proper controls, BUP metabolism using a wider range of gestational age placentas (specifically, from first and early second trimesters), and identification of additional BUP metabolites. Clinically, periodic measurement of estriol levels in pregnant women undergoing BUP therapy should be a routine test due to the reported in vitro inhibition of estrogen formation via CYP19 by BUP. Such information would provide a framework for interpreting clinical assessments of potential negative long-lasting effects of in utero exposure to BUP.

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Curriculum Vitae

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- ## Educational Qualifications:

B.S. (Biology) Texas A&M University, College Station, Texas (2003)

M.S. (Pharmacology and Toxicology) University of Texas Medical Branch Galveston, Texas (2007)

- ## Research Experience:

Apr. 2000-Aug. 2001:	Vet Technician, Weslayan Animal Clinic, Houston, Texas
Nov. 2002-May 2004:	Research Assistant, Department of Veterinary Pathobiology, Texas A&M University, College Station, Texas
May 2004-Feb. 2007:	Research Assistant, Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, Texas
Feb. 2007- Oct. 2007:	Research Technician, Hyperion Biotechnology, Inc. Brooks City-Base, San Antonio, Texas
Nov. 2007- present:	Quality Control Scientist, Mission Pharmacal, Boerne, Texas

- ## Expertise:

1. Enzyme kinetics
2. Drug metabolism

3. Proficient with HPLC-UV
4. Experienced in developing methodology to separate drug metabolites using HPLC-UV
5. Familiar with LC-MS identification of compounds
6. Proficient in analysis of LC-MS chromatograms
7. Preparation of subcellular fractions
8. Experienced in immunohistochemistry
9. Proficient in Western blotting
10. Familiar with RT-PCR
11. Experienced in animal handling, treatment, and surgery
12. Data analysis and statistical analysis using Microsoft Excel and SPSS

- **Abstracts:**

1. **McRee, R.**, Ravindran, S., Zharikova, O., Vargas, R., Nanovkaya, T., Hankins, G., Ahmed, M. Buprenorphine metabolism by preterm human placentas. The College on Problems of Drug Dependence 68:133 2006.

2. Apte, U. M., **McRee, R.**, Nguyen, J., and Ramaiah, S. K. Role of calpain in endotoxin-mediated hepatic injury. *Toxicologist*, 2004

3. Apte, U. M., **McRee, R**., Ramaiah, S. K. Osteopontin induction: possible mechanism of increased sensitivity of steatotic hepatocytes to alcohol-induced liver damage *Internat. J. Toxicol*. 2003

4. Apte, U. M., **McRee, R.**, Ramaiah, S. K. Osteopontin induction: possible mechanism behind hepatic neutrophil infiltration during alcoholic steatohepatitis *Hepatology* 38:228, 2003.

5. Apte, U. M., **McRee, R.**, Ramaiah, S. K. Increased hepatocyte division leads to transient decrease in liver injury following chronic ethanol consumption *Veterinary Pathology* 40:134, 2003.

- **Manuscripts:**

1. Apte, U. M., **McRee, R.,** and Ramaiah, S. K. Hepatocyte proliferation is the mechanism for the decrease in liver injury during steatosis stage of alcoholic liver disease. *Toxicol. Pathol.* 32:567-76, 2004.

2. Apte, U.M., Banerjee, A., **McRee, R**., Wellberg, E., Ramaiah, S.K. Role of osteopontinin hepatic neutrophil infiltrationduring alcoholic steatosis. *Toxicol Appl Pharmacol.* 207:25-38, 2005.

- ## Previous Research:

Discovery of salivary biomarkers: Currently working on developing methods for discovery of biomarkers in human saliva using HPLC/mass spectrometry.

Buprenorphine metabolism by human placenta: Investigation of differences between term and preterm placental metabolism of Buprenorphine, a μ-opioid partial agonist used for maintenance of the opiate addict.

Mechanisms of alcoholic steatohepatitis: Role of extracellular matrix and matricellular proteins, especially osteopontin, in neutrophil attraction.

Interaction of alcohol consumption and environmental toxicants: Mechanisms behind potentiation of toxicity of environmental compounds. Interaction of alcohol with bacterial endotoxin (LPS) due to chronic alcohol consumption.

- ## Committee Membership:

UTMB Institutional Animal Care and Use Committee (2004- 2006)

- ## Academic Societies:

Society of Toxicology (SOT)