REGULATION OF GLUCURONIDATION (UGT1A9) PATHWAY DURING PREGNANCY

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Pregnancy is associated with many physiological changes. During pregnancy, the pharmacokinetics of certain drugs are altered. In particular, the clearance of drugs undergoing glucuronidation has been reported to drastically increase during pregnancy. Pregnancy-mediated changes in the plasma concentrations of estrogens and progesterone have been suggested to influence the expression and activity of certain phase I metabolic enzymes. Limited information, however, is available on the effect of pregnancy-related hormones on the activity of glucuronide-conjugating enzymes in humans. In this study, we determined the optimal conditions for evaluating glucuronidation in human liver microsomal systems. We also determined the effects of progesterone and estradiol on the expression of UGT1A9 mRNA, and the activity UGT1A9 (formation of mycophenolic acid glucuronide (MPAG) from mycophenolic acid (MPA)) using primary cultures of human hepatocytes. The results showed a non-significant increase in the expression of UGT1A9 and in the formation of mycophenolic acid glucuronide from MPA in human hepatocytes treated with a combination of progesterone and estradiol. Progesterone and estradiol do not appear to significantly alter the metabolism of the substrates of UGT1A9. Future studies should evaluate additional factors that may account for the observed alterations in UGT1A9 activity during pregnancy.
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PREFACE

In name of Allah, the Most Beneficent, the Most Merciful. All praise and thanks are due to Allah, and peace and blessings be upon His Messenger. I wish to express my love and gratitude to my parents for their patience and prayers and to my great family, my wife, Fatimah, and my daughter, Raseel, for their understanding and endless love through the duration of this work.

I wish to express my sincere gratitude to my advisor Dr. Raman Venkataramanan for the valuable guidance and advice and who taught me the value of hard work and an education. Without him, I may never have gotten to where I am today. I sincerely thank my committee members, Dr. Michael Zemaitis and Dr. Regis Vollmer, for their valuable comments and help in completing this project. I also wish to thank my great colleague, Mohammed Shawaqfed, for his great help and assistance throughout this work. Not forgetting my technical support by HPLC expert, Wench Zhao, for his help and kindness. I would also like to convey thanks to King Khalid University for providing me with the scholarship and supporting my graduate studies.
Eighty-three percent of pregnant women are prescribed at least one prescription drug, with most of them being antibiotics or analgesics (Mitchell et al., 2011). Other frequently prescribed medications during pregnancy include antidepressants, antihistamines, antiepileptics, diuretics and medications that are used to treat pregnancy-induced conditions such as hypertension, gestational diabetes and emesis (Mitchell et al., 2011)

**Pharmacokinetics in Pregnancy**

Pharmacokinetics of drugs are not well characterized during pregnancy because of the exclusion of pregnant women from clinical trials (Blackburn, 2012). Pregnancy is known to be associated with many physiological changes. These changes can alter the absorption, distribution, metabolism, and excretion of various drugs (Anderson, 2005). Several factors contribute to the altered absorption of drugs during pregnancy. Increased cardiac output during pregnancy has been associated with an increase in the rate of absorption of certain drugs from the gastrointestinal tract (Yaffe & Aranda, 1992). Increase in gastric pH during pregnancy tends to reduce absorption of weak acids and lead to increase $T_{\text{max}}$ and decrease $C_{\text{max}}$ (Dawes & Chowienczyk, 2001). Nausea and vomiting, which are common in pregnant women, may interfere with absorption of orally administered drugs (Dawes & Chowienczyk, 2001). In such
cases, administration of drugs via inhalation could enhance the absorption of drugs due to increase in cardiac output and tidal volume that leads to increase in alveoli uptake (Dawes & Chowienczyk, 2001). Drug absorption from the intramuscular route has been reported to be increased due to increased tissue blood perfusion (Dawes & Chowienczyk, 2001).

Several factors contribute to the altered distribution of drugs during pregnancy. The maternal blood volume increases during pregnancy to supply the uteroplacental unit and the fetus (Lee, 1991; Whittaker, Macphail, & Lind, 1996). The total body water and plasma volume increase by approximately 50% by the end of gestation (Cunningham & Williams, 2010). These changes will increase the volume of distribution of hydrophilic drugs (Anderson, 2005). Concentrations of some of the plasma proteins (e.g., albumin and alpha-1 acid glycoprotein) are decreased during pregnancy resulting in an increase in the unbound fraction of highly protein-bound drugs, and corresponding increase in the apparent volume of distribution (Anderson, 2005) and (Little, 1999; Perucca & Crema, 1982). The drugs that are influenced to the greatest extent by changes in plasma protein binding are low extraction ration drugs such as phenytoin, and high extraction ration drugs that have a narrow therapeutic window (Anderson, 2005). In addition, body fat increases during pregnancy causing an increase in the volume of distribution of lipophilic drugs (Little, 1999). Increase in volume of distribution typically leads to a decrease in maximum concentrations of the drugs in plasma (Dawes & Chowienczyk, 2001).

Pregnancy is also associated with increased clearance of certain drugs. The increase in renal blood flow and decrease in renal vascular resistance leads to an increase in glomerular filtration rate in pregnant women (Anderson, 2005; Pavek, Ceckova, & Staud, 2009). Other physiological changes associated with pregnancy include an increase in heart rate, blood
pressure, blood volume, tidal volume, gastric emptying time and other changes in other body systems such as the kidney and the liver (Feghali & Mattison, 2011; Hill & Pickinpaugh, 2008; Yeomans & Gilstrap, 2005). Drug metabolism has also been reported to be altered during pregnancy (Jeong, Choi, Song, Chen, & Fischer, 2008; Tracy, Venkataramanan, Glover, & Caritis, 2005; Tsutsumi et al., 2001).

Cytochrome P450 (CYPs), uridine diphosphate glucuronosyltransferases (UGTs), sulfonil transferases and N-acetyltransferase (NAT) (Anderson, 2005) are some of the common drug metabolizing enzymes in humans. The activity of certain cytochrome P450 and UGT isoforms has been reported to be altered in pregnancy (Anderson, 2005; Pavek et al., 2009). The activity of cytochrome P450 3A4 (CYP3A4), the most highly expressed CYP enzyme in the human liver and gastrointestinal tract (Anderson, 2005), is increased during pregnancy (Pavek et al., 2009). The metabolism of methadone, nifedipine and indinavir, which are substrates of CYP3A4, have been reported to increase in pregnancy (Pavek et al., 2009). Likewise, cytochrome P450 2A6 (CYP2A6) activity increases in the second and third trimesters of pregnancy (Pavek et al., 2009). Clearance of nicotine and cotinin, substrates of CYP2A6, increases during pregnancy (Anderson, 2005; Pavek et al., 2009). The activity of cytochrome P450 2C9 (CYP2C9) and cytochrome P450 2D6 (CYP2D6) also increases during pregnancy (Anderson, 2005; Pavek et al., 2009). A recent study has shown an increase in the activity of cytochrome P450 2D6 (CYP2D6) during pregnancy, which was responsible for the increased clearance of clonidine (Claessens et al., 2010). On the other hand, a decrease in the activity of cytochrome P450 1A2 (CYP1A2) in all three trimesters has also been reported (Tsutsumi et al., 2001). The activity of cytochrome P450 1A2 (CYP1A2) decreases throughout pregnancy (Pavek
Correspondingly, the metabolism of caffeine, a CYP1A2 probe, is decreased during pregnancy (Pavek et al., 2009).

Some studies have shown an increase in glucuronidation during pregnancy (Chen, Yang, Choi, Fischer, & Jeong, 2009; Miners, Robson, & Birckett, 1986). UDP-glucuronosyltransferase 1A4 (UGT1A4) is the most well-studied UGT isoform during pregnancy. Studies have shown a significant increase in the clearance of lamotrigine in pregnant women compared to non-pregnant women (Anderson, 2005; Chen et al., 2009; Pavek et al., 2009). The clearance of morphine and zidovudine, which are substrates of UGT2B7, has been reported to increase during pregnancy suggesting an increase in the activity of UGT2B7 (Pavek et al., 2009). An increase in the oral clearance of labetalol, which is a substrate of UGT1A1, during pregnancy, has also been reported (Jeong et al., 2008).

The mechanism underlying alteration in the activity of various drug-metabolizing enzymes is not completely clear. Various pregnancy-related hormones have been implicated as potential agents that contribute to altered enzyme activity during pregnancy. Progesterone levels at third trimester average nearly 150 ng/ml, representing an approximately ten-fold increase over normal levels in menstrual cycle (Pfaff, 2002). Estradiol levels range from 10 to 15 ng/ml, representing a fifty-fold increase over normal levels (Pfaff, 2002). Given that the blood levels of female hormones such as estradiol and progesterone are increased during pregnancy, it is logical to expect a potential role of these hormones in regulating the metabolism and the pharmacokinetics of various drugs (Chen et al., 2009). Recent observations have suggested a role for estrogens and progesterone as regulators of altered activity of CYP enzymes in pregnancy (Choi, Koh, & Jeong, 2012). Nothing is known about what factors regulate the expression and
activity of UGTs during pregnancy. In this document, we focus on the activity of one of the UGTs and evaluate the role of estrogens and progesterone on the activity of the UGTs. The following is a brief discussion of various UGT pathways.

UDP-glucuronosyltransferases (UGTs) are phase II metabolic enzymes that catalyze the conjugation of many endogenous (e.g., bilirubin, bile acids, steroid hormones, thyroid hormones), and exogenous compounds (e.g. mycophenolic acid, morphine, buprenorphine and acetaminophen) with glucuronic acid (Radominska-Pandya, Czernik, Little, Battaglia, & Mackenzie, 1999) (Table 1). This conjugation makes compounds more hydrophilic and facilitates renal and biliary excretion (Williams & Lemke, 2002). UGTs are located in the endoplasmic reticulum (ER) of the liver, endothelial cells of intestine and other tissues (Williams & Lemke, 2002). They are classified into two major groups, UGT1 and UGT2, based on nucleotide and amino acid sequences (Luquita et al., 2001) and are then subdivided into further subfamilies (King, Rios, Green, & Tephly, 2000). The major UGTs isoforms expressed in the liver include UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B15, 2B17 and 2B28 (Miners, Smith, Sorich, McKinnon, & Mackenzie, 2004). Some UGT isoforms are also expressed in other tissues such as lung, small intestine, kidney, olfactory epithelium, ovary, mammary gland, testis, and brain (Miners et al., 2004). However, UGT1A8 and UGT1A10 are expressed predominately in gastrointestinal tract (Mojarrabi & Mackenzie, 1998). UGT1A1 and UGT1A6 are absent from fetal liver, while UGT1A3 is present in fetal liver (McCarver & Hines, 2002).

Although a majority of glucuronidated compounds are inactive, some glucuronides exhibit higher activity than the parent drugs (Liston, Markowitz, & DeVane, 2001). For instance, morphine 6- glucuronide is found to be more potent than parent drug morphine and digitoxin-
and digoxin-glucuronides have positive inotropic effects (Pasternak, Bodnar, Clark, & Inturrisi, 1987; Scholz & Schmitz, 1984). Various isoforms of UGTs exhibit overlapping selectivity which makes studying one particular isoform somewhat difficult (Miners et al., 2004).

An exhaustive literature search revealed that drug metabolism in general has not been well-studied in pregnancy; in particular, the regulation of the glucuronidation pathway has not been well characterized in pregnancy. Most of studies are done in animals and cannot be extrapolated to humans. So it is important to use a human-based system such as human hepatocytes or human liver microsomes. Therefore, more research on factors that regulate the expression and/or the activity of UGTs is necessary in humans in order to optimize drug therapy in pregnancy. This study is a first attempt at characterizing the effect of estrogen and progesterone on the activity of UGTs using primary cultures of human hepatocytes.

Selection of Method to Study UGT Activity

The active site of UGT is hidden within the lumen of ER (Chang, Yoo, Lee, Klopf, & Takao, 2009). Therefore, the activity of UGTs in vitro exhibits latency. Disruption of the membrane by chemical (e.g., brij58, alamehticin) (Dutton, 1980; Engtrakul, Foti, Strelevitz, & Fisher, 2005; Fisher, Campanale, Ackermann, VandenBranden, & Wrighton, 2000; Kilford, Stringer, Sohal, Houston, & Galetin, 2009) and mechanical (e.g., sonication, freeze/thaw and vortexing) means have been used to characterize the activity of UGTs (Dutton, 1980). The activity of UGTs has been shown to be higher with such treatment (Chang et al., 2009). The most
widely used membrane-disturbing agents are alamethicin and brij58. Alamethicin, a pore forming 20 amino acid peptide (Bechinger, 1997), is widely used to study the activity of UGTs. It forms pores in the ER membrane, which facilitates the passage of substrate and the cofactor, UDP-glucuronic acid (UDPGA), through the membrane (Chang et al., 2009). Alamethicin showed the highest activation of UGTs compared with other tested detergents (Fisher, Paine, Strelevitz, & Wrighton, 2001; Soars, Ring, & Wrighton, 2003; Vashishtha, Hawes, McCann, Ghosheh, & Hogg, 2002). Another commonly used detergent is brij58 (polyoxyethylene monocetyl ether). It is a non-ionic surfactant with high hydrophilic-lipophilic balance (HLB) and low critical micelle concentration (CMC) (Rai, Tan, & Michniak-Kohn, 2011). It is used to enhance membrane permeability to improve measurement of the activity of UGT. In this study we compared the effects of alamethicin and brij58 on UGT1A9 activity in microsomes.

Selection of UGT and a Probe for Measuring its Activity

UDP-glucuronosyltransererase (UGT) 1A9 is expressed in the liver, kidney and colon (McGurk, Brierley, & Burchell, 1998; Strassburg, Manns, & Tukey, 1998; Wooster et al., 1991). It conjugates planar and nonplanar phenols, anthraquinons, flavones, aliphatic alcohols, aromatic carboxylic acids and steroids (Liston et al., 2001). It metabolizes many drugs including propranolol, labetalol, acetaminophen, mycophenolic acid, ibuprofen and dapsone and endogenous compounds such as estrone, 4-hydroxyestrone and retinoic acid (Liston et al., 2001). An extensive literature search revealed that no study has investigated the activity of UGT1A9
during pregnancy. UGT1A9 glucuronidates a wide range of substrates (Ebner & Burchell, 1993) and is predominantly expressed in major metabolic tissues, liver and kidney, it is necessary to study its activity during pregnancy. Given that the activity of UGT1A1 has been reported to be increased by progesterone (Jeong et al., 2008) and the activity of UGT1A4 is increased by estrogen (Chen et al., 2009), it was of interest to evaluate the potential effects of estrogen and progesterone on the activity of UGT1A9.

Mycophenolic acid (MPA), an active metabolite of mycophenolate mofetil (MMF), an immunosuppressant agent, acts by inhibition of inosine monophosphate dehydrogenase (IMPDH) in lymphocytes (Bullingham, Nicholls, & Kamm, 1998). Mycophenolic acid is mainly metabolized by UGT1A9 to the hydroxyl-β-glucuronide (MPAG) and to other two metabolites, MPA-acyl-glucuronide (AcMPAG) and MPA-phenyl-glucuronide (Picard, Ratanasavanh, Premaud, Le Meur, & Marquet, 2005). Most of the oral dose of MMF is excreted in the urine as MPAG (Bullingham et al., 1998). Mycophenolic acid serves as a good marker substrate of UGT1A9.

The overall objective of this study is to set up a system for measuring the activity of UGT1A9 in human microsomes and hepatocytes. We hypothesized that treatment with alamethicin will lead to the highest measured activity of UGT1A9 in human liver microsomes, and that the expression of and activity of UGT1A9 will be increased by estradiol and progesterone in human hepatocytes. The specific aims of this study were a) to determine the effect of various surfactants (alamethicin and brij58) on the activity of UGT1A9 in microsomal systems; and b) to evaluate the effect of progesterone and estradiol on the expression and activity of UGT1A9 in primary cultures of human hepatocytes.
Table 1: Some endogenous and exogenous substrates for major human UGT isoforms

<table>
<thead>
<tr>
<th>UGT isoform</th>
<th>Endogenous substrates</th>
<th>Exogenous substrates</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>UGT1A1</td>
<td>Bilirubin, thyroxine</td>
<td>Estradiol, paracetamol</td>
<td>(Bock, 2010; Court, 2005)</td>
</tr>
<tr>
<td>UGT1A4</td>
<td>Androsterone</td>
<td>Lamotrigine, imipramine, nicotine</td>
<td>(Bock, 2010; Chen et al., 2009; Picard et al., 2005)</td>
</tr>
<tr>
<td>UGT1A9</td>
<td>Thyroxine, estrogens</td>
<td>Mycophenolic acid, propofol, paracetamol</td>
<td>(Bock, 2010; Court, 2005; Radominska-Pandya et al., 1999)</td>
</tr>
<tr>
<td>UGT2B4</td>
<td>Androsterone, bile acids, arachidonic acid</td>
<td>Fibrates</td>
<td>(Bock, 2010; Court, 2005; Radominska-Pandya et al., 1999)</td>
</tr>
<tr>
<td>UGT2B7</td>
<td>4-hydroxyesterone, progesterone, retinoids</td>
<td>Morphine, zidovudine</td>
<td>(Bock, 2010; Court, 2005; Radominska-Pandya et al., 1999)</td>
</tr>
</tbody>
</table>
2.0 MATERIALS AND METHODS

2.1 CHEMICALS

Mycophenolic acid (MPA), mycophenolic acid glucuronide (MPAG), uridine 5’ diphospho-glucurinic acid (UDPGA), brij58, rifampicin, testosterone, saccharolactone, magnesium chloride (MgCl₂) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO). Alamethicin was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Bovine serum albumin (BSA) was purchased from Fluka (Buchs, Switzerland). 6β-hydroxytestosterone was purchased from Steraloids (Waltham, MA). Reagents for real time PCR were purchased from Promega (Madison, WI). Methanol and water (HPLC Grade) were purchased from Thermo Fisher Scientific (Waltham, MA). All tissues were obtained from the liver tissue cell distribution system, from the Hepatocyte Transplantation Laboratory at the University of Pittsburgh (Pittsburgh, PA).
2.2 EVALUATION OF MPAG FORMATION IN HUMAN LIVER MICROSOMES

2.2.1 Microsomal Preparation

Microsomes were prepared by differential centrifugation as previously described (Charpentier et al., 1997). Briefly, liver tissues were homogenized with three volumes of homogenization buffer (50 mM Tris–HCl buffer, 1% KCl and 1mM EDTA, pH 7.4) using an electrical homogenizer (Polytron, Brinkmann Instruments, Westbury, NY). The homogenate was centrifuged at 10,000g for 20 minutes at 4°C. The supernatant was further centrifuged at 105,000g for 65 min at 4°C. The microsomes were reconstituted with 50mM Tris-HCl buffer containing 20% glycerol. Aliquots were stored at -80°C until used. The protein concentration was determined by the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951).

2.2.2 Microsomal Incubation

Glucuronidation of MPA was characterized as previously described (Picard et al., 2005) with some modifications. The reaction mixture consisted of 0.5 mg/ml microsomal proteins (prepared in Tris-HCl buffer), 0.1M Phosphate buffer (pH 7.4), brij58 (0.015%
w/v final concentration protein) or alamethicin (50 µg/mg protein), 10mM MgCl₂, 6mM saccharolactone, 0.05 to 2mM mycophenolic acid, and 2mM uridine 5′-diphosphoglucuronic acid (UDPGA). The final volume of the reaction mixture was 500µl. Microsomes were preincubated in ice with buffer and brij58 or alamethicin for 15 min. MgCl₂ and saccharolactone and MPA were added to the mixture and pre-incubated at 37°C. The microsomal incubation was warmed up to 37°C, and, to initiate the reaction, UDPGA was added and the entire mixture was incubated for 30 min at 37°C. The reaction was stopped by the addition 25µl of perchloric acid (24% v/v). After centrifugation, the supernatant was stored at -20°C, until analysis of MPAG by high performance liquid chromatography (HPLC). The concentrations of alamethicin and brij58 used based on the concentrations that provided maximal activation of UGT (Chang et al., 2009; Court et al., 2001; Soars et al., 2003).
2.3 EVALUATION OF THE EFFECT OF PROGESTERONE AND ESTRADIOL ON
THE FORMATION OF MPAG IN HUMAN HEPATOCYTES

2.3.1 Preparation of Human Hepatocytes

Human hepatocytes were obtained from Hepatocyte Transplantation Laboratory at the University
of Pittsburgh (Pittsburgh, PA). Hepatocytes were isolated by a three-step collagenase perfusion
method (Strom et al., 1996). The viability of the cells used ranged from 80 to 91% as measured
by a trypan blue exclusion test. Briefly, trypan blue (0.4%) and cell suspension were mixed and
then placed on a hemocytometer. The cells were observed under a light microscopy and the
number of living and dead cells was counted. For study purpose, cells were diluted to a final
volume of 1×10⁶ cells/ml in hepatocyte medium. Hepatocytes were plated on Falcon 6-well
culture plates previously coated with rat tail collagen at a density of 1.5×10⁶ cells, and
maintained in hepatocytes maintenance medium (HMM, Lonza Walkersville, Inc.) supplemented
with 0.1 µM insulin, 0.1 µM dexamethasone, 0.05% streptomycin, 0.05% amphotericin B and
10% bovine calf serum. After allowing 4-6 h for the cells to attach, the medium was replaced
with serum free medium (HMM+). Cells were maintained in culture at 37 °C in an atmosphere
containing 5% CO₂ and 95% air. After 24 h in culture, unattached cells were removed by gentle
agitation and the medium was changed every 24 hours.
The concentrations of progesterone and estradiol were chosen based on their levels in subjects during the third trimester of pregnancy (Becker, 2001). Two days after plating, the cells were treated for four days with HMM+ containing rifampin (10 µM, estradiol (0.1, 1 µM), progesterone (1, 10 µM) or a combination of progesterone and estradiol at low and high concentrations. Stock solutions of progesterone and estradiol were prepared in DMSO. The final concentration of DMSO in the medium used for incubation was less than 0.1%. The cells were observed daily under microscope to monitor cell morphology and attachment. On the fifth day, the medium was changed and fresh HMM+ containing MPA (100 µM) or testosterone (250 µM) was added and incubated for 30 min. After the end of the incubation period, the media were collected into 1.5 ml Eppendorf tubes. Testosterone served as a positive control for assessment of the functional capacity of the hepatocytes as measured by CYP3A activity. Treatment of hepatocytes with rifampicin served as a positive control to document the responsiveness of the hepatocytes to external stimulus. Experiments were performed in duplicate in human hepatocytes from three different donors. The characteristics of human liver donors are listed in Table (2).
Table 2: Human liver donor Characteristics

<table>
<thead>
<tr>
<th>Donor</th>
<th>Sex</th>
<th>Age</th>
<th>Medical History</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH1956</td>
<td>M</td>
<td>ND</td>
<td>Explanted liver</td>
<td>80</td>
</tr>
<tr>
<td>HH1958</td>
<td>F</td>
<td>ND</td>
<td>Steatosis</td>
<td>80</td>
</tr>
<tr>
<td>HH1962</td>
<td>F</td>
<td>62</td>
<td>Metastatic tumor</td>
<td>91</td>
</tr>
</tbody>
</table>

M, male; F, female; ND, no data

2.3.3 UGT1A9 mRNA Expression in Human Hepatocytes

Total RNAs were isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and used as a template for cDNA synthesis. RNA concentration was determined by UV absorbance at 260/280 nm (µQuant Microplate 25 Spectrophotometer). RNA integrity was measured by 0.5 % agarose gel electrophoresis with ethidium bromide staining. The cDNA was prepared as previously described (Komoroski et al., 2004). RT-PCR was performed using TaqMan system and conditions designated by Assays on Demand, Gene Expression Products (Applied Biosystems, Foster City, CA). The primers sequences used were provided by Assays on Demand, Gene Expression assay ID Hs99999904_m1 to detect cyclophilin A and Hs02516855_sH to detect UGT1A9.
mRNA expression levels were measured in triplicate and normalized to mRNA content of cyclophilin A.

### 2.4 ANALYTICAL METHODOLOGY

#### 2.4.1 Testosterone Analysis

Stock solution of 6β-hydroxytestosterone was prepared in methanol and further diluted with HMM and used for standard curve. Standard curve samples and hepatocyte medium samples were treated with methanol and vortexed. The samples were centrifuged at 13000 rpm for 5 min. One hundred µl samples were injected on to high-pressure liquid chromatographic system (HPLC). Testosterone and 6β-hydroxytestosterone were analyzed using Alliance HPLC system (Waters, Milford, MA). A reverse phase Hibar® RT 250-4 pre-packed column (4.6_250 mm, 0.5 µm) (Merck KGaA, Darmstadt, Germany) was used. The mobile phase consisted of 60% methanol: 40% water (v/v) and the run time were 20 minutes. The retention time for 6β-hydroxytestosterone was 5 min. Empower 2 software was used for data acquisition and analysis. The standard curve was linear in the concentration range of 0.2 – 10 µg/ml for 6β-hydroxytestosterone. Concentrations of the metabolites observed in all samples were within this concentration range. The coefficient of variation (CV %) for the assay was less than 6%.

Mycophenolic acid glucuronide was analyzed on an Alliance HPLC system (Waters 2695, Milford, MA) attached to a photodiode array detector (PDA, Waters 2998) set at 250 nm.
The mobile phase consisted of (A) 20 mM ammonium acetate in 95% water 5% (B) methanol, (C) 95% water 5% methanol and (D) acetonitrile. Various components were separated using a Symmetry® C18 (4.6×250 mm, 5µm) column and the retention time of MPAG was 8.8 min. The concentration of MPAG was determined from a linear standard curve of containing known concentrations of MPAG (0.0675-10µg/ml) (Figure 1). The coefficient of variation of this assay was less than 10% at all concentrations tested.
Figure 1: Standard curves of mycophenolic acid glucuronide (MPAG)

a) Standard curve used for estimation of MPAG concentrations in human liver microsomes,

b) Standard curve used for estimation of MPAG concentrations in primary culture human liver hepatocytes.

2.5 STATISTICAL ANALYSIS

The data were analyzed by Student’s t-test and one-way ANOVA using Microsoft Excel 2007. The Michaelis-Menten kinetic parameters, $K_m$ and $V_{max}$, were determined by GraphPad Prism 5 software. The results were considered statistically significant at $p \leq 0.05$. 
3.0 RESULTS

3.1 VALIDATION OF HEPATOCYTE FUNCTION

The baseline activity of CYP3A was greater than 0.5 µg/ml in all the cultures tested as measured by the formation of 6-β (OH) testosterone (Table 3). In HH1956, rifampicin (10µM) increased 6-β (OH) testosterone formation by twofold, but this increase wasn’t statistically significant ($p=0.28$) (Figure 2). In HH1958 and HH1962, rifampicin (10µM) significantly increased 6-β (OH) testosterone formation by two- ($p=0.02$) and fivefold ($p=0.002$) respectively, indicating the responsiveness of the hepatocytes to inducers (Figure 2).

Table 3: Effect of rifampicin on the activity of CYP3A4 as measured by the formation of 6β-hydroxytestosterone. Basal activity of greater than 0.5 µg/ml and a change in activity of at least 50% are considered acceptable.

<table>
<thead>
<tr>
<th>Liver no.</th>
<th>Untreated (µg/ml)</th>
<th>Treated with 10 µM rifampicin (µg/ml)</th>
<th>Fold change</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HH1956</td>
<td>0.84</td>
<td>1.74</td>
<td>2</td>
<td>0.28</td>
</tr>
<tr>
<td>HH1958</td>
<td>3.8</td>
<td>8.8</td>
<td>2</td>
<td>0.02</td>
</tr>
<tr>
<td>HH1962</td>
<td>1.5</td>
<td>7.7</td>
<td>5</td>
<td>0.002</td>
</tr>
</tbody>
</table>
Human liver hepatocytes prepared from donors indicated in the figure were treated with rifampicin (10µM) for 4 days. On the fifth day, fresh media including testosterone (250µM) were added and incubated for 30 min. After 30 min of incubation, aliquots of the media were removed and testosterone 6β-hydroxylase formation was determined as described in Materials and Methods section. Each value represents means of duplicate treatments. Abbreviations: TST, testosterone; RIF, rifampicin; TST+RIF, incubation of testosterone in presence of rifampicin. (*) Significantly different from cells not treated with rifampicin ($p \leq 0.05$) (Student’s $t$-test).
Using the standard incubation conditions described above, enzyme kinetic parameters for the formation of MPAG were determined in human liver microsomes treated with different surfactants. The formation of MPAG was consistent with Michaelis-Menton kinetics at the substrate concentrations used in the experiment (Figure 3). Treatment with alamethicin showed an increase in the formation rate of MPAG; however, we didn’t detect any statistical significance using one-way ANOVA test ($p=0.16$). $V_{max}$ following treatment with alamethicin was higher than following treatment with brij58; however, it was not significant ($p=0.45$) (Table 4). The $K_m$ values were relatively same for all treatments (Table 4). A two fold increase in $V_{max}$ with alamethacin and brij58 suggest that is better to use these surfactants with microsomes, when UGT1A9 activity is to be measured.

**Table 4: Kinetic parameters of MPA glucuronidation in alamethacin and brij58- pretreated mixture of human liver microsomes**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Alamethicin</th>
<th>Brij58</th>
<th>$p$-value (one-way ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$ (nmol/min/mg protein)</td>
<td>4137</td>
<td>11460</td>
<td>7193</td>
<td>0.45</td>
</tr>
<tr>
<td>$K_m$ ($\mu$M)</td>
<td>9498</td>
<td>1185</td>
<td>1119</td>
<td>0.83</td>
</tr>
</tbody>
</table>
Human liver microsomes were preincubated with brij58 (0.015% w/v final concentration protein) or alamethicin (50 µg/mg protein) for 15 min. Different concentrations of mycophenolic acid were added to the mixture and incubated at 37°C. After 30 min of incubation, aliquots of the media were removed and mycophenolic acid glucuronide (MPAG) formation rate was determined by high performance liquid chromatography (HPLC) as described in the materials and methods section. Data were analyzed by one-way ANOVA to detect statistical significance. Each value represents the means of duplicate treatments (n=2).

Figure 3: Effect of alamethicin and brij 58 on the rate of formation of MPAG
3.3 HUMAN HEPATOCYTE INCUBATION

The potential regulation of UGT1A9 by estradiol and progesterone was examined using primary cultures of human hepatocytes. The formation rate of MPAG served as a measure of the activity of UGT1A9. Figure 4 shows the effect of estradiol 0.1 µM / progesterone 1 µM, estradiol (0.1, 1 µM), progesterone (1, 10 µM) and estradiol 1 µM / progesterone 10 µM on the activity of UGT1A9 as measured by the formation of MPAG. Incubation with estradiol 1 µM / progesterone 10 µM and estradiol 0.1 µM / progesterone 1 µM increased MPAG rate formation by 40% and 33%, respectively compared to the untreated cells. Combination of estradiol and progesterone showed the maximum effect over incubation of these compounds separately.

Although incubation of combination of estradiol and progesterone showed the highest increase, there was no statistical significant as determined by one-way ANOVA ($p=0.99$).
Figure 4: Effect of estradiol and progesterone on mycophenolic acid glucuronidation

Human liver hepatocytes were incubated with estradiol and progesterone or vehicle control for four days. On the fifth day, the media were changed to contain mycophenolic acid (100µM) and incubated for 30 min. After 30 min, the media were collected and concentrations of mycophenolic acid glucuronide were determined by HPLC. Data were analyzed by one-way ANOVA to detect statistical significance. Each value represents the mean of four treatments (n=4). EST, estradiol; PROG, progesterone (all units are in µM).
3.4 EFFECT OF ESTRADIOL AND PROGESTERONE ON UGT1A9 mRNA

EXPRESSION

For further analysis of the effect of the hormones on the expression of UGT1A9, mRNA was examined using real time polymerase chain reaction (PCR). Figure 5 summarizes the effect of progesterone and estradiol on the expression of UGT1A9. Addition of estradiol 0.1 µM/progesterone 1 µM did not have any effect on the expression of UGT1A9 mRNA. Treatment the human hepatocytes with estradiol 1 µM/ progesterone 10 µM increased the expression by 2.7-fold, but this increase was not significant ($p=0.25$).
Figure 5: Effect of estradiol and progesterone on the UGTA1A9 mRNA expression

Human liver hepatocytes were treated with estradiol 0.1µM+ progesterone 1 µM and estradiol 1 µM+ progesterone 10 µM or vehicle control for five days. On the fifth day of treatment, mRNA levels were determined by RT-PCR and normalized with cyclophilin A results represent percentage changes relative to the vehicle control. Data were analyzed by one-way ANOVA detect statistical significant. Each value represents the means of duplicate treatment (n=2).
4.0 DISCUSSION

Glucuronidation plays a major role in metabolizing various endogenous and xenobiotic molecules (Radominska-Pandya et al., 1999). Many factors have been shown to affect glucuronidation including disease states, age, diet, gender, cigarette smoking, alcohol consumption, ethnicity, genetic polymorphism, drug-drug interaction (Liston et al., 2001; Miners et al., 2004). Pregnancy is one important factor that has been reported to affect drug metabolism. Pregnancy is accompanied by many physiological changes that can alter the metabolism of drugs being taken during pregnancy (Anderson, 2005). Only a few studies have been conducted to determine changes in drug metabolism during pregnancy (Chen et al., 2009; Jeong et al., 2008; Miners et al., 1986).

Understanding physiologic changes during pregnancy and how these changes affect drug disposition could avoid sub therapeutic or over dosing of drugs in the pregnant subject. Most studies to date have been carried out in animal models and the results cannot be extrapolated to humans. Thus, establishing a human-based system such as human liver hepatocytes and human liver microsomes is important to evaluate the mechanism behind altered activity of UGTs during pregnancy. This study describes the first step in that direction.
UGT1A9 is an important enzyme involved in conjugation of many endogenous and exogenous molecules (Liston et al., 2001; Radomska-Pandya et al., 1999). The first objective of this study was to optimize the conditions to measure the activity of UGT1A9 in human liver microsomes. Studying UGTs requires the use of surfactant to permeabilize the membrane to allow interaction of the substrate with the active site of the enzyme (Banhegyi, Garzo, Fulceri, Benedetti, & Mandl, 1993). The most common detergents used to evaluate UGTs are alamethicin and brij58. We evaluated the effect of alamethecin and brij58 on the formation of MPAG in hepatic microsomes. The results showed that alamethicin and brij tended to give higher activity estimates for UGT1A, which is in agreement with previous results (Soars et al., 2003).

In the current study, we hypothesized that estradiol and progesterone at high concentrations seen in pregnancy will have a regulatory effect on the expression of UGT1A9. Thus, the second objective was to evaluate the changes in the activity of UGT1A9 in human hepatocytes following treatment of human hepatocytes with estradiol and progesterone to mimic physiologic conditions of pregnancy. We used MPA as a substrate to determine the activity of UGT1A9. The data presented here show that primary cultures of human hepatocytes were able to metabolize MPA to MPAG. The data show that a combination of estradiol and progesterone at 1 and 10 µM respectively increase the MPAG rate formation by 40% (Figure 4). Real-time RT-PCR results confirmed that estradiol and progesterone together increased UGT1A9 mRNA expression by 2.7-fold. However, there was no significant difference in the increase in the MPAG rate formation and UGT1A9 mRNA expression. Estradiol (0.1, 1 µM) and progesterone (1, 10 µM) alone showed an increase in the MPAG formation rate only minimally (13 - 22%) (Figure 5). The findings suggested that both progesterone and estradiol together do not have major effects on the activity of UGT1A9 under the conditions tested.
It has been hypothesized that the dramatic increase in female hormones contributes in alteration of drug metabolism in pregnant women (Jeong et al., 2008). Female hormones have been suggested to have different functions from their primary functions as gonadal hormones at high concentrations seen in pregnancy (Jeong et al., 2008). Studies have shown that estradiol and progesterone regulate hepatic metabolic enzyme expression and transporters through pregnane X receptors (PXR) and estrogen receptors (ERα) (Wang et al., 2006; Yamamoto et al., 2006). Using ERα-transfected HepG2 cells treated with different female sex hormones has revealed that UGT1A1 expression can be up-regulated by high concentration of progesterone through PXR (Jeong et al., 2008). The effect of estradiol and progesterone on the UGTs enzymes has been intensively studied for lamotrigine. Lamotrigine clearance increased when combined with oral contraceptives (Christensen et al., 2007; Sabers, Buchholt, Uldall, & Hansen, 2001; Sabers, Ohman, Christensen, & Tomson, 2003). This increase in lamotrigine clearance has been linked to an increase in the concentration of female hormones (Chen et al., 2009). A study has reported that estrogen containing contraceptives were responsible for increased clearance of lamotrigine and not progesterone (Reimers, Helde, & Brodkorb, 2005). An earlier clinical study conducted in pregnant women at the third trimester has shown that the clearance of paracetamol was increased in pregnant women compared to the controls (Miners et al., 1986). Another study has shown an increased in bilirubin, a UGT1A1 substrate, glucuronidation after treatment of 3-MC (Ritter et al., 1999). In contrast, another study reported that no induction was noted in glucuronidation mediated by UGT1A1 after treatment of 3-MC (Li, Hartman, Lu, Collins, & Strong, 1999). These contradictory results outline the difficulty of studying UGT enzyme family due to complex regulatory mechanisms and some factors that should be taken in consideration such as enzyme latency (Banhegyi et al., 1993; Jeong et al., 2008). Our observations indicate that
unlike UGT1A1 and UGT1A4, UGT1A9 is unlikely to be affected by high concentrations of progesterone and estradiol.

Progesterone and estradiol concentrations in this study were chosen based on their levels at the third trimester. The physiological levels of progesterone and estradiol are 0.1 and 1 µM, respectively (Becker, 2001). It is known that liver is the major metabolism organ receiving about 30% of cardiac output (Jeong et al., 2008). Progesterone and estradiol concentrations in liver are reported to be tenfold (Carlson, Brandes, Pomper, & Katzenellenbogen, 1988) and threefold (Schleicher, Tauber, Louton, & Schunack, 1998) respectively, higher than the hormone levels in the blood (Jeong et al., 2008). Therefore, selection of 10 µM progesterone and 1 µM estradiol will simulate levels observed in pregnant women’s livers (Jeong et al., 2008).

There are other factors such as drug transporters and polymorphism that can also alter the pharmacokinetics of drugs during pregnancy. Glucuronide conjugates typically require transporters to get out of the cells (Williams & Lemke, 2002). Studies have shown that pregnancy can also alter drug transporters (Meyer zu Schwabedissen et al., 2005; Wang et al., 2006). A study has reported an increased in the Bcrp expression in the liver of pregnant mice at mid-gestational ages, suggesting that the systemic exposure of drugs could be affected due to changes in the activity of transporters during pregnancy (Wang et al., 2006). Thus, it is possible the alteration in drug transporters activity could also influence the elimination of glucuronides. Genetic polymorphism has been reported in the UGT families (Testa & Krämer, 2010). Crigler-najar and Gilbert syndrome are caused by the mutations in UGT1A1 (Testa & Krämer, 2010). Genetic polymorphism is another factor that plays a role in the regulation of the activity of UGTs. The UGT1A9 isoforms has been reported to have genetic polymorphism (Levesque et al., 2007).
Some limitations of this study must be taken in consideration while interpreting the results. This study was conducted in a small number of hepatocytes that, due to variation in activity, may not lead to statistically significant activity differences. Also, the effect of transporters was not investigated which may not be ruled out as a potential factor that changes drug metabolism during pregnancy.

Future studies to determine the change in drug transporters is highly recommended. Also, further studies should be conducted to evaluate all the major UGTs isoforms to overcome the ambiguity that arises from limited understanding a single glucuronidation pathway. Screening for genetic polymorphism for UGT1A9 in the hepatocytes is recommended in the future to exclude any effect that might interfere with the accuracy of the results.

In conclusion, we have shown that estradiol and progesterone do not have the potential to regulate the expression of UGT1A9 using primary cultures of human hepatocytes under the conditions tested. In addition, we have shown that alamethicin shows no superiority over brij58 in the activation effect for UGT1A9.
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