PRE-CLINICAL AND CLINICAL PHARMACOLOGY OF 17ALPHA-HYDROXYPROGESTERONE CAPROATE (17-OHPC): AN AGENT FOR THE PREVENTION OF PRETERM BIRTH

by

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Submitted to the Graduate Faculty of

The School of Pharmacy in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2010

UNIVERSITY OF PITTSBURGH

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Pre-Clinical and Clinical Pharmacology of

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Preterm birth (PTB), birth prior to 37 weeks of gestational age, is a major cause of early childhood mortality and morbidity in the United States. 17alpha-hydroxyprogesterone caproate (17-OHPC) has recently been documented to reduce the incidence of preterm birth. A complete understanding of the pharmacokinetics of 17-OHPC will help in improving clinical outcome.

The goal of this dissertation research was to evaluate the preclinical and clinical pharmacology of 17-OHPC in order to optimize the use of this drug in preventing preterm birth.

Studies in human liver microsomes and human hepatocytes indicate that 17-OHPC is metabolized by CYP3A. Significant transplacental transfer of 17-OHPC (cord blood to maternal plasma ratio of 0.3) has been observed in pregnant subjects. Studies were performed in fetal hepatocytes to evaluate the metabolism of 17-OHPC. Fetal hepatocytes demonstrate the ability of human fetal liver to metabolize 17-OHPC to fetal specific metabolites, with oxidation being the major metabolic pathway. Further, 17-OHPC and/or its metabolites inhibit bile salt transport in both adult and fetal hepatocytes.

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To understand the clinical pharmacology of 17-OHPC, pregnant women who received 17-OHPC for clinical reasons were studied and blood samples collected periodically. Wide-interindividual variation was observed in the pharmacokinetics of 17-OHPC in pregnant subjects. The half life of 17-OHPC was 9 days and plasma concentrations of 17-OHPC did not achieve steady state. The race and body mass index of the pregnant subjects affect the plasma levels of 17-OHPC.

In conclusion, since CYP3A is involved in the oxidative metabolism of numerous commonly used drugs; 17-OHPC may be involved in clinically relevant metabolic drug interactions with co-administered CYP3A inhibitors or inducers. Since 17-OHPC crosses the placental barrier and reaches the fetus, use of higher doses of 17-OHPC should be approached with caution. The clinical effectiveness of 17-OHPC in preventing preterm birth has been observed in only 33% of the patients. Given the wide interindividual variability, modification of the starting dose based on BMI and race alongwith monitoring of plasma levels and adjustment of subsequent doses accordingly may be needed to improve therapeutic outcomes in the treatment of preterm birth with 17-OHPC.

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PREFACE

I would like to express my sincere appreciation to Prof. Raman Venkataramanan, my dissertation advisor, for his professional and scientific support during my graduate training at University of Pittsburgh. His scientific input, guidance and enthusiasm for research were indispensable to completing this work and understanding the various aspects of this project.

I would like to thank Dr. Stephen Strom for providing invaluable support and sustained guidance throughout my PhD. His constant optimism and encouragement has been key to shaping my outlook towards research and life in general.

I would like to express my sincere gratitude to Dr. Steve Caritis for providing me the opportunity and guidance to understand pharmacokinetics from a clinician's perspective.

I would like to thank my committee members Dr. Billy Day for his help with the LC-MS studies, Dr. Samuel Poloyac for the Radio-HPLC studies and Dr. Wen Xie for his guidance with the gene reporter assays. I would also like to thank Dr Balwant Dixit for his constant encouragement during this endeavor.

Thanks are also due to my colleagues Shimin Zhang and Wenchen Zhao who provided technical support for my PhD projects. A special word of thanks to Susan Price for providing administrative and personal support during my years as a gradate student.

I would like to acknowledge my classmates and friends Nisa Ghonem, Jennifer & Jeremy Bonner, Alex Sassi, Haitao Yang, Ashish Sharma, Je Young An, Ken Dorko, Aarti Ranade,

Fabio Marongiu, Roberto Gramignoli, Ewa Ellis and Veysel Tahan for all the wonderful times we had in Pittsburgh.

Lastly, I would like to thank my wife Shalini and my parents for their unwavering support and patience through all these years. Their confidence in my abilities has been highly instrumental in reaching this stage of life.

1.0 Introduction

1.1 Preterm birth and its significance

Preterm birth (PTB) as the name suggests is birth before term i.e. 37 weeks of gestation. The gestational length of a normal pregnancy is around 40 weeks and the babies born between 37-42 weeks of gestation are termed full term. In Europe and other industrialized nations the rate of pre-term birth has been reported to be between 5 and 9% (Slattery and Morrison, 2002; Hamilton et al., 2006). In the United States, about 12.8 % or a little more than half a million of all births are premature. The rate of preterm birth in the U.S. has increased by ~30% since the early 1980s i.e. from 9.5% (% of all births) in 1981 to 12.8% in 2006 (Goldenberg et al., 2008; Martin et al., 2008). This increase in the rate of PTB (32-36 weeks) has been largely associated with an increase in the age at which women become pregnant (>35 yrs) and the use of assisted reproductive technologies (Martin et al., 2008; Sunderam et al., 2009).

Preterm birth is a serious health problem as it is reported to be one of the major causes of neonatal mortality and early childhood morbidity in the United States (McCormick, 1985). The perinatal mortality rate has been shown to be associated with the gestational age at birth. In developed countries, perinatal survival ranges from <5% at 22 weeks to almost 100% above 31 weeks gestation and can be attributed to the recent advances in neonatal medicine (Slattery and Morrison, 2002). Babies born premature are at an increased risk for a number of health problems like low birth weight, respiratory and breathing difficulties, cerebral palsy, vision and hearing loss, underdeveloped organs/organ systems and even death. Most prematurely born infants require admission to intensive care units equipped with specialized medical staff and equipment trained to deal with the issues related to PTB. Recent reports in literature have indicated an increased risk of autism (Limperopoulos et al., 2008; Schendel and Bhasin, 2008) alongwith certain adult health problems, such as diabetes (Hovi et al., 2007), and heart disease

(http://www.nichd.nih.gov/womenshealth/research/pregbirth/preterm.cfm) in individuals who were born premature. Preterm birth has an impact on the long term health of the infants including mental or physical disability in 1 out of 4 babies born at less than 25 weeks' gestation (Wood et al., 2000). Further, higher healthcare costs are incurred due to longer hospital stays as babies born at less than 28 weeks spend 85 times longer in hospital as compared to term babies in the first five years of life (Petrou et al., 2003). In premature babies, even if the gestational age at birth is >32 weeks, educational and behavioural problems occur in 1 in 3 children at the age of 7 and these children require support from non-teaching assistants at school (Huddy et al., 2001).

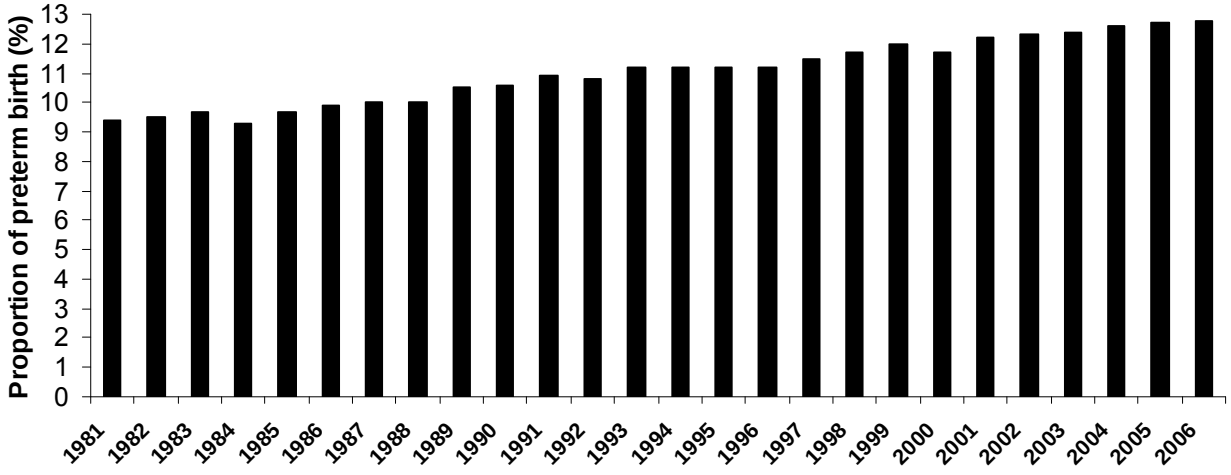


Figure 1. Increase in preterm births (% of all births) in the USA (1981-2006)

Reference: (Goldenberg et al., 2008; Martin et al., 2008)

1.2 Epidemiology of preterm birth

Preterm birth can be divided into two categories (based on the nature of obstetric precursors leading to labour), namely, indicated and spontaneous. Preterm birth is classified as indicated when labour is induced or the infant delivered via caesarean section for maternal and fetal indications. Preterm births that occur when labour occurs spontaneously, irrespective of whether the method of delivery is vaginal or caesarean section, is termed as spontaneous PTB (Tucker et al., 1991). Indicated PTB can result due to medical conditions that put the mother (maternal sepsis or hypoxia), fetus (maternal diabetes or intrauterine growth restriction) or both (maternal hypertension, placenta previa, or abruption) at risk should the pregnancy continue its normal course. About 30-35% of all preterm births are indicated. The most common pathological conditions that led to indicated preterm births in one study reported by Meis et al. were preeclampsia (~40%), fetal distress (25%), intrauterine growth restriction (10%), placental abruption (7%), and fetal demise (7%) (Meis et al., 1998). Other risk factors for indicated preterm birth include maternal proteinuria, chronic hypertension, Mullerian duct fusion, age over 30 years, nulliparity, maternal pulmonary disease, drug (cocaine) use and maternal asthma (Cherukuri et al., 1988; Meis et al., 1998; Dombrowski, 2006).

Spontaneous PTB can be further divided into two categories - spontaneous preterm labour with intact membranes and preterm premature rupture of membranes (PPROM). About 40-45% of all preterm births follow spontaneous preterm labour and 25-30% follow PPROM (remaining 30-35% being indicated PTB). Preterm premature rupture of membranes is defined as the rupture of the amniotic membranes with release of the amniotic fluid more than 1 hour prior to the onset of labour (Simhan and Canavan, 2005). Spontaneous PTB has been reported to follow two different patterns which can be distinguished on the basis of the gestational age at

delivery and the likelihood of repeated preterm birth in future pregnancies (Goldenberg et al., 1998). Preterm births that occur before 32 weeks of gestation, also known as early PTB, are more likely to be associated with infection. This type of PTB is observed more commonly in African- Americans and Caribbean Hispanic women and less so in African, non-Caribbean Hispanic and Caucasian women. Early preterm births have been reported to be associated with long-term morbidity in infants and also is associated with a greater likelihood of recurrence in subsequent pregnancies. However, births that happen after 32 weeks of gestation are less likely to be associated with clinical or sub-clinical intra-amniotic infection, are less likely to be followed by long-term morbidity in infants and are associated with a lower risk of recurrence in subsequent pregnancies. This type of PTB is often associated with increased uterine contraction frequency, increased uterine volume caused by hydramnios or multiple gestation (Iams, 2003).

Preterm birth has been classified as a syndrome since it is initiated by multiple mechanisms which include, infection, inflammation, uteroplacental ischaemia or hemorrhage, uterine overdistension, stress and other immunologically mediated processes (Romero et al., 2006). Since an exact explanation of the mechanism behind PTB has not been established to date, factors that put the pregnant women at an increased risk for the occurrence of PTB have been sought to explain its pathophysiology. Identifying risk factors can help in 1) determining when to initiate risk-specific treatments, 2) defining a population that can be used to study the specific treatment options and 3) explaining the mechanisms leading to PTB. A number of risk factors have been reported which include –

Obstetric history: A history of PTB has been associated with an increased risk of early preterm early in subsequent pregnancies. Risk factors differ among women with recurrent versus nonrecurrent PTBs. For nonrecurrent PTBs the risk factors include second trimester bleeding,

abnormal amniotic fluid volume, multiple gestation, substance abuse and trauma. For recurrent PTBs the risk factors include genitourinary infection, maternal ethnicity, and the gestational age of prior PTB (Mercer et al., 1999).

Cervical length: Before 16 weeks, the relationship between cervical length, as measured by transvaginal ultrasonography and PTB is not evident. Between 18 and 32 weeks the risk of PTB increases with decrease in the cervical length (Urbaniak et al., 2005). Iams et al. (Iams et al., 1996) have reported that the relative risks of preterm delivery in women with shorter cervixes (as measured at 24 weeks) were as follows: 1.98 for cervical lengths at or below the 75th percentile (40 mm), 2.35 for lengths at or below the 50th percentile (35 mm), 3.79 for lengths at or below the 25th percentile (30 mm), 6.19 for lengths at or below the 10th percentile (26 mm), 9.49 for lengths at or below the 5th percentile (22 mm), and 13.99 for lengths at or below the 1st percentile (13 mm). For the lengths measured at 28 weeks, the corresponding relative risks were 2.80, 3.52, 5.39, 9.57, 13.88, and 24.94.

Infection: Substantial evidence exists in the literature showing the association of PTB with infection caused mainly by bacteria of vaginal origin which reside in the uterus (Hillier et al., 1988; Romero et al., 1989b; Andrews et al., 2000). These include: Ureaplasma urealyticum, Mycoplasma hominis, Gardnerella vaginalis, peptostreptococci, and bacteroides species. Micro-organisms such as Chlamydia trachomatis, trichomonas, Escherichia coli, and group B streptococcus, are less commonly recovered. Bacterial vaginosis, a pathological conditon caused mainly by gram negative bacteria (Gardnerella vaginalis, Bacteroides, Prevotella, Mobiluncus, and Mycoplasma species), has been shown to be associated with more than two fold increased risk of spontaneous PTB (Meis et al., 1995). Infections outside the genital tract have also been

reported to be associated with PTB and include urinary tract infections, pyelonephritis and appendicitis (Romero et al., 1989a).

Maternal Race: The rate of PTB before 37 weeks in African-American women is twice that in women of other races and for delivery before 32 weeks the rate is three times higher than in white women (Iams, 2003). However, it is interesting to note that the black women born outside the United States have a lower risk of preterm delivery than those in the US (Martin et al., 2002).

Bleeding: Vaginal bleeding during the second trimester due to placenta previa or placental abruption has been reported to be associated with PTB with a relative risk of 15.1 (Ekwo et al., 1992).

Uterine Anomalies: Preterm births have been reported to occur in 25-50% of women diagnosed with uterine abnormalities like Mullerian fusion anomalies, implantation of the placenta on the uterine septum or a T-shaped uterus (Raga et al., 1997; Kaufman et al., 2000).

Uterine contractions: An increased frequency of uterine contractions has been linked to preterm delivery before 35 weeks with a relative risk (RR) of 1.62 (Mercer et al., 1996).

Multiple pregnancies: A strong association exists between the multiple gestation and PTB with the rate approaching almost 100% for quadruplets. The increased rate is caused in part because of overdistension of the uterus. As the incidence of multiple gestation has increased in recent years because of assisted reproductive technologies (ART), so has the rate of PTB (MMWR, 2000).

Behavioural factors: Sexual practices (Moore et al., 1994), maternal stress, maternal cigarette smoking (Savitz et al., 2001; Kyrklund-Blomberg et al., 2005) and the duration and intensity of work (Mamelle et al., 1984) has also been linked to the incidence of preterm birth.

The abovementioned epidemiologic characteristics of pregnant women who deliver their infants before term provide us with clues as to the cause of this most important obstetric problem.

1.3 Mechanisms of preterm birth

A number of mechanisms are responsible for preterm birth due to the initiation of preterm labor.

Infection and Inflammation: Studies have shown that infection accounts for 25-40% of all preterm births (Romero et al., 1992). Microbial infection in the amniotic fluid is associated with inflammation as evidenced by the high concentration of IL-6, a marker for intra-amniotic inflammation (Wenstrom et al., 1998). Further, a significantly higher concentration of other markers including, matrix metalloproteinase (MMPs) 8 (Yoon et al., 2001), tumor necrosis factor-α (Heyborne et al., 1992), and angiogenin (Spong et al., 1997) has also been reported in women who deliver prior to term. However, the concentration of IL-6 in the maternal serum was not associated with the incidence of preterm birth (Wenstrom et al., 1996).

A gene-environment interaction has also been reported for the occurrence of preterm birth. The genotype of interest was TNF-alpha allele 2. The host factor which constituted the environmental aspect of the interaction was the presence of bacterial vaginosis in the pregnant subject. The presence of this gene leads to hyperresponsiveness of the individual to genital tract infection, thus, promoting preterm premature rupture of the fetal membranes and subsequent preterm delivery (Roberts et al., 1999).

Microbial infections can produce an inflammatory response primarily through the recognition of bacterial toxins (lipopolysaccharide, peptidoglycans and lipoglycans) by pattern

recognition receptors including, C-reactive protein (CRP) and Toll-like receptors (TLRs). Using a TLR knockout mouse model, Wang et al (Wang and Hirsch, 2003) have shown that the likelihood of preterm delivery due to lipopolysaccharide administration is significantly reduced in comparison to the wild type thus confirming the role of TLRs in preterm birth.

Uteroplacental ischaemia: Maternal vascular lesions in the placenta including, atherosis and thrombosis of the spiral arteries can lead to uteroplacental ischaemia. Uteroplacental ischaemia leads to the generation of thrombin which stimulates the generation of MMP-1, urokinase type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). These products can cause the degradation of collagen and fibronectin which are important constituents of the extracellular matrix in the chorioamniotic membranes (Aplin et al., 1985) thus causing preterm delivery.

Uterine overdistension: Intra-amniotic pressure remains relatively constant throughout pregnancy. This is due to the progressive relaxation (stretching) of the myometrium by progesterone or nitric oxide. However, rapid stretching can induce increased myometrial contractility (Kloeck and Jung, 1973), prostaglandin release (Laudanski and Rocki, 1975), expression of gap junction protein (Sladek et al., 1999) or connexin (Ou et al., 1997), and increased oxytocin receptor which can lead to preterm delivery.

Immunological phenomena: Some studies support the hypothesis that suppression of immune response occurs during pregnancy which enables the body to treat the feto-placental unit as an allograft. Recognition of the fetus as a foreign antigen by the immune system of the mother causes production of mediators like natural killer cells and macrophages leading to preterm birth (Aksel, 1992) . There is also evidence suggesting the involvement of an allergic phenomenon in preterm birth. The human fetus is exposed to common allergens like house dust mite and this

compound has been detected in both amniotic fluid and fetal blood. The uterus is a rich source of mast cells—the effector cells of allergic-like immunological reactions. Upon coming in contact with the allergen, mast cell degranulation occurs, the products of which have been reported to induce myometrial and cervical contractility (Padilla et al., 1990). Thus, this type of allergic response can be implicated as one of the mechanisms causing preterm labour and preterm birth.

The current scientific literature considers preterm birth as a syndrome. Multiple pathological processes (indicated above) may lead to myometrial contractions, membrane/decidual activation, cervical ripening and hence preterm birth. Since, preterm birth is caused by multiple etiologies; the treatment should be aimed at ameliorating the symptoms associated with the multiple mechanisms mentioned above.

1.4 Interventions for the prevention of preterm birth

Interventions to reduce the incidence of preterm labour can be classified, based on the goals of the therapy, into primary (initiated before or during pregnancy in all women to prevent or reduce risk), secondary (only in women known to have the risk factors associated with PTB) and tertiary (initiated once the labour has started to prolong the delivery or improve the neonatal outcome).

Primary Intervention: Primary prevention strategies have shown success in the treatment of cancer (cervical cancer) and vascular disease and thus form a desirable strategy in the treatment of PTB (Iams et al., 2008). These strategies include:

Public educational interventions – involving educating the public about potentially avoidable risk factors (mentioned previously). For example, awareness of the association between repeated

uterine trauma due to curettage/ endometrial biopsy or use of assisted reproductive technologies resulting in singleton or multiple gestations and PTB.

Public and professional policies – to improve work related conditions like providing minimum maternity leave of 14 weeks, time off for prenatal visits and protection from workplace hazards (working in a standing position or lifting objects).

Nutritional Supplements – multivitamins (Vahratian et al., 2004) especially vitamins C (Rumbold et al., 2006) and E (Rumbold and Crowther, 2005) and calcium (Crowther et al., 1999) supplementation has been recommended both prior to and during pregnancy. However, the effectiveness of nutritional supplementation in reducing the rate of PTB or improving the neonatal outcome for infants born premature is not clear.

Prenatal care – with the objective to prevent prematurity within prenatal care has shown promise in Europe but has received little attention in USA. In France, prenatal care has been shown to be associated with reduced PTB rates over time (Papiernik et al., 1985).

Periodontal care – has been suggested in pregnancy as it has been demonstrated that maternal periodontal disease may increase the relative risk for preterm or spontaneous preterm births (Offenbacher et al., 2006). However, the association between periodontal care and PTB needs to be confirmed in larger clinical trials.

Screening of low-risk women – for signs of infections (bactriuria, bacterial vaginosis), routine cervical examination and testing for fetal fibronectin can help in identifying these subjects and help reduce the risk of PTB (Goldenberg et al., 1998).

Secondary Intervention: The efforts under this strategy are directed at women who are at risk of PTB as indicated by their obstetric history (previous PTB or uterine anomaly) or present pregnancy risk factors. The interventions include both preconception and postconception

strategies. Under the preconception interventions, control of risk factors like presence of diabetes, seizures, asthma and hypertension identified based on careful evaluation of patient history, correction of uterine anomalies, and antibiotic treatment can reduce the risk of PTB. Postconceptional interventions include control of risk factors like pre-eclampsia with low dose aspirin (Sibai et al., 1993). Treatment options for pre-eclampsia including multivitamins (Poston et al., 2006), antioxidants (Rumbold et al., 2008), fish oil (Olsen et al., 1992), calcium, omega-3 polyunsaturated fatty acids (Olsen et al., 1992) have been shown to be either marginally effective or ineffective. Other prevention strategies include modification of maternal activity (bed rest, sexual activity) (Goldenberg et al., 1994; Sosa et al., 2004), improved prenatal care (social support, home visits, education) (Bryce et al., 1991; Kitzman et al., 1997), antibiotic treatment (clindamycin or metronidazole for bacterial vaginosis) (Lamont, 2005), ***progesterone supplementation*** (modulation of inflammation or cervical ripening) (Keirse, 1990; da Fonseca et al., 2003), and cervical cerclage (in women with short cervix) (Berghella et al., 2005; Mancuso and Owen, 2009).

Tertiary Intervention: The main goal here is to improve the outcome for women (and the infant) in whom symptoms of preterm birth are detected proximate to delivery. The treatment options include antibiotics (ampicillin or amoxicillin/clavulanic acid plus erythromycin) to reduce neonatal morbidity (Kenyon et al., 2001b; Kenyon et al., 2001a; Schrag et al., 2002; Law et al., 2005) and corticosteroids (to reduce neonatal morbidity or mortality from respiratory distress, hemorrhage, necrotizing enterocolitis and patent ductus arteriosus) (Roberts and Dalziel, 2006; Wapner et al., 2006). Further, tocolytic drugs i.e. drugs that prolong the delivery long enough to enable to transfer of the pregnant subject to a specialist care facility, are also used. These include calcium channel blockers (nifedipine, nicardipine), beta agonists (ritodrine,

terbutaline) (Anotayanonth et al., 2004) and prostaglandin inhibitors (Indomethacin) (Niebyl, 1981).

Most interventions intended to reduce preterm birth do not show consistent results when tested in randomized trials, which, in turn can mostly be attributed to the insufficient data available at the time of their design. However, prophylactic treatment with progestogens (e.g. progesterone, 17-OHPC, etc.) is one of the options that has shown promise in preventing PTB associated morbidity and cost.

1.5 Progesterone and preterm birth

Progesterone is essential in maintaining pregnancy. Progesterone has been reported to promote myometrial quiescence, down-regulate gap junction formation, inhibit cervical ripening, and decrease the production of chemokines (i.e. IL-8) by the chorioamniotic membranes (Gorodeski et al., 1987; Chwalisz, 1994; Stjernholm et al., 1996; Mesiano, 2001; Brown et al., 2004). Further, administration of progesterone receptor antagonists (Mifepristone) to pregnant women has been shown to induce labour (Bygdeman et al., 1994). In many species (rats, mice, rabbits, goats and pigs) a fall in circulating levels of progesterone occurs prior to parturition; however, a similar decline has not been reported in humans. Thus, the concept of "functional withdrawl" of progesterone has been suggested to explain the role of progesterone in human pregnancy or lack thereof in causing preterm birth. This concept explains the loss or decline in sensitivity to progesterone prior to parturition at term or preterm on the basis of: 1) reduced bioavailability of progesterone by binding to a high affinity protein; 2) increased cortisol concentration in late pregnancy that may compete with progesterone for binding to the

glucocorticoid receptor (GR) which results in suppression of the immunosuppressive activity associated with progestins; 3) conversion of progesterone to an inactive form within the target cell by 5alpha-reductase and 20alpha-hydroxysteroid dehydrogenase; 4) quantitative and qualitative changes in progesterone receptor isoforms (PR-A, PR-B, PR-C) with an increase in PR-A/PR-B ratio being associated with reduced mymetrial response to progeserone; 5) changes in progesterone receptor co-regulators (Steroid hormone Receptor Coactivator, SRC-1,2,3) ; and 6) a functional progesterone withdrawal through NF-kB wherein the latter, generated as a result of inflammation, interacts directly with progesterone receptor and reduces its transcriptional activity (Brown et al., 2004). Based on this concept of functional withdrawal and to remedy the situation, progestational agents (17alpha-hydroxyprogesterone caproate, medroxyprogesterone acetate) have been used in the prophylactic treatment of preterm birth, although with varying rates of success.

1.6 17α-hydroxyprogesterone caproate: Clinical history & Effectiveness

The Food and Drug Administration (FDA) approved the use of 17α-hydroxyprogesterone caproate (17-OHPC; Fig 3A) in pregnancy in 1956 (Delalutin®). It was indicated for the treatment of habitual and recurrent abortion, threatened abortion, and post-partum “after pains.” This approval was based largely on safety consideration in that it occurred prior to the FDA Drug Amendment of 1962, which required drug manufacturers to provide proof of the effectiveness and safety of their drugs before approval. In 2000, the FDA withdrew approval for Delalutin® at the request of the holder of the New Drug Application (NDA), Bristol-Myers Squibb Co,

because the company was no longer marketing the drug. The action was not taken because of safety concerns.

The published literature includes several studies evaluating the efficacy of 17-OHPC in preventing preterm birth (Table 1).

Table 1. Studies of the Efficacy and Safety of 17-OHPC in Preventing Preterm Birth

Investigator	**Drug:Dose**	**Inclusion Criteria**	**Study Design**	**Subjects**	**Start**	**Stop**	**Outcome % PTB[#]**	**No. of SAB[+]**
(Levine, 1964)	17OHPC: 500 mg weekly vs. Placebo	3 SABs	RCT, DBC Placebo 1:1	17OHPC: 15 Placebo: 15	< 16 wks	36 wks	17OHPC: 7/15 (46%) Placebo: 10/15 (66%)	17OHPC: 3/15 Placebo: 7/15
(Papiernik-Berkhauer, 1971)	17OHPC: 250 mg q 3 days vs. Placebo	High risk of preterm birth	RCT Placebo 1:1	17OHPC: 50 Placebo: 49	28 – 30 wks	8 doses	17OHPC: (4%) Placebo: (19%)	
(Johnson et al., 1979)	17OHPC: 250 mg weekly vs. Placebo	2 SABs or 1PTB + 1 SAB or hx 2 PTBs	RCT, DBC Placebo 1:1	17OHPC: 18 (4 cerclage) Placebo: 22 (3 cerclage)	< 24 wks	37 wks	17OHPC: 0/18 (0%) Placebo: 9/22 (41%)	17OHPC: 3/23 Placebo: 0/27
(Hartikainen-Sorri et al., 1980)	17OHPC: 250 mg weekly vs. Placebo	Twin gestation	RCT, DBC Placebo	77 subjects	28-33 wks	37 wks	17OHPC: 31% Placebo: 24%	
(Yemini et al., 1985)	17OHPC: 250 mg weekly + cerclage vs. Placebo	2 SABs or 2 PTBs	RCT, DBC Placebo 1:1	17OHPC: 39 (39 cerclage) Placebo: 40 (40 cerclage)	12 wks	37 wks	17OHPC: 5/31 (16%) Placebo: 14/37 (38%)	17OHPC: 8/39 Placebo: 3/40
(Suvonnakote, 1986)	17OHPC: 250 mg weekly vs. no treatment	1 PTB or 2 late SABs	Non-randomized	17OHPC: 36 No Rx: 39	16 – 20 wks	37 wks	17OHPC: 5/35 (14%) No Rx: 19/39 (49%)	

Investigator	Drug:Dose	Entry Criteria	Design	Subjects	Start	Stop	Outcome % PTB[#]	No. of SAB[+]
(Hauth et al., 1983)	17OHPC: 1000 mg weekly vs. Placebo	Military	RCT, DBC	17OHPC: 80 Placebo: 88	16 – 20 wks	36 wks	17OHPC: (6.3%) Placebo: (5.7%)	
(Meis et al., 2003)	17OHPC: 250 mg weekly vs. Placebo	1 PTB	RCT, DBC	17OHPC: 310 Placebo: 153	16 – 20 wks	36 wks	17OHPC: (36%) Placebo: (55%)	
(Rouse et al., 2007)	17OHPC: 250 mg weekly vs. Placebo	Twin gestation	RCT, DBC	17OHPC: 325 Placebo: 330	16 – 20 wks	36 wks	17OHPC: (42%) Placebo: (38%)	
(Caritis et al., 2009)	17OHPC: 250 mg weekly vs. Placebo	Triplet gestation	RCT, DBC	17OHPC: 71 Placebo: 63	16 – 20 wks	35 wks	17OHPC: (83.0 %) Placebo: (84.0 %)	

PTB=Preterm Births
+ SABs=Spontaneous Abortions
RCT, DBC=Randomized Controlled Trial, Double Blind

At present, the body of evidence available in the literature on the efficacy of 17-OHPC in preventing preterm birth in women at high risk has shown the drug to be effective in singleton gestation, but ineffective in multiple gestation. No other progestational drugs have shown efficacy in randomized trials and insufficient data exists for these other compounds such as Medroxyprogesterone Acetate. The dose of 17-OHPC used in most of the reported trials has been 250 mg/week. Little data exists from pharmacokinetic or pharmacotherapeutic studies to argue for or against this dose. Doses of 1000 mg/wk and upto 2000 mg of 17-OHPC have been used but no adverse effect has been reported thus deeming this drug to be safe. However, there is a signal for embryo–fetal toxicity associated with 17-OHPC in the largest clinical trial (Meis et al., 2003) conducted to date and there is also a signal for embryo–fetal toxicity with 17-OHPC in rhesus monkeys (Hendrickx et al., 1987). The relationship between these signals is unclear given the absence of state-of-the-art reproductive toxicology studies and human pharmacokinetic studies. Treatment of preterm birth with 17-OHPC has also been reported to be associated with

significant medical cost savings. It has been estimated that treatment of eligible women could result in direct medical cost reductions (exclusive of 17P treatment costs) per treated individual of between $1000 and $3800 in initial neonatal hospital care, $7500 in medical care through age 15 years, and $15,900 in medical care through age 75 years. Overall, treatment of all eligible women with 17-OHPC would achieve $2 billion in annual direct medical cost reductions (Armstrong, 2007). Thus, understanding the pharmacokinetics of 17-OHPC in humans is essential 1) to optimize the current fixed dosing regimen for maximum therapeutic effectiveness, 2) to reduce the medical cost associated with management of preterm birth, and 3) to elucidate the mechanism of action of 17-OHPC in preventing preterm birth and any associated risk of adverse event. Understanding the pharmacokinetics of 17-OHPC (a steroid analog), or any other compound for that matter, involves elucidating the metabolic pathways (mediated mainly by Phase I and II enzymes) and transport processes (Phase III) involved in its disposition.

1.7 Drug metabolism pathways

The major organ responsible for metabolism of drugs in humans is the liver although some metabolism also occurs in the gut wall, lungs and kidneys. Hepatocytes, one of the primary functional cells of the liver, contain the enzymes necessary for the metabolism of xenobiotics and endogenous compounds. The xenobiotic-metabolizing enzymes convert drugs and xenobiotics into hydrophilic derivatives that are more easily eliminated through excretion into the aqueous compartments of the tissues (bile and urine). Thus, the process of drug metabolism that facilitates elimination of compounds plays a major role in determining the biological activity of a drug (Woolf, 1999). Historically, xenobiotic metabolizing enzymes have

been grouped into the phase 1 and phase 2 enzymes. Phase 1 enzymes catalyze reactions which include primarily, oxidation, reduction, and hydrolytic reactions, whereas phase 2 enzymes catalyzed reactions, in which a conjugate of the substrate (drug or its phase 1 product) is formed.

The phase 1 enzymes lead to the introduction of functional groups (-OH, -SH, -NH2 or –COOH) making the metabolite more polar than the parent compound (Gibson and Skett, 2001). The addition of functional groups may not increase the water solubility of the drug significantly, but can dramatically alter its biological properties. Phase 1 reactions, also classified as the functionalization phase of drug metabolism, can not only lead to the inactivation of an active drug but in certain instances, can also cause bioactivation of a drug (e.g. cyclophosphamide). Phase 1 enzymes include cytochrome P450s (CYP), flavin-containing monooxygenases (FMO), and epoxide hydrolases (EH). The CYPs are the most important phase I enzymes and are essentially a superfamily of heme containing enzymes. Some CYPs (CYP11A1, CYP11B1, CYP11B2, CYP24, CYP27A1, CYP27B1, and CYP27C1) expressed mainly in the adrenal are found in the mitochondrial inner membrane and are responsible for steroid biosynthesis and Vitamin D metabolism). However, the greatest amount of CYPs (CYP1A, CYP2A, 2B, 2C, 2D, 2E and CYP3A) are expressed in the liver and the intestine. In liver and most other tissues, they are present mainly in the membranes of the smooth endoplasmic reticulum (ER), which constitute part of the microsomal fraction when tissue is subjected to subcellular fractionation. These CYPs are responsible for the metabolism of a variety of xenobiotics and endobiotics (Woolf, 1999). Human CYP isoforms involved in the metabolism of xenobiotics include CYP1A1/2, CYP2B6, CYP2C8/9/19, CYP2D6, CYP2E1 and CYP3A4/5, of which CYP3A is the most abundant isoform (30 % of the hepatic CYPs) in human livers (Shimada et al., 1994; Eagling et al., 1998; Woolf, 1999). Functionally, CYP3A is responsible for the metabolism of

about 53% of commonly prescribed drugs, followed by CYP2D6, CYP2C, CYP1A2 and CYP2E1 accounting for 25%, 18%, 3% and 1% of drug metabolism, respectively (Shimada et al., 1994; Woolf, 1999). The subfamily of CYP3A includes CYP3A4, CYP3A5 and CYP3A7. CYP3A4 and CYP3A5 are expressed in adult livers, while CYP3A7 is expressed mainly in fetal liver (Venkatakrishnan et al., 2000).

Phase 2 enzymes are responsible for the elimination of drugs and the inactivation of electrophilic and potentially toxic metabolites produced by phase 1 pathway like oxidation. Unlike phase 1 reactions, phase 2 reactions produce a metabolite with significantly improved water solubility and increased molecular weight, which helps in the elimination of the drug from the tissue (urine or bile). Phase 2 enzymes include glutathione-S-transferases (GST), UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT), N-acetyltransferases (NAT), and methyltransferases (MT). These conjugation reactions usually require the substrate to have oxygen (hydroxyl or epoxide groups), nitrogen, and sulfur atoms that serve as acceptor sites for a hydrophilic moiety, such as glutathione, glucuronic acid, sulfate, or an acetyl group, that is covalently conjugated to an acceptor site on the molecule. Uridine diphosphate glucuronosyltransferases (UGTs) are the important phase II enzymes located in the membrane of endoplasmic reticulum. They are responsible for the glucuronidation of endogenous and exogenous compounds, wherein glucuronic acid is delivered to the functional group, forming a glucuronide metabolite that is now more water soluble with a higher molecular weight that is targeted for excretion either in the urine or bile (Mackenzie et al., 2003). Sixteen different UGT isoforms have so far been identified in humans and have been classified into 1A or 2B subfamilies (Tukey and Strassburg, 2000). Among the UGT1A family, UGT1A1 is the most important isoform involved in the glucuronidation of endogenous compounds like bilirubin,

estradiol as well as xenobiotics like acetaminophen and irinotecan (Cheng et al., 1998; Court et al., 2001; Tukey et al., 2002). UGT1A6 and UGT1A9 are other isoforms involved in the metabolism of catechols, acetaminophen and 4-methylumbelliferone (Court et al., 2001). Table 2 lists selected drug metabolizing enzymes along with their respective substrates, inducers and inhibitors (Bowen et al., 2000; Sueyoshi and Negishi, 2001; Asghar et al., 2002; Kostrubsky et al., 2003; Chandra and Brouwer, 2004).

Table 2. Selected substrates, inducers and inhibitors for drug metabolizing enzymes and drug transporter.

CYP3A4/5	BSEP
SUBSTRATES	
Testosterone	Taurocholate
Midazolam	
Erythromycin	
FK506	
Cyclosporine	
Amlodipine	
Cisapride	
INDUCERS	
Rifampin	CDCA
Hyperforin	
Phenobarbital	
Carbamazepine	
Troglitazone	
INHIBITORS	
Ketoconazole	Cyclosporin
Ritonavir	Troglitazone
Itraconazole	Rifampin
Verapamil	Glyburide

Source: FDA Document on Drug Development and Drug Interactions: Table of Substrates, Inhibitors and Inducers.

Steroids are lipophilic, low-molecular weight compounds derived from cholesterol that play a number of important physiological roles. Steroids are known to occur in several

biologically active forms. This is not only due to the large range being synthesized by the body but also because of their extensive metabolism in the liver and in their target tissues, where conversion to an active form is sometimes required before they can elicit their biological responses. Most of the peripheral metabolism occurs in the liver and to some extent in the kidneys, which are the major sites of hormone inactivation and elimination, or catabolism. For certain classes of hormones and particular target tissues, steroids (progestins and androgens) must be converted in situ to an active form (metabolic activation) before they can interact with their specific receptor(s). For example unlike its parent compound, the progesterone metabolite 5α-dihydroprogesterone has no effect on the uterus, but is more effective than progesterone itself in the facilitation and/or inhibition of GnRH-induced LH release *in vitro* (Gower, 1979). It also has a barbiturate-like action on brain GABAA receptors, as the other metabolite pregnanolone (5β-pregnane-3β-ol-20-one).

Inactivation can occur at various stages of hormone action. Peripheral inactivation, which is the metabolic conversion of a biologically active compound into an inactive one, is required to ensure steady-state levels of plasma hormones as steroids are more or less continuously secreted into the bloodstream. The main site of peripheral steroid inactivation and catabolism is the liver, but some catabolic activity also occurs in the kidneys. Usually, steroids are eliminated once they have been inactivated. This elimination (e.g. as a urinary excretion products) requires conversion to hydrophilic compounds in order to ensure their solubility in biological fluids at rather high concentrations. Depending on the structure of the starting steroid, the following reactions may be involved (Karavolas, 1990): 1) Reduction of a double bond at C-4 and reduction of an oxo(keto) group at C-3 to a secondary alcoholic group, 2) Reduction of an oxo group at C-20 to a secondary alcoholic group, 3) Oxidation of a 17β-hydroxyl group, 4) Further hydroxylations at

various positions of the steroid nucleus (e.g. 7-hydroxylation of 5α-reduced androgens), and 5) Conjugation (sulphate and/or glucuronide derivatives).

A number of studies have shown that CYP3A4 is one of the major P450 enzymes involved in the oxidation of steroid hormones in human liver microsomes. The substrates that are catalyzed by CYP3A4 included testosterone (6β-, 15β -, and 2β-hydroxylations), progesterone (6β- and 16α-hydroxylations), 17β-estradiol (2- and 4-hydroxylations), 17α-ethinylestradiol (2-hydroxylation), and dehydroepiandrosterone 3-sulfate (16α-hydroxylation) (Guengerich, 1988; Brian et al., 1990; Guengerich, 1995; Yamazaki and Shimada, 1997). In human liver microsomes, metabolism of progesterone generates six metabolites including, 6β-hydroxyprogesterone (-OHP) which was the predominant metabolite, followed by 16α- and 21-OHP (Swinney, 1990). Similarly, studies by Kobayashi et al. (Kobayashi et al., 2000) in human liver microsomes and expressed enzymes demonstrated CYP3A4 as the major enzyme involved in the metabolism of Medroxyprogesterone Acetate (MPA), a progesterone analog, used in the treatment of breast and endometrial cancer. The major metabolites generated were identified to be hydroxylated-MPA with preferred sites of hydroxylation at 2-, 6-, and 21-positions (Zhang et al., 2008a; Chen et al., 2009). Thus, it is likely that 17alpha-hydroxyprogesterone caproate, a progesterone analog, will be metabolized in the human liver with CYP3A4 being the major enzyme involved and that hydroxylation will be the main reaction involved in 17-OHPC metabolism. Further, we predict that metabolism of 17-OHPC will lead to the cleavage of the caproate side chain and production of 17-OHP which is hypothesized to be responsible for the prolonged and more potent action of 17-OHPC over progesterone. In addition to metabolism, various organs also take advantage of the presence of transporters to move chemicals in and out of tissues. Liver in addition to its metabolic capability is also rich in several transporters.

1.8 Hepatic transport systems

Transport of endogenous compounds and xenobiotics across hepatocyte plasma membranes serves a vital role in their hepatic clearance and usually occurs through different carrier-mediated systems. The transport process also known as hepatobiliary transport involves uptake of compounds by passive diffusion or membrane transporters, conversion to a pharmacologically inactive, active or sometimes toxic metabolite(s) and finally efflux via plasma membrane transporters. Hepatobiliary transport and bile flow play numerous vital roles including maintenance of cholesterol and lipid homeostasis, the removal of endogenous and exogenous substances from the body, and adequate bile salt flow and recycling (Faber et al., 2003).

The membrane transporters involved in the uptake of the chemicals are expressed on the sinusoidal (basal) membrane of the hepatocyte and include Na+-dependent (driven by the transmembrane Na+ gradient, unidirectional) or Na+-independent (mediated by facilitated exchange with intracellular anions such as glutathione) anionic and some cationic transporters (Kullak-Ublick et al., 2000b; Lecureur et al., 2000). These transporters (e.g. NTCP, OATP, OCT1, OAT2) are involved in the transfer of amphipathic and polar organic compounds, as well as some lipophilic molecules from the sinusoidal plasma to hepatic cytosol. The membrane transporters (efflux transporters) that are involved in the transfer of chemicals from the hepatic cytosol to the biliary space and subsequently into bile are expressed on the apical side of the hepatocyte (Muller and Jansen, 1997). These transporters are members of the 'ATP binding cassette' (ABC) family, characterized by the presence of an ATP-binding site and require ATP hydrolysis for their transport function e.g. MDR1, BSEP, MRP2. Figure 2 shows a schematic

diagram of the uptake and efflux transporters in human liver and their localization in human hepatocytes. Table 2 lists selected drug transporters, their substrates, inducers and inhibitors.

Basal Membrane Uptake Transporters:

NTCP (Na+-taurocholate co-transporting polypeptide): belongs to the solute carrier family (SLC) and is also known as SLC10A1 (Hagenbuch et al., 1991; Kullak-Ublick et al., 2000a). NTCP is primarily involved in the Na+-dependent transport of conjugated bile salts (TC, tauroursodeoxycholate, taurochenodeoxycholate), although it also transports unconjugated bile salts (cholate) to a lesser extent (Karpen et al., 1996; Kouzuki et al., 2000; Chandra and Brouwer, 2004). Dehydroepiandrosterone sulfate (DHEAS) (Meier et al., 1997), 3, 3', 5-triiodo-L-thyronine (T3), thyroxine (T4) (Friesema et al., 1999), bromosulfophthalein (BSP), and estrone-3-sulfate are non-bile salt substrates for this protein (Meier et al., 1997).

OATPs (organic anion transporting polypeptides): are sodium-independent, bi-directional transporters involved in the hepatic uptake of substrates driven by countertransport of reduced glutathione present within the hepatocyte (Kullak-Ublick et al., 2000b). The major transporters that form a part of this family include: OATP1A2 (SLCO1A2), OATP1B1 (SLCO1B1), OATP1B3 (SLCO1B3), and OATP2B1 (SLCO2B1) (Li et al., 1998). OATP1B1 is the primary Na^+-independent bile salt uptake system in human liver, with OATP1A2 and OATP1B3 playing a less extensive role (Kullak-Ublick et al., 2001). However, OATP2B1 does not transport bile salts. OATPs have been reported to transport various other substrates including bromosulphophthalein, glycocholate, prostaglandin E2, organic anions like estradiol-17β (β-D-glucuronide) and anionic peptides ([D-penicillamine2,5]-enkephalin (DPDPE) and BQ-123 (Meier et al., 1997; Tamai et al., 2000).

Apical Membrane Efflux Transporters:

MDR1 (P-gp, ABCB1): The multidrug resistance transport protein is the most widely recognized member of the ABC family of proteins and plays a major role in the hepatic excretion of a vast number of endogenous and exogenous compounds, including many drugs and metabolites. Development of resistance to chemotherapeutic agents by cancer cells has been attributed to the over expression of this transporter (Pastan and Gottesman, 1987). MDR1 is primarily involved in the transport of compounds with following physicochemical characteristics - hydrophobic, molecular weight >400 (Schmid et al., 1999), and a log partition coefficient >2 (Oude Elferink et al., 1995). 17-OHPC is a hydrophobic molecule having a molecular weight of 428.7, a LogP value of 5.63 with no apparent charges on the molecule.

Thus, it is likely that 17-OHPC will be a P-gp substrate. Further, steroids have been reported to behave both as inhibitors (Progesterone, Dehydroepiandrosterone) and substrates (Hydrocortisone, Flunisolide) of P-gp.

BSEP (bile salt export pump, ABCB11): is a unidirectional, ATP-dependent transport protein expressed on the canalicular membrane responsible for the excretion of conjugated and unconjugated bile salts into the canalicular space (Hyde et al., 1990; Gerloff et al., 1998). Though this protein does not appear to play a key role in hepatic excretion of xenobiotics, drug interactions leading to clinical hepatotoxicity have been reported (Kostrubsky et al., 2006).

MRPs (multidrug resistance–associated protein): consists of at least 6 members, known as MRP1-6. MRP2 is the most studied member of this family and is involved in the biliary transport of organic anions including LTC4, divalent bile salts, and glutathione, glucuronide, and sulfate conjugates (Konig et al., 1999a). Deficiency in the expression of MRP2 has been known to cause Dubin-Johnson syndrome in humans (Konig et al., 1999b). Further, it has also been

implicated in the producing resistance to chemotherapy (anthracyclines, vinca alcaloids, methotrexate, SN38, SN38-glucuronide and cisplatin) by cancer cells (Chu et al., 1997; Masuda et al., 1997; Cui et al., 1999).

BCRP (Breast cancer resistance protein, ABCG2): is a transmembrane half-transporter, overexpression of which confers multidrug resistance (MDR) to tumor cells and often limits the efficacy of chemotherapy (mitoxantrone, doxorubicin, daunorubicin, and sulfated conjugates). BCRP has been reported to play an important role in the biliary excretion of the sulfated conjugates of steroids and xenobiotics (Doyle et al., 1998; Suzuki et al., 2003).

Alterations in hepatic transporters, through inhibition or induction, can affect the intracellular concentration of xenobiotics, resulting in altered pharmacokinetic and pharmacodynamic profiles, or of endogenous substances, altering normal physiological processes in the liver.

The compound under investigation, 17-OHPC, is a highly lipophillic molecule with low solubility. The highly hydrophobic nature makes this compound highly permeable to lipid membranes. Based on these properties it can be classified under BCS Class II (Wu and Benet, 2005). For BCS class II compounds being administered orally, as stated by Wu et al., efflux transporter effects are important clinically because they affect the oral bioavailability and the rate of absorption.

In the case of intramuscular administration, low concentrations are expected at the hepatocyte-blood interface due to the diluting effect of volume of distribution and thus, saturation of hepatic transporters (uptake and efflux) is unlikely. Thus, with the intramuscular route of administration (assuming no transporter is involved in the absorption of 17-OHPC at the

depot-plasma interface), both uptake and efflux transporters can be important in determining the overall disposition of 17-OHPC.

In this dissertation, we will evaluate the metabolic pathways and transport processes involved in the disposition of 17-OHPC using human liver microsomes and primary hepatocyte culture system.

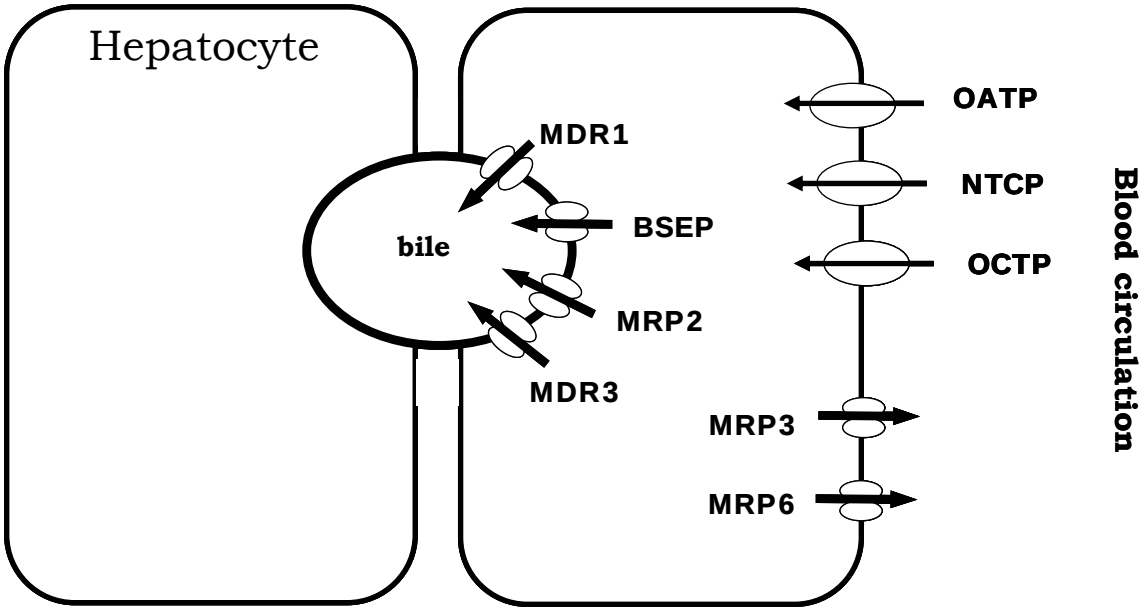


Figure 2. Hepatic uptake and efflux transporters in human liver

Source: (Faber et al., 2003; Chandra and Brouwer, 2004)

1.9 Use of human hepatocytes to study drug metabolism and drug transport

Hepatic drug metabolism and transport has been studied in a variety of *in vitro* or *in vivo* animal or human systems (Table 3). The similarity to the *in vivo* situation increases as the complexity of the model increases e.g. from immortalized cell lines to animals. Studies performed in humans are believed to be a more accurate indicator of a drug's pharmacokinetics, as they comprise all of

the biological process that will interact with a compound in one contained system. However, due to ethical considerations it is not practical to carry out studies in-humans routinely. In-vivo animal studies are also considered as a reasonable alternative for the prediction of a drug's pharmacokinetics, but have the limitation of ethical issues, cost involved and extrapolation to humans. Liver slices and whole perfused livers have the advantages of maintaining the *in vivo* liver architecture but have the disadvantage of being viable for only 4-6 hours and thus show poor reproducibility. Human liver microsomes, a subcellular preparation made from endoplasmic reticulum membranes, are one of the most widely models to evaluate drug metabolism. The main advantage is their long storage life (in years at -80ºC) without loss of enzyme activity. However, the disadvantages include: inapplicability for studying the effect of inducers, role of transporters and enzyme-transporter interplay, and problems with extrapolation to the in-vivo situation due to variables like preparation process, cofactors and substrate concentrations, and incubation conditions.

Recombinant enzyme systems (expressed enzymes) are genetically transformed insect cells expressing a specific human metabolizing enzyme. They are widely used to answer questions regarding specific enzyme(s) and metabolic pathway(s) involved in the metabolism of a molecule. The disadvantages of this system are similar to human liver microsomes.

Human hepatocytes, the functional units of liver, form the next best model systems for studying drug metabolism and transport. Hepatocytes provide a physiological environment that contains normal concentrations of enzymes and cofactors, as well as the cellular machinery necessary to regulate the synthesis of new proteins. Further, similar to the in-vivo situation, xenobiotics must cross a biological membrane, interact with cellular organelles and receptors, and compete with endogenous substrates for biotransformation. Thus, hepatocytes provide a system which

replicate the in-vivo conditions more closely than isolated enzymes, subcellular fractions or homogenates, and liver slices.

Primary cultures of human hepatocytes (PCHH) are viable for up to 2 weeks, or one month if placed in a three-dimensional culture, and express the majority of drug metabolizing phase 1 and 2 enzymes as well as several active transport mechanisms (uptake and efflux), thus, making them a versatile *in vitro* system to study induction and inhibition of drug metabolism and transport (Gebhardt et al., 2003). Hepatocytes have been used to study various aspects of drug metabolism including, metabolic profiling (formation of metabolites for identification purposes), biotransformation pathways, and inter-species comparisons of metabolism to determine which animal species best represents the metabolism in humans. Further, they have also been used to elucidate the physiological response of cofactors, regulatory biomolecules, or xenobiotics on hepatocyte function. Hepatocytes have been widely used by the pharmaceutical industry for studying the detoxification or toxification of xenobiotics derived through metabolism.

As the use of PCHH has advanced, modified culturing techniques have enabled the examination of other processes involved in drug metabolism, namely the uptake and efflux of drugs and their metabolites by hepatic drug transporters. The loss of tight junctions secondary to the hepatocyte isolation procedure results in a loss of cellular polarity, or depolarization, and results in changes in hepatically expressed genes. Normal monolayered PCHH show reduced albumin secretion over time, increased levels of alpha-fetoprotein, a protein that is associated with depolarization and dedifferentiation of hepatocytes, dephosphorylation of cell surface receptors responsive to growth factors and, in the case of rat hepatocytes, a rapid loss of drug metabolizing activity and MDR1 expression (Luttringer et al., 2002; Richert et al., 2002; Boess et al., 2003).

Table 3. Systems to study hepatic drug metabolism

System	Complexity	Ease of use
Subcellular fractions Supersomes Microsomes Cytosol Expressed Enzyme Systems		
Human hepatocyte cultures Suspended cultures Tumor derived cell lines Cryopreserved hepatocytes Primary hepatocytes 3D cultures		
Liver slices		
Whole Liver perfusion		
***In vivo* animal model**		
Human		

Source: Adapted from Brandon et al. (2003). *Tox App Pharmacol* 199:233-246.

The application of extracellular 3D matrix prevents the loss in albumin synthesis, leads to the phosphorylation of hepatocyte growth factor receptors and results in cuboidal, polar hepatocyte structure. This has been shown to result in relocalization of MDR1 in hepatic canalicular membrane (Sidhu and Omiecinski, 1995; Kudryavtseva and Engelhardt, 2003; Engl et al., 2004). Hepatocytes in 3D culture have been utilized to document the effects of a variety of compounds on MDR1, NTCP, MRP2 and BSEP expression and activity (Liu et al., 1999a; Luttringer et al., 2002; Kostrubsky et al., 2003; Hoffmaster et al., 2004). Thus, primary hepatocytes (in culture or suspension) are an excellent in-vitro tool for not only evaluating the role of metabolic pathways and transporters in the disposition of a compound and any interplay between metabolizing enzymes (Phase 1 and 2) and transporters (uptake and efflux) but also provide data that can predict the in-vivo situation with better accuracy.

1.10 Summary and introduction to dissertation

Preterm birth (PTB), birth prior to 37 weeks of gestational age, is the leading cause of neonatal mortality (infant death <28 days of life) and is a major cause of early childhood mortality and morbidity in the United States.

17alpha-hydroxyprogesterone caproate (17-OHPC) has recently been documented to reduce the incidence of preterm birth. 17-OHPC is administered intramuscularly once a week at a fixed dose of 250 mg. Currently very limited documentation is available on the pharmacokinetics of 17-OHPC in humans or animals. The purpose of this dissertation work is to elucidate the pharmacokinetics of 17-OHPC in both in-vitro (preclinical) and in-vivo (clinical) conditions in order to optimize the dosing regimen of this agent for preventing preterm birth.

The progestational effect of 17-OHPC is expected to be related to its concentration in the pregnant subjects. In earlier clinical studies 17-OHPC was demonstrated to be effective in only about 33% of patients. It is possible that this variability in clinical response may be related to the concentration of 17-OHPC. Inter-individual variability in the metabolism is an important factor contributing to the variability in the pharmacokinetics of several drugs. The metabolic fate of 17-OHPC is not completely understood. The metabolism of 17-OHPC in human has not been characterized. The role of the metabolites of 17-OHPC on the progestational effects of 17-OHPC or in mediating any side effects of 17-OHPC is also not known. A complete understanding of the pharmacokinetics of 17-OHPC will help in designing an optimal dosing regimen for this agent and improve therapeutic outcomes.

The following research will evaluate the preclinical and clinical pharmacology of 17-OHPC. We hypothesize based on the structural similarity between Medroxyprogesterone Acetate (MPA), a CYP3A4 substrate, and 17-OHPC (a progesterone analog) that CYP3A4 will

be the major enzyme involved in the metabolism of 17-OHPC by adult human liver. Further, given the substrate overlap between CYP3A4, CYP3A5 and CYP3A7, we hypothesize that fetal liver will metabolize 17-OHPC with CYP3A7 being the major isoform involved in the metabolism. Additionally, we hypothesize that metabolism of 17-OHPC will lead to the cleavage of the caproate side chain and production of 17-OHP which is responsible for the prolonged and more potent action of 17-OHPC over progesterone. We also hypothesize based on the fact that progesterone and its metabolites interfere with bile salt transport via inhibition of bile salt export pump (BSEP) that 17-OHPC, a structural analog of progesterone, will also inhibit bile salt transporter (BSEP) in adult and fetal human hepatocytes. Finally, since 17-OHPC is administered as a fixed dosing regimen consisting of 250mg weekly injection and a high inter-individual variability has been reported in CYP3A4 expression, we hypothesize that a large variability will be observed in the exposure of 17-OHPC in pregnant subjects.

Full descriptions of the methods used in this research project are outlined in Chapter 2. The aim of Chapter 3 was to investigate the metabolizing enzymes and metabolic pathways involved in the disposition of 17-OHPC in adult human liver using human liver microsomes and adult human hepatocyte primary cultures. The purpose of Chapter 4 was to elucidate the metabolizing enzymes and metabolic pathways involved in the disposition of 17-OHPC by fetal human liver. Primary cultures of fetal hepatocytes were used as the model system to study the metabolism and enzyme kinetics. The goal of Chapter 5 was to evaluate the role of transporters in the disposition of 17-OHPC and the effect of 17-OHPC on bile salt transport. Primary cultures of human fetal hepatocyte overlaid with Matrigel® were used for this study. The purpose of Chapter 6 was to evaluate the pharmacokinetics of 17-OHPC in human pregnant subjects with multiple gestation.

2.0 Material & Methods

2.1 Chemicals

17α-hydroxyprogesterone caproate (17-OHPC, Mol. Wt. 428.6) was a gift from Diosynth Inc, Chicago. The radioactive isotope of 17α-hydroxy [1, 2, 6, 7-3H]-progesterone [1-14C] caproate was custom synthesized by RTI International (Research Triangle Park, NC). Quinidine, sulfaphenazole, coumarin, ketoconazole, methimazole, α-naphthoflavone, testosterone, 6-β-hydroxytestosterone, medroxyprogesterone acetate, rifampin, phenobarbital, cyclosporine and NADPH were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and HPLC grade water were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Microsomes derived from baculovirus-infected insect cells expressing human CYP isoforms (CYP3A4, 3A5 and 3A7) were purchased from BD-Gentest (Woburn, MA, USA). EMEM (Eagle's Minimum Essential Medium), HBSS (Hank's balanced salt solution), HMM (Hepatocyte. Maintenance Medium), DMEM (Dulbecco's Modified Eagle Medium), Dexamethasone, Insulin were purchased from Lonza (Lonza Walkersville Inc., MD). (Lonza Walkersville, MD). Matrigel™ Matrix was purchased from BD Biosciences, Bedford, MA. Penicillin G/streptomycin was acquired from GIBCO Laboratories (Grand Island, NY). ^{3}H-Taurocholic acid (2 Ci/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA). BSA standard for total protein measurement by Lowry was obtained from Sigma. Reagents for reverse transcription were purchased from Promega (Madison, WI). TaqMan analysis was used for all the genes analyzed with primers and conditions designated by Assays on Demand, Gene Expression Products (Applied Biosystems). Forward and reverse primers for CYP3A4, CYP3A7, MDR1, MDR3, OATP1B1, OATP1B3, OATP2B1, BSEP, NTCP, MRP2, MRP3, MRP4, BCRP and cyclophillin were synthesized by Applied Biosystems. Primers for all transporters and Taqman probe were purchased from Applied Biosystems. Assay IDs for each gene are listed in Table 4. All solvents and other chemicals used were of HPLC grade or the highest purity available.

Table 4. Real-Time PCR Assay IDs for genes detected by Taqman gene expression assays

GENE SYMBOL	GENE NAME	ASSAY ID
OATP1B1	Solute carrier organic anion transporter family, member 1B1	Hs00272374_m1
OATP1B3	Solute carrier organic anion transporter family, member 1B3	Hs00251986_m1
OATP2B1	Solute carrier organic anion transporter family, member 2B1	Hs00200670_m1
P-gp	ATP-binding cassette, B1 (ABCB1 - MDR1)	Hs00184500_m1
MDR3	ATP-binding cassette, B4 (ABCB4)	Hs00251620_m1
BSEP	ATP-binding cassette, B11 (ABCB11)	Hs00184824_m1
MRP2	ATP-binding cassette, C2 (ABCC2)	Hs00166123_m1
MRP3	ATP-binding cassette, C3 (ABCC3)	Hs00358656_m1
MRP4	ATP-binding cassette, C4 (ABCC4)	Hs00195260_m1
BCRP	ATP-binding cassette, G2 (ABCG2)	Hs00184979_m1
NTCP	Sodium/bile acid cotransporter 1 (SLC10A1)	Hs00914889_m1
PPIA	Cyclophilin A (peptidylprolyl isomerase A)	Hs99999904_m1
CYP 3A4	Nifedipine oxidase	Hs00430021_m1
CYP 3A7	Aryl hydrocarbon hydroxylase	Hs00426361_m1

2.2 Preparation of human liver microsome

Liver pieces were dissected and kept in cold saline on ice. Liver microsomes were prepared by a standard differential centrifugation procedure with minor modifications (Court and Greenblatt, 1997; Nelson et al., 2001). Briefly, liver pieces were homogenized with three volumes of a homogenization buffer (50 mM Tris-HCl buffer, 1.0% KCl and 1 mM EDTA, pH 7.4), using an electrical homogenizer (Polytron, Brinkman Instruments, N.Y., U.S.A.). The crude homogenate was centrifuged (Optima XL-100K ultracentrifuge, Beckman Instruments, Palo Alto, CA, USA) at 10,000g for 20 min at 4ºC. The supernatant was further centrifuged at 105,000g for 65 min at 4ºC to sediment the microsomes. The microsomes were reconstituted using a manual homogenizer (Wheaton, Millville, NJ, USA) in twice their weight of Tris HCl buffer (50 mM Tris-HCl buffer, pH 7.4) containing 20% glycerol. Aliquots (1.0 ml) were immediately kept in storage at -80ºC until used. The protein content was determined by Lowry's method (Lowry et al., 1951) using bovine serum albumin (Fluka, USA, 98% pure) as a standard.

2.3 Adult Hepatocyte Isolation

Adult human liver tissue was procured under an Institutional Review Board approved protocol and with support from the liver tissue cell procurement and distribution system (LTCDS). Primary cultures of human hepatocytes (PCHH) were prepared by a three-step collagenase perfusion technique (Strom et al., 1996). Viability of cells was determined by the trypan blue

exclusion method and cells were used only when the viability was at least 65%. Briefly, equal volumes of trypan blue (0.4%) and cell suspension were mixed and a portion of this suspension was then placed on a hemocytometer. The cells were observed under a light microscopy and the numbers of live and dead cells (stained blue), were counted in two fields. Concentration of cells (number of cells / ml) was determined using the following formula: Live cells in two fields x 10,000 = # of cells/ml. Cells were diluted to final volume of 1 x 106 cells per ml. Hepatocytes were plated on Falcon 6-well culture plates (1.5 x 106 cells), previously coated with rat tail collagen in HMM supplemented with 0.1 μM insulin, 0.1 μM dexamethasone, 0.05% streptomycin, 0.05% penicillin, 0.05% amphotericin B and 10% bovine calf serum. After allowing the cells to attach for 4 to 6 hours, medium was replaced with serumfree medium containing all of the supplements described above. Cells were maintained in culture at 37°C in an atmosphere containing 5% CO2 and 95% air. After 24 hours in culture, unattached cells were removed by gentle agitation and the medium was changed. For transporter studies, cells were overlayed with Matrigel™ (0.233 mg/mL) at this time. The medium was changed every 24 hours and the hepatocytes were maintained in culture between 5 and 9 days depending on the experimental design.

2.4 Fetal hepatocyte isolation

Fetal livers were obtained from planned termination of pregnancy procedures, having human fetal tissues with a gestational age between 18-23 weeks. The tissues were obtained from Mercy Women's Hospital, Pittsburgh, PA after obtaining informed consent. The fetal tissue was kept in EMEM and transported on ice. The hepatic tissue was gently scraped with a sterile cell scraper in

a 100 mm cell culture dish. HBSS (30ml) containing 0.1M EGTA was added to the tissue and the contents were pipetted up and down few times with a 25 ml pipette to get a homogenous mix. Tissue was transferred to a 50 ml falcon tube and gently rotated by hand to suspend red cells. The tubes were centrifuged at 60x g for 5 minutes and the supernatant poured off. Cell pellets were washed with 30 ml HBSS and again centrifuged using the same settings. The supernatant was poured off and the volume of pellet was recorded. The pellet was then resuspended in 25 ml EMEM (at 37ºC) containing 1 mg/ml collagenase XI, 0.2 mg/ml DNase I and penicillin/ streptomycin. The suspension was rotated on a rotary shaker at 37ºC, approximately 25 minutes. After shaking the tissue was redispersed by pipetting up and down with a 10 ml pipette. The suspension was spun down at 50 – 100 G for 5 minutes. The supernatant was poured off and resuspended in 50 ml EMEM (at 4ºC). The resulting suspension was spun down again using the previously described settings and supernatant poured off. The pellet was resuspended in 40 ml cell culture media (DMEM+) containing insulin (0.1µM), dexamethasone (0.1µM) and fungizone (25 µg/ml) and cell viability estimated using the trypan blue exclusion method. The cells were diluted to the desired concentration with DMEM (+) and 10% bovine calf serum was added. The cells were plated at a cell density of 0.3 - 0.5 x 10^6 cells per well in 12-well plates previously coated with 0.2 mg/ml type I collagen. Media was changed to serum free DMEM (+) after 3-5 hours. The media was changed once every day for the next 3-4 days until the cells achieved the desired confluency. The media was subsequently aspirated and the cells overlaid with Matrigel® (final concentration of 0.233 mg/ml) diluted in HMM (+) containing insulin (0.1µM), dexamethasone (0.1µM) and fungizone (25 µg/ml)

2.5 General hepatocyte treatment

Briefly, hepatocytes were maintained in culture in the presence of an inducer or vehicle control (DMSO 0.1%) On the day of the experiment, cells were washed with HMM devoid of insulin, dexamethasone, antibiotics and antifungal drugs. It is assumed that this one hour period is sufficient to remove residual chemical from the enzyme active site. Following this period, media containing the appropriate probe substrate was applied to the cells with media sampled at the appropriate time points. Table 5 summarizes the enzymes studied with the respective probe substrates, concentrations used and sampling times. A number of variations on the traditional methods used to assess drug metabolizing enzyme activity are discussed in the subsequent chapters.

2.6 Analytical methods

Only the analytical methods used to assess enzyme activity that are used in subsequent chapters are described below.

2.6.1 HPLC measurement of CYP3A4 activity

The concentration of 6β-hydroxytestosterone in the medium was measured by HPLC as previously described, with the following modifications (Kostrubsky et al., 1999). One hundred microliters of medium were diluted with an equal volume methanol and centrifuged at 13,000 r.p.m. for 5 minutes. One hundred microliters of this solution was injected onto a LiChrospher

100 RP-18 column (4.6 x 250 mm, 5 μm). 6β-hydroxytestosterone was eluted with a mobile phase of methanol/water (60:40, v/v) at a flow rate of 1.2 ml/min and the eluents were monitored at 242 nm. The concentration of the metabolite was quantitated by comparing the peak areas in samples to a standard curve containing known amount of the metabolite.

Table 5. Probe substrates to study enzyme and transporter activity in human hepatocyte cultures

Enzyme/ Transporter	**Probe Substrate**	**Probe Conc. (μM)**	**Metabolite**	**Standard Inducer**	**Inducer Conc. (μM)**	**Incubation Time**	**Analysis**
CYP3A4	Testosterone	250	6β(OH)-testosterone	Rifampin	10	30 min	HPLC-UV
BSEP	^{3}[H]-Taurocholate	1	NA	NA	NA	20 min	Scintillation counter

2.6.2 HPLC measurement of 17-OHPC and its metabolites

Analysis of the unmetabolized drug and the potential metabolites obtained from HLM based incubations was performed using a HPLC system equipped with UV detection. The HPLC system comprised of an autosampler (712 WISP, Waters) and solvent delivery system (Waters 501) attached to a UV detector (Waters 486). Chromatography was performed with a 4.6 x 250 mm, 100Å, 5μm Symmetry C18 (Waters, Milford, MA, USA) column. Isocratic elution was

performed with a mobile phase of 90% (v/v) methanol in water at a flow rate of 0.8 ml/min, column temperature of 25°C and eluent monitored at 242 nm. The intra- and interday variation expressed as coefficient of variation did not exceed 10% in any of the assays. The concentration of 17-OHPC in samples was quantitated by comparing the peak areas in samples to a standard curve of the pure drug. The metabolites were quantitated by expressing them in terms of 17-OHPC equivalents.

2.6.3 Radio- HPLC measurement of 17-OHPC and its metabolites

Analytical separations were achieved using conditions similar to above mentioned HPLC method. The metabolites were analysed using a Radiomatic Model 525TR/FLO-ONE flow-through radioactivity detector (PerkinElmer Life Sciences, USA), and peak areas were integrated with Windows-based FLO-ONE version 3.61.

2.6.4 LCMS measurement of 17-OHPC and its metabolites

The HPLC system used for the analysis of 17-OHPC and its metabolites was a Waters 2695 Model (Waters Corporation, MA, USA). Separation was performed on Waters C18 Symmetry (3.5 μm, 2.1 x 150 mm) analytical column at 40ºC with a Waters C18 Symmetry (2.1 x 10 mm) guard column. The mobile phases used were: [A] - 5% methanol in water containing 0.1% formic acid and [B] - methanol containing 0.1% formic acid. The total run time was 45 min at a flow rate of 0.2 ml/min. A gradient profile was used starting from a mobile phase containing 50% solution [B], increased linearly to 95% [B], and followed by returning to the initial condition of 50% [B] to achieve the base line.

Analysis was performed on a Micromass Quattro Micro triple quadrupole mass spectrometer (Waters, Milford, MA, USA) with positive electrospray ionisation mode. Medroxyprogesterone acetate was used as the internal standard. For all analytes and internal standard, MS settings used were as follows: capillary voltage 3.5 kV; source temperature 100ºC; desolvation temperature 300ºC; cone gas flow (L/hr) 50; desolvation gas flow (L/hr) 550; argon pressure 20±10 psig; nitrogen pressure 100±20 psig. The LC-MS system was controlled and data collected with Masslynx® version 4.1.

2.6.5 LCMS measurement of 17-OHPC in plasma samples

The method used for the analysis of 17-OHPC in plasma samples has been published previously (Zhang et al., 2007; Zhang et al., 2008b). Briefly, routine daily calibration curves, controls and the clinical samples were thawed at room temperature. Exactly 400 µl of plasma was diluted with 1 ml of water, after addition of 25 µl of IS (1µg/ml), the entire solution was passed through SPE cartridges, previously conditioned with methanol and water, under vacuum. After washing with 1ml of 50% methanol, 17-OHPCwas eluted with 1ml of methanol and the eluent was evaporated to dryness under air. The residue was reconstituted in 100 µl of starting mobile phase and 20 µl was injected into the HPLC system connected to the mass spectrometer. The HPLC unit used for the analysis of 17-OHPC was a Waters 2695 model (Waters Corporation, MA, USA). Separation was performed on Waters C18 Symmetry (3.5 µm, 2.1 x 50 mm) analytical column at 40ºC with a Waters C18 Symmetry (2.1 x 10 mm) guard column. The mobile phases used were: [A] - 5% methanol in water containing 0.01% ammonium hydroxide and [B] - methanol containing 0.01% ammonium hydroxide. The total run time was 7 min at a flow rate of 0.3 ml/min. A gradient profile was used starting from a mobile phase containing

65% solution [B], increased linearly to 97% [B], held for 2.5 min, followed by returning to the initial condition of 65% [B] to achieve the base line.

Analysis was performed on a Micromass Quattro Micro triple quadrupole mass spectrometer (Waters, Milford, MA, USA) with positive electrospray ionisation mode using multiple reaction monitoring (MRM). For all analytes and internal standard, MRM setting used was as follows: capillary voltage 3.5 kV; source temperature 100ºC; desolvation temperature 450ºC; cone gas flow (L/hr) 50; desolvation gas flow (L/hr) 550; argon pressure 20±10 psig; nitrogen pressure 100±20 psig. The extracted ions monitored following MRM transitions were m/z 429.24→313.25 for 17-OHPC and m/z 387.15→327.25 for MPA (IS). The cone and collision energy for 17-OHPC and MPA are shown in Table 6. The LC-MS system was controlled by Masslynx® version 4.1, and data collected with the same software.

Table 6. The cone and collision energy set in LCMS for 17-OHPC and MPA (IS)

	Parent (m/z)	Daughter (m/z)	Dwell (sec)	Cone Energy (V)	Collision Energy (V)
17-OHPC	429.24	313.25	0.1	24	13
MPA	387.15	327.25	0.1	25	13

2.7 Determination of total protein

After sampling of the medium for metabolite measurements, the remainder of medium (0.5 mL) was aspirated from each well. Cells were then harvested in 150 μL of phosphate buffer and

stored at -80°C for protein determination by the method of Lowry (Lowry et al., 1951). Briefly, the proteins were dissolved in SDS/sodium hydroxide, then 1% sodium tartarate and 1% copper sulfate were added, followed by the addition of Folin's reagent. The tubes were mixed gently and the color was allowed to develop for 45 min. At the end of 45 minutes, 200 µL aliquotes were transferred to 96-well plates and the absorbance was measured at 490 nm. The concentration of the protein was calculated using bovine serum albumin as a protein standard.

2.8 Measurement of mRNA expression

Total RNA was extracted from 1 x 10^6 cells plated on 6-well plates using 1 mL Trizol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. RNA was quantified spectrophotometrically and subjected to agarose gel electrophoresis to assess the integrity of RNA. Following treatment with RNase-free DNase (Promega, Madison, WI), 2 µg of RNA was mixed with 0.5 µg of Random Hexamers (Promega) heated to 70°C for 5 minutes then cooled to 4°C. A reaction mixture containing 200 U MMLV-Reverse transcriptase, 1 mM dNTPs and 25 U RNasin (Promega) was added to the previous mixture and incubated at 37°C for 60 minutes. The resulting cDNA was diluted 10-fold and stored at -20°C.

2.9 Real-Time PCR

Real-time quantitative RT-PCR was conducted on an ABI Prism 7700 (Applied Biosystems, Foster City, CA). DNase-I treated total RNA of each sample was transcribed and mixed with

specific primer sets and PCR master mix (Applied Biosystems). TaqMan analysis was used for all the genes analyzed with primers and conditions designated by Assays on Demand, Gene Expression Products (Applied Biosystems). Data were analyzed with the ABI Prism 7700 SDS software (version 1.0). Expression of specific genes was normalized to an internal control (cyclophillin) mRNA expression.

3.0 Elucidation of the metabolizing enzymes and metabolic pathways involved in the disposition of 17-OHPC in adult human liver.

[Sharma S, Ou J, Strom S, Mattison D, Caritis S and Venkataramanan R (2008) Identification of enzymes involved in the metabolism of 17alpha-hydroxyprogesterone caproate: an effective agent for prevention of preterm birth. *Drug Metab Dispos* **36:**1896-1902]

3.1 Abbreviations

6β(OH)TE - 6β(OH) testosterone
CYP - cytochrome P450
CYP3A4 - cytochrome P4503A4
HMM - hepatocyte maintenance medium
FHH - primary cultures of human hepatocytes
TE - testosterone
17-OHPC - 17α-hydroxyprogesterone caproate
17-OHP - 17α-hydroxyprogesterone
P – Progesterone
MPA – Medroxyprogesterone acetate
FHH - Fresh human hepatocytes
HLM - Human liver microsomes
FMO - Flavin containing monooxygenase

3.2 Abstract

Aims: Preterm delivery, that is delivery before 37 completed weeks of gestation, is the major determinant of neonatal morbidity and mortality. In a recent multicentered trial, 17α-hydroxyprogesterone caproate (17-OHPC) reduced the rate of preterm birth by 33% in a group of high risk women. Limited pharmacologic data exist for this drug. The recommended dose is empiric, the metabolic pathways are not well defined especially in pregnant women, and the fetal exposure has not been quantified. The purpose of this study was to define the metabolic pathways of 17-OHPC in human liver.

Methods: The model systems used were human liver microsomes, fresh human hepatocytes and expressed enzymes. 17-OHPC was quantified using both HPLC-UV and LC-MS systems. Protein content and mRNA were also measured.

Results: Human liver microsomes (HLM) in the presence of NADPH generated three metabolites; whereas five metabolites were observed with fresh human hepatocytes (FHH). Metabolism of 17-OHPC was significantly inhibited by the CYP3A4 inhibitors, ketoconazole and troleandomycin, in HLM and FHH. Metabolism of 17-OHPC was significantly greater in FHH treated with the CYP3A inducers, rifampin and phenobarbital. Further, studies with expressed enzymes demonstrated that 17-OHPC is metabolized exclusively by CYP3A4 and CYP3A5. The caproic acid ester was intact in the major metabolites generated indicating that 17-OHPC is not converted to the primary progesterone metabolite, 17-α hydroxyprogesterone (HP).

Conclusions: This study demonstrates that 17-OHPC is metabolized by CYP3A. Since, CYP3A is involved in the oxidative metabolism of numerous commonly used drugs; 17-OHPC may be involved in clinically relevant metabolic drug interactions with co-administered CYP3A inhibitors or inducers.

3.3 Introduction

Preterm delivery — that is, delivery before 37 completed weeks of gestation — is the major determinant of infant mortality in developed countries (Paneth, 1995). Preterm delivery is more common in the United States than in many other developed countries and is the factor most responsible for the relatively high infant mortality in this country (Mattison et al., 2001). However, little is known about the mechanisms especially at the molecular level which are responsible for this pathophysiology.

In animal (sheep, rabbits, cows and pigs) models, decline in progesterone and rise in estrogen levels in plasma has been shown to precede induction of labor (Liggins et al., 1973; Anderson et al., 1975; Young, 2001). Thus, it was hypothesized that preterm delivery or spontaneous abortion occurred when body levels of progesterone dropped below the required level to maintain pregnancy (Keirse, 1990). These hormonal fluctuations led to various molecular changes especially in the myometrium (increases in gap junctions, oxytocin receptors and COX-2) (Gorodeski et al., 1987; Kelly et al., 1992; Chwalisz, 1994; Stjernholm et al., 1996) which highly increased its sensitivity to stimuli. However, the fluctuations in plasma progesterone levels were not observed in humans, monkeys and guinea pigs. This inter-species contradiction led to the concept of "functional progesterone withdrawal".

According to this concept, progesterone has a regulatory role in initiating labor; however, it is not reflected by the fluctuating plasma levels. Several models have been proposed in support of this concept involving downregulation of progesterone receptors, change in expression levels of various progesterone receptor phenotypes (Progesterone receptor A, B, C), chemical induced inhibition of progesterone receptor function or inhibition mediated by loss of interaction of

progesterone receptor with the responsive genes (Brown et al., 2004). However, the evidence so far is not conclusive in favor of any of the proposed models.

One drug that has shown promise in cases of preterm birth is 17 alpha-hydroxyprogesterone caproate (17-OHPC) (Meis, 2005; Petrini et al., 2005; Sanchez-Ramos et al., 2005). 17-OHPC is a synthetic hormone (Fig.3A) similar to the natural female sex hormone progesterone (Fig.3C) and has been proven effective in the prevention of spontaneous abortion in women with a history of recurrent miscarriage (habitual abortion).

It was hypothesized that the mechanism of action of 17-OHPC may involve maintenance of body's progesterone levels at desired threshold by being metabolised into hydroxy-progesterone or progesterone (http://www.tiscali.co.uk, 2005). However, based on above mentioned reasons and the fact that progesterone levels required to bind to progesterone receptors are 1000 times less than the basal maternal levels, there is little reason to believe that progesterone supplementation will reduce preterm birth. In addition, administration of 17-OHPC did not appear to increase plasma progesterone concentrations in singletons or twins (Hartikainen-Sorri et al., 1980). Therefore, any benefit of 17-OHPC on preterm birth rates does not appear to involve an increase in plasma or tissue progesterone concentrations.

In animal studies, 17-OHPC produces a longer lasting and more robust progestational effect on the endometrium than progesterone (Wu and Allen, 1959). When compared to injections of free progesterone, the same amount of 17-OHPC has twice the progestational activity and is twice as prolonged thus, favoring its use over the former in pregnancy (Junkmann, 1953). 17-OHPC also appears to be more effective than progesterone in providing luteal support in patients undergoing *in vitro* fertilization-embryo transfer cycles (Abate et al., 1997; Costabile et al., 2001; Unfer et al., 2004).

Thus, we postulate that whatever leads to this enhanced effect of 17-OHPC over progesterone may be responsible for the benefit in preterm birth prevention.

Currently undergoing extensive clinical trials, 17-OHPC is usually administered intramuscularly once a week at a dose of 250 mg (Meis et al., 2003). However, the optimal dosing regimen of 17-OHPC has not yet been determined and the currently used dose is arbitrary (based on studies in 1950-60s, (Castelazo Ayala et al., 1959), (Eichner, 1958; Gold and Cohen, 1958; Reifenstein, 1958) and does not have a pharmacological basis. The progestational effect of 17-OHPC is expected to be related to its concentration in the pregnant subjects. It is possible that one or more metabolites of 17-OHPC may also be active.

Limited documentation is available on the pharmacokinetics of 17-OHPC in humans or animals. The only study that had evaluated the pharmacokinetics of 17-OHPC was carried out in patients with endometrial carcinoma and used a nonspecific immunoassay to measure 17-OHPC (Onsrud et al., 1985). This study reported a large variability in the serum concentration vs. time profile after a fixed intramuscular dose to different patients and the apparent half life of 17-OHPC appeared to be several days in these patients. Additionally, in earlier clinical studies 17-OHPC was not demonstrated to be effective(Hartikainen-Sorri et al., 1980; Hauth et al., 1983) and only about 33% - 60% of patients (depending on gestational age) in the recent trials appear to benefit from 17-OHPC administration (Spong et al., 2005).

It is possible that this variability in clinical response to 17-OHPC may be related to the variability in drug metabolism in different individuals in addition to other factors like gestational age. Further, the issue of drug-drug interactions needs to be addressed prior to its regular clinical use. Thus, it is important to identify the various enzymes (phase 1 and phase 2) involved in 17-OHPC metabolism for not only designing an optimal dosing regimen for this agent but also to

determine the role of the metabolites of 17-OHPC on the progestational effects or in mediating any side effects. However, to date there are no literature reports available on enzymatic pathways involved in the metabolism of this progestational compound and the various metabolites that are produced as a result.

The purpose of the present study was to identify the various enzymes (cytochrome P450 and FMO enzymes) involved in 17-OHPC metabolism and to characterize the metabolites of this compound.

Medroxyprogesterone Acetate (Figure 3D) is a structural analog of 17-OHPC with an acetate ester at the C-17 position. It has been reported that CYP3A4 is mainly involved in the overall metabolism of MPA in human liver microsomes (Kobayashi et al., 2000). Further, oxidation (mono- and dihydroxy MPA metabolites) has been reported as the major metabolic pathway involved in the metabolism of Medroxyprogesterone Acetate (Sturm et al., 1991). Thus, based on structural similarity between MPA and 17-OHPC we hypothesize that CYP3A4 will be the major enzyme involved in the metabolism of 17-OHPC by adult human liver. Further, we believe that the oxidation will be the major metabolic pathway involved in 17-OHPC metabolism with the generation of monohydroxy-17OHPC as the major metabolite.

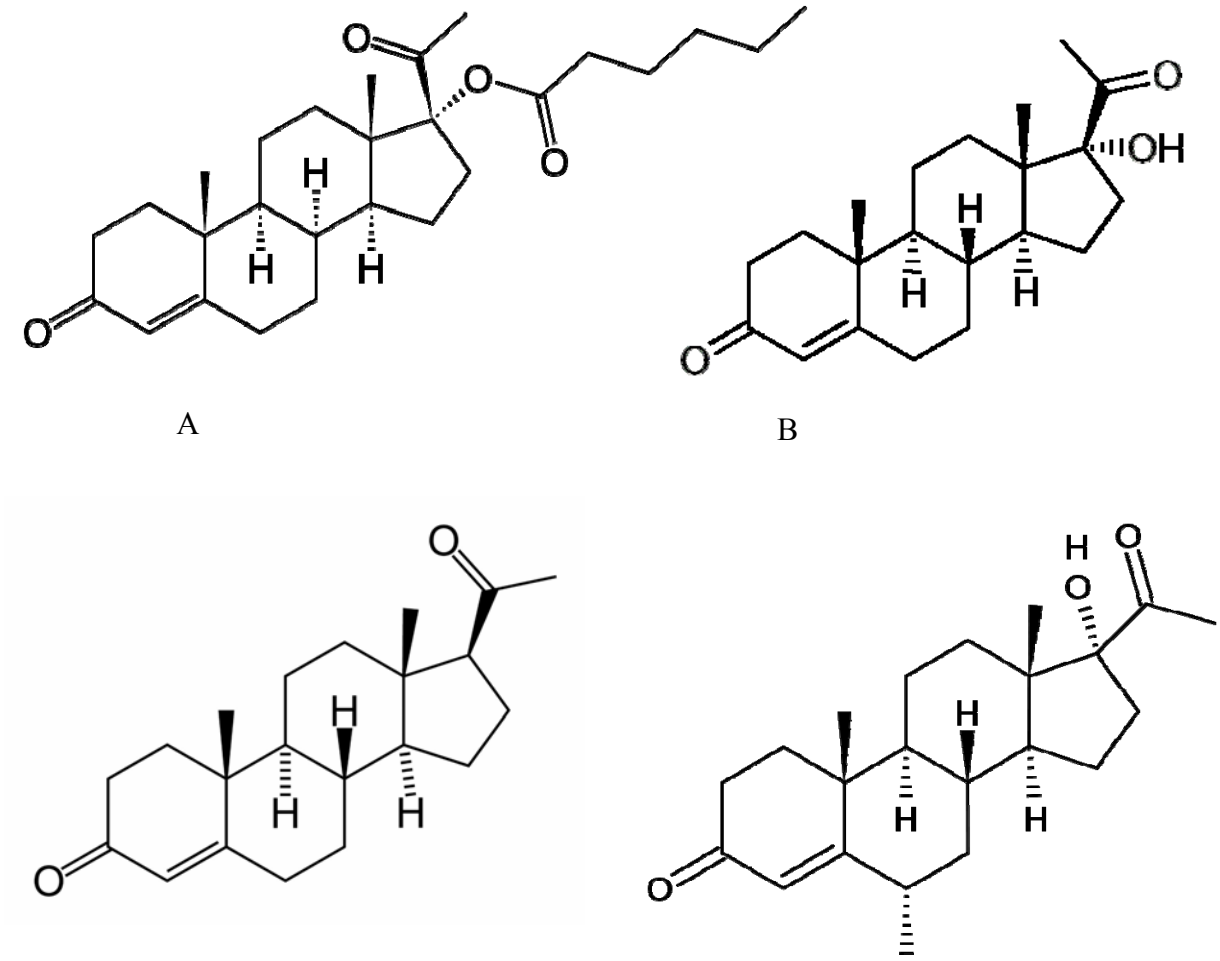


Figure 3. Chemical Structures of 17-OHPC, 17-OHP, P and MPA

A) Product Name: 17α-Hydroxyprogesterone caproate (17alpha- hydroxy-4-pregnene-3, 20-dione hexanoate)
B) Product Name: 17α-Hydroxyprogesterone (17alpha- hydroxy-4-pregnene-3, 20-dione)
C) Product Name: Progesterone (4-pregnene-3, 20-dione)
D) Product Name: Medroxyprogesterone Acetate (Pregn-4-ene-3,20-dione, 17-(acetyloxy)-6-methyl).

3.4 Methods

3.4.1 Microsomal incubations

Optimal conditions for the evaluation of the metabolism of 17-OHPC were selected by varying the time of incubation (0 - 180 min) and the microsomal protein concentrations (0 – 2 mg/ml). Different concentrations of 17-OHPC (0 - 200 µg/ml in methanol, final concentration 0.5%) were incubated with human liver microsomes (0.5 mg/ml, optimum protein concentration) and $MgCl_2$ (10 mM) in 0.1mM phosphate buffer (pH 7.4). The final volume was allowed to equilibrate in a shaking water bath for 5 min at 37 ºC. The reaction was initiated with the addition of NADPH (1 mM). In additional experiments, the incubations were also carried out in the absence of either NADPH or protein. After 60 min of incubation (optimum incubation time), the reaction was stopped by immediately adding equal volume of cold methanol. The mixture was centrifuged at 700 RCF for 20 min and supernatant was injected into the HPLC.

Incubations of [^{3}H], [^{14}C] - 17-OHPC were also performed with human liver microsomes. Radiolabeled 17-OHPC was incubated with three individual human liver microsomal preparations for 10 mins using the above mentioned method and the samples analyzed directly by HPLC connected to a radioactivity detector.

3.4.2 Chemical Inhibition Studies

Various CYP inhibitors were used in the study to identify CYPs which might be involved in the metabolism of 17-OHPC. Human liver microsomal preparations (n=3) were used for the studies with the incubations being performed in triplicate. Experiments were done at different 17-OHPC

concentrations (0-200μg/ml expressed as 0-467 μM) spanning the concentrations seen clinically in order to identify the type of inhibition keeping the inhibitor concentration constant. CYP isoform selective chemical inhibitors were used at the following concentrations based on values reported in the literature (Newton et al., 1995): CYP1A2 (α – naphthoflavone, 10 μM), CYP2A6 (coumarin, 20 μM), CYP2C9 (sulfaphenazole, 5 μM), CYP2D6 (quinidine, 5μM), CYP3A (ketoconazole, 1.0 μM), and FMO3 (methimazole, 200μM). Additionally, inhibition studies were also carried out by incubating 17-OHPC (25μM) with different concentrations of ketoconazole (0.01 to 10 μM) to determine the IC_{50} for the reaction. The formula used to determine the IC_{50} for microsomal incubations involving 17-OHPC and ketoconazole involves estimating % inhibition which was calculated as follows:

% Inhibition = [(17-OHPC $_{\textbf{without inhibitor}}$ - 17-OHPC $_{\textbf{with inhibitor}}$**) / (**17-OHPC $_{\textbf{without inhibitor}}$**)] * 100**

where, 17-OHPC $_{\text{without inhibitor}}$ is amount of 17-OHPC metabolized in the absence of ketoconazole relative to the total amount of 17-OHPC. 17-OHPC $_{\text{with inhibitor is}}$ amount of 17-OHPC metabolized in the presence of ketoconazole relative to the total amount of 17-OHPC. Subsequently, IC50 values or the inhibitor concentration resulting in 50% inhibition of 17-OHPC metabolism, were determined from a plot of the % inhibition versus the logarithm of ketoconazole concentration.

In the case of mechanism based inhibitor like troleandomycin (TAO; 100μM), pre-incubation was done for 30 min at 37°C prior to adding the substrate (17-OHPC, 25μM). In inhibition experiments with ketoconazole (1, 10 μM) the substrate was co-incubated with inhibitor.

3.4.3 Expressed enzyme microsomal incubations

The incubations (n=3) were carried similar to the method described for human liver microsomes. To evaluate the involvement of CYP isoforms in 17-OHPC metabolism, 20 pmole of each expressed enzyme tested was incubated for 60 mins with 17-OHPC and the samples analyzed using HPLC-UV.

3.4.4 Incubations with fresh human hepatocytes

Briefly, hepatocytes were maintained in culture in the presence of the chemical under study or vehicle control (DMSO 0.1% or MeOH 0.1 %). On the day of the experiment, cells were washed with HMM devoid of insulin, dexamethasone, antibiotics and antifungal drugs. Drug stocks were prepared in methanol at 1000-fold incubation concentration (100 mM). Ten microliters of this 100 mM stock was added to a vial containing 10 ml of hepatocyte maintenance media (HMM). Reactions were started by incubating 6-well cell culture plates containing human hepatocytes (1.5 million cells/well) with the drug (17-OHPC or TE) in HMM solutions for 60 min. At the end of that time, 1 mL of medium was sampled and stored at -80°C analysis. The remaining media was aspirated, the cells were harvested in phosphate buffer (0.1 M, pH 7.4) and stored at -80°C for protein determination.

For acute inhibition experiments, human hepatocytes (n=4) were co-incubated with 17-OHPC (25μM) in the presence and absence of inhibitors (troleandomycin and ketoconazole). The samples were incubated for 30 mins and collected as described above.

The induction experiments were initiated 24 hrs after plating the cells. The hepatocytes (n=3) were incubated with the inducers (rifampin and phenobarbital) for 4 days prior to adding

HMM/ 17-OHPC (50μM) or Testosterone (250μM) to estimate the effect of CYP3A induction on 17-OHPC metabolism and TE metabolism. Cells were also harvested for mRNA by adding 1 mL of Trizol® reagent to each well of a 6-well plate. The RNA samples were stored at -20°C for Real Time PCR analysis. Primers for CYP3A4 and cyclophillin and the PCR procedure have been described in Chapter 2. The mRNA expression for all genes was normalized to cyclophillin in each sample and expressed as fold change over control treatment.

3.4.5 Correlation studies:

Testosterone 6β-hydroxylation was used as the marker for CYP3A activity (Chiba et al., 1996). Formation rates of 17-OHPC metabolite (M2) were measured using microsomes (n=7) and fresh human hepatocytes (n=7) at a substrate concentration of 100 μM. These rates were correlated with 6β(OH)TE formation activity to assess the involvement of CYP3A isoforms.

3.4.6 Data Analysis

Data are expressed as the mean ± SD. Student's t-test was used to assess the significance of results. IC_{50} was calculated using GraphPad Prism 4.0 (GraphPad Software Inc., USA).

3.5 Results

3.5.1 Metabolism of 17-OHPC by CYPs in human liver microsomal and fresh human hepatocyte preparations.

Incubation of 17-OHPC with human liver microsomes resulted in the generation of three (M1-$M3_{HLM}$) main metabolites wherein ($M2_{HLM}$) was the major metabolite (Figure 4). The formation of metabolites was observed to increase upto 60 minutes using a microsomal protein concentration of 0.5 mg/ml. Hence, the abovementioned conditions were used for all incubations unless specified otherwise. The formation of metabolites ($M1_{HLM}$ and $M3_{HLM}$) was low compared to $M2_{HLM}$, thus, accurate data could not be obtained for these minor metabolites and the paper focuses on the major metabolite ($M2_{HLM}$). Incubation of 17-OHPC with fresh human hepatocytes resulted in the generation of five metabolites (M1-$M5_{HH}$). The major metabolite generated was $M2_{HH}$ which was used to characterize 17-OHPC metabolism in fresh human hepatocytes. Fig. 4 (A, B) shows the metabolism of 17-OHPC in microsomal incubates in the presence/ absence of NADPH detected using LC-UC analysis. All the metabolites were formed in a NADPH-dependent manner only. Fig. 5 (A) shows the metabolism of 17-OHPC in fresh human hepatocytes incubated for 1 hour detected using LC-MS analysis.

3.5.2 Incubation of [^{14}C, ^{3}H] 17-OHPC with Human Liver Microsomes

Incubation of radiolabeled 17-OHPC with HLM generated one major and two minor metabolite peaks (Figure 6). The recovery of radioactivity from the incubations was 85-97%.

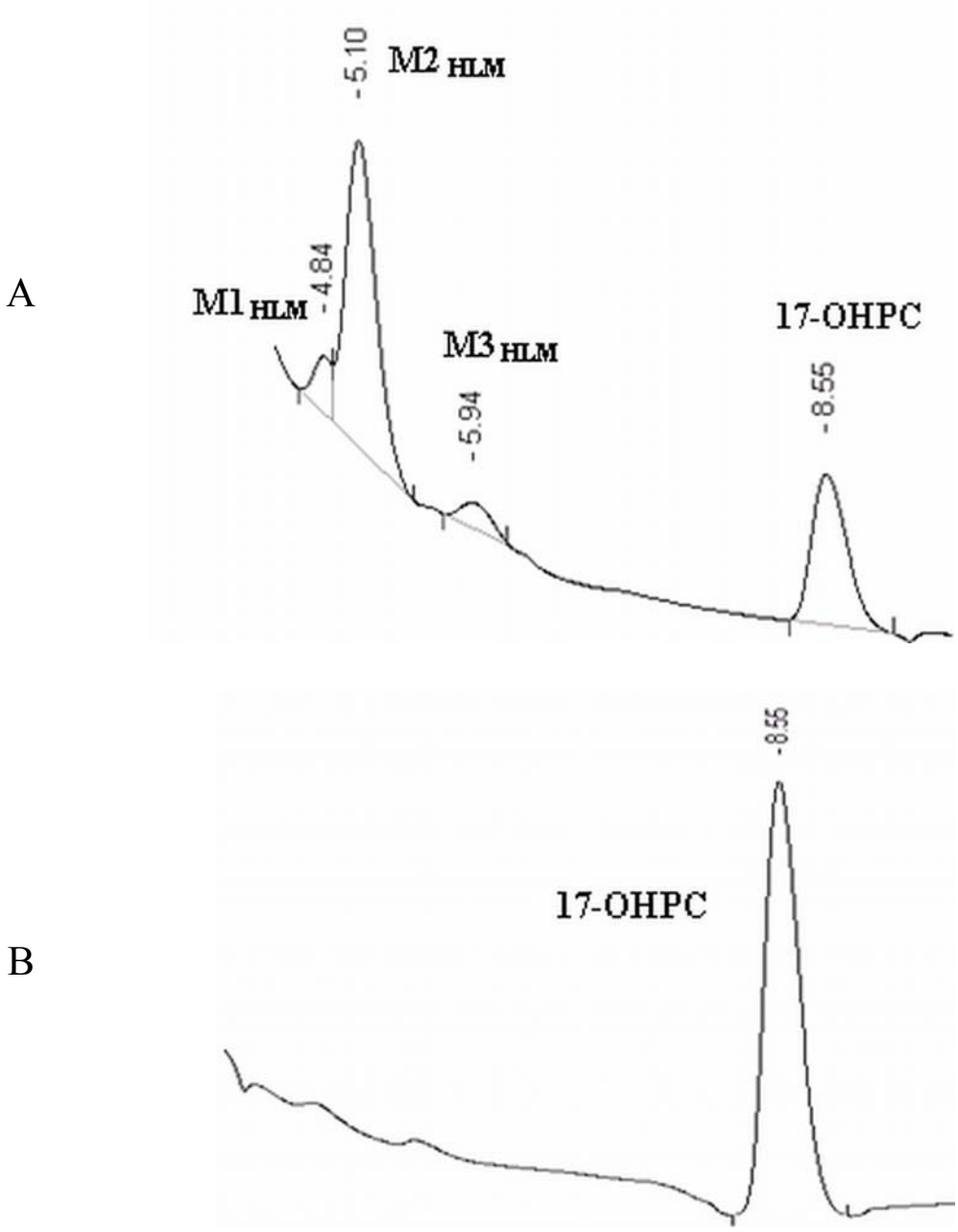


Figure 4. Chromatograms (HPLC-UV) depicting 17-OHPC metabolism by HLM

A) Chromatogram (HPLC-UV) showing retention times and absorption peaks for metabolites 1, 2, 3 ($M1_{HLM}$, $M2_{HLM}$, $M3_{HLM}$) and 17-OHPC having retention times of 4.84, 5.10, 5.94 and 8.55 respectively, after microsomal incubation in the presence of NADPH.
B) Chromatogram (HPLC-UV) depicting incubation of 17-OHPC with HLM in the absence of NADPH.

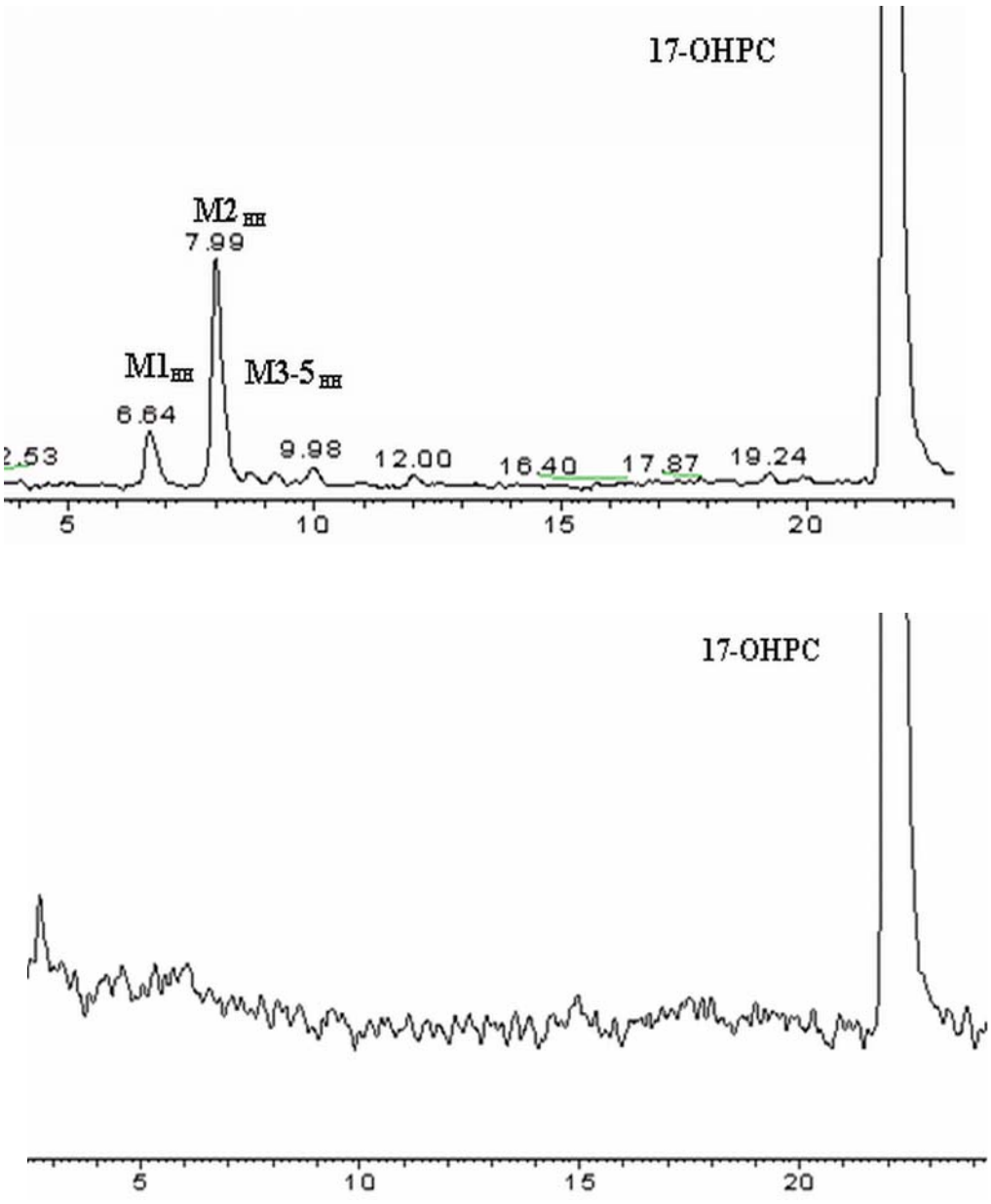


Figure 5. Chromatograms (LC-MS) depicting 17-OHPC metabolism by HLM

A) Chromatogram (LC-MS) showing absorption peaks for metabolites ($M1_{HH}$, $M2_{HH}$, $M3\text{-}5_{HH}$) and 17-OHPC after incubation with Fresh Human Hepatocytes (FHH) for 60 minutes. The retention times for M1, M2 and 17-OHPC were observed to be 6.64, 7.99 and 21.71 respectively.
B) Chromatogram (LC-MS) showing absorption peak for 17-OHPC after incubation with Fresh Human Hepatocytes (FHH) for 1 minute. No metabolite formation was detected. The retention time for 17-OHPC was 21.97.

The major metabolite constituted approximately 60-65% of the metabolized 17-OHPC and the two minor metabolites were estimated to be approximately 15%. The remaining 20% of the metabolite could not be accounted for due to the limitations of analytical method used. The two ([^{14}C, ^{3}H]) labels on the 17-OHPC molecule were observed to remain intact in the metabolites generated confirming that the caproate side chain or the ring structure was not cleaved during metabolism in human livers.

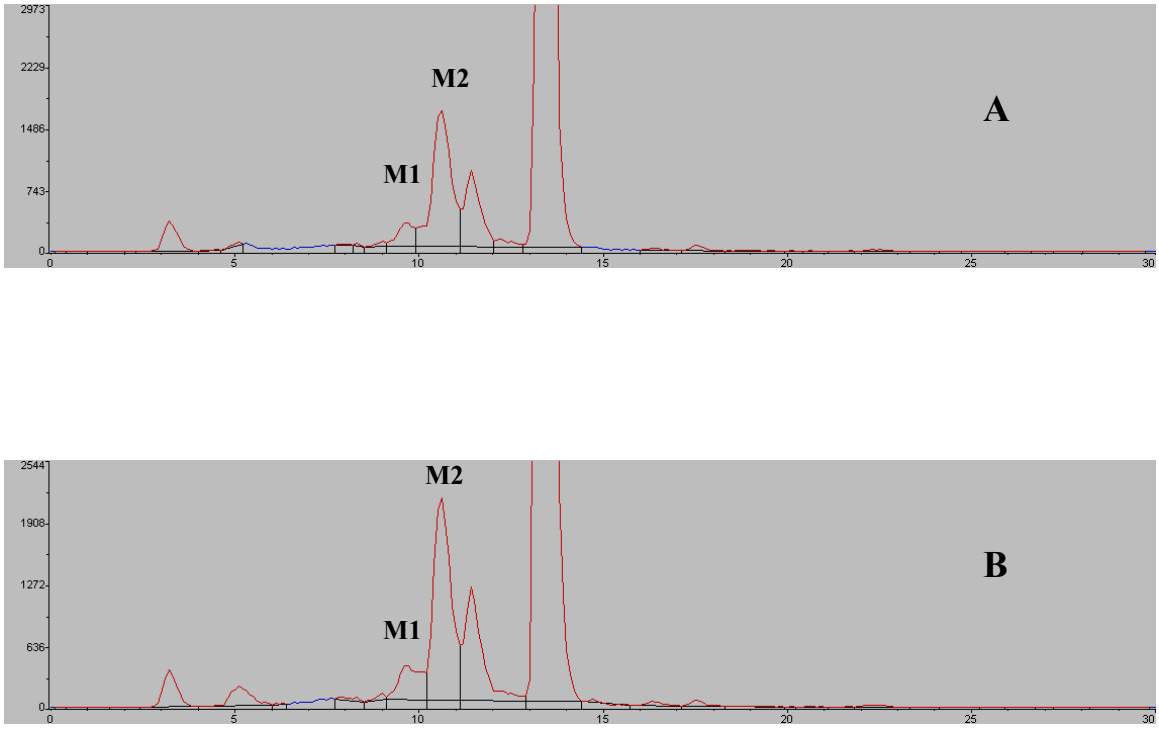
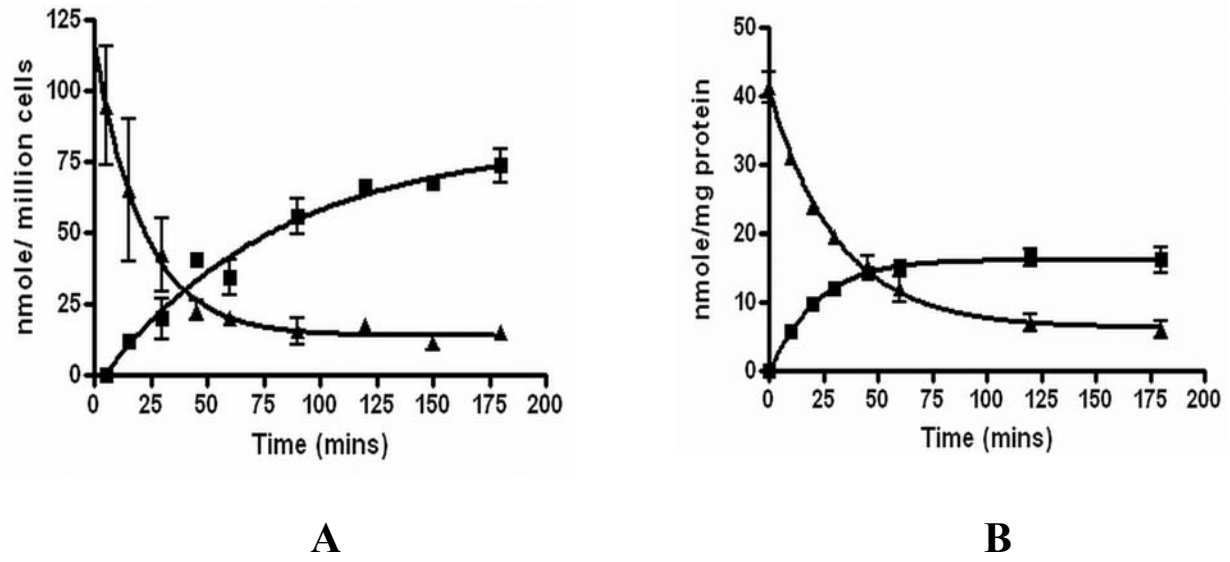


Figure 6. Radio-HPLC tracing of metabolites generated on incubating radiolabeled 17-OHPC with HLM.
(A) - [^{14}C] label and (B) – [^{3}H] label was observed on the major metabolites (M1, M2) on incubation with HLM, thus, confirming that the caproate moiety is not cleaved during metabolism.

3.5.3 Metabolic Profiles in Human Liver Microsomes and Hepatocytes

Metabolism of 17-OHPC was evaluated over time using human liver microsomes and hepatocytes. The concentrations of unmetabolized 17-OHPC and the major metabolite (expressed in terms of 17-OHPC equivalent) were determined over a time period of 0 – 180 mins incubation. Fig. 7 (A, B) shows that the concentration of 17-OHPC decreased in a time-dependent manner and the concentration of the metabolite increased proportionately. Approximately, 60% of the parent drug (17-OHPC) was metabolized within 60 minutes and $M2_{(HLM, HH)}$ accounted for almost 50% of metabolized 17-OHPC.



A **B**

Figure7. Metabolism of 17-OHPC and generation of major metabolite (M2) by human hepatocytes and human liver microsomes
A) Incubation of 17-OHPC (150µM) with Fresh Adult Human Hepatocytes generated five metabolites (M1-5HH). The major metabolite ($M2_{HH}$, ■) showed a time dependent increase throughout the incubation which corresponded with a decrease in the amount of 17-OHPC (▲).
B) Incubation of 17-OHPC (50µM) with Human Liver Microsomes generated metabolites 1, 2 and 3. Metabolite 2 ($M2_{HLM}$, ■) was the major metabolite which depicted time dependent increase in concentration corresponding to a decrease in 17-OHPC concentration (▲).
The amount of metabolite has been expressed in terms of 17-OHPC equivalents.

3.5.4 Identification of Human P450 Isoforms

3.5.4.1 Incubations with human liver microsomes

17α-hydroxyprogesterone caproate was incubated with microsomes for 60 min in the presence of various representative inhibitors of P450 isoforms. A similar reaction was performed in the absence of the inhibitors, and the rate of major metabolite ($M2_{HLM}$) generated was compared between the two conditions to identify the isoforms responsible for 17-OHPC metabolism. As summarized in Fig. 8, α-naphthoflavone, sulfaphenazole, coumarin, quinidine and methimazole did not inhibit the metabolism of 17-OHPC by the corresponding CYPs. In contrast, the amount of $M2_{HLM}$ was significantly decreased in the presence of ketoconazole, indicating that the metabolism of 17-OHPC was markedly inhibited. The Vmax (0.29±0.02 nmole/min/mg) and Km (77.94±19.4μM) values for $M2_{HLM}$ were observed to decrease significantly in the presence of ketoconazole (Vmax=0.03±0.002, Km=15.38±3.9). An IC50 (0.17 μM) value for the inhibition of 17-OHPC metabolism by ketoconazole in human liver microsomes (n=3) was also calculated (Fig. 9).

3.5.4.2 Incubations with baculovirus expressed human CYP isoforms

Studies were also performed in baculovirus-infected insect cells expressing various CYPs. The metabolizing activity of each CYP3A isoform for 17 α-hydroxyprogesterone caproate was compared to control microsomes, which were devoid of any CYP activity. The results indicate the involvement of CYP3A4/5 in the metabolism of 17-OHPC and the formation of the major metabolite ($M2_{HLM}$) was a result of CYP3A4/5 pathway (Table 7).

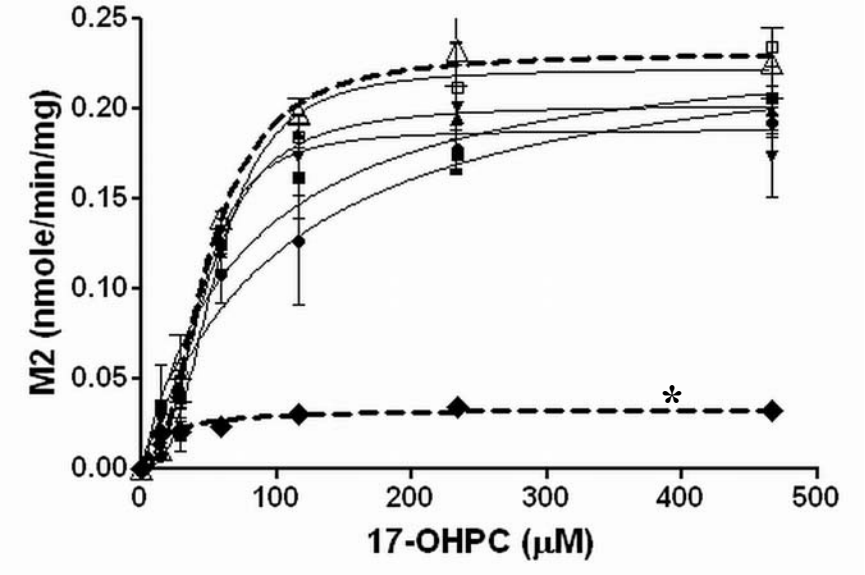


Figure 8. Ketoconazole inhibits 17-OHPC metabolism

Of the various inhibitors evaluated, namely, Coumarin (CYP2A6, ■), α-Naphthoflavone (CYP1A2, ▲), Sulfaphenazole (CYP2C9, ▼), Quinidine (CYP2D6, □), Ketoconazole (CYP3A, ♦), and Methimazole (FMO3, ●), only Ketoconazole (1μM) demonstrated significant inhibition of 17-OHPC (0-200 μg/ml expressed as 0-467μM) metabolism in human liver microsomes (n=3) when compared to control (No Inhibitor, Δ). This indicated the role of CYP3A isoforms in metabolizing 17-OHPC and the potential involvement of CYP3A4. Approx. 80% inhibition of 17-OHPC was observed at 1 μM Ketoconazole (* $p < 0.05$). V (μmoles/min/mg microsomal protein) denotes the major metabolite formation ($M2_{HLM}$) expressed in terms of 17-OHPC equivalents.

3.5.4.3 Inhibition studies with Human Hepatocytes

Results obtained from inhibition experiments performed in HLM were confirmed in fresh adult human hepatocytes (Fig. 10) using chemical inhibitors for CYP3A (ketoconazole and troleandomycin). Troleandomycin and ketoconazole inhibited 17-OHPC metabolism ($M2_{HH}$ formation) by 75 and 89%, respectively, indicating involvement of CYP3A4/5.

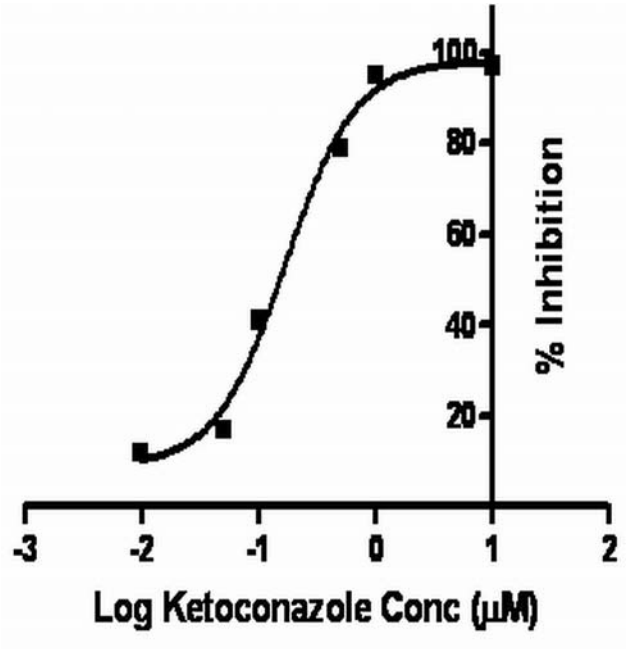


Figure 9. Estimation of IC50

IC50 value for the ketoconazole mediated inhibition of 17-OHPC (25µM) metabolism was calculated to be 0.17 µM in human liver microsomes (n=3). Inhibition of 17-OHPC metabolism was evaluated by estimating the formation of $M2_{HLM}$.

3.5.4.4 Induction Studies with Human Hepatocytes

Induction studies were performed in fresh human hepatocytes to further confirm CYP3A to be the primary enzyme responsible for 17-OHPC metabolism. CYP3A inducers like RIF and PB increased $M2_{HH}$ formation (Fig. 11). RIF showed the maximum (2.2 fold) increase in 17-OHPC metabolism and PB showed a 2.1 fold increase in human hepatocytes. The increase in CYP3A4 expression by the inducers was confirmed by RT-PCR and the induction of CYP3A activity by TE metabolism (formation of 6β(OH)TE). Treatment with these inducers, RIF (10 µM) and PB (2000 µM), resulted in 10.2 ± 5, 18.5 ± 4 - fold increase in the mRNA expression of CYP3A (Fig. 12A) and a 7.1± 1.8, 8.5 ± 3 fold increase in the formation of 6β(OH)TE (Fig. 12B), respectively.

Table 7. Identification of cytochrome P450s involved in the metabolism of 17-OHPC

Expressed CYPs	Metabolite ($M2_{HLM}$) (pmole/pmole CYP/min)	17-OHPC Metabolized (pmole/pmole CYP/min)
CTRL	ND	ND
1A1	ND	ND
1A2	ND	ND
1B1	ND	ND
2A6	ND	ND
2B6	ND	ND
2C8	ND	ND
2C9*1	ND	ND
2C9*2	ND	ND
2C9*3	ND	ND
2C18	ND	ND
2D6*1	ND	ND
2D6*10	ND	ND
2E1	ND	ND
19	ND	ND
FMO1	ND	ND
FMO3	ND	ND
FMO5	ND	ND
CYP3A4	27.4±1.2	64.5±0.1
CYP3A5	57.1±3.7	68.4±0.4

Mean ± SD of triplicate in-vitro preparations. Amount of metabolite is expressed in terms of HPC equivalent. Significant levels of metabolite were detected on incubation of 17-OHPC (100 μM) with expressed CYP3A4/5 for 120 mins, whereas no metabolite could be detected for other CYPs.

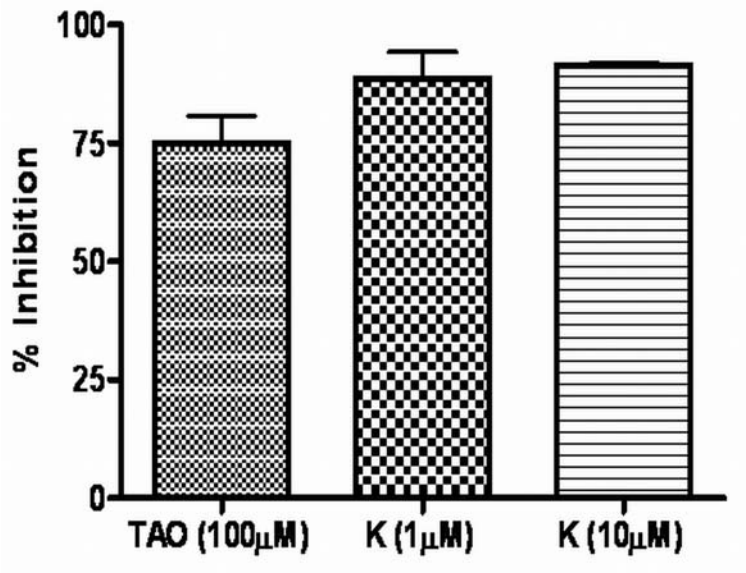


Figure 10. CYP3A4/5 metabolize 17-OHPC in Human Hepatocytes

Ketoconazole (K, 1 and 10μM) and Troleandomycin (TAO, 100μM) demonstrated significant ($p<0.05$) inhibition of 17-OHPC (25μM) metabolism in Fresh Human Hepatocytes (n=4). This indicated the role of CYP3A isoforms in metabolizing 17-OHPC and the potential involvement of CYP3A4. Inhibition of 17-OHPC metabolism was evaluated by estimating the formation of M2HH.

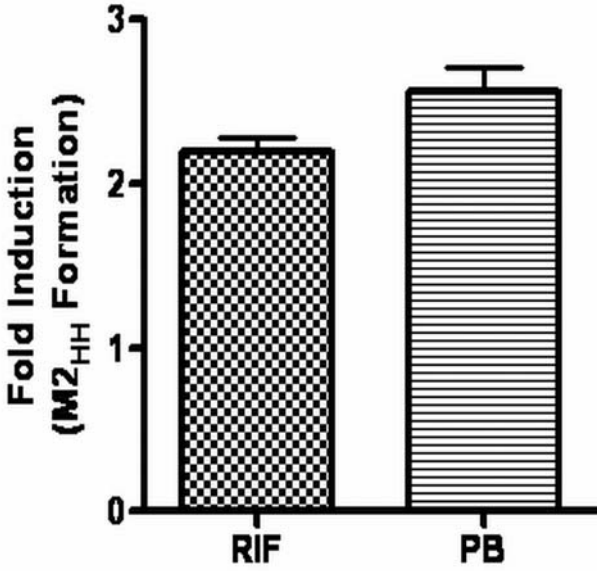
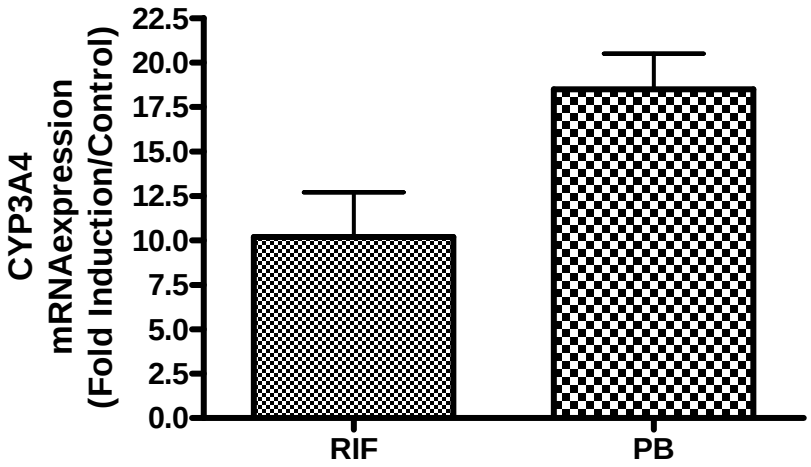
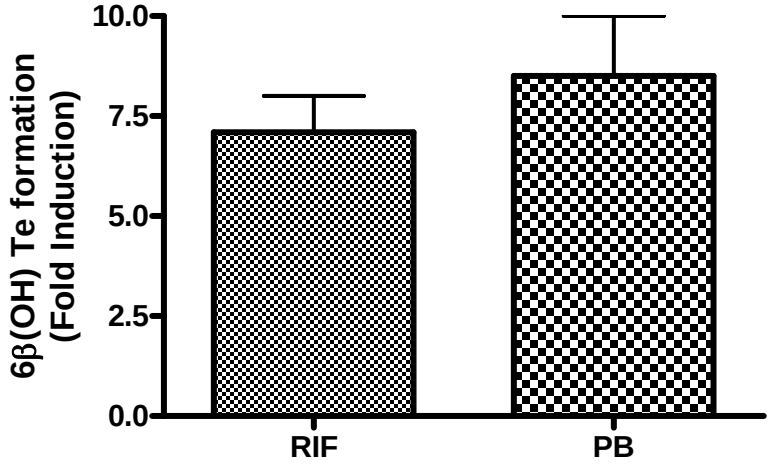


Figure 11. CYP3A inducers increase 17-OHPC metabolism in Human Hepatocytes

Incubation of fresh human hepatocytes (n=3) with specific CYP3A4 inducers, namely, Rifampin ($p<0.05$) and Phenobarbital ($p<0.05$) increased the metabolism of 17-OHPC (50μM) as compared to control (DMSO). The estimation of induction was based on the generation of the major metabolite ($M2_{HH}$) in hepatocytes.



A



B

Figure 12. Effect of CYP3A inducers on CYP3A4 mRNA expression (A) and activity (B) in Human Hepatocytes

Hepatocytes were exposed to Rifampin (RIF) and Phenobarbital (PB) for 72 hours and mRNA expression and activity were determined.

(A) An increase in CYP3A4 expression was observed in the presence of the inducers as compared to control (DMSO).

(B) An increase in CYP3A4 activity was observed in the presence of the inducers as determined by estimating the formation of 6beta-hydroxytestosterone.

3.5.5 Correlation studies

Rates of formation of 17-OHPC metabolite (M2) with liver microsomes and adult human hepatocytes were measured at a substrate concentration of 100 μM. There was considerable interindividual variation in the values of metabolite generated (Fig. 8). Formation of $M2_{(HLM)}$ and $M2_{(HH)}$ correlated significantly with the testosterone 6-β-hydroxylation activity (CYP3A4) in both microsomes (r=0.89) and hepatocytes (r=0.91), respectively.

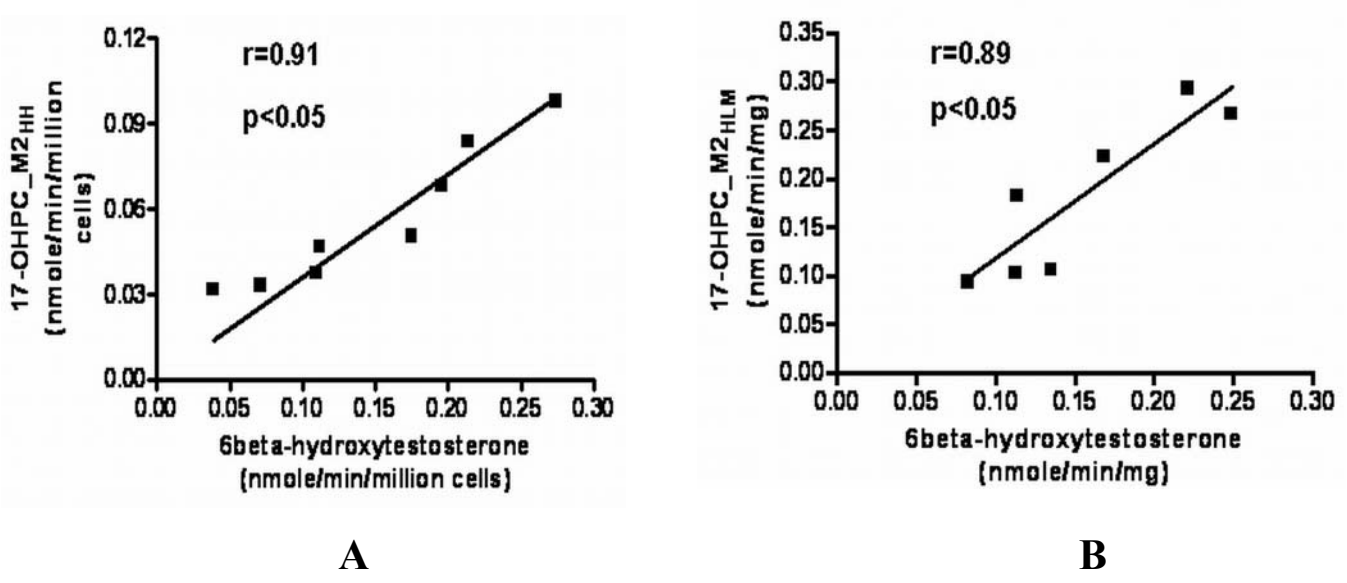


Figure 13. Correlation analysis of 17-OHPC metabolite M2 with CYP3A dependent 6β-hydroxytestosterone formation

(A) Correlation analysis in fresh human hepatocytes (FHH); n=7
(B) Correlation analysis in human liver microsomes (HLM); n=7

CYP3A activity was estimated by incubating 17-OHPC (100μM) and Testosterone (100 μM) in individual human liver microsomal preparations and fresh human hepatocyte cultures. The experiment was carried out in triplicate in both cases.

3.6 Discussion

To the best of our knowledge, this is the first report identifying the human hepatic enzymes, which play a major role in the metabolic pathway of 17-OHPC. The metabolism of 17-OHPC was evaluated using human liver microsomes, fresh human adult hepatocytes and recombinant systems expressing cytochrome P450s and FMOs. Incubation of 17-OHPC with heat-inactivated microsomes did not result in any loss of 17-OHPC or generation of any 17-OHPC metabolites.

Significant metabolism of 17-OHPC however was seen in all the three systems that were tested. Generation of metabolites as well as loss of parent drug confirmed this observation. No metabolites were observed in the absence of NADPH thus confirming the metabolism to be CYP mediated. Incubation of 17-OHPC with human liver microsomes under the conditions for direct generation of conjugates did not alter 17-OHPC concentrations or yield any 17-OHPC metabolites, ruling out a direct role of UGT mediated pathway. However, studies evaluating the role of UGT as a sequential pathway in 17-OHPC metabolism are being carried out currently.

Multiple approaches were used to identify the cytochrome P450 enzymes involved in the metabolism of 17α-hydroxyprogesterone caproate. Metabolism of MPA, a potent progestogenic compound, has been reported in literature (Kobayashi et al., 2000) to be catalyzed mainly by CYP3A4. Since, MPA is structurally similar to 17-OHPC it was expected that the metabolic pathways for both compounds would likely be similar. The results obtained in this study confirmed this expectation. In human liver microsomes, 17α-hydroxyprogesterone caproate was metabolised to one major and two minor metabolites. CYP inhibition experiments indicated CYP3A4/5 to form the main metabolic pathway. Ketoconazole (CYP3A inhibitor) at 1.0 μM inhibited 90% of 17-OHPC biotransformation in human liver microsomes. These findings and the lack of effect of α – naphthoflavone, quinidine, coumarin, sulphaphenazole (inhibitors for

CYP1A2, CYP2D6, CYP2A6, and CYP2C9) suggest a major role for CYP3As in 17-OHPC metabolism.

In fresh human adult hepatocytes, five ($M1\text{-}5_{HH}$) metabolites were observed on incubation of 17-OHPC with $M2_{HH}$ being the major metabolite. Ketoconazole and Troleandomycin (a known CYP3A inhibitor) significantly inhibited 17-OHPC metabolism. Inducers of CYP3A namely; rifampicin, phenobarbital and clotrimazole significantly increased 17-OHPC metabolism in comparison to control. Thus, the results in human liver microsomes were successfully reproduced in fresh human hepatocytes. This showed CYP3A to play the major role in 17-OHPC metabolism in hepatocytes as well.

To further confirm our results we conducted experiments to check the ability of expressed enzyme systems to catalyze the biotransformation of 17-OHPC. CYP3A isoforms were the only enzyme systems that metabolized 17-OHPC significantly. The enzyme activity for 17-OHPC metabolism was observed to be higher for CYP3A5 isoform than CYP3A4. The reason for the differential activity is not known at this time.

The CYP3A subfamily is known to be expressed most abundantly (i.e., from 10-60% of total CYPs) in human liver and plays a pivotal role in the oxidative metabolism of many clinically important drugs. Among the CYP3A isoforms tested (i.e., CYP3A4 and 3A5), CYP3A4 is the major isoform in adult humans. CYP3A5 is polymorphically expressed in approximately 10 to 20% of the adult liver (Wrighton et al., 1990). Overall, we can predict that CYP3A4 would be the major CYP isoform responsible for the hepatic metabolism of 17-OHPC in the majority of adult patients given 17-OHPC. Given that CYP3A5 has higher activity for 17-OHPC, genetic polymorphism in CYP3A5 may have a significant role in 17-OHPC metabolism and pharmacokinetics, but this remains to be evaluated. It has been proposed that the prolonged

and more potent action of 17-OHPC over progesterone involves the cleavage of 17-OHPC molecule to 17-OHP (Fig.1B) and release of free caproic acid. It was also suggested that caproic acid could affect genomic pathways and hence have an effect of progesterone signaling pathways (Attardi et al., 2007). However, results of radio-HPLC based method confirmed that the structure of 17-OHPC remained intact during metabolism by human enzymes. Our observation does not support the hypothesis that 17-OHPC is a prodrug that gets metabolized to progesterone or hydroxyprogesterone and thus prevents pre-term labor. Further, LC-MS based analysis of the major metabolite ($M2_{HLM}$, m/z=445) generated from incubations in human liver microsomes indicated possible mono-hydroxylation or an oxidation product. Identity of the major metabolite ($M2_{HH}$) generated from human hepatocytes needs to be elucidated although preliminary data based on retention time and LCMS data analysis indicates $M2_{HH}$ (m/z=445) to be similar to $M2_{HLM}$.

Our study demonstrates that the metabolism of 17-OHPC is predominantly mediated by CYP3A isoforms, mainly CYP3A4. Given that the activity of CYP3A enzyme is known to vary between subjects, one would expect large variation in the pharmacokinetics of 17-OHPC in pregnant subjects. CYP3A4 plays a major role in the metabolism of various drugs due to its abundance in the liver and its broad substrate specificity. Numerous clinically important drugs are known as substrates of CYP3A4 (Rendic and Di Carlo, 1997). Thus, further in-vitro and clinical studies are required to assess 17-OHPC associated clinically relevant metabolic drug interaction with any co-administered CYP3A4 substrates/ inhibitors.

Pregnancy is a dynamic state of the human body which is characterized by significant variations in the physiology and metabolism. Changes in the metabolizing activity of CYPs especially CYP3A4 has been reported in literature (Tracy et al., 2005). Activity of CYP3A4 has

been shown to increase significantly in all trimesters in humans. Further, the expression level of CYP3A isoforms varies from individual to individual. Thus, we expect significant inter-individual fluctuations in 17-OHPC plasma concentrations in pregnant patients over time. In clinical studies, 17α-Hydroxyprogesterone Caproate is administered as a fixed dose regimen of 250mg weekly. The therapy was observed to be effective in 33% of the patients in the study of Meis et al. It is possible that the low success rate of 17-OHPC may be attributable to the significant variation in CYP3A mediated metabolism of 17-OHPC in these patients. On the basis of the abovementioned facts, it may be necessary to investigate various dosing regimen and individualize the therapy with 17-OHPC.

Clinical Implications:

1) CYP3A4 is the major isoform (of total CYPs) expressed in the human intestine. Since, 17-OHPC is a CYP3A4 substrate and likely a BCS Class II compound; the oral bioavailability of this drug is expected to be low.
2) Significant interindividual variability (10 fold) in the plasma concentrations of CYP3A4 substrates has been reported. Thus, monitoring 17-OHPC plasma levels and adjustment of dose accordingly may be needed to improve therapeutic outcomes.
3) Potential for drug interactions with other CYP3A substrates exists when multiple medications that are CYP3A substrates are co-administered.

Acknowledgements:

This project was supported in part by the Obstetrical-Fetal Pharmacology Research Units Network (OPRU). This work is supported in part by NICHD grant HD-047905-2.

4.0 Elucidation of the metabolizing enzymes and metabolic pathways involved in the disposition of 17-OHPC in fetal human liver

[Sharma S, Ellis E, Dorko K, Zhang S, Mattison D, Caritis S, Venkataramanan R and Strom S (2010). Metabolism of 17α-hydroxyprogesterone caproate, a new agent for preventing pre-term birth, by fetal hepatocytes. *Drug Metab Dispos* **38:**723-727]

[Sharma S, Zhang S, Mattison D, Caritis S, Strom S and Venkataramanan R (2009). Hepatic Metabolism of 17α-hydroxyprogesterone caproate in Mice, Rats, Dogs, Pigs, Rabbits and Baboons. A comparison with human liver microsomes and human hepatocytes. Submitted to Xenobiotica]

4.1 Abbreviations

6β(OH)TE - 6β(OH) testosterone
CYP - cytochrome P450
CYP3A7 - cytochrome P4503A7
CYP3A4 - cytochrome P4503A4
FHH - primary cultures of human hepatocytes
TE - testosterone
17-OHPC - 17α-hydroxyprogesterone caproate
MPA – Medroxyprogesterone acetate
EMEM - Eagle's Minimum Essential Medium
HBSS - Hank's balanced salt solution
HMM - Hepatocyte Maintenance Medium
DMEM - Dulbecco's Modified Eagle Medium.

4.2 Abstract

Aims: Weekly injections of 17-OHPC are commonly utilized for the prevention of preterm birth (PTB). Recent evidence of transplacental transport of 17-OHPC raises questions about how the fetus might respond to this agent. In adults, hepatic mono-oxidation via the cytochrome P450 enzymes CYP3A4 and 3A5 is the primary metabolic pathway. Whether or not the human fetal liver can metabolize 17-OHPC is not known. In this study, we evaluated the metabolism and kinetics of 17-OHPC in adult and fetal human hepatocytes and in expressed CYP enzymes.

Methods: The model systems used were expressed enzymes and primary hepatocyte cultures isolated from human adult and fetal livers. 17-OHPC and metabolites were quantified using LC-MS systems. Protein content and mRNA were also measured.

Results: 17-OHPC was metabolized by expressed CYP3A7 and by fetal hepatocytes. Expressed CYP3A4 demonstrated a significantly higher capacity to metabolize 17-OHPC than CYP3A7. The metabolite profile was qualitatively different between expressed CYP3A4 and CYP3A7. Based on retention times, two unique metabolites were observed in fetal hepatocytes as compared to adult hepatocytes along with one metabolite which was common to both. The intrinsic clearance of 17-OHPC by fetal hepatocytes was similar to that in adults.

Conclusion: This study demonstrates that fetal hepatocytes and in particular fetal form of CYP3A i.e. CYP3A7 can metabolize 17-OHPC. Further, fetal specific metabolites (M1 and M3) were generated, which were not observed in the adult hepatocyte system. Future studies are needed to investigate the effect of accumulation of these 17-OHPC metabolites on the fetal hepatic elimination processes and any associated toxicity.

4.3 Introduction

Fetal drug exposure is an unavoidable risk associated with drug therapies in pregnant subjects. The role of metabolism, via phase I and II pathways, in regulating fetal exposure is relatively lesser known. Fetal metabolism and the presence of cytochrome P-450s in the fetal liver has been previously documented in the literature (Ackermann and Richter, 1977; Aranda et al., 1979; Rollins et al., 1979; Rane and Tomson, 1980; Wiebkin et al., 1985; Komori et al., 1990; Chiba et al., 1997; Ladona et al., 2000).

Since, almost all lipid-soluble xenobiotics enter the fetal system through placental transfer there is a high probability that 17-OHPC will penetrate through the placental barrier and gain access to the fetal circulation. Studies performed using the dual perfused placental lobule (DPPL) technique have shown that 17-OHPC is transferred to the fetal circulation (Hemauer et al., 2008). Further, analysis of fetal cord blood has shown significant 17-OHPC levels (unpublished data) thus confirming the transplacental transport from the mother to the fetus.

Although teratology studies have shown the safety of 17-OHPC in rodents and non-human primates, difference in drug metabolism between animals and humans is well known. For example, the drug oxidizing capacities have been reported to develop relatively early in human fetuses whereas this capability is only reported postnatally in experimental animals, especially non-primates (Rane and Tomson, 1980). Thus, it is important to investigate the pathway(s) involved in the metabolism of 17-OHPC in both the adult and fetal liver. Further, the major pathways by which the fetus can eliminate drugs and their metabolites include placental diffusion and active transport back to the maternal circulation. Since the permeability of placenta to relatively polar metabolites is lower, these metabolites may accumulate in the fetus; especially in the liver where the drug is metabolised. This may be disadvantageous to the fetus if these

metabolites induce cellular toxicity or interfere with the various hepatic transport processes in liver and kidney. Thus, studies are needed to identify the metabolites of drugs administered during pregnancy and their concentrations in fetal circulation. Identification of the metabolic pathways will help to make informed decisions regarding drug therapy for pregnant women.

The objectives of the study presented here are 1) to investigate the metabolism of 17-OHPC in fetal hepatocyte cultures, 2) identify the major enzyme and metabolic pathway(s) involved, and 3) to compare the kinetics and metabolism of 17-OHPC in fetal hepatocytes with adult human hepatocytes in culture.

In chapter 1, we have shown that CYP3A4 is the major enzyme involved in the metabolism of 17-OHPC. Further, monohydroxylated-17-OHPC constituted the major metabolites generated by its metabolism. Cytochrome P450 3A family consists of four genes CYP3A4, CYP3A5, CYP3A7 and CYP3A43, encoded at an approximate 270-kbp locus on human chromosome 7q21.1. CYP3A4, CYP3A5 and CYP3A7 share at least 85% sequence identity which has imparted considerable overlapping substrate specificities to these isoforms although the catalytic efficiency has been reported to vary significantly for the common substrates. Many substrates which are metabolized by both CYP3A4 and CYP3A7 have been reported in the literature including, testosterone, dextromethorphan, dehydroepiandrosterone and codeine (Hines, 2008). It has also been observed that CYP3A4 is the major isoform (10 to 50% of total CYP) expressed in the adult human liver whereas CYP3A7 is the dominant enzyme in the fetal liver. Thus, based on the overlap in substrate specificities we hypothesize that the fetal liver will metabolize 17-OHPC with CYP3A7 being the major isoform involved in its metabolism.

4.4 Methods

4.4.1 Hepatocyte Incubations

Adult and fetal hepatocytes (n=4) were incubated with 17-OHPC (5 μM methanolic solution) in HMM for clearance studies. Samples were taken at different time points (15-120 minutes) to assess 17-OHPC metabolism. Kinetic analysis in adult and fetal hepatocytes (n=4) was evaluated by incubating different 17-OHPC concentrations (0-80 μM methanolic solutions) in HMM with cells for 30 and 60 mins, respectively. Control cultures were exposed to HMM containing methanol alone. The final concentration of methanol in all incubations was 0.1%. The supernatant media was collected and stored at -80ºC until analysis by LC-MS. The remaining cells were harvested in phosphate buffer for protein determination.

The induction experiments were initiated 24 hrs after plating the cells. The fetal hepatocytes (n=2) were incubated with the inducers (rifampin and phenobarbital) for 4 days prior to adding HMM/ 17-OHPC (50μM) or Testosterone (250μM) to estimate the effect of CYP3A induction on 17-OHPC and TE metabolism. TE was used as a positive control in these experiments since it is widely used as a CYP3A probe substrate. Cells were harvested for mRNA by adding 1 mL of Trizol® reagent to each well. The RNA samples were stored at -20°C for Real Time PCR analysis. Primers for CYP3A4, CYP3A7 and cyclophillin and the PCR procedure have been described in Chapter 2. The mRNA expression for all genes was normalized to cyclophillin in each sample and expressed as fold change over control treatment.

4.4.2 Expressed enzyme microsomal incubations

Expressed CYP3A4/3A7 (20pmole) microsomes were incubated with 17-OHPC (100µM) and $MgCl_2$ (10 mM) in 0.1mM phosphate buffer (pH 7.4). The final volume of 0.25 ml was allowed to equilibrate in a shaking water bath for 5 min at 37 ºC. The reaction was initiated by the addition of NADPH (1 mM). After 120 mins of incubation, the reaction was stopped by adding 0.25ml of cold methanol. The mixture was centrifuged at 3000 rpm for 20 min and supernatant was injected into the HPLC. The final concentration of methanol was less than 2%. For elucidating the kinetics of 17-OHPC metabolism, incubations of expressed CYP3A4 and 3A7 (20 pmole, 10 mins) were carried out using the abovementioned method with increasing concentrations of 17-OHPC (0-200µM).

4.4.3 Data Analysis

Data are expressed as the mean ± SD. Apparent kinetic parameters for 17-OHPC metabolism were estimated by the non-linear least square regression analysis using GraphPad Prism 4.0 (GraphPad Software Inc.). Half life estimates were obtained by non-compartmental analysis with WinNonlin 4.1 (Pharsight Corp.). Intrinsic clearance was calculated using the equation - Clint = $(\ln 2/t_{1/2})$*(ml incubation/10^6cells).

4.5 Results

4.5.1 Metabolism of 17-OHPC by human fetal hepatocytes

Incubation of 17-OHPC with fresh human fetal hepatocytes (Fig. 14A) generated three major metabolites (M1-17.60, M2-17.86, M3-19.18) as identified using LC-MS. Incubation of 17-OHPC with fresh human adult hepatocytes (Fig. 14B) also generated three major metabolites (M2-17.84, M4-16.55 and M5-16.84). Metabolite M2 was common to adult and fetal hepatocytes. Two metabolites (M1, M3) were generated by fetal hepatocytes that were either absent or below the detection limit in the adults. All the metabolites observed in the fetal hepatocyte based incubations displayed the same m/z ratio (m/z=445.0, Fig.15B). A significant amount of sodium adduct formation (m/z=467.0) was observed at the same retention time as the major metabolites (M1-M3, Fig. 15A). Induction studies were performed in fetal hepatocytes to investigate the role of CYP3A (primarily CYP3A4 or CYP3A7) as the primary enzyme responsible for 17-OHPC metabolism in the fetal liver. CYP3A inducers, RIF and PB, increased the formation of all 17-OHPC metabolites (Fig. 16). RIF showed a 1.5 fold increase in 17-OHPC metabolism and PB showed a 1.8 fold increase in fetal hepatocytes. The increase in CYP3A4 and CYP3A7 expression by the inducers was also confirmed by RT-PCR and the induction of CYP3A activity by TE metabolism (formation of 6β(OH)TE). Treatment with these inducers, RIF (10 μM) and PB (2000 μM), resulted in 4.0 ± 1.5, 2.5 ± 0.8 - fold increase in the mRNA expression of CYP3A4 and a 2.2 ± 0.7, 1.8 ± 0.3 - fold increase in the mRNA expression of CYP3A7 (Fig. 18). Further, both RIF and PB showed ~ 2 fold increase in TE metabolism in fetal hepatocytes (Fig. 17).

4.5.2 Metabolism of 17-OHPC by expressed CYP3A7

Incubation of 17-α-hydroxyprogesterone caproate (17-OHPC) with baculovirus expressed CYP3A7 (60pmole/ml) generated three (M1, M2, M3) major metabolites (Fig. 19A). The major metabolites accounted for 90 % of the total substrate (17-OHPC) metabolized by the enzyme. Further, studies were carried out to identify and compare the metabolites generated by expressed CYP3A4. The metabolites - M2, M4 and M5 were generated by expressed CYP3A4 (Fig. 19B). The common metabolite, based on similar retention time, which was observed in both CYP3A isoforms, was M2 with a mass/charge (m/z) value of 445.0 (Fig. 20B). Metabolites (M1 and M3) generated by CYP3A7 were observed to have similar m/z value (445.0) to CYP3A4 metabolites (M4 and M5), however, the relative retention times were different in both cases and thus, were concluded to be fetal specific metabolites. Further, a significant sodium adduct (m/z=467.0) was observed at the same retention time as the major metabolites (M1-M3, Fig. 20A).

4.5.3 Relative Metabolic Capacities of CYP3A isoforms

To evaluate the relative 17-OHPC metabolizing capabilities of CYP3A isoforms (CYP3A4 and CYP3A7), incubations of 17-OHPC and TE (100μM and 250 μM, equimolar quantities, respectively) were carried out in individual expressed enzyme systems. Comparison of the isoforms' 17-OHPC metabolizing capability showed CYP3A7 to metabolize ≈7% of the substrate (Fig. 21A) at the rate of 3.2 pmole/min/pmole CYP3A7. CYP3A4 metabolized ≈83% of total 17-OHPC added to the incubation at a rate of 47.1 pmole/min/pmole CYP3A4. The results were calculated based on estimating the metabolism of 17-OHPC (loss of parent drug) during incubations.

Further, a comparison of the amount of major metabolites generated (M1-M5) by various isoforms was done (Fig. 21B). The total major metabolites (M1+M2+M3) generated by CYP3A7 isoform were ≈6 times less than those observed for the CYP3A4 (M2+M4+M5) isoform. Similarly, comparison of the isoforms' TE metabolizing capability showed CYP3A7 to metabolize ≈7% of the substrate whereas CYP3A4 metabolized ≈92% of total TE (Fig. 22A). The total major metabolites generated by CYP3A7 isoform were ≈15 times less than those observed for the CYP3A4 isoform. Thus, CYP3A7 consistently depicted a lower intrinsic capacity to metabolize substrates than CYP3A4.

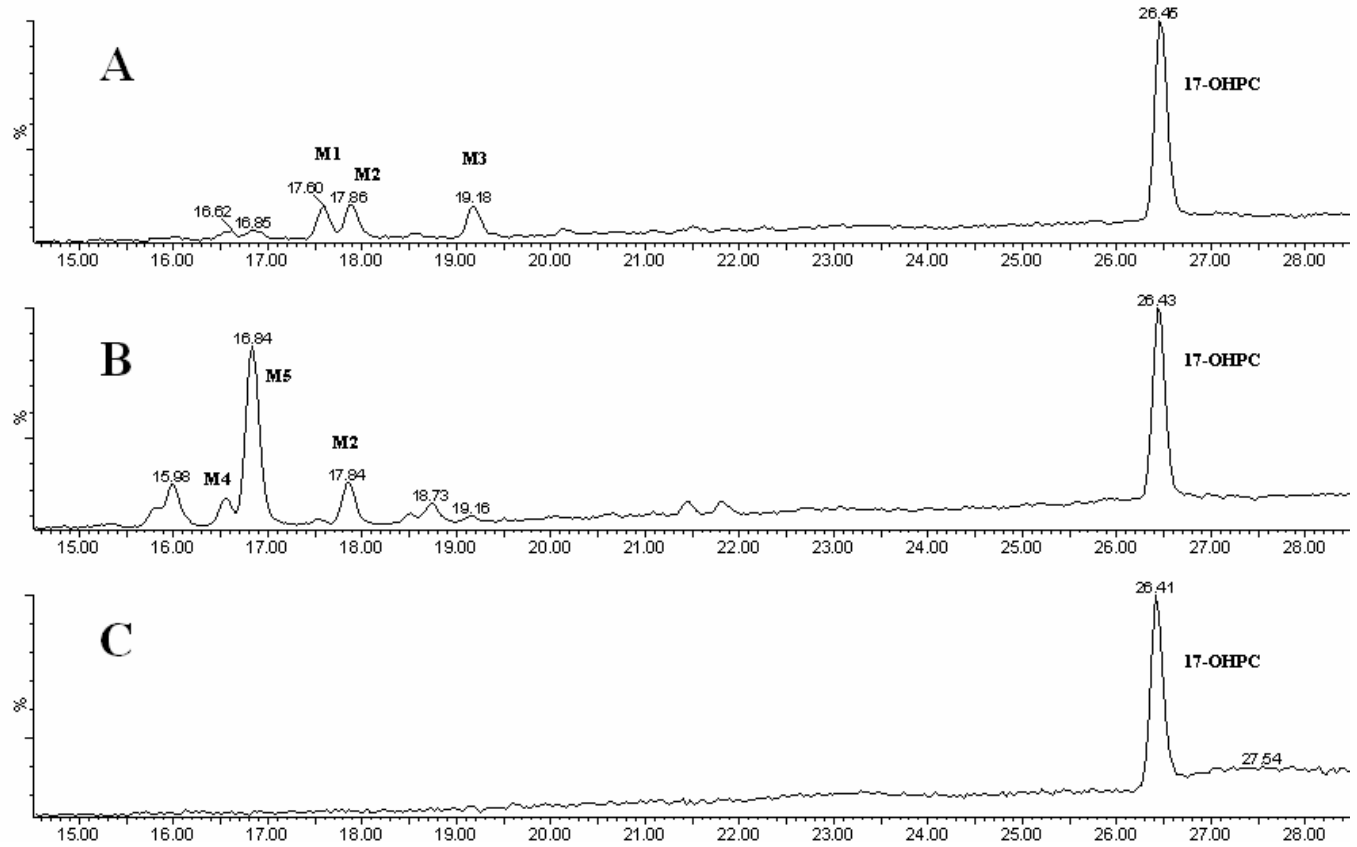


Figure 14. LC-MS chromatograms of 17-OHPC and its metabolites derived from incubation of 17-OHPC with fresh human fetal and adult hepatocytes

A: Incubation of 17-OHPC with fresh human cultured fetal hepatocytes. The incubation generated 6 metabolites of which 3 were the major metabolites (M1, M2 and M3). The retention times of 17-OHPC and its metabolites- M1, M2 and M3 in this analytical condition were 26.45, 17.60, 17.86 and 19.18 min, respectively.

B: Incubation of 17-OHPC with fresh human cultured adult hepatocytes. The incubation generated 3 major metabolites (M2, M4 and M5). The retention times of 17-OHPC and its metabolites- M4, M5 and M2 in this analytical condition were 26.43, 16.55, 16.84 and 17.84 min, respectively.

C: Incubation of 17-OHPC in media without hepatocytes. No metabolites were generated. The retention time of 17-OHPC was 26.41.

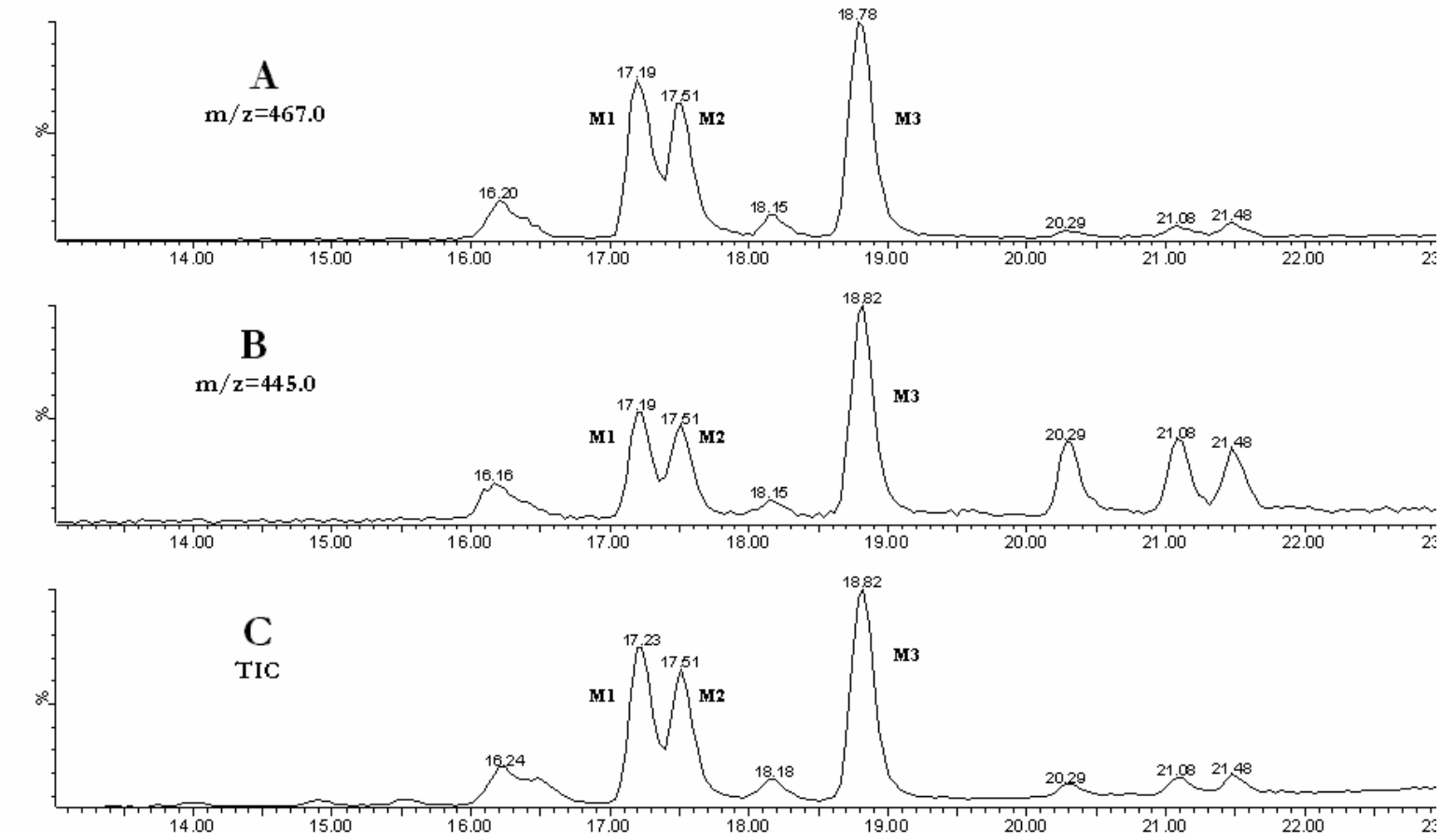


Figure 15. LC-MS chromatograms of 17-OHPC metabolites derived from incubation of 17-OHPC with fresh human fetal hepatocytes The incubation generated a number of metabolites of which 3 were the major metabolites (M1, M2 and M3).
A: The extracted ion chromatogram depicting the major metabolites (M1, M2 and M3) having a m/z = 467.0. This m/z value depicts a sodium adduct of the mono-hydroxylated products of 17-OHPC metabolism.
B: The extracted ion chromatogram depicting the major metabolites (M1, M2 and M3) having a m/z = 445.0. This m/z value depicts a mono-hydroxylated product of 17-OHPC metabolism.
C: The total ion chromatogram (TIC) depicting the major metabolites (M1, M2 and M3).

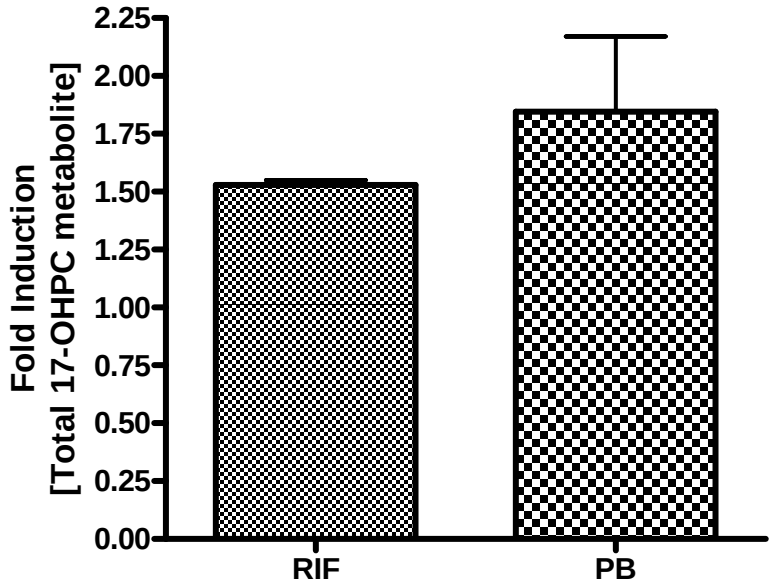


Figure 16. CYP3A inducers increase 17-OHPC metabolism in Fetal Hepatocytes

Incubation of fetal hepatocytes (n=2) with specific CYP3A4 inducers, namely, Rifampin and Phenobarbital increased the metabolism of 17-OHPC (50μM) as compared to control (DMSO). The estimation of induction was based on the total metabolites generated in hepatocytes.

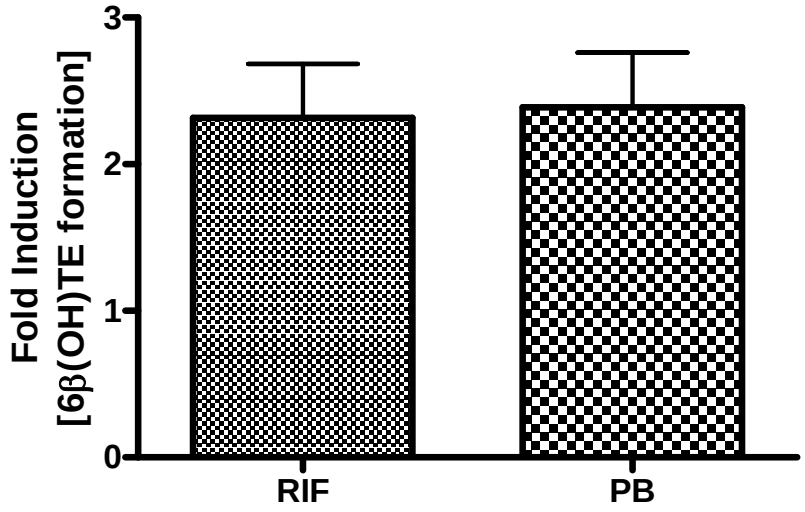


Figure 17. Effect of CYP3A inducers on Testosterone metabolism in Fetal Hepatocytes

Fetal Hepatocytes (n=2) were exposed to Rifampin (RIF) and Phenobarbital (PB) for 72 hours and mRNA expression and activity were determined. An increase in CYP3A4 activity was observed in the presence of the inducers as determined by estimating the formation of 6beta-hydroxytestosterone.

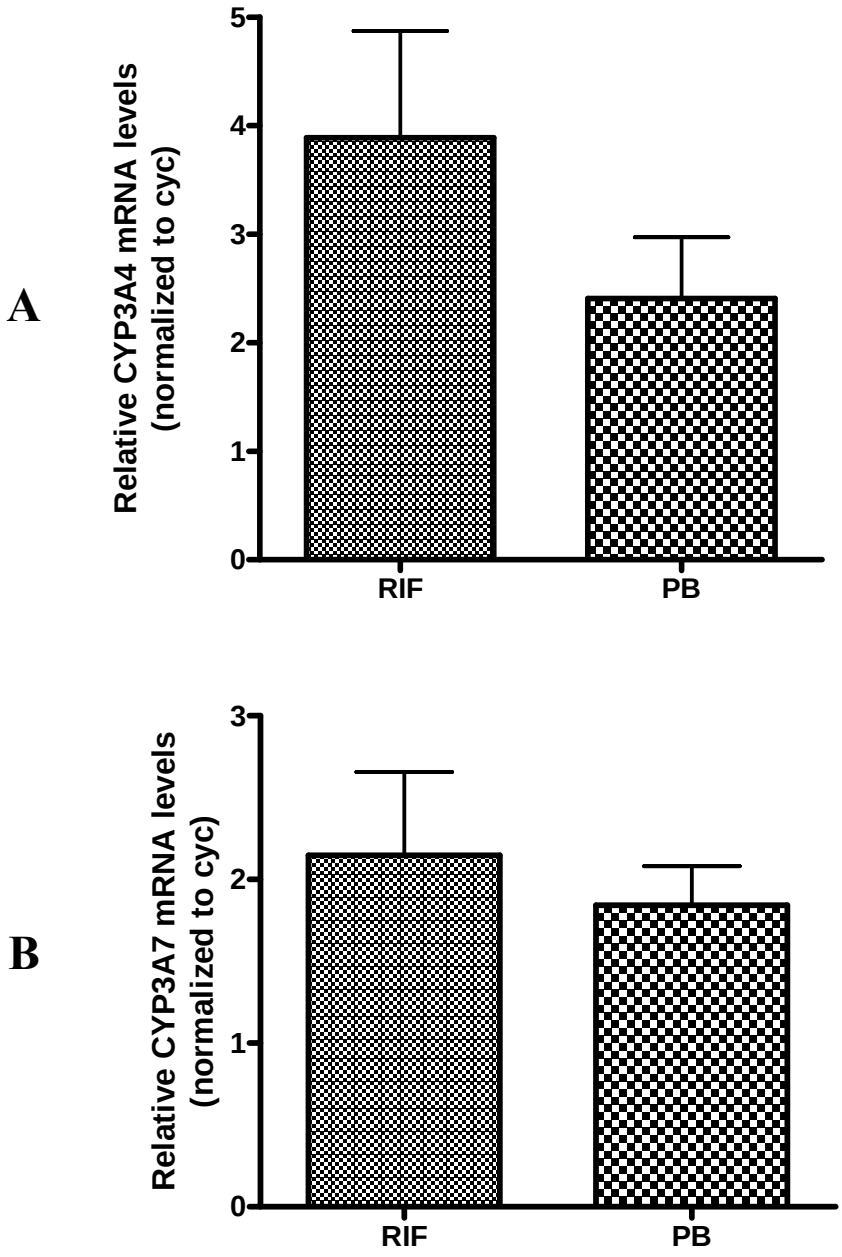


Figure 18. Effect of CYP3A inducers on CYP3A4 and CYP3A7 mRNA expression in Fetal Hepatocytes

Fetal Hepatocytes (n=2) were exposed to Rifampin (RIF) and Phenobarbital (PB) for 72 hours and mRNA expression and activity were determined.

(A) An increase in CYP3A4 expression was observed in the presence of the inducers as compared to control (DMSO).

(B) An increase in CYP3A4 expression was observed in the presence of the inducers as compared to control (DMSO).

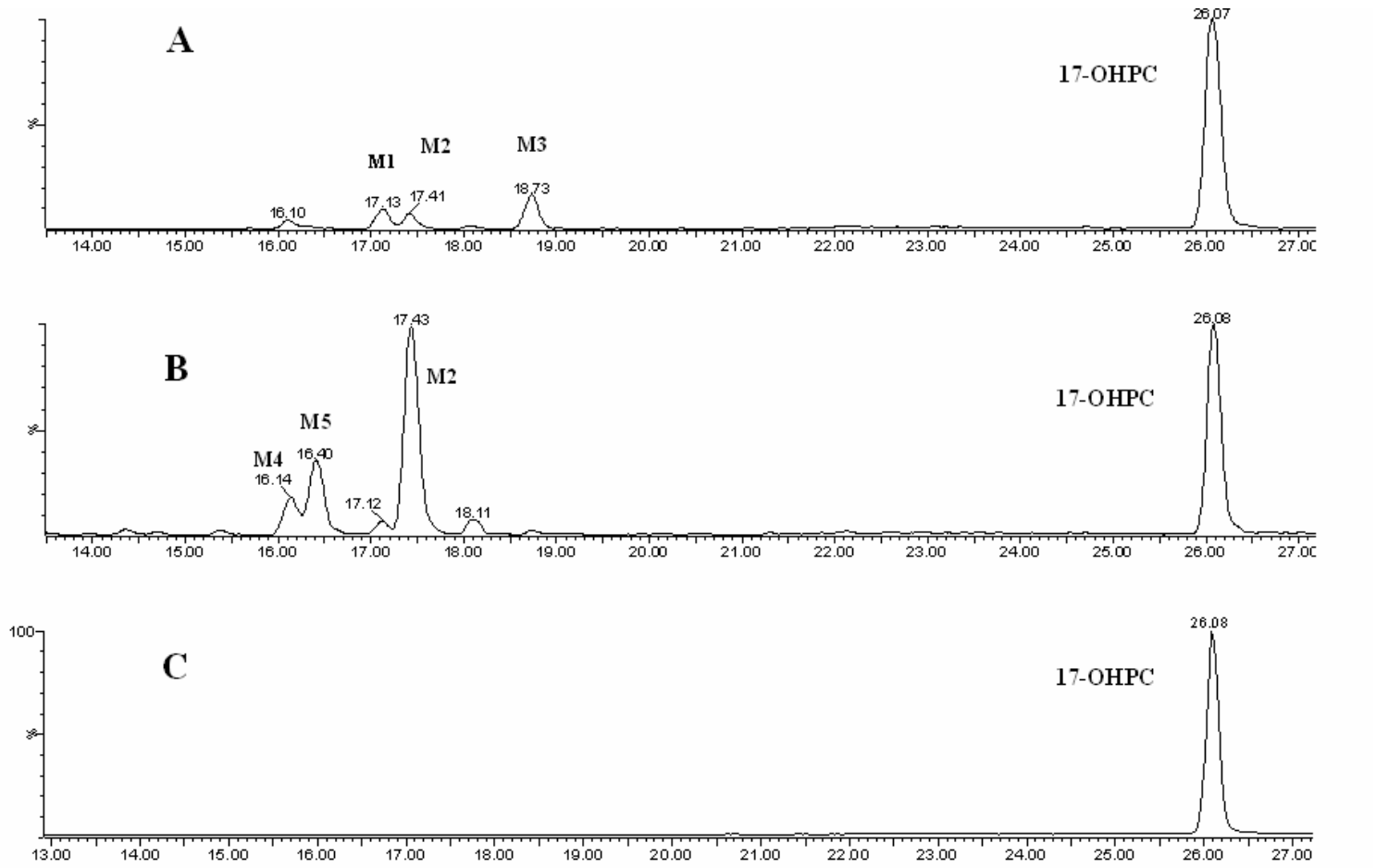


Figure 19. LC-MS chromatograms of 17-OHPC and its metabolites (M1-M5) derived from incubation with expressed CYP3A isoforms

A: The retention times (mins) observed for CYP3A7 mediated metabolic pathway were – 26.07 (17-OHPC), 17.13 (M1), 17.41 (M2) and 18.73 (M3).
B: The retention times (mins) observed for CYP3A4 mediated metabolic pathway were – 26.08 (17-OHPC), 17.43 (M2), 16.14 (M4) and 16.40 (M5).
C. The retention time (mins) observed for 17-OHPC in the absence of NADPH – 26.08.

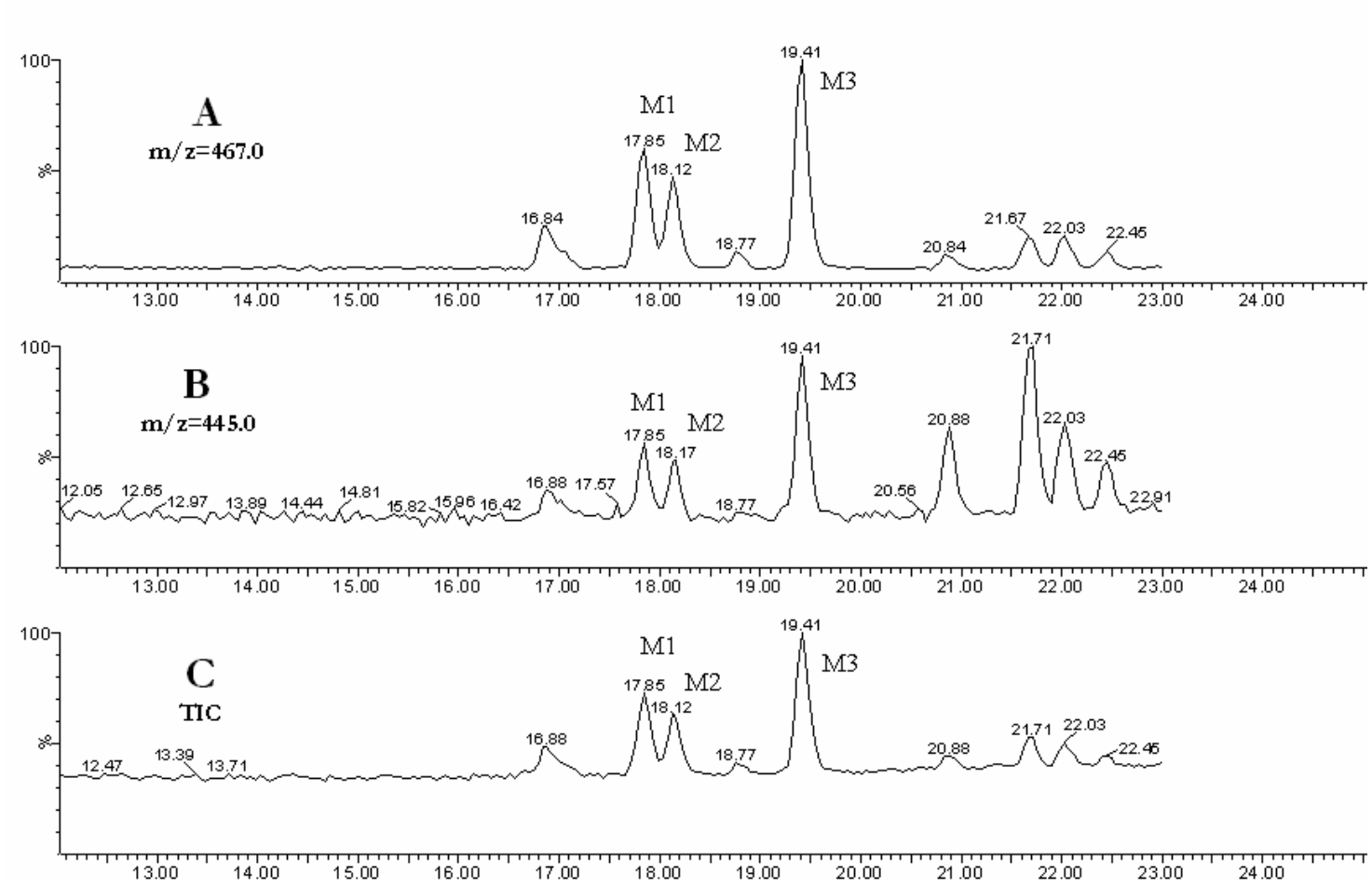


Figure 20. LC-MS chromatograms of 17-OHPC and its metabolites derived from incubation of 17-OHPC with expressed CYP3A7.

A: The extracted ion chromatogram depicting the major metabolites (M1, M2 and M3) having a m/z = 445.0. This m/z value depicts a mono-hydroxylated product of 17-OHPC metabolism.
B: The extracted ion chromatogram depicting the major metabolites (M1, M2 and M3) having a m/z = 467.0. This m/z value depicts a sodium adduct of the mono-hydroxylated products of 17-OHPC metabolism.
C: The total ion chromatogram (TIC) depicting the major metabolites (M1, M2 and M3).
D: The total ion chromatogram (TIC) generated on incubation of 17-OHPC in the absence of NADPH.

4.5.4 Estimation of 17-OHPC clearance in fetal human hepatocytes in comparison with adult human hepatocytes

The time course of 17-OHPC metabolism in freshly isolated human fetal and adult hepatocytes has been illustrated in Fig. 23A. 17-OHPC was incubated for a total period of 120 mins and samples taken at various time points. At each sampling time point, the amount of 17-OHPC unmetabolized decreased and corresponded to an increase in total major metabolites generated (Fig. 23B). The drug depletion profiles at 5 μM depicted a linear log concentration decline, thus, showing that the metabolism follows first-order kinetics under these conditions. The result has been reported in terms of 17-OHPC equivalents for the metabolites since the identity of the metabolites has not been confirmed. Similar profiles were observed in both fetal and adult hepatocytes although the amount of metabolites varied depending on the metabolic efficiency of individual cases. The half life and intrinsic clearance for the drug depletion in the case of fetal hepatocytes (Table 8) was not significantly different from that observed for adult hepatocytes.

4.5.5 Kinetics of 17-OHPC metabolism by Fetal and Adult Human Hepatocytes

The kinetics of metabolism of 17-OHPC was characterized in human fetal and adult hepatocytes (n=4). Kinetics of 17-OHPC was evaluated using the rate of generation of metabolite expressed in terms of 17-OHPC equivalent. An incubation time of 60 mins was used in fetal hepatocytes since all three metabolites were readily detectable beyond this time point in hepatocytes. M2 was the major metabolite followed by M3 and M1. The generation of all three major metabolites of 17-OHPC by fetal hepatocytes displayed Michaelis -Menten kinetics and the kinetic parameters

for adult and fetal hepatocytes are reported in **Table 9.** Michaelis menten kinetics was also observed for the major metabolites generated by expressed CYP3A7 and the calculated kinetic parameters for CYP3A7 and CYP3A4 are reported in **Table 10**.

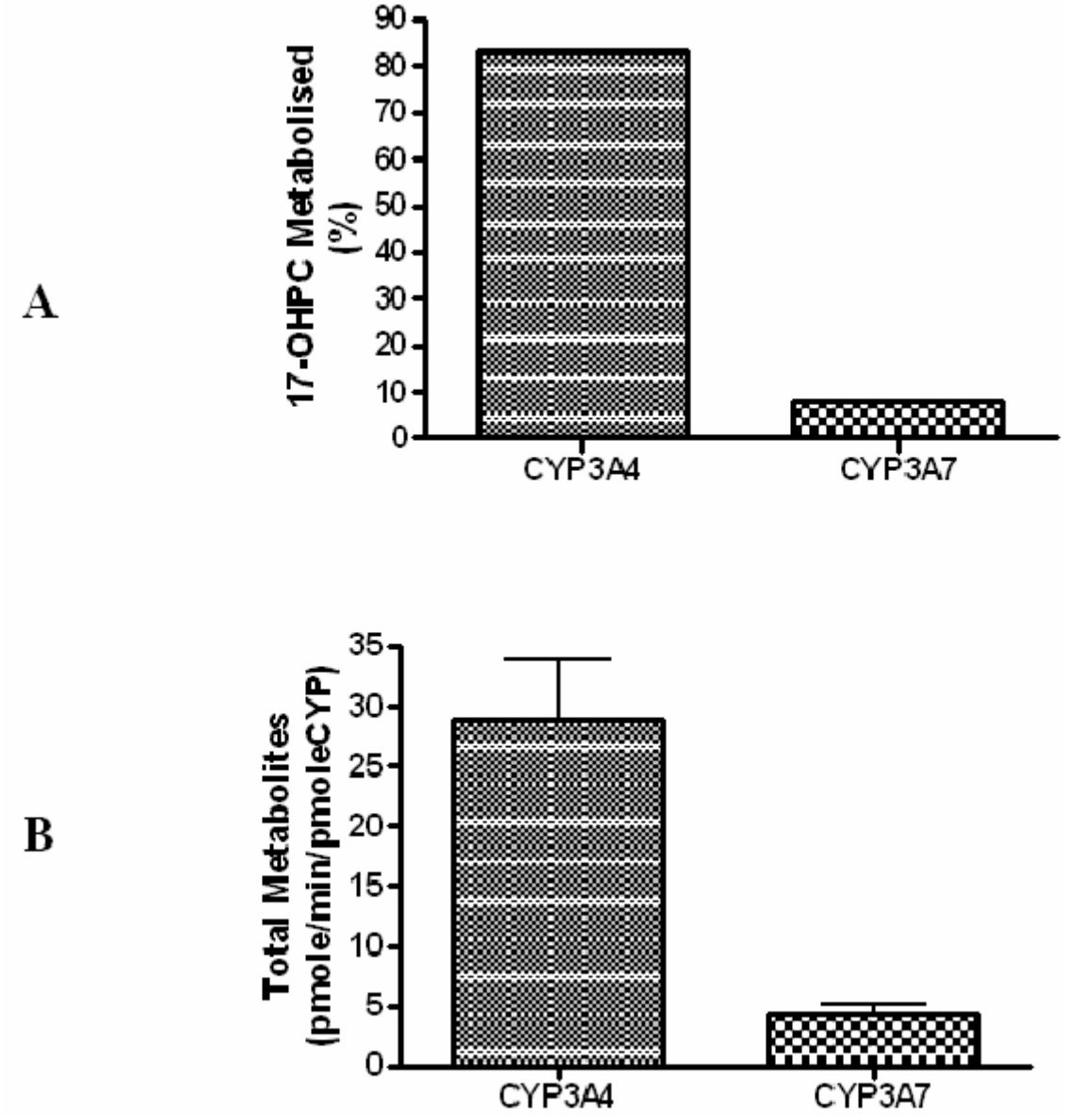


Figure 21. Comparative evaluation of (A) 17-OHPC metabolism and (B) total major metabolites (M1-M4) generated by expressed CYP3A4 and CYP3A7 isoforms

Recombinant P450 isozymes (80 pmol/ml) were incubated with 17-OHPC (100μM) at 37°C for 120 min. The incubation was performed in the presence of NADPH. Each column in the bar diagram represents the mean of two independent analyses. The amount of metabolites generated is expressed in terms of 17-OHPC equivalents.

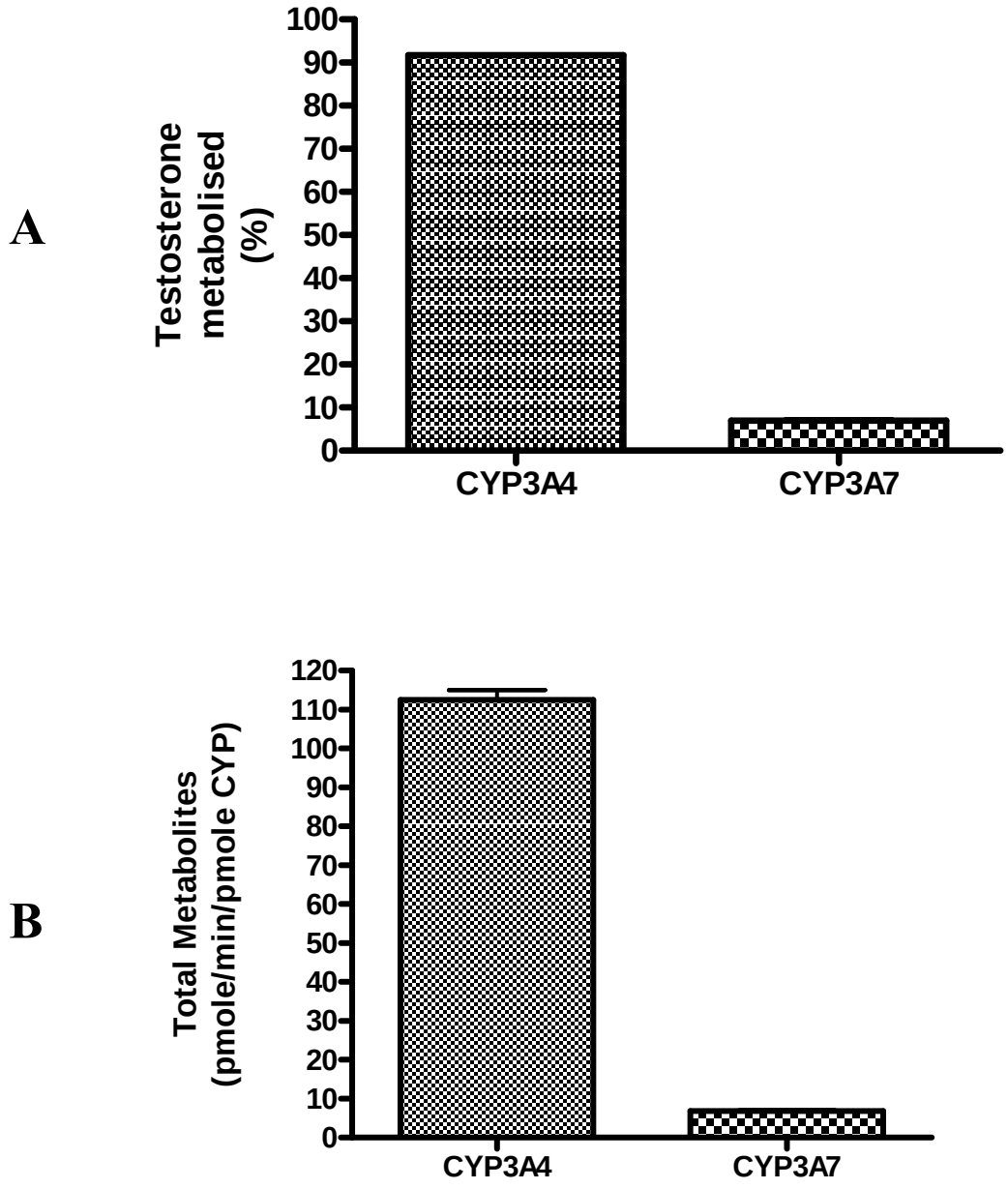


Figure 22. Comparative evaluation of (A) TE metabolism and (B) total major metabolites generated by CYP3A4 and CYP3A7

Recombinant P450 isozymes (80 pmol/ml) were incubated with Testosterone (250μM) at 37°C for 120 min. The incubation was performed in the presence of NADPH. Each column in the bar diagram represents the mean of two independent analyses.

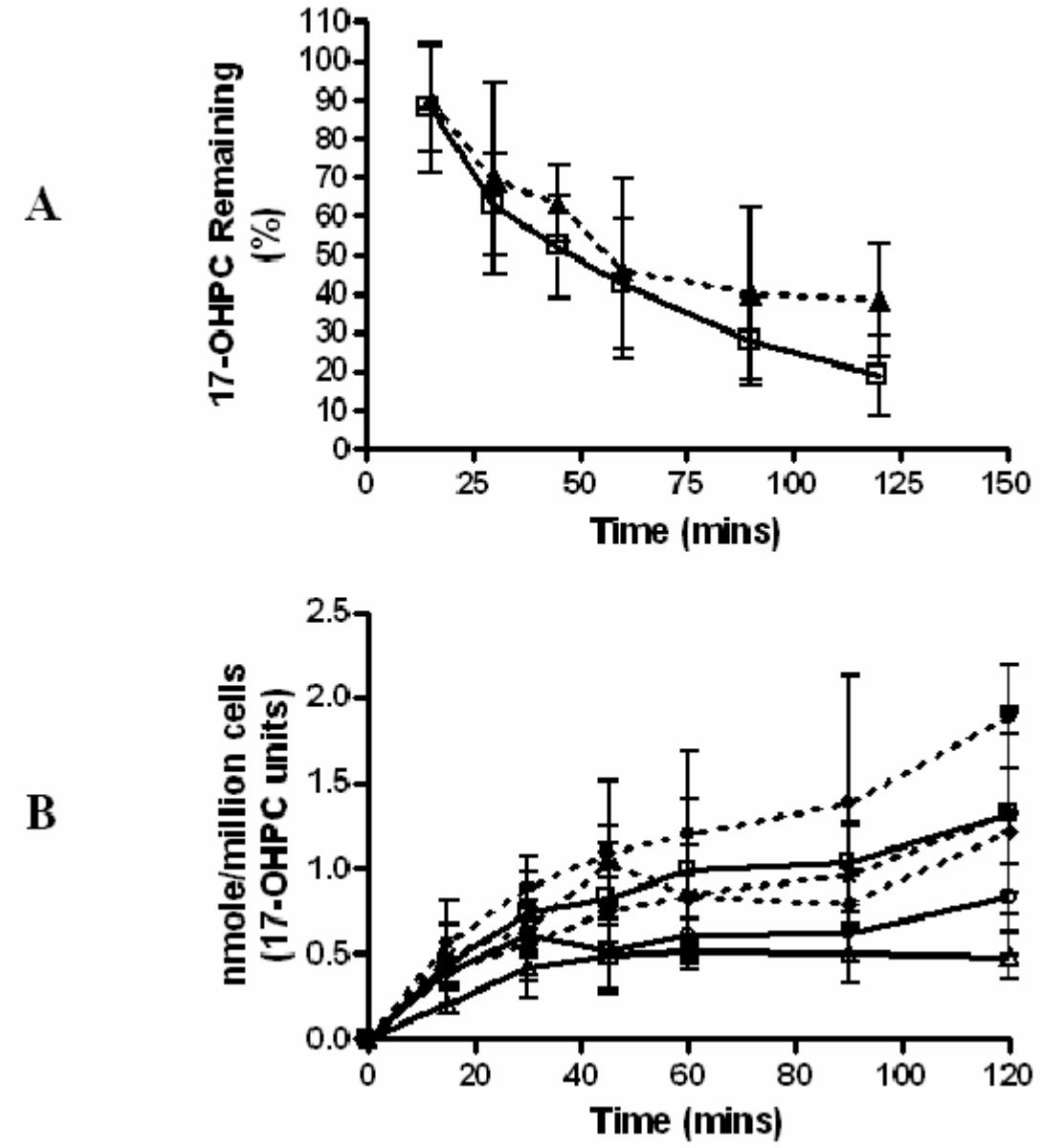


Figure 23. Metabolism of 17-OHPC and generation of major metabolites by fetal (dashed line, n=4) and adult hepatocytes (solid line, n=4)

Fresh fetal and adult hepatocytes were incubated with 17-OHPC (5μM) and all three metabolites quantified by sampling at various time points. The graph depicts depletion of 17-OHPC with time corresponding to a similar increase in the metabolite levels. The major metabolites generated have been expressed in terms of 17-OHPC equivalents.

A) The figure depicts the amount of 17-OHPC (expressed as % remaining) metabolized as a function of incubation time.

B) The figure depicts the amount of major metabolites generated by fetal hepatocytes [M1 (✳), M2 (●) and M3 (♦)] and adult hepatocytes [M2 (□), M4 (Δ) and M5 (○)] as a function of incubation time.

Table 8. Half Life (T-Half) estimation of 17-OHPC metabolism by human fetal and adult hepatocytes

	T-HALF (mins)	Clint (ml/min/million cells)
ADULT	**57±31**	**0.041±0.02**
FETAL	**66±40**	**0.035±0.01**

Table 9. Kinetics of 17-OHPC metabolism in fetal and adult hepatocytes

Metabolite #	Vmax (nmole/min/million cells)	Km (μM)
Fetal_M1	**0.46 ± 0.16**	**12.3 ± 5.1**
Fetal_M2	**0.50 ± 0.11**	**11.7 ± 5.3**
Fetal_M3	**0.65 ± 0.10**	**25.2 ± 11.0**
Adult_M2	**1.3±0.52**	**32.60±49.4**
Adult_M4	**0.35±0.23**	**33.10±25.1**
Adult_M5	**0.79±0.50**	**38.80±25.7**

Table 10. Kinetics of 17-OHPC metabolism in recombinant CYP3A7 and CYP3A4

Metabolism of 17-OHPC demonstrated a Michaelis-Menten type kinetics.

Metabolite #	Vmax (nmole/min/pmoleCYP)	Km (µM)
CYP3A7_M1	0.0103 ± 0.002	1.8 ± 0.90
CYP3A7_M2	0.0104 ± 0.001	1.7 ± 0.50
CYP3A7_M3	0.0130 ± 0.002	5.2 ± 2.1
CYP3A4_M2	0.20±0.02	57.5±14.7
CYP3A4_M4	0.04±0.006	40.1±16.1
CYP3A4_M5	0.032±0.003	10.5±4.8

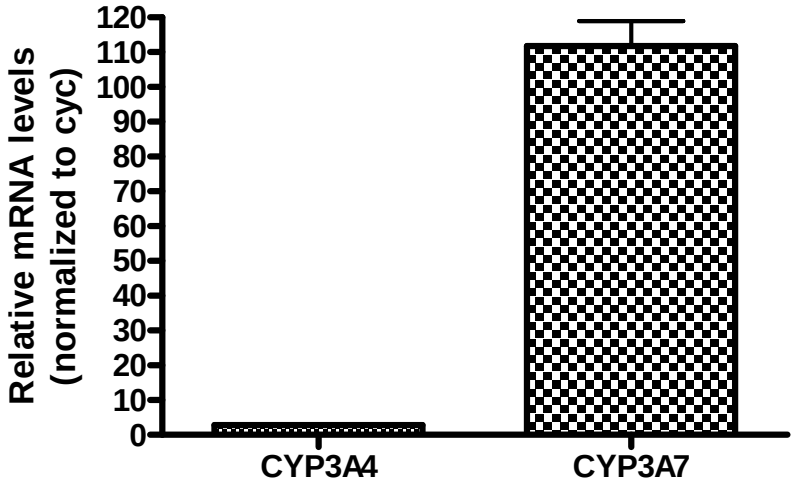


Figure 24. Expression of CYP3A4 and CYP3A7 in primary fetal hepatocyte cultures (n=3)

The results are expressed as Mean ± SD. The expression levels of CYP3A7 were observed to be ~100 fold higher than the CYP3A4 expression thus making CYP3A7 the major isoform in fetal liver.

4.6 Discussion

The present study shows that 17-OHPC is metabolized by hepatocytes obtained from human fetuses in the second trimester of gestation. This report adds to, and confirms the existing data on the ability of the human fetus to metabolize some drugs administered during pregnancy (Ackermann and Richter, 1977; Aranda et al., 1979; Rollins et al., 1979; Chiba et al., 1997; Ladona et al., 2000).

Incubation of 17-OHPC with fresh human fetal hepatocytes in culture generated three major metabolites (M1, M2 and M3) having a similar m/z values of 445.0 but different retention times. These m/z values suggest the formation of mono-hydroxylated metabolites (429+16=445) which were isomers having the hydroxylation at different carbon positions. Increase in the metabolism of 17-OHPC in the presence of CYP3A inducers, RIF and PB, provided the first indication that CYP3A (primarily 3A7 or 3A4) isoforms are likely to be involved. Further, metabolism of 17-OHPC by expressed CYP3A7 to three major metabolites (M1, M2 and M3) confirmed the role of CYP3A7.

Metabolites (M1 and M3) generated by CYP3A7 were observed to be isoform specific based on the relative retention times and preliminary fragmentation data. These CYP3A7 specific metabolites are likely to be structural isomers of CYP3A4 metabolites. Metabolite structure elucidation involving “Ion Trap” based fragmentations and NMR studies are currently being carried out to confirm the structure.

CYP3A7 is the major CYP3A isoform expressed in the fetal liver (Komori et al., 1990; de Wildt et al., 1999). The metabolite profile in fetal hepatocytes was observed to match the profile generated on incubating 17-OHPC with expressed CYP3A7. Further, metabolism of 17-OHPC by fresh adult human hepatocytes generated a metabolite profile which was similar to the

profile observed in incubations with expressed CYP3A4. It can be concluded that CYP3A7 expressed in fetal hepatocytes and CYP3A4 in adult hepatocytes form the major pathway in the metabolism of 17-OHPC. Comparison of the two expressed CYP3A isoforms' 17-OHPC metabolizing capacity showed CYP3A7 to have a much lower metabolic capacity (based on % 17-OHPC metabolised) than CYP3A4. RT-PCR based analysis of mRNA expression in the fetal livers that were utilized in our study has shown CYP3A7 expression level to be ~100 times higher than CYP3A4 (Figure 24). Thus, inspite of the lower 17-OHPC metabolizing capability, the results suggest that CYP3A7 may play a major role in the fetal disposition of 17-OHPC. Although CYP3A5 has been observed to metabolize 17-OHPC (Sharma et al., 2008) much more efficiently than CYP3A4, it is less likely to play a significant role in fetal metabolism since the expression level of CYP3A5 is almost negligible in the fetal liver (Hakkola et al., 2001).

Metabolism of 17-OHPC by human fetal hepatocytes displayed a regular Michaelis-Menten profile. The estimated Vmax values for the major metabolites (M1-M3) in fetal hepatocytes were observed to be ~3-4 fold lower than adult human hepatocytes (M2 – 2.0, M4 - 0.4, M5 – 1.0). Similar results were also observed in studies involving expressed CYP3A4 and CYP3A7. The total metabolites generated by incubating 17-OHPC (100μM) with CYP3A7 (major isoform in fetal liver) were observed to be ~5 fold less than CYP3A4 (major isoform in adult liver).

The relative metabolic capacities of adult and fetal hepatocytes was evaluated by estimating the half-life and intrinsic clearance of 17-OHPC (5μM) based on loss of parent drug experiments. The results indicated the adults to have a similar capacity to metabolize 17-OHPC as compared to the fetus. The intrinsic clearance (Vmax/Km) for fetal hepatocytes for the major metabolites (M1=0.04, M2=0.05, M3=0.02) was also comparable to that calculated for adult

hepatocytes (M2=0.04, M4=0.02, M5=0.03). Similar results were observed in experiments with expressed CYP3A isoforms. The intrinsic clearance (Vmax/Km) for expressed CYP3A7 (M1=0.006, M2=0.006, M3=0.002), the major isoform in fetal liver, was comparable to expressed CYP3A4 (M2=0.004, M4=0.001, M5=0.003), the major isoform in adult liver.

In summary, the intrinsic clearance of 17-OHPC was similar in adult and fetal hepatocyte cultures. Further, the kinetics of 17-OHPC metabolism was also observed to be similar in hepatocytes and expressed enzyme systems.

A number of factors can affect the kinetics of a drug in fetal hepatocytes. It is known that both adult and fetal livers express similar set of transporters which are responsible for the uptake and efflux of various endogenous and exogenous chemicals but differ in their level of expression (Chen et al., 2005; Cizkova et al., 2005). Thus, kinetic differences (or lack thereof) observed between adult and fetal hepatocytes for a drug could be related to the levels of expression of these transport proteins. For example, clearance of a drug by hepatocytes at non-saturating concentrations (<1μM) may be more dependent on the total uptake or other factors that influence the intracellular concentration rather than simply on the intrinsic metabolic capacity of the CYPs expressed. Thus, evaluation of the transport processes that may be involved in the disposition of a drug in adult and fetal cells needs to be investigated as well. Other possibilities could include differences in tissue protein binding between adult and fetal hepatocytes.

The capacity of the fetus to metabolize drugs is a critical component in understanding the maternal and fetal bioavailability of drugs administered during pregnancy. The amount of drug or metabolite accumulation in the fetal circulation largely depends on the placental permeability or transport and fetal nonplacental clearance pathways. Hence, determining the contribution of metabolism to fetal nonplacental clearance pathways (hepatic metabolism) is essential in

understanding fetal drug disposition. We report here that human fetal hepatocytes are capable of significantly metabolizing 17-OHPC with CYP3A7 being the major enzyme involved. Further, fetal specific metabolites (M1 and M3) were generated, which were not observed in the adult hepatocyte system. Level of 17-OHPC in the cord blood from pregnant subjects undergoing treatment for preterm birth was observed to range from 1-8 ng/ml (<20 nM). Thus, given the capacity of fetal hepatocytes to metabolize 17-OHPC (Km>1μM), it is likely that metabolism by human fetal liver will be a significant contributor to fetal drug disposition of 17-OHPC. Further, 17-OHPC metabolism will also lead to the generation and possible accumulation of the monohydroxylated metabolites in the fetal circulation.

The ratio of 17-OHPC in the maternal plasma to cord blood was observed to range from 0.1 to 0.3. Thus, as the amount of 17-OHPC transferred to the fetal compartment is significantly lower as compared to the mother, the contribution of fetal metabolism to the overall disposition is likely to be insignificant. However, studies in suitable animal models (using infusions of the 17-OHPC and its major metabolites) are needed to evaluate and confirm the role of fetal metabolism in the overall disposition of 17-OHPC.

Clinical Implications:

1) Human fetus can metabolize 17-OHPC to 3 major metabolites. Accumulation of these metabolites can affect the fetal hepatic elimination processes and be a potential cause for any associated toxicity.
2) A short half life of ~ 57 mins was observed in adult hepatocyte cultures. Thus, a short in-vivo half life is expected of this intermediate clearance compound.

Acknowledgements:

This project was supported in part by the Obstetrical-Fetal Pharmacology Research Units Network (OPRU). This work is supported in part by NICHD grant HD-047905-2 and the Liver Tissue and Cell Distribution System, funded by NIH Contract # N01-DK-7-0004/HHSN26700700004C (SCS).

5.0 Evaluation of the role of transporters in the disposition of 17-OHPC and the effect of 17-OHPC on bile salt transport.

[Sharma S, Ellis E, Marongiu F, Mattison D, Caritis S, Venkataramanan R and Strom S (2009). Expression and activity of transporters in fetal human hepatocytes: A comparison with adult human hepatocytes. Submitted to DMD]

5.1 Abbreviations

FHH - primary cultures of human hepatocytes
17-OHPC - 17α-hydroxyprogesterone caproate
EMEM - Eagle's Minimum Essential Medium
HBSS - Hank's balanced salt solution
HMM - Hepatocyte Maintenance Medium
DMEM - Dulbecco's Modified Eagle Medium
RIF – Rifampin
CYC – Cyclosporine
VER - Verapamil

5.2 Abstract

Aim: 17-OHPC has been reported to traverse the placental barrier and gain access to fetal circulation. Further, 17-OHPC is metabolized by the fetal liver. Little information is available in literature regarding the expression and activity of transporters in fetal human liver or cultured cells. In this study, the role of transporters in the disposition of 17-OHPC in fetal and adult human hepatocytes was examined. Progesterone metabolites have been reported to induce trans-inhibition of BSEP. Thus, the purpose of the study was to investigate the effect of 17-OHPC (a structural analog of progesterone) or its metabolites on [^{3}H]-taurocholic acid transport.

Methods: The transport activity was evaluated using sandwich cultured human fetal and adult human hepatocytes. Aliquots of media were counted in a liquid scintillation counter to estimate the activity. Protein content and mRNA were also measured.

Results: 17-OHPC was taken up rapidly into the cells, mostly by passive diffusion. The drug was transported out of hepatocytes partially by an active efflux process which was significantly inhibited by cyclosporine, rifampin, verapamil and cold temperature. Active efflux was observed in both adult and fetal hepatocyte cultures. Further, we evaluated the expression of various hepatic transporters (MDR1, MDR3, OATP1B1, OATP1B3, OATP2B1, BSEP, NTCP, MRP2, MRP3, MRP4 and BCRP) in fetal and adult hepatocytes. With the exception of MDR3 and NTCP, all transporters evaluated were found to be expressed in significant amounts in fetal hepatocytes as compared to adults. 17-OHPC produced a concentration-dependent inhibition of taurocholate (TC) efflux into canaliculi in fresh cultures of adult and fetal human hepatocytes.

Conclusion: The study demonstrates the functional expression of both sinusoidal and canalicular transporters responsible for transport of taurocholate and 17-OHPC in fetal human hepatocytes.

Further, results from a competition assay show the inhibitory potential of 17-OHPC towards taurocholate transport.

5.3 Introduction

Drug therapy during pregnancy exposes both the mother and fetus to the potential adverse effects associated with therapy. Despite the presence of a placental barrier to drug transfer, certain drugs, depending on their physicochemical properties, are able to gain access to the fetal circulation. The fetus like the adult has detoxifying mechanisms in the form phase 1, 2 and 3 pathways to eliminate these xenobiotics. Little is known regarding the expression and activity of various enzymes and transporters in fetal liver which mediate xenobiotic elimination. Fetal metabolism and the expression of cytochrome P-450s in the fetal liver has been documented in the literature (Ackermann and Richter, 1977; Aranda et al., 1979; Rollins et al., 1979; Rane and Tomson, 1980; Wiebkin et al., 1985; Komori et al., 1990; Chiba et al., 1997; Ladona et al., 2000). However, little to no information is available regarding the expression (Chen et al., 2005) and activity of hepatic transporters in fetal human hepatocyte cultures. Biliary excretion of therapeutic drugs plays an important role as one of the detoxification mechanism and for certain drugs, inhibition of biliary transport can explain the adverse hepatic effects associated with them. The hepatic transporter, bile salt export pump (BSEP, ABCB11), is responsible for the transport of unconjugated bile acids such as taurocholic acid. Inhibition of the BSEP activity can lead to the accumulation of these toxic unconjugated bile acids leading to pathological conditions like intrahepatic cholestasis of pregnancy (Fattinger et al., 2001; Funk et al., 2001; Kroumpouzos, 2002). Besides BSEP, other efflux and uptake hepatic transporters like MDR1, MRP2, OATP

and BCRP are also expressed in the adults and have been implicated in hepatotoxicity due to transporter based drug-drug interactions.

Our first aim was to establish the functional activity of hepatic transporters (mainly NTCP and BSEP) in primary cultures of fetal hepatocytes using sodium taurocholate a substrate of sodium dependent uptake transporter, NTCP, and the efflux transporter, BSEP. Our second aim was to determine if transport processes were involved in the disposition of 17alpha-hydroxyprogesterone caproate (17-OHPC), a new agent administered to pregnant subjects to prevent pre-term birth. The final aim was to evaluate the interaction between 17-OHPC and taurocholate to determine if there was the potential for hepatotoxicity due to transporter mediated drug-drug interactions involving 17-OHPC or its metabolites in the fetus. This was achieved via minor modification of a well established transport assay (Kostrubsky et al., 2006) which identified competition between the 17-OHPC and radioactively labeled Taurocholic Acid. For the present study we used sandwich cultures of fresh human adult and fetal hepatocytes which were overlaid with Matrigel®. The use of sandwich cultures has been widely used for the evaluation of uptake and efflux processes involved in bile and xenobiotic transport. Sandwich cultures of hepatocytes overlaid with Matrigel® establish tight junction complexes and cell polarity leading to the formation of canalicular spaces which have been characterized by the transport of various radiolabeled or fluorescent substrates in adult hepatocytes.

We have previously demonstrated the ability of fetal and adult hepatocytes to metabolize 17-OHPC. We have also shown the presence of novel fetal metabolites that were not observed in adult hepatocytes. Further, we have reported the transplacental transfer of 17-OHPC and the presence of significant levels of this drug in the fetal circulation (unpublished data). The cholestatic effect of certain steroids, such as estradiol 17β-D-glucuronide (E217βG), has been

suggested to be due to trans-inhibition of the major mechanism accounting for bile acid (BA) transport across the canalicular membrane of hepatocytes, i.e. the bile salt export pump (BSEP, gene symbol ABCB11) (Kroumpouzos, 2002). Further, the role of progesterone metabolites (PMs) in the etiology of Intrahepatic Cholestasis of Pregnancy (ICP) has been suggested. Rise in the serum concentrations of PMs has been associated with impaired biliary excretion. Progesterone metabolites have been reported to inhibit BSEP activity (Kroumpouzos, 2002; Vallejo et al., 2006). Also, progesterone is reported to be an inhibitor of Pgp, a transporter expressed on the canalicular membrane of hepatocytes (Barnes et al., 1996). Thus, we hypothesize that 17-OHPC and/or its metabolites will inhibit bile salt transporter (BSEP) in adult and fetal human hepatocytes.

5.4 Methods

5.4.1 Uptake and Release Studies

1. Transport assay for 17-OHPC and taurocholate was conducted in sandwich cultures of hepatocytes as described by Kostrubsky et al., (Kostrubsky et al., 2003) with the exception that rather than collagen, the cells were overlaid with Matrigel® at a concentration of 0.233 mg/ml. The hepatocytes were incubated for different time intervals in medium (HBSS (+) – HBSS supplemented with Ca^{2+}/Mg^{2+}) containing test compound, 17-OHPC - 1µM and Taurocholic acid - 1µM, in the presence or absence of inhibitors, Cyclosporine - 40µM, Rifampin - 100µM and Verapamil – 40µM (Loading Phase). The transport was stopped by removing the buffer and washing cells with ice-cold media. The efflux of the test compound was initiated by adding

HBSS (-) i.e. HBSS depleted of Ca^{2+}/Mg^{2+}, with and without inhibitor to the cells. Aliquots of media were harvested at different time intervals and counted in a liquid scintillation counter to estimate the efflux. Subsequently, the remaining media was aspirated and cell lysis buffer was added to the cells. The cell lysates were collected and counted in a liquid scintillation counter to estimate the uptake. Results were normalized to total cellular protein.

2. Transport of the test compounds was also evaluated in warm (37ºC) and cold (4ºC) media using a method similar to the one mentioned above. Briefly, the hepatocytes were incubated in warm or cold media (HBSS+) containing test compound, 17-OHPC - 1μM and Taurocholic acid - 1μM, for different time intervals (Loading Phase). The transport was stopped by removing the buffer and washing cells with ice-cold media. The efflux of the test compound was initiated by adding (warm or cold) buffer (HBSS-) to the cells. Aliquots of media and cell lysates were harvested and counted in a liquid scintillation counter to estimate efflux and uptake as described before. Results were corrected for total protein content.

5.4.2 Inhibition of Bile Acid Transport

Inhibition of bile acid transport in sandwich cultures of hepatocytes was conducted according to previously described protocol (Kostrubsky et al., 2003) except that cells were overlaid with Matrigel® rather than collagen. Briefly, 1 μM [^{3}H]-taurocholic acid, with or without increasing concentrations of test compound (17-OHPC, CYA) was added to hepatocytes for different time intervals at 37^{o}C. The transport was stopped by removing the buffer and washing cells with cold media. The taurocholate efflux from canalicular spaces was initiated by adding HBSS (+) or HBSS (-) to the cells. Aliquots of media were collected and counted in a liquid scintillation counter. The difference in amount of radioactivity between two buffer conditions in the absence

of test compound was defined as a 100% taurocholate efflux in canaliculi. In the presence of compound, this difference became smaller and was used to calculate the percent inhibition of bile acid efflux (Kostrubsky et al., 2003). All values were normalized to total cellular protein.

5.4.3 Hepatobiliary transport in adult and fetal human hepatocytes

Hepatobiliary transport of drugs in human hepatocytes was assessed based on the protocol described previously (Bi et al., 2006; Kalgutkar et al., 2007). Briefly, fresh human hepatocytes in culture were rinsed twice with 1ml of HBSS (+) or HBSS (-) and then equilibrated in the same buffers for 10 min at 37ºC. [^{3}H]-taurocholic acid (1μM) or [^{3}H] 17-OHPC (1μM) in HBSS (+) was then added to both sets of cultures. Aliquots of media were collected after 15 mins and counted in a liquid scintillation counter. Taurocholate transport was used as a positive control for active hepatic uptake and efflux and also to quantify the activity of bile transporters in fetal hepatocytes. All values were normalized to total cellular protein. The equation (1) used to calculate biliary excretion index (BEI) is shown below (Liu et al., 1999b). In the presence of Ca^{2+}/Mg^{2+}, the integrity of the biliary canaliculi remains intact, whereas in the absence of Ca^{2+}/Mg^{2+}, the canalicular tight junctions are disrupted. Therefore, quantifying the accumulation of a test compound in the presence and absence of Ca^{2+}/Mg^{2+} allows one to determine the amount of test compound in the bile canaliculi.

$$BEI = \frac{\text{Accumulation}_{(+Ca^{2+}/Mg^{2+})} - \text{Accumulation}_{(+Ca^{2+}/Mg^{2+}-\text{free})}}{\text{Accumulation}_{(+Ca^{2+}/Mg^{2+})}} * 100 \qquad \text{.....(1)}$$

5.4.4 Reverse Transcription–Polymerase Chain Reaction and Real-Time Quantitative Polymerase Chain Reaction

Primers for MDR1, MDR3, OATP1B1, OATP1B3, OATP2B1, BSEP, NTCP, MRP2, MRP3, MRP4 and BCRP and the Real Time PCR procedure were described in Chapter 2. The relative cDNA content was determined from standard curves constructed from serially diluted cDNA and all genes were normalized to an internal control (cyclophillin) mRNA in each sample.

5.4.5 Data Analysis

Data are expressed as the mean ± SD. Student's t-test was used to assess the significance of the results (17-OHPC transport in the presence vs absence of cyclosporine, verapamil, rifampin and temperature OR Taurocholate transport in the presence or absence of 17-OHPC (GraphPad Software Inc., USA).

5.5 Results

5.5.1 Effect of Cyclosporin on the transport of 17-OHPC and Taurocholate in primary human hepatocytes

To confirm the involvement of an active, transporter mediated process in the disposition of [^{3}H]-17-OHPC in primary human hepatocytes, Cyclosporin, a well known inhibitor, was co-incubated with fetal and adult human hepatocytes.

The amount of [^{3}H]-17-OHPC transported into the media (Figure 25A), estimated by counting the media aliquots, was observed to be significantly ($p<0.05$) lower in the presence of Cyclosporin for both fetal and adult human hepatocytes. In fetal hepatocytes, the % inhibition of [^{3}H]-17-OHPC transport into the media was observed to vary from 17 to 45% whereas it ranged from 30 to 52% in the adult human hepatocytes. The results indicate the involvement of a significant transport mediated process in the efflux of [^{3}H]-17-OHPC in both adult and fetal human hepatocytes.

The total uptake of [^{3}H]-17-OHPC was calculated by taking sum of the amount of radiolabeled drug in the cell lysate (Figure 25B) and the amount effluxed (Figure 25A). The total amount of [^{3}H]-17-OHPC accumulated by adult and fetal human hepatocytes was not observed to be significantly ($p>0.05$) higher in the presence of Cyclosporin. These observations suggest the presence of a significant efflux process rather than active uptake in the disposition of [^{3}H]-17-OHPC in both adult and fetal human hepatocytes.

We also carried out timed incubations of [^{3}H]-17-OHPC with Cyclosporin in fetal and adult hepatocytes to further characterize the uptake and efflux process. Data presented in Figure 26A shows the increase in efflux, with time, into the media in the presence and absence of Cyclosporine. Data presented in Figure 26B shows the change in the amount of intracellular drug with time. A time dependent decrease in intracellular levels of 17-OHPC was observed in the absence of cyclosporine, whereas, an increase with time was seen in co-incubations with Cyclosporine. In fetal hepatocytes, the % inhibition of [^{3}H]-17-OHPC efflux observed with Cyclosporine varied from 5% at 5 mins to 30% at 30 mins whereas the intracellular accumulation increased from 0.8 fold at 5 mins to 1.8 fold at 30 minutes. These observations

confirm the results from previous experiments indicating the role of active efflux transporters in the disposition of 17-OHPC in fetal hepatocytes.

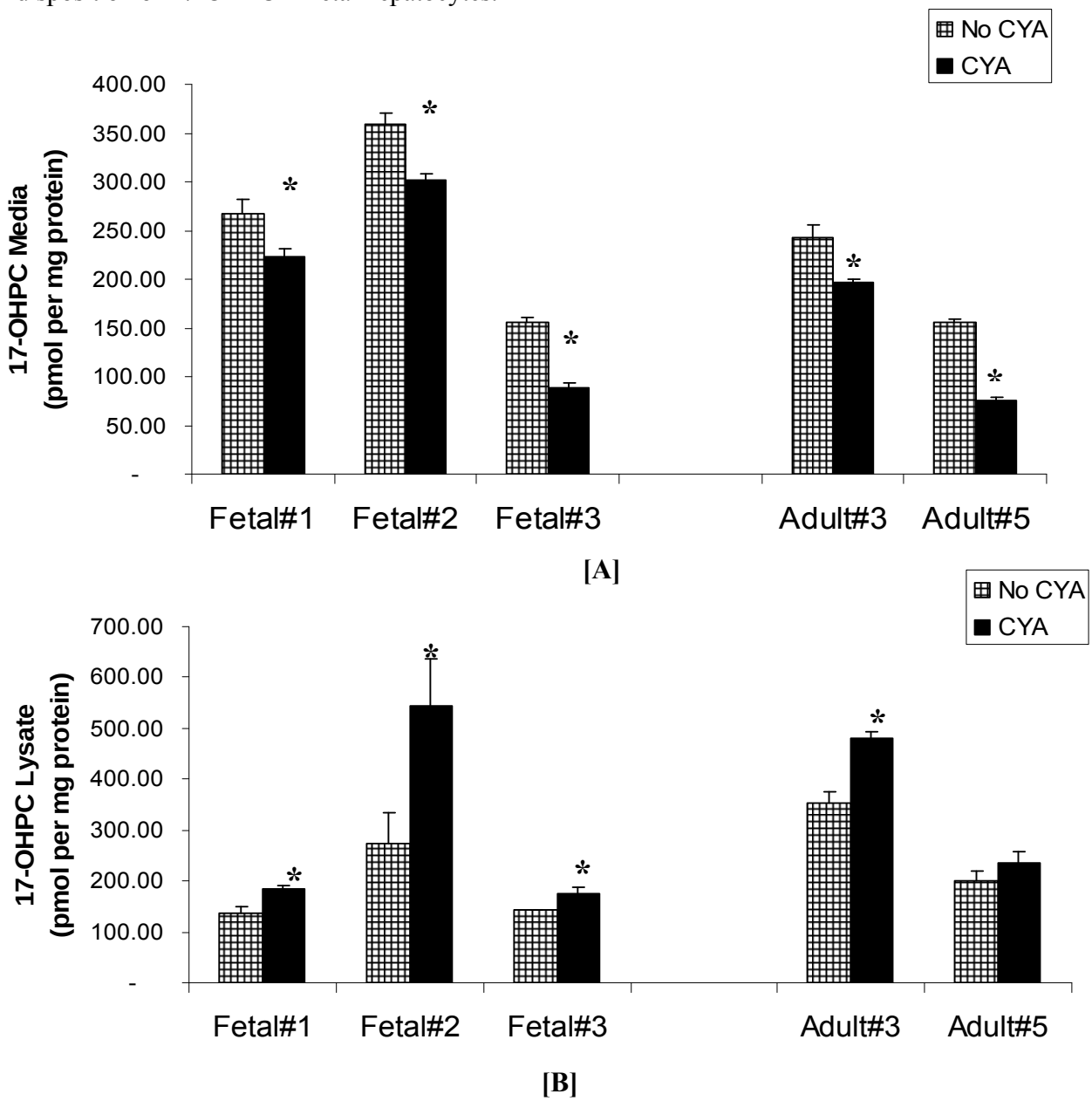


Figure 25. Transport of 17-OHPC in the presence (solid bars) and absence of Cyclosporine (CYA) in primary human fetal and adult hepatocytes

The cells were incubated for 15 mins with 17-OHPC in the presence and absence of CYA during the loading phase. DMSO (0.1%) in buffer was used as the control. Cells were then washed with ice-cold media and reincubated in standard buffer in the presence or absence of CYA. The total efflux and accumulation were estimated by scintillation counting and expressed on the basis of total protein content. Each bar represents the mean of triplicate treatments (±SD). * - $p<0.05$

(A) Media: Significant ($p<0.05$) inhibition of 17-OHPC efflux was observed in the presence of 17-OHPC.

(B) Cell Lysate: Cyclosporine significantly ($p<0.05$) increased the accumulation of 17-OHPC.

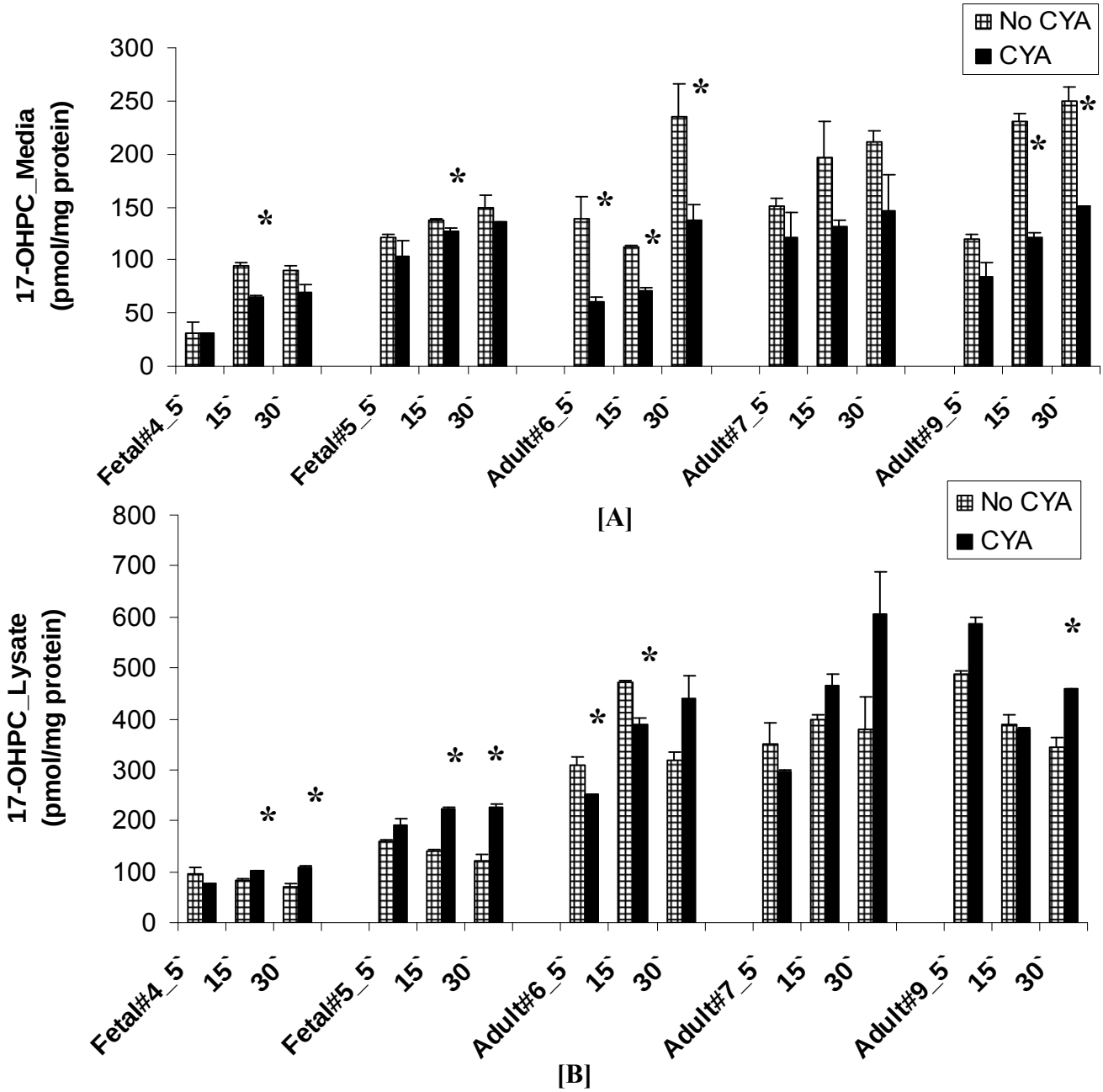


Figure 26. Effect of Cyclosporine (CYA) on the time course of transport of 17-OHPC in primary human fetal and adult hepatocytes

DMSO (0.1%) in buffer was used as the control. Each bar represents the mean of duplicate treatments (±SD). * - $p<0.05$

(A) Efflux of 17-OHPC into the media was observed to increase with time. Cyclosporine significantly ($p<0.05$) inhibited the efflux of 17-OHPC at all time points except at 5 mins.

(B) Accumulation of 17-OHPC was observed to decrease with time in the absence (0.1%DMSO) of Cyclosporine. However, a significant ($p<0.05$) increase in the accumulation of 17-OHPC with time was observed in the presence of Cyclosporine.

Hepatic taurocholate transport is well characterized with its uptake being mediated by NTCP (~80%) and OATP (~20%) and efflux into the canalicular space by BSEP (Kemp et al., 2005). The role of transporters in the hepatic uptake and efflux of taurocholate was estimated by measuring the radioactivity levels in the media and cell lysate, respectively, as mentioned above for 17-OHPC.

The amount of canalicular transport of taurocholate into the media (Figure 27A) and the total cellular accumulation (Figure 27B) was observed to be significantly ($p<0.05$) lower in the presence of Cyclosporin in fetal and adult human hepatocytes. The % inhibition of taurocholate transport into the media was observed to vary from 30 to 60% for fetal cells and ~ 10% in the adult human hepatocytes. Also, the % inhibition of taurocholate accumulation was observed to range from 60-90% for fetal hepatocytes and >90% for adult human hepatocytes. Thus, the results not only confirm the presence of active taurocholate transporters (BSEP and NTCP) in fetal hepatocytes but also suggest that drugs that interfere with normal bile transport function in the fetal liver might be hepatotoxic.

5.5.2 Effect of Rifampin on the transport of 17-OHPC in primary human hepatocytes

The organic anion transporting family of proteins (OATPs; SLCO gene family) has been reported to play a role in the uptake transport of bile salts and xenobiotics. Rifampin has been reported to inhibit human liver OATPs and thus interfere with carrier mediated organic anion uptake (Fardel et al., 1995). To evaluate the involvement of an active, transporter mediated uptake process in the disposition of 17-OHPC in primary human hepatocytes, Rifampin was co-incubated with fetal and adult human hepatocytes.

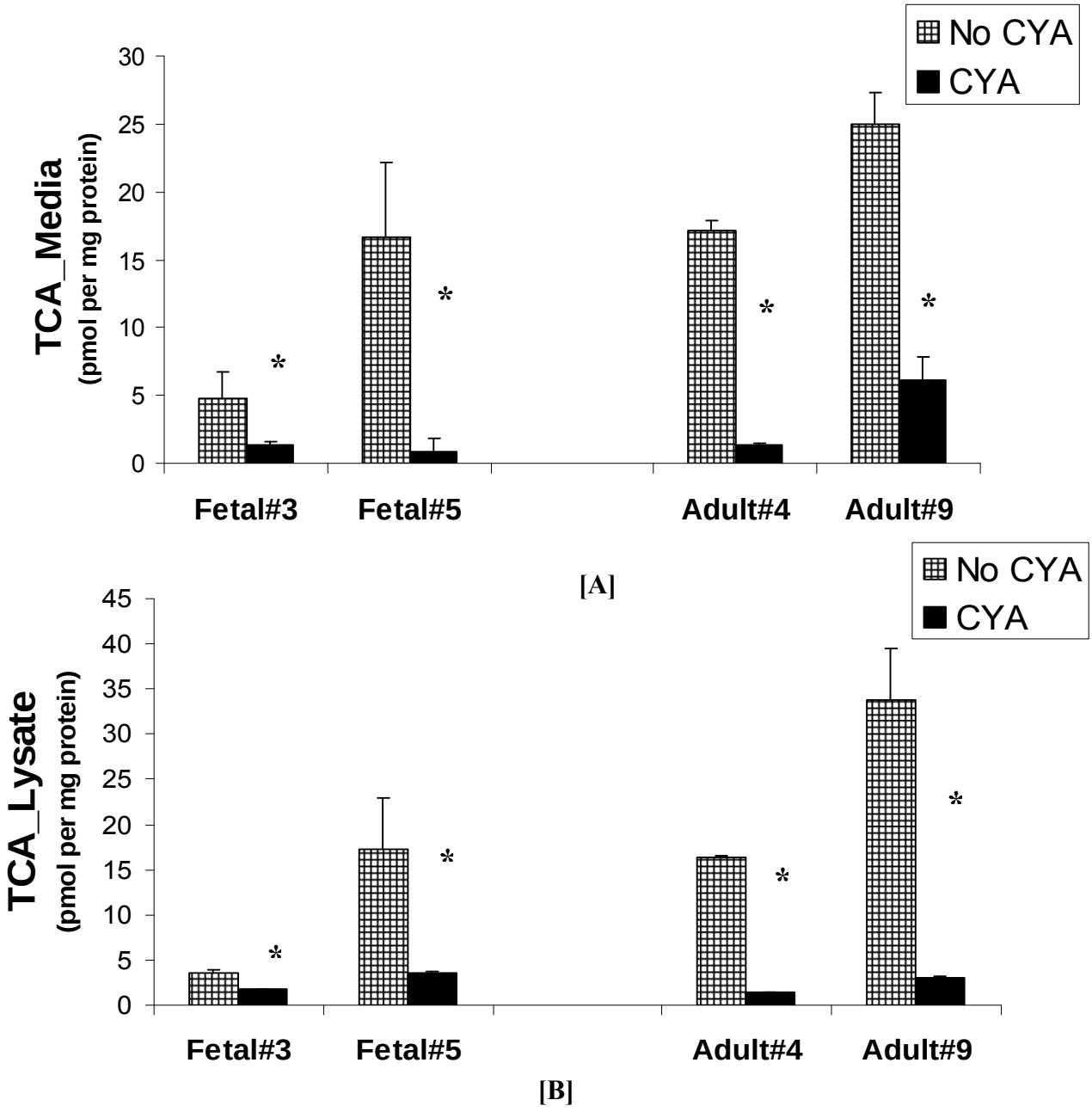


Figure 27. Transport of Taurocholate (TCA) in the presence (solid bars) and absence of Cyclosporine (CYA) in primary human adult and fetal hepatocytes

Hepatocytes were incubated for 15 minutes during the loading phase, washed with ice-cold buffer followed by efflux for 15 minutes. DMSO (0.1%) in buffer was used as the control. Each bar represents the mean of triplicate treatments (±SD). * - $p<0.05$

(A) Significant ($p<0.05$) inhibition of TCA efflux was observed in the presence of CYA.

(B) Cyclosporine significantly ($p<0.05$) reduced the accumulation of TCA.

In fetal hepatocytes, time dependent inhibition of 17-OHPC transport into media was observed in the presence of Rifampin with the % inhibition ranging from ~40% at 5 mins to ~60% at 30 mins (Figure 28A). Further, a time dependent increase in the intracellular amount of 17-OHPC was observed in the presence of Rifampin. The accumulation increased from ~1.6 fold at 5 mins to ~2.3 fold at 30 minutes (Figure 28B). In adult hepatocytes, similar results to fetal hepatocytes were observed. In the presence of Rifampin the % inhibition was observed to range from ~25% at 5 mins to ~50% at 30 mins (Figure 28A). Also, a higher intracellular amount of 17-OHPC was observed in the presence of Rifampin. The accumulation varied from ~1.01 fold at 5 mins to ~1.4 fold at 30 minutes (Figure 28B).

5.5.3 Effect of Verapamil on the transport of 17-OHPC in primary human hepatocytes

P-glycoprotein (P-gp) is a member of the ATP-binding cassette transporter superfamily that has been shown to be involved in the efflux of various chemical compounds. Verapamil has been reported to inhibit the drug efflux activity mediated by P-gp (Zong and Pollack, 2003). To evaluate the involvement of an active, transporter mediated efflux process in the disposition of 17-OHPC in primary human hepatocytes, Verapamil was co-incubated with fetal and adult human hepatocytes. In adult hepatocytes, inhibition of 17-OHPC efflux was observed in the presence of Verapamil with the % inhibition ranging from ~35% at 10 mins to ~45% at 40 mins (Figure 29A). Further, a time dependent increase in the intracellular amount of 17-OHPC was observed in the presence of Verapamil. The accumulation increased from ~1.01 fold at 10 mins to ~1.6 fold at 40 minutes (Figure 29B). In fetal hepatocytes, similar results to adult hepatocytes were observed.

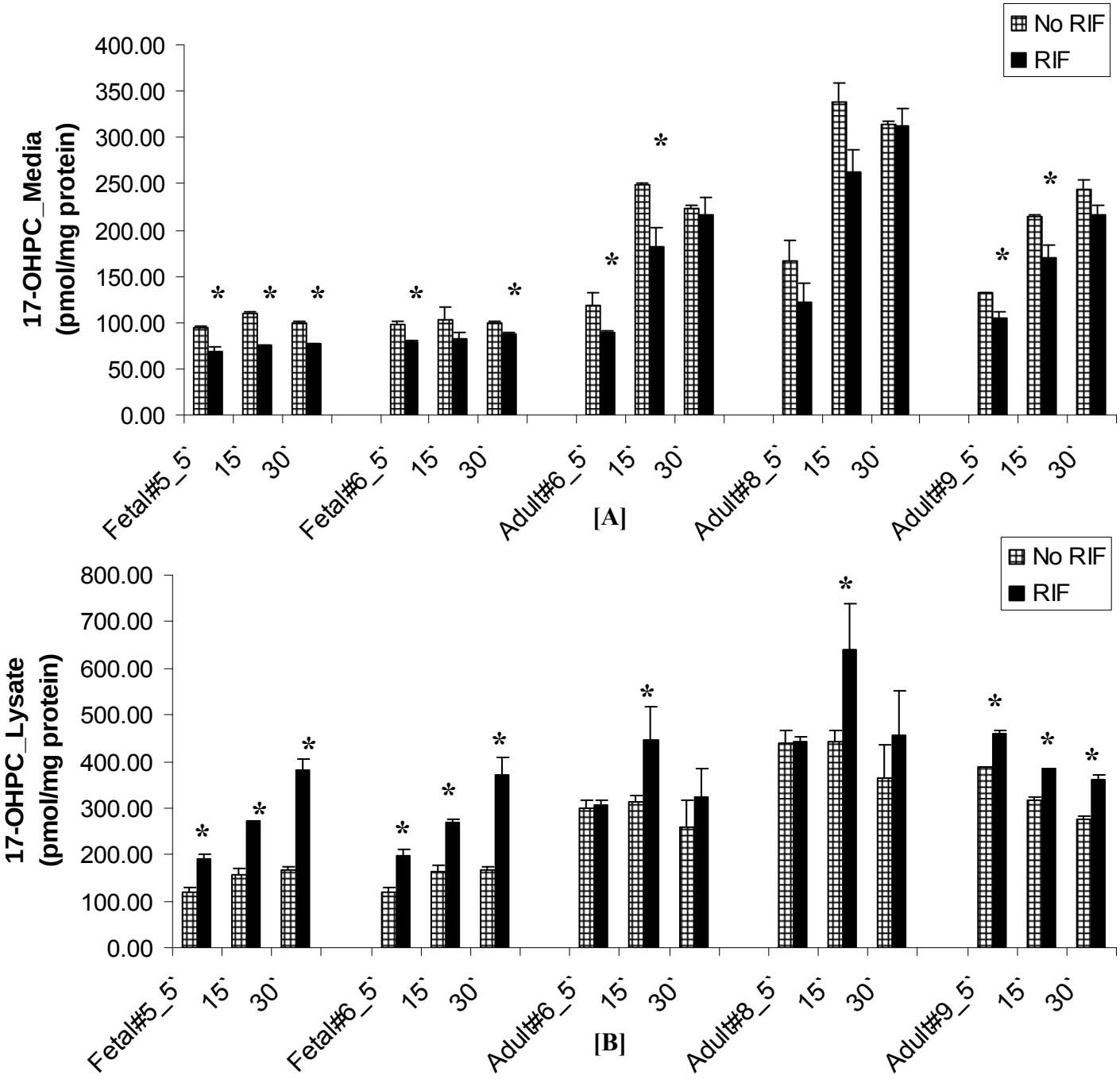


Figure 28. Effect of Rifampicin (RIF) on the time course of 17-OHPC efflux from primary human fetal hepatocyte cultures

The cells were incubated for different time intervals with 17-OHPC in the presence and absence of Rifampicin during the loading phase, washed with ice-cold media and subsequently reincubated in standard buffer in the presence or absence of Rifampicin (RIF). DMSO (0.1%) in buffer was used as the control. Each bar represents the mean of duplicate treatments (±SD). * - $p<0.05$

(A) Significant ($p<0.05$) inhibition of 17-OHPC efflux was observed in the presence of RIF.

(B) RIF significantly ($p<0.05$) increased the accumulation of 17-OHPC.

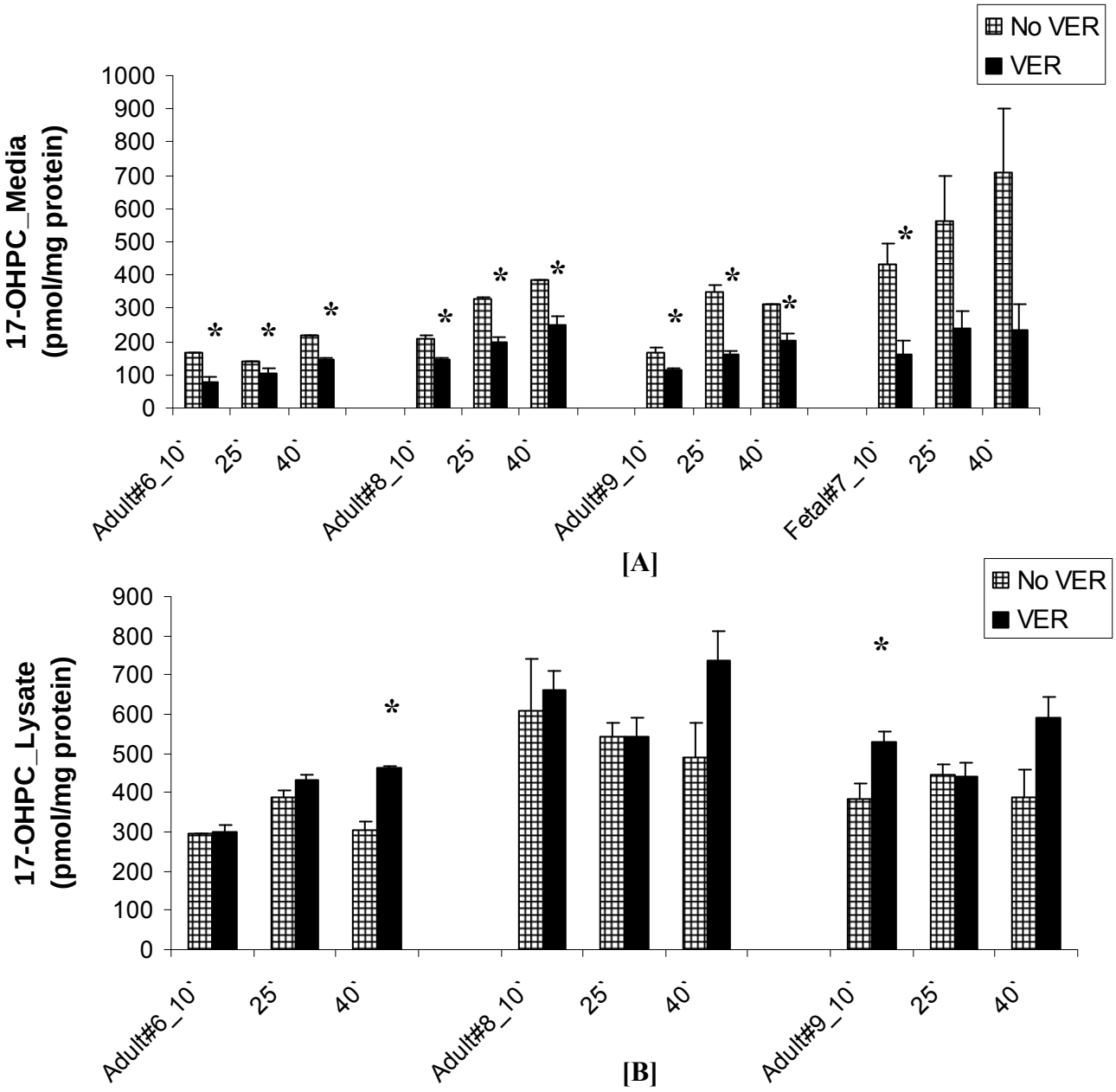


Figure 29. Effect of Verapamil (VER) on the time course of 17-OHPC efflux from primary human fetal hepatocyte cultures

The cells were incubated for different time intervals with 17-OHPC in the presence and absence of Verapamil during the loading phase, washed with ice-cold media and subsequently reincubated in standard buffer in the presence or absence of Verapamil (VER). DMSO (0.1%) in buffer was used as the control. Each bar represents the mean of duplicate treatments (±SD) in adults and a single treatment in fetal. * - $p<0.05$

(A) Significant ($p<0.05$) inhibition of 17-OHPC efflux was observed in the presence of VER.

(B) VER significantly ($p<0.05$) increased the accumulation of 17-OHPC.

In the presence of Verapamil the % inhibition was observed to range from ~60% at 10 mins to ~70% at 40 mins (Figure 29A). No data was available for estimating the counts in the cell lysate.

5.5.4 Effect of temperature on the transport of 17-OHPC and taurocholate in primary human hepatocytes

To further investigate the mechanisms of hepatic transport of 17-OHPC (passive diffusion or active uptake process), the effect of temperature (4ºC vs. 37ºC) on the transport of 17-OHPC into hepatocytes was evaluated.

In fetal hepatocytes, significant ($p<0.05$) inhibition of 17-OHPC efflux was observed in cold media (4ºC) as compared to warm media (37ºC). Further, a time dependent decrease in the efflux of 17-OHPC was observed in cold media. The % inhibition due to cold temperature was observed to be 10-48% at 5 mins and increased to 20-60% at 30 mins (Figure 30A). The total uptake of 17-OHPC in hepatocytes was 1.6 fold higher at 37ºC at 5 minutes and decreased to 0.9 fold at 40 minutes. A time dependent increase in the intracellular amount was observed at 4ºC whereas the intracellular amount showed a net decrease at 37ºC. The accumulation at 4ºC was observed to be less than or equal to 37ºC at 5 mins and increased to 1.2-2 fold at 30 mins (Figure 30B).

Similar results were observed in adult hepatocytes (Figure 30A). The % inhibition of 17-OHPC efflux observed at 4ºC was ~45% at 5 mins and increased to ~60% at 30 mins. The intracellular amount of 17-OHPC at 4ºC was observed to increase from 1.3 fold at 5 mins to >3

fold at 30 mins. The total uptake of 17-OHPC in hepatocytes was 0.9 fold lower at 37ºC (than 4ºC) at 5 minutes and decreased to 0.8 fold at 40 minutes. The results suggest the presence of an active efflux process in the disposition of 17-OHPC and further confirm the results from previous experiments. Effect of temperature on the transport of Taurocholate was also evaluated in fetal hepatocytes. The efflux of taurocholate was observed to be less at 4ºC than 37ºC and showed a time dependent inhibition profile. The % inhibition of efflux was observed to increase from no significant inhibition at 5 mins to ~20% inhibition at 30 mins (Figure 31A). The intracellular accumulation of taurocholate was also observed to be inhibited at 4ºC with a % inhibition of 30-75% at 5 mins which increased to 60-90% at 30 mins (Figure 31B). The total uptake (media + lysate) was also inhibited ranging from 30-80% at 5 mins to 60-90% at 30 mins. These results further confirm the observations from previous chemical inhibitor based experiments and indicate the presence of an active transport process for the uptake (NTCP, OATP) and efflux (BSEP) of taurocholate in fetal hepatocytes.

5.5.5 Effect of 17-OHPC on the bile salt transport in adult and fetal hepatocytes

To evaluate the inhibitory potential of 17-OHPC on bile salt efflux, we pre- and co-incubated different concentrations of the drug with taurocholate. In fetal hepatocytes, 17-OHPC was observed to reduce the efflux of taurocholate in a concentration dependent manner with a significant decrease observed at 0.5 μM, 1.0 μM and 10.0 μM concentrations (Figure 32A). The total uptake of taurocholate was not significantly inhibited by 17-OHPC at any concentration (Figure 32B). Cyclosporine (CYA; 40μM) was used as the positive control and depicted a significant ($p<0.05$) inhibition of both efflux and the total uptake of taurocholate.

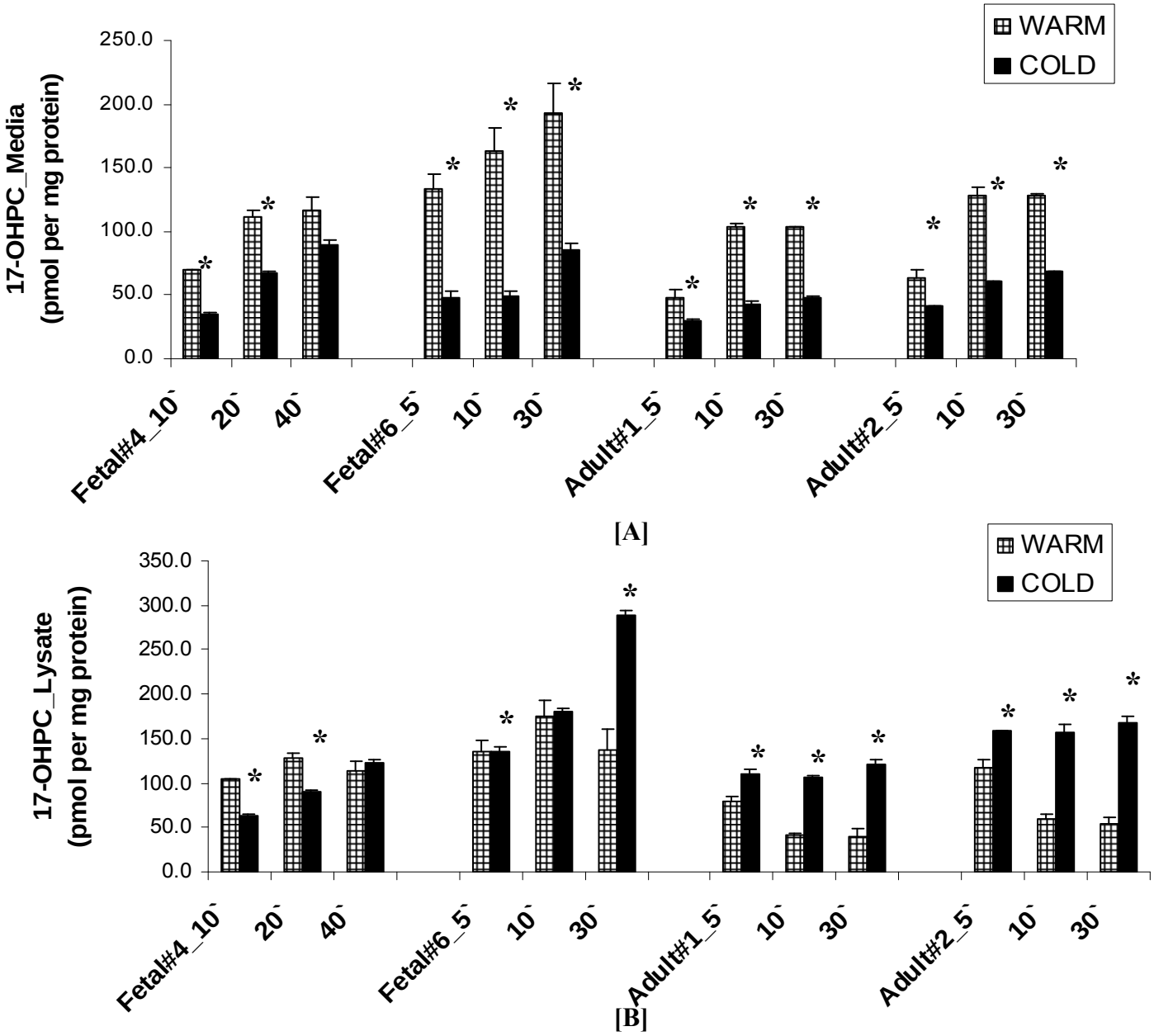


Figure 30. Effect of temperature on the time course of 17-OHPC transport from primary human fetal and adult hepatocytes

Each bar represents the mean of duplicate treatments (±SD). * - $p<0.05$

(A) Efflux of 17-OHPC into the media was observed to increase with time. The efflux of 17-OHPC was significantly ($p<0.05$) inhibited at all time points in cold media.

(B) Accumulation of 17-OHPC was observed to increase significantly ($p<005$) or remain constant with time in cold media. Further, the accumulation was observed to decrease in warm media conditions.

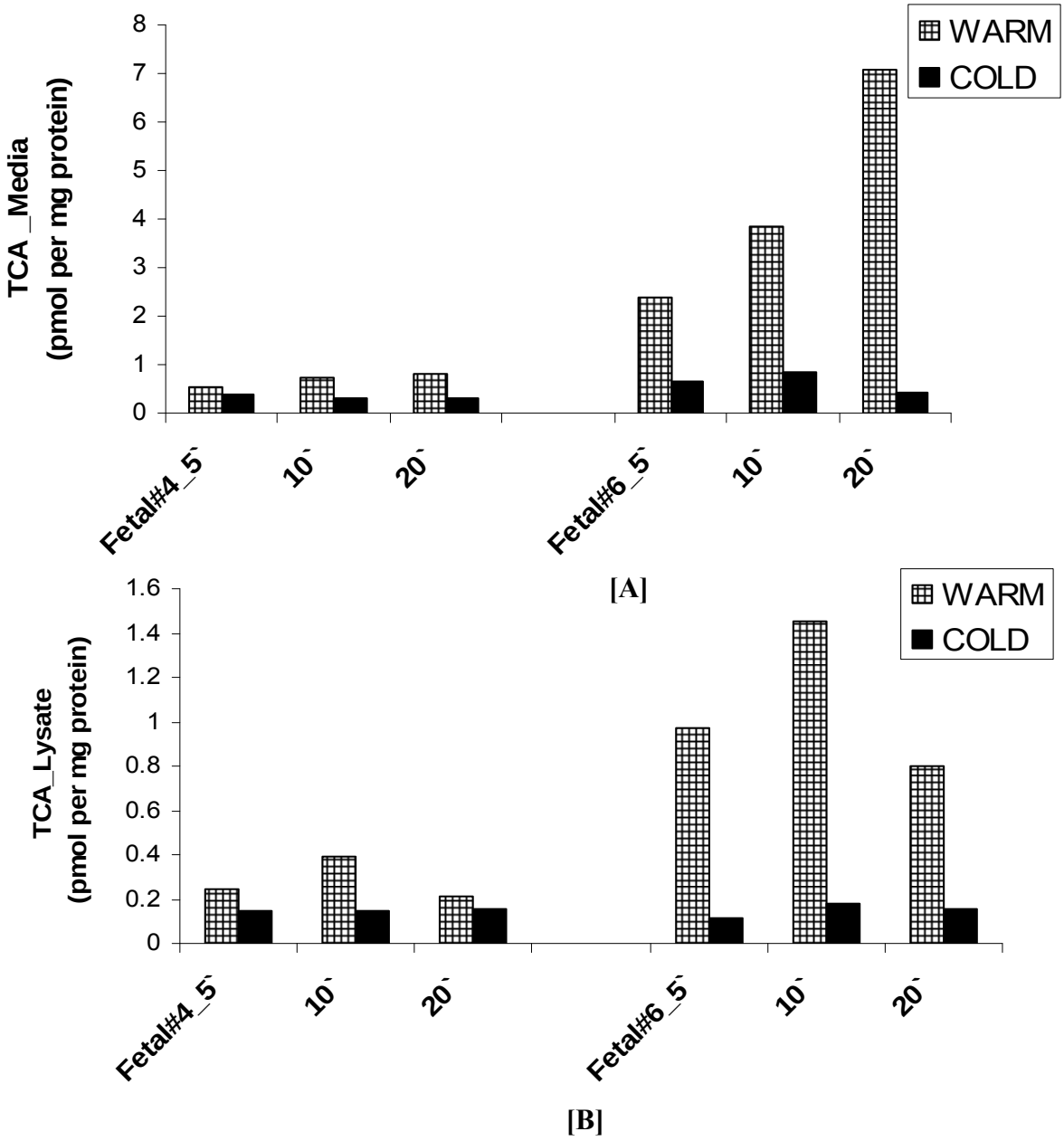


Figure 31. Effect of temperature on the time course of Taurocholate (TCA) transport from primary human fetal hepatocytes

The cells were incubated for different time intervals with TCA in WARM (37ºC) and COLD (4ºC) media during the loading phase, washed with ice-cold media and subsequently reincubated in the warm and cold media. Each bar represents a single treatment (±SD).

(A) Efflux of TCA into the media was observed to increase with time. The efflux of 17-OHPC was inhibited in cold media.

(B) Accumulation of 17-OHPC was also observed to decrease or remain constant with time in cold media as compared to warm media.

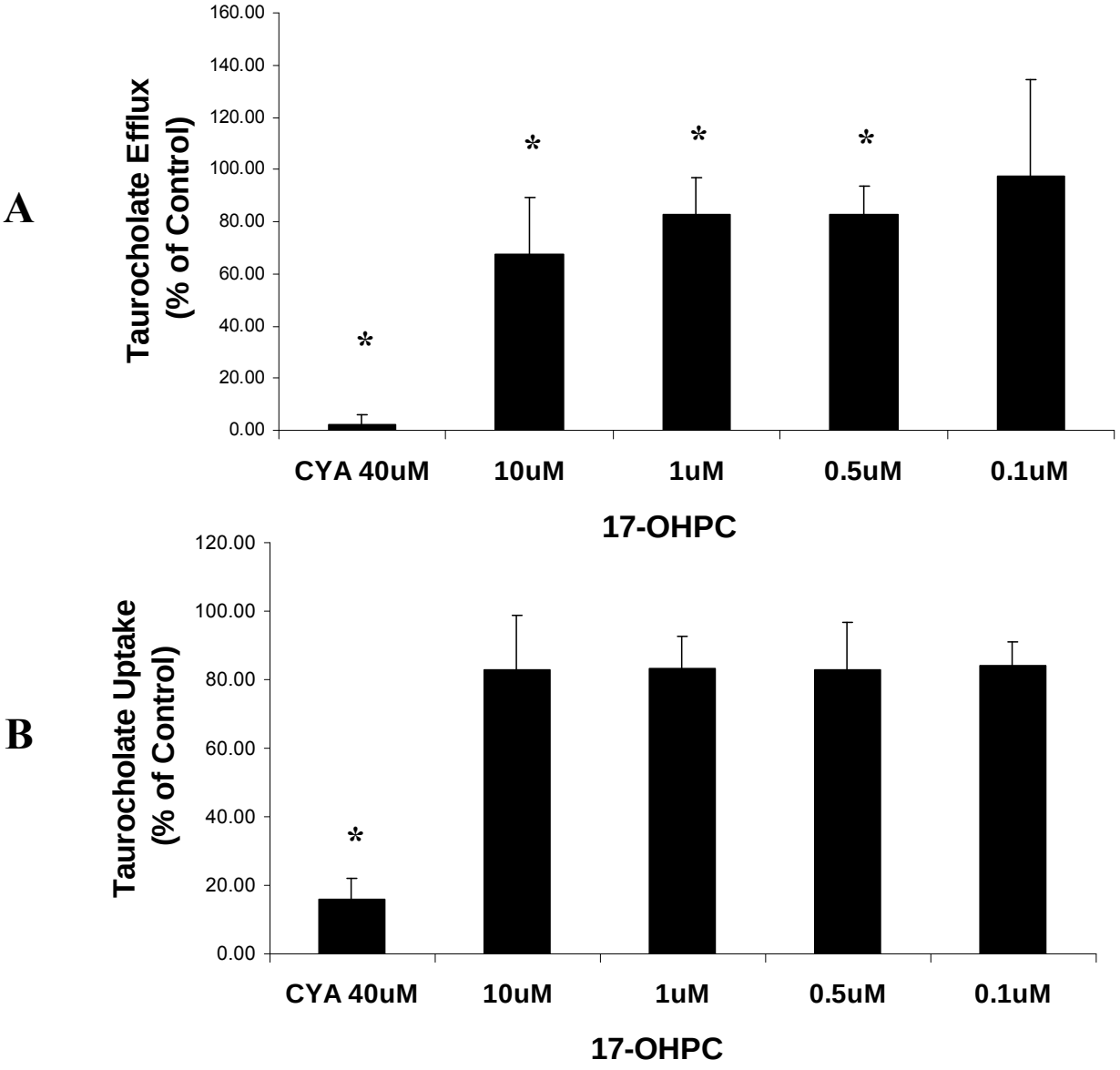


Figure 32. Effect of 17-OHPC on taurocholate disposition in cultured FETAL human hepatocytes (n=8)

Taurocholate efflux or uptake in the absence of 17-OHPC or CYA was defined as control having 100% efflux or uptake. DMSO (0.1%) in buffer was used in the control. Each bar represents treatments in triplicate (±SD). CYA was employed as the positive control. * - $p<0.05$

(A) Significant decrease ($p<0.05$) in taurocholate efflux was observed at 0.5 μM, 1.0 μM and 10.0 μM 17-OHPC concentration and depicted a dose dependent inhibition. CYA significantly ($p<0.05$) decreased taurocholate efflux.

(B) No significant decrease ($p<0.05$) in taurocholate uptake was observed at all 17-OHPC concentrations, 10 μM ($p=0.07$), 1 μM ($p=0.062$) and 0.5 μM ($p=0.053$) due to the large inter-individual variations in the total uptake of 17-OHPC. CYA significantly ($p<0.05$) decreased taurocholate uptake.

In adult hepatocytes, 17-OHPC decreased the efflux of taurocholate in a concentration dependent manner. Significant decrease in the efflux was observed at 100μM, 50μM and 5μM 17-OHPC concentrations (Figure 33A). The total uptake of taurocholate was also inhibited by 17-OHPC with significant inhibition at 100μM concentration (Figure 33B). Similar to fetal hepatocytes, cyclosporine (CYA; 40μM) was used as the positive control and showed a significant ($p<0.05$) inhibition of both taurocholate efflux and uptake.

Biliary excretion index (BEI) represents the percentage of substrate accumulated in the monolayer hepatocyte culture that is localized in bile canaliculi. A large biliary excretion index (as high as taurocholate) indicates extensive excretion of substrate into the canalicular space via transporters (Liu et al., 1999b). The BEI for 17-OHPC in adult and fetal hepatocyte cultures were similar (Figure 34A). The BEI for taurocholate in adult and fetal hepatocyte cultures were significantly ($p<0.05$) different (Figure 34B). The biliary excretion for taurocholate was observed to be ~ 2 fold higher in adult cultures as compared to fetal cells.

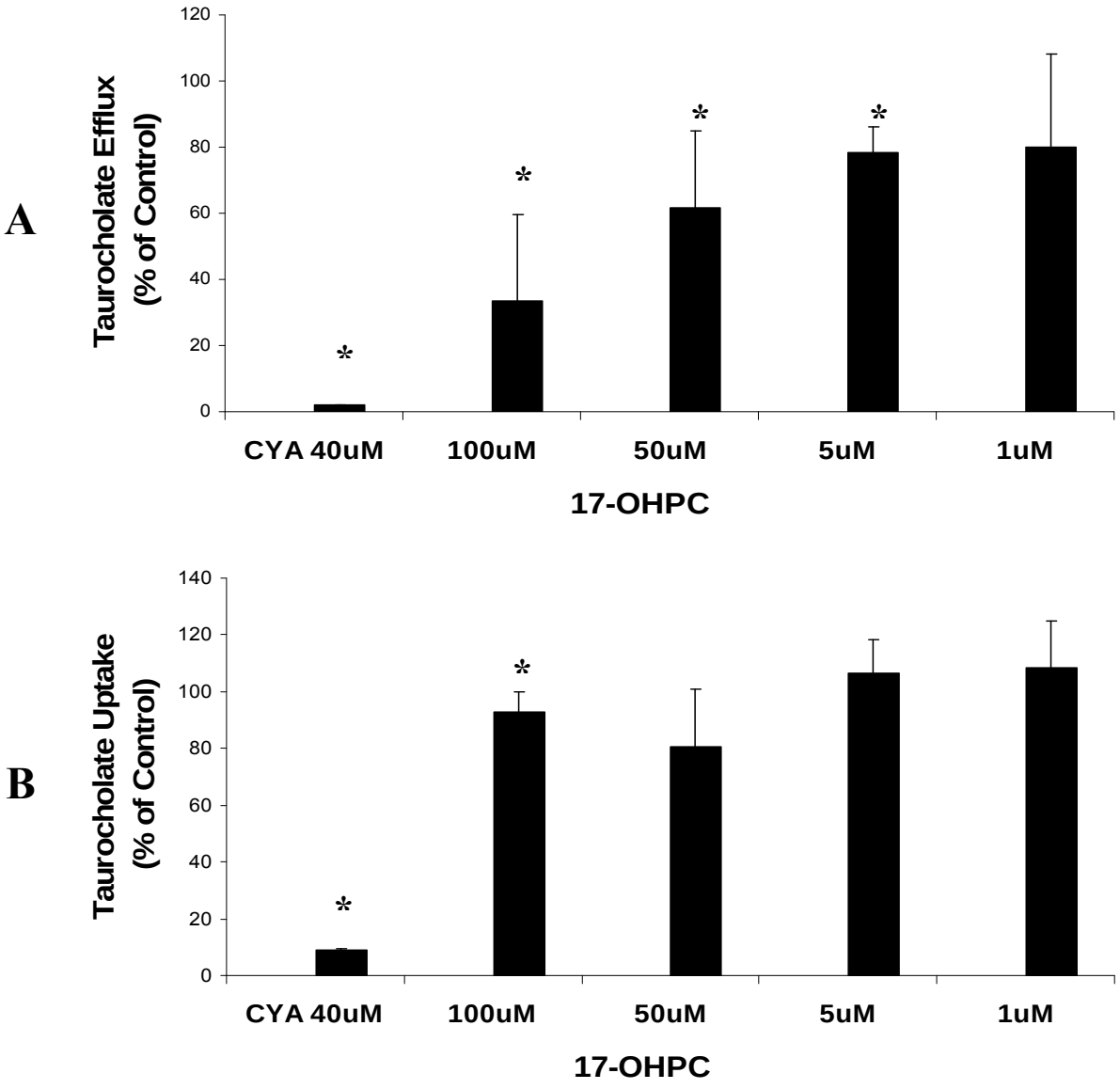


Figure 33. Effect of 17-OHPC on taurocholate disposition in cultured ADULT human hepatocytes (n=5)

Taurocholate efflux or uptake in the absence of 17-OHPC or CYA (0.1%DMSO) was defined as control having 100% efflux or uptake. DMSO (0.1%) in buffer was used in the control. Each bar represents treatments in triplicate (±SD). CYA was employed as the positive control. * - $p<0.05$

(A) Significant decrease ($p<0.05$) in taurocholate efflux was observed at 100 μM, 50.0 μM and 5.0 μM 17-OHPC concentration and depicted a dose dependent inhibition. CYA significantly ($p<0.05$) decreased taurocholate efflux.

(B) Significant decrease ($p<0.05$) in taurocholate uptake was observed only at 100.0 μM 17-OHPC concentration. Significant difference was not observed at 50 μM ($p=0.051$) due to the large inter-individual variations in the total uptake of 17-OHPC. CYA significantly ($p<0.05$) decreased taurocholate uptake.

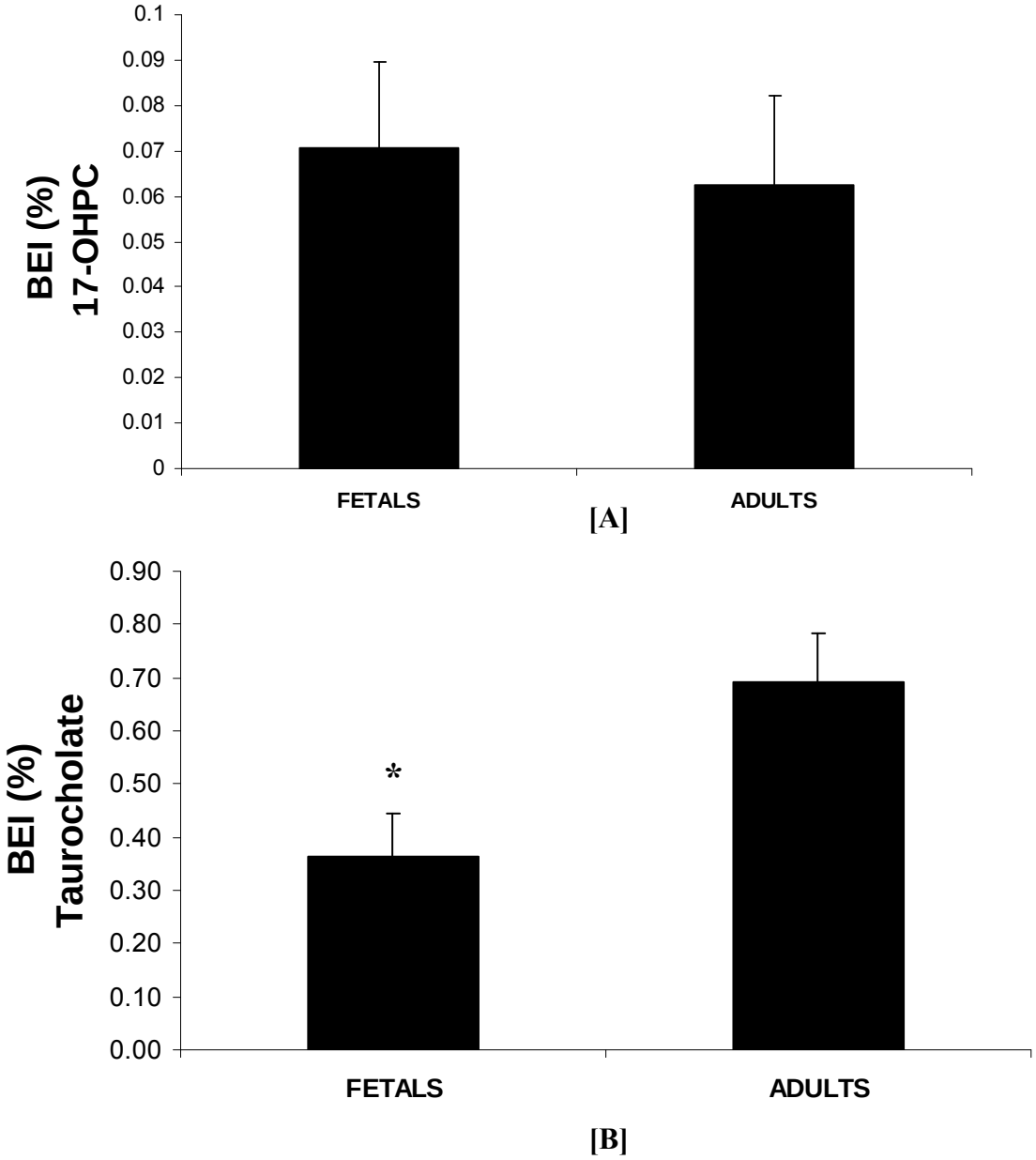


Figure 34. Comparison of the biliary excretion index (BEI) in fetal and adult human hepatocytes cultures

BEI was calculated based on equation 1. Each bar represents treatments in triplicate (±SD). * - $p<0.05$

(A) BEI estimate for 17-OHPC in adult human (n=3) and fetal (n=3) hepatocyte cultures. No significant difference was observed between adults and fetal cells.

(B) BEI estimate for taurocholate in adult human (n=3) and fetal (n=3) hepatocyte cultures. Significant ($p<0.05$) difference was observed between adults and fetal cells.

5.5.6 Expression of transporters in Fetal and Adult hepatocytes

Quantitative-real time RT-PCR based estimates of the mRNA levels of transporters in fetal hepatocyte cultures were measured and compared with results with adult hepatocytes (Figure 35). The relative mRNA levels were normalized to the endogenous reference, cyclophillin. The mean expression for all the transporters was significantly higher ($p<0.05$) in adults than fetals except OATP1B3 ($p=0.1$) and MRP4 ($p=0.09$). In adult hepatocytes, MRP2 showed the highest expression level followed by MDR1 and BSEP. In fetal hepatocytes, a similar profile was observed with MRP2 transporter depicting the highest expression (~30% of adults) followed by MDR1 (~45% of adults) and BSEP (~30% of adults). MDR3 was the only transporter that was not detected on a consistent basis in the fetal hepatocytes. Relative to adults, the transporters with the lowest expression in fetal liver were MDR3 (~2% of adults) and NTCP (~4% of adults).

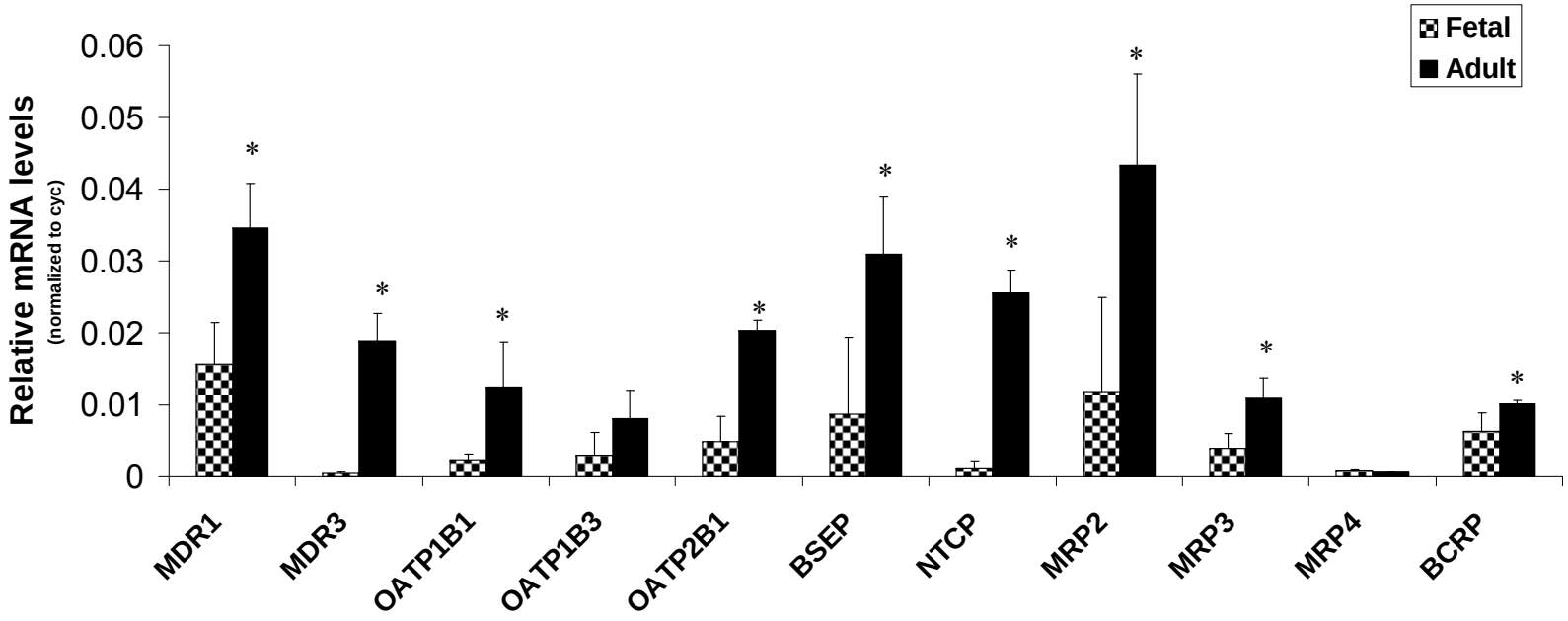


Figure 35. Expression of transporters based on RT-PCR analysis in adult (n=3) and fetal (n=3) hepatocytes
Expression of hepatic transporters in adults was observed to be significantly higher ($p<0.05$) as compared to fetals except for OATP1B3 ($p=0.1$) and MRP4 ($p=0.09$). Each bar represents the mean of triplicate observations (±SD). * - $p<0.05$

5.6 Discussion

The current study was designed to evaluate the expression and functional activity of sinusoidal and canalicular membrane transporters in primary cultures of fetal human hepatocytes. Parallel studies were also performed in adult human primary hepatocyte cultures to provide a fetal vs adult comparison. Treatment with 17-OHPC for preventing pre-term labor is initiated in pregnant subjects at the start of second trimester in the current regimen. Thus, primary cultures of hepatocytes isolated from fetal livers at 21-23 weeks of age were studied. Cholestatic liver disease affects not only the mother but also the fetus and the neonate due to the immaturity of the biliary excretion system. Thus, it is important to investigate the uptake and efflux of drugs and xenobiotics which can gain access to the fetal circulation. The dual-labeled 17-OHPC used in our study was observed to retain both (^{14}C, ^{3}H) labels on being metabolized to mono-, di-, and tri-hydroxy metabolites. These metabolites were observed to be the major metabolites generated on incubations with adult and fetal hepatocyte cultures. Thus, the findings in our study refer to the disposition of 17-OHPC and its metabolites and in its current form the study cannot differentiate between the two products.

Cyclosporin, a known inhibitor, was used to evaluate the role of active transporters in the disposition of 17-OHPC. Significant inhibition of 17-OHPC efflux and an increase in the total uptake of 17-OHPC was observed in both adult and fetal hepatocytes. The results indicate that an active efflux process seems to be involved in the disposition of 17-OHPC. Similar results were also observed with time based incubations. In presence of cyclosporin, the total amount of 17-OHPC accumulated in hepatocytes was observed to increased significantly with time whereas a

consistent decrease in the intracellular drug concentration was seen in the absence of inhibitor, which can be ascribed to an uninhibited efflux process.

Hepatic transport of taurocholate, a known substrate for NTCP (uptake) and BSEP (efflux) was characterized to evaluate the functional activity of these transporters in fetal hepatocytes. Significant inhibition taurocholate efflux was observed in the presence of cyclosporin in both adult and fetal hepatocytes. Also, the total accumulation of taurocholate was significantly inhibited in the presence of cyclosporine in both adult and fetal hepatocytes. This observation was in contrast to what was observed for 17-OHPC. The results indicate that functional uptake and efflux processes are involved in the transport of taurocholate in fetal hepatocytes, whereas with 17-OHPC only an efflux process was observed.

The organic anion-transporting polypeptide (OATP1B1, 2B1, 1B3) transporters are uptake proteins with broad substrate specificities including bile salts, prostaglandins, and steroid conjugates. Recently, it was reported that the sulfate conjugate of pregnenolone, precursor to endogenous hormones including dehydroepiandrosterone (DHEA) and progesterone, is a substrate of OATP2B1. To investigate the involvement of an uptake process mediated by the OATPs in addition to the predominant efflux process in the transport of 17-OHPC, a structural analog of pregnenolone, we used rifampin which is a known inhibitor of OATPs. If OATPs played a role in the disposition of 17-OHPC or its metabolites we expected to observe a decrease in the total intracellular accumulation of 17-OHPC in the presence of rifampin. However, a time dependent increase in the total uptake of 17-OHPC was observed along with a similar decrease in the % efflux which was contrary to the expected results. Previous literature reports (Fardel et al., 1995) have indicated that rifampin has the ability to bind to Pgp-drug binding sites and thus cause an inhibition in the Pgp mediated basolateral efflux of substrates (digoxin, vinblastine).

Thus, the results suggest the involvement of Pgp as the efflux transporter in the disposition of 17-OHPC.

In support of the previous experiments using chemical inhibitors, we evaluated the effect of temperature (4ºC vs. 37ºC) on the transport of 17-OHPC in hepatocytes. A decrease in transport into the media and a time dependent increase in the intracellular amount of 17-OHPC was observed in both adult and fetal hepatocytes at cold temperature (4ºC). In warm temperature (37ºC), a time dependent increase in the efflux of 17-OHPC and a decrease in the intracellular concentration was observed which again can be explained by an uninhibited efflux process. The results suggest that an active efflux process is likely to be the main mechanism involved the transport of 17-OHPC. However, on evaluating the effect of temperature on taurocholate transport in fetal hepatocytes, the results were in contrast to 17-OHPC. At 4ºC, significant inhibition of both total uptake and efflux of taurocholate was observed. Thus, unlike 17-OHPC, the effect of temperature on taurocholate confirms the involvement of functional uptake (NTCP, OATP) and efflux (BSEP) processes involved in the transport of taurocholate.

In addition to evaluating the functional activity of hepatic transporters in fetal hepatocytes, we performed RTPCR based analysis to compare the mRNA expression of various hepatic transporters (MDR1, MDR3, OATP1B1, OATP1B3, OATP2B1, BSEP, NTCP, MRP2, MRP3, MRP4 and BCRP), known to be important for the disposition of xenobiotics and bile salts, in adult liver and isolated hepatocytes. With the exception of MDR3 and NTCP, all transporters examined were found to be expressed in fetal hepatocytes as well as adults.

Hepatic clinical toxicity due to various xenobiotics in humans has been reported to be associated with the inhibition of bile salt transporters, especially NTCP and BSEP. Kostrubsky et

al., using a sandwich culture inhibition model, have reported three factors that are associated with clinical hepatotoxicity:

1) high-molecular weight compounds with expected biliary excretion of parent and metabolites,
2) affinity for biliary transporters as shown by a concentration-dependent inhibition of bile acid transport *in vitro*, and
3) plasma concentration at or above 1 μg/ml.

The maximum plasma concentrations reported for 17-OHPC is 30ng/ml in adults and 10ng/ml in the cord blood. These concentrations are much lower than the 1μg/ml threshold reported for clinically significant hepatotoxicity. However, the abovementioned factors are based on evaluations carried out in adult human hepatocytes and the model had to be validated for fetal human hepatocyte cultures. To the best of our knowledge, this is the first report that tests the applicability of the in-vitro hepatotoxicity model in fetal hepatocyte cultures. In experiments conducted previously, the expression and function of transporters involved in the disposition of taurocholate and 17-OHPC in fetal hepatocytes was confirmed. Subsequently, we decided to evaluate the potential of 17-OHPC to interfere with the transport of taurocholate.

To test the role of biliary excretion pathway in the disposition of 17-OHPC (Mol. Wt. 428.7) or its metabolites, we estimated the biliary excretion index (BEI) for 17-OHPC in both adult and fetal hepatocytes and compared it with taurocholate. Biliary excretion index (BEI) represents the percentage of substrate accumulated in the monolayer hepatocyte culture that is localized in bile canaliculi. A large biliary excretion index (as high as taurocholate) indicates extensive excretion of substrate into the canalicular space via transporters (Liu et al., 1999b). Biliary excretion of 17-OHPC was observed to be similar in fetal and adult hepatocytes although

it was 5-10 times less than the BEI for taurocholate. The BEI for taurocholate was significantly ($p<0.05$) higher in adults than fetal hepatocytes which can be explained by the higher expression level of NTCP and BSEP proteins in adult cells. The results indicate that biliary excretion pathway might play a limited role that in the disposition of 17-OHPC.

To evaluate the affinity of 17-OHPC for the bile salt transporters, we tested the ability of 17-OHPC to inhibit taurocholate transport in a concentration dependent manner. Significant inhibition of taurocholate transport by 17-OHPC was observed at ≥ 0.5 μM in fetal hepatocytes indicating the potential for inhibiting bile transport. The uptake was observed to decrease with increasing concentrations of 17-OHPC (although not statistically significant) thus indicating potential for inhibition of both canalicular uptake and basolateral efflux processes. In adult hepatocytes, similar trend was observed with both efflux and total uptake decreasing with increasing 17-OHPC concentrations. The IC50 calculated for 17-OHPC mediated inhibition of taurocholate efflux in fetal hepatocytes was ~20μM which was ~ 4 fold lower than the IC50 (80μM) observed in adult hepatocytes. This observation highlights the fact that the fetal liver will develop toxicity to xenobiotics more readily than the adult liver, largely due to its lower expression of bile salt and other sinusoidal/ canalicular transporters which play an important role in the detoxification mechanism. Cyclosporine, used as positive control, showed significant inhibition of both the uptake and efflux of taurocholate in adult and fetal hepatocytes thus confirming the functional activity of bile transporters. Although, inhibition of taurocholate transport was observed in fetal and adult hepatocytes, the inhibition is not likely to be clinically significant given the very low 17-OHPC levels observed in human plasma under the current dosing regimen.

In conclusion, the study demonstrates the functional expression of both sinusoidal and canalicular transporters responsible for transport of taurocholate and 17-OHPC in fetal human hepatocytes. Results from a competition assay show the inhibitory potential of 17-OHPC on taurocholate transport. It is likely that the presence of an active efflux process in fetal hepatocytes prevents the accumulation of 17-OHPC or its metabolites in fetal hepatocytes thus preventing any adverse event. The bile transport inhibition assay is a proven indicator of hepatotoxicity with adult hepatocytes and can also be used in fetal hepatocytes to evaluate drugs which traverse the placental barrier and gain access to the fetal circulation after being administered to pregnant subjects.

Clinical Implications:

1) Pgp is likely to be the major efflux transporter involved in the transport of 17-OHPC in adult and fetal liver. Direct competition of 17-OHPC with other Pgp substrates (xenobiotics or endogenous hormones like progesterone) can lead to clinically significant drug-drug interactions, especially, in the fetus.

2) Induction and/or Inhibition of Pgp (and CYP3A7) in the fetal liver can affect the elimination of 17-OHPC and/or its metabolites, thus, leading to their accumulation in the fetal circulation. Accumulation of these xenobiotics alongwith the increased level of endogenous compounds like progesterone metabolites can work in an additive (or synergistic) manner leading to significant inhibition of bile salt transport mediated by BSEP. Thus, the potential of an adverse event like cholestasis in the fetus is very likely under this scenario.

Acknowledgements:

This project was supported in part by the Obstetrical-Fetal Pharmacology Research Units Network (OPRU). This work is supported in part by NICHD grant HD-047905-2 and the Liver Tissue and Cell Distribution System, funded by NIH Contract # N01-DK-7-0004/HHSN26700700004C (SCS).

6.0 Clinical Pharmacology of 17alpha-hydroxyprogesterone caproate in women with twin gestation

[Caritis S, Sharma S, Zhang S, Carpenter M and Venkataramanan R. Pharmacokinetics of 17-hydroxyprogesterone caproate (17-OHPC) in multifetal gestation (2009). Under Review]

6.1 Abbreviations

17-OHPC - 17α-hydroxyprogesterone caproate
PTB – Preterm Birth
17-OHP - 17α-hydroxyprogesterone
P – Progesterone
MPA – Medroxyprogesterone acetate
BMI – Body Mass Index
AUC – Area Under Curve
Cmax – Maximum plasma concentration
Tmax – Time to reach Cmax
Cl – Clearance
T1/2 – Half life

6.2 Abstract

Aim: 17alpha-hydroxyprogesterone caproate (17-OHPC) reduces the rate of preterm birth in women with a prior preterm birth. Despite the widespread clinical use of this agent, no data exist describing the pharmacokinetics of 17-OHPC in pregnancy or the plasma concentrations achieved during therapy for preterm birth prevention. In this study, we evaluated the pharmacokinetics (PK) of 17-OHPC in a group of women with either twin (Trial 1) or triplet (Trial 2) gestation who were receiving 17-OHPC in two separate placebo controlled trials aimed at determining the utility of this agent in reducing preterm birth rates.

Methods: Samples from women with twins (n=71) were studied in the trial. Subjects received weekly intramuscular injections of 250 mg 17-OHPC in 1 ml castor oil from the time of enrollment (16 0/7 weeks – 20 6/7 weeks) until delivery. The subjects were studied twice i.e. two blood samples were drawn during pregnancy, one between 24-28 weeks (epoch1) and one between 32-35 weeks (epoch 2) for measurement of plasma concentration of 17-OHPC. Data analysis was performed by non-linear mixed effects modeling implemented by the Monolix® software.

Results: The average Cmax observed in the pregnant subjects was 18ng/ml. Steady state concentrations were not achieved during epoch 1 since the plasma concentrations at period 2 (32-35 weeks) were higher than at period 1 (24-28 weeks). The apparent half-life of 9.1 days suggested that depot release from the castor oil regulated the apparent terminal half-life of the drug. The pharmacokinetics of 17-OHPC was best described by a one-compartment model with first-order absorption and elimination. BMI and plasma levels of progesterone and hydroxyprogesterone were observed to be significant covariates. Simulations of plasma concentration time profiles showed that by starting the regimen with a loading dose of 1000 mg,

steady state concentrations could be achieved within the first week of therapy as compared to 11-12 weeks with the current schedule. Further, administering a dose of 500 mg once in 2 weeks was observed to achieve steady state concentrations similar to the current regimen although with lower trough concentrations. Race, although not significant in this study, may play a role in the pharmacokinetics of 17-OHPC. African American subjects may require a higher dose as compared to subjects of other ethnicities due to their higher metabolic capacity for 17-OHPC as evident by their higher average clearance values.

Conclusions: This is the first report of plasma concentrations of 17-hydroxyprogesterone caproate in pregnant women. This report defines for the first time the pharmacokinetic behavior of 17-hydroxyprogesterone caproate in twins.

6.3 Introduction

17alpha-hydroxyprogesterone caproate (17-OHPC) has been shown to reduce the rate of PTB in women with a prior history of preterm birth (Meis et al., 2003). The effectiveness of 17-OHPC in preventing PTB has been evaluated in other associated conditions including multifetal gestation (Rouse et al., 2007; Caritis et al., 2009) and cervical cerclage (Rebarber et al., 2008). Although this agent has been widely used clinically, no data describing the pharmacokinetics of 17-OHPC in pregnancy or the plasma concentrations achieved during therapy exists in the literature. Only a single study by Onsrud et al. has reported the drug concentrations achieved during administration of this agent (Onsrud et al., 1985). However, in that study 17-OHPC was indicated for the treatment of endometrial cancer in non-pregnant women. A complete understanding of the pharmacokinetics of 17-OHPC will help in designing an optimal dosing

regimen for this agent. In the current study, our objective was to evaluate the pharmacokinetics (PK) of 17-OHPC in a group of women with twin gestation who were receiving 17-OHPC.

We have previously shown that 17-OHPC is metabolized by human liver microsomes and primary hepatocyte cultures with CYP3A4 being the major enzyme involved. Highly variable expression and function of CYP3A has been observed between individuals. Intersubject variability in CYP3A-mediated activity has been reported to be large (approximately 10-fold difference). Some studies have reported variability of greater than 20-fold (Lin et al., 2001; Zhu et al., 2003; Chen et al., 2006). In earlier clinical studies 17-OHPC was not demonstrated to be effective and only about 33% of patients in the recent trials appear to benefit from 17-OHPC administration. Inter-individual variability in the metabolism is an important factor contributing to the variability in the pharmacokinetics of several drugs. Thus, it is possible that the variability in clinical response to 17-OHPC may be related to the concentration of 17-OHPC. Further, based on in-vitro studies in adult human liver microsomes, hepatocytes cultures (Chapter 1) and suspensions, we have shown 17-OHPC to be an intermediate clearance compound (Hepatic Clearance, CL_H (in-vitro) ~ 4ml/min/kg) with an average half life of ~ 70 mins. However, steroidal intramuscular injections (Medroxyprogesterone acetate and Nandrolone decanoate, vehicle – castor oil), have been reported to have half lives of 50 and 20 days, respectively. This has been attributed to their half life being absorption rate limited. 17-OHPC is administered as an intramuscular injection (vehicle - castor oil) once a week at a dose of 250 mg.

We hypothesize that a large variability due to interindividual variability in CYP3A and long half life due to slow release from the intramuscular depot will be observed for 17-OHPC in pregnant women receiving i.m. injections of 17-OHPC for treatment of preterm birth.

6.4 Methods

6.4.1 Patients and Drug administration

A total of 71 women with twins were studied. Subjects received weekly injections of 250 mg 17-OHPC in 1 ml castor oil from the time of enrollment (16 0/7 weeks – 20 6/7 weeks) until 35 weeks or until delivery. Data recorded for each patient included maternal age, gestational age at each blood sampling and delivery, plasma levels of 17alpha-hydroxyprogesterone (17-OHP), progesterone (P), race and BMI. These data were evaluated as covariates in the pharmacokinetic analysis.

6.4.2 Pharmacokinetic sampling schedule

Sparse sampling - Two blood samples were drawn during pregnancy from each subject, one between 24-28 weeks **(EPOCH1)** and one between 32-35 weeks **(EPOCH 2)** for measurement of plasma concentration of 17-OHPC.

Intensive sampling - For the study of 17-OHPC pharmacokinetics (PK), six women were recruited to have a single blood sample removed daily for seven consecutive days between 24-28 weeks. The first blood sample was drawn minutes prior to the next scheduled injection. These six subjects had already received a minimum of four weekly injections from the time of enrollment in anticipation that steady state concentration would be achieved by the start of the PK study.

6.4.3 Sample Analysis

For all 17-OHPC measurements, blood was collected in 10 ml tubes with heparin and centrifuged within one hour at 3500 x g for 10 minutes. The supernatant plasma was aliquoted to 1 ml tubes and was frozen at -70ºC until analysis. Analyses of 17-OHPC was performed using high performance liquid chromatography with tandem mass spectrometry (LC-MS). The assay methodology, developed in our lab, has been reported elsewhere (Zhang et al., 2007). At the time of the analysis, the analyst and the clinical centers involved in recruitment were blinded to the treatment assigned. The lower assay limit of detection was 1 ng/ml, inter and intra assay variability at 10 ng/ml was 7.9 and 5.2% respectively.

6.4.4 Noncompartmental Pharmacokinetic Analysis

Pharmacokinetic parameters for the 6 subjects undergoing ‘’intensive sampling” were estimated using the standard noncompartmental approach implemented using WinNonlin (ver 4.0). Maximum concentration (Cmax) and time to maximum concentration (Tmax) were determined from the observed data. The elimination rate constant (λz) was determined by log-linear regression of terminal linear disposition phase. Half-life (T1/2) was estimated by 0.693/ λz. Area under curve $(AUC)_0^T$ was calculated using the linear trapezoidal method. Since, the trough concentrations between the 2 consecutive doses during the pharmacokinetic study were not significantly different; the area was calculated for one dosing interval. Apparent clearance (CL/F) was estimated by Dose/$(AUC)_0^T$.

6.4.5 Compartmental Pharmacokinetic Analysis

For sparse sampling data, analysis was performed by means of non-linear mixed effects modeling implemented by the Monolix® software. MONOLIX provides an estimate of the parameters (fixed effects and variance of the random effects) as well as an estimate of the estimation error via the Fisher information matrix.

Preliminary analysis for evaluating the structural model was performed by comparing one-compartment model with first-order absorption and elimination with two and three compartment models. Further, first order absorption under the one compartment model was also compared to mixed order absorption. A proportional variance model was used for the residual error. The parameters that were evaluated by the model included ka, Cl/F and V/F. Interindividual variability was assumed to have a log-normal distribution. The covariance between clearance and volume of distribution was examined by testing a full covariance matrix.

The patient characteristics (BMI and race) were evaluated as covariates during the model building process as described by Lavielle and Mentre (Lavielle and Mentre, 2007). Briefly, the effect of each covariate was tested on each individual parameter and evaluated for significance using both a Wald test and a LRT test. All population models with all combinations of covariates found significant in the first analysis were fitted and the best ones were subsequently chosen according to the Bayesian Information Criteria (BIC). Finally, each covariate remaining in the model was tested for significance using both a Wald test and a LRT. Further, the change in inter-patient and residual variability in the presence and absence of this covariate was also taken into account before deciding on the final model.

The goodness of fit of the final model was evaluated by inspecting the following charts including, scatterplots of predictions (population and individual) versus individual observations;

population weighted residuals versus predictions and independent variable (time); absolute individual weighted residuals versus individual predictions. In addition, model validation was performed using prediction distribution errors and visual predictive check, obtained by performing 1000 simulations of the data set for the final model.

Further, simulations were carried out based on the final model to evaluate the effect of changes in BMI and dosing regimen on the plasma concentration time profiles of 17-OHPC. To evaluate the effect of BMI on plasma concentration time profiles we selected 3 BMI values (highest, average and lowest) based on the range observed in our sample set which were 45, 27 and 18, respectively. Possible modifications in dosing regimen (for achieving the same therapeutic effect) were evaluated, including:

a) the effect of increasing the dose from 250 mg once weekly to 500mg or 750 mg once weekly,
b) the effect of adding a loading dose of 1000 mg to the regimen in order to rapidly achieve the intended steady state concentrations, and,
c) the effect of changing the dosing schedule from 250 mg once weekly to 500 mg every two weeks.

6.4.6 Statistical Analysis:

GraphPad Prism (4.01) was used for performing the statistical tests for significance. Non-parametric (Wilcoxon signed rank and Mann Whitney U) tests were used for group comparisons. Kruskal-Wallis test with Dunn's multiple comparison was used for testing equality of population medians in multiple groups. We considered p-values < 0.05 to be significant. All results are reported as (±) standard deviation (s.d.).

6.5 Results

6.5.1 Pharmacokinetics

Figure 36 depicts the mean plasma concentration (±SD) of 17-OHPC in the six women with twins who had blood removed daily for seven days after an injection (intensive sampling). Each woman had received at least 4 injections at a weekly interval from the time of randomization prior to performance of the PK study. Average peak concentrations of 18 ng/ml were noted at the first post injection sample at 24 hours. Over the ensuing 6 days, plasma concentrations declined slowly to a nadir of 9 ng/ml at 7 days post injection. Selected pharmacokinetic parameters (mean, SD and range) for 17-OHPC in these 6 subjects are summarized in Table 11. The peak concentration (Tmax) occurred 1.2 days after the injection. The wide range of AUC values indicates considerable interindividual variation in release and metabolism of 17-OHPC. The apparent half-life of 9.1 days suggests that depot release from the castor oil regulates the apparent half-life of the drug. Clearance (apparent) varied seven fold with a mean of 1924 l/day.

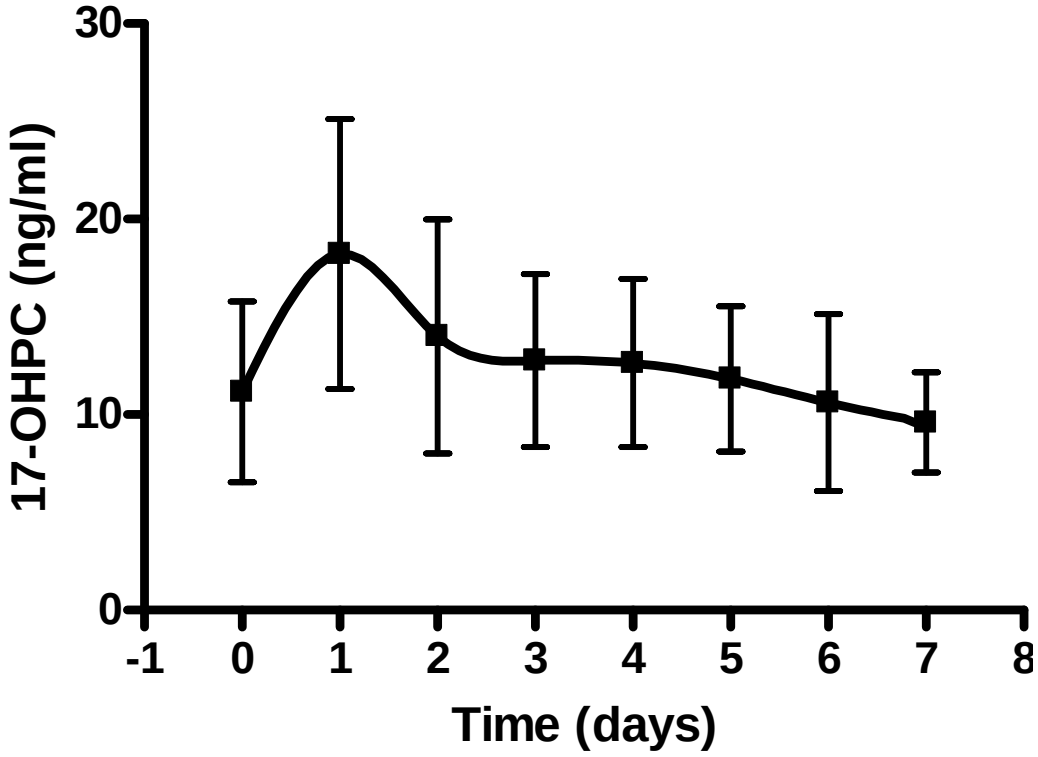


Figure 36. Average 17-OHPC concentration–time profiles for 6 subjects with twins who had sampling done daily for seven days after an injection

Error bars represent (±) standard deviations.

Table 11. Mean pharmacokinetic parameters (± sd) of 17-OHPC in pregnant subjects with twins

These subjects had sampling done daily for seven days after an injection. Non-compartmental analysis was used for the estimation of the parameters. The observed range for each parameter calculated is reported below in parenthesis.

AUC ng/ml/day (range)	**t ½ (days) (range)**	**CMAX ng/ml (range)**	**TMAX (Days) (range)**	**Cl/F (l/day) (range)**
67.2 ± 25.3 (35-105)	9.1 ± 4.2 (5-34)	13.3 ± 6.2 (9-24)	1.2 ± 0.45 (1-2)	1924 ± 1000 (534-3500)

6.5.2 Impact of BMI and Race on 17-OHPC concentrations

We evaluated the impact of BMI and race on plasma 17-OHPC concentrations in women with twins. Figure 37 depicts the relationship between plasma 17-OHPC concentrations and body mass index (BMI - recorded at the time of registration for prenatal care) during epoch 1 and 2 in women with twins. A significant linear relationship was observed for plasma 17-OHPC and BMI in the pregnant women. The race of the subjects was not observed to affect 17-OHPC levels significantly (Figure 38) although the average concentrations in African-Americans (AA) were observed to be lower than Caucasians (CA) during epoch 2 ($p=0.052$).

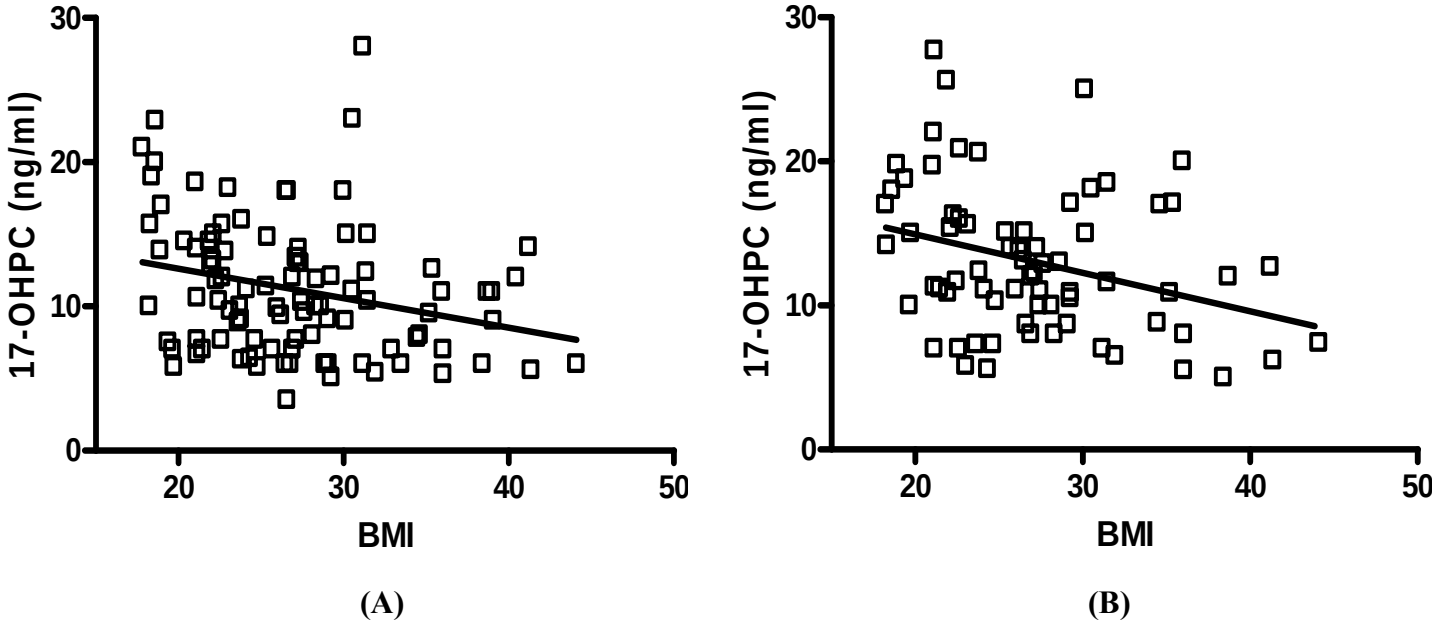


Figure 37. Relationship between the plasma levels of 17-OHPC and BMI
(A): Epoch 1 - A significant correlation was observed with $p<0.01$, $R^2 = 0.08$ for epoch 1
(B): Epoch 2 - A significant correlation was observed with $p<0.01$, $R^2 = 0.10$ for epoch 2

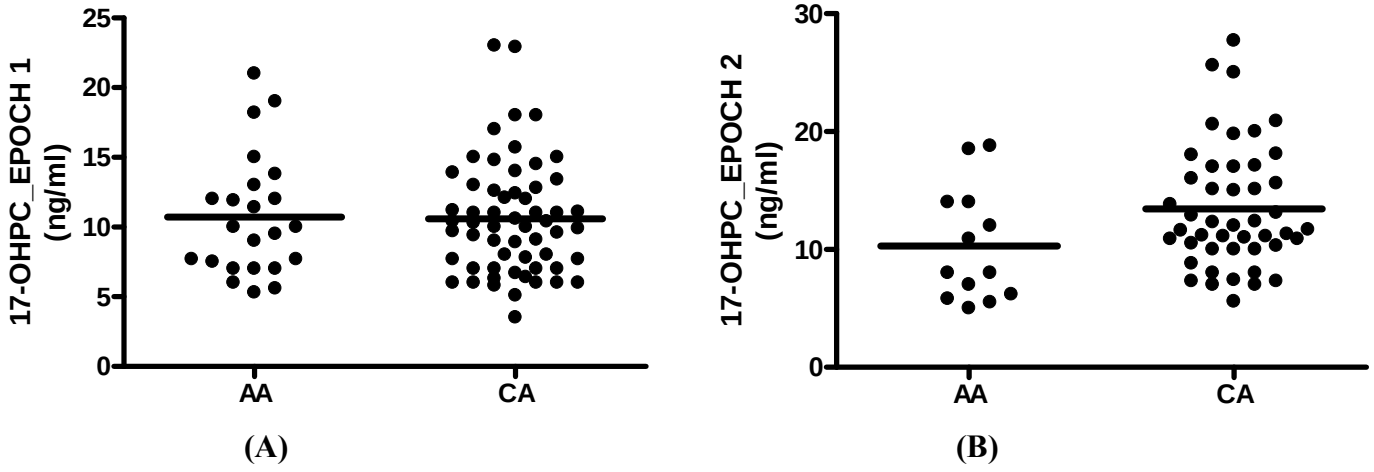


Figure 38. Relationship between the plasma levels of 17-OHPC and Race
AA – African-Americans, CA - Caucasians
(A): Epoch 1 – No significant difference was observed between AA and CA for epoch 1
(B): Epoch 2 – The plasma levels of 17-OHPC were higher in CA than AA although the difference was not significant ($p=0.052$). Mann Whitney U test was used for the comparisons.

6.5.3 Steady State Concentration

We also evaluated whether steady state concentrations of 17-OHPC were achieved over the course of therapy. For this analysis only women who received all their scheduled injections and remained undelivered through period 1 (24-28 weeks) and period 2 (32-35 weeks) were included. In 71 pregnant women with twins that were studied, the plasma concentrations of 17-OHPC were significantly higher ($p<0.05$) in period 2 than in period 1 (Figure 39). The mean time from first injection to first blood draw was 42.9 (sd 12.6) days and the mean time between first and second blood draw was 46 (sd 8.2) days. Clearly, since plasma concentrations at period 2 (32-35 weeks) were higher than at period 1 (24-28 weeks) steady state concentrations were not achieved.

6.5.4 Population Pharmacokinetics (POP-PK)

A total of 71 patients, who had at least two samples drawn during the study, were included in the POP-PK model building process. The population (POP-PK) consisted of only subjects with twin gestation; four racial groups were in the cohort: Caucasians (46), African-Americans (15), Hispanics (9) and Asians (2). A total of 190 observational data points were collected and utilized for the population analysis.

The pharmacokinetic behavior of 17-OHPC was best described by a one-compartment model with first-order absorption and elimination. The scatter plot of the observed 17-OHPC concentrations vs model predicted individual concentrations is shown in Figure 40. Visual predictive check generated from Monte Carlo simulations (n = 1000) showed that the estimated

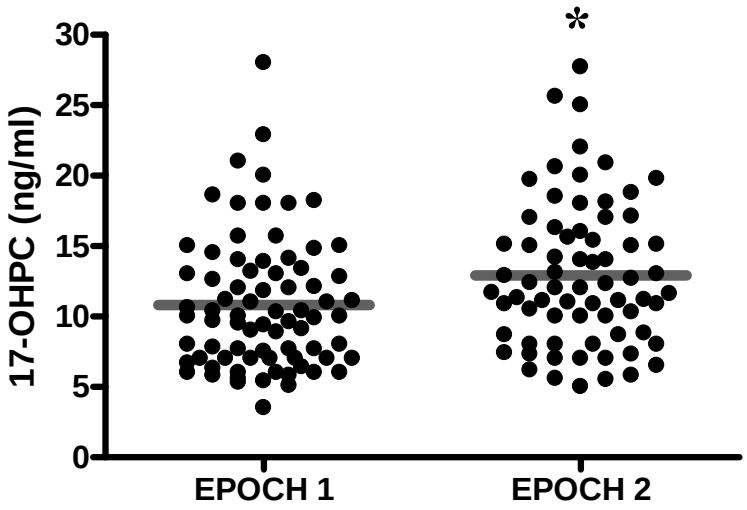


Figure 39. Mean (±sd) 17-OHPC concentrations in subjects with twins (n=71)

Mean 17-OHPC concentration in subjects with twins in epoch 2 (32-35 weeks) was observed to be significantly ($p<0.05$, *) higher than epoch 1 (24-28 weeks) suggesting that the steady state concentrations were not achieved.
Mann Whitney U test was used for the comparisons.

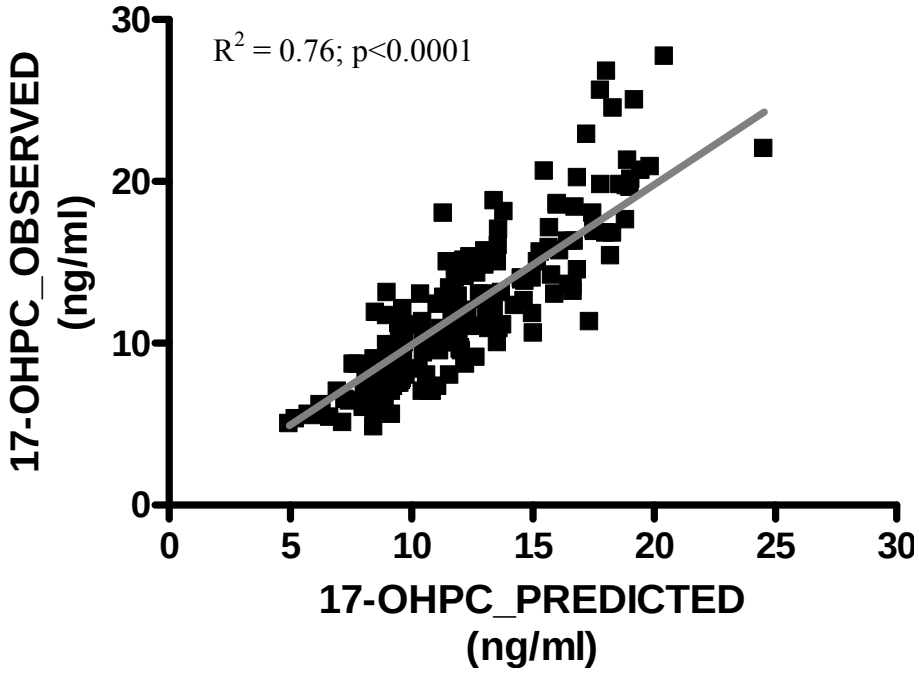


Figure 40. Scatter plot of the individual predicted vs observed concentrations of 17-OHPC**.**
The correlation coefficient was 0.87.
The figure shows the individual data points for the entire population and the line of identity.

population PK model had adequate predictive performance. The estimates of the pharmacokinetic parameters and their respective standard errors are shown in Table 12. The inter-individual variability (IIV) was observed to be 20% for clearance and 51% for volume of distribution estimation, indicating wide inter-subject variability in the pharmacokinetics of 17-OHPC.

Correlation between pharmacokinetic estimates of 17-OHPC and patients' demographic variables was examined. Of the various covariates tested in building the final model, BMI and

Table 12. Population pharmacokinetic parameter estimates obtained from the final model

Ka: absorption rate constant, V/F: volume of distribution/ bioavailability, CL/F: clearance/bioavailability, IIV: Interindividual variability in estimated pharmacokinetic parameters, SE, standard error of the estimate, r.s.e.: relative SE (%).

Parameter	Mean	SE (r.s.e. %)
Ka (day^{-1})	**0.41**	**0.29 (72)**
V/F ($x10^3$ ltr)	**58.9**	**7.9 (13)**
Cl/F ($x10^3$ ltr/d)	**2.09**	**0.47 (23)**
IIV_ka	**1.04**	**1 (97)**
IIV_V	**0.51**	**0.13 (26)**
IIV_Cl	**0.20**	**0.04 (20)**

plasma levels of progesterone and 17-hydroxyprogesterone were observed to have a significant effect on the estimation of 17-OHPC clearance as indicated by the Wald test, LRT test and BIC value.

Although race was not observed to be a significant covariate in the final model, however, individual estimate of plasma clearance in African Americans was significantly higher than in Caucasians ($p<0.05$) (Fig.41).

Simulations were carried out to explore the effect of changes in BMI and dosing regimen on the plasma concentration time profiles of 17-OHPC (Figure 42). The plasma concentration of 17-OHPC varied significantly with BMI (Figure 42A) showing a 2.5-fold concentration difference over a BMI range of 18-45. The relationship between BMI and 17-OHPC concentration was linear. When we evaluated the impact of dose on plasma 17-OHPC concentrations without considering BMI, plasma concentrations were linearly related to dose. A 500 mg dose of 17-OHPC increased plasma concentrations two-fold over the 250 mg dose and a 750 mg weekly dose increased 17-OHPC concentration three-fold (Figure 42B).

The effect of a loading dose on time needed to reach steady state concentrations was also evaluated. A loading dose of 1000 mg reached steady state and maintained it from the beginning (Figure 42C). The final steady state concentrations achieved by a loading dose of 1000 mg were comparable to those observed without a loading dose. We also evaluated the effect of changing the dosing schedule on the concentration time profile. Administering a dose of 500 mg once every 2 weeks achieved steady state concentrations similar to those with a 250 mg weekly injection although peaks were ≈ 15% higher and trough concentrations were ≈ 15% lower with the 500 mg dose (Figure 42D).

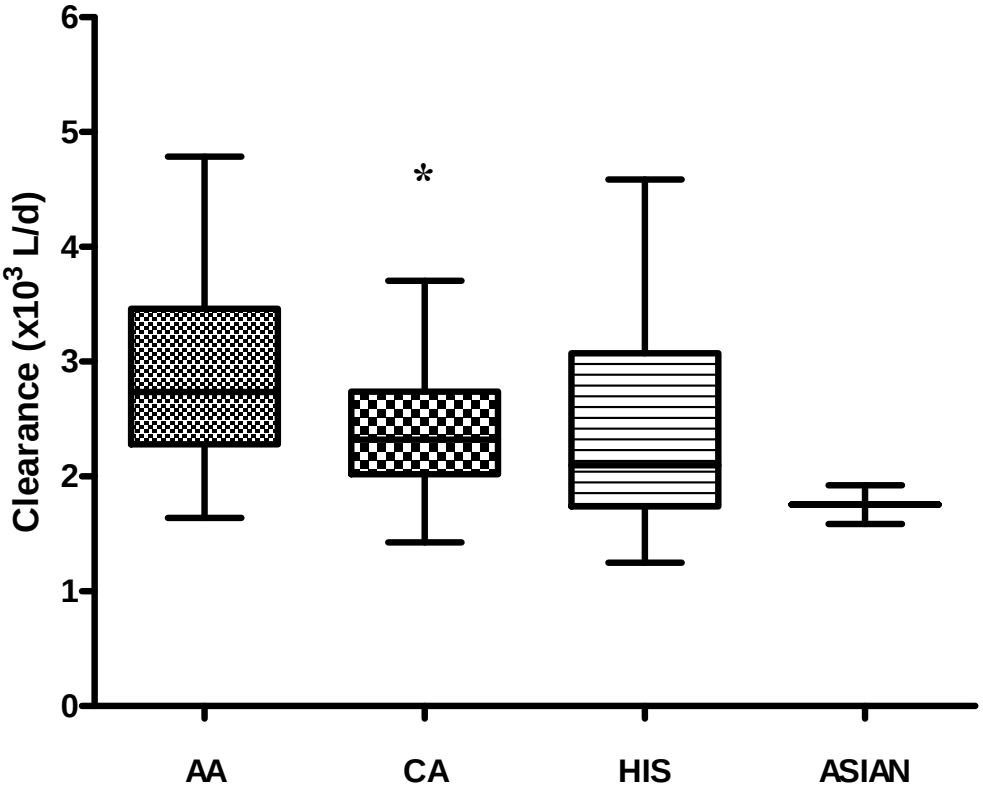


Figure 41. A boxplot depicting the median 17-OHPC clearance (individual) estimates associated with different ethnicities.
The bars represent (±) standard deviation.
AA – African Americans (n= 15), CA – Caucasians (n=46), HIS – Hispanics (n=9) and Asians (n=2).
The mean clearance in the Caucasians was observed to be significantly (p=0.025, *) less than African Americans. No other group was found to differ significantly. The individual estimates were obtained with the final model which included the covariates – BMI, 17-OHP and Progesterone.
Kruskal-Wallis one-way analysis of variance with Dunn's post test was used for the comparison.

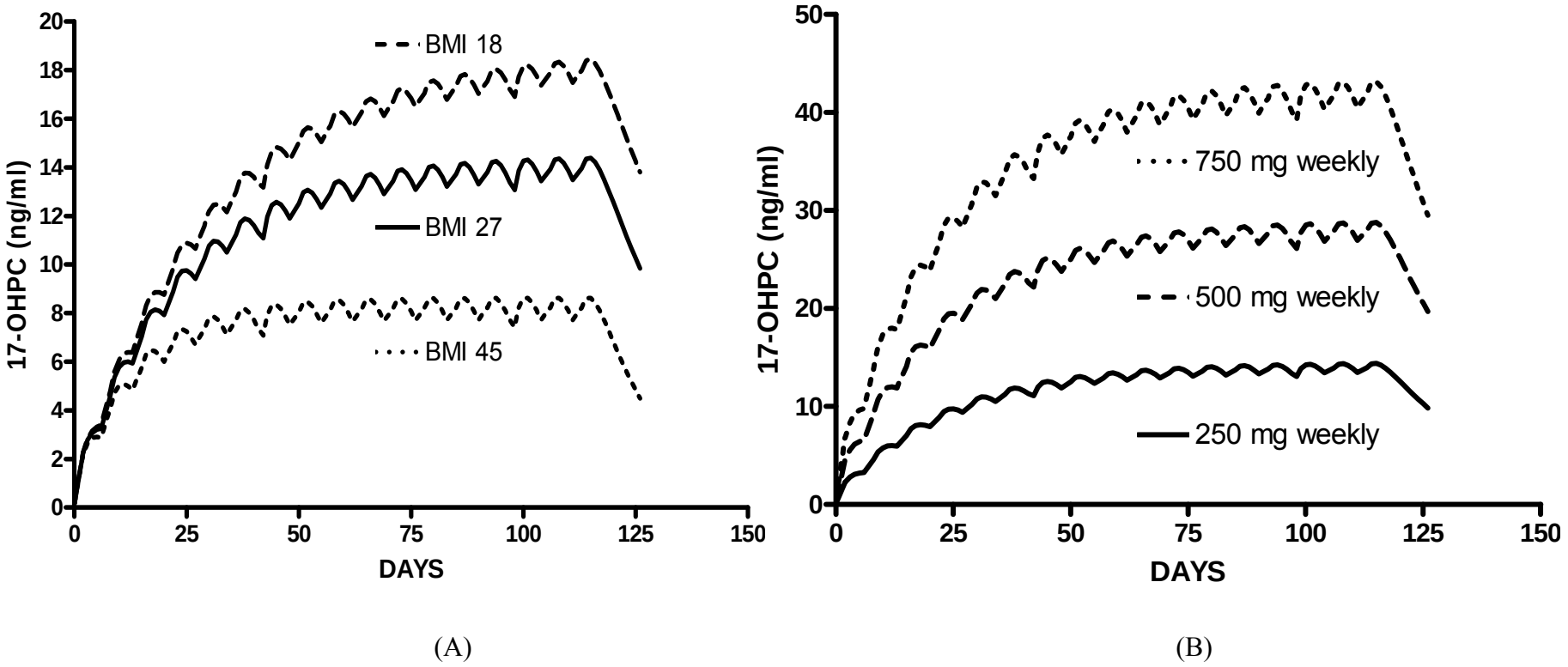


Figure 42. Characteristics of simulated plasma concentration time profiles of 17-OHPC in pregnant subjects

The bold line is the current standard therapeutic regimen involving a 250 mg once weekly i.m. depot of 17-OHPC.

(A) effect of BMI (18, 45 and 27) on plasma concentration time profiles,
(B) effect of increasing dose from 250 mg once weekly to 500mg or 750 mg once weekly,

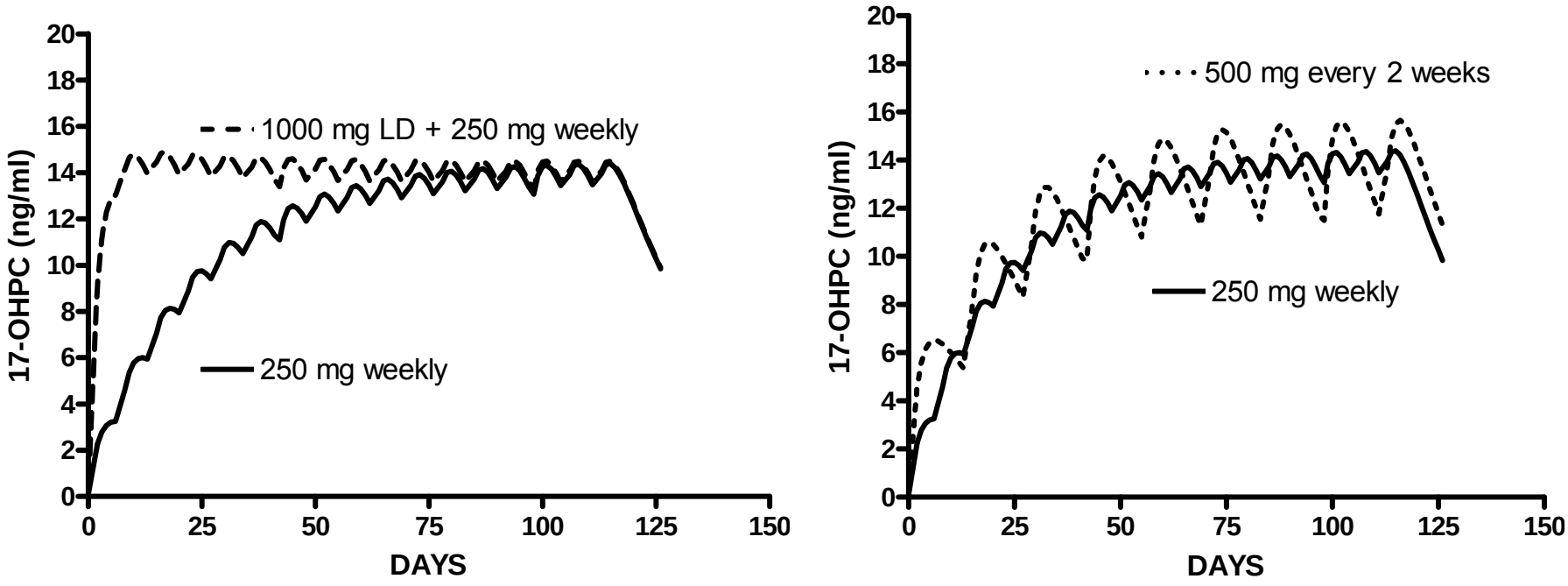


Figure 42 contd. Characteristics of simulated plasma concentration time profiles of 17-OHPC in pregnant subjects

The bold line is the current standard therapeutic regimen involving a 250 mg once weekly i.m. depot of 17-OHPC.
(C) effect of adding a loading dose of 1000 mg to the regimen, and,
(D) effect of changing the dosing schedule from 250 mg once weekly to 500 mg once in 2 weeks.

6.6 Discussion

Here we report the results of a multiple dose pharmacokinetic study of 17-OHPC conducted in pregnant women with twin gestation. The average peak plasma concentration of 17-OHPC observed in the six subjects who were sampled intensively for seven days was 18 ng/ml (%CV~40). The interindividual variability in the plasma concentrations was also reflected in the area under the concentration-time curve (AUC) measurement (%CV~40). In the sparsely sampled subjects, the trough concentration of 17-OHPC were observed to range from 3 ng/ml to 28 ng/ml. Furthermore, wide interindividual variability (Epoch 1 - 8 fold, Epoch 2 – 6 fold) was also observed in the trough concentrations of 17-OHPC measured in 71 pregnant subject who were sparsely sampled.

We have previously reported on the role of CYP3A4 as the major enzyme involved in the metabolism of 17-OHPC (Sharma et al., 2008). Thus, given the widely reported interindividual variability (10-20 fold) observed with CYP3A4 substrates (e.g. Midazolam), we can explain the variability in plasma levels of 17-OHPC by concluding that the pharmacokinetics of 17-OHPC in humans is affected by its hepatic clearance, mediated largely by CYP3A4.

The apparent half life of 17-OHPC, estimated by non-compartmental analysis of the intensively sampled data set, was observed to be 9.1 days. The half life of a compound in the human body is determined by its total body clearance (and volume of distribution) which is basically the sum of hepatic (primarily metabolism) and extra-hepatic (primarily renal) elimination pathways. The pharmacokinetics of 17-OHPC using radiolabeled drug has been reported in rats (Kimbel et al., 1958; Wiener et al., 1961). The study observed that 85% of the

drug (or its metabolite) was eliminated within the first 8 days of injection of 17-OHPC (100mg/kg) via the subcutaneous route. Almost 85% of the excreted drug (or its metabolite) was found in the feces and the remaining in the urine. Further, during the first 2 days of the study, most of the radioactivity (due to the drug or its metabolite) was observed in the liver and kidney. However, analysis of urine samples obtained from pregnant human subjects did not show detectable 17-OHPC levels. Thus, the role of renal elimination pathway in the overall clearance of 17-OHPC in humans is considered to be limited in comparison to the hepatic pathway.

In-vitro estimation of the clearance and half life of 17-OHPC in primary human hepatocyte cultures depicted 17-OHPC as an intermediate clearance compound with a half life of ~ 70 minutes. However, the in-vivo estimate of half life (9 days) suggests otherwise. This observation can be explained using the concept of flip-flop kinetics. In the case of “normal” kinetics (e.g. intravenous injection), the rate of elimination is much smaller than the rate of absorption i.e. Ke <<< Ka. Thus, the half life of a compound in the body depends on the rate at which it is eliminated. However, with certain routes of administration or formulations (intramuscular depots, slow release tablets) the elimination a drug is much faster than the rate at which it is absorbed or Ka <<< Ke. In such cases, the decline of the terminal slope depends on how fast the absorption is taking place. Thus, the terminal slope reflects Ka rather than Ke and is termed as "flip-flop" kinetics. We believe that a similar mechanism is involved in the pharmacokinetics of 17-OHPC which is administered as an intramuscular depot injection constituted in castor oil. Based on the plasma concentration-time profile depicted in figure 36, it is likely that two different mechanisms, simultaneous zero and first order or two parallel first order processes, maybe involved in the absorption of 17-OHPC from the i.m. depot. Between day 0 (time of injection) and day 1, zero order or fast first order absorption will be the primary

mechanism (characterized by rapid absorption i.e. Ka>>Ke) followed by the slower first order release between day 1 and the next dose (characterized by slow absorption or Ka <<< Ke). However, due to insufficient sampling time points between day 0 and 1, these absorption models were found to be statistically insignificant in a non-linear mixed effects model and a simpler first order absorption model was chosen.

Based on the abovementioned discussion we conclude that despite hepatic metabolism being the primary mechanism involved in the overall clearance of 17-OHPC, the apparent half life observed in-vivo is also regulated by the release rate of the drug from the intramuscular depot. However, to confirm that flip-flop kinetics is indeed responsible for the prolonged half life, a single dose pharmacokinetic study comparing the terminal slope of 17-OHPC after intravenous and intramuscular administration needs to conducted. It is important to note that since absorption of 17-OHPC from the depot is slower than its elimination by hepatic pathway, especially in the terminal phase of the sampling, the release from the depot will affect the plasma levels and possibly contribute to the observed inter-individual variability. However, as noted previously, a pilot study involving administration of a single dose of 17-OHPC needs to be conducted to accurately determine the pharmacokinetics (Cmax, Tmax, Half-life) of 17-OHPC.

Estimation of clearance in intensively and sparsely sampled subjects depicted a value of ~ 2000 l/d. This estimated value is uncharacteristically high for 17-OHPC which has a long half life of 9 days. The primary reason for this observation is the fact that despite being administered a high dose of 250 mg, a peak plasma level of only 18ng/ml was observed in the clinical study. The reasons behind this discrepancy can be: 1) the first sample from the pregnant subjects was taken one day after administration of the dose. However, it is likely that a Cmax, much higher than 18ng/ml, was achieved between day 0 and day 1. Thus, the lack of a true estimate of Cmax

may explain the high estimated clearance value. 2) It is also likely that the stability of 17-OHPC in the muscle tissue at body temperature is affected. Degradation of 17-OHPC in the depot will lead to very low bioavailability with less amounts of the drug being released into the circulation and can explain the low plasma levels observed.

In the women who were sparsely sampled, steady state concentrations were not achieved as indicated by the significant difference in the average plasma concentrations of 17-OHPC (Epoch 1 – 10.8 ng/ml vs Epoch 2 – 13 ng/ml). Although steady state concentrations are usually achieved in 4-5 half lives, however, this was not observed with 17-OHPC. This is likely due to the continuous, slow release of the drug from the castor oil depots that were augmented with each successive injection.

Body mass index (BMI) was observed to have a significant impact on the plasma levels of 17-OPHC. A decrease in the plasma levels was observed with increasing BMI which can be explained by the associated change in volume of distribution.

Although race was not a significant covariate in the final model, however, the estimated individual 17-OHPC clearance was observed to be higher in African Americans than Caucasians. Based on in-vitro estimations in expressed enzyme systems, the intrinsic clearance (Vmax/Km) of 17-OHPC in CYP3A5 was observed to be ~10 fold of CYP3A4. CYP3A5 protein has been reported to represent more than 50% of the total CYP3A content in ~33% of Caucasian livers and > 50% of African American livers (Kuehl et al., 2001). Thus, CYP3A5 represents a significant proportion of total CYP3A content and is more frequently expressed in African Americans. Thus, CYP3A5 can contribute significantly to CYP3A content and catalytic activity in African Americans as compared to Caucasians and explain not only the interracial differences

in CYP3A-dependent 17-OHPC clearance (based on the higher metabolic capacity of CYP3A5 than 3A4) but also the interindividual variability observed.

Simulations were conducted to evaluate the effect of different doses of 17-OHPC on plasma concentrations. The results showed a dose dependent increase in the plasma 17-OHPC levels thus providing an estimate for doses required for achieving a certain target concentration. However, caution needs to be exercised in case of dose escalation as the resulting increase in the fetal 17-OHPC concentrations can lead to hepatotoxicity, primarily due to inhibition of BSEP as demonstrated in Chapter 3.

Simulations with different dosing regimens showed that by starting the regimen with a loading dose of 1000 mg followed by maintenance doses of 250 mg once a week, steady state concentrations could be achieved within the first week of therapy. This could help achieve the intended "therapeutic" concentrations more rapidly and possibly improve the clinical outcome. Further, a dosing schedule of 500 mg injection administered once every two weeks could also be utilized to achieve plasma concentrations comparable with the currently utilized regimen of 250 mg weekly. This could improve patient acceptability and reduce the number of visits to healthcare providers. However, this schedule needs to be evaluated carefully since the peak/ trough fluctuations were greater in this case as compared to the standard regimen.

17alpha-hydroxyprogesterone caproate is widely used to reduce the rate of preterm birth. Despite the widespread use of this agent, very little pharmacologic information exists as to its mechanism of action and the proper dosing regimen. The optimization of 17-OHPC dosing (from the current fixed dose of 250mg) is severely constrained because the target organ of 17-OHPC in reducing preterm birth rates is not known. However, the pharmacokinetic study

reported here and the subsequent simulation based dosing recommendations represent the first step in understanding and optimizing 17-OHPC therapy for preventing preterm birth.

Clinical Implications:

1) Variability observed in the plasma levels of 17-OHPC suggests that individualization of 17-OHPC dosing regimen can help in enhancing the therapeutic effectiveness.
2) Individualization of the dosing regimen can be done on the basis of BMI which was observed to have a significant impact on the plasma levels of 17-OHPC.
3) Individualization of the dosing regimen can be also be done on the basis of race as higher clearance of 17-OHPC was observed in African-Americans. However, further studies with more subjects need to be done to confirm this observation.
4) Adjustments in dosing regimen including, addition of a loading dose and changes in dosing frequency are recommended to achieve better clinical outcome.

7.0 Conclusions and Future Directions

7.1 Discussion and Summary

The objective of the work carried out in this dissertation was to evaluate the pre-clinical and clinical pharmacology of 17alpha-hydroxyprogesterone caproate (17-OHPC), a new agent that has been used for the prevention of preterm birth. Currently very limited documentation is available on the pharmacokinetics of 17-OHPC in humans or animals.

In this research work, we studied the metabolism and transport of 17-OHPC in adult and fetal (human) model systems. Extending our findings from the pre-clinical experiments, we also investigated the pharmacology of 17-OHPC in a clinical study. Several key findings were generated in this work, which are summarized in the following section.

We have used primary cultures of human hepatocytes (PCHH) isolated from adult and fetal livers, which provide several advantages over other systems used to study drug metabolism and drug transporters. The PCHH contain all the necessary cofactors for oxidative, reductive and conjugative metabolism as well as various regulatory elements required to maintain and induce/inhibit the expression of enzymes. Our research on investigating the interaction between 17-OHPC and hepatic transporters in human adult and fetal hepatocytes is limited to BSEP. While other investigators have had success measuring the activity of MRP2 and MDR1 in rat hepatocytes (Hoffmaster et al., 2004), the lower expression of these transporters in human hepatocyte cultures has

prevented us from characterizing their activity in PCHH using known substrates like rhodamine 123 and 5,6-dicarboxy-2',7'-dichlorofluorescein diacetate, respectively.

In the first part of the study, we studied the metabolism of 17-OHPC in human liver preparations in order to elucidate the pathways involved in its disposition. This was evaluated using human liver microsomes, fresh human adult hepatocytes and recombinant systems expressing cytochrome P450s. We demonstrated that the metabolism of 17-OHPC is predominantly mediated by CYP3A isoforms, mainly CYP3A4. Results of radio-HPLC and LC-MS analysis of the metabolites confirmed that the structure of 17-OHPC remained intact during metabolism by human enzymes with no cleavage of the caproate side chain. Further, LC-MS based analysis of the major metabolites indicated mono-hydroxylation or oxidation as the major metabolic pathway. These findings suggest that 1) biotransformation of 17-OHPC to the endogenous hormone, hydroxyprogesterone, is not the mechanism by which 17-OHPC works, 2) caution needs to be exercised when co-administering CYP3A4 substrates/ inhibitors with 17-OHPC to prevent any associated clinically relevant drug interactions, and 3) significant inter-individual variability in the pharmacokinetics of 17-OHPC is likely to be observed with the fixed dosing regimen.

In the second part of the study, we evaluated the metabolism and enzyme kinetics of 17-OHPC in the human fetal liver and compared the results with adult liver. This was done using primary cultures of human fetal hepatocytes and adult hepatocytes and recombinant enzyme systems. We demonstrated that 17-OHPC is metabolized by fetal human hepatocytes with CYP3A7 being the major enzyme involved. Incubation of 17-OHPC with fresh human fetal hepatocytes generated three major monohydroxylated metabolites of which two were observed to be fetal specific. The intrinsic clearance of 17-OHPC was observed to be similar in adult and fetal hepatocyte cultures. Further, the kinetics of 17-OHPC metabolism was also observed to be

similar in hepatocytes and expressed enzyme systems. Our findings suggest that 1) metabolism by human fetal liver will be a significant contributor to fetal drug disposition of 17-OHPC, and 2) 17-OHPC metabolism will lead to the accumulation of metabolites in the fetal circulation that can affect the fetal hepatic elimination processes and be a potential cause for any associated toxicity.

In the third part of the study we evaluated the role of transporters in the disposition of 17-OHPC and the effect of 17-OHPC on bile salt transport. This was done using sandwich culture preparations of primary human adult and fetal hepatocytes. We demonstrated, for the first time, the functional expression of both sinusoidal and canalicular transporters responsible for transport of taurocholate and 17-OHPC in fetal human hepatocytes. The results indicated an active efflux process as the main mechanism involved in the transport of 17-OHPC, possibly mediated by Pgp. Further, we also confirmed the expression of various hepatic transporters (MDR1, OATP1B1, OATP1B3, OATP2B1, BSEP, MRP2, MRP3, MRP4 and BCRP) in fetal hepatocytes in comparison with adults. Results from the competition assay demonstrated the inhibitory potential of 17-OHPC towards taurocholate transport. Our findings suggest that the presence of an active efflux process in fetal hepatocytes prevents the accumulation of 17-OHPC or its metabolites in fetal hepatocytes thus preventing any adverse event due to the ability of 17-OHPC and/or its metabolites to inhibit BSEP. However, inhibition of the efflux process (possibly Pgp) by xenobiotics or endogenous compounds like progesterone can lead to the accumulation of 17-OHPC or its metabolites which can subsequently lead to hepatotoxicity.

In the final part of this study we evaluated the pharmacokinetics of 17-OHPC in pregnant subjects with twin gestation. This study is the first report of plasma concentrations of 17-hydroxyprogesterone caproate in pregnant women and defined for the first time the

pharmacokinetic behavior of 17-hydroxyprogesterone caproate in twins. Wide inter-individual variation in the pharmacology of 17-OHPC was observed. Plasma concentrations of 17-OHPC did not achieve steady state concentrations but rather continued to increase with added injections. A long half life of 9.1 days was observed in the subjects which coupled with the rapid metabolism of 17-OPHC *in vitro* by human hepatocytes and liver microsomes suggested that the slow release from the castor oil depot was the half-life determining step rather than the drug's metabolic or elimination characteristics. Body mass index (BMI) was observed to have a significant impact on the plasma levels of 17-OPHC. Also, the clearance of 17-OHPC was observed to be higher in African-Americans than in Caucasians. These findings indicate that the therapeutic effectiveness can be improved by individualizing the drug administration regimen based on BMI and race of the subject which will minimize the inter-individual variation in pharmacokinetics. Further, changes in the dosing schedule frequency and addition of a loading dose can also improve the outcome by affecting patient compliance and medical costs.

7.2 Clinical Implications

1) CYP3A4 is the major isoform (of total CYPs) expressed in the human intestine. Since, 17-OHPC is a CYP3A4 substrate; the oral bioavailability of this drug is expected to be low.
2) Significant interindividual variability (10 fold) in the plasma concentrations of CYP3A4 substrates has been reported. Thus, significant inter-individual variation in the plasma concentrations of 17-OHPC is expected and monitoring of plasma levels and adjustment of dose accordingly may be needed to improve therapeutic outcomes.

3) Potential for drug interactions with other CYP3A substrates exists when multiple medications are being administered.
4) Human fetus can metabolize 17-OHPC to 3 major metabolites. Accumulation of these metabolites can affect the fetal hepatic elimination processes and be a potential cause for any associated toxicity.
5) A short half life of ~ 57 mins was observed in adult hepatocyte cultures. Thus, a short in-vivo half life is expected of this intermediate clearance compound when administered IV or PO.
6) Pgp is involved in the transport of 17-OHPC in adult and fetal liver. Direct competition of 17-OHPC with other Pgp substrates (xenobiotics or endogenous hormones like progesterone) can lead to clinically significant drug-drug interactions, especially, in the fetus.
7) Induction and/or Inhibition of Pgp (and CYP3A7) in the fetal liver can affect the elimination of 17-OHPC and/or its metabolites, thus, leading to their accumulation in the fetal circulation. Accumulation of these xenobiotics alongwith the increased level of endogenous compounds like progesterone metabolites can work in an additive (or synergistic) manner leading to significant inhibition of bile salt transport mediated by BSEP. Thus, the potential of an adverse event like cholestasis in the fetus is very likely under this scenario.
8) Inspite of the short half life of 57 mins observed in adult hepatocyte cultures, a long half life of 9.1 days was observed in the pregnant subjects. This suggests that the slow release from the castor oil depot is the half-life determining step rather than the drug's metabolic

or elimination characteristics. The long time needed for the elimination of this compound needs to be taken into account when designing a multiple dose regimen.

9) Variability observed in the plasma levels of 17-OHPC suggests that individualization of 17-OHPC dosing regimen can help in enhancing the therapeutic effectiveness.
10) Individualization of the dosing regimen can be done on the basis of BMI which was observed to have a significant impact on the plasma levels of 17-OHPC.
11) Individualization of the dosing regimen can be also be done on the basis of Race as higher clearance of 17-OHPC was observed in African-Americans. However, further studies with more subjects need to be done to confirm this observation.
12) Adjustments in dosing regimen including, addition of a loading dose and changes in dosing frequency are recommended to achieve better clinical outcome.

7.3 Limitations and Future Directions

1) Limited information is available regarding the molecular mechanisms that cause preterm birth. Extensive research needs to be done to elucidate these mechanisms which will help not only in better predicting preterm birth but also in designing effective and targeted therapies.
2) The mechanism of action of 17-OHPC leading to the prevention of preterm birth is unknown. The success rate of 17-OHPC in preventing preterm birth is only 33% as per a recent trial by Meis et al. Conflicting reports exist regarding its effectiveness. Thus, studies done to understand the mechanisms that cause preterm birth can help us

understand how 17-OHPC works. Without the background knowledge, the optimal therapeutic concentration and dose cannot be determined.

3) The potential for clinically relevant drug-drug interactions between 17-OHPC and other CYP3A4 substrates needs to be studied in human hepatocytes using clinically relevant concentrations of 17-OHPC. Primary cultures of human hepatocytes are an intact cellular system that is valuable in characterizing the regulation and activity of a variety of drug metabolizing enzymes and transporters. Though much has been done to standardize this model, further work is needed to optimize variables that enable for the better prediction of *in vivo* situation.
4) Synthesis of the various metabolites of 17-OHPC and confirmation of their chemical structure needs to be done by LC-MS and NMR studies. It is likely that 17-OHPC works via a bioactivation mechanism wherein the metabolite(s) generated are the active products.
5) Confirmation of Pgp as the transporter involved in the disposition of 17-OHPC needs to be carried out in cell lines selectively expressing the MDR1 protein. Further, hepatocyte based assays for Pgp, MRP and other transporters using transporter specific substrates needs to be set up and optimized for both adult and fetal cultures.
6) LC-MS based analysis needs to be set up for estimating the intracellular concentrations of 17-OHPC and its metabolites in hepatocyte cultures. This will help in understanding the mechanism of inhibition of BSEP by these xenobiotics in taurocholate assays that is important especially in the case of the fetus.
7) To provide better estimates of the pharmacokinetic parameters of 17-OHPC in pregnant subjects, a pilot study needs to be conducted designed to include more intensive sampling during the first dosing interval in order to capture the absorption phase of the plasma concentration-time curve.

APPENDIX A

Pharmacokinetic Behavior of 17 Alpha-Hydroxyprogesterone Caproate on Multifetal Gestation

A. Principal Investigator: Steve N. Caritis, MD

Co-Investigators: Hyagriv Simhan, MD; Raman Venkataramanan, PhD; Ashi Daftary, MD; Peg Watt-Morse, MD; Arundhathi Jeyabalan, MD; Kristiina Parviainen, MD; Margaret Cotroneo, RN, CCRC

B. Protocol Title:

Pharmacokinetic Behavior of 17α-Hydroxyprogesterone Caproate on Multifetal Gestation

Research Protocol Abstract: This study is an ancillary study for an NIH-sponsored Maternal-Fetal Medicine Units Network trial entitled "A Randomized Trial of 17 Alpha-Hydroxyprogesterone Caproate for Prevention of Preterm Birth in Multifetal Gestation" (STTARS Trial, IRB #0412048), which is a randomized, double-masked, placebo-controlled, multi-centered clinical trial of 17 alpha-hydroxyprogesterone caproate (17OPHC) for prevention of preterm birth in multifetal gestation. The primary objective of this study is to define the pharmacokinetic behavior of 17OHPC after intramuscular injection in women with twin or triplet gestation. We will obtain blood prior to and daily for one week after injection of 17OHPC (8 samples total) for determination of 17OHPC concentrations. Additionally, blood samples will be drawn at 24-28 weeks and at 32-35 weeks to measure concentrations of 17OHPC, estradiol, progesterone, and 17- hydroxyprogesterone. These measurements will define the pharmacokinetic behavior of 17OHPC in multifetal gestation for the first time, enable the determination of steady state 17OHPC concentrations after repeated doses, and evaluate the effect of 17OHPC on other pregnancy hormones. Subjects must be participating in the IRB #0412048 study (whose eligibility criteria includes multifetal gestation between 16 weeks and 0

days to 20 weeks and 6 days) to qualify for participation in this ancillary study. Approximately 20 subjects (ages 15-45) will be accrued at Magee-Womens Hospital (MWH) or one of the other 13 MFMU Network sites. Study treatment will be administered through 35 weeks gestation or delivery. The total duration of this multi-center study is 5 years.

C. Hypothesis and Specific Aims:

The objective of this study is to provide preliminary information about the pharmacokinetic behavior of 17 OHPC so that the optimal dose of the drug for women pregnant with twins or triplets eventually can be determined. Specifically, we hope to:

- Determine the pharmacokinetic profile of 17OHPC among women with twin or triplet gestation who are receiving 17 OHPC (or placebo) as part of a multicenter study
- Determine if the weekly injection of 17OHPC increases plasma progesterone, estradiol, or plasma 17-hydroxyprogesterone.

The specific aims for this study include:

a) Measuring the concentration of 17OHPC 7 times over one week after an injection of 17 OHPC (or placebo) in women with twin or triplet gestation.

b) Measuring steady state concentrations of 17OHPC and pregnancy-specific hormones (progesterone, 17-hydroxyproesterone, and estradiol) at 24 to 28 weeks and 32 to 35 weeks in women receiving weekly injection of 17OHPC (or placebo).

D. Background Information and Significance:

In a recently completed multicenter study (MWH #97-051) by the National Institute of Child Health and Human Development (NICHD) sponsored Maternal-Fetal Medicine Units (MFMU) Network, a marked reduction in preterm birth was noted among high risk women who received 17 alpha-hydroxyprogesterone caproate (17OHPC) rather than placebo.[1] A one-third reduction in preterm birth was observed regardless of whether an endpoint of 37, 35 or 32 weeks gestation was used. The mechanism by which 17OHPC exerts its beneficial effect is unclear, particularly since there is little evidence that progesterone deficiency has a causal role in preterm birth or that injections of 17OHPC increase plasma progesterone concentrations either in singletons or twins.[2,3] The high and rising rate of twin pregnancy, and the disproportionate perinatal morbidity and mortality attributable to preterm twin birth, argue for a preterm birth prevention trial in multifetal pregnancies. Moreover, the promising results of the cited MFMU Network 17OHPC trial suggest the value of evaluation of 17OHPC in pregnancies at high risk of preterm birth for reasons other than prior preterm birth. Given the beneficial effect of progesterone supplementation in singleton gestations, it is reasonable to consider that a similar benefit may occur in multifetal gestations. Hence, the MFMU Network opened the multicenter study entitled "A Randomized Trial of 17 Alpha-Hydroxyprogesterone Caproate for Prevention of Preterm Birth in Multifetal Gestation" (STTARS Trial, IRB #0412048) in 2003.

The pharmacokinetic behavior of 17OHPC has not been evaluated in pregnancy. Only a single study has measured concentrations of 17OHPC in non-pregnant women receiving this agent intramuscularly.[5] The dose of 17OHPC used to prevent preterm birth in singleton gestation has varied from 250 mg to 1000 mg every week and there are no data to provide guidance as to what the appropriate dose should be either in singletons or in multifetal gestation.[1, 4, 6] In general,

metabolism of pharmacologic agents in pregnancy differs considerably between pregnant and non-pregnant women. For example, the volume of distribution, renal blood flow and hepatic metabolism are markedly affected by pregnancy and especially in women with multifetal gestation.

17OHPC has potent progestational effects[7], which occur without alterations in the plasma progesterone concentrations and may be key to the agent's effectiveness. Progestational effects that may be relevant to preterm birth prevention include an effect on the inflammatory response associated with term and preterm labor, binding to nuclear progesterone receptors in the myometrium or to other non-myometrial tissue.[2, 8, 9]

Labor is characterized by an inflammatory response in the lower uterine segment and cervix.[10-12] Leukocytes are found at these sites in increasing numbers as labor nears. This leukocytic infiltration is associated with increased cytokine production and with cervical ripening. Progesterone inhibits the leukocytic infiltration of the cervix and prevents the increase in cytokine concentration. Progesterone also inhibits CRH (which is associated with preterm birth) and expression of oxytocin receptors and gap junctions.[2, 13] Thus, if 17OHPC binds to nuclear progesterone receptors more avidly than endogenous progesterone, a biologically plausible explanation for the beneficial effect of 17OHPC therapy can be proposed.

Significance: The significance of this study is that it will establish the appropriate dose of 17-hydroxyprogesterone caproate to be used in multifetal gestation.

E. Progress Report and Preliminary Studies: Not applicable

F. Research Design and Methods:

Drug/Device Information: 17 Alpha-Hydroxyprogesterone Caproate (17OHPC)(IND #69,094) is a synthetic hormone produced from progesterone that is being utilized in the main study (IRB #0412048). The FDA has not approved this drug for use in this setting. Subjects participating in IRB #0412048 already will have been randomized to receive either study drug or placebo (inert castor oil). The dose of 17P (or placebo) is 250 mg and will be given by the study nurse as a 1 ml IM injection into the upper quadrant of the gluteus maximus. No study drug (or placebo) will be administered in this ancillary study.

Study Design: This study is an ancillary study for an NIH-sponsored Maternal-Fetal Medicine Units Network trial entitled "A Randomized Trial of 17 Alpha-Hydroxyprogesterone Caproate for Prevention of Preterm Birth in Multifetal Gestation" (STTARS Trial, IRB #0412048), which is a randomized, double-masked, placebo-controlled, multi-centered clinical trial of 17 alpha-hydroxyprogesterone caproate (17OPHC) for prevention of preterm birth in multifetal gestation. Subjects participating in this ancillary study will have blood drawn immediately prior to 17OHPC injection and once a day for seven days after injection for determination of 17OHPC concentrations (Specific Aim #1).

A secondary objective of this study is to evaluate the effect of 17OHPC on selected pregnancy hormones (Specific Aim #2). Blood will be drawn from subjects on two occasions (once between 24 and 28 weeks and once between 32 and 35 weeks) to determine the steady

state concentration of 17OHPC on pregnancy-specific hormones (progesterone, 17-hydroxyproesterone, and estradiol).

Subjects must be participating in the IRB #0412048 study (whose eligibility criteria includes multifetal gestation between 16 weeks and 0 days to 20 weeks and 6 days) to qualify for participation in this ancillary study. Approximately 20 subjects (ages 15-45) will be accrued at Magee-Womens Hospital (MWH) or one of the other 13 MFMU Network sites. Study treatment will be administered through 35 weeks gestation or delivery. The total duration of this multi-center study is 5 years.

Screening Procedures for Eligibility and Consent: There are no screening procedures for this study. Only subjects participating in "A Randomized Trial of 17 Alpha-Hydroxyprogesterone Caproate for Prevention of Preterm Birth in Multifetal Gestation" (STTARS Trial, IRB #0412048) are eligible for participation in this study. Subjects participating in IRB #0412048 who express an interest in this ancillary study will be told about this study and asked to sign an informed consent form. Subjects ideally will be consented for this study when they are consented for participation in IRB #0412048 because we hope to perform the pharmacokinetic (PK) analysis in conjunction with the first injection of 17OHPC (or placebo) in order to evaluate accurately the absorption and effect on hormonal and inflammatory markers. No study procedures will be performed unless/until informed consent in obtained.

Study Procedures: The following procedures will be performed during this study:

- A baseline blood sample (6cc) will be obtained at a study visit prior to an IM injection of 250 mg 17OHPC (or placebo). Since the half-life of 17OHPC in non-pregnant subjects is

- Samples will be centrifuged at 3000 x g for 15 minutes and the supernatant aliquotted into 1 ml tubes, labeled and frozen at -80oC until analyzed. For 17OHPC, analysis will be by HPLC which has a sensitivity of <1 ng/ml and will differentiate 17-OHPC from progesterone and 17-OH progesterone. Expected concentrations will be 20 ng/ml at peak and 5 ng/ml at trough. The results of the PK analysis will be blinded from the investigators as to group assignment (study drug or placebo) and all assays will be performed at the University of Pittsburgh, Pittsburgh, PA.

- Approximately 10 cc blood will be drawn for measurement of 17OHPC, 17-hydroxyprogesterone, progesterone, and estradiol during study visits between 24-28 weeks and 32-35 weeks (please reference Specific Aim #2).
    - Samples will be centrifuged at 3000 x g for 15 minutes and the supernatant aliquotted into 1 ml tubes, labeled and frozen at -80oC until analyzed. Analysis will be by radio immunoassay or HPLC. All assays will be performed at the University of Pittsburgh, Pittsburgh, PA, and results will be blinded from the investigators as to group assignment (study drug or placebo).

Storage of Samples: Analysis of blood samples will be performed at the University of Pittsburgh. These samples will be stored for 5 years and then discarded. The principal investigator will be responsible for control of the storage area. Samples will be stored to include assigned code

numbers (using bar-coding software entitled "Freezerworks"), and the information linking these code numbers to the corresponding subject's identity will be kept in a separate, secure location. De-identified samples will be made available to secondary investigators and may be used for future research on preterm birth or other pregnancy problems. If subject decides to withdraw consent for this study, her samples will be destroyed within 5 business days of receipt of notice of withdrawal.

These study visits and blood draws will be completed as outpatient procedures in the Ambulatory Care Research Center (ACRC) of the General Clinical Research Center (GCRC) of MWH (subjects also have the option of having blood samples after the injection of 17OHPC [or placebo] drawn in their home or office if more convenient than attending MWH), should take approximately 10 minutes to complete, are considered research-only procedures.

At the completion of the study, the investigators may contact the subject to discuss a follow-up study on her and her baby.

Endpoints for Discontinuation of Study Treatment: Regarding "A Randomized Trial of 17 Alpha-Hydroxyprogesterone Caproate for Prevention of Preterm Birth in Multifetal Gestation" (STTARS Trial, IRB #0412048), there are no recognized reasons (including preeclampsia, hypertension, abruptio placenta, or preterm labor) not to administer 17OHPC to pregnant women based on the specific medical condition, unless the subject has a reaction to 17OHPC, which would be an indication to stop. Unless the subject has a reaction to 17OHPC, withdraws consent from IRB #0412048, or withdraws consent from this proposed ancillary study, the study procedures (blood draws) will occur as scheduled. There are no dose modifications since study drug is not being administered in this ancillary study.

Adverse Event Reporting: Subjects will be questioned in a non-directed manner at each study visit regarding side effects or symptoms associated with study procedures. The data will be captured on the Study Visit Form. In addition, an Adverse Event Form will be completed for any event that is serious, deemed related to the study, and/or unexpected in nature, severity, or frequency. The Adverse Event Form will be sent by facsimile to the NICHD program scientists, Biostatistical Coordinating Center (BCC) of George Washington University, and University of Pittsburgh IRB according to guidelines (within 24 hours in the case of maternal, fetal, or neonatal death). If a death is reported, a copy of the subject's medical record will be made, and in the case of a miscarriage or intrauterine demise, the pathology report on examination of the fetus and placenta must be included and forwarded to the BCC.

G. Biostatistical Design and Analysis:

Sample size estimation

1. Specific Aim #1 (measuring the concentration of 17OHPC 7 times over one week after an injection of 17 OHPC [or placebo] in women with twin or triplet gestation): There are limited studies of 17OHPC in humans and no studies in pregnant women in which pharmacokinetic data are reported.5 Steady state concentration among five non-pregnant subjects following injections of 1000 ml ranged from 30 to 75 ng/ml. The variation in steady state concentrations was approximately 40%. Sample size estimates for pharmacokinetic studies depend on the degree of variation tolerated. Given only 40% variation with 5 subjects, approximately 16 patients should be sufficient in order to define, with an adequate level of confidence, the average pharmacokinetic behavior of 17OHPC in pregnancy assuming that half of these women would be

receiving placebo. We will recruit at least 16 subjects pregnant with twins and as many as 4 subjects pregnant with triplets. We may enroll too few subject pregnant with triplets for any comparisons, but even 1-2 such subjects will provide useful pilot information.

2. Specific Aim #2 (measuring steady state concentrations of 17OHPC and pregnancy-specific hormones [progesterone, 17-hydroxyproesterone, and estradiol] at 24-28 weeks and 32-35 weeks in women receiving weekly injection of 17OHPC [or placebo]): Blood samples will be obtained at 24-28 and at 32-35 weeks for the measurement of 17OHPC steady state concentrations. In order to demonstrate that 17OHPC concentrations are not zero, only 4 subjects receiving 17OHPC are needed because the assay sensitivity is <1 ng/ml. For 17OHPC progesterone, 17-hydroxyprogesterone, and estradiol, we will use a simple t-test to compare differences between groups. Given the variation in hormone concentrations the sample size will allow us to detect differences of 40% or greater.

Feasibility: At the University of Pittsburgh, there were 134 twin gestations delivered in 1999. Approximately 600 patients with twin gestation and 120 with triplet gestation will be recruited to the main study (IRB #0412048). There are 14 centers participating in the main study, thus, each center will be expected to recruit 40-50 twins and 8 triplets. Given the large number of multifetal gestation, it is our expectations that MWH will recruit above an average amount of patients to this main study. Therefore, a sufficient subject population should be available for this ancillary study.

All sample size estimates are for subjects pregnant with twins. When possible, we will also collect data on subjects pregnant with triplets. For Specific Aim #1, sample size is small and therefore recruitment should not be difficult. Blood samples will be drawn once a day for

seven days. This may limit our recruitment, but with appropriate incentives (such as offering to draw blood in the subject's home), we hope to overcome this potential problem. For Specific Aim #2, an adequate sample size can be achieved if the assumed recruitment occurs at the projected rate. There is a possibility that a number of enrolled subjects will be placebo (i.e., a bad distribution of subjects), but no statistical difference will be noted in this preliminary study. Even if bad distribution occurs, pharmacokinetic analysis will be possible, and any information collected from pharmacokinetic analysis will be of value to investigators. The MFMU Network has approved this sample size estimate.

The Biostatistics Coordinating Center (BCC) of George Washington University is responsible for all aspects of biostatistical design, analysis, and data management of the study, in addition to the interim and final statistical analyses and preparation of publications based on the study results. The Principal Investigator of the BCC reports to the Steering Committee and the Data Monitoring and Safety Committee.

H. Human Subjects:

1. *Subject Population:* The racial, gender, and ethnic characteristics of the proposed subject population reflects the demographics of Pittsburgh and the surrounding areas and/or the patient population of the University of Pittsburgh Medical Center/MWH. We shall attempt to recruit subjects in respective proportion to these demographics. No exclusion criteria shall be based on race, ethnicity, or HIV status. Since this study involves pregnancy, only women will be included. Approximately 20 subjects (ages 15-45) will be accrued at Magee-Womens Hospital (MWH). Pregnant children from ages 15-17 may be included as long as parental consent is obtained. In other multicenter studies, most subjects have presented ≥ 15 years of age; therefore,

age 15 is the minimum age for inclusion. This research study meets the standards for Criterion 1 of permitted research for minors, whereby the research involves no greater than minimal risk to subjects. MWH and the investigators listed on this research study have extensive experience in treating pregnant minors. The risk-benefit ratio for minor subjects is the same as described in this protocol for adult subjects.

Eligibility Criteria:

- Inclusion Criteria: 1) Subjects enrolled in the main study, entitled "A Randomized Trial of 17 Alpha-Hydroxyprogesterone Caproate for Prevention of Preterm Birth in Multifetal Gestation" (STTARS Trial, IRB #0412048). 2) Subjects willing to undergo additional blood draws.
- Exclusion criteria: 1) Subjects unwilling to sign informed consent. 2) Pregnant minors < 15 years of age or pregnant women > 45 years of age.

2. *Targeted/Planned Enrollment Table (20 subjects to be accrued at MWH):*

Ethnic Category	Females	Males	Total
Hispanic or Latino	1	0	1
Non Hispanic or Latino	19	0	19
Ethnic	20	0	20
Racial Categories			
American Indian	0	0	0
Asian	0	0	0
Native Hawaiian or other Pacific Islander	0	0	0

Black or African American	4	0	4
White	16	0	16
Racial Categories: total of all Subjects*	20		20

*Must be equal to the Racial Categories Total of all subjects

3. Sources of Research Material: This research study will involve obtaining medical information from hospital and/or other health care provider (e.g. physician office) records. The information that will be obtained will be limited to information concerning treatment of the subject's pregnancy. This information will be used to determine the subject's eligibility for this study and to follow their study response. The confidentiality of collected specimens will be maintained through adherence to HIPAA standards, and the information obtained from subject interview and chart review is illustrated in the full protocol and Manual of Operations. The following procedures will be performed as part of this research study:

Procedure	Research-Only	Standard of Care
Blood draws (x10), study visits (x10)	☒	☐

4. Recruitment Methods and Consent Procedure: Subjects will be recruited for this ancillary study only from the population participating in the main study, entitled "A Randomized Trial of 17 Alpha-Hydroxyprogesterone Caproate for Prevention of Preterm Birth in Multifetal Gestation" (STTARS Trial, IRB #0412048). Information about subjects' family members is not required for this ancillary study. Subjects for the main study (IRB #0412048) will be recruited from the MWH outpatient clinics and private physician offices in the following manner: 1) Subjects that have signed up for the Women and Infant's Research Registry (MWH #23-072)

will be approached/contacted by the research staff to determine if they are interested in the study; 2) Clinicians directly involved in the patient's/potential subject's clinical care will approach all patients/potential subjects. If the clinician is also an investigator, he/she will obtain written informed consent. If the clinician is not an investigator, it will be noted that he/she and the researcher to whom the information is being provided both work for UPMC. The referring clinician will document the verbal permission of the patient for the sharing of her protected health information with the researchers. The sharing of the patient's/potential subject's contact information (linked to medical diagnosis or condition) between the referring health care provider and the researchers involves no more than a minimal risk to the privacy of the patient since the referring health care provider, who introduces the research study to his/her patient, will obtain and appropriately document the verbal permission of the patients to provide the patient's contact information to the researchers. To further ensure that the risk to the privacy of the involved patients remains minimal: (A) the patient's/potential subject's recorded contact information will be stored by the researchers in a secure manner (e.g., locked file cabinet, password protected database) accessible only to the researcher who was provided this information and other members of the research team involved in the conduct of the research study (studies) for which this information was originally provided; and (B) the patient's recorded contact information will be destroyed immediately after the researchers have contacted the patient to discuss the research study (studies) for which this information was originally provided; unless, upon such contact, the patient/potential subject indicates a further interest in study participation. For patients/potential subjects who indicate a further interest in study participation, the patient will be engaged in an informed consent process to further determine research study eligibility and/or study interest. The patient's recorded contact information will be destroyed immediately after ascertaining that

the patient is not eligible for or declines participation in the research study (studies). We hereby provide our assurance that the recorded patient contact information will not be reused or redisclosed to any other person or entity (i.e., other than the members of the research team involved in the conduct of the research study [studies] for which the contact information was originally provided) except as required by law or for authorized oversight of the research study. Other subjects are referred in specifically for possible participation in a particular study, which they or their physician are aware of based on publications, which list clinical trials at UPMC/MWH. "Cold-calling" will not be used to recruit subjects. Potential subjects will be approached by clinicians who are directly involved in their care. "Finder's fees" for referring a potential subject for participation in a research study are prohibited.

Once a subject is identified as a potential participant in a research study as indicated above, she is screened for eligibility. No research-related procedures (including review of the subject's medical records) will be performed until the subject has provided written informed consent. The consent process will be carried out as a joint effort among the subject's physician, the study coordinator, and a physician who is listed as an investigator on the study and the Informed Consent Form will be signed by an investigator who is a physician.

5. Potential Risks and Discomforts:

Risks of Intravenous Blood Draw:

Likely (occurs in more than 25% of subjects, or more than 25 out of 100 subjects): bruising, bleeding, swelling, and discomfort at the injection site

Rare (occurs in less than 1% of subjects, or less than 1 out of 100 subjects): infection at injection site; fainting; lightheadedness

6. Risk Management Procedures: Risk will be managed by having blood draws performed by certified phlebotomists. Risks will be evaluated at each study visit through observation of the subject and by eliciting a verbal description of the subject's comfort level. The local research team meets every day when possible (at minimum, on a monthly basis) and all aspects of the studies are discussed, including adverse events, recruitment, and subject retention. There are no alternative procedures not already utilized in managing the subject with multifetal gestation. These subjects are at substantially increased risk of preterm birth and there is no therapy that is recognized as beneficial in such cases. No attempt will be made to alter or mandate clinical management of the subjects. The principal investigator will terminate the involvement of a given subject in the research study if said subject develops a condition that precludes her from having a blood draw. This ancillary study will be terminated if the risk-to-benefit ratio changes, i.e., risk outweighing benefits.

All records pertaining to the subject's involvement in this research study will be stored in a locked file cabinet in the office of the research staff. A case number will indicate the subject's identity on these records. This information will be accessible to the investigators and their research study staff listed on the first page of this document. Individuals from the agencies funding this research may review the subject's records as part of their ongoing audit of this project. Any information about the subject or the subject's hospital treatment will be handled in a confidential (private) manner consistent with other hospital medical records. The subject will not be specifically identified in any publication or research results. However, in unusual cases, the subject's research records may be inspected by appropriate government agencies, such as the US Food and Drug Administration and the Office for Human Research Protections or be released

in response to an order from a court of law. All research records will be kept for a minimum of seven years following closure of this study.

Data and Safety Monitoring Plan

The multicenter Data and Safety Monitoring Committee (DSMC), a group of individuals not affiliated with any of the participating institutions was established by the NICHD. Before the trial can begin, the protocol must be approved by the committee. During the conduct of the study, the committee is charged with monitoring the emerging results for efficacy and safety, in addition to center performance and protocol adherence. Recommendations by the committee can include protocol modification, early termination for efficacy, or for unexpected safety problems. Recommendations are made to the NICHD and disseminated to the Steering Committee. Any changes in the risk level ascertained by the DSMC would be recommended for change immediately. Such changes would be reported to the IRB in a timely fashion. Local study staff will be responsible for reporting any adverse events to the IRB and sponsor. A summary of DSMC findings will be provided to the IRB upon receipt, but no later than at the time of annual renewal.

7. Evaluation of Risk/Benefit Ratio: There is minimal risk to subjects participating in this study. The subject may not benefit directly from participation in this study. The privacy of all subject information will be maintained as per HIPAA requirements by de-identification of subject information. A unique code, consisting of a computer-generated network number, check digit, and the first digit of the subject's first name, will be used in lieu of any personal identifiers. The code letter/number combination assigned by the BCC is the only link to the subject information and may not be identified, other than to MWH research staff. All data that is sent to the BCC is

backed up on tape prior to transmission. Edits, audits and queries are performed weekly as per the data sent.

8. Costs and payments: Neither the subject nor the subject's insurance company will be charged for any tests, procedures, or study visits performed in this research study. The cost of the blood draws and study visits performed in this research study will be covered by the study budget. The subject will receive $30 for food/inconvenience and/or bus/cab fare for completion of the first 8 scheduled blood draws (a total of $240). If the subject misses any one of the first 8 scheduled blood draws, she will be removed from the study. The subject will receive an additional $30 for food/inconvenience and/or bus/cab fare for completion of each of the last two scheduled blood draws (between 24-28 weeks and 32-35 weeks, for a total of $60). The subject will receive an additional $100 for completing all scheduled blood draws. If the subject misses one of the last two scheduled blood draws, she will not collect the additional $100. Hence, the subject will be compensated a total of $400 if all scheduled blood draws are completed. The subject also will receive free parking at Magee-Womens Hospital for all study visits. If the subject delivers prior to completion of the scheduled study visits, she will be compensated as though she had completed all study visits and blood draws.

I. Justification for Utilization of GCRC Resources: All subjects for this trial will be seen in the outpatient clinic of the GCRC to allow them to have a centralized area to which to report for visits.

J. Study Size and GCRC Resources: 1) Number of Research Subjects = 20; 2) Total Number of Inpatient Days = 0; 3) Total Number of Outpatient Days: (10 subjects per year) x (10

visits per subject) = 100 outpatient visits per year; 4) Estimated GCRC Inpatient Ancillary Cost of the Study = No requested ancillary funding; 5) Estimated GCRC Outpatient Ancillary Cost of the Study = No ancillary costs for outpatient visits; 6) No other GCRC resources requested other than those described in section I.

K. Research Needs to be Provided by Investigators' Laboratory or Outside Laboratory: None

L. Funding Support: National Institutes of Health Grant #HD21410-17

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N. Qualification of Investigators:

Steve N. Caritis, MD, Professor, OB/GYN/RS, has been a contributing member of the Maternal Fetal Medicine Units Network at Magee-Womens Hospital for the past 19 years. He is the MFM Division Director and has been conducting research at the University of Pittsburgh since 1975.

Drs. Simhan, Daftary, Watt-Morse, and Jeyabalan are faculty members who conduct their own research studies and are well acquainted with MFMU Network trials. All have completed Masters programs in either Public Health or in Clinical Research.

Raman Venkataramanan, PhD, Professor of Pharmaceutical Sciences, University of Pittsburgh, has served as the kineticist on several projects with Dr. Caritis. He was a subcontractor for the Drug Metabolism in Pregnancy Study, an MFMU Network trial. He has completed extensive post-doctoral work in pharmacokinetics and has a total of 176 publications to date.

Dr. Parviainen is a fellow who has presented projects at the MFMU Network and is working on her own research projects.

Margaret Cotroneo, RN, CCRC, has been the Clinical Research Coordinator for the Maternal Fetal Medicine Units Network at Magee-Womens Hospital since 1988. She has served on several subcommittees for the Network and is certified to perform all study-related tasks for all of the trials.

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