

**ONTOGENY OF HEPATIC DRUG METABOLIZING ENZYMES IN
SHEEP**

by

Manoja Pretheeban

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Abstract

Administration of therapeutic, supplemental and social drugs to the mother during pregnancy exposes the fetus to these medicines and their metabolites, which can cause defects in nervous, renal, cardiac and metabolic functions. Hepatic metabolic enzymes such as cytochrome P450 (CYP) 2A6, 2C19, 2D6 and UDP glucuronosyltransferases (UGT) 1A6, 1A9, 2B7 are important for the metabolism of many drugs. In order to understand whether these drugs are safe enough during pregnancy and in postnatal period, knowledge of the expression levels of these enzymes in different developmental stages is important.

Therefore we proposed to clone the CYP and UGT genes and quantify these enzymes at gene and protein levels at different developmental stages such as fetus, newborn and adults. This study was performed in sheep (adult n=4; newborn n=3; and fetus n=3) liver to compare the mRNA and protein expression levels of the above enzymes and a regulatory factor, Hepatic Nuclear Factor 4 α (HNF4 α). The effect of antenatal glucocorticoid on these enzymes was also studied by infusion of cortisol (0.45mg/h; 80h) to another group of fetuses (n=5). Sheep sequences were cloned and real time PCR was performed to analyze the relative mRNA expression levels in the above four groups. Microsomes were prepared and western blot analysis was performed using human or rat antibodies to measure the relative protein expression levels.

In terms of mRNA and protein expression of the above mentioned enzymes, fetal and newborn levels were very low compared to the adult. Some CYP proteins (CYP2A, CYP2C) were absent in the fetus and even in newborns (CYP2A). Sheep UGT protein levels were not measured since the human UGT antibodies did not work. Glucocorticoid plays a role in up regulating both the mRNA and protein expression of CYP2D6. Moreover the correlation observed between the above enzymes and HNF4 α indicates a possible regulatory role in sheep similar to that in humans.

The findings of this study follow a similar pattern found in the human and indicate that fetal and newborn lambs have a reduced ability to metabolize drugs that are substrates of these CYP isoforms.

Preface

All use of animals in this study conformed to the guidelines of the Canadian Council on Animal Care and were approved in advance by the University of British Columbia Committee on Animal Care (A07-0302).

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Abbreviations

1NP	1-naphthol
4MU	4-methylumbelliferone
AhR	Aryl hydrocarbon receptor
ATP	Adenosine Triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BCRP	Breast Cancer Resistance Protein
Bis	N, N'-methylene-bis-acrylamide
bp	base pairs
BSA	Bovine serum albumin
CAR	Constitutive androstane receptor
cDNA	Complimentary Deoxyribonucleic acid
CIP	calf intestinal \square hosphatise
CNS	Central Nervous System
COS-1	cells being CV-1 (simian) in Origin, and carrying the SV40 genetic material
CYP	Cytochrome P450
Da	Dalton
dg	Days of gestation
DEPC	Diethylpyrocarbonate
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
EDTA	ethylene diamine tetraacetic acid
ENT1	Equilibrative nucleoside transporter-1
F/M	Fetal maternal ratio
FDA	Food and Drug Administration
FMO	Flavin-containing mono-oxygenases
FX	Fluoxetine
g	Gram
GAPDH	Glyceraldehyde 3- phosphate Dehydrogenase
GH	Growth hormone
GR	Glucocorticoid receptor
GST	Glutathione-S-transferases
H	Hour
HCl	Hydrochloric acid
HNF4 α	Hepatic nuclear factor 4 α
HREs	DR-5 hormone response elements
KCl	Potassium chloride
LB	Luria Broth
LTG	lamotrigine
M	Molar
MCTs	Monocarboxylate transporters
MDR1	Multidrug Resistance Protein
mg	milligram
MgCl ₂	Magnesium Chloride
ml	\square hosphatis
mRNA	Messenger Ribonucleic Acid

MRP	Multidrug Resistance-related Proteins
n	Number
NADPH	Nicotinamide adenine dinucleotide phosphate
NAT	N-acetyltransferases
NBT	p-nitro-blue tetrazolium chloride
NET	Norepinephrine transporter
ng	nanogram
NSAIDs	Non Steroidal Anti Inflammatory Drugs
OAT4	Organic anion transporter-4
OATP-B	Organic anion transporting polypeptide-B
OCTN2	Carnitine transporter
OD	Optical Density
ORF	Open reading frame
PAH	Polycyclic aromatic hydrocarbons
PCN	Pregnenolone 16 α -carbonitrile
PCR	Polymerase Chain reaction
PPAR	Peroxisome proliferator-activated receptors
PXR	Pregnane X receptor
RACE	Rapid Amplification of cDNA Ends
RFT1	Reduced folate transporter
RT	Reverse transcriptase
SDS-PAGE	Sodium dodecyl suiphate-polyacrylamide gel electrophoresis
SER	Smooth endoplasmic reticulum
SERT	Serotonin transporter
SIDS	Sudden infant death syndrome
SNP	single nucleotide polymorphisms
SSRI	Selective Serotonine Reuptake Inhibitor
STs	Sulfotransferases
TCDD	Tetrachlorodibenzo- <i>p</i> -dioxin
TEMED	N,N,N',N'-tetramethylethylenediamine
TFP	trifluoperazine
Tris	tris-(hydroxymethyl) aminoethane
UGT	Glucuronosyltransferases
UTR	untranslated region
UV	Ultraviolet
VPA	Valproic acid
μ g	microgram
μ l	microliter

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“This Thesis is dedicated to my parents and husband”

1. INTRODUCTION

1.1 Drugs in pregnancy

Administration of medicines has increased in modern day medical practice. Numerous prescription and over the counter drugs are used during pregnancy for the treatment and prevention of various disease conditions or as supplements and/or for pleasure (social or illicit drugs). Many pregnant women require drug therapy because of pregnancy-induced conditions such as nausea and vomiting, chronic conditions diagnosed before pregnancy, or acute conditions during pregnancy or conditions that require surgical treatment with the use of anesthetic agents (reviewed by Chin and Lao, 1987; Nosten et al., 2006; Cono et al., 2006; Sachdeva et al., 2009). Every year, many new drugs are approved and marketed. But before marketing a new drug, the manufacturers almost never test the products in pregnant women to determine effects on the fetus. Also most drugs are not labeled for use during pregnancy. Since the potential benefits to the mother outweigh the risks to the fetus, a number of drugs have been used in pregnant women. Several circumstances put large numbers of women and their physicians in difficult situations. One is that at least half of the pregnancies in North America are unplanned (Khanna et al., 1992). Therefore every year, hundreds of thousands of women expose their fetuses to drugs before they know they are pregnant (Koren et al., 1989; Koren and Pastuszak, 1990). Another reason is that with the recent increase in the age at which women have children, conditions that necessitate long-term drug therapy are diagnosed in larger numbers of women before pregnancy. Furthermore, for pregnant women with certain conditions once believed to be incompatible with pregnancy, such as systemic lupus erythematosus and heart diseases, the outcome of pregnancy has improved dramatically in the past few decades (Newton et al., 1989).

Commonly there are several groups of drugs used in pregnancy. They are anticonvulsants (phenytoin, carbamazepine, valproate) (reviewed by Adab et al., 2004), antimicrobials (penicillins, erythromycin, cephalosporins, and quinolones), psychotropic drugs (benzodiazepines, lithium, general psychotropic, selective serotonin reuptake inhibitors, pimozide, tricyclic antidepressants), antiemetics (dimenhydrinate, promethazine, meclizine, prochlorperazine, metoclopramide) (reviewed by Chin and Lao, 1987), anti-inflammatory drugs, NSAIDs (Aspirin, ibuprofen), anticoagulants (coumarin) etc. The US Food and Drug Administration (FDA) classifies these drugs into five categories of safety for use during pregnancy on the basis of data from humans and animals, ranging from class A drugs, which are designated as safe for use

during pregnancy, to class X drugs, which are contraindicated during pregnancy because of proven teratogenicity.

Even though all medications are not harmful to the fetus, some can cause deleterious effects. Several drugs have been identified to cause adverse effects during pregnancy; antibacterials (aminoglycosides, chloramphenicol, sulfanamides, tetracyclines, trimethoprim, fluoroquinolones, primaquine) (Nosten et al., 2006), anticoagulants (heparin, warfarin) (Vitale et al., 1999), anticonvulsants (carbamazepine, phenytoin, phenobarbital, valproate) (reviewed by Adab et al., 2004), antihypertensives (enalapril, captopril, lisinopril) (Hanssens et al., 1991), antidepressants (SSRI) (Oberlander et al., 2002, 2004, 2005, 2006, 2007; Moses-Kolko et al., 2005), antineoplastic, opioids, NSAIDs (aspirin), thyroid drugs, vaccines (for measles, mumps, rubella, polio, chickenpox, and yellow fever) etc (reviewed by Koren et al., 1998). However, influenza vaccine is recommended for all pregnant women in the second or third trimester during the influenza season.

Despite widespread concern about drug safety, exposure to therapeutic drugs accounts for only 2 to 3% of all fetal congenital malformations; most malformations result from genetic, environmental, or unknown causes (Brown, 2007). All maternal drugs do not cross the placenta and enter the fetus but, the drugs which enter the fetus, can cause harmful effects. However, those that do not cross the placenta may still harm the fetus by constricting placental vessels and thus impairing gas and nutrient exchange, by producing severe uterine hypertonia resulting in anoxic injury, or by altering maternal physiology (e.g., causing hypotension). Furthermore, usage of social and illicit drugs, such as nicotine, alcohol, marijuana, cocaine and large amounts of caffeine during pregnancy can cause significant problems in fetuses and neonates including placental abruption, stillbirth, preterm deliveries, low birth weight, and spontaneous abortions (Brown, 2007).

Most of the drugs administered during the pregnancy are metabolized by hepatic phase I and phase II enzymes. Several studies in sheep and humans have shown that preterm and term fetuses and newborns have either limited or no abilities to eliminate these compounds (Kim et al., 2004, 2006; Oberlander et al., 2005, 2006; Wong et al., 2000a, 2000b, 2001; Kumar et al., 2000a, 2000b, 2000c). In pregnant woman, drug metabolizing capacity increases (Anderson, 2005; Prowler and Kim, 2009) due to the hormonal and blood volume changes occur during pregnancy and may therefore alter maternal drug concentrations. There is an increase of five to eight liters in maternal total body water and 30%-50% of plasma volume during pregnancy

(Little, 1999; Cono et al., 2006). This may result in a higher volume of drug distribution in the mother, which could affect the drug levels in the fetus (i.e., by lowering maternal drug concentration).

Pharmacokinetics and enzyme activities have been studied in fetal, newborn and adult sheep for several drugs such as diphenhydramine (Au-Yeung et al., 2007; Kumar et al., 1997, 1999a, 1999b, 2000c), valproic acid (Wong et al., 2000b, 2001; Kumar et al., 2000a, 2000b; Yu et al., 1995; Gordon et al., 1995) and labetalol (Yeleswaram et al., 1993). Adverse effects were observed in sheep fetus and newborn with some of these drugs including diphenhydramine (Rurak et al., 1988).

To minimize the fetal risk, drug doses at the lower end of the therapeutic range should be prescribed during pregnancy. However, because of increased body weight, volume of distribution and more rapid clearance of many drugs (e.g., lithium, digoxin, and phenytoin) during late pregnancy, some women may need higher-than-normal doses (Loebstein et al., 1997). However, drug effects on the human fetuses and neonates can not be assessed easily and the manufacturers do not test this before release of drug to the market. Therefore, the pharmacokinetics of the drugs are analyzed using animal models such as sheep, rats etc. Since the drug metabolism of sheep is similar to human, the data from sheep studies can be, in some cases, extrapolated to human. In such animal studies, it would be more appropriate if the pharmacokinetics and *in vitro* enzyme activity studies were combined with the quantification of the drug metabolic enzymes at the transcript and protein levels.

1.2 Fetal drug exposure

During pregnancy, the fetus may be exposed to many maternally administered therapeutic agents that could affect the cardiovascular, nervous and metabolic functions of the fetus (Rurak et al., 1991), yet the ability of the fetus to tolerate this exposure is poorly understood. Many xenobiotics, including a number of clinically used drugs, are known to cause unwanted effects in the embryo or fetus, including in utero death, initiation of birth defects, and production of functional abnormalities (Hakkola et al., 1998). It is likely that most drugs have effects on fetal development, although many escape detection. Even though some of these effects may be beneficial, most cause adverse effects (Garland, 1998). There are no direct methods of measuring fetal drug exposure in the clinical setting. Identification of exposure is made by a careful maternal history, evaluating associated risk factors and obtaining a maternal urine

toxicologic screen (Plessinger and Woods, 1993) which gives only qualitative information. The only means to assess exposure quantitatively is by measuring drug effects in the mother, fetus, or both, or by predicting fetal exposure based on a careful review of drug intake and applying general principles of drug distribution. Prior to delivery, drug measurements can be obtained from the fetus only by percutaneous umbilical blood sampling. Because of the potential risks of fetal sampling, this procedure is not indicated for evaluation of fetal drug exposure alone.

Exposure of the fetus to drugs is dependent on the placental permeability of the drugs and the ability of the fetus to eliminate them. Most drugs cross the placenta by passive diffusion (Reynolds, 1989) and it is the concentration, size of the drug molecule and the properties of the barrier (thickness and size of the pores of the placenta) that determine how readily the drug will pass. Fetal maternal (F/M) ratios in the sheep tend to be lower than in primates for hydrophilic drugs, as the placental permeability in the sheep for such compounds is lower (Riggs et al., 1990) than in primates because of the structural differences of the placenta (Perry, 1981; Schneider et al., 1985). In the hemochorial human placenta, hydrophilic compounds up to a molecular weight of 600 Dalton (Da) can cross readily (Ostrea et al., 2004), whereas in the multilayered cotyledonary sheep placenta, transfer becomes limited for hydrophilic compounds up to 60 to 180 Da (Stulc, 1996). However for lipophilic compounds, placental permeability is not the limiting factor for transplacental transfer; rather the efficiency of transfer is determined by maternal and fetal placental blood flows and their relative orientations (Battaglia and Meschia, 1986).

During the normal course of events, the combination of increasing placental surface area, placental thinning and enhanced maternal and fetal placental blood flows keeps pace with the growth of the fetus and its nutritional and energy requirements (de Swiet et al., 1992). A similar balance may also exist between drug delivery and fetal clearance mechanisms such that fetal drug levels change little over gestation. Placental transfer of drugs diminishes when the surface area of placenta decreases and thickness increases. The first fetal organ to encounter the drugs that cross the placenta is the placenta itself. Thus, the placenta can exert a considerable first-pass metabolic effect on some drugs (Juchau and Dyer, 1972) and hence decrease fetal drug exposure.

The placenta expresses numerous transporters, which are mainly grouped into efflux and influx transporters. These transporters function to facilitate the transport of endogenous compounds across the placenta, exogenous compounds with structural resemblance to endogenous substrates (e.g., drugs and other xenobiotics) also interact with these transporters.

Therefore, if drugs are present in maternal blood, influx placental transporters may facilitate the transfer of these compounds from mother to fetus and lead to the toxicity of such drugs towards the fetus (Unadkat et al., 2004). Similarly, there are transporters in placenta that mediate the efflux of endogenous substrates from fetus to mother and these transporters may prevent entry of exogenous compounds into the fetal compartment by acting as a barrier (Unadkat et al., 2004). There are several drug transporters in the brush border (maternal-facing) membrane of the placenta. They are: P-Glycoprotein (MDR1), Placenta-specific ABC transporter (BCRP), multidrug resistance-related proteins (MRP1, MRP2, and MRP3), serotonin transporter (SERT), norepinephrine transporter (NET), carnitine transporter (OCTN2), equilibrative nucleoside transporter-1 (ENT1) and monocarboxylate transporters (MCTs). Several drug transporters are found in the basal membrane (fetal-facing) of the placenta: Reduced folate transporter (RFT-1), monocarboxylate transporters (MCTs), organic anion transporter-4 (OAT4) and organic anion transporting polypeptide-B (OATP-B) (reviewed by Ganapathy and Prasad, 2005). Multidrug resistance protein MDR1 and the breast cancer resistance protein BCRP are widely regarded as drug transporters because much is known about their role in drug transport. These two transporters mediate the efflux of substrates from the cell via coupling to ATP hydrolysis. Hydrolysis of ATP leads to export of the substrates. Thus, MDR1 and BCRP remove the substrates from the placenta and transfer them into maternal circulation. A wide variety of drugs including antivirals and chemotherapeutic agents are substrates for these transporters. Since MDR1 and BCRP are efflux transporters, they protect the developing fetus from exposure to these drugs (Young et al., 2003). Therefore, the location of P-gp in placenta helps to protect the fetus against drugs (Lankas et al., 1998).

Drug that is not metabolized by the placenta would then enter the umbilical vein where it encounters the next fetal organ, the fetal liver. Under normal conditions, about 40% of umbilical venous blood flow will bypass the liver via the ductus venosus to enter the thoracic inferior vena cava. However, Kumar et al. (1997) demonstrated in pregnant sheep that fetal hepatic first pass uptake of the antihistamine, diphenhydramine removes 44% of the drug delivered from the placenta via the umbilical vein. Under pathologic conditions, such as intrauterine growth restriction, first pass metabolism of the fetal liver may change due to the alteration in hepatic blood flow patterns (Bellotti et al., 2004). Other organs that are often rich in drug metabolic activity include kidneys, gut, and adrenal glands (Dutton and Leakey, 1981). Although the routes of fetal elimination are not fully elucidated, the rate of non-placental clearance of morphine,

methadone, acetaminophen, metoclopramide, ritodrine and diphenhydramine in the fetal lamb is comparable to that in the ewe (Szeto et al., 1982; Wang et al., 1986b; Riggs et al., 1987, 1990; Olsen et al., 1988; Wright et al., 1991; Kumar et al., 1999a, 1999b).

1.2.1 Drug metabolizing capacity in the fetus and neonate

Drug disposition in the fetus differs from that of adult because of the differences in absorptive, distributive, metabolic and excretory capacities. Metabolism occurs mainly in liver and non-hepatic organs (gut, CNS) and hepatic drug metabolism depends on the amount/concentration of unbound fraction of the drug, hepatic blood flow and the activity of drug metabolizing enzymes especially the phase I and phase II enzymes. There is a unique geometry of the fetal and neonatal circulations (arterial and venous shunts) and in liver function, especially synthesis of binding proteins and drug metabolizing enzymes.

1.2.2 Fetal renal drug excretion

The anatomic and functional immaturity of the kidney in pre-term and term neonates is another factor that can affect drug elimination, particularly for drugs excreted by the kidney (van den Anker, 1996). Only around 4% of fetal cardiac output goes to the fetal kidneys and glomerular filtration rate is low in term and especially in pre-term human infants and there are also reduced tubular secretory and reabsorptive capacities (van den Anker, 1996; Warner, 1986; Drukker, 2002). These features are also present in perinatal sheep (Wong et al., 2001; Smith and Lumbers, 1989; Lumbers, 1983). Nonetheless in spite of these functional immaturities, numerous drugs have been measured in fetal urine (Szeto, 1993) as unchanged drug and/or drug glucuronide metabolites. Thus, drugs can reach the amniotic fluid cavity by fetal urinary excretion and also via excretion of lung fluid (Riggs et al., 1987). It is also possible that drug may diffuse from amniotic fluid across the fetal chorionic membranes, and the fetal membranes overlying the chorionic plate of the placenta (Brace., 1995 and 2004; Adams et al., 2005). The role of these routes in determining the amniotic fluid concentration of drugs is not known, although in pregnant sheep diphenhydramine exits amniotic fluid largely via the intra-membranous pathway and fetal swallowing (Rurak et al, 1991).

1.2.3 Developmental anatomy of the liver

Fetal and adult livers differ anatomically (Healey and Sterling, 1963; Wilson et al., 1963; Edelstone et al., 1978; Bristow et al., 1981; deLemos and Kuehl, 1987) and biochemically (Battaglia, 1978; Jones and Rolph, 1985; Nakagawa and Setchell, 1990; Battaglia, 1992; Detmer et al., 1992; Devi et al., 1992). However the human fetal liver has a significant capacity for *in vitro* drug metabolism (Pelkonen, 1980; Krauer and Dayer, 1991; Carpenter et al., 1996). In the human fetus, liver growth is rapid, beginning in the fourth week of gestation and the basic liver elements and structure are formed by the end of the first trimester of gestation (Moore, 1982). Even though the left lobe is larger in size before birth, it changes after birth, and the lobes become similar in size in adults (Gray, 1980). The anatomy of the adult and fetal sheep livers is very similar to that of their human counterparts (Rudolph, 1983).

1.2.4 Hepatic circulation of fetus

The fetal hepatic circulation is substantially different from that of adult. The fetal liver has an extra input, the umbilical vein, which supplies the liver in addition to the hepatic portal vein and hepatic artery. The fetal hepatic artery supplies only about 10% of the total hepatic blood flow, compared with 25% in adult human and dog (Greenway and Stark, 1971) and 5% in adult sheep (Rosenfeld, 1977; Edelstone et al., 1978). The umbilical vein delivers 70% and the portal vein 20% of total hepatic flow (Rudolph, 1983) compared with the portal flow contribution around 75% in adult human and dog (Greenway and Stark, 1971) and 95% in the sheep (Rosenfeld, 1977; Edelstone et al., 1978). There is a large venous shunt, the ductus venosus, which diverts around 50% of umbilical venous flow past the liver and directly to the inferior vena cava (Edelstone et al., 1978). After birth this shunt closes gradually to result in the adult blood circulatory pattern (Zink and van Petten, 1980).

1.2.5 Hepatic lobe differences in metabolic activity

Recent work suggests that there is a difference in functional activity between right and left lobes of the fetal liver and this may be due to the difference in oxygenation of the lobes (Ring et al., 1998). Differences between the lobes with respect to metabolic activity were observed by Chianale et al. (1988) in mice; Cytochrome P450 (CYP) mRNA levels are higher in the left lobe than that of right in the fetal and in the immediate postnatal period liver in mice. This is also observed in the fetal sheep (Mihaly et al., 1982) due to more highly oxygenated

blood in the left lobe compared to the right lobe, this in turn being due to more umbilical venous blood perfusing the left lobe (Ring et al., 1998). There also are differences between the lobes with respect to metabolic activity. For example, levels of malondialdehyde (an index of lipid peroxidation), oxygen consumption and cytochrome P450, are higher in the left lobe than the right lobe of the fetal sheep liver, whereas there are no differences in these levels between the lobes of the adult sheep liver (Bristow et al., 1983; Rudolph, 1983; Germain et al., 1987). During acute hypoxemia in the fetal lamb, there are decreases in hepatic blood flow and oxygen consumption with greater changes in the right compared to left lobes (Bristow et al., 1983). Thus, fetal hypoxia could adversely affect the drug metabolizing capacity of the fetal liver and thereby increase fetal drug exposure, a phenomenon that is observed even with the mild hypoxia in adult rats with propranolol metabolism (Jones et al., 1984; Elliott et al., 1993). However, the effect of hypoxia of fetal drug disposition does not appear to have been examined.

1.2.6 Development of hepatic drug metabolic enzymes

In the adult liver, constitutive CYP enzymes predominate in the perivenous hepatocytes, where they are associated with large amounts of hepatocyte smooth endoplasmic reticulum (SER). This pattern of localization appears to develop only after birth (Chianale et al., 1988; Murray et al., 1992; Okajima et al., 1993; Watanabe et al., 1993; Kitada and Kamataki, 1994). For example, in the human fetal liver, CYP1A was shown to be expressed along the entire length of the acinus, but after birth, it was observed only in the perivenous zone (Murray et al., 1992). This pattern of development has also been demonstrated for CYP3A (Ratanasavanh et al., 1991) and NADPH-CYP 450 reductase (Kanai et al., 1992). In the adult liver, glutathione *S*-transferase and sulfotransferases (STs) are localized predominantly in the periportal area of the acinus, whereas glucuronosyl transferases (GTs) are located predominantly perivenously (Gebhardt, 1992). While there is *in vitro* evidence demonstrating the activity of these enzymes in mammalian fetal liver, no studies show their precise location (Pacifci et al., 1988, 1989, 1993; Barker et al., 1994; Gilissen et al., 1994).

The rate and pattern of postnatal CYP enzyme development may have a significant impact on therapeutic efficacy and toxicant susceptibility in the newborn and developing infant. Significant drug metabolism is now recognized to occur in the mammalian fetus, mostly by a range of Phase I and Phase II processes (Juchau et al., 1980; Pelkonen, 1980; Krauer and Dayer, 1991; Rurak et al., 1991; Raucy and Carpenter, 1993). Although considerable *in vitro* data are

available on the expression and activity of human fetal drug-metabolizing enzymes (Pacifci et al., 1982; Perucca, 1987; Burchell et al., 1989; Krauer and Dayer, 1991; Jacqz-Aigrain and Cresteil, 1992; Hakkola et al., 1994; Cazeneuve et al., 1994; Shimada et al., 1996), the *in-utero* role of these enzymes in drug metabolism and the resulting effects on drug and metabolite exposure in the human cannot be examined in detail due to obvious practical and ethical limitations. There are numerous studies that demonstrate the presence of drug metabolizing enzymes in human fetus and neonate (Hines and McCarver, 2002; McCarver and Hines, 2002) and the ontogenetic differences among various phase I and phase II drug metabolizing enzymes. But the few studies in sheep have shown the in utero functional development of fetal drug metabolism such as propranolol (Mihaly et al., 1982), labetalol (Yeleswaram et al., 1993), ritodrine (Wright et al., 1991), acetaminophen (Wang et al., 1986b), morphine (Olsen et al., 1988), and valproic acid (Kumar et al., 2000a; Wong et al., 2000b).

The extent to which fetal hepatic drug metabolizing capabilities develop qualitatively and quantitatively and the ontogeny of these systems remains poorly understood (Chauhan et al., 1991; Miners and Mackenzie, 1991; Raucy and Carpenter, 1993; Coughtrie et al., 1994). The concentration of hepatic microsomal protein of sheep fetal liver is significantly lower than that of maternal liver during early pregnancy, but it increases in concentration as gestation progresses thus suggesting that the increase of endoplasmic reticulum is a function of developmental maturation (Homeida et al., 1993). Similar results were obtained in postnatal goat liver (El Sheikh et al., 1988). In vitro assessments in the human have revealed significantly lower levels of CYP enzyme protein and activity in the fetus (total CYP enzyme protein levels are one-third adult levels) (Cresteil et al., 1985; Shimada et al., 1996, Treluyer et al., 1996).

1.3 Drug metabolic enzymes

Drugs that are administered to humans or animals undergo enzymatic conversion. Most of the drugs are converted from lipophilic chemical compounds into more readily excreted polar products to facilitate their excretion in urine or bile. However some drugs are eliminated intact. Usually, drug metabolism generates metabolites that are inactive and relatively non-toxic; however, metabolites may occasionally be the source of toxic effects. Drug metabolism occurs in several organs such as liver, gut, brain, lung and kidney. Among these, liver contributes to a major portion.

Hepatic drug metabolism of xenobiotics occurs mainly by the actions of phase I and phase II drug metabolizing enzymes (Ionescu and Caira, 2005). Some drugs undergo phase I or phase II metabolism, however most of the drugs undergo phase I and then phase II metabolism sequentially. Phase I reactions introduce a functional group to the parent drug and this process occurs mainly by oxidation and also by hydrolysis, reduction and hydration (Ionescu and Caira, 2005). Cytochrome P450 (CYP) is the major enzyme family involved in phase I metabolism and responsible for metabolism of most of the drugs and endogenous compounds. In addition, flavin-containing monooxygenases (FMO), alcohol dehydrogenases, aldehyde dehydrogenases and monoamine oxidases also participate in xenobiotic oxidation reactions. Further, NADPH-cytochrome P450 reductases are involved in phase I metabolism at a lower level through reduction reactions. Phase II reactions conjugate a water soluble entity such as acetate, sulfate or glucuronate onto a parent drug or a phase I metabolite at newly created or preexisting sites (Ionescu and Caira, 2005). Phase II metabolism occurs predominantly through glucuronidation and also by sulfation, glutathione conjugation, and acetylation. Even though UDP-glucuronosyltransferase (UGTs) is the major enzyme group, sulfotransferases, glutathione S-transferases, N-acetyltransferases and amino acid N-acyltransferases also play a minor role in phase II metabolism.

1.3.1 Cytochrome P450

Cytochrome P450 enzymes are a very large super family of heme proteins and are primarily membrane-bound proteins. P450 enzymes give a unique 450nm absorption peak, accounting for the name cytochrome P450 (Omura, 1999). CYP enzymes catalyze aromatic hydroxylation, aliphatic hydroxylation, N-, O- and S-dealkylation, N-hydroxylation, N-oxidation, sulfoxidation, deamination and dehalogenation (Ghosal et al., 2005). In addition to drug metabolism, CYPs are involved in vascular auto regulation, particularly in the brain (Gebremedhin et al., 2000). Furthermore, CYPs are vital to the formation of cholesterol, steroids and arachidonic acid metabolites such as prostaglandins, prostacyclins and thromboxanes (De Montellano, 1986; Guengerich, 1991; Porter and Coon, 1991; Nebert, 1991). They also play important roles in the metabolism of steroids and vitamin D. To cope with the wide diversity of substrates, numerous isoforms of cytochrome P450 exist and display overlapping substrate specificity (Nelson et al., 1993). CYP isoforms are involved in drug interactions that generally result from enzyme inhibition or enzyme induction. Mutations in CYP genes or deficiencies of

the enzymes are responsible for several human diseases (Okuyama et al., 1996). Moreover, some CYP enzymes mediate cancers by converting the pro-carcinogens into carcinogens (Yamazaki et al., 1995; Shimada et al., 1994a).

1.3.1.1 Nomenclature of cytochrome P450

To date, approximately 4000 CYP enzymes have been identified in different species (Denisov et al., 2005; Lewis, 1996; Nelson et al, 1996, 2004). Since, there is a wide range of CYP enzymes discovered by different investigators in different time periods, it is very important to impose a systematic classification. The nomenclature of the CYP super family has been developed based on the degree of similarity of their primary protein structure (Nebert et al, 1987). The nomenclature of the CYP enzymes designated with the abbreviation “CYP”, followed by an Arabic numeral indicating the gene family (eg:- CYP1, CYP2, CYP3), a capital letter indicating the subfamily (eg:- CYP2A, CYP2B, CYP2C), and another number for the individual gene (CYP2C8, CYP2C9, CYP2C18) (Nelson et al., 1996). For example, the enzyme belonging to the CYP family 2, subfamily D and individual enzyme 6 is denoted as CYP2D6. According to the classification, CYP enzymes with amino acid homology greater than 40% were assigned to the same family (Nelson et al., 1993, 1996). When the enzymes exhibited greater than 55% amino acid homology within a family, they were grouped as same subfamily (Nelson et al., 1993, 1996).

In humans, 57 CYP genes have been identified and they belong to 18 families and 43 subfamilies (Hasler et al., 1999; Hoffman et al., 2001; Ingelman-Sundberg, 2004) (Table 1.1). In addition, more than 50 pseudo CYP genes have been discovered. CYP1, CYP2, CYP3 and CYP4 are the major CYP enzyme families that participate in most of the drug metabolizing reactions (Rendic and Di Carlo, 1997; Rendic, 2000; Lewis, 2001). Other CYP families such as CYP5, CYP7, CYP8, CYP17, CYP24, CYP27 and CYP51 participate in the synthesis and catabolism of endogenous compounds (Lewis, 2004).

1.3.1.2 Distribution of CYPs

CYP enzymes are present in most organisms including mammals, birds, fishes, reptiles, amphibians, insects, plants, worms, fungi and bacteria (Guengerich et al., 2005). In vertebrates, they are found in several tissues such as liver, intestine, lung, kidney, stomach, brain, adrenal glands, gonads, heart, nasal mucosa and skin (Krishna and Klotz, 1994; Ding and Kaminsky, 2003; Pavek and Dvorak, 2008). The concentration and amount of CYP enzyme vary with different tissues (Table 1.2). Liver contains the largest amount of CYP enzymes which

participate in drug metabolism (Gonzalez, 1990). Smooth endoplasmic reticulum is the organelle that contains the highest amount of CYP enzymes. When CYP enzymes are isolated from tissues, they re-form into vesicles called microsomes. In rats, total microsomal protein represents 1.8-2% of the liver wet weight (Lewis, 1996) and CYP enzymes comprise 4-6% of total microsomal protein, giving a specific content of 0.8-1.1 nmol of total CYP per milligram of microsomal protein. However, in the human, CYP content of liver is lower, comprising 1.5-3% of total microsomal protein (Table 1.2).

According to Ivan et al. (2007), 12 CYP enzymes are predominantly hepatic among the 23 CYP isoforms studied; CYP1A2, 2A6, 2A7, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4 and 3A43. Interestingly, based on mRNA data, CYP1B1 was the predominant CYP in seven extrahepatic tissues (bone marrow, kidney, mammary gland, prostate, spleen, thyroid and uterus) and CYP2J2 in four extrahepatic tissues (heart, placenta, salivary gland and skeletal muscle). Some CYPs are expressed in a single extra hepatic tissue exclusively; CYP2R1 in testis, CYP2U1 in thymus and CYP2F1 in the respiratory tract (lung and trachea) (Ivan et al., 2007).

The amount of individual CYP enzymes differs greatly in liver microsomes (Table 1.3). CYP3A4 is the most predominant CYP enzyme in human liver and intestines (Wrighton and Stevens, 1992). There is significant inter-individual variation in the level of CYP enzymes due to the allelic variation exhibiting gene polymorphisms and, also, that resulting from differences in regulatory mechanisms of CYP gene expression (Guengerich, 1995). Table 1.4 shows different CYP genes and proteins identified in different tissues in human.

1.3.2 CYP2 family enzymes

Within the P450 super family, the CYP2 family of enzymes is the largest and most diverse (Nelson et al., 1993), as it contains many isoforms such as CYPs 2A6, 2A7, 2A13, 2B6/2B7, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1 2F1, 2J2, 2R1, 2S1, 2U1. The members of this family are mainly expressed in the liver, yet many are reported in extra hepatic tissues as well. In human adult liver CYPs 2A6/2A7, 2B6/2B7, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1 are expressed while CYP2F1 is absent (Hakkola et al., 1994). However significant differences in expression are observed in the individual enzymes of this family. Some CYP enzymes of this family are involved in the metabolism of endogenous substrates as well (see review Henderson and Wolf, 1992).

Table 1.1: CYP enzymes in humans

Family	Subfamily	Individual enzymes
CYP1	CYP1A	CYP1A1, CYP1A2
	CYP1B	CYP1B1
CYP2	CYP2A	CYP2A6, CYP2A7, CYP2A13
	CYP2B	CYP2B6
	CYP2C	CYP2C8, CYP2C9, CYP2C18, CYP2C19
	CYP2D	CYP2D6
	CYP2E	CYP2E1
	CYP2F	CYP2F1
	CYP2J	CYP2J2
	CYP2R	CYP2R1
	CYP2S	CYP2S1
	CYP2U	CYP2U1
	CYP2W	CYP2W1
CYP3	CYP3A	CYP3A4, CYP3A5, CYP3A7, CYP3A43
CYP4	CYP4A	CYP4A9, CYP4A11
	CYP4B	CYP4B1
	CYP4F	CYP4F2, CYP4F3, CYP4F8, CYP4F11, CYP4F12
	CYP4V	CYP4V2
	CYP4X	CYP4X1
	CYP4Z	CYP4Z1
CYP5	CYP5A	CYP5A1
CYP7	CYP7A	CYP7A1
	CYP7B	CYP7B1
CYP8	CYP8A	CYP8A1
	CYP8B	CYP8B1
CYP11	CYP11A	CYP11A1
	CYP11B	CYP11B1, CYP11B2
CYP17	CYP17	CYP17
CYP19	CYP19	CYP19
CYP20	CYP20	CYP20

Family	Subfamily	Individual enzymes
CYP21	CYP21A	CYP21A1
CYP24	CYP24	CYP24
CYP26	CYP26A	CYP26A1
	CYP26B	CYP26B1
	CYP26C	CYP26C1
CYP27	CYP27A	CYP27A1
	CYP27B	CYP27B1
	CYP26C	CYP27C1
CYP39	CYP39	CYP39
CYP46	CYP46	CYP46
CYP51	CYP51	CYP51

Source: Adapted from Nelson, 2006 (<http://drnelson.utmem.edu/CytochromeP450.html>).

Table 1.2: Total CYP Content in Various Human and Rat Organs

Organ	Human (nmol/mg microsomal protein)	Rat (nmol/mg microsomal protein)
Liver	0.30-0.60	0.8-1.1
Adrenal	0.23-0.54	0.5
Small Intestine	0.03-0.21	0.02-0.13
Kidney	0.03	0.05-0.2
Lung	0.01	0.035-0.05
Brain	0.10	0.025-0.05
Testis	0.005	0.07-0.12
Skin	not determined	0.05
Mammary	<0.001	0.001-0.003

Taken from Hrycay and Bandiera, 2008.

Table 1.3: CYP Enzyme Levels in Human Liver Microsomes

CYP enzyme	Content (pmol CYP/mg microsomal protein)	Percentage of total CYP
CYP1A1	n.d.	0
CYP1A2	<1-45	<0.5-15
CYP1B1	n.d.	0
CYP2A6	<1-68	4-17
CYP2B6	1-39	<1-7
CYP2C8	1-60	12-15
CYP2C9	9-100	14-18
CYP2C18	<1	<0.5
CYP2C19	0.5-19	1-17
CYP2D6	1.5-10	1-4
CYP2E1	7-49	3-9
CYP3A4	40-140	18-29
CYP3A5	<1	<0.5
CYP4A11	not determined	10

CYP enzyme levels were measured in individual human liver samples by immuno quantification.

Values represent the approximate range of CYP levels reported. N.d., not detected.

Taken from Hrycay and Bandiera, 2008.

Table 1.4: CYP Genes Expressed in Various Human Organs

Organ	CYP genes expressed
Liver ^a	<i>Protein:</i> 1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2J2, 2S1, 3A4, 3A5, 3A7, 3A43, 4A11, 4F2, 4F8, 4F11, 4F12, 5A1, 7A1, 8B1, 27A1, 51A1 <i>mRNA:</i> 1A1, 1A2, 1B1, 2A6, 2A7, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 2J2, 2R1, 2S1, 2U1, 3A4, 3A5, 3A7, 3A43, 4A11, 4A22, 4F2, 4F3B, 4F11, 4F12, 4F22, 4X1, 4Z1, 5A1, 7B1, 8A1, 8B1, 17A1, 20A1, 26A1, 26C1, 27A1, 27B1, 39A1, 46A1, 51A1
Small intestine ^a	<i>Protein:</i> 1A1 (5.6), 2C9 (8.4), 2C19 (1), 2D6 (0.5), 2J2 (0.9), 3A4 (43), 3A5 (16) <i>mRNA or protein:</i> 1B1, 2A13, 2B6, 2C8, 2C18, 2E1, 2R1, 2S1, 2U1, 2W1, 3A7, 4A11, 4B1, 4F2, 4F3A, 4F3B, 4F8, 4F12, 4F22, 4X1, 5A1, 7B1, 8A1, 8B1, 19A1, 24A1, 26A1, 26B1, 27A1, 27B1, 39A1, 46A1, 51A1
Nasal mucosa	2A6, 2A13, 2B6, 2C ^b , 2J2, 2S1, 3A ^b
Trachea	1A1, 1B1, 2A6, 2A7, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 2J2, 2R1, 2S1, 2U1, 2W1, 3A5, 3A7, 4F3A, 4F3B, 4X1
Lung	1A1, 1A2, 1B1, 2A6, 2A7, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 2J2, 2R1, 2S1, 2U1, 2W1, 3A4, 3A5, 3A7, 4A11, 4B1, 4F2, 4F8, 4X1, 5A1, 7B1, 8A1, 8B1, 19A1, 24A1, 26A1, 26C1, 27A1, 27B1, 39A1, 46A1, 51A1
Esophagus	1A1, 1A2, 2A ^b , 2E1, 2J2, 2S1, 3A5
Salivary gland	1A1, 2A13, 2D6, 2E1, 2J2, 2R1, 2S1, 2U1
Stomach	1A1, 1A2, 2C ^b , 2J2, 2S1, 2U1, 3A4, 4F8, 4F12
Colon	1A1, 1A2, 1B1, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2J2, 2R1, 2S1, 2U1, 2W1, 3A4, 3A5, 4B1, 4F12, 4X1, 7B1, 24A1, 26B1, 27B1, 51A1
Kidney	1A1, 1B1, 2A6, 2A13, 2B6, 2C8, 2C9, 2C18, 2D6, 2E1, 2J2, 2R1, 2S1, 2U1, 2W1, 3A4, 3A5, 3A7, 3A43, 4A11, 4A22, 4B1, 4F2, 4F3B, 4F8, 4F11, 4F12, 4F22, 4X1, 4Z1, 5A1, 7B1, 8A1, 8B1, 17A1, 24A1, 26B1, 27A1, 27B1, 39A1, 46A1, 51A1
Heart	1A1, 1B1, 2A6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2J2, 2R1, 2S1, 2S1, 2U1, 4A11, 4B1, 4F11, 4F12, 4X1, 7B1, 8A1, 11A1, 26A1
Brain	1A1, 1B1, 2E1, 2A6, 2A7, 2A13, 2B6, 2C8, 2C9, 2C18, 2D6, 2E1, 2J2, 2R1, 2S1, 2U1, 3A4, 3A5, 3A7, 4A11, 4F2, 4F11, 4F22, 4X1, 5A1, 7B1, 8A1, 8B1, 19A1, 20A1, 24A1, 26A1, 26B1, 26C1, 27A1, 27B1, 39A1, 46A1, 51A1
Skin	1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2J2, 2S1, 2U1, 2W1, 3A4, 3A5, 4A11, 4B1, 4F2, 4F8, 4F12, 4F22, 4X1, 11A1, 17A1, 21A2, 24A1, 26A1, 26B1, 26C1, 27A1, 27B1
Spleen	1A1, 1B1, 2A13, 2B6, 2C8, 2D6, 2E1, 2J2, 2R1, 2S1, 2U1, 2W1, 4B1, 4X1, 5A1, 26B1, 26C1, 27A1, 51A1
Thymus	1A1, 1B1, 2A13, 2C8, 2C18, 2D6, 2E1, 2J2, 2R1, 2S1, 2U1, 2W1, 3A4, 3A5, 4B1, 4X1, 5A1, 26A1, 26B1, 51A1
Pancreas	1A1, 1B1, 2E1, 2J2, 2R1, 2S1, 2U1, 2W1, 4B1, 4X1, 7B1, 26A1, 26B1, 27B1

Organ	CYP genes expressed
Skeletal muscle	1A1, 1B1, 2A6, 2A7, 2B6, 2C8, 2C9, 2C18, 2D6, 2E1, 2J2, 2R1, 2S1, 2U1, 3A4, 3A5, 4B1, 4F11, 4F22, 4X1, 4Z1, 8A1
Eye	1B1, 4B1, 4F8, 4V2
Thyroid	1A1, 1B1, 2A13, 2C8, 2C18, 2D6, 2E1, 2J2, 2R1, 2S1, 2U1, 3A5
Adrenal	1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2D6, 2E1, 2J2, 2R1, 2S1, 2U1, 3A4, 3A5, 3A7, 4A11, 4B1, 4F8, 5A1, 7B1, 8A1, 8B1, 11A1, 11B1, 11B2, 17A1, 19A1, 21A2, 26A1, 27A1, 27B1, 39A1, 46A1, 51A1
Prostate	1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C18, 2D6, 2E1, 2J2, 2R1, 2S1, 2U1, 2W1, 3A4, 3A5, 3A7, 3A43, 4A11, 4B1, 4F2, 4F3A, 4F3B, 4F8, 4F12, 4X1, 5A1, 7B1, 8A1, 8B1, 11A1, 19A1, 24A1, 26B1, 27A1, 27B1, 39A1, 46A1, 51A1
Urinary bladder	1A1, 1B1, 2D6, 2E1, 2J2, 2R1, 2S1, 2U1, 2W1, 3A5, 3A7, 3A43, 4B1, 4F8
Testis	1A1, 1A2, 1B1, 2A6, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 2J2, 2R1, 2S1, 2U1, 2W1, 3A4, 3A5, 3A7, 3A43, 4A11, 4B1, 4F2, 4F8, 4F22, 4X1, 5A1, 7B1, 8A1, 8B1, 11A1, 17A1, 19A1, 24A1, 26A1, 26B1, 26C1, 27A1, 27B1, 39A1, 46A1, 51A1
Ovary	1A1, 1B1, 2A6, 2A13, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2F1, 2J2, 2R1, 2S1, 2U1, 2W1, 3A4, 3A5, 3A7, 3A43, 4F11, 4F12, 4V2, 4X1, 4Z1, 7B1, 8A1, 11A1, 17A1, 19A1, 24A1, 26A1, 26B1, 26C1, 39A1, 51A1
Uterus	1A1, 1A2, 1B1, 2A6, 2A7, 2B6, 2C8, 2C18, 2D6, 2E1, 2J2, 2R1, 2S1, 2U1, 3A4, 3A5, 4A11, 4B1, 4F8, 4X1, 5A1, 7B1, 8A1, 8B1, 11A1, 17A1, 19A1, 24A1, 26A1, 27A1, 27B1, 39A1, 46A1, 51A1
Mammary	1A1, 1B1, 2A6, 2A7, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2J2, 2R1, 2S1, 2U1, 3A4, 3A5, 4B1, 4X1, 4Z1, 24A1, 27B1
Placenta	1A1, 1B1, 2A6, 2A7, 2A13, 2C8, 2C9, 2C18, 2D6, 2E1, 2F1, 2J2, 2R1, 2S1, 2U1, 2W1, 3A4, 3A5, 3A7, 4A11, 4B1, 4F11, 4F12, 4F22, 4X1, 5A1, 7B1, 8A1, 8B1, 11A1, 17A1, 19A1, 24A1, 26A1, 26B1, 27A1, 27B1, 39A1, 46A1, 51A1

^a CYP genes in liver and small intestine were detected by mRNA or protein expression while CYP genes in other organs were detected mainly by mRNA expression. CYP enzyme levels in small intestine were determined by immuno blot analysis using intestinal microsomes from 31 individuals. The numbers shown in parentheses are mean CYP enzyme levels, expressed as pmoles CYP/mg of intestinal microsomal protein. CYP1A1 was detected in 3, and CYP3A5 was detected in 11, of the 31 samples while CYPs 1A2, 2A6, 2B6, 2C8 and 2E1 were below the limit of detection (Lewis, 1996; Shimada et al., 1994b).

^b In cases where the precise identity of the CYP gene/enzyme is unknown, only the CYP subfamily is shown.

(Taken from Hrycay and Bandiera, 2009)

1.3.3 CYP2A6

The CYP2A subfamily consists of three isoforms, CYP2A6, CYP2A7 and CYP2A13 in human. Even though CYP2A6 represents a relatively minor component (~4%) of the human CYPs, it is a highly important CYP member because it metabolizes a number of clinically used drugs, several procarcinogens, such as nitrosamines (Topcu et al., 2002) and toxicants (Liu et al., 1996).

Levels of hepatic CYP2A6 mRNAs and proteins are well characterized (Pelkonen et al., 1993; Fernandez-Salguero and Gonzalez, 1995). All three CYP2A genes are transcribed in human liver (Ivan et al., 2007). An approximate 1:1 ratio of CYP2A6 and CYP2A7 mRNAs in human liver was reported (Ding et al., 1995; Koskela et al., 1999). CYP2A13 mRNA is present at negligible levels in the liver.

In the human liver, there is mRNA and protein expression of CYP2A6 (Shimada et al., 1996; Nishimura et al., 2003) while fetal liver fails to express CYP2A6 protein, enzyme activity and mRNA (Maenpaa et al., 1993; Shimada et al., 1996; Gu et al., 2000; Hakkola et al., 1994) (Table 1.5). In contrast, other studies have reported gene expression of this enzyme in the fetus (Nagata et al., 2003; Ivan et al., 2007) although expression is 0.01 that of the expression in the adult (Nishimura et al., 2003). Moreover, low protein levels are observed in the perinatal period with adult capacities achieved only after the first year of life (Tateishi et al., 1997). CYP2A6, CYP2A13, have been detected in fetal nasal mucosa (Walters et al., 1993). In addition, mRNA and protein levels of CYP2A6 and CYP2A13 were detected in nasal mucosa of 13 to 18 weeks old human fetuses. However, fetal levels were only 1 to 5% of adult levels and tended to be higher in the older samples (Gu et al., 2000). Additional studies are needed to clarify the CYP2A hepatic and extrahepatic developmental expression pattern.

CYP2A6 catalyzes the hydroxylation of coumarin (Miles et al., 1990; Pelkonen et al., 1993) and the resulting metabolite, 7-hydroxycoumarin, is excreted in adults and in children of 6-13 years of age similarly (Pelkonen et al., 1997). This coumarin 7 hydroxylase activity serves as a marker for this enzyme. In addition, it metabolizes methoxyflurane (Kharasch et al., 1995), nicotine (Nakajima et al., 1996a) and cotinine (Nakajima et al., 1996b). CYP2A6 enzyme also mediates the oxidative metabolism of several procarcinogens, which primarily target the liver. In human hepatocellular carcinoma samples, over expression of CYP2A6 protein was found to be associated with the presence of chronic inflammation and cirrhosis (Raunio et al., 1997).

1.3.4 CYP2C

The CYP2C subfamily is an important class of enzymes composed of four highly homologous isoforms (CYP2C9, CYP2C19, CYP2C8, and CYP2C18) in the human. It accounts for about 18% of the total adult liver cytochrome P450 content (Shimada et al., 1994b). In adult human, CYP2C9 comprises the major portion, followed by CYP2C19 and CYP2C8 (Goldstein et al., 1994; Edwards et al., 1998). But CYP2C19 represents the dominant enzyme in most prenatal samples (Koukouritaki et al., 2004). There is more than 80% identity among the CYP2C isoforms in terms of amino acid sequence.

Adult human expresses CYP2C8, CYP2C9, CYP2C18 and CYP2C19 genes (Hakkola et al., 1994) and proteins (Hines, 2007) in liver. Several human studies showed that the fetal liver expresses low levels of CYP2C9 (Nagata et al., 2003; Nishimura et al., 2003; Ivan et al., 2007), CYP2C19 (Nishimura et al., 2003) and CYP2C8 mRNA (Hakkola et al., 1994; Nishimura et al., 2003) (Table 1.5). In contrast, a few studies demonstrated that gene expression of CYP2C9 (Hakkola et al., 1994), CYP2C19 (Hakkola et al., 1994; Ivan et al., 2007), CYP2C8 (Ivan et al., 2007) and CYP2C18 (Ivan et al., 2007) is undetectable in the fetus.

However, CYP2C9 protein was not detected in fetal liver by immuno-blotting or by hydroxylation of tolbutamide, a marker for CYP2C9 activity (Shimada et al., 1996; Treluyer et al., 1997). Enzyme levels and parallel activity were extremely low in newborn, greater in the first month after birth and reached the adult levels by one year of age. This suggests that CYP2C maturation largely depends on postnatal factors (Treluyer et al., 1997). Protein expression of CYP2C19, CYP2C8 and CYP2C18 was also undetectable in fetal liver (Oesterheld, 1998). However, a few recent studies detected low CYP2C9 and CYP2C19 protein levels and enzyme activity in many fetal samples (Koukouritaki et al., 2004; Hines, 2007). This was supported by a few other studies (Ratanasavanh et al., 1991; Maenpaa et al., 1993). CYP2C9-specific content was significantly greater in one month old neonates than in fetuses (older than 24 weeks of gestation), suggesting that increased postnatal CYP2C9 expression is linked to birth. In contrast, CYP2C19 protein levels were not significantly greater in the neonates when compared to fetuses. CYP2C9 and CYP2C19-specific content was dissimilar among most gestational age samples. Koukouritaki et al., (2004) showed that the measurements of both CYP2C9-specific content and catalytic activity during the first trimester of fetal development are consistent with hepatic expression of this enzyme at 1 to 2% of mature values with progressive increases in expression during the second and third trimesters to levels approximately 30% of mature values. CYP2C19

protein and catalytic activities were found to be 12 to 15% of mature values in hepatic tissue samples as early as 8 weeks of gestation. There might be several contributors to the inconsistent results between the above two reports (Treluyer et al., 1997; Koukouritaki et al., 2004) including sample size within specific age brackets, sample quality, and assay sensitivity. Also the study performed by Treluyer et al. (1997) did not describe the developmental expression pattern of individual CYP2C isoforms.

Increases in CYP2C9 and CYP2C19 expression occur in the first few weeks after birth, irrespective of gestational age (Ratanasavanh et al., 1991; Treluyer et al., 1997). Within the first month of postnatal life, CYP2C enzyme activity surges to 50% adult levels (Treluyer et al., 1996, 1997) and declines slightly for the first year of life and adult levels are reached sometime after one year of age (Treluyer et al., 1996, 1997; Tateishi et al., 1997; Koukouritaki et al., 2004). Expression levels during five months to eighteen years were greater than for other age groups (Koukouritaki et al., 2004). The postnatal CYP2C19 expression pattern was dissimilar to that of CYP2C9, with no significant change in expression between the fetal and immediate neonatal periods. CYP2C19 protein values increased somewhat linearly over the first five months of postnatal age and were highly variable from five months to ten years. Further they observed a significant but modest increase in CYP2C19-specific content from ten years through eighteen years postnatal age that approached previously reported adult values (Koukouritaki et al., 2004). Nagata et al. (2003) demonstrated that CYP2C9 gene expression increased by thirteen fold postnatally (5-65 years).

Members of the CYP2C subfamily account for the metabolism of about 20% of clinically important drugs (Goldstein, 2001), including the anticoagulant warfarin (Kaminsky and Zhang, 1997; Takahashi et al., 2000), the H₂ receptor antagonist omeprazole (Andersson et al., 1993; Kearns et al., 2003), the antiepileptic agent phenytoin (Bajpai et al., 1996; Loughnan et al., 1977; Giancarlo et al., 2001), diazepam (Treluyer et al., 1997), the antihyperglycemia sulfonylureas tolbutamide (Wester et al., 2000) and glipizide (Kidd et al., 1999), many nonsteroidal anti-inflammatory agents (Leemann et al., 1993; Yasar et al., 2001; Koukouritaki et al., 2004) and endogenous compounds such as arachidonic acid (Capdevila et al., 1992).

CYP2C8, CYP2C9, and CYP2C19 have been reported to be inducible in humans by prior exposure to drugs, leading to another possible source of variability in the metabolism of substrates (Gerbal-Chaloin et al., 2001; Raucy et al., 2002). Elevated expression of CYP2C19 was observed in fetuses with maternal administration of glucocorticoids which was given to

enhance fetal lung maturation during preterm labor (Chen et al., 2003). In utero and/or postnatal exposure to certain exogenous or endogenous compounds may cause rapid enzyme induction or inhibition in the fetus, newborn and infant (Morselli, 1989). This will further exacerbate the variable rate and pattern of enzyme maturation. For example, newborns treated concomitantly with barbiturates, a CYP2C inducer (Treluyer et al., 1997), exhibited a marked reduction in diazepam (a CYP2C substrate) half-lives as compared with newborns treated with diazepam alone (Perrot et al., 1989; Treluyer et al., 1997). Transcriptional regulation of both CYP2C9 and CYP2C19 is known to involve the constitutive androstane receptor (CAR) and pregnane X receptor (PXR) as well as the glucocorticoid receptor (GR) (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002; Chen et al., 2003).

All members of CYP2C subfamily show genetic polymorphisms. These polymorphisms have clinical consequences resulting in toxicity of some drugs in the affected individual, and may alter the efficacy of other drugs. Moreover, significant drug interactions have been demonstrated in individuals with polymorphisms in CYP2C enzymes. With CYP2C19, two predominant null alleles and at least seven different inactivating mutations are present (Goldstein, 1996; Ferguson et al., 1998; Ibeanu et al., 1998a, 1998b). Rare defective alleles have been described for CYP2C9 (Rettie et al., 1994; Sullivan-Klose et al., 1996). Polymorphisms have also been described for CYP2C18 (Komai et al., 1996; Tsuneoka et al., 1996) and recently for CYP2C8 as well (Dai et al., 2000).

A higher hepatic content of CYP2C isoenzymes is found in infants dying from sudden infant death syndrome (SIDS), which exceeded adult values as early as one month of age (Treluyer et al., 1996). It was also associated with increased production of epoxyeicosatrienoic and dihydroxyeicosatrienoic acids from arachidonic acid (Treluyer et al., 1996, 2000). As these compounds are vasodilators, it has been suggested that the increased expression of CYP2C could be involved in the pathogenesis of this condition (Treluyer et al., 2000). It is also suggested that since epoxyeicosatrienoic acids act as a relaxant of pulmonary smooth muscles in neonates, the consequence might be the induction of fatal respiratory depression or apnea which leads to SIDS.

1.3.5 CYP2D6

Human CYP2D6 is known to play a major role in the metabolism of a wide range of clinically important drugs (Daly et al., 1993), although it contributes only 2% of total adult

hepatic CYP P450 (Shimada et al., 1994b). Even though it is mainly expressed in the liver, expression of CYP2D6 has been identified in extrahepatic tissues (Ivan et al., 2007) as well. A single report also has described the expression of CYP2D6 in human fetal brain (Gilham et al., 1997).

Both CYP2D6 protein and gene are expressed in adult human liver (Table 1.5). Previous studies have shown the expression of gene at the mRNA level (Hakkola et al., 1994; Ivan et al., 2007) and protein level (Treluyer et al., 1991; Jacqz-Aigrain et al., 1993) in the fetus but at a lower level. Only 15% of the adult level was observed in about 50% of the fetal samples that were obtained at greater than thirty weeks gestational age (Treluyer et al., 1991). In contrast, a few other studies have demonstrated that the protein and the activity of enzyme are not detectable until birth (Ladona et al., 1991; Shimada et al., 1996). Further work is needed to clarify this issue. The enzyme level rises dramatically after birth (Hines and McCarver 2002; Treluyer et al 1991; Oesterheld 1998), and this is unrelated to the gestational age at birth (Treluyer et al., 1991). This suggests that factors that are present during parturition might participate in the rise of CYP2D6. It has also been suggested that its expression in the fetus might be repressed by maternal hormones, since the CYP2D6 protein is absent until the first week after birth (Treluyer et al., 1991). In the newborn period a developmentally determined low level of enzyme activity occurs, independent of genotype that results in all neonates being poor metabolizers. Hence, the clearance of CYP2D6 substrates is expected to be low in the neonatal period, thus requiring the individualization of dosing to prevent drug accumulation and toxicity. CYP2D6 protein increases progressively in postnatal life. At four weeks, levels of activity are 20% of those in adults and reach two-thirds of the adult level at ~5 years (Treluyer et al., 1991). Available data suggest that the adult CYP2D6 catalytic activity is not fully developed until at least 10 years of age.

CYP2D6 mediates the metabolism of several drugs, including antitussives, antihypertensives, tricyclic and nontricyclic antidepressants, β -blockers, anti-arrhythmic drugs and other compounds such as codeine, cinnarizine, deprenyl, captopril and ondansetron (Rogers, 1994; Parkinson, 1996).

1.3.6 CYP2E1

CYP2E1 enzyme comprises ~7% of total hepatic CYP enzymes in human. It is involved in the metabolism of several drugs (acetaminophen, isoniazid) and halogenated anesthetics

(halothane and enflurane) (Raucy et al., 1989; Thummel et al., 1993). It also participates in the metabolism of alcohols, aldehydes, ketones and plays a key role in gluconeogenesis from endogenous ketone bodies (Casazza et al., 1984; Koop and Casazza, 1985; Eknstrom et al., 1989). CYP2E1 expression is known to be elevated by ethanol consumption (Takahashi et al., 1993), obesity (O'Shea et al., 1994), and type II diabetes (Wang et al., 2003).

CYP2E1 mRNA expression has been reported in fetal liver (Ivan et al., 2007). However, two other studies (Jones et al., 1992; Hakkola et al., 1994) were unable to detect the enzyme in 6 to 24 week fetuses. The presence of the CYP2E1 protein expression was not detected in the fetus in several reports (Shimada et al., 1996; Vieira et al., 1996; Hines, 2007). In contrast, it has been detected in fetal liver even at 16-24-weeks gestational age (Carpenter et al., 1996) and its level increases progressively from the second trimester (Johnsrud et al., 2003). Moreover, Vieira et al., (1996) showed that the CYP2E1 protein was absent in the fetal liver while very low catalytic activity was observed. The activity and protein level surge immediately after birth, regardless of the gestational age (Vieira et al., 1996), whereas, there was only a moderate increase in gene expression throughout early postnatal development (Vieira et al., 1996). This suggests the involvement of a post-translational event during the early neonatal period. Protein and catalytic activity steadily increased during the first year and reached the adult value in infants aged 1 – 10 years (Vieira et al., 1996). Gene expression reached 50% of the adult value in infants aged 3-12 months. The CYP2E1 enzyme has been detected in mouse and rat (Raucy and Carpenter, 1993) as well.

1.3.7 Other important CYPs

In addition to the CYPs described above, there are several other enzymes which participate in the metabolism of several drugs.

1.3.7.1 CYP1A

The mean CYP450 1A2 protein level comprises ~13% of liver microsomal proteins in the adult human (Shimada et al., 1994b). CYP1A1 and CYP1A2 are essential for the metabolic disposition of environmental polycyclic and halogenated aromatic hydrocarbon, aromatic amines, estradiol and several therapeutics (Parkinson, 1996). CYP1A1 protein and enzyme activity were identified in fetal liver samples (Pasanen et al., 1987; Yang et al., 1995; Shimada et al., 1996). However, a few studies were unable to detect CYP1A1 and 1A2 mRNA in the fetus (Hakkola et al., 1994; Ivan et al., 2007). In contrast, Omiecinski et al. (1990) identified CYP1A1

mRNA in fetal liver (6 and 12 weeks), lung (8 and 21 weeks), and adrenal (11 and 17 weeks), but not in kidney tissue. In each case, CYP1A1 mRNA levels declined with increasing age. In contrast, CYP1A1 expression is not generally detectable in adult tissues. Thus, the suppression of this activity must occur sometime late in prenatal, perinatal, or early childhood development.

CYP1A2 enzyme activity remains very low after birth and significant *in vitro* activity is detected only by 1–3 months of age (Sonnier and Cresteil, 1998). By the first year of life CYP1A2 enzyme activity levels are only 50% of adult values and mature to adult activity levels sometime after this (Tateishi et al., 1997; Sonnier and Cresteil, 1998).

1.3.7.2 CYP2B

CYP2B6 is a very minor CYP isoform in human liver microsomes; the levels of this protein were less than 1% of total hepatic CYP in human (Shimada et al., 1994b). A few studies have reported the expression of CYP2B6 enzymes in fetal liver in human (Ivan et al., 2007), mouse and rat (CYP2B: Chianale et al., 1988). In contrast a few other studies have demonstrated negligible or no expression of CYP2B6 and CYP2B7 in the human fetal liver (Hakkola et al., 1994).

1.3.7.3 CYP3A

The major drug metabolizing CYP enzymes appear to be primarily members of the CYP3A subfamily in all stages of development. CYP3A comprises 30% of total human hepatic CYP enzymes. The CYP3A subfamily is constituted by three major isoforms: CYP3A4, CYP3A5 and CYP3A7. They are structurally closely related and the amino acid and nucleotide sequence homology among them is more than 83%. The balance between different members of this subfamily, however, undergoes significant changes from the fetal predominant CYP3A7 to the major adult form CYP3A4. The major change on appears to occur mainly after birth. CYP3A7 is very active in the fetal liver and its activity is maximal during the first week after birth and then progressively declines to a very low level in adult. Conversely, the activity of CYP3A4 was extremely weak in the fetus and began to rise after birth and reached 50% of adult levels between 6 and 12 months of age (de Wildt et al., 1997; Lacroix et al., 1997; Stevens et al., 2003). However, total CYP3A protein levels do not change from fetus to adult. In contrast, a few studies reported the maximal expression of CYP3A7 at different ages such as in the 20-week old fetus (Kitada et al., 1987) and during the first trimester (Hines, 2007).

Several studies have reported that the expression of CYP3A4, CYP3A5 and CYP3A7 in the human fetus (Hakkola et al., 1994 and 2001; Ivan et al., 2007; Schuetz et al., 1994; Hines,

2007; Kitada et al., 1985; Wrighton and Vandenbranden, 1989). CYP3A5 mRNA average level was 700-fold lower than that of CYP3A7 and CYP3A4 was expressed least (Hakkola et al., 2001). CYP3A7 protein was also detected in the fetus (Hakkola et al., 2001). In contrast, CYP3A4, CYP3A5 proteins were not observed. (Hakkola et al., 2001).

CYP3A family enzymes metabolize many pesticides, carcinogens, drugs (Conney, 1982; Guengerich et al., 1986a and 1986b; Pichard et al., 1990; Wrighton et al., 1990) fatty acids, inflammatory mediators, and hormones (Ladona et al., 1989) such as cortisol, testosterone, androstendione, and progesterone (Berliner and Salhanick, 1956; Lipman et al., 1962; Tukey et al., 1985; Fabre et al., 1988; Yamazoe et al., 1989). Substrate specificity was found to be overlapping between the individual enzymes of this subfamily.

1.3.8 Regulation of CYP enzymes

The expression of CYP genes varies with age, sex, nutritional status, disease conditions, pregnancy, dietary components, endogenous compounds, xenobiotic intake and regulators such as hormones and nuclear receptors. Most regulatory effects on CYP expression occurs at the level of transcription. However, the induction of CYP2E1 by ethanol is a post-transcriptional mechanism (Koop and Tierney, 1990).

1.3.8.1 Hormones

The expression of some CYP enzymes can be modulated by gender due to the presence of different amounts of endogenous steroid and peptide hormones in males and females (Denison and Whitlock, 1995; Mode, 1993). Several hepatic CYP enzymes are expressed in a sexually dimorphic and age-dependent manner in rats (Kato and Yamazoe, 1992). Female rats have 10% to 30% less total CYP enzymes when compared to male rats (Mugford and Kedderis, 1998). Particularly, enzymes belonging to the CYP2 and CYP3 families exhibit sex- and age-dependent expression (Waxman et al., 1985). Growth hormone (GH) is an important regulator of the expression of several members of the CYP2C subfamily in rat liver. In rodents, the sexually dimorphic expression of certain CYP enzymes is controlled by the sexually differentiated pattern of GH secretion (Mode, 1993). Female rats have the continuous presence of GH in serum, associated with a high expression of CYP2C12 and a low expression of CYP2C11. In contrast, the male has high expression of CYP2C11 form due to the intermittent pattern of GH secretion (Mode, 1993). In addition, sex steroids such as testosterone and estrogen regulate the sexual dimorphic and age-dependent expression of hepatic CYP enzymes (Pampori and Shapiro, 1999;

Wang and Strobel, 1997; Waxman et al., 1985). Moreover, some studies suggest that some of the CYP enzymes (e.g., CYP1B1, CYP2C11, CYP2E1, CYP11A1, CYP17, CYP19, and CYP21) present in extra hepatic tissues (e.g., adrenal gland, testis, ovary, kidney and lymphoid tissues) are primarily regulated by the pituitary and sex hormones (Chen et al., 1999a; Imaoka et al., 1992; Leung et al., 2009; Payne and Hales, 2004; Thangavel et al., 2007). However, there is no report on the hormonal regulation of CYP enzymes in the lung. Estrogen suppresses the expression of CYP17A1, a key enzyme in steroidogenesis in the adrenal gland, testis and ovary (Payne, 1990; Payne and Youngblood, 1995). The expression of CYP17A1 in mouse and rat Leydig cells is suppressed by testosterone (Georgiou et al., 1987; Hales et al., 1987).

Both agonists and antagonists of glucocorticoid receptors induce P450 enzymes, particularly CYP3A11 and 3A13 in mice and CYP3A4 in humans (Watkins et al., 1985; Wrighton et al., 1985). Although glucocorticoids induce the expression of many genes by ligand-mediated activation of the glucocorticoid receptor, glucocorticoid and, paradoxically, antiglucocorticoid induction of CYP3A reflect a nonclassic glucocorticoid-receptor induction process (Schuetz and Guzelian, 1984). Importantly, glucocorticoids are not selective inducers of human CYP3A, as they also induce other CYP isozymes, such as CYP2B6 (Strom et al., 1996) and activities associated with CYP2C19 (Zhou et al., 1990) in human liver. A functional glucocorticoid response element has been identified in the rat CYP2B2 gene promoter (Jaiswal et al., 1990).

Homeostasis of hormones especially glucocorticoids is important for fetal growth and maturation of many fetal organ systems (Liggins et al., 1994; Fowden et al., 1996). The expression of some hepatic drug metabolizing enzymes increases at birth. This suggests that factors associated with parturition such as parturition surge in fetal plasma cortisol concentrations, could be involved. A recent study illustrated that antenatal administration of cortisol slightly increases the mRNA levels of CYP2D6 in fetal sheep (May et al., 2007).

1.3.8.2 Xenobiotics

Xenobiotics comprise mainly drugs and environmental compounds. Altered CYP expression both at protein and gene levels occurs in humans after exposure to therapeutic agents (e.g., rifampicin, phenobarbital, tamoxifen) and environmental toxicants (Ickenstein and Bandiera, 2002; Urquhart et al., 2007). Rifampin and phenobarbital induce CYPs, particularly CYP3A11 and 3A13 in mice and CYP3A4 in humans (Watkins et al., 1985; Wrighton et al., 1985; Yanagimoto et al., 1997; Rendic and Di Carlo, 1997). The CYP2C subfamily is induced

by phenobarbital, rifampicin and dexamethasone in humans (Gerbal-Chaloin et al., 2001). These drugs can up-regulate CYP2C8, CYP2C9, and CYP2C19 transcript levels in primary human hepatocytes (Gerbal-Chaloin et al., 2001). However, an earlier study reported that CYP2C9 and CYP2C19 are not inducible by phenobarbital or rifampicin (Runge et al., 2000). In addition, metronidazole inhibits CYP2C9 and causes increased concentrations of the anticonvulsant, phenytoin (Levy, 1995).

Moreover, various synthetic chemicals including polycyclic aromatic hydrocarbons (PAH) (e.g., 3-methylcholanthrene and benzo(*a*)pyren), induce several CYP enzymes, mainly CYP1A1/1A2 and 1B1 (Okey, 1990; Whitlock, 1993; Quattrochi et al., 1994; Savas et al., 1994; Sutter et al., 1994; Edwards et al., 2007) both in human and rat. These PAHs act through the aryl hydrocarbon receptor (Fujisawa-Sehara et al., 1988). The environmental contaminant 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) is also a potent inducer of CYP1A1 (Poland and Knutson., 1982). In addition, Sutter et al., (1994) have demonstrated that levels of CYP1B1 mRNA are highly induced by TCDD in primary cultures of normal human epidermal keratinocytes.

1.3.8.3 Endogenous compounds

Endogenous compounds (e.g., bile acids) are also involved in the modulation of CYP expression (Chiang, 2002). Bile acids have the ability to decrease CYP protein levels in hepatic and gonadal tissues (Chiang, 2002). For example, bile acids decrease hepatic CYP7A1 expression in humans and mice. CYP7A1 catalyzes the rate-limiting step in the biosynthesis of bile acids from cholesterol (Chiang, 2002).

Overall, differential exposure to xenobiotics or endogenous compounds may lead to variability in CYP-mediated metabolism, thereby leading to altered pharmacokinetic and toxicological profiles of therapeutic agents due to drug-drug interactions (Fuhr, 2000; Moore and Kliewer, 2000).

1.3.8.4 Disease conditions

Certain disease conditions alter the expression of CYP enzymes. For example, diabetes, obesity, rheumatoid arthritis, multiple sclerosis, cholestasis, and viral infections (e.g., hepatitis, influenza) can suppress the expression of CYP enzymes and thereby drug biotransformation (Morgan, 1997; Renton, 2004). During inflammatory and viral disease conditions, the circulating levels of inflammatory mediators (e.g., tumor necrosis factor, interleukins and interferons) are increased significantly (Aukrust et al., 1994; Drachenberg et al., 1999; Nawata et al., 1989; Waehre et al., 2004). This leads to a decrease in total hepatic CYP content and the levels of some

hepatic CYP subfamilies such as CYP1A, CYP2B, CYP2C and CYP3A (Christen et al., 2001; Hopkins, 2003; Renton, 2001; Sharief and Hentges, 1991).

1.3.8.5 Nuclear receptors

Nuclear receptors are a family of molecules found within cells that are responsible for the regulation of the expression of specific genes thereby controlling the development, homeostasis, and metabolism of the organism (see review Mangelsdorf et al., 1995). There are several orphan nuclear receptors (NR) which participate in the CYP regulation identified in vertebrates (Giguere, 1999). These are hepatic nuclear factor 4 α (HNF4 α), peroxisome proliferator-activated receptors (PPAR), pregnane x receptor (PXR) (Kliwer et al., 2002), constitutive androstane receptor (CAR) and aryl hydrocarbon receptor (AhR). Glucocorticoid receptor (GR), a nuclear receptor, also participates in the regulation of CYP genes (Lewis, 2001).

There are three PPAR isoforms identified in mammals: PPAR α , β and γ (Reviewed in Lemberger et al., 1996). Each PPAR gene shows a characteristic expression pattern in different tissues. PPAR α is highly expressed in tissues such as heart, liver, kidney, intestine, and brown fat where higher fatty acid β oxidation occurs (Issemann and Green., 1990, Braissant et al., 1996). In contrast, PPAR β is widely expressed in adult tissues including brain, kidney, small intestine, and Sertoli cells (Braissant et al., 1996, Amri et al., 1995). PPAR α regulates the expression of the CYP4 family enzymes (Lewis, 2001).

The AhR is a cytosolic ligand-mediated transcription factor and expressed in most organs and tissues. In rats it is expressed in lung, liver, placenta, and thymus (Carver et al., 1994). AhR is conserved across many species including vertebrates and invertebrates (Fujii-Kuriyama and Mimura, 2005) and mediates CYP1 enzyme induction (Schmidt and Bradfield, 1996). Induction occurs due to the binding of a heterodimer of AhR in the promoter region of CYP1. AhR regulates both the constitutive and inducible expression of CYP1A1, CYP1A2 and CYP1B1 enzymes in liver and extrahepatic tissues such as lung, kidney, stomach, small intestine, and placenta (Whitlock, 1999; Shimada et al., 2002; Shimada et al., 2003; Riddick et al., 2003; Pavek and Dvorak, 2008).

The nuclear receptor PXR is predominantly expressed in the liver and intestine of fetus and adult animals. It mediates the induction of CYP3A4 and CYP3A5 in response to glucocorticoids and rifampicin (Schuetz et al., 1998). Mouse and human PXR are ligands activated by high concentrations of glucocorticoid agonists (e.g., dexamethasone) and phenobarbital and in a species-specific manner by the glucocorticoid antagonist (pregnenolone

16 α -carbonitrile; PCN) and by rifampin (Kliewer et al., 1998; Lehmann et al., 1998). Ligand-stimulated PXR transcriptionally activates hormone response elements found in rat CYP3A1 (Kliewer et al., 1998) and human CYP3A4 genes (Blumberg et al., 1998; Lehmann et al., 1998) that are also conserved in the mouse CYP3A11 gene (Toide et al., 1997).

CAR activates reporter genes driven by promoters containing DR-5 hormone response elements (HREs) (Baes et al., 1994, Choi et al., 1997) or a complex HRE present in the CYP2B gene (Honkakoski et al., 1998). While the constitutive activity of CAR can be suppressed by androstanes, various phenobarbital-type inducers have been shown to reverse the negative effect of androstanes on the human CYP2B6 promoter (Sueyoshi et al., 1999). Phenobarbital is the prototype for xenobiotics that induce CYP2B genes. This observation suggests that CAR, together with PXR and PPAR α , may participate in a nuclear receptor based regulatory pathway controlling the expression of CYP genes in response to exogenous xenochemicals and endogenous compounds such as steroids and lipids.

Genetic variability within the CYP2C has been well documented (Goldstein, 2001). Multiple factors have been implicated in regulating CYP2C9 and CYP2C19 genes. Both CYP2C9 and CYP2C19 are known to be regulated by PXR, CAR, and GR (Ferguson et al., 2002; Gerbal-Chaloin et al., 2002; Chen et al., 2003). However, only the proximal CYP2C19 CAR binding site is active, in contrast to both the distal and proximal CAR binding sites being functional in CYP2C9. This observation has been offered as a probable cause for lower constitutive CYP2C19 expression in adult human liver (Chen et al., 2003). CAR expression is markedly lower in the fetal and neonatal versus adult liver (Wei et al., 2002; Huang et al., 2003). Thus, it would appear that CAR has a minimal role in regulating fetal and neonatal hepatic CYP2C expression.

1.3.8.6 HNF4 α

There are three genes encoding HNF4 subtypes in vertebrates: two in humans and rodents (HNF4a and g) (Sladek et al., 1990, Chartier et al., 1994, Kritis et al., 1996) and one in *Xenopus* (HNF4b) (Holewa et al., 1997). Each gene product differs significantly in its expression pattern and transactivation potential (Chartier et al., 1994, Kritis et al., 1996, Drewes et al., 1996). There are several transcriptional regulatory factors that regulate some metabolic enzymes. HNF4 α is the major one among them and therefore studying this regulatory factor in fetal, newborn and adult sheep will be of importance.

HNF4 is regulated by a phosphorylation signal-dependent pathway (Ktistaki et al., 1995). HNF4 α is highly expressed in liver, kidney, intestine, and pancreas and at low levels in the testis (Sladek et al., 1990, Drewes et al., 1996, Miquerol et al., 1994). It is found to be an important regulator of hepatic and pancreatic gene expression, development and function, including hepatic intermediary metabolism (Watt et al 2003; Odom et al., 2004, 2006).

Several transcriptional regulatory factors have been identified in the liver of mice from as early as day 14 of gestation, particularly HNF4 α which increases by 20-fold at 20 days of gestation. In this species, HNF4 α regulates the induction of genes encoding xenobiotic metabolizing enzymes by controlling hepatic PXR transcription, and the up-regulation of PXR during fetal development is due to an increase in HNF4 α expression (Kamiya et al., 2003). If a similar role is demonstrated in humans, HNF4 α may directly regulate CYP2C9 and CYP2C19 developmental expression as well as indirectly regulate these two genes through its action on PXR. HNF4 α has also been shown to be an important modulator of PXR- and CAR-dependent CYP3A4 induction in the liver (Tirona et al., 2003) and, as such, may be playing a similar role for CYP2C expression.

In more than 20 CYP2 family genes, the HNF4 binding element is conserved in the proximal promoter regions (Chen et al., 1994; Ibeanu and Goldstein, 1995). HNF4 α plays a general role in the regulation of major P450 genes, including CYP3A4, CYP3A5, CYP2A6, CYP2B6, CYP2C9, and CYP2D6, in human hepatocytes (Jover et al., 2001). These findings indicate that HNF4 α may act as a common regulator of the liver-specific transcription of many CYP genes.

A recent study showed that inter-individual variation in the expression level of CAR, HNF4 α , and POR probably determines the variation in expression and activity of xenobiotic metabolic genes and thereby the clearance rate of a variety of xenobiotics (Wortham et al., 2007).

1.3.9 Uridine 5'-diphospho-glucuronosyltransferase

The UGT enzymes are a group of isoenzymes of 50-60 kDa localized primarily in hepatic endoplasmic reticulum and nuclear envelope (Tephly and Burchell, 1990). Hepatic phase II metabolism involves the generation of more water soluble drug metabolites compared to the parent drugs and this facilitates the biliary and renal elimination in adults. However in the fetus the production of water soluble metabolites may paradoxically increase the fetal exposure to the

metabolite, as the biliary and renal routes are not definitive routes of elimination (Rurak et al., 1991). Water soluble compounds have reduced ability to cross the placenta for disposal by the mother, and substances are excreted in fetal bile and urine can accumulate in the amniotic fluid and be swallowed and/or taken up by the intramembranous pathway and thereby recirculated to the fetus (Krauer and Dayer, 1991; Rurak et al., 1991). Hence, an understanding of Phase II drug metabolism is important in understanding the potential for fetal drug exposure.

UGTs are responsible for the glucuronidation of several hydrophobic endogenous (e.g., bilirubin, bile acids, thyroxine and steroids) and exogenous (e.g., morphine, acetaminophen and NSAIDs) compounds (Burchell et al., 1991). In addition, UGTs detoxify potentially carcinogenic or teratogenic xenobiotics that enter the body as components of the diet or as airborne pollutants (de Wildt et al., 1999). The expression of UGT enzymes is regulated by several factors such as xenobiotics, hormones and developmental processes, such that different tissue specific profiles of activities are observed (Burchell and Coughtrie, 1989). Variations in drug glucuronidation reported here focused essentially on variations due to physiological factors, induction, drug interactions and genetic factors in disorders such as Gilbert's Syndrome and Crigler-Najjar type I and II diseases (Batt et al., 1994).

There is less information on the development of phase II conjugation reactions (glutathione, glucuronide, acetate and sulphate conjugates) during the fetal and neonatal periods and these pathways have received less attention compared to the phase I reactions (de Wildt, 1999; McCarver and Hines, 2002). The pathways studied most extensively are glucuronidation and sulfation and less is known of other fetal conjugation processes, such as glutathione conjugation and methylation (Besunder et al., 1988).

Burchell et al., (1991) proposed the nomenclature of UGT genes. UDP-glucuronosyl transferases were represented by the root symbol "UGT" for human (Ugt for mouse and rat). It is followed by an Arabic number denoting the family, a letter designating the subfamily, and an Arabic numeral representing the individual gene within the subfamily e.g., human UGT1A5 and murine Ugt1a5. While the gene and cDNA of UGT should be italicized (human – *UGT1A5*, mouse – *Ugt1a5*), protein and enzyme activity should not be written with lowercase or in italics. To date, 15 individual UGT enzymes have been identified in humans (Tukey and Strassburg, 2000). According to the sequence homology, it is mainly subdivided into two broad families named UGT1 (phenol/bilirubin family) and UGT2 (steroid/bile salt family) (Burchell et al., 1991). Several studies have demonstrated the developmental expression of UGTs in human (de

Wildt, 1999; McCarver and Hines 2002) and rats (Burchell, 1980; Weatherill and Burchell, 1980; Falany and Tephly, 1983; von Meyerinck et al., 1985). Parturition triggers an increase in UGT enzyme activity and this enzyme activity reaches 25% of adult levels by 3 months of age (Coughtrie et al., 1988).

These enzymes are responsible for the formation of glucuronide conjugates of endogenous and exogenous compounds and glucuronidation activity is low in the fetus (Leakey et al., 1987; Coughtrie et al., 1988). UGT proteins have demonstrated a broad overlap of substrates for glucuronidation. For example, UGT1A1, UGT1A6 and UGT1A9 metabolize 1-naphthol.

1.3.10 UGT1A subfamily

At least nine UGT1A proteins and three pseudogenes are encoded on chromosome 2. These UGT enzymes are predominantly found in liver and also present in extrahepatic tissues. But the UGT activities are significantly lower in colon than in liver tissues (Strassburg et al., 1999a). Five UGT1A genes (UGT1A1, UGT1A3, UGT1A4, UGT1A6, and UGT1A9) are expressed in human liver (Strassburg et al., 1997a, 1997b and 1998). The remaining UGT genes are also expressed in extrahepatic sites (Strassburg et al., 1999 and 2000; Tukey and Strassburg, 2000). UGT1A10 has been identified in biliary, gastric and colon tissues (Strassburg et al., 1997b), UGT1A7 in gastric tissue (Strassburg et al., 1997b) and UGT1A8 in colon tissues (Strassburg et al., 1998). These three UGT1A enzymes are not present in liver. All other isoforms are present in liver. The carboxy terminus of 245 amino acids is identical in all UGT1A enzymes in human, whereas the 280 amino acids of the amino terminal are unique.

All UGT1A transcripts are absent in the fetal liver at 20 weeks of gestation using RT-PCR whereas UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9 mRNAs were identified in all liver samples from adults and children (Strassburg et al., 2002). A number of developmental studies have shown that glucuronidation activity towards bilirubin and other compounds is very low in fetal liver (Onishi et al., 1979; Kawade and Onishi, 1981; Leakey et al., 1987; Burchell et al., 1989; Pacifici et al., 1989). UGT1A1 enzyme, which is the main iso-enzyme responsible for the glucuronidation of bilirubin, is very low in the fetus and increases after birth (Burchell et al., 1989; Coughtrie et al., 1988; Kawade and Onishi, 1981). Moreover, UGT1A1 expression is triggered by processes associated with birth and activity reaches adult levels by 3 to 6 months postnatal age (Burchell et al., 1989). Leakey et al (1987) observed that the activity is very low

(<1%) in the pre-term fetus (16-25 weeks) with slightly higher levels (6%) in the term fetus (Onishi et al., 1979), when compared to adult. UGT activities towards bilirubin and testosterone are absent in fetal rats; conversely UGT activity for 2-aminophenol is present in the rat fetus five days before birth (Coughtrie et al., 1988).

In contrast, UGT1A3 (Burchell et al., 1989), which catalyzes morphine glucuronidation is present in fetal liver, but at a lower level than in the adults. Fetal hepatic microsomes (15–27 weeks gestation) catalyze morphine glucuronidation at only 10–20% the efficiency of adult hepatic microsomes (Pacifci et al., 1982 and 1989). The *in vivo* postnatal elimination pattern of morphine is consistent with the *in vitro* studies. Premature and full-term infants have a markedly reduced and variable capacity to eliminate morphine, and the adult capacity is achieved by 6–30 months of age (McRorie et al., 1992; Mikkelsen et al., 1994).

A moderate activity towards estrone (Leakey et al., 1987) and estradiol (Burchell, 1974) glucuronidation is present in the term fetal and neonatal human liver whereas the activity towards 5-hydroxytryptamine was at adult levels (Leakey et al., 1987). These observations contrast markedly with those from rat and rhesus monkey fetuses at term, in which the activities towards 1-naphthol, 2-aminophenol and 4-nitrophenol in addition to activity towards 5-hydroxytryptamine all are similar to adult values (Wishart, 1978; Leakey et al., 1983).

A relative deficiency in glucuronide conjugation of salicylamide and paracetamol is still observed in 7 to 10 year old children compared to adults (Widdowson, 1974). Acetaminophen is metabolized mainly by UGT1A6 and, to a lesser extent, by UGT1A9 and UGT1A1 (Bock et al., 1993; Curry et al., 1999). Acetaminophen glucuronidation appears to be absent or negligible in the fetus, low at birth and appears to approach full competence by 9-12 months of postnatal life (Alam et al., 1977; Rollins et al., 1979; Behm et al., 2003). Even though the mRNA and protein levels were similar across the different age groups, hepatic glucuronidation activity in children aged 13–24 months was found to be lower than that in adults for ibuprofen, amitriptyline, 4-*tert*-butylphenol, estrone, and buprenorphine. This deficiency in glucuronidation seems to be compensated by the early development of sulfation activity in the human fetus. Further, activities towards bilirubin, androsterone, testosterone, 1-naphthol, 2-aminophenol and 4-nitrophenol were all present from the fetal and term age groups at values less than 14% of those of adults.

UGT1A9 participates in the glucuronidation of phenols, (Wooster et al., 1991) anthraquinones, flavones, coumarins (Wooster et al., 1993; Ebner and Burchell, 1993), and amines (Nowell et al., 1999; Strassburg et al., 1999). Of the UGT enzymes expressed in human

liver, UGT1A9 mRNA and protein levels increased with age (Strassburg et al., 2002) and exhibited the highest specific activity towards phenols (Tukey and Strassburg, 2000). However, levels of UGT1A9 mRNA increased in an age dependent fashion with significantly lower levels in the age groups 6-12 months and 13-18 months (Strassburg et al., 2002). These findings demonstrate an age dependent differential up-regulation of the human UGT1A9 gene. There are no differences in protein levels for UGT1A1 and UGT1A6 between child and adult. Moreover, the transcript levels for all UGT1A isoforms with the exception of UGT1A9 were similar in the adult and pediatric groups and exhibited comparable inter-individual fluctuation (Strassburg et al., 2002).

In Crigler-Najjar type 1 syndrome, a single loss of the UGT1A isoform abolishes the ability of efficient bilirubin glucuronidation and detoxification (Bosma et al., 1994).

1.3.11 UGT2B7

Five UGT2B genes (UGT2B4, UGT2B7, UGT2B10, UGT2B11 and UGT2B15) are expressed in human liver (Beaulieu et al., 1997; Belanger et al., 1998; Strassburg et al., 1999; Tukey and Strassburg, 2000). The remaining UGT genes are expressed in extra hepatic sites (Strassburg et al., 1999 and 2000; Tukey and Strassburg, 2000). There is no UGT2B gene expression in 20 week old fetuses. However, UGT2B4, UGT2B7, UGT2B10, and UGT2B15 are present in adult and child liver (Strassburg et al., 2002).

UGT2B7 expression increases at birth, reaching adult levels by 2 to 6 months of age (Choonara et al., 1989). UGT2B7-dependent glucuronidation is an important pathway for epirubicin detoxification where UGT2B7 is the responsible enzyme (Innocenti et al., 2001). An increase in epirubicin glucuronidation activity was observed with increasing age, with a positive correlation between UGT2B7 levels and postnatal age suggesting a progressive increase in UGT2B7 protein levels (Zaya et al., 2006).

Morphine is also extensively metabolized by UGT2B7 with formation at both the 3- and 6- hydroxyl positions (M3G and M6G) (Coffman et al., 1997; Stone et al., 2003). Morphine glucuronidation by UGT2B7 is present in fetal liver, but at lower levels than in the adults (Pacifci et al., 1982). Studies on the metabolism of morphine in children and premature neonates have shown that morphine glucuronidation capacity is enhanced after the neonatal period. Morphine clearance was 5-fold higher in children 1 to 16 years of age versus neonates,

with clearance values matching adults being attained between 6 and 30 months (Choonara et al., 1989).

Chloramphenicol is an important example of a drug where lower glucuronidation capacity in the pediatric population can lead to toxicity. As glucuronidation is markedly deficient in most premature infants and some full-term babies due to the delayed onset of UGT2B7 enzyme activity, high concentrations of unchanged chloramphenicol may accumulate in a neonate receiving 'usual' doses on the basis of bodyweight and may cause Gray baby syndrome (reviewed by McCarver and Hines, 2002).

1.3.12 Other phase II metabolic pathways

Sulfotransferases (ST) are another group of enzymes which have substrate specificities that demonstrate significant overlap with the UGT enzymes (Coughtrie et al., 1994). Although changes in activity of the individual ST enzymes with development occur, the data on their development are limited and confusing. In general, liver from fetal, newborn and infant human was found to express significant ST activity, and sulfate conjugation is a relatively efficient pathway at birth (Pacifici et al., 1988 and 1993; Richard et al., 2001).

Glutathione-S-transferases (GST) are responsible for the detoxification of a number of potentially toxic drug or drug metabolites (Mannervik et al., 1992). As with the ST enzymes, the GST enzymes demonstrate age related expression of individual enzymes in the liver (Pacifici et al., 1981; Strange et al., 1989; Vos and Van Bladeren, 1990). For example, liver from preterm newborn babies exhibited 60% greater activity towards chloramphenicol than fetal livers, but showed similar activity levels towards chlorodinitrobenzene (Holt et al., 1995).

Very limited data exists on the developmental appearance of N-acetyltransferases1 (NAT1) and NAT2 enzyme activity. Fetal livers show activity towards several NAT enzyme substrates, but at much lower levels than the adult (Peng et al., 1984; Pacifici et al., 1986). Consequently, the newborn exhibits a limited capacity to acetylate substrates. The acetylation status of the infant may reflect that in the adult only after the first year of life (Szorady et al., 1987; Pariente-Khayat et al., 1991 and 1997).

Table 1.5: Summary of ontogenesis of CYPs and UGTs in human liver.

Genes & Proteins	Fetus		New Born	Infants <1yr	Children <12yrs	Adults >12yrs	Reference
	<20w	>20w					
CYP1A1 g	-/+	+/-				++	2, 3,7,12
CYP1A1 p	+/-	-				++	1, 9, 10, 11
CYP1A2 g	-	-				++++	2, 3,7
CYP1A2 p	-	-		++	+++ /++++	++++	1, 4, 9,13
CYP2A6 g	-	+				++++	2, 3, 6, 7
CYP2A6 p	-			+++	+++ /++++	++++	1, 4, 5, 8
CYP2B6 g	-	+				+++	3, 7
CYP2B6 p							
CYP2C9 g	-	+				+++	2, 3, 6, 7,21
CYP2C9 p	-/+	+	++	+++	+++ /++++	++++	1,4,5,14,15,16,17,18, 19,20
CYP2C19g	-	-/+				+++	2, 3, 7
CYP2C19p	-	-/+	+	+	++	+++	4, 14,16,17,18
CYP2D6 g	+	+	++	+++	+++	+++	2, 3,7,22
CYP2D6 p	-/+	+	++	+++	+++	++++	1,4,17,22,23,24
CYP2E1 g	-	+				++++	2, 3, 6, 7,25,27
CYP2E1 p	-/+	+	++	+++	++++	++++	1,4,16,26,27,28
CYP3A4 g	+	+				++++	2, 3, 6, 7
CYP3A4 p	-	+	++	+++	+++ /++++	++++	1,16,29,30,31
CYP3A7 g	++++	+++				+	2, 3,7
CYP3A7 p	++++	++++ / +++	++ / ++++	++	+ /-	-	1,4,16
UGT1A1 g	-			++++	++++	++++	32,33,34,35
UGT1A1 p	-/+	+	++	+++	++++	++++	35,36,37,38,39,40,55
UGT1A3 g	-			+++	++++	++++	32,33,34,35
UGT1A3 p	+	+	++	+++	++++	++++	36,41,42,43,44
UGT1A4 g	-			++++	++++	++++	32,33,34,35
UGT1A4 p							

Genes & Proteins	Fetus	New Born	Infants <1yr	Children <12yrs	Adults >12yrs	Reference	
UGT1A6 g	-		+++	++++	++++	32,33,34,35	
UGT1A6 p	-	-	+	++	+++/++++	++++	35,45,46,47,48,56,57,58
UGT1A9 g	-	-	-	++	+++	++++	32,33,34,35
UGT1A9 p	+	+	++	+++	++++	++++	35,39,
UGT2B7 g	-			++/+++	+++/++++	++++	35,51,52,53,54
UGT2B7 p		+	++	+++	++++/+++++	++++	35,41,49,50,59,60

w – week, m – month, yr – year, yrs – years, g – gene, p – protein; +++++ - very high expression; +++++ - high expression; +++ - moderate expression; ++ - low expression; + - very low expression; - - no or negligible expression.

(Shimada et al., 1996 – 1; Nishimura et al., 2003 – 2; Hakkola et al., 1994 – 3; Tateishi et al., 1997 – 4; Maenpaa et al., 1993 – 5; Nagata et al., 2003 – 6; Ivan et al., 2007 – 7; Pelkonen et al., 1997 – 8; Cazeneuve et al., 1994 – 9; Pasanen et al., 1987 -10; Yang et al., 1995 -11; Omiecinski et al., 1990 -12; Sonnier and Cresteil, 1998 -13; Klose et al., 1999 -14; Treluyer et al., 1997-15; Hines, 2007 -16; Oesterheld, 1998-17; Koukouritaki et al., 2004-18; Ratanasavanh et al., 1991-19; Cresteil et al., 1985 -20; Umberhauer et al., 1987 -21; Treluyer et al., 1991 -22; Jacqz-Aigrain et al., 1993 – 23; Ladona et al., 1991 -24; Jones et al., 1992-25; Vieira et al., 1996-26; Carpenter et al., 1996-27; Johnsrud et al., 2003-28; de Wildt et al., 1997- 29; Lacroix et al., 1997 -30; Stevens et al., 2003 -31; Strassburg et al., 1997a -32, Strassburg et al., 1997b -33; Strassburg et al., 1998 – 34; Strassburg et al., 2002 -35; Burchell et al., 1989 -36; Coughtrie et al., 1988 -37; Kawade and Onishi, 1981 -38; Leakey et al., 1987 -39; Onishi et al., 1979 -40; Pacifici et al., 1982 -41; Pacifici et al., 1989 -42; McRorie et al., 1992 -43; Mikkelsen et al., 1994 -44; Widdowson et al., 1974-45; Alam et al., 1977-46; Rollins et al., 1979-47; Behm et al., 2003-48; Choonara et al., 1989 -49; Zaya et al., 2006 -50; Beaulieu et al., 1997 -51; Belanger et al., 1998 -52; Strassburg et al., 1999 -53; Tukey and Strassburg, 2000 -54; Leakey et al., 1983 -55; Alam et al., 1977 -56; Court et al., 2001 -57; Miners et al., 1988-58; Rowland et al., 2006 -59; Posner et al., 1991 -60).

1.3.13 Sheep metabolic enzymes

1.3.13.1 Cytochrome P450

Much less information is available on the identity and the developmental profiles of phase I drug metabolizing enzymes in sheep compared to that of humans and rats. Until now, only a few cytochrome genes have been cloned and identified in sheep. Full length sequences are available for CYP1A1, CYP2J2, CYP4F21, CYP11A1, CYP11B1, CYP17A1, CYP19 and CYP21, but these do not participate in the metabolism of drugs (Messina et al., 2010). No drug metabolizing cytochrome genes were cloned so far except CYP3A24 and a small piece (117bp) of CYP2E1 in sheep (Nelson, 2006; National Center for Biotechnology Information – NCBI). No gene expression studies have been performed in different age groups.

In contrast, some protein studies were reported in several age groups using Western blots or enzyme activity assays. In adult sheep, proteins from the CYP3A, 2B, and 2C subfamilies have been identified to date (Pineau et al., 1990; Murray, 1991 and 1992; Kaddouri et al., 1992). Furthermore, CYP3A may be the predominant fetal sheep CYP enzyme (Kaddouri et al., 1992). *In vitro* assessments of the metabolism of various substrates and use of immunologic methods have demonstrated the presence of CYP3A and CYP2B in postnatal sheep, but only CYP3A was detected in the fetus and the levels were lower than in the adults (Kaddouri et al., 1990 and 1992; Galtier and Alvinerie, 1996; Dacasto et al., 2005). Machala et al., (2003) compared the specific activities and protein levels of major CYP enzymes in several ruminant species including male sheep. Orthologs to human CYP2A6, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 proteins were identified in rams using polyclonal human antibodies (Machala et al., 2003). Moreover, Szotakova et al., (2004) also reported orthologs to human CYP2C9, CYP2E1 and CYP3A4 protein and enzyme activity in adult sheep while CYP1A2 was undetectable.

1.3.13.2 Glucuronidation

The availability of information on the identity and the developmental profile of phase II drug metabolizing enzymes in sheep are scarce compared to humans. Similar to human, differences in the ontogenesis of the various UGTs are observed in sheep glucuronidation *in vivo*. Several drugs form glucuronide conjugates in the adult, but this activity found to be less in the perinatal period in sheep (Wong et al., 2000b and 2001; Kim et al., 2004). Production of glucuronides conjugates of acetaminophen (Wang et al., 1985, 1986a and 1986b), ritodrine (Wright et al., 1991), labetalol (Yeleswaram et al., 1993), morphine (Olsen et al., 1988; Dvorchik et al., 1986), VPA (Wong et al., 2000b and 2001; Kumar et al., 2000a) and

indomethacin (Krishna et al., 2002) has been demonstrated in fetal and neonatal lambs but this is less than that of adult for acetaminophen and markedly less for VPA (Wang et al., 1985; Wong et al., 2000b and 2001). Fluoxetine (FX) glucuronides are present in adult sheep, but not in the fetus (Kim et al., 2004; Morrison et al., 2005). Recently UGT1A6 and UGT1A7 were isolated from sheep (Kobayashi et al., 1999) and both these isoenzymes metabolize phenolic substrates such as paracetamol, but their expression and ontogeny in fetal and neonatal sheep are unknown. Glucuronidation of morphine has also been demonstrated *in vitro* in fetal lamb in hepatocytes, suggesting the presence of UGT1A and UGT2B isoforms (Dvorchik et al., 1986). And mRNA expression of UGT1A6 isoform has been detected in the liver of adult sheep (Kobayashi et al., 1999).

1.4 Significance

Since the usage of drugs in pregnant women is increasingly common, a larger number of fetuses could be exposed to drugs and some may cause deleterious effects (Rurak et al., 1991; Hakkola et al., 1998). Since most drugs are lipophilic they cross the placenta readily, the extent of exposure depends largely on the fetal ability to metabolize them. Moreover, since the metabolic clearance of some drugs in the mother increases during pregnancy, higher drug doses are required to achieve the desired therapeutic effects (Loebstein et al., 1997). Consequently, this might further increase the extent of fetal drug exposure and the incidence and/or severity of adverse effects. Therefore a detailed study of hepatic drug metabolism at various stages of development especially in fetus and newborn, through the quantitative and qualitative analysis of enzymes involved, using transcript and protein profiles is of considerable importance, particularly if this can be combined with *in vivo* estimates of fetal drug metabolizing capacity and fetal drug effects.

Although there have been numerous studies of maternal-fetal drug disposition and fetal drug effects in pregnant sheep, there is little information available on the specific hepatic CYP and UGT enzymes in sheep and their ontogenetic profiles (Rurak et al., 1988; Kumar et al., 1997, 1999a, 2000a, 2000b; Wong et al., 2000a, 2000b, 2001). Obtaining such data will allow for a better assessment for the usefulness of sheep as a model for the ontogenetic development of drug metabolizing capacity in the human. Studies on the *in vivo* drug elimination at different age groups of sheep are being conducted by our research group. The studies reported in this thesis

increase understanding of the ontogeny of specific CYPs and UGTs, some of which are involved in the metabolism of the drugs being studied.

There are several advantages to the use of pregnant sheep for studies of maternal-fetal drug disposition and fetal drug effects. The longer gestational length and larger size of fetus, when compared with other smaller laboratory mammalian species allows for the placement and maintenance of chronic indwelling catheters for serial sampling of blood and other fluids that allow for estimation of fetal, maternal, and placental drug clearances. Moreover, detailed monitoring of fetal physiologic and metabolic variables is entirely feasible and the studies of individual fetuses can be continued into the postnatal period. This is less feasible in the human fetus for practical and ethical reasons, although non-invasive monitoring methodologies have greatly increased our ability to obtain physiologic data from the human fetus. Comparison of the human fetal physiologic data with that obtained from fetal lambs has indicated many similarities between the two species. Therefore in many respects pregnant sheep are useful model for human pregnancy.

It is also difficult to obtain normal tissues from the human. Normally, fetal samples are obtained from aborted or dead fetuses adult tissues are collected from patients who undergo liver resection or following death. Thus the underlying pathological conditions could alter CYP and UGT gene expression, and these could also be altered by the time period between death and obtaining the tissue samples.

1.5 Hypotheses

1. The pattern of hepatic expression of these enzymes in liver from fetal, newborn and adult sheep will be similar to that reported for the liver from fetal, newborn and adult humans.
2. Antenatal glucocorticoid administration will increase the hepatic expression of some of these enzymes.

1.6 Objectives

1. To determine the full length nucleotide sequences of several cytochrome P450 (orthologs to human CYP2C9, CYP2C19, CYP2A6, CYP2D6), glucuronosyltransferase (orthologs to human UGT1A6, UGT1A9, UGT2B7) isoforms and HNF4 α in sheep and determine the extent of homology with other species including the human.

2. To measure the mRNA and protein levels of the CYPs, UGTs and HNF4 α in the liver of fetal, newborn and adult sheep.
3. To determine the effect of antenatal glucocorticoid administration on these enzymes.

2. MATERIALS AND METHODS

2.1 Chemicals

Acrylamide 99.9%, N,N'-methylene-bis-acrylamide (Bis), sodium dodecyl sulphate, 2-mercaptoethanol, N,N,N',N'-tetramethylethylenediamine (TEMED), tris (hydroxymethyl) aminoethane (Tris base), p-nitro-blue tetrazolium chloride (NBT), bovine serum albumin (BSA), sodium chloride, sodium phosphate, potassium dihydrogen phosphate, sodium carbonate, sodium bicarbonate and sucrose were obtained from Fisher Scientific (Vancouver, BC, Canada).

Random primers, 1Kb Plus Ladder, PCR super mix, Superscript III Reverse Transcriptase, Oligo(dT)₁₂₋₁₈ Primer, dNTP set, One Shot TOP10 Chemically Competent E.coli, One shot TOPO cloning kit, EcoRI, Accuprime Pfx supermix, Rnase out, PureLink Quick Plasmid Miniprep Kit, Agarose, Trizol and RACE kit were purchased from Invitrogen Canada (Burlington, ON, Canada).

LB Broth, LB agar Kanamycin-50 plates, Kanamycin solution from *Streptomyces kanamyceticus* and Gel loading buffer were obtained from Sigma Aldrich (Oakville, Ontario, Canada). GelRed nucleic acid gel stain was purchased from Biotium (Hayward, CA). Precision Plus Protein Standard dual colour was purchased from Bio Rad Laboratories Inc. (Hercules, CA, USA). SYBR Green PCR master mix, MicroAmp Fast 96 Well Reaction Plate and the cover were obtained from Applied Biosystem Inc. (CA, USA). Nitrocellulose membrane was obtained from the Pall Corporation (Pensacola, FL, USA). QIAquick PCR purification kit and QIAquick gel extraction kit were purchased from Qiagen (Mississauga, Ontario).

2.2 Animal treatment and tissue collection

Sheep fetuses were instrumented with catheters in carotid artery, jugular vein, trachea and amniotic fluid at 120 days of gestation (dg). Around 126 dg, one group of fetuses was infused with cortisol (0.45mg/h) for 80 h and other group obtained saline. Then the fetuses were delivered by hysterotomy under anesthesia at around 129 dg.

In this study, all newborn lambs and ewes were sacrificed by an overdose of sodium pentobarbitone, administered intravenously. Liver tissues were collected from non pregnant adult (n=4), fetal lambs (126-128 dg) which received cortisol infusion (n=5), control fetal lambs (126-128dg) which received saline (n=3) and newborn lambs (<1 post natal day) (n=4) within 15 minutes of death. Tissues were stored at -80°C until microsome preparation and RNA extraction.

2.3 Preparation of liver microsomes

Microsomes were prepared from frozen liver using a standard differential ultracentrifugation technique at 5°C (Lu and Levin, 1972). Briefly, frozen liver samples were thawed, minced and then homogenized in Tris/KCl buffer (0.05M Tris, 1.15%KCl, pH 7.4) using a Potter-Elvehjem tissue grinder (Talboys Engineering Corp., Emerson, NJ, USA). The homogenate was centrifuged at 9,000g for 20 min at 5°C using a Beckman J2-21 centrifuge (Beckman Instrument, Palo Alto, CA, USA) and the collected supernatant was then centrifuged at 105,000g for 60 min at 5°C using a Beckman LE-80 ultracentrifuge. The resulting microsomal pellet was resuspended in EDTA/KCl buffer (10mM EDTA, 1.15%KCl, pH 7.4) using the homogenizer and centrifuged again at 105,000g for 60 min. Microsomal pellets were resuspended in 0.25M sucrose and aliquots were stored in cryo vials at -80°C. Protein concentrations were measured according to Lowry et al. (1951) and BSA was used as standard. Protein concentrations were measured in duplicate at 750 nm using a Shimadzu UV-160 UV-visible recording spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

2.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Microsomal proteins (1-100µg) were resolved by 7.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) using a Hoefer SE 600 vertical slab gel unit (Hoefer Scientific Instruments, San Francisco, CA, USA). The discontinuous SDS-polyacrylamide gel consisted of a 3.0% acrylamide stacking gel (0.75 mm thick, 1 cm long, 0.125 M Tris-HCl, pH 6.8) and a 7.5% acrylamide separating gel (0.75 mm thick, 12.5 cm long, 0.375 M Tris-HCl, pH 8.8). The final concentrations of reagents in the stacking gel were 3% acrylamide:Bis (22.2%:0.6% w/w), 0.125 M Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.08% (w/v) of ammonium persulphate, and 0.05% (v/v) TEMED. The final concentrations of reagents in the separating gel were 7.5% acrylamide:Bis (22.2%:0.6% w/w), 0.375 Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.042% (w/v) ammonium persulphate, and 0.03% (v/v) TEMED. The electrophoresis buffer contained 0.1 M Tris base, 0.767M glycine and 0.4% (w/v) SDS. Microsomes were prepared (1-100 µg) for loading by diluting in sample dilution buffer containing 0.062 M Tris-HCl (pH 6.8), 1% (w/v) SDS, 0.001% (w/v) bromophenol blue, 10% (v/v) glycerol, and 5% (v/v) mercaptoethanol and were boiled for 2 minutes. Denatured microsomal proteins and molecular weight marker (4µl) were subjected to electrophoresis with constant cooling. A constant current setting of 11.5 mA/gel was used for approximately 1 h to

allow migration of the dye front through the stacking gel and a setting of 23 mA/gel for approximately 2 h or until the dye front reached the bottom of the separating gel.

2.5 Western blot analysis

Protein levels of CYP2A, CYP2C, CYP2D, UGT1A and UGT2B enzymes were assessed by immuno-blot analysis with polyclonal antibodies against rat and human CYP proteins obtained from mainly from Dr. Bandiera (Faculty of Pharmaceutical Sciences, University of British Columbia) and BD Biosciences (Bedford, MA) and also from Abcam (Cambridge, MA, USA) and Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) (Table 2.1). Separated proteins by SDS- PAGE were transferred electrophoretically onto nitrocellulose membranes using a Hoefer Transphor Apparatus (model TE 52) at a setting of 0.4A for 2 h at 4°C (Towbin et al., 1979). Nitrocellulose membranes were incubated individually with the following primary antibodies at 37°C for 2 h with shaking.

After washing three times with wash buffer (137 mM sodium chloride, 2.6 mM potassium chloride, 8.1 mM sodium phosphate, 1.5 mM potassium dihydrogen phosphate, 0.2 mM EDTA, 0.05% Tween 20; pH 7.4), the membranes were incubated with alkaline phosphatase conjugated goat F(ab')₂ anti-rabbit IgG or rabbit anti sheep antibody or swine anti-goat IgG at a dilution of 1:3000 at 37°C for 2 h with shaking. The nitrocellulose membranes were then washed three times with wash buffer and then washed with distilled water thoroughly.

Cytochrome P450 and UDP-Glucuronosyl transferease proteins were visualized with buffered alkaline phosphatase (0.1M Tris HCl buffer, pH 9.5; with 0.5 mM MgCl₂) substrate containing 0.03% nitro blue tetrazolium (NBT) and 0.015% 5-bromo-4-chloro-3-indolyl phosphate (BCIP) under subdued light. The reaction was stopped by rinsing the membrane with an ample amount of distilled water when the bands were visible (<5 min). Quantitation of the strong bands was performed by densitometric analysis using a pdi 4200e scanning densitometer (pdi Inc., Huntington Station, NY, USA) connected to a personal computer and pdi Quantity One 3.0 software. Purified human CYPs and UGTs were loaded onto each gel as an internal standard. Intensities of the protein bands were calculated as optical density multiplied by the stained area, a measure known as contour quantity (OD X mm²). All densitometry data were standardized to one mg of protein and to the human standards.

Frozen sheep liver sample quality was compared with fresh adult sheep liver samples which were obtained from an abattoir.

Table 2.1: Details of antibodies used in western blot

Anti body	Raised in	antigen	description	Dilution factor/final concentration	company
CYP 2A6	rabbit	human CYP2A6		500	abcam
CYP 2C11	rabbit	rat CYP2C11, purified protein	IgG	25µg/ml	Dr. Bandiera
CYP 2D6	rabbit	human CYP2D6	serum	1000	BD bioscience
UGT 1A	rabbit	human UGT1A, a peptide	whole serum	500	BD bioscience
UGT 2B7	rabbit	human UGT2B7, a peptide	whole serum	500	BD bioscience
CYP 2A1	sheep	rat CYP2A1, purified protein	IgG	500	Dr. Bandiera
UGT 1A	Goat	human UGT1A, a peptide of C-19	IgG	200	Santa Cruz

2.6 RNA isolation and cDNA synthesis

Total RNA was extracted from the liver samples using Trizol reagent according to the manufacturer's instruction. The quantity of RNA was assessed by measuring optical densities using a Nanodrop ND-1000 (Thermo scientific) spectrophotometer. RNA quality was measured by evaluation of the 28s and 18s ribosomal bands on a 1% agarose gel and A260/A280 ratio. RNA preparations with an A260/A280 ratio greater than 1.8 were used for subsequent experiments. Extracted RNA was stored at -80°C until processing.

Complementary deoxyribonucleic acid (cDNA) was synthesized according to the manufacturer's instructions. Total RNA (2µg) was incubated with 0.8 µl random primers (240ng), 1µl dNTP (10mM) and sterile water up to 13 µl at 65°C for 5 min followed by at least one minute on ice. Then 5XFS buffer (4 µl), 0.1 M DTT (1µl), Rnase out (1µl) and 200 units Superscript III (1µl) were added to each tube. Tubes were incubated at 25°C for 5 min and then 50°C for 45 min. Finally the reaction mixture was inactivated by heating at 70°C for 15 min. cDNA was used immediately or stored at -20°C.

2.7 Primer design

Ovine sequences were not available for the CYP2C9, CYP2C19, CYP2A6, UGT1A9, UGT2B7 and HNF4α genes. Therefore genes from different animal species (human, bovine, chimpanzee, olive baboon, rhesus monkey, rat and mouse) were aligned using the ClustalW2 program to identify conserved regions. Forward and reverse primers were designed manually in the conserved areas. The unique regions of each enzyme were considered when designing the primers because of the high homology between these enzymes. The program Sequence Manipulation Suite was used to avoid primer dimers and hair pin formations. A short sequence of sheep ortholog to human CYP2D6 mRNA (unpublished data from May, Hammond and Rurak) and full length UGT1A6 sequence (Kobayashi et al., 1999) were obtained from previous studies in sheep.

2.8 Reverse transcriptase polymerase chain reaction (RT-PCR)

Polymerase Chain Reaction (PCR) was performed in a final volume of 35µl containing 2µl of cDNA template, 1µl, 10µM of each primer (Table 2.2) and 31µl of PCR Super Mix. The typical reaction cycles consisted of an initial denaturation step at 94°C for 2 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55 to 60°C for 30 sec and elongation at

72°C for 45 sec with a final elongation step at 72°C for 7 min. Glyceraldehyde 3-phosphate Dehydrogenase (GAPDH) was used as a positive control. Amplicons were visualized on 1% agarose gels containing ethidium bromide (0.2 µg/ml) and imaged under ultraviolet illumination.

2.9 PCR purification

PCR product was purified using a Qiagen PCR purification kit according to the manufacturer's protocol. All centrifugation steps were at 13000 rpm in a conventional tabletop micro centrifuge. Five times the volume (100µl) of buffer PBI was added to a one time volume (20µl) of PCR reaction and mixed well. The QIAquick column was placed in a provided 2ml collection tube. The sample was applied to the column for DNA binding and centrifuged for 1 min. The eluted volume was discarded and the column was placed back in the same tube. Buffer PE (750µl) was added to the column for washing and centrifuged for 1 min. Again flow through was discarded and the column was placed back in the same tube. The column was centrifuged again for 1 min and placed in a clean 1.5ml micro centrifuge tube. Buffer EB (50µl) was added to the center of the QIAquick membrane and the column was let stand for 1 min. Again the column was centrifuged for 1 min.

The concentration and the quality of the purified DNA were analyzed using the spectrophotometer by 260/280nm ratio. Then purified DNA was mixed with loading dye and run in a 1% agarose gel to analyze the quality and desired size of DNA. Then it was subjected to TOPO cloning.

2.10 Gel extraction

When there was more than one band present, the desired DNA fragment was excised from the agarose gel with a clean sharp razor blade. The gel slice was weighed in an eppendorf tube. Three times the volume of buffer QG was added to one volume of gel. The tube was incubated at 50°C for 10 min and mixed by vortexing every 2-3 min during the incubation, to ensure that the gel completely dissolved. Then isopropanol (one gel volume) was added to the tube and mixed well. A QIAquick spin column was placed in a 2ml collection tube. The sample was added to the column and centrifuged at 13000 rpm for 1 min. The eluted volume was discarded and the column was placed back into the same tube. Buffer PE (750µl) was added to the column and centrifuged at 13000 rpm for 1 min. Again the eluted volume was discarded and the column was placed back in the same tube. The column was centrifuged again at 13000 rpm

for 1 min and placed in a clean 1.5ml micro centrifuge tube. Buffer EB (50µl) was added to the center of the QIAquick membrane and the column was let stand for 1 min. Again the column was centrifuged for 1 min. The concentration and quality of the purified DNA and the proper size of bands were analyzed using the methods described above.

2.11 Cloning

Cloning reactions were performed using the TOPO cloning kit with 4µl of PCR product, 1µl salt solution (1.2M NaCl and 0.06M MgCl₂) and 1µl of PCR II Blunt TOPO (vector) by incubating at room temperature for 10 min. This was followed by transforming One Shot Competent cells. TOPO cloning reaction (2µl) was added into a vial of One Shot Competent Ecoli and incubated on ice for 30min. Cells were heat shocked for 30s at 42°C and immediately transferred to ice. After the addition of Super Optimal broth with Catabolite repression (S.O.C) medium, tubes were horizontally shaken at 200 rpm, 37°C for 1 hour. The mixture (100µl) was placed on a culture plate containing kanamycin and cultured overnight at 37°C to get visible colonies. Four individual larger colonies were randomly selected and cultured overnight in LB medium (3ml) containing kanamycin at 37°C with shaking.

2.12 Plasmid purification and DNA sequencing

Plasmid was purified using the Purelink Mini Purification kit. Briefly, a cell lysate was prepared from the culture by centrifugation (1500 rpm, 15min), followed by addition of the resuspension solution (240µl), lysis buffer (240µl) and neutralization buffer (340µl). After gentle mixing, it was centrifuged at maximum speed for 10min.

Cell lysate was filtered through a spin column by centrifuging at room temperature at 14000 rpm for 1 min and the column was re-centrifuged to remove the residual buffer. Fifty µl of elution buffer was added to the column and centrifuged to get the purified DNA in an elution tube. Concentrations of the plasmids were measured using a spectrophotometer at 260nm. Around 200-500ng plasmid DNA was digested to confirm the presence of the desired gene sequence. An enzyme mixture (10µl) containing buffer (1µl), EcoR1 (0.3 µl) and sterile water (up to 10µl) was added to the purified plasmid (10µl) and incubated at 37°C for 1 hour. The product size was identified on a 1% agarose gel and the appropriate size was confirmed. Isolated plasmids were sequenced in Center for Molecular Medicine and Therapeutics (CMMT) (Vancouver, BC).

Table 2.2: Details of primers used in regular PCR

Gene (orthologs)	Forward (F) and Reverse I Primers 5' - 3'	Ta °C	Amplicon size (bp)
CYP2A6	F – GCG GGA CTT CGG GGT GGG R – TTCTCATTCAGGAAGTGCTGGGG	58	854 (430-1280)
CYP2C9	F – AGG AGC CAC ATG CCC TAC AC R – TTC CTG CTG AGA AAG GCA TGA AG	55	271 (1024-1294)
CYP2C19	F – AGG ATT GTA AGC ACC CCC TGG R – TTTCCACCTTCATCCAGAAAGTGAC	55	638 (615-1250)
UGT1A9	F – ATG GGA GCC ACT GGT TCA CC R – AAAGGATCGAGAAACACTGCATC	55	349 (175-525)
UGT2B7	F – TGG GAA TAA ACC AGA TAC CTT AGG R – CGA AGG TGT TTA GCT CCT TTG TG	55	410 (1070-1477)
HNF4 α	F – CCT CAA AGC CAT CAT CTT CTT TGA CCC C R – GTG GAC ATC TGT CCG TTG CTG A	55	393 (953-1313)
GAPDH	F – TGTCCGTTGTGGATCTGACC R – CGTACCAGGAAATGAGCTTGAC	60	240 (Cui et al., 2002)

F – Forward; R – Reverse; Ta – Annealing temperature; bp – base pairs size of the amplicon and the position in the human sequence within brackets.

2.13 RACE PCR

2.13.1 Primer design

Using the small piece of sheep sequence I obtained, outer and nested gene specific primers were designed for both 3' and 5' RACE (Table 2.3). Primers were designed at both ends of the sequences according to the manufacturer's instructions and a high annealing temperature was maintained to be similar to the GeneRacer primers. At least 50bp difference between outer and nested product was considered when designing the primers to differentiate both PCR products.

2.13.2 Purification of RNA for 5' RACE

High quality RNA was extracted using the Trizol method and analyzed. RNA was re-suspended in DEPC-treated water before using the GeneRacer™ Kit.

Total RNA was treated with calf intestinal phosphatase (CIP) to dephosphorylate non-mRNA or truncated mRNA. A dephosphorylation reaction (10µl) was set up in a 1.5 ml sterile microcentrifuge tube on ice using the reagents in the kit. Total RNA (3µg) was used with 10X CIP Buffer (1µl), 1µl RnaseOut™ (40 U/µl), 1µl CIP (10 U/µl) and DEPC treated water to a volume of 10µl and mixed gently by pipetting gently. The tube was centrifuged and then incubated at 50°C for 1 hour. After incubation, it was centrifuged briefly and placed on ice.

To precipitate RNA, 90 µl DEPC treated water and 100µl phenol: chloroform was added and vortexed vigorously for 30 seconds. The tube was centrifuged at maximum speed (14000rpm) in a micro centrifuge for 5 minutes at room temperature. The aqueous (top) phase was transferred to a new micro centrifuge tube (~100µl). Mussel glycogen (2µl; 10 mg/ml) and 10µl 3 M sodium acetate (pH 5.2) were added and mixed well. Ethanol (220µl; 95%) was added and vortexed briefly. Then it was frozen on dry ice for 10 minutes. The next step took place on the same day.

To pellet RNA, the sample was centrifuged at maximum speed in a micro-centrifuge for 20 minutes at 4°C. The position of the pellet was noted and the supernatant was removed by pipette without disturbing the pellet. 70 % ethanol (500µl) was added, and the tube was inverted several times and vortexed briefly. Then it was centrifuged at maximum speed in a micro-centrifuge for 2 minutes at 4°C. Then ethanol was carefully removed without disturbing the pellet. Again it was centrifuged to collect the remaining ethanol. This was removed carefully

and the pellet was air-dried for 1- 2 minutes at room temperature. The pellet was resuspended in 7 μ l DEPC treated water. Next, steps were taken to remove the mRNA Cap Structure.

After dephosphorylating and precipitating the RNA, the decapping reaction was performed by the following steps. The 10 μ l decapping reaction was set up on ice in a 1.5 ml sterile micro-centrifuge tube using the reagents in the kit. 10X TAP Buffer 1 μ l, RnaseOut™ (40 U/ μ l) 1 μ l, TAP (0.5 U/ μ l) 1 μ l were added to the dephosphorylated RNA (7 μ l) and mixed gently by pipetting. Then the tube was centrifuged briefly to collect fluid and incubated at 37°C for 1 hour. After incubation, the tube was once more centrifuged briefly and placed on ice. Again the RNA was precipitated and pelleted as described above.

Once the RNA was decapped, it is ready to ligate the GeneRacer™ RNA Oligo to the 5' end of the RNA. Seven μ l of dephosphorylated, decapped RNA was added to the tube containing the pre-aliquoted, lyophilized GeneRacer™ RNA Oligo (0.25 μ g). The solution was pipetted several times to mix and resuspend the RNA Oligo. The tube was centrifuged briefly to collect the fluid in the bottom of the tube and then it was incubated at 65°C for 5 minutes to relax the RNA secondary structure. Then the tube was placed on ice to chill (~2 minutes) and centrifuged briefly. The following reagents were added to the tube, mixed gently by pipetting, and centrifuged briefly: 10X Ligase Buffer 1 μ l, 10mM ATP 1 μ l, RnaseOut™ (40 U/ μ l) 1 μ l and T4 RNA ligase (5 U/ μ l) 1 μ l. The tube was incubated at 37°C for 1 hour, centrifuged briefly and placed on ice. Again RNA was precipitated as described above.

2.13.3 Reverse transcribing mRNA

For the 5' RACE, the following reagents were added to the 10 μ l of ligated RNA: Random primers 1 μ l, dNTP Mix 1 μ l and sterile distilled water 1 μ l. The tube was incubated at 65°C for 5 minutes to remove any RNA secondary structure. Then it was chilled on ice for at least 1 minute and centrifuged briefly. 5X First Strand Buffer (4 μ l), 0.1M DTT (1 μ l), RnaseOut™ (40 U/ μ l; 1 μ l) and SuperScript™ III RT (200 U/ μ l; 1 μ l) were added to the RNA mixture and mixed well by pipetting gently up and down. The reaction mix was incubated at 25°C for 5 minutes and then incubated at 50°C for 45 minutes. The RT reaction was inactivated at 70°C for 15 minutes and chilled on ice for 2 minutes. One μ l of Rnase H (2U) was added to the reaction mix and incubated at 37°C for 20 minutes. cDNA was immediately used or stored at -20°C.

For the 3' RACE, cDNA synthesis can be performed on the original, unligated RNA or total RNA. OligodT primers were used instead of random primers and 2µg of RNA was used. Except the incubation at 25°C for 5 minutes, all other steps were same as 5' RACE cDNA synthesis.

2.13.4 Amplification of cDNA ends

For both 3' and 5' RACE, outer gene specific primers (0.75µl; 10mM) and GeneRacer primers (0.75µl; 10mM) were added to 1µl of the RT reaction. Then 22.5µl pfx accuprime supermix was added and the tube gently tapped at the bottom to mix it. The tube was centrifuged to bring all fluids to the bottom. All the steps were performed on ice.

The typical reaction cycle consisted of an initial denaturation step at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 60 to 65°C for 30 sec and elongation at 68°C for 1 min with a final elongation step at 68°C for 10 min. Amplicons were visualized on 1% agarose gels containing GelRed (0.2 µg/ml) and imaged under ultraviolet illumination.

Nested PCR was performed with the above PCR product. The amount of template was determined according the band thickness and the concentration of DNA which was present. When the DNA concentration was low, 1µl of template was used, if not, it was diluted with sterile distilled water to make it 1µl. Nested gene specific primers (0.75µl; 10mM) and GeneRacer nested primers (0.75µl; 10mM) were added to the template. Then 22.5µl pfx accuprime supermix was added and gently tapped at the bottom to mix it. The PCR conditions and visualization of bands were the same as above. PCR product was purified, cloned and sequenced as mentioned above.

2.14 Full sequences

For some of the genes (orthologs to human CYP2A6, CYP2C19, CYP2E1 and UGT2B7) full sequences were obtained with the mid and 3' and 5' end pieces that were obtained. These sequences were arranged according to their corresponding positions in the human sequence. The ends of the pieces were overlapping to avoid false sequences since the primers were designed in an overlapping manner.

For other genes, several small pieces were cloned to get a full sequence especially HNF4α and UGT1A. These small pieces (Table 2.4) were aligned together to get a full sequence.

2.15 Real time PCR

2.15.1 Primer design for real time PCR

Two real time primer sets were designed (Table 2.5, Table 2.6) for each enzyme using the sheep gene sequences obtained from our study. Sequences in the non coding region were used to avoid obtaining other CYP P450 or UGT genes, since there is high sequence similarity between CYP and UGT enzymes. A BLAST search was conducted to ensure that primers were not constructed from any homologous regions that would encode for other proteins. The specificity of the PCR product was confirmed by gel electrophoresis to verify that the transcripts were of the predicted molecular size. Dissociation curves were obtained for both sets of primers in each enzyme. According to the dissociation curve, one primer set was selected for real time PCR for each enzyme (Table 2.5).

2.15.2 Validation of internal control

Generally GAPDH is often used as a house keeping gene in several studies since it is constant in several tissues. A previously designed GAPDH primer set was used in our study (Cui et al., 2002). But, in our study, GAPDH mRNA concentrations varied among the liver samples. Therefore 18S ribosomal RNA primers were designed and used as an internal control. It served as a good internal control for liver tissues.

2.15.3 Template preparation

Just prior to the cDNA synthesis, the concentration and quality of all RNA samples were measured using Nanodrop ND-1000 at 280nm wave length. All the RNA samples used in our experiment were of good quality with a A260/A280 ratio of more than 1.8. RNA quality was also measured by evaluation of the 28s and 18s ribosomal bands on 1% agarose gel. The cDNA of all samples were synthesized together to reduce the experimental variability. Templates were diluted 50 times for CYPs and HNF4 α and 25 times for UGTs with TE buffer.

Table 2.3: Nucleotide sequences of 5' and 3' race primers

Gene (orthologs)	Primer name	Primers	Ta °C	Amplicon size (bp)
CYP 2A6	5' O	GCT TCC CAG CAT CAT TCG CAG TAG TGA	65	650
	5' I	TCA GGA AGA AGG TGG GAT CGA TGA AGG T	65	550
	3' O	CGC AGA GTC ACC AAG GAT ACC AAG TT	65	645
	3' I	CCT ATG CTG GGT TCC GTG CTG AGA	65	580
CYP 2C19	5' O	TATTGAGGTCCAGGGATTCTTGGTGTT	64	850
	5' I	CGTAGTAAAGAGGGGAAAGCATTGAAGA	64	750
	3' O	AGCAATCTGCCCATGTAGCAACTCA	64	700
	3' I	GGCACAGCCATATTAACATCTCTGACT	64	640
CYP 2D6	5' O	TCCTCGGTCACCCACTGCTCCA	63	514
	5' I	CCCATATCGCGCCAGGATCACTCC	63	421
	3' O	CGACACTCATCACCAACCTGTCGTCA	65	450
	3' I	TCAAGCAGGAGGCCTTCATACCCTTCT	65	350
CYP 2E1	5' O	AAGAGCAGGTCCGCCACAGTCACAG	63	928
	5' I	AAGGTGTACATGGGGTCCGCACTGTGT	63	892
	3' O	ACTGTTTCGGAGAAGGCCT	60	330
UGT 2B7	5' O	ATCAGCAAACAAAGGAATGCCACCAT	63	1210
	5' I	CGTAAATGCCGTTGGTTCCACCATGAGT	65	1160
	3' O	TATGAAGGCCAAGGGAACAGCTGTCAG	63	680
	3' I	CCTTGACAGAGCAGTCTTCTGGATTGAGT	64	530
UGT 1A9	5' O	CGTTCAAATCCTCCAGAGTGT	60	380
UGT 1A	5' O	GAGTCTTTGGGTGACCAAGCAGATCA	60	1185
	3' O	ACG GCT GGT TCT CAA TTA TGC TTG TGT	63	305
	3' I	GATGTGTCTTC CTTGTCCTTG ATGTGA	63	250
HNF 4 α	5' O	ACC TGG GAA CGC AGC CTC TTG AT	63	938
	3' O	TCCTCGATTTCTTGTGATGACCAAGCCA	63	293
	3' I	AGCAGTTCTGAGAGTGGGCTGGCAT	63	195

O – Outer primer; I – inner primer; Ta – annealing temperature; bp – base pairs

Table 2.4: Nucleotide sequences of other primers for the full sequence, annealing temperature, position in the human sequence and the amplicon size.

Gene (orthologs)	Primer name	Primers	Ta °C	Position	Amplicon size (bp)
CYP2E1	Mid F	GTTC AATGAGA ACTTCTACCTGCTCAG	60	528 – 1415	887
	Mid R	CTTCAGGTTAAAGTGCTGCAGGATG			
CYP2D6	5' mid F	TGG ACG CCG GTA GTC GTG CTC AA	63	269 – 1061	800
	5' mid R	GAT CAC CTC ATC GAT TTC CTG CTG GA			
UGT1A9	1F	CCTCAGACAGTCCTGTGGCG	55	1035 – 1506	470
	1R	GACCTCACCTGGTACCAGTAC			
	2F	GCTGTAAGAGCTTGTGTTGAGGACAAGA	60	418 – 1147	730
	2R	GAGTCTTTGGGTGACCAAGCAGATCA			
	End F	GACCTGGCTGTATTCTGGGTGGAGT	60	1450 – 2270	820
	End R	CCAGTCACTATAATTTAGGCATCTGTC			
HNF4α	1F	CTCTCCAAAACCCTCGTCGACAT	60	1 – 938	938
	1R	ACC TGG GAA CGC AGC CTC TTG AT			
	2F	CAC ATG GGC ACC AAC GTC ATT GTT	57	1182 – 2105	920
	2R	ACC CTG GAT TGT TCT TTC ATG TTC A			
	3F	TTT GTG AGA GAG GCA GGA AGG GTA	57	3468 – 4378	910
	3R	GGA CAG CAC AGG ACC CGA TAC AT			
	4F	GTCTCTCCCTTCTTCTGGGACT	60	2020 – 2933	913
	4R	TGAGGCTTCCAAGGCTGCTGAAG			
5F	TGGA ACTCACACAGCTAGTAAGTGA	57	2773 – 3723	950	
5R	CAATGACCAGAAATGATCCCAGCTTC				

F – Forward; R – Reverse; Ta – annealing temperature; bp – base pairs

Table 2.5: Nucleotide sequences of forward and reverse primers used in Real Time PCR

Gene (orthologs)	Forward (F) and Reverse I Primers 5' - 3'	Amplicon size (bp)
CYP2A6	F – CCT GGC CAT CAC TCC CAG TT R – GCA GGT AGT TCC CGA TGA AG	167
CYP2C19	F – CCT CAC ACA AAG TGC ACC AAT GGA R – AGG TGG CAG CTT CCC TTT GCC AT	112
CYP2D6	F – TGC AGC ACT TGC TTC TCG GT R – ATT GAG CAT CAG GGC TAT GG	164
UGT1A6	F – TGGTGGTGCCCAAAGTCAACC R – CGATCATGTTGTTTCCTATACTCCAC	180
UGT1A9	F – CTGATGGCTCCTGCAATGTTAATG R – TCTTTGGGAGAGTTTCTCCACC	155
UGT2B7	F – GCTGACTTGCTACTTCAACTCT R – ACCTCATGACCCCTTGAGACT	120
HNF4 α	F – ACC CAG GCA AGA TCA AGA GG R – CCG AAG AGC TTG ATA AAC TGG	174
18S RNA	F – AAA ACC AAC CCG GTC AGC CT R – CTT CCT TGG ATG TGG TAG CC	240

Table 2.6: Nucleotide sequences of the second set of forward and reverse primers designed for Real Time PCR

Gene (orthologs)	Forward (F) and Reverse I Primers 5' - 3'	Amplicon size (bp)
CYP2A6	F – TGG CTT TGC TGG CCT GTC TGA R – CGT AGC GCT CGC TGA TCT TCA T	170
CYP2C19	F – ACA TTC TTG AGC CCT GCC TCT TCT R – AAG GTG CCC TCC ATT TGG CAT ACA	163
CYP2D6	F – ACC GTC TGG GAG AAG CCC TT R – GTC ACC AGG AAG GCA AAG AC	240
UGT1A6	F – TCC TCC AGC ATG GCT TGC CT R – CCTTTCTCGCTGAGACGTTCA	158
UGT1A9	F – TGC TGC TGA CAC CAG GCT TTG R – GGATGAATTGCTCAGGTGCCAAC	164
UGT2B7	F – AGGAAAGAAGAAGAGGGAGTAGC R – GAGGTGCTAAACTGAGACCAGC	143

2.15.4 Real-time PCR

Levels of all CYPs, UGTs and HNF4 α transcripts were evaluated in all four groups by real time PCR using the ABI 7300 system. 18S RNA was used as an internal control. PCR products were detected using SYBR Green (Applied Biosystem). Each reaction mix (25 μ l) contained SYBR Green master mix (12.5 μ l), sterile distilled water (8 μ l), forward and reverse primers (10mM, 2 μ l) and 2.5 μ l of diluted cDNA. Thermal cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 60°C for 60s. Melting curve analysis was performed after the real-time PCR reaction to monitor PCR specificity.

2.15.5 Quantitative expression analysis.

Quantifying the relative expression of all enzymes was performed using the $\Delta\Delta$ Ct method (Livak and Schmittgen, 2001). In this method, the Ct values (threshold cycle where the fluorescence is first detected above background noise) are used to measure fold differences in target mRNAs between samples. Then, the relative levels of the mRNAs were calculated using the formula: relative expression of target mRNA = $2^{-\Delta\Delta$ Ct}, where $\Delta\Delta$ Ct = Δ Ct sample - Δ Ct reference. Here, Δ Ct sample is the Ct value for any sample normalized to the endogenous housekeeping gene (18S) and Δ Ct reference is the Ct value for the calibrator also normalized to the endogenous housekeeping gene.

2.16 Statistical analysis.

Expression data was analyzed using the JMP 7.0.2 (SAS Institute, Inc., Cary, NC) software package. Relative hepatic mRNA and protein levels of the enzymes were compared between experimental groups using one-way analysis of variance (ANOVA) and the differences were analyzed by Student t-test. The results were reported as the mean values for each set of data \pm SEM and the level of statistical significance was defined at probability level (p) <0.05. Linear regression analysis was performed using the least squares method to identify the relationship between the relative mRNA expressions of HNF4 α and CYP or UGT enzymes.

3. Results

3.1 cDNA sequences

The full mRNA sequences of orthologs to human CYP2C19, CYP2D6, CYP2E1, UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B7 and HNF4 α were cloned. In addition, partial cDNA sequences of orthologs to human CYP2A6 and CYP2C18 were also obtained. Amino acid sequences were obtained by translating the nucleotide sequences using Sequence Manipulation Suite website (<http://www.bioinformatics.org/sms2/translate.html>).

Cytochrome P450 2A6 cDNA was obtained only up to 1505bp in length in sheep. We were only able to clone the 27bp 5' untranslated region (UTR) and 1478bp of the open reading frame (ORF) encoding 492 amino acids (Fig 3.1). We could not clone the rest of the coding region (7bp) and the 3' UTR. Sheep CYP2A6 nucleotide sequence showed 87% identity with human CYP2A6, 89% identity with pig CYP2A6 and 98% identity with cow CYP2A6, of which 231bp of the full length sequence is available (Table 3.1). The sheep CYP2A6 amino acid sequence also showed a similar identity with human (89%), pig (92%) and cow (98%).

The cloned sheep CYP2C19 cDNA was 1966bp long and contained a 1473bp ORF, encoding 490 amino acids as in the human and cow. The 5' UTR was 21bp and 3' UTR was 472bp (Fig 3.2). The sheep CYP2C19 nucleotide sequence has 95% identity to cow CYP2C19 and 77% homology to human CYP2C19 whereas the sheep CYP2C19 amino acid sequence has 93% homology with cow and 67% with human.

Only the partial (1384bp) sequence of orthologs to human CYP2C18 was obtained in sheep. It consists of 139bp 5'UTR and 1245bp ORF which encodes 415 amino acids (Fig 3.3). The rest of the ORF and the 3'UTR regions were not cloned. Sheep CYP2C18 nucleotide sequence revealed 88% homology with cow and 73% with human CYP2C18.

A 1682bp cDNA sequence was cloned for ortholog to human CYP2D6, containing a 79bp 5'UTR, 100bp 3'UTR and a 1503bp coding region which is translated to 500 amino acids (Fig 3.4). Several other species (cow, horse, mouse CYP2D22 and rat CYP2D4) also have 500 amino acids in CYP2D6 except for the human (497 amino acids). The cloned CYP2D6 nucleotide sequence revealed 92% homology with cow and 80% homology with human CYP2D6. The homology of the amino acid sequence was also similar to that of cDNA sequence (with cow 92%, with horse 80%, with human 76%).

The full cDNA sequence of CYP2E1 was 1718bp and contained a 23bp 3'UTR, 207bp 5'UTR and 1488bp ORF in sheep (Fig 3.5). The sheep sequence contains 495 amino acids like

the cow and pig but the human, rat and mouse sequences are 2 residues shorter. The nucleotide sequence of sheep CYP2E1 showed 93% identity with cow and 73% identity with human CYP2E1, whereas the sheep amino acid sequence revealed homology of 93% with cow, 77% with human, 85% with pig and 78% with mouse.

The full cDNA sequence of sheep HNF4 α was also cloned. It consists of a total of 4465 nucleotides with a 21bp 5'UTR, 3076bp 3'UTR and 1368bp ORF (Fig 3.6). The sheep amino acid sequence of 455 residues is similar to the cow but the human amino acid sequence is 19 residues longer. Even though the sheep nucleotide homology with cow and human is 95% and 74% respectively, the amino acid identity with these species is very high (99% and 97%). Surprisingly, only one amino acid of sheep, glutamine at position of 386 differs from cow (proline).

Ortholog to human UGT2B7 cDNA is 2086bp in length and it contained a 21bp 5' UTR, 469bp 3' UTR and a coding region of 1596bp (Fig 3.7). The sheep nucleotide sequence has 81% identity with cow UGT2B10 and 74% identity with human UGT2B7. This UGT2B7 enzyme encoded 531 amino acids and has a homology of 75% with cow and 71% with human.

Five UGT1A family members have been cloned in our sheep study (orthologs to human UGT1A1, UGT1A3, UGT1A4, UGT1A6 and UGT1A9) (Figs 3.8-3.12). UGT1A6 and UGT1A07 have previously been cloned in sheep (Kobayashi et al., 1999). All UGT1A enzymes have common carboxyl terminal sequences (245 amino acids in length) and unique amino terminal sequences (285-289 amino acids). Homology among these UGT1A members ranges between 65-90% in amino acids and 72-95% in nucleotide sequence. The length of the 5' UTR varies: they are 33bp, 27bp, 23bp, 53bp and 51bp in UGT1A1, UGT1A3, UGT1A4, UGT1A6 and UGT1A9, respectively. But the 3' UTR is constant in length and sequence in all the UGT1A enzymes that were cloned (668bp). The length of the amino acid sequence varies slightly; UGT1A1, UGT1A3 and UGT1A4 have 533, UGT1A6 has 529 and UGT1A9 has 532 amino acids. Nucleotide homology of sheep enzymes are 78%, 76%, 77%, 74% and 74% with human UGT1A1, UGT1A3, UGT1A4, UGT1A6 and UGT1A9, respectively and 95% with cow UGT1A6. The UGT1A9 nucleotide that we cloned has 98% identity with sheep UGT1A07 and the UGT1A6 that we cloned has 99% identity with sheep UGT1A6.


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1 M L A S G L L L V A L L A C L T I M V L M S V W R
1 ATGCTGGCCTCAGGGCTGCTCCTCGTGGCTTTGCTGGCCTGTCTGACCATCATGGTCTTGATGTCTGTCTGGCGG
26 Q R N L K G K L P P G P T P L P F I G N Y L Q L N
76 CAAAGGAACCTCAAAGGGAATTCCTCCAGGGCCACTCCTCTGCCCTTCATCGGGAACCTGCAGCTGAAC
51 T E Q M Y N S L M K I S E R Y G P V F T I R L G T
151 ACCGAGCAGATGTACAACCTCCCTCATGAAGATCAGCGAGCGCTACGGCCCCGTTCACGATCCGCTGGGACC
76 R Q I V V L C G Y D A V K E A L V D Q A E E F S G
226 CGGAGATCGTGGTGCTGTGCGGCTATGATGCTGTGAAGGAGGCTCTGGTGGACCAGGCTGAAGAATTCAGTGGG
101 R G E Q A T F D W L F K G Y G V A F S N G G E R A K
301 CGAGGCGAGCAGGCTACCTTTGACTGGCTCTTCAAAGCTATGAGAGTGGCTTTTACGCAACGGGAGCGGCCAAG
126 Q L R R F S I T T L R D F G V G K R G I E E R I Q
376 CAGTCCGCGCTTCTCAATACGACGCTGCGGACTTCGGCGTGGGCAAGCGCGCATCGAAGAGCGATCCAG
151 E E A G F L I E A F R G T R G A F I D P T F F L S
451 GAGGCGAGGCTTCTCTCATCGAGGCTTCCGGGCACTCGCGCGCTTCATCGATCCCACTTCTCTGAGC
176 R T V S N V I S S I V F G D R F D Y E D K E F L S
526 CGAAGGCTCCCAATGTCTATCAGCTCCATGTCTTCGGGACCGCTTTGACTACGAGGACAAAGATTCTGTCA
201 L L R M M L G S F Q F T A T S T G Q L Y E M F Y S
601 CTAGTGCAGATGATGCTGGGAAGCTTCCAGTTACCGTACGCTACCGGCGAGCTCTACGATGTTCTACTCA
226 V M K Y L P G P Q Q Q A F K E L Q G L E D F I A K
676 GTGATGAAATATCTGCCAGGGCCACAGCAGCAGGCTTTAAGGAGCTGCAGGGACTGGAGGACTTCATAGCCAAG
251 K V E Q N Q R T L D P N S P R D F I D S F L I R M
751 AAGTGGAAACAAAACCAGCGCACACTGGACCCCACTCCCGGACTTCATCGACTCCTTCCTCATCGCAGC
276 Q E E K E N P N T E F Y M K N L V L T T L N L F F
826 CAGGAGGAGAAGGAGAATCCCAACCGGAGTTCATACGAAGAACCTGGTGCTAACAACCTGAACCTTCTCTTT
301 A G T E T V S T T M R Y G F L L L M K H P D V E A
901 GCGGCACTGAGACGGTCAGCACGACCATGCGGTATGGCTTCCCTGCTGCTCATGAAGCACCAGATGTGGAGGCC
326 K I H E E I D R V I G K N R Q P K F E D R A K M P
976 AAAATCCATGAGGAGATTGACCGGTGATCGGCAAGAACCGTCAGCCCAAGTTTGAGGACCGGCCAAGATGCC
351 Y T E A V I H E I Q R F G D M I P M G L A R R V T
1051 TACACAGAGCTGTGATCCAGATCCAGAGATTCGGAGACATGATCCCATGGGCTGGCCCGCAGAGTCACC
376 K D T K F R D F L L P K G T E V F P M L G S V L R
1126 AAGGATACCAAGTTTCGAGACTTCTCCTCCCCAAGGGCACCGAAGTGTCCCTATGCTGGGTTCCGTGCTGAGA
401 D P K F F S N P R D F N P Q H F L D E K G Q F K K
1201 S A C C C A A G T T C T C C A A C C C G A G A T T T C A A T C C C C A G C A C T T C C T G G A C G A G A A G G G C A A T T T A A G A A G
426 S D A F V P F S I G K R Y C F G E G L A R M E L F
1276 AGTGATGCTTTTGTGCCCTTCTCCATCGGAAAGCGGTACTGTTTCGGAGAAGGCTCGCCAGAATGGAGCTCTTC
451 L F F T T I M Q N F R F K S P Q S P Q D I N V S P
1351 CTCTTCTTACCACCATCATGCAGAACTTCCGCTTCAAGTCCCCACAGTCGCCCCAGGACATCAATGTGTCCCC
476 K L V G F A T I P P N Y T M S F L
1426 AAACCTCGTGGGCTTTGCCACTATCCCTCAAACCTACACCATGAGCTTCCTGCC

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Fig 3.1: mRNA and deduced amino acid sheep sequence ortholog to human CYP2A6.

The numbers at the left of each line denote the positions of the start nucleotide and amino acid. The initiation codon is highlighted in grey. The bold and larger font letters denote the potential glycosylation site.

```

-21
1 M D L F V V L V I C L S I L I F L F L W N Q K H G
1 ATGGATCTCTTTGTAGTCTTGGTGTCTGTCTTTCTATTTTGATTTTCCTTTTTCTATGGAATCAGAAGCATGGC
26 K G K L P P G P T P L P I V G N I L Q I N I K N I
76 AAAGGGAAGTGCCACCTGGGCCACTCCTCTCCCAATTTGTTGAAATATTCTCCAGATAAATATTAAGAATATC
51 S K C I S K L A E D Y G P V F T L Y F G M K P T V
151 AGCAAATGCATAAGCAAGCTAGCAGAAGATTATGGCCCTGTGTTCACTCTGTATTTTGGCATGAAGCCCCTGTG
76 V L H G Y E A V K Q V L I D Q S E E F S G R G S L
226 GTGTTGCATGGATATGAAGCAGTGAAGCAAGTCTGATGATCAGAGTGAAGGAATTTTCTGGCAGAGGTAGTTTA
101 P V A D N I N Q G L G I V F S N G E K W K Q T R R
301 CCAGTGGCAGACAACATCAACCAAGGATTAGGAATGTTTTCAGCAATGGGGAATAAATGAAGCAAACCTGACGT
126 F S L M V L R N M G M G K R T I E H R I Q E E A L
376 TTCTCCCTCATGGTTTTGAGGAATATGGGGATGGGGAAGAGGACTATTGAACATAGAATTCAAGAGGAAGCCTTG
151 C L V E A L K K T N G S P C D P T L L L S C A P C
451 TGTTTGGTGAAGCATTAAAAAACCCTGATGATCCTTTGATGATCCTTTACTTTCTGAGCTGTGCTCCCTCGC
176 N V I C S I V F R N R F E Y S D E K L L T L I K Y
526 AATGTGATCTGCTCCATTGTTTCCGGAATCGTTTTGAGTATAGTGTGATGAGAAATGTCTAACCTTGATAAAATAT
201 F N E N A M L A S T P W I E L F N A F P S L T L R H
601 TTTAATGAAAATGCAATGCTTGCAAGCACCCCTGGATAGAGCTTTCAATGCTTTCCCTCTTTACTACGTCA
226 F P G S H N T I F K N M T E Q R K F I L E E I K K
676 TTCCAGGAAGTCATAATACAATATTTAAAAACATGACTGAGCAAAGAAAGTTTATTTTGGAGGAAATAAAGAAA
251 H Q E S L D L N N P Q D F I D Y F L I K M E K E K
751 CACCAAGAATCCCTGGACCTCAATAACCCTCAAGATTTTATTGATTACTTTCTGATTAATAAATGGAAAAGGAAAA
276 H N K H S E F T M D N L I T T V W D V F S A G T E
826 CACAATAAACATTCTGAATTTACCATGGACAACCTGATCACCCTGTATGGGATGTATTTAGTGTGGAACAGAG
301 T T S L T L R Y G L L L L L K H P E V T A K V Q E
901 ACAACGAGCTCACTCTGAGATATGGGCTCCTGCTCCTGCTGAAGCACCCAGAGGTCACAGCTAAAGTTTCAGGAA
326 E I D H V V G R N R S P C M Q D R S R M P Y T D A
976 GAAATGACCATGTGGTTGGCAGAAACCGAAGCCCTGATGATGACAGGACAGGAGCCGCATGCCCTACACGGATGCT
351 V L H E I Q R Y I D L V P S N L P H V A T Q D V K
1051 GTGCTGCATGAGATCCAGAGATACATTGATCTTGTCCCCAGCAATCTGCCCATGTAGCAACTCAGGATGTTAAG
376 F R E Y L I P K G T A I L T S L T S V L H D G K E
1126 TTCAGAGAATACCTCATTCCCAAGGCACAGCGATATTAACATCTCTGACTTCTGTCTGCACGATGGTAAGGAG
401 F P N P G Q F D P A H F L D E S G N F K K T D H F
1201 TTTCCCAACCCAGGCAGTTTGATCCTGCTCATTCTCTGGATGAAAGTGGCAACTTTAAGAAGACTGACCACTTC
426 M A F S A G K R V C V G E G L A R M E L F L L L V
1276 ATGGCTTTTTTCAGCAGGAAAAAGGTTTTGTGTTGGAGAAGGCCTGGCCCGCATGGAGCTGTTTTTACTCTGGTC
451 S I L Q H F T L K P V V D P K H I D I A P S F K G
1351 AGCATTTTACAGCATTTTACCTTGAAACCTGTGTTGATCCAAAGCACATTGATATTGCACCAAGTTTCAAAGGG
476 I L S I P P F C E M C F I P V
1426 ATACTCTCTATTCCACCCCTTCTGTGAGATGTGTTTCATTCCAGTCTGAAGAAAGGGCTGACTGCCAGGTTGCAGA
1501 GTGAGCCTCTCCAGCCCCATCTACACAAGCTGATGCTCTCTATCCTGGCTGTTAGCCATTGTCGCCCTCTCCCTT
1576 ACCTTTAGGAGTGACATTCTTGAGCCCTGCCTCTTCTAATTTTCTTTAAGATTCACTTCAAGTTTTTTCTTGCA
1651 GAAATACAACAGTTTTGTCCCAATAATTTTTTTTAAATTAAGTCTGCTGAGATCTGAAGCTACATCCAATTTAT
1726 TGTGTATGCCAATGGAGGGCACCTTATAAATGTCATATTTTATTAATAAATAAAGTTGGCAATTATCCCATGTG
1801 ATAAAATTTTTTAAATGCACTCATCTCTACTCTATTTATCTCTAGGGGAATGATCTCTTGATAGTTTGGGA
1876 AAATAATTTTGTCTTTTCCCAATGTTTATTAATAAATAAATTATGTGAAAAAAAAAAAAAAAAAAAAAAAAA

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Figure 3.2: mRNA and deduced amino acid sheep sequence ortholog to human CYP2C19.

The numbers at the left of each line denote the positions of the start nucleotide and amino acid. The initiation codon and termination codon are highlighted in grey. The bold and larger font letters denote the potential glycosylation site.

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-139          AAAAAAAAAAGAGAGATTAAGTAGACCTGGCTGACTGTTCACTTTCTGAGTTAGAGGCCAAAG
-75 TTGAATTAGCAGGGACTGTTATAAAAGCTTTAGGTTTCAGGCTCACAGTCGTCTGAGTGAGAAGGAAGGCACCA
  1 M D L A V V L V L C L S C L L L L S L W K Q S S G
  1 ATGGATCTGGCTGTGGTCTCGTGCTCTGCCTCTCCTGCTTCTCCTCTCACTCTGGAACAGAGCTCTGGG
 26 K G K L P P G P T P L P I L G N I L Q L D V K N I
 76 AAAGGAAGCTCCGCCGGGTCCCACTCCTCTCCCGATTCTTGAAATATCCTGCAGCTAGATGTTAAGAACATT
 51 G K S L S N L S N T Y G P V F T V Y F G L R P T V
151 GGCAAATCCTTAAGCAATCTCTCAAACACCTATGGCCCTGTGTTACAGTGTATTTGGCTTGAGGCCACTGTT
 76 V L H G Y E A V K E A L I D Q G E E F S G R G S L
226 GTGTTGCATGGATATGAAGCAGTAAAGAACCCCTAATTGATCAGGGAGAAGAGTTTTCTGGAAGAGGCAGTCTC
101 P M S Q R V N K G Y G I L F G N G K R W K E I R R
301 CCAATGTCTCAAAGAGTTAATAAAGGATATGGAATCCTCTTCGGCAACGGGAAGAGATGGAAGGAGATCCGACGC
126 F S L M T L R N F G M G K R S I E D R V Q E E A R
376 TTCTCCCTAATGACCCTGCGGAATTTCCGGATGGGGAAGAGGAGCATCGAGGACCGGTTCAAGAGGAAGCGCGC
151 C L V E E L R K T N G S P C D P T F L L G C A P C
451 TGCCTAGTGGAGGAGTTGAGAAAAACGAACGGGTACCTTGTGATCCCACTTTCTGCTGGGCTGTGCTCCCTGC
176 N V I C S I I F Q N R F D Y T D Q N F R N L L E K
526 AATGGATCTGCTCCATTATTTCCAAAATCGGTTGATTATACAGATCAGAATTTTCGTAACCTGTAGAGAAA
201 F N E N L R I M S S P W I Q I C N S F P I L I D Y
601 TTTAATGAAAATCTCCGGATTATGAGCTCCCCATGGATACAGATCTGCAACAGTTCCCTATTCTCATGACTAT
226 F P G S H N K V F K N F A Y V R S Y V L E E V K E
676 TTCCAGGGAGTCATAACAAAGTATTTAAAAATTTGCTTATGTGAGAAGCTATGTTTTGGAGGAAGTAAAAGAA
251 H Q A T L D I N N P R D L I D C F L I K M E Q E K
751 CATCAAGCAACTCTGGACATTAACAATCCTCGGGACCTCATTGATTGTTTCCTGATCAAATGGAACAGGAAAAG
276 H N Q E M E F T F E N L I A S V F D L F G A G T E
826 CACAATCAAGAGATGGAGTTACTTTTGAAAACCTGATAGCATCTGTGTTGATTGTTTGGCGCTGGGACAGAG
301 T T S T T L R Y G L L L L L L K H P E V T A K V Q E
901 ACAACAAGCACCACGCTGAGATATGGGCTCCTCCTGCTGCTGAAACACCCAGAGGTCACAGCTAAAGTCCAGGAA
326 E I D R V I G R H R S P C M Q D R S H M P Y M D A
976 GAGATTACCGTGTGATCGGCAGACATCGGAGCCCTGCATGCAGGACAGAAGCCACATGCCATACATGGATGCT
351 V V H E I Q R Y I D L V P T N L P H A V T R D V K
1051 GTGGTGCACGAGATCCAGAGATACATTGACCTCGTCCCACCAATCTGCCCCACGCGGTGACCCGTGACGTTAAA
376 F R N Y L I P K G T T I V T S L S S V L H D D K E
1126 TTCAGAACTACCTTATCCCAAGGCACGACTATAGTAACATCACTGAGTTCCTGTGCTACATGATGACAAAGAA
401 F P N P E V F D P A H F L D E
1201 TTCCCAACCCAGAGGTATTTGACCCTGCTCACTTCTGGATGAA

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Figure 3.3: mRNA and deduced amino acid sheep sequence ortholog to human CYP2C18.

The numbers at the left of each line denote the positions of the start nucleotide and amino acid. The initiation codon is highlighted in grey. The bold and larger font letters denote the potential glycosylation site.

```

-75 GTGGAGTGAGAGTGCCGCGCTGGGCTGGTCTGGGTGCCAGTGTGGTGAGGAACGCAGCGCCACAGAGGCAGCC
 1 M G L L S G D T L G P L A V A V L I F L L L L D L
 1 ATGGGGTCTGTCTGGGGACACGCTGGGGCCCTGGCCGTGGCCGTGCATCTTCTTGCTCTGTGGACCTG
26 M H R R S R W A P R Y P P G P T P L P V L G N L L
76 ATGCACCGGCGCTCACGCTGGGCCCCACGCTACCCACCAGGCCCCACGCGCTGCGGGTGTGGGCAACCTGCTG
51 Q V D F D D P C P S F N Q L R R R F G N V F S L Q
151 CAGGTGGACTTTGACGACCCGTGTCCCAGCTTCAACCAGCTGCGGGCGCGCTTCGGGAACGTGTTGAGCTGCAG
76 Q V W T P V V V L N G L A A V R E A L V Y C N Q D
226 CAGTCTGGACGCCGGTAGTCTGTCTCAACGGGCTGGCCGCTGTGCGGAAGCGTGGTGTACTGCAACCAGGAC
101 T S D R P P P A V Y E H V G Y G P R A E G V I L A
101 ACTTCCGACCGTCCACCTCCGGCTGTCTACGAGCAGTGGGTTACGGGCGCGCGCCGAGGAGTGCATCTGGCG
126 R Y R K A W R E Q R R F S L S T L R N F G L K K
376 CGATATGGGAAGGCTGGCGAGAGCAGCGCGTTTCTCCCTGTCCACCTGCGCAACTTCGGCTGGGGAAGAAG
151 S L E Q W V T E E A S C L C A A F A D Q A G R P F
451 TCACTGGAGCAGTGGGTGACTGAGGAGGCTCGTGCCTCTGTGCCGCTTCGCAGACCAGGCCGGACGCCCTTT
176 S P K L L H K A V P M L R I P G L A A K V F P G L A F
526 AGCCCAAGGACCTCCTGCATAAAGCAGTGAGCAACGTGATTGCCTCCTTGACCTTCGGATGTCGATTGAGTAC
201 N D P R I V K L L D K L E D L L K E E S G L V R Q
601 AACGATCCTCGCATCGTCAAGTTGTTGGACAAGTTGGAGGACCTGTTGAAAGAAGAGTCTGGCTTAGTGCCGAC
226 V V E A V P M L R I P G L A A K V F P G L A F
676 GTGGTGGAAAGCTGTGCCCATGCTCCTGCGCATCCCAGGGCTGGCCGCAAGTCTTCCCGGGCTGAAGGCTTC
251 M A L I D E L I T E Q K M T R D P T Q P P R H L T
751 ATGGCCCTGATTGATGAGTTGATCACCGAGCAGAAGATGACCCGGGACCCGACCAGCCACCCCGACCTGACC
276 D A F L D E M K E A K G N P E S S F N D E N L R L
826 GACGCCTTCTGGATGAGATGAAGGAGGCAAGGGGAACCCGAGAGCAGCTTCAATGATGAGAACCTGCGCCTG
301 V V T D L F T A G M V T T S T T L A W A L L L M I
901 GTGGTGACCGACCTGTTCACTGCCGGGATGGTCACCACCTCGACCACGCTGGCCTGGGCCCTCCTCCTCATGATC
326 L H P D V Q R R V Q Q E I D E V I G K V R R P E M
976 CTGCAACCCAGACGTGCAGCGACGGTCCAGCAGGAAATCGATGAGGTGATCGGAAAGGTGAGGCGACCAGAGATG
351 G D Q A F M P F T V A V V H E V Q R F A D I I P L
1051 GGGGATCAGGCCTTCATGCCCTTACCCTGGCCGTGGTCCATGAGGTGCAACGCTTTGCGGACATCATCCCCCTG
376 G L P H M T S R D I E L Q G F Y V P K G T T L I T
1126 GGATTGCCCCACATGACATCCCGTGACATCGAGCTGCAGGGCTTCTACGTGCCAAAGGGGACGACACTCATCACC
401 N L S S V L K D E T V W E K P F R F H P E H F L D
1201 AACCTGTCTGTCAGTGCTGAAGGATGAGACCGTCTGGGAGAAGCCCTTCCGCTTCCACCCGGAGCACTTCTGGAT
426 A Q G R F V K Q E A F I P F S A G R R A C L G E P
1276 GCCCAGGGCCGCTTCGTCAAGCAGGAGGCTTACACCTTCTCCGACGGCCGCGCATGCCTCGGGGAGCCC
451 L A R M E L F L F F T S L L Q H F S F S V P A G Q
1351 CTGGCCCGCATGGAGCTCTCCTTTTTTTCACCAGCCTCCTGCAGCACTTCAGCTTCTCGGTGCCTGCCGGGCG
476 P R P S D H G V F A F L V T P A P Y Q L C A V P R
1426 CCCCAGCCAGCGACACGGTGTCTTTGCCCTTCTGGTGACCCAGCCCTACCAGCTCTGTGCGGTGCCCGC

1501 TAGAGGCAGGCACCTGCCCAACCCGCTCCACAGCCATAGCCCTGATGCTCAATAAACCACTGTCTGGTGGCTC
1626 CAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

```

Figure 3.4: mRNA and deduced amino acid sheep sequence ortholog to human CYP2D6.

The numbers at the left of each line denote the positions of the start nucleotide and amino acid. The initiation codon and termination codon are highlighted in grey. The putative polyadenylation signal (AATAAA) is indicated with a solid underline. The bold and larger font letter denotes the potential glycosylation site.

TCGGCAGATCCCCACGGGCGCC

-23

```

1 M A A L G I T V A L L V W M A A L L L I S I W K H
1 ATGGCCGCGCTGGGCATCACGGTCGCCCTGCTGGTGTGGATGGCCGCGCTGCTGCTCATCTCCATCTGGAACAC
26 I Y S S W K L P P G P F P L P I I G N L L Q L D I
76 ATCTACAGCAGTGGAACTGCCCCCTTTCCCACTGCCCATCATGGGAATCTTTTGCAACTGGATATT
51 K N I P K S F T R L A E R F G P V F T L Y L G S R
151 AAGAACATCCCCAAGTCCTTACCAGGCTGGCAGAGAGTTTCGGGCCGGTGTTCACCCTGTACCTGGGTTCTCGG
76 R V V V V H G Y K P V K E V L L D Y K N E F S G R
226 CGCGTCGTGGTGCACGGCTACAAGCCGTGAAGGAGTTCCTGCTTGACTACAAGAACGAGTTTCTGGCAGA
101 G E N P G F Q V H K N N G I I F N N G P T W R D T
301 GGAGAAAACCTGGGTTCCAGGTGCACAAAACAACGGGATCATTTTCAACAATGGACCAACCTGGCGGGACACC
126 R R F S L T I L R D L G M G K Q G N E Q R I Q R E
376 CGGCGTTCTCGTTGACCATCTCCGTGACTTAGGATGGGCAAACAGGGCAACGAGCAGCGGATCCAGAGGAG
151 A H F L L D V L R K T Q G Q P F D P T F V I G F A
451 GCCCACTTCTGCTGGACGTGCTCAGGAAGACCCAGGGCCAGCCCTTTGACCCACATTTGTTCATCGGCTTCGCT
176 P Y N V I S D I L F H K R F D Y K D K T G L R L M
526 CCTTACAATGTCTATCTGACATCTTCCACAAGCGCTTTGACTATAAAGACAAGACGGGGCTGAGGCTGATG
201 S L F N E N F Y L L S T P W I Q L Y N N F S D Y L
601 AGTCTGTTCAATGAGAACTTCTACTGCTCAGCACCCCTGGATCCAGCTTTATAATAATTTCTCAGACTACCTG
226 K Y L P G S H R K L L K N V S E V K D Y A L E R T
676 AAGTACCTGCTGGAAGCCATAGAAAGCTACTGAAAAACGTGTCTGAAGTAAAAGATTACGCCTTAGAGAGAACG
251 K D H Q K S L E P S C P R G F L D T M L I E M A K
751 AAGGATCACAGAAAGTCCCTGGAGCCAGCTGCCCCGAGGCTTCTCGACACCATGCTGATAGAAATGGCTAAG
276 E K H S A D P M Y T L E N I A V T V A D L L F A G
826 GAAAACACAGTGGCGGACCCATGTACACCTTAGAAAACATCGCTGTGACTGTGGCCGACCTGCTCTTTGAGGG
301 T E T T S T T L R Y G L L I L M K Y P E V E E K L
901 ACGGAGACCACGACCCACCTGCGATATGGGTCCTGATTCTCATGAAATACCCAGAGGTTGAGAGAAACTT
326 H E E I D R V I G P S R I P A I K D R L D M P Y L
976 CATGAAGAAATTGACAGGTAATTGGGCCAAGCCGAATCCCTGCCATCAAGGACAGGCTGGACATGCCCTACCTG
351 D A V V H E I Q R F I D L I P S N L L H E A T Q D
1051 GATGCTGTGGTGCATGAGATTGAGGATTCAGCGATTTCAGCTCATTCCCTCCAATCTGCTCCATGAAAGCAACCCAGGAC
376 T V F R G Y V I P K G T L I I P T L D S V L Y D K
1126 ACAGTGTTCAGAGGATATGTCATCCCCAAGGGCACACTCATAATCCGACGCTGGACTCTGTCTGTATGATAAG
401 Q E F P E P E E F K P E H F L N E S G K F K Y S D
1201 CAAGAGTTTCTGAAACCAGAGGAGTTTAAGCCAGAGCACTTTCTGAATGAAAGTGGAAAGTTCAAGTACAGTGAC
426 H F K A F S A G K R V C V G E G L A R M E L F L F
1276 CATTTCAAGGCGTTTCTGCAGGAAAGCGGGTGTGTGTTGGAGAAGGCCTGGCTCGCATGGAAGTGTCTCTGTTT
451 L A A I L Q H F N L K S L V D P K D I D L S P I A
1351 TTGGCCGCATCCTGTCAGCACTTTAACCTGAAGTCACTTGTGACCCCAAGGATATCGACCTCAGCCCCATTGCA
476 I G F G K I P P H Y K L C L I P R S E V
1426 ATTGGGTTTGGCAAGATCCCGCCCCATTACAAACTCTGTCTCATTCCCGCTCAGAAGTGTGAAAACCCTGGGCTC
1501 CCCTGCATCCCCTCTGCGAGGACTCTGTGAGCCAGGCTCCGGAGGGAACCAGCCGGGCCAGAGACACTGCTTTC
1576 CAGCCCACGTTCTCAGACAGAGTTTGAAGCAAAGTGCGAAAAGATTGTGTCAAACCTAATTAATACTAATTAGAG
1651 CAACTAAAAAAAAAAAAAAAA

```

Figure 3.5: mRNA and deduced amino acid sheep sequence ortholog to human CYP2E1.

The numbers at the left of each line denote the positions of the start nucleotide and amino acid. The initiation codon and termination codon are highlighted in grey. The putative polyadenylation signal (AATAAA) is indicated with a solid underline. The bold and larger font letters denote the potential glycosylation site.

1 M D M A D Y S A A L D P A Y T T L E F E N V Q V L
1 ATGGACATGGCCGACTACAGTGC CGCGCTGGACCCAGCCTACACCACCCTGGAGTTTGAGAATGTGCAGGTGTTG
26 T M G N D T S P S E G A N L N A P N S L G V S A L
76 ACGATGGGCAATGACACATCCCCATCAGAGGGTGCCAACCTCAATGCACCCAACAGCCTGGGTGTGACGCGCCCTG
51 C A I C G D R A T F G K H Y G A S S C D G C K G F F
151 TGCCCATCTGCGGGGACCGGGCCAGGCAAGCACTACGGTGCCTCGAGCTGTGACGGCTGCAAGGGCTTCTTC
76 R R S V R K N H M Y S C R F S R Q C V V D K D K R
226 CGGAGAAGTGTGAGGAAGAACCACATGTACTCCTGCAGGTTTAGCCGGCAGTGCCTGGTGGACAAAGACAAGAGG
101 N Q C R Y C R L K K C F R A G M K K E A V Q N E R
301 AACCAAGTGCCTTATTCAGGCTCAAGAAGTGCTTCCGGGCTGGCATGAAGAAGGAGCTGTACAGAATGAACGG
126 D R I S T R R S S Y E D S S L P S I N A L L Q A E
376 GACCGGATCAGCACTCGCCGGTCCAGTACGAGGACAGCAGCCTGCCCTCCATCAATGCGCTCCTGCAGGCTGAG
151 V L S Q Q I T S P V I L D E R A I N G D I R A K K I G F F
451 GTCCTGTCCCAGCAGATCACCTCCCCTGTCTCTGGAATCAATGGCGACATTCGGGCGAAGAAGATCGCCAGCATC
176 A D V C E S M K E Q L L V L V E W A K Y I P A F C
526 GCAGATGTGTGAGTCCATGAAAGAGCAGCTGTGCTGCTCGTCGAGTGGGCAAGTACATCCCAGCCTTCTGT
201 E L S R Y S Q V R I L D E R A H A G E H L L L G D E R
601 GAGTCCCCTCGGATGACCAGGTGGCCCTGCTCAGAGCCCACGCGGCGAGCACCTGCTTCTCGGAGCCACCAAG
226 R S M V F K D V L L L G N D Y I V P R H C P E L A
676 CGTCCATGGTGTCAAGGATGTTCTGCTTCTAGGCAATGACTACATCGTCCCCTCGGCACTGCCCCGAGCTGGCA
251 E M S R V S R I L D E R A L V L P F Q E L Q I D D E R
751 GAGATGAGTFCGAGTGTCCGTGCGCATCCTTGATGAGTTGGTGTGCTGCCCTTCAAGAGTGCAGATTGATGACAAT
276 E Y A C L K A I I F F D P D A K G L S D P G K I K
826 GAGTACGCCTGCCTCAAAGCCATCATCTTCTTTGACCCGATGCCAAGGGGCTGAGTGACCCAGGCAAGATCAAG
301 R L R S Q V Q V S L E D Y I N D R Q Y D S R G R F
901 AGGCTGCGTTCAGGAGTGCAGGTGAGCCTGGAAGACTACATCAACGACCCAGTACGACTCGCGTGGCCGCTTC
326 G E L L L L L P T L Q S I T W Q M I E Q I Q F I K
976 GGCAGCTGTGCTGCTGCTGCTGCCACCCTGCAGAGCATCACCTGGCAGATGATTGAGCAGATCCAGTTTATCAAG
351 L F G M A K I D N L L Q E M L L G G S S S E A P H
1051 CTCTTCGGATGGCCAAGATCGACAACCTGCTGAGGAGATGCTGCTGGGAGGGTCCCTCCAGTGAAGCCCTCAT
376 A H H P L H P H L M Q E H M G T N V I V A N T M P
1126 GCCCACCACCCCTGCACCTCACCTGATGCAGGAACACATGGGCACCAACGTCATTGTTGCCAACACGATGCCT
401 A H L S N G Q M S T P E T P Q P S P P G S P G S E
1201 GCTCACCTCAGCAACGACAGATGTCACCCCTGAGACTCCACAGCCTTCACCGCCCGGTGGTTCAGGTCGAG
426 P Y K L L P G A I A T I V K P P S A I P Q P T I T
1276 CCCTACAAGCTTCTGCCCCGAGCTATTGCTACCATCGTCAAGCCCCCTCCGCCATCCCGAGCCGACCATCAC
451 K Q E V I
1351 AAGCAGGAAGTCACTAGCAAGTCACTGGGGGCGGGGCTCTGCTGATCCCCCTCGCCCCCTCCGAGAGCCCTTG
1426 ATGGTCACTTGGTCACAGTGAAGCAAGCTGTGACCACGGGCCAGTCCCAGAGCAGTGATGGGAAAGGCGAGGGCC
1501 CTTGAACTCGGGCTGTGGGTTGCGTCTCACTGCCCCCTTACCACCTTGCTCTGGATATCGGGGCTGTGACTTGG
1576 GGAGACCCAGCTGGAAAATCCTCTGCTGCTTGAGCAGCTTTTCTCCTGCTGAAAACCACTGCCTTCCCCTCATCC
1651 TTTGCCTCACTTCTTCAACCCTAAAGGGCTGCCGCTGGAGACGACACGTGACCTTGCTGAAGCACCCTCCCTC
1726 CCCGTTAGACTGGTGAATGTTGGACATAGAGCCCTTCTGGGCTGGGCCACATGGCGGCTCCTCCCACATCCCT
1801 GTTCTGCTGCCGAGCCACCCTGTCCCCCTTCTTATGTCATCCTCGTTTCTCCAAAGCCACCTTCCAGAGGCT
1876 GAGGAAGACATGCAAAACACCCCCAGTCATCCAGGAGCGTTTGAAAAGCACTGACGAGCTCGCTGCAGTGTG
1951 GGATGCTCTCGAGCAGGTGGACGCCGTTTCTCCTTAACCCCTGCTCTTCCCTTCTTCTGGACTTAGGAGGT
2026 CCTTCGGGTCAGAGTGATGGTACCTATAGGCTGCTGAACATGAAAGAACAATCCAGGGTGGACTGCAGCCAGG
2101 ACCTATGGGAGGCTGGGAGGGGTCAGAGAAGGCGAGGAAGGGGCGACCATCCATTAGGCCTTTAAGGAAGGGCT
2176 GGATCTGCTGCTGTTGGAACAAGTGAAGCAGATTTCTAAGGAAGAGCAGATAGCCTAGACCAGGCGGGGAGGTC
2251 AGAAGGAGGCCATGGAAGGAGGTGATGAAAGACGGGGCTCTCTAGAGGAGAGGGGGTGCAGTGGACAGCCTGGGG
2326 ATCTTGCACGCCAGACGGAGCATGGCGACTTGGGTCCCAGGCTGTGAGTAGCTGGAGAGAGATGTGCAGGAGC
2401 CGAGCTTTCAGAACTGCAAGATCACAGTCTCCAGAGGTCAGAAGTTTGGGAGAGAGCAGGAAGGTGAGATGGG
2476 GTCTGATGGTGTATCCAGTGAAGGAGATGAAGCTGGGGATGGAGAAGACAGATGGGTGAACCTCAGGTGAGAG
2551 CAGCAATGAGGAGGAATTATCCCACCAGGCTGTCTCGAGCATTCTCCTGTTACTGTCTTAAGGCACTTGACC
2626 TGCCCCATCTCCTTTCATCCTCCTTATTACCCAGAAATCATTACTGAAGAAGGAATTGAGATCTCAGTGGTTAAA
2701 TCATTTTTCTGAACTCACACAGCTAGTAAGTGACCCAGATTTGATCCCAGATCTGTGACGGCTCAGGCACTCTTA
2776 GCCTCAGGTTGAACATCTCTTAAAAGGCCCCCTCTGCTCTTACTGGGATTGAGTTAAGAACCCTTCTACCTGCTT
2851 CAGCAGCCTTGAAGCCTCATTCTCCCCAGAAGCCTGCCACTCCCAGGCTAAGATTGGCCTGAGGCTGCACT
2926 AAAGTCCAGTTTGGGTCCAGAATCCTGTTTGTGATAAACATCGAGAAGTTGTGGTTCCTAGACCCTTTTGTGTT
3001 TCAGTCCCAAGTGTGGGAAGGGGCAATCAGCAGTTCATTGTGAGAGGGACCAATGTGACCTGGCATTCCGGAC
3076 GTGGATGGTGTGAGTAGGGGCGGGGAGAGGTAAGGAGAAGTGGGTTTTCAGACCCGCTCTGTGGAGCATCACTC

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3151 AGGGTACCTTCTGAAACCATTAAACCCAGAAATTGAGCAGCCAGTACTCATCCATGCAGAATTTTAGAGGCTTTG
3226 TGAAAATTCTGCAAAGTGCCACTGGCTAGTGGGAATTAAGGGATGTGTCTCGAACTGGAGCTTAGGGAGGAGA
3301 GTGAGCAGTCAGACTGACTACCTACCCCCATGCAGGCTTCTGAGTGGATGCAGGCAGCACCCCAGTGCCCCCA
3376 AATTCTACCATTAGGGACTGCCTTTTGTGAGAGAGGCAGGAAGGTATTGGGACAGGATGTCCGAGAACAATCAC
3451 CACATCTGCAGTCTGTGAGAGCTGGGGTTTCCGCTTCCACCAAGCCAAGTCTCAGTCCCAGGAAAGGTTGTTC
3526 CCTGCTCAGGGCAGCCAGCCTCCCTCAGCCGCCCTCATTCCGGGGCGTTCCCCCAGAAACCCCATCCAGGGC
3601 GCTCAGATGGGTCTGGGGCAAGGTGGGGAAGCTGGGATCATTCTGGTCATTGTCATGCCCTCTTTGCCACCCC
3676 GACCCCTCACCCCTGCCTCACTGTGCCCAGACTTCTGCCATGGGAACAAAGCTGAGATAAGAAGTTCCTGTGGG
3751 GTGGGGTGGGGTAGCTGTGATTTTTAACGGTAGCCAATCTTCTGGGCCAGGGGCAATTGTCGGCCCCAAATCAA
3826 CCTCCCTGGAGGAGAAATCCATGCCACTTTAATAATTTTATTGCCACGCAGGAAACACAAAGAGAATTTACTTTT
3901 CTGACTTTAAACTGAGGCCACCAGTTGGAAAAATCTTTATTTTCACTACCTGAGTTGTGATCCTAAAACGTAAG
3976 ATCTCATGGACTCAAAGAGGGTTGGAGGGCAAGCATGGACCCTGTAATTCCTCCACTGAGACAACCTCTTCTCTG
4051 GGTGTCTCTCAGTAGCTTCTGGAACCCACACAGCTCTCCTGAGGGTGGACAAATGGGAGCAGAGCCTTGAAGTG
4126 GACGCTCCTCGATTTCTTGTGATGACCAAGCCAATTACTTTTGCCAAATTGCTCTGTGATTTGCCTTGATTGTGA
4201 TGAAGTTAGAAATTCACATTGAGCAGTTCTGAGAGTGGGCTGGCATCCC GCCAGACTGATCAACATCTTCCCATG
4276 GCGATGTCATCGGGTCCTGTGCTGTCCCCGAGCCCCGTGCACCGCCATCCAATGGGGCAAAGAAGACAAAAA
4351 GATTTCTTACTCTTGTGCATTTTAAACAAAAGTTTAATAAAAAGAAATAAATGGCGCCTATGTTTTCTAAAAA
4426 AAAAAAAAAAAAAAAAAA

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Figure 3.6: mRNA and deduced amino acid sheep sequence ortholog to human HNF4 α .

The numbers at the left of each line denote the positions of the start nucleotide and amino acid. The initiation codon and termination codon are highlighted in grey. The two putative polyadenylation signals (AATAAA) are indicated with a solid underline. The bold and larger font letter denotes the potential glycosylation site.

-21 GATTTTGGAAAAGTTCTGCAT

1 M K M M T M K W L S F L L L L Q L T C Y F N S G N
1 ATGAAAATGATGACTATGAAATGGCTCTCGTTTCTGCTGCTGCTACAGCTGACTTGCTACTTCAACTCTGGAAAT
26 C G K V L V W P V E Y S H W I N I K T I L D E L V
76 TCGGAAAAGGTGCTGGTGTGGCCAGTGGAAATAGTCATTGGATAAACATAAAGACAATCTTGATGAACTAGTC
51 S R G H E V T V M T S S A S I F I N T R K E F G I
151 TCAAGGGGTGATGAGGTGACTGTTATGACATCTTCAGCTTCCATTTTTATTAATACCAGAAAAGAATTTGGTATC
76 K F E N F P V S L T K D D F E Y I F K D L L D K W
226 AAATTTGAGAATTTCCCTGTTTCTTTAACTAAAGATGATTTTGGATATATCTTCAAGGATTTGGTTGATAAATGG
101 T Y M E K D S F F S Y F P A L Q S L F W K Y S D V
301 ACATATATGGAAAAGATTCTTTTTTTTCATATTTCCAGCATTGCAAAGTTTATTTTGGAAATATTCTGATGTG
126 T M K M C K E A V S N K K L I T K L Q E S S F D I
376 ACCATGAAGATGTAAAGAAGCTGTTTCAAACAAGAACTTATAACAAAACACTACAGGAATCAAGCTTTGACATC
151 L L A D A I G P C G E L L A E I L K V P L V Y S L
451 CTTCTGACAGATGCCATTGGACCCTGTGGTGGAGCTGCTAGCTGAGATACTTAAAGTGCCTTTAGTGTACAGTCTC
176 R F S P G F S I E K Y S G G L S L P P S Y V P V I
526 CGCTTCTCCTGGTTTTTCAATTGAAAAGTATAGTGGGGGCCTTTCACTCCCACCGTCTACGTACCTGTTATT
201 M S E L S D Q M T F V F L I R T Y W D F Q F P H P L D P
601 ATGTCGGAATTAAGTGATCAAATGACATTCGTGGAGAGGGTAAAAATATGATATATGTACTTTATTTTGACTTT
226 W F Q T F N E K K W N Q F Y S E V L G R Q T T L S
676 TGGTCCAGACATTTAATGAGAAGAAATGGAATCAATTTTACAGTGAAGTACTAGGAAGACAGACTACATTATCA
251 E T M R K A E M W L I R T Y W D F Q F P H P L D P
751 GAGACAATGAGAAAAGCTGAAATGTGGCTCATTGAACTACTGGGATTTTCAATTTCCCTCACCCTACTACCA
276 N F E F V G G L H C K P A K P L P K E M E E F V Q
826 AATTTTGAATTTGTTGGAGGCCTTCACTGCAAACCTGCCAAAACCCCTGCCTAAGGAAATGGAAGATTTTGTCCAG
301 S S G E N G I V V F T L T G S M I T **N** M T E E R A N
901 AGCTCGGGAGAAAATGGTATTGTGGTGTTTACTTTGGGGTCGATGATCACTAACATGACAGAAGAAAAGAGCCAAT
326 T I A S A L A Q I P Q K V L W R Y S G K K P D T L
976 ACAATGGCATCAGCCCTTGCCAGATTCACAGAGGTGTATGGAGATACAGTGGCAAAAAACCGGACACCTTA
351 G P N T R L Y D W I P Q N D L L G H P K T K A F I
1051 GGACCTAATACTCGCTGTATGACTGGATCCCCAGAAAGTACCTTCTGGTCACTCAAAAAACCAAGCCCTTTATA
376 T H G G T N G I Y E A I Y H G I P M V G I P L F A
1126 ACTCATGGTGAACCAACGGCATTACGAGGCCATCTACCATGGAATCCCTATGGTGGGCATTCTTTGTTTGTCT
401 D Q P D N I A H M K A K G T A V S L D F E K M S T
1201 GATCAACCTGATAACATCGCTCATATGAAGGCCAAGGGAACAGCTGTCAGCTTGACTTCGAAAAATGTCAACA
426 R D L L N A L N E V I N N P S Y K E N V M W L S T
1276 AGAGATTTGCTTAATGCATGAAACGAAGTCATTAATAATCCTTCATACAAAGAGAATGTAATGTGGTTATCAACC
451 I Q H D Q P M K P L D R A V F W I E F V M R H K G
1351 ATTCAACATGATCAGCCTATGAAGCCCCTTGACAGAGCAGTCTTCTGGATTGAGTTTGCATCGCCACAAAAGGA
476 A K H L R P A A H D F T W Y Q Y H S L D V I G F L
1426 GCTAAGCACTGCGCCAGCCGCCACGACTTACCTGTTACCAGTACCCTCTCTGGATGTGATGGGTTCCCTG
501 L A C V A T A A F V I T K C Y L F C Y R K F L K T
1501 CTAGCATGTGTGGCAACTGCTGCATTTGTCATCACAAAATGTTATCTATTTTGTACCAGAAAGTTTCTTAAAAACA
526 G K K K R E
1576 GGAAAAGAAGAAGAGGGAGTAGCTGGATGTGAGGCTGAAGCAAATATGCCCATAGCTGGAACCTTCTAGCTAA
1651 TTCCAACAGGAAGGAATTAAGGCTCCCATCCTCCTGTGACAAAATGGCTGGTCTCAGTTTAGCACCTCAGATCAA
1726 AATTAGTTTTTAAAGGATTTTCCGTGCTTAAGAGTAGAAATCTGTCATGCCAGAGGAAGTTAAATAAAAAATAAAA
1801 CTCTATGACTATATATTGAAATTCATAGTTTTATTTTATTAATTTTGATTTTAATTCAAAACATTATACAAAAAAT
1876 TATTGAATATAACAATGTTTTCACATATTGTGCACAGCAACAAGTTTATGCATAGTATCCATGTTATTTGTAGAT
1951 ATCCAGAATATTTTAAATTCATTTTGATGCAGAATTTTATAGTTTATCTTGCTACAGAACTGTACAATAATCA
2026 GAATTGTGTTGATTCAAAAAAAAAAAAAAAAAAAAAAAAAA

Figure 3.7: mRNA and deduced amino acid sheep sequence ortholog to human UGT2B7.

The numbers at the left of each line denote the positions of the start nucleotide and amino acid. The initiation codon and termination codon are highlighted in grey. The putative polyadenylation signal (AATAAA) is indicated with a solid underline. The bold and larger font letter denotes the potential glycosylation site.

-33 ACTCCGGCAGGTGACCATCACGGCAGAGCAAGC

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1 M T A G S Q G E R P V I L L L L L C T L G P S V S
1 ATGACAGCAGGGTCCCAGGGGAACGCCAGTCATCCTGCTCCTGCTGTGTGCACGCTCGGCCCTCTGTGTCC
26 Q A G K L L V V P V D G S H W L S L V G P L Q P L
76 CAGGCCGGAAGCTGTGGTGGTCCCAGGTAGATGGCAGCCACTGGCTGAGTTTGGTCCGACCCCTCAGCCCTG
51 Q Q R G H D I V V L A P D A S V Y I K E E A F Y T
151 CAGCAGAGGGGACATGACATAGTGGTCTGGCACCTGACGCCTCTGTGTACATTAAGAGGAAGCATTTTACACC
76 L K R Y P V P F Q R E D L E E T F I S L G R T V F
226 TTGAAGAGGTACCCTGTGCCATTCCAAGGGAGGACTTGAAGAGACTTTATCAGCCTCGGGCTACTGTTTTT
101 E D E P F L K R A I K T Y Q K I K K D S A L L L S
301 GAGGATGAGCCTTTCTTGAAGCGCGGATCAAAACATACCAGAAAATCAAAAAGGACTCGGCTCTGCTCTTATCC
126 A C S H L L H N K E L M A S L T A S S F D A V L T
376 CCTGTCTCCCACTTACTGCACAACAAGGAGCTGATGGCTCCCTGACGGCAAGTAGCTTCGATGCCCTTTTGACA
151 D P F L P C G P I V A Q Y L S V P A V F F L N G L
451 GACCTTTCTCTCCCTGCGGCCCATCGTGGCCAGTACCTGTCTGTGCCTGCTGTGTTCTTCTGAATGGACTG
176 P C G L D F Q G T Q S P S P P S Y V P R Y L S F N
526 CCATGTGGCCTGGACTTTCAGGGTACCCAGAGCCCCAGTCCACCATCCTATGTGCCAGGTATCTGTCCTTTAAC
201 S D H M T F L Q R V K N D F V F N F P R P I M P N I V F V
601 TCAGATCACATGACCTTCTGCAGCGGGTGAAGAACATGTTTCATCACCTTGTCCAGAGAGTTTCTGTGCGATGTG
226 V Y S P Y R L L A S E F L Q K D M T V Q D L M S S
676 GTTTATTCCCCGTACAGGCTACTTGCCTCCGAATTCTTCAGAAAACATGACTGTTCCAGGATCTCATGAGCTCT
251 G S V W L L R N D F V F N F P R P I M P N I V F V
751 GGGTCTGTTTGGCTTCTCAGAAATGACTTTGTGTTAATTTCCCGAGGCCCATCATGCCAACATAGTGTGTTGTT
276 G G I N C A S K K P L S Q E F E A Y V N A S G E H
826 GGTGGGATCAACTGCGCTAGCAAAAAGCCACTCTCTCAGGAATTTGAAGCCTATGTAAATGCTTCTGGAGAACAT
301 G I V I F S L G S M V S E I P E Q K A M E I A D A
901 GGAATTTGTGATTTTCTCTTGGGCTCAATGGTCTCAGAGATTCCGGAGCAGAAAAGCTATGAAAATTTGATGCT
326 L G K I P Q T V L W R Y T G T P P P N L A K N T K
976 TTGGGCAAAATACCTCAGACAGTCTGTGGCGGTACACTGGGACTCCACCACCGAATCTTCCGAGAATACGAAA
351 L V L K W L P Q N D L L G H P K T R A F I T H S G S
1051 CTTGTCAAGTGGCTGCCCAAAATGATCTGCTTGGTCAACCCAAAAGACTCGGGCCTTTATCACACATTCGGCTCC
376 H G V Y E G I C N G V P M V M M P L F G D Q M D N
1126 CATGGCCTTTATGAAGGAATATGCAATGGCGTTCCCATGGTGATGATGCCCTTGTGTTGGTGATCAGATGGACAAT
401 A K R M E T R G A G I T L N V L E M S S G D L E N
1201 GCAAAGCGCATGGAGACCCGGGAGCTGGAATAACCTTGAATGTCTCGAAAATGAGTTCCGGAGATTTAGAAAAAT
426 A L K A V I N E K S Y K E N I M R L S R L H K D R
1276 GCCCTGAAAAGCTGTCAATGAAAAAGCTATAAGGAAAACATCATGCGCCTCTCCAGACTTACAAGGACCGC
451 P I E P L D L A V F W V E F V M R H K G A S H L R
1351 CCCATAGACCTCTGGACCTGGCTGATTTCTGGTGGAGTTTGTGATGAGGCACAAGGGGGCATCCACCTGCCG
476 P A A H D L T W Y Q Y H S L D V I G F L L A V T L
1426 CCTGCAGCCCATGACCTCACCTGGTACCAGTACCCTCTCTGGATGTAATTTGGCTTCCCTCCTGCGGTGACGCTG
501 T V I F I T F K A C A F T F R K C F G K K E R V K
1501 ACAGTCATCTTCATCACCTTTAAGGCCTGTGCCCTTCCCGAAAATGCTTTGGGAAGAAAGACGAGTCAAG
526 K S H K S K T H
1576 AAATCTCATAAATCCAAGACACACTGAGAAGTGGGTTGAAAATTTAGAGAAAGGTTAAATTCATGTTATGCTTA
1651 CTCAAGAATACTTTGCCAGGAAATGATGCCCCAGGGTGTGTTTTGAAAATAAAAATGACCCAGTCCCTAGTCG
1726 CACACACAGGTAGAGATATGTGAGTCTCTTTGCTGCTCCCTCCAGTACTTTAATCCGGACTTCACTCGCTTTTCA
1801 AATGACTTAAGAGAACTGGTAGGTCCTTCCCTTCAACCTACAGTACTATTCTAGCCAGAAATGAGACCTGTCTGG
1876 GGACCATGTCTTCTCCCTCCCTCCAGGAATGTGCCAGCATCGGTAGATACATTCCTCCACCGATTACGGCTG
1951 GTTCTCAATTATGCTTGTGTTTCTCAGATGCCTGTGTGCATCTAAGAAGATGTGTCTTCTTGTCTTGTGATGTG
2026 ATATGAATGAATGACATGGAGTTCTTCTGAGACAGTGTATAATTTCTGACTTTAGTGGGGAAAGAATGATGTATG
2101 AAATGGTGGGTGATATGCCTGAGTAGATAATCATTTGCTTGTACCAACAGATCTGAATTTGACAGATGCCTAAA
2176 TTATAGTGACTGGAAAGTATATTTTTTCTGATGTGATAACTAAAATACATTAATAAAATTTATGTTAATTATAAA
2251 AAAAAAAAAAAAAAAAAAAAAA

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Figure 3.8: mRNA and deduced amino acid sheep sequence ortholog to human UGT1A1.

The numbers at the left of each line denote the positions of the start nucleotide and amino acid. The initiation codon and termination codon are highlighted in grey. The two putative polyadenylation signals (AATAAA) are indicated with a solid underline. The bold and larger font letter denotes the potential glycosylation site.

AGACTGTAACCTCTGGTTTGTGCAGGG

-27

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1 M A L G L Q L R R Q V L A G L L L C L C V G R W A
1 ATGGCCCTGGGACTGCAGCTTCGGCGGCAGGTGCTGGCTGGACTCCTGCTCTGTCTGTGCGTTCGGGCGATGGGCC
26 E A G K V L V V P M E G S H W L S M R E A V R E L
76 GAGGCTGGGAAGGTGCTGGTCCCTATACACAGGAGCAAGCCACTGGCTCAGCATGCGGGAGGCCGTGCGGGAGCTC
51 H A R G H Q A V V V A P E V N M H I K A E D F F T
151 CATGCCAGAGGTACCAGGCAGTGGTTCGTTGCTCCGGAGGTGAATATGCACATCAAGGCAGAGGACTTTTTCCACC
76 M K T Y G I P Y T Q E K L D S L M K T H V Q L L F
226 ATGAAAACCTACGGTATTCCTATACACAGGAGAACTGGATTCCCTCATGAAGACTCATGTACAGCTCTCTTT
101 E K V H W V T L F L K S M A S L K N A S L L F E R
301 GAAAAAGTACATTGGGTAACACTGTTTTGAAAAGTATGGCATCTTTGAAAAATGCATCTTTGCTCTTTGAAAGG
126 S C E G L L Y N K D L I R N L N A S S F D V V L T
376 TCTTGTGAGGGCTGCTGTATAACAAGGATCTTATCAGGAACCTGAATGCCAGTTCCTTTGATGTGGTTTTAATC
151 D P V H P C G A V L A K Y L S I P A V F F L R Q I
451 GACCTGTTACCCCTGTGGAGCAGTGGCTAAGTACCTGTCCATTCTCTGCTGTGTTTTCTTGAGGCAAATT
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526 CCCTGTGACTTAGATGTTGAGGGCACAGCGTGCCCAAACCTTTTTCTTATGTTCCCTCGGTTGTAACAATGAAT
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751 GGATCCGTGTGGCTGTTTCAGAGGAGACTTTGTGATGGACTACCCGCGGCCGATCATGCCCAACATGGTGTTCATT
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826 GGGGCATCAACTGTGCCAACAGGAAACCGTTATCTCAGGAATTTGAAGCCTATGTAATGCTTCTGGAGAACAT
301 G I V I F S L G S M V S E I P E Q K A M E I A D A
901 GGAATGTGATTTTTCTTGGGCTCAATGGTCTCAGAGATTCCGGAGCAGAAAGCTATGGAATTTGATGATGCT
326 L G K I P Q T V L W R Y T G T P P P N L A K N T K
976 TTGGGCAAAATACCTCAGACAGTCTGTGGCGGTACACTGGGACTCCACCACCGAATCTTCCGAGAATACGAAA
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1051 CTTGTCAAGTGGCTGCCCAAAATGATCTGCTTGGTCAACCCAAAGACTCGGGCCTTTATCACACATCCGGCTCC
376 H G V Y E G I C N G V P M V M M P L F G D Q M D N
1126 CATGGCGTTTATGAAGGAATATGCAATGGCGTTCCCATGGTGTGATGATGCCCTTGTGGTGTGATGAGATGACAAT
401 A K R M E T R G A G I T L N V L E M S S G D L E N
1201 GCAAAGCGCATGGAGACCCGGGGAGCTGGAATAACCTTGAATGTCTCGAAATGAGTTCGGGAGATTTAGAAAAAT
426 A L K A V I N E K S Y K E N I M R L S R L H K D R
1276 GCCCTGAAAAGCTGTCAATGAAAAAGCTATAAGGAAAACATCATGCGCCTTCCAGACTTACAAGGACCGC
451 P I E P L D L A V F W V E F V M R H K G A S H L R
1351 CCCATAGCCCTTGGACCTGGCTGATTTCTGGTGGAGTTTGTGATGAGGCACAAGGGGGCATCCACCTGCCG
476 P A A H D L T W Y Q Y H S L D V I G F L L A V T L
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2101 AAATGGTGGGTGATATGCCTGAGTAGATAATCATTTGCTTGTACCAACAGATCTGAATTTGACAGATGCCTAAA
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2251 AAAAAAAAAAAAAAAAAAAAAA

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Figure 3.9: mRNA and deduced amino acid sheep sequence ortholog to human UGT1A3.

The numbers at the left of each line denote the positions of the start nucleotide and amino acid. The initiation codon and termination codon are highlighted in grey. The two putative polyadenylation signals (AATAAA) are indicated with a solid underline. The bold and larger font letter denotes the potential glycosylation site.

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26 E A G K V L V V P M E G S H W L S M R E A V Q E L
76 GAGGTGGGAAGGTGCTGGTCCCCATGGAGGCAGCCACTGGCTCAGCATGCGGGAGGCCGTGCAGGACTT
51 H A R G H Q A V V L S P E V N V H I K A E D F F T
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451 GACCTGTTTACCCTGCGGGCAGTGTGGCTAAGTACCTGTCCATTCTGTGTGTTTTTCTTGCCTTCCCT
176 P C D L D V E G T A C P N P F S Y V P K L L T R N
526 CCCTGTGACTTAGATGTTGAGGGCACAGCATGCCCAAACCTTTCTCTTATGTTCCCTAAGTTGTAACAAGGAAT
201 S D H M T F F Q R V K N M L Y P P S L K Y L R F I
601 TCAGACCACATGACATTCTCCAGAGGGTCAAGAACATGCTCTACCCTCCGAGCCTGAAGTACCTTTGCCATTTT
226 S F T P Y A R M A S E L L Q R D V S L G E I F G S
676 TCTTTCACTCCTTATGCACGTATGGCCTCTGAGCTGCTTCAGAGAGACGTGCTACTGGGGGAGATTTTTGGCTT
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751 GCATCTGTGTGGCTGTTCCAGAGGAGACTTTGTGATGGACTACCCGCGCCGATCATGCCAACATGGTGTTCATT
276 G G F N C G N K K P L S Q E F E A Y V N A S G E H
826 GGGGTTTTCAACTGTGGCAACAAGAAACCGTTATCTCAGGAATTTGAAGCCTATGTAAATGCTTCTGGAGAACAT
301 G I V I F S L G S M V S E I P E Q K A M E I A D A
901 GGAATGTGATTTTTCTTGGGCTCAATGGTCTCAGAGATTCCGGAGCAGAAAGCTATGGAATTTGGATGCT
326 L G K I P Q T V L W R Y T G T P P P N L A K N T K
976 TTGGGCAAAATACCTCAGACAGTCTGTGGCGGTACACTGGGACTCCACCACCGAATCTTCCGAGAATACGAAA
351 L V L K W L P Q N D L L G H P K T R A F I T H S G S
1051 CTTGTCAAGTGGCTGCCCAAAATGATCTGCTTGGTCAACCCAAAGACTCGGGCCTTTATCACACCTCCGGCTCC
376 H G V Y E G I C N G V P M V M M P L F G D Q M D N
1126 CATGGCGTTTATGAAGGAATATGCAATGGCGTTCCCATGGTGTGATGATGCCCTTGTGGTGTGATGAGACAAAT
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1201 GCAAAGCGATGGAGACCCGGGAGCTGGAATAACCTTGAATGTCTCGAAATGAGTTCGGGAGATTTAGAAAAAT
426 A L K A V I N E K S Y K E N I M R L S R L H K D R
1276 GCCCTGAAAAGCTGTCAATGAAAAAGCTATAAGGAAAACATCATGCGCCTTCCAGACTTACAAGGACCGC
451 P I E P L D L A V F W V E F V M R H K G A S H L R
1351 CCCATAGCCCTTGGACCTGGCTGATTTCTGGTGGAGTTTGTGATGAGGCACAAGGGGGCATCCACCTGCCG
476 P A A H D L T W Y Q Y H S L D V I G F L L A V T L
1426 CCTGCAGCCCATGACCTCACCTGTTACCAGTACCCTCTCTGGATGTAATTTGGCTTCCCTCCTGCGGTGACGCTG
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526 K S H K S K T H
1576 AAATCTCATAAATCCAAGACACACTGAGAACTGGGTTGAAAACCTTAGAGAAAGGTTAAATTCATGTTATGCTTA
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1876 GGACCATGTCTTCTCCCTCCCTCCAGGAATGTGCCAGCATCGGTAGATACATTCCTCCACCAGTTACGGCTG
1951 GTTCTCAATTATGCTTGTGTTTCTCAGATGCCTGTGTGCATCTAAAGAAGATGTGTCTTCTTGTCTTGTGATGTG
2026 ATATGAATGAATGACATGGAGTTCTTCTGAGACAGTGTATAATTTCTGACTTTAGTGGGGAAAGAATGATGTATG
2101 AAATGGTGGGTGATATGCCTGAGTAGATAATCATTTGCTGTACCAAACAGATCTGAATTTGACAGATGCCTAAA
2176 TTATAGTGACTGGAAAGTATATTTTTTCTGATGTGATAACTAAAAATACATTAATAAATTTATGTTAATTATAAA
2251 AAAAAAAAAAAAAAAAAAAAA

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Figure 3.10: mRNA and deduced amino acid sheep sequence ortholog to human UGT1A4.

The numbers at the left of each line denote the positions of the start nucleotide and amino acid. The initiation codon and termination codon are highlighted in grey. The two putative polyadenylation signals (AATAAA) are indicated with a solid underline. The bold and larger font letter denotes the potential glycosylation site.

-53 ATTTTTGGAGCTGTA AATTATTCTTGTGTGCTATCTGCCATCCTCCTCCAGC

1 M A C L L R R V S V A V F F L A L W G F A L G D R
1 ATGGCTTGCCTTCTTCGCAGAGTTTCTGTGGCAGTTTTCTTCTTAGCACTTTGGGGCTTCGCCCTGGGGGACAGG

26 L L V V P Q D G S H W L S M K D I T E R L S E K G
76 CTGCTGGTGGTCCCTCAGGATGGAAGCCACTGGCTCAGCATGAAGGACATCACTGAACGTCTCAGCGAGAAAGGG

51 H E I V V V V P K V N L L L Q E S K H Y T R R I H
151 CATGAGATCGTGGTGGTGGTGC CCAAAGTCAACCTGCTCCTCCAAGAGTCCAAGCACTACACAAGGAGAAATCCAC

76 P V P Y D Q E E L E A R Y R S F G K H H F S P R W
226 CCAGTGGCTACGACCAGGAGGCTGGAAGCCGTTACCGTTCCCTTCGGGAAACACCCTTTTCTCCACGCTGG

101 L V T A P M V E Y R N N M I V I N M Y F L N C Q S
301 CTGGTACTGCCCTATGGTGGAGTATAGGAACAACATGATCGTCATCAACATGTACTTTCTCAACTGCCAGAGT

126 L L R H S D T L R F L R E S K F D A L F T D P A L
376 CTCCTGAGGCACTCGGACACCCTGCGCTTCCTCCGGGAGAGCAAGTTCGATGCCCTGTTACAGACAGCCGGCCTTA

151 P C G V I L A E Y L N L P S V Y L F R G F P C A L
451 CCTTGGGGGTGATCCTGGCCGAGTACCTGAACCTGCCCTCTGTGTATCTCTCAGGGGCTTTCCCTGTGCCCTG

176 E N T F T R T P S P L S Y V P R Y Y T Q F S D K M
526 GAGAACACGTTACCAGGACTCCAAGCCCTTTGTCCTACGTCCCAGGTACTACACTCAGTTTTCAGACAAGATG

201 T F L R Y D F V A N F L V S Y L E N I L L Y A L G S S
601 ACCTTCTCCAAGGGTGGCCAACCTTCCTGGTGAGTTACCTGGAGAACATTTTGTCTATGCGCTGTATTCCAAG

226 Y E D L A E E V L G R Q V H L P A L Y Q K A S I W
676 TACGAAGACTGGCGGAGGAAGTCTTGGGAGACAGGTGCACTTGCCGGCCTTGATCAGAAGGCCTCCATTTGG

251 L L R Y Q R V F Y F E Y P R V M P N M V F I G C S S
751 CTGCTAAGATACGACTTTGTGTTCCGAGTATCCCAGACCAGTATGCCCAACATGGTCTTCATCGGAGGGTCCAGC

276 C K K Q G I L P R E F E A Y V **N** A S G E H G I V I
826 TGCAAGAAACAAGGCATCCTGCCTCGGGAAATTTGAAGCCTATGTAATGCTTCTGGAGAACATGGAATTTGTGATT

301 F S L G S M V S E I P E Q K A M E I A D A L G K I
901 TTCTCTTTGGGCTCAATGGTCTCAGAGATTCGGAGCAGAAAAGCTATGGAATGCTGATGCTTTGGGCAAAAATA

326 P Q T V L W R Y T G T P P P N L A K N T K L V K W
976 CCTCAGACAGTCTGTGGCGGTACTGGGACTCCACCACCGAATCTTGCGAAGAATACGAAACTTGTCAAGTGG

351 L P Q N D L L G H P K T R A F I T H S G S H G V Y
1051 CTGCCCCAAAATGATCTGCTTGGTCACCCAAGACTCGGGCCTTTATCACACATTCGGCTCCCATGGGCTTTTAT

376 E G I C N G V P M V M M P L F G D Q M D N A K R M
1126 GAAGGAATATGCAATGGCGTTCCCATGGTATGATGCCCTTGTGTTGGTATCAGATGGACAATGCAAAGCGCATG

401 E T R G A G I T L N V L E M S S G D L E N A L K A
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451 L D L A V F W V E F V M R H K G A S H L R P A A H
1351 CTGGACCTGGCTGTATTCTGGGTGGAGTTTGTGATGAGGCACAAGGGGGCATCCACCTGCGCCCTGCCACCCAT

476 D L T W Y Q Y H S L D V I G F L L A V T L T V I F
1426 GACCTCACCTGGTACCAGTACCCTCTCTGGATGTAATGGCTTCTCCTGCGGTGACGCTGACAGTATCTTC

501 I T F K A C A F T F R K C F G K K E R V K K S H K
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526 S K T H
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2026 GACATGGAGTTCTTCTGAGACAGTGTATAATTTCTGACTTTAGTGGGGAAAGAATGATGTATGAAATTTGGTGGGT

2101 GATATGCCTGAGTAGATAATCATTGCTTGTACCAACAGATCTGAATTTGACAGATGCCTAAATTTATAGTGACTG

2176 GAAAGTATATTTTTTCTGATGTGATAACTAAAAATACATTAATAAAATTTATGTTAATTATAAAAAAAAAAAAAA

2251 AAAAAAA

Figure 3.11: mRNA and deduced amino acid sheep sequence ortholog to human UGT1A6.

The numbers at the left of each line denote the positions of the start nucleotide and amino acid. The initiation codon and termination codon are highlighted in grey. The two putative polyadenylation signals (AATAAA) are indicated with a solid underline. The bold and larger font letter denotes the potential glycosylation site.

Table 3.1: The length of sheep, cow and human full nucleotide sequences and the sheep homology with cow and human

Gene	Sheep sequence cloned (bp)	Cow/Human available data (bp)	Cow homology (%)	Human homology (%)
CYP2A6	1505*	231*/1775	98	87
CYP2C19	1966	2048/1473	95	77
CYP2D6	1682	1794/1673	92	80
CYP2E1	1718	1921/1667	93	73
CYP2C18	1384*	1896/2562	88	84
HNF4 α	4465	4515/3209	95	74
UGT1A1	2303	-/2357	-	78
UGT1A3	2297	-/2345	-	76
UGT1A4	2293	-/2374	-	77
UGT1A6	2311	1590/2339	95	74
UGT1A9	2318	-/2333	-	74
UGT2B7	2086	1848(2B10)/1899	81 (2B10)	74

bp – base pairs; * indicates partial sequence, - - not available

3.2 mRNA levels

Using the full sheep sequences, primers were designed and real time PCR was performed in all four experimental groups to identify the relative mRNA levels of CYP enzymes (CYP2A6, CYP2C19, CYP2D6) (Fig 3.13), UGT enzymes (UGT1A6, UGT1A9, UGT2B7) (Fig 3.14) and HNF4 α (Fig 3.13) in liver tissues.

Adult ewes showed high mRNA levels of CYP2C19, CYP2D6 and HNF4 α in liver followed by newborn and then the fetus. In the case of CYP2A6, adult level of mRNA was significantly higher compared to the newborn and fetus, but no significant difference was observed between the fetus and newborn due to high inter-individual variability. Only CYP2D6 showed a significant difference between the fetuses treated with saline and fetuses treated with cortisol. But HNF4 α also tends to show a cortisol effect ($P < 0.076$). High inter individual variability was observed in all cytochrome genes but CYP2A6 showed the highest variability within all groups especially in the newborns.

Adult mRNA levels for all three UGT enzymes were significantly higher than in the newborn and fetus. Thus, UGT genes are expressed at very low levels in the fetus when compared to adults. No significant cortisol effects on UGT mRNAs were observed in the fetus. But there was a trend towards cortisol effect with UGT1A9 and UGT2B7 mRNA ($P < 0.1$).

Correlations between mRNA levels of HNF4 α and CYPs (CYP2A6, CYP2C19, CYP2D6) and UGTs (UGT1A6, UGT1A9, UGT2B7) enzymes were analyzed (Fig 3.15). Coefficient of determination (r^2) values were more than 0.64, with a significant correlation observed for all enzymes ($P < 0.05$). Also the correlation between HNF4 α mRNA and 18S was analyzed to provide additional evidence as to whether the above significant correlations between HNF4 α and the drug metabolizing enzymes were due to direct HNF4 α effects on the expression of the CYPs or UGTs or due rather to maturational changes affecting CYPs, UGTs and HNF4 α independently. No correlations were observed between HNF4 α and 18S ($r = 0.50$, $P > 0.05$) (Fig 3.16).

3.3 Protein levels

The quality of the frozen sheep liver samples was checked using fresh adult sheep liver, which was obtained from an abattoir before starting the Western blot studies (Fig 3.17). It was analyzed with a polyclonal antibody against human CYP2D6. Since the appearance and the

density of the bands were similar to fresh sample, quality of the frozen samples was confirmed (Table 3.2).

Western blot analysis was performed to measure the protein levels of CYP2D, CYP2C, CYP2A, UGT2B and UGT1A in adult, newborn and fetal sheep. Figure 3.18 shows representative blots for enzymes studied. Optical densities of the stronger bands were measured and the data analyzed. Antibody against human CYP2A6 gave a faintly-stained and a strongly-stained band in adults but only a single faintly-stained band in the newborn and no bands in fetuses (Fig 3.18A). In this blot, the strongly-stained band (47Kda) might be CYP2A and the faintly-stained band (49Kda) might be another CYP2A enzyme or a non specific protein. We could not rule out this band. However, this faintly-stained band disappeared with a low protein load (2 µg) compared to 4µg. Human microsomes showed a band at 46Kda with an antibody against human CYP2A6. Antibody against rat CYP2C11 also gave a faintly-stained and a strongly-stained band in adults but only a single faintly-stained band in the newborn and no bands in fetuses (Fig 3.18B). In this blot, the two bands in sheep (47.5Kda, 47Kda) could be due to the presence of two different CYP2C enzymes, since there are several CYP2C proteins identified in different species. The observed molecular weight of the human CYP2C9 band was 52Kda. The antibody against human CYP2D6 cross reacted with three proteins in the adult and two in the newborn and fetuses (Fig 3.18C). In this blot the strongly-stained top band in sheep (50Kda) may be CYP2D. The second band (49Kda) might be CYP2C and third band (47Kda) might be CYP2A according to the antibody specificity blots. The human CYP2D6 (47.5Kda) protein was visualized with lower molecular weight than sheep CYP2D (50Kda). Antibodies against human UGT1A and UGT2B7 did not give any bands with sheep proteins. The experiment was repeated several times and different antibodies against UGT were tried with sheep microsomes. However, we could not visualize UGT protein bands with antibodies against human UGT.

Adult sheep had higher protein levels than newborn both with CYP2C and CYP2D (Fig 3.19). Protein levels in the fetus were very low when compared with adult CYP2D proteins. Fetuses treated with cortisol showed a higher CYP2D protein levels ($P < 0.05$) when compared to control fetuses. High inter-individual variability was observed in all CYP enzymes and in all groups of animals.

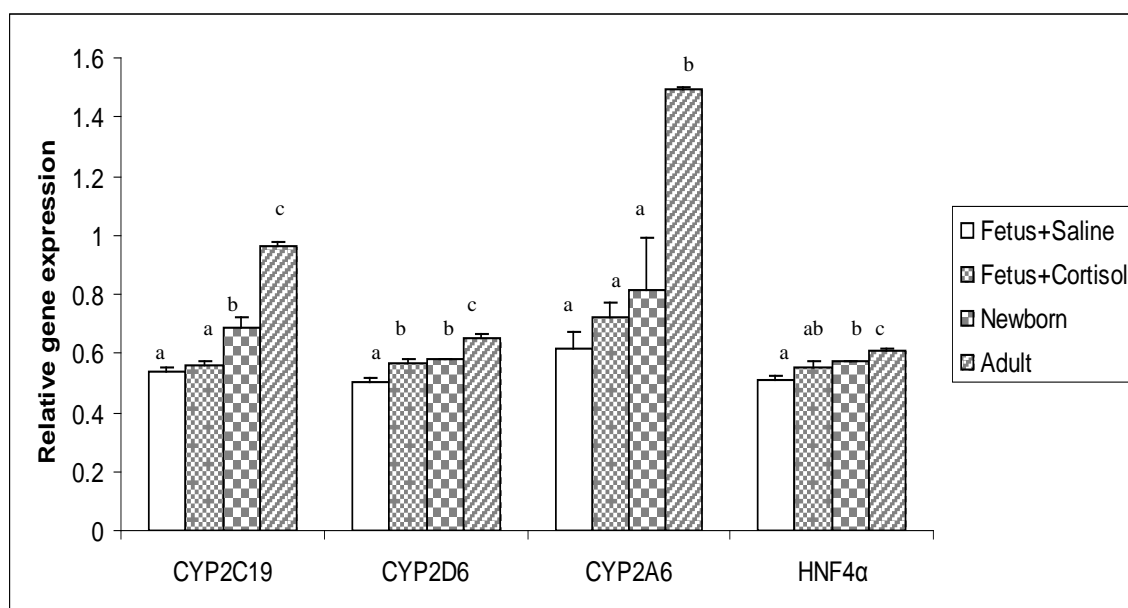


Fig 3.13: Relative hepatic mRNA levels of CYP2C19, CYP2D6, CYP2A6 and HNF4 α in fetuses treated with saline (n=3) or cortisol (n=5), newborns (n=3) and adults (n=4) sheep. Different letters indicate a significant difference (P<0.05) between groups.

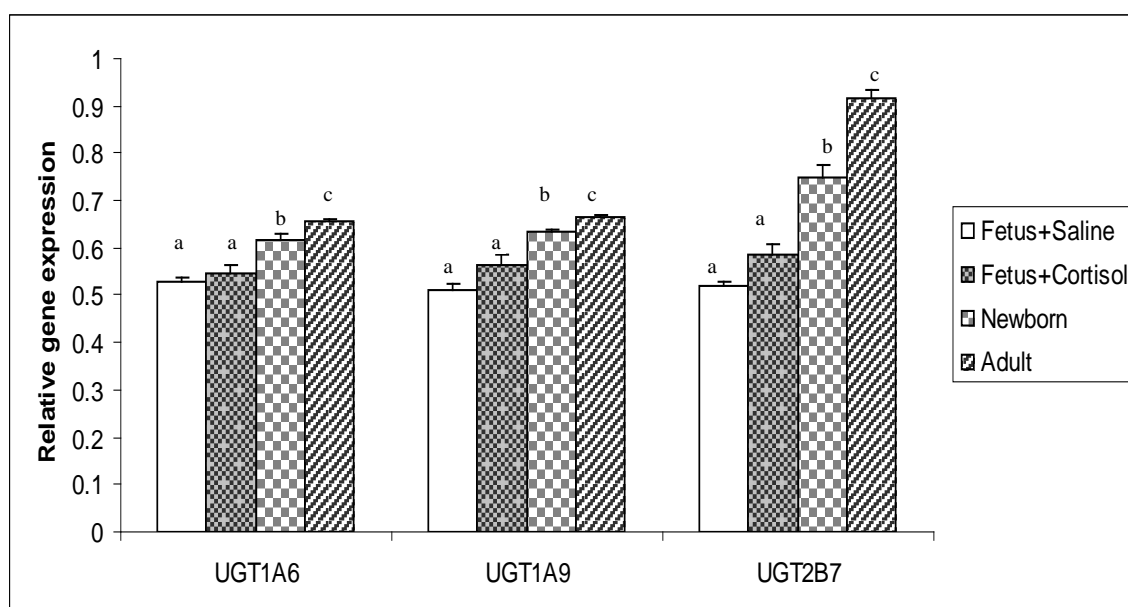


Fig 3.14: Relative hepatic mRNA levels of UGT1A6, UGT1A9 and UGT2B7 in fetuses treated with saline (n=3) or cortisol (n=5), newborns (n=3) and adults (n=4) sheep. Different letters indicate a significant difference (P<0.05) between groups.

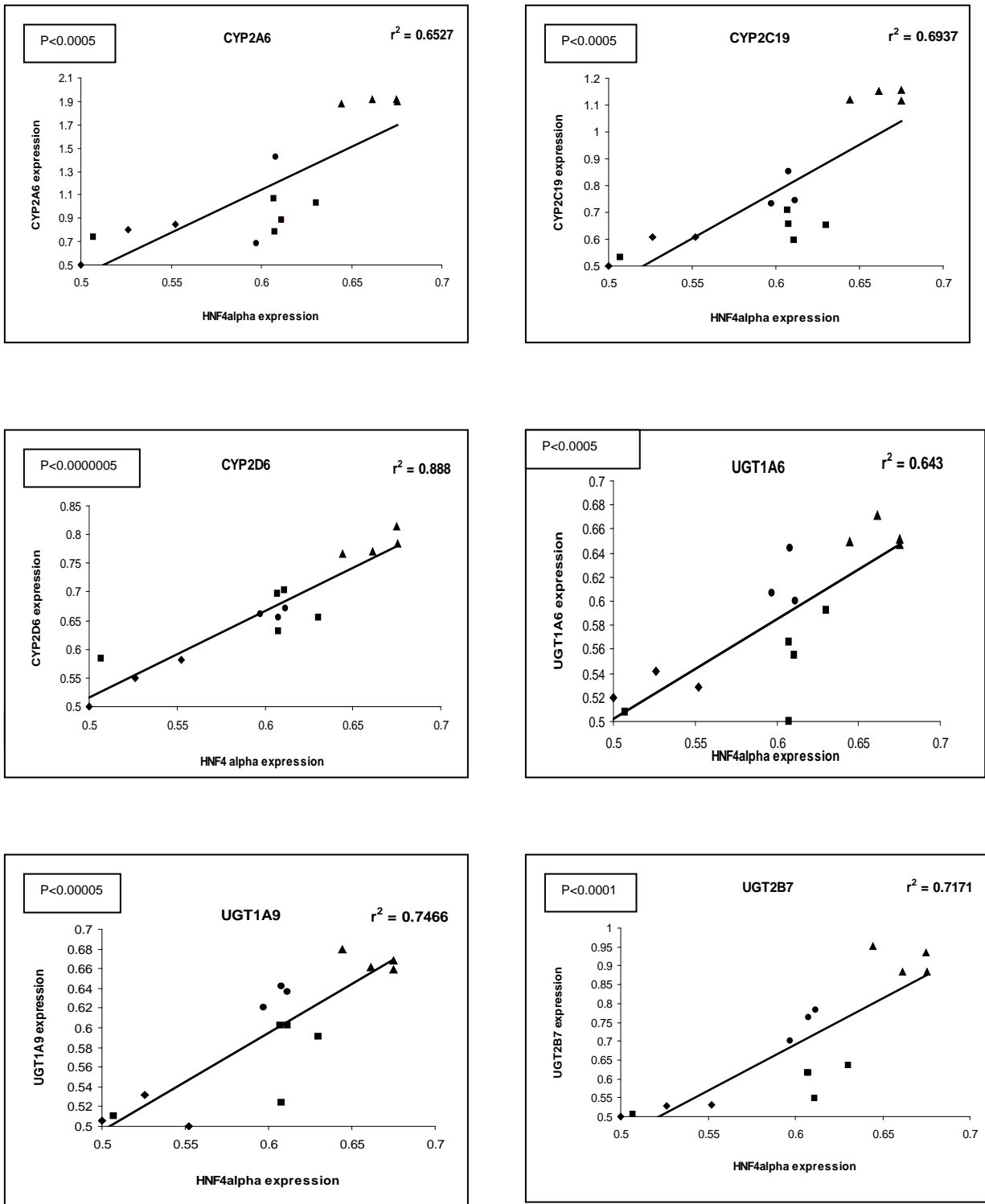


Fig 3.15: Correlation between relative mRNA levels of HNF4 α and CYP (CYP2A6, CYP2C19, CYP2D6) and UGT (UGT1A6, UGT1A9, UGT2B7) enzymes in sheep liver. Coefficient of determination (r^2) and significance value (p) for each correlation is shown.

Fetus (saline treated) - \blacklozenge Fetus (cortisol treated) - \blacksquare New born - \bullet Adult - \blacktriangle

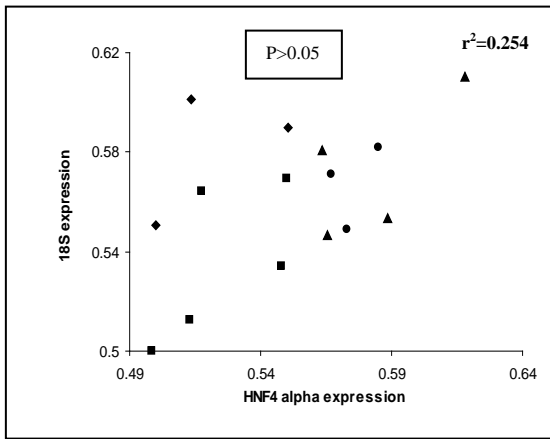
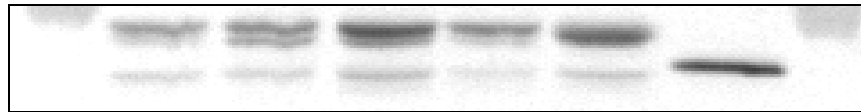


Fig 3.16: Correlation between relative levels of HNF4 α and 18S in sheep liver. Coefficient of determination (r^2) and significant value (p) are shown.

Fetus (saline treated) - \blacklozenge Fetus (cortisol treated) - \blacksquare New born - \bullet Adult - \blacktriangle



Lanes 1 2 3 4 5 6 7 8

Fig 3.17: Immuno-blot detection of CYP2D proteins in adult and abattoir sheep liver. Microsomal proteins (4 μ g) were analyzed with anti human CYP2D6. Lanes 1 and 8 – molecular weight marker (50kDa), Lanes 2-5 adult liver, Lane 6 – abattoir liver, Lane 7 – human CYP2D6 standard (0.025pmol/20 μ l).

Table 3.2: CYP2D protein expression in terms of optical density /mg protein of bands in adult and abattoir liver

CYP2D protein	Band 1	Band 2	Band 3
Adult 1	98.53	11.24	9.45
Adult 2	123.62	43.49	5.70
Adult 3	218.57	57.49	29.48
Adult 4	126.38	10.42	3.26
Abattoir (n=1)	216.29	24.10	15.96

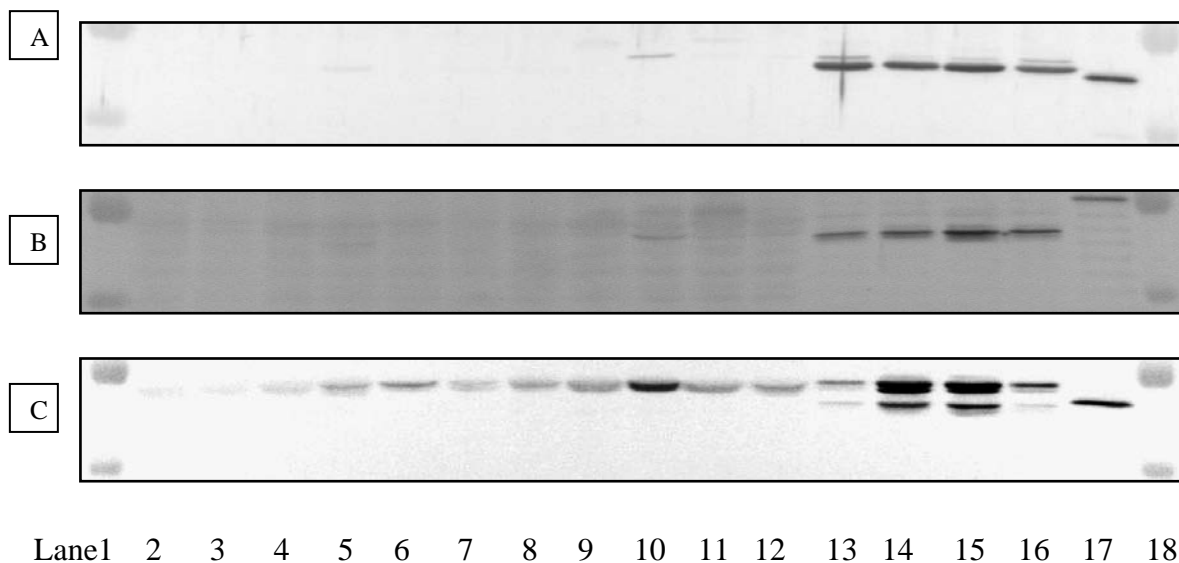


Fig 3.18: Western blots (A) with antibody against human CYP2A6, (B) with antibody against rat CYP2C11 and (C) with antibody against human CYP2D6. Lanes 1 and 18 – molecular weight marker (Top-50Kda, Bottom -37Kda), Lanes 2-4 – fetus treated with saline, Lanes 5-9 – fetus treated with cortisol, Lanes 10-12 – newborn, Lanes 13-16 – adult, Lane 17 – human standards (A – CYP2A6, B – CYP2C9, C – CYP2D6). Different amount of hepatic microsomes were loaded in different groups (Fetus treated with saline, fetus treated with cortisol – 80,100 40 μ g; newborn – 10, 100, 40 μ g; adult sheep – 4, 2, 1 μ g in CYP2A, CYP2C and CYP2D blots respectively).

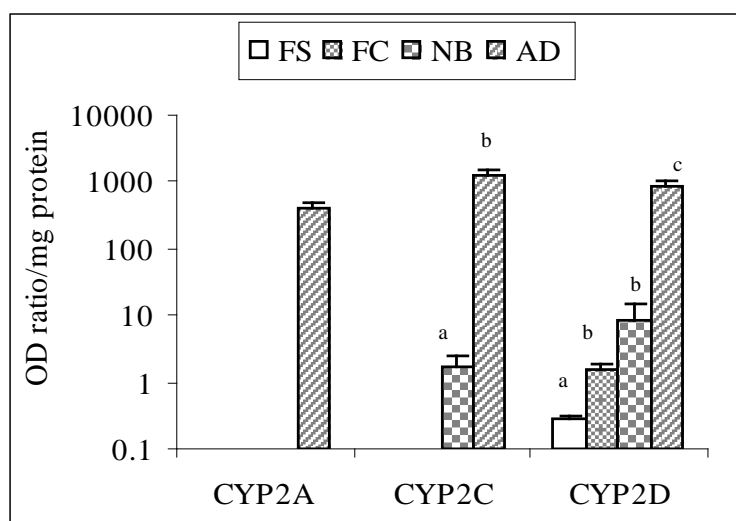


Fig 3.19: Western blot OD ratio/mg of CYP2A, CYP2C and CYP2D bands in adult, newborn and fetal sheep liver normalized to the human CYP2A6, CYP2C9 and CYP2D6 standards respectively. FS – Fetuses treated with saline (n=3), FC – Fetuses treated with cortisol (n=5), NB – Newborn (n=3), AD – Adult (n=4). Different letters indicate significant differences between groups ($P < 0.05$).

3.4 Validation of antibodies

The specificities of the antibodies used in the Western blots were analyzed (Fig 3.20) with the same amounts (0.2pmol/20µl) of several human CYP proteins. Optical densities of these bands were measured but it was not possible to quantify the weaker bands (Table 3.3). Antibody against human CYP2A6 reacted with the human CYP2A6 protein standard in addition to human and sheep microsomes. It also reacted with human CYP2D6 protein but very weakly. Antibody against rat CYP2C11 gave bands with all human CYP2C proteins (CYP2C8, CYP2C9, CYP2C18 and CYP2C19) and rat CYP2C11. In addition, it also gave a band with CYP2D6 protein and very weak bands with CYP1A1, CYP2A6 and CYP2E1. Antibody against human CYP2D6 reacted very well with human CYP2D6 but gave very weak bands with CYP2A6 and CYP2C8 proteins.

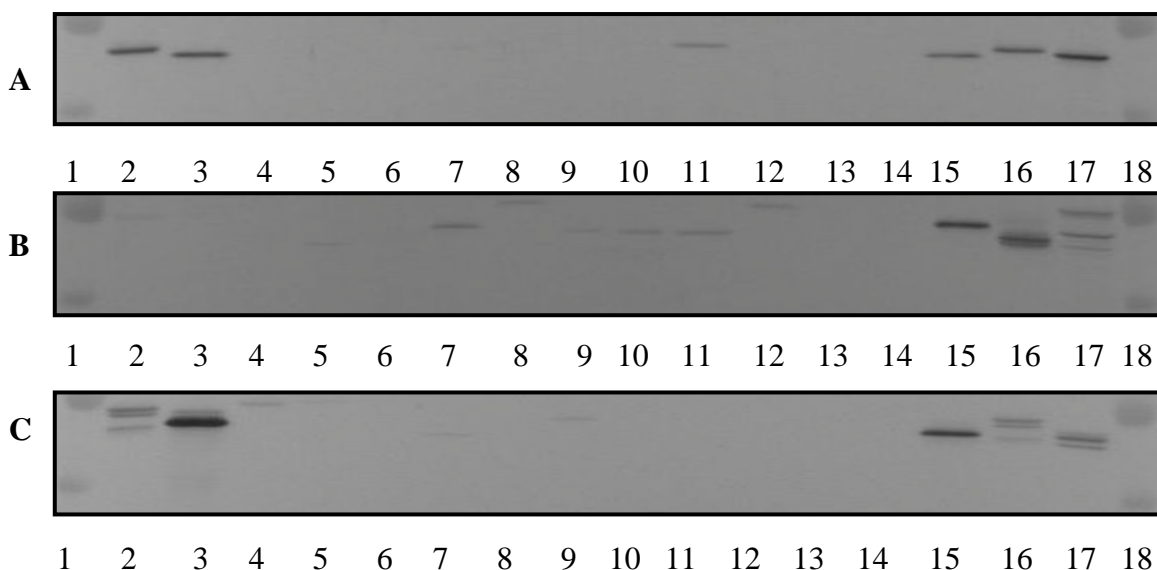


Fig 3.20: Antibody specificity blots (A) Anti human CYP2A6, (B) Anti rat CYP2C11, (C) Anti human CYP2D6. Lane 1 and 18 – molecular weight marker (50Kda, 37Kda). All the human standards are 0.2pmol/20µl unless otherwise stated. In A, Lane 2- sheep liver microsomes (4µg), Lane 3- CYP2A6, Lane 4 – CYP1A1, Lane 5 – CYP1A2, Lane 6 – CYP1B1, Lane 7 – CYP2B6, Lane 8 – CYP2C8, Lane 9 – CYP2C9, Lane 10 – CYP2C19, Lane 11 – CYP2D6. In B, Lane 2 – CYP1A1, Lane 3 – CYP1A2, Lane 4 – CYP1B1, Lane 5 – CYP2A6, Lane 6 – CYP2B6, Lane 7 – CYP2C8, Lane 8 – CYP2C9, Lane 9 – CYP2C18, Lane 10 – CYP2C19, Lane 11 – CYP2D6. In C, Lane 2- sheep liver microsomes (4µg), Lane 3- CYP2D6, Lane 4 – CYP1A1, Lane 5 – CYP1A2, Lane 6 – CYP1B1, Lane 7 – CYP2A6, Lane 8 – CYP2B6, Lane 9 – CYP2C8, Lane 10 – CYP2C9, Lane 11 – CYP2C19. In A, B, C, Lane 12 – CYP2E1, Lane 13 – CYP3A4, Lane 14 – CYP4A11, Lane 15 – standards (A – 0.1pmol/20µl human CYP2A6; B – 0.2pmol/20µl rat CYP2C11; C – 0.05pmol/20µl human CYP2D6), Lane 16 – sheep liver microsomes (A-2µg, B-4µg, C-2µg), Lane 17-human liver microsomes (20µg, 40µg, 20µg).

Table 3.3: OD values of the antibody specificity blots. A – Antibody against human CYP2A6, B – Antibody against rat CYP2C11, C – Antibody against human CYP2D6; ln – lane number, SM – Sheep microsomes, HM – Human microsomes.

Ln	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
A	SM 1.4	2A6 1.34	1 A 1 -	1A 2 -	1B1 -	2B6 -	2C8 -	2C9 -	2C19 -	2D6 0.1	2E1 -	3A4 -	4A11 -	2A6 0.66	SM 0.79	HM 1.59
B	1A1	1A2	1 B 1	2A 6	2B6	2C8 0.14	2C9 0.03	2C18 0.01	2C19 0.04	2D6 0.06	2E1 0.02	3A4	4A11	2C11 2.01	SM 0.23 1.11 0.48	HM 0.29 0.48 0.07
C	SM 0.66 0.35 0.11	2D6 7.16	1 A 1	1A 2	1B1	2A6 0.01	2B6	2C8 0.01	2C9	2C19	2E1	3A4	4A11	2D6 3.24	SM 0.37 0.16 0.03	HM 0.05 0.82 0.25

4. DISCUSSION AND CONCLUSIONS

4.1 Discussion

4.1.1 mRNA sequences of hepatic drug metabolizing enzymes in sheep

Sheep cDNA sequences orthologs to human CYP2A6, CYP2C18, CYP2C19, CYP2D6, CYP2E1, UGT1A1, UGT1A3, UGT1A4, UGT1A9, UGT2B7 and HNF4 α were for the first time cloned in this study. To date, only a few CYP gene sequences have been cloned in sheep: CYP1A1, CYP2J2, CYP4F21, CYP11A1, CYP11B1, CYP17A1, CYP19 and CYP21 (Messina et al., 2010). However, the CYPs which are important for the drug metabolism had not been cloned in sheep prior to this study, except CYP3A24 and a 117bp section of CYP2E1 (Nelson, 2006; National Center for Biotechnology Information – NCBI).

Of the CYP cDNAs that I tried to identify in sheep, I was unable to clone a sheep ortholog of human CYP2C9 even though cloning was tried using several different sections of the aligned sequences. This suggests that the sheep and human CYP2C9 show less homology between them or that there is not a CYP2C9-like gene present in sheep. However, there were several small pieces of CYP2C gene sequences cloned in our study, but identification of the authentic CYP2C9 sequence among them was not possible. Therefore the full sequence was not cloned and the gene expression study of CYP2C9 was also not performed.

Only two UGT genes, UGT1A07 in the small intestine and UGT1A6 in the liver have been cloned until now in sheep (Kobayashi et al., 1999). Full mRNA sequences of orthologs to human UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9 and UGT2B7 were obtained in this study. The UGT1A6 nucleotide sequence that I cloned revealed 99% similarity with the UGT1A6 already cloned in sheep by Kobayashi et al. (1999) and 19 nucleotides differ from each other. This may be due to two different alleles. Interestingly, cDNAs encoding the sheep UGT1As share an identical C-terminal region with 245 amino acid residues, indicating that sheep have the typical structure found in the human and rat UGT1As (Burchell et al., 1991; Emi et al., 1995; Ritter et al., 1992). A variable N-terminal region was also obtained with 285-289 amino acid residue sequences.

4.1.2 Structure-activity relationships in CYP and UGT enzymes

Numerous studies of structure-activity relationships in various human CYP enzymes have identified the amino acid residues that are important for substrate binding and catalytic activity. Given that this study has provided sequence information on several CYPs in sheep, it is of

interest to see if the same critical amino acid residues identified in the human CYPs are also present in sheep. This might give an idea whether the sheep CYPs has the same activity as the human.

In the CYP2A subfamily, several complementary amino acid residues appear to be involved in binding and orientating the substrate relative to the haem moiety such that 7-hydroxylation is favoured. In human CYP2A6, Gln104, Arg128, Phe209 and His477 were reported to be active sites for coumarin 7 hydroxylase activity (Lewis et al., 1999; Kitagawa et al., 2001; Lewis and Lake, 2002) which is the marker for CYP2A6 activity (Table 4.1). Arg128 represents one of the key binding residues which form a salt bridge with a haem propionate moiety in CYP2A6, according to a previous modeling study (Lewis and Lake, 2002). All the above active sites are conserved in sheep CYP2A6 except His477. This His477 variation might be due to the species variation. In cow and sheep, Leu (codon CTC) is found in the amino acid position of 477 instead of His (codon CAC) which is found in human and pig (Lin et al., 2004). Therefore, the activity of coumarin 7 hydroxylase might be the same as human or lesser than human. However, Coumarin 7-hydroxylase activity was readily detectable in calf liver microsomes similar to human and Cynomolgus monkey (Lake et al., 1995). This suggests that calf liver microsomes contain P4502A isoenzyme(s) similar to human 2A6. Since all four active sites are the same in cow and sheep, sheep should show similar coumarin 7 hydroxylase activity as cow and human. An enzyme activity assay for coumarin 7 hydroxylase using sheep hepatic microsomes would confirm this. In addition to the above four active sites, Thr212 could be involved in directing the access of coumarin to the binding site as this residue lies close to the mouth of the haem pocket, with the hydroxyl side chain of this residue being able to form a hydrogen bond with the substrate (Lewis and Lake, 1995). This Thr212 is conserved in sheep. Moreover, Asn297 and Phe107 which form hydrogen bonds and facilitate coumarin metabolism (Lewis et al., 2006), are conserved in sheep, human and pig (Yano et al., 2005).

The above binding sites responsible for coumarin metabolism are shared by other substrates such as fadrozole, losigamone, cotinine, 4-nitroanisole, 4-nitrophenol, 2, 6-dichloro benzonitrile, aflatoxin B1, nicotinine, diethylnitrosamine and methoxyflurane (Lewis et al., 1999). In addition to the sites responsible for coumarin binding, there are other important sites identified for the metabolism of other substrates. Thr303 may be involved in hydrogen bonding to the hydroxyl group of 4-nitroanisole (Lewis et al., 1999). Ile366 and Leu298 are also important for the diethylnitrosamine metabolism. In the human sequence, Asp108 is one of the

residues responsible for methoxyflurane metabolism (Lewis et al., 1999). Moreover, the Ile471 amino acid residue is responsible for substrate specificity of nicotine C-oxidase ability (Ariyoshi et al., 2001). These CYP2A6 substrate activities might be present in sheep because sheep also have the same amino acid residues at these sites.

With human CYP2C19, three key amino acids at positions 99, 220 and 221, are critical determinants of omeprazole 5-hydroxylase activity (Ibeanu et al., 1996; Wada et al., 2008) in human CYP2C19. Mutation of all three residues eliminated omeprazole 5-hydroxylase activity whereas mutation of one (e.g., His99 responsible for a 50% reduction in activity) or two decreased the activity. Residues His99 and Thr221 are substituted with Ser not only in sheep but also in cow CYP2C19 (Table 4.1). However, Pro220 is conserved in sheep and cow CYP2C19 and CYP2C18. Arg410 also plays an important role in omeprazole 5-hydroxylase activity (Wada et al., 2008) and it is substituted with Ala in sheep. Therefore, the omeprazole 5-hydroxylase activity level in sheep might not be same as human CYP2C19. There might be partial activity in sheep CYP2C18 as well since it also has Pro220.

(*S*)-Mephenytoin 4'-hydroxylation is regarded as a marker activity for CYP2C19 (Ibeanu et al., 1996). Residues His99, Pro220, Thr221, Leu237, Asn286, Ala292 and Leu295 play a role in mephenytoin 4'-hydroxylation activity by influencing substrate access or binding indirectly (Tsao et al., 2001; Wada et al., 2008; Niwa et al., 2002). Among them, the residue Asn286 plays an important role in determining the substrate specificity to *S*-mephenytoin (Tsao et al., 2001). Also Val288 and Ile289 contribute to the catalytic activity and the mechanism may involve alteration of the microenvironment of the binding site (Tsao et al., 2001). However, at all of the positions there are different amino acids in sheep CYP2Cs except for Pro220 and Asn286. Therefore, metabolism of *S*-mephenytoin might be very low by CYP2C19 as well as CYP2C18 in sheep.

Arg442, which is conserved in sheep CYP2C19 and CYP2C18, is also important for CYP2C19 catalytic activity (*S*-mephenytoin 4'-hydroxylase and omeprazole 5-hydroxylase) and tolbutamide *p*-methylhydroxylase and diclofenac 4'-hydroxylase activities (Wada et al., 2008). A single nucleotide polymorphism of CYP2C19 was found in which Arg442 was substituted to Cys (Morita et al., 2004). This substitution reduced the *in vivo* capacity for mephobarbital 4'-hydroxylation, which is catalyzed by CYP2C19. Therefore, residue 442 appears to be involved in the transfer of electrons, rather than exerting an effect on substrate specificity.

In addition to omeprazole 5-hydroxylase and *S*-mephenytoin 4'-hydroxylase activities, Arg97, Arg108, Ser220 and Pro221, Val237, Lys241, Ser286, Asn289, Asp293, Lys423, which are present in CYP2C9, are important for the oxidation of flurbiprofen (Wester et al., 2004), diclofenac hydroxylation (Klose et al., 1998; Ridderstrom et al., 2000; Wester et al., 2004; Dickmann et al., 2004; Wada et al., 2008), oxidation of *S*-warfarin (Jung et al., 1998; Dickmann et al., 2004; Wester et al., 2004), tolbutamide *p*-methylhydroxylase activity (Wada et al., 2008), ibuprofen hydroxylase activity (Klose et al., 1998), amino pyrine *N*-demethylation (Niwa et al., 2002) and testosterone 17-oxidation (Niwa et al., 2002). Among these residues, only Arg97 and Asp293 are conserved in both sheep CYP2C19 and CYP2C18 sequences. This suggests that these sheep CYP2Cs might show very low CYP2C9 activities similar to human CYP2C19.

A cluster of three residues (Ala113, Ser115 and Lys118) are important for progesterone 21-hydroxylase activity by CYP2C5 (Kronbach et al., 1989). Sheep CYP2C19 and CYP2C18 might also have at least partial activity for 21 hydroxylation of progesterone due to its sequence identity (CYP2C19- Ser115, CYP2C18- Lys118) with CYP2C5 especially in the substrate binding sites. Ala113 is substituted by Val113 in human as well as in sheep.

The structure of CYP2D6 has a well defined active site cavity that contains many important residues, which are implicated in substrate recognition and binding. These include Asp301, Glu216, Phe481, Phe483, and Phe120 (Rowland et al., 2006; Wang et al., 2009) in the human. Kirton et al. (2002) showed that Glu216 was the more likely binding residue. Furthermore, the two rotameric states of the aspartate molecule can account for all the various pharmacophoric models, and therefore Glu216, which sits at the top of the active site cavity, is more likely to act as a recognition residue that attracts basic ligands to the pocket. Since these substrate binding residues are conserved in sheep CYP2D6, activity of this enzyme in sheep may be similar to the human. Phe120 play a role in controlling the orientation of the aromatic ring found in most substrates with respect to the heme (Rowland et al., 2006), however, it is not present in sheep and cow where it is replaced by isoleucine and also not present in rat orthologs, CYP2D4 and CYP2D2, where it is replaced by a valine. Phe483 is also notably absent in the rat orthologs. Rat CYP2D4 is essentially inactive toward debrisoquine, although metabolism by CYP2D2 occurs (Hiroi et al., 2002). Met374 forms a hydrophobic cleft on the loop and plays a role in metoprolol metabolism (Ellis et al., 1996). Since all amino acid residues except Phe120 are conserved in sheep, CYP2D6 activity might be present in sheep. However, even with a single amino acid mutation, the substrate specificity is altered (Raija et al., 1989).

Some of the marker activities of CYP2E1 enzyme (demethylation of nitro sodimethylamine, deethylation of 7-ethoxy-4-trifluoromethylcoumarin and hydroxylation of *p*-nitrophenol and chlorzoxazone) involve significant interactions with residues Pro213, Ala299, Thr303, Val364, Pro365, Ser366, Asn367, Leu368, Phe478 and Gly479 (Park and Harris, 2003). All of the above residues are conserved in sheep except for Val364, which has been replaced with Ile. Thr303 in CYP2E1 plays an important role in hydroxylation of fatty acids as well by determining its substrate specificity (Fukuda et al., 1993). Moreover, Phe209 is a responsible site for the metabolism of coumarin (a substrate of CYP2E1) and is conserved in sheep (Lewis et al., 2006).

There are several residues that are very important for the function of HNF4 α . For example, Ser304 plays an important role in phosphorylation of HNF4 α by AMP-activated protein kinase (Leff, 2003). Acidic residues Glu261 and Asp262 create a bulge in α helix 5 (α 5) that may be important for dimerization (Gampe et al., 2000). Moreover, glutamic acid 276 is a critical residue for DNA binding, transcriptional activation, and protein stability in vivo (Navas et al., 1999). R226 (Dhe-Paganon et al., 2002) and V255 (Navas et al., 1999) are important for HNF4 α to bind with fatty acids (Yuan et al., 2009). All of these important residues are conserved in sheep. Therefore, the function of the HNF4 α should be similar in human and sheep.

The substitution of Pro for His35 in UGT2B7 abolished 4-methylumbelliferone (4MU), 1-naphthol (1NP) and naproxen glucuronidation and lacked activity toward the tertiary amines (Kerdpin et al., 2009). Miley et al. (2007) reported recently that the His to Ala substitution at position 35 of UGT2B7 abolished activity toward androsterone, hyodeoxycholic acid and tetrachlorocatechol. Moreover, exchanging the amino terminal 298 and 299 residues of UGT2B7 results in proteins that are devoid of enzyme activity (Lewis et al., 2007). Exchanging the carboxyl terminal 230-232 residues of UGT2B7 generally resulted in the synthesis of hybrid proteins that retained enzyme activity (Lewis et al., 2007). Glucuronidation activities were also low when the carboxyl terminal residues from position 385 (Tyr) were exchanged (Lewis et al., 2007). Arg338, His 374 and Gly379 are key residues that may have functional significance in UGT2B7 (Miley et al., 2007). Several mutants (e.g., S308A, R338S, W356H) exhibited stronger effects on glucuronic acid transfer to some substrates (e.g., tetrachlorocatechol and hyodeoxycholic acid) (Miley et al., 2007). All important residues in sheep UGT2B7 are the same as in the human, except the S308 which is replaced with Thr in sheep. Therefore, sheep UGT2B7 activity might be similar to human.

Several amino acid residues are important for the function of UGT1A enzymes. A conserved motif, YXXXXXXPP is observed in the phenol binding proteins of several enzymes including UGT1A6 and sulfotransferases that participate in the phenol metabolism in human (Huang and Miller, 1991; Chen et al., 1999b). In UGT1A6, this conserved domain is localized between amino acids 71-81, a region postulated as part of the aglycon binding site of all UGTs (Battaglia et al., 1997; Meech and Mackenzie, 1997). This is the most variable part of all UGTs (Meech and Mackenzie, 1997). This region is also postulated as the substrate binding site (Senay et al., 1997). This is conserved in several species. In sheep, 2 amino acids are conserved among the four important residues similar to rabbit.

Two sites Arg52 and His54 located at the amino-terminal end of UGT1A6 are important for the optimal function and structure of the protein (Senay et al., 1997). His54 is conserved in several species including rat, rabbit, cow, mouse and sheep. Whereas, Arg52 is conserved in rat, rabbit and mouse. However in the sheep and cow it is replaced with Lys.

Pro40 is critical for the UGT1A4-catalyzed conversion of the tertiary amines lamotrigine (LTG) and trifluoperazine (TFP) (Kubota et al., 2007). A recent study showed that a substitution of His for Pro altered the activity to these amines. In addition, with UGT1A1, UGT1A6, and UGT1A9, substitution of histidine for proline at positions 39, 38, and 37 of UGT1A1, UGT1A6, UGT1A9 and UGT2B7, respectively, showed alteration in the glucuronidation of LTG and TFP (Kerdpin et al., 2009). In sheep also, this proline is substituted by histidine in all UGTs that we cloned and therefore an alteration in the glucuronidation of LTG and TFP might be observed in sheep as well.

Asp143 in UGT1A9 protein may stabilize the transition state during the glucuronidation of the primary and secondary amine functional groups of 4-aminobiphenyl and retigabine (Patana et al., 2008). H37 is critical in O-glucuronidation (Patana et al., 2008). The D256N (256Asp to Asn) variant of UGT1A9 is essentially nonfunctional with regard to SN-38 (7-ethyl-10-hydroxycamptothecin) glucuronidation (Jinno et al., 2003). Asp143, H37 and Asp256 are conserved in sheep UGT1A9 and therefore similar glucuronidation of the above substrates might be observed in human and sheep. A study involving analysis of recombinant UGT1A1 mutants in COS-1 cells revealed that Phe170 is critical for enzymatic activity (Radominsky-Pandya et al., 1999). Since it is conserved in sheep, UGT1A1 activity in sheep may be similar to human UGT1A1. Residual activities are very low in the UGT1A1 polymorphisms: UGT1A1*6 (G⁷¹R), UGT1A1*27 (P²²⁹Q), UGT1A1*35 (L²³³R), and UGT1A1*7 (Y⁴⁸⁶D) (Gagne et al., 2002).

Table 4.1: Important amino acid residues in human enzymes and comparison with sheep

Enzyme	Human	Sheep
CYP2A6	Gln104, Phe107, Asp108, Arg128, Phe209, Thr212, Asn297, Leu298, Thr303, Ile366, Ile471, His477	Gln104, Phe107, Asp108, Arg128, Phe209, Thr212, Asn297, Leu298, Thr303, Ile366, Ile471, Leu477
CYP2C19	His99 , Val113, Ser115, Lys118 , Pro220, Thr221 , Leu237 , Asn286, Val288 , Ile289 , Ala292 , Leu295 , Arg410 , Arg442	Ser99 , Val113, Ser115, Glu118 , Pro220, Ser221 , Met237 , Asn286, Ile288 , Thr289 , Trp292 , Phe295 , Ala410 , Arg442
CYP2C18	Val113 , Ser115 , Lys118	Leu113 , Gly115 , Lys118
CYP2D6	Asp301, Glu216, Phe481, Phe483, Phe120	Asp301, Glu216, Phe481, Phe483, Ile120
CYP2E1	Pro213, Ala299, Thr303, Val364 , Pro365, Ser366, Asn367, Leu368, Phe478, Gly479, Phe209	Pro213, Ala299, Thr303, Ile364 , Pro365, Ser366, Asn367, Leu368, Phe478, Gly479, Phe209
HNF4 α	Ser304, Glu261, Asp262, Glu276, R226, V255	Ser304, Glu261, Asp262, Glu276, R226, V255
UGT2B7	His35, Lys230, Lys231, Trp232, Ser298, Ser299, Ser308 , Arg338, Trp356, His 374, Gly379, Tyr385	His35, Lys230, Lys231, Trp232, Ser298, Ser299, Thr308 , Arg338, Trp356, His374, Gly379, Tyr385
UGT1A1	Gly71 , Phe170, Pro229, Leu233, Tyr486	Glu71 , Phe170, Pro229, Leu233, Tyr486
UGT1A4	Pro40	His40
UGT1A6	Arg52 , His54, Tyr72 , Lys76 , Pro79, Pro81	Lys52 , His54, His72 , Arg76 , Pro79, Pro81
UGT1A9	His37, Asp143, Asp256	His37, Asp143, Asp256

Different amino acid residues between human and sheep are highlighted in bold. The positions of amino acids are according to human amino acids numbers.

Human	KYYTRKIYPVP
Rat	KYYRRKSFVP
Rabbit	RFYTRRIYPVP
MouseUGT1A6a	KYYRRKIFSVT
MouseUGT1A6b	KYYRRKIFSVP
Cow	KHYTRKIHPVP
Sheep	KHYTRRIHPVP

Fig 4.1: A motif, responsible for phenol binding proteins of several UGT1A6 enzymes in many species.

Amino acid residues of human and sheep are same except G71. It is replaced by Glu in sheep.

Sheep sequences showed higher homology with goat sequences compared to any other species. For example, goat CYP2C31 gene sequence showed 97% homology with sheep CYP2C19 whereas cow CYP2C19 showed 95% homology. Pig sequences also showed a higher sequence identity with sheep than human. But, only very few CYP and UGT sequences have been cloned in goat, cow and pig (<http://www.ncbi.nlm.nih.gov/PubMed>). It is relatively easy to clone the sequences of sheep or any other species from a species with similar sequences or vice versa. Therefore the sequences of sheep that have been determined in this study will facilitate the cloning of CYPs and UGTs in related species including the goat, cow and pig.

4.1.3 Expression of hepatic drug metabolizing enzymes

The expression of CYP2A6, CYP2C19, CYP2D6, UGT1A6, UGT1A9, UGT2B7 and HNF4 α was investigated in the present study and the relative protein and mRNA levels of these enzymes in the fetus, newborn and adult sheep liver are reported for the first time.

4.1.3.1 CYP2A6

There is evidence for the presence of several hepatic CYP2A isoforms in different species. From the CYP2A family, only one CYP2A cDNA was cloned in sheep in this study. Also, Western blot analysis with an antibody against human CYP2A6 revealed only one prominent CYP2A protein (47Kda) in adult liver which is in agreement with a previous study (Machala et al., 2003). But our findings also showed a very weak protein band (49Kda) and this could be due to a non specific protein or another CYP2A protein. To rule out this weaker band, the specificity of the antibody against human CYP2A6 was analyzed with several human CYP proteins. In addition to the CYP2A6 protein standard, the antibody cross-reacted with CYP2D6 protein standard but the OD value of CYP2D6 was very low when compared to CYP2A6. Moreover, the top weaker band (49Kda) in the sheep microsomes with the antibody against human CYP2A6 may not be a CYP2D protein, because, the molecular weight of sheep CYP2D protein was 50Kda in our study. Therefore, this band was ignored. Sheep microsomes gave only one weak CYP2A band with antibody against rat CYP2A1 (figure not shown).

Sheep, cows, monkey and dog show a single CYP2A protein in liver, and cow CYP2A has a similar molecular weight to the CYP2A protein identified in sheep liver extracts (Sivapathasundaram et al., 2001, Machala et al., 2003; Shimada et al., 1997). According to our and a previous study, the molecular weight of CYP2A was slightly higher than that of the human (Machala et al., 2003). In the human, three CYP2A mRNAs are expressed in liver; CYP2A6,

CYP2A7 and CYP2A13 (Ivan et al., 2007, Koskela et al., 1999). CYP2A6 and CYP2A7 are expressed at equally high levels in liver (Ding et al., 1995). In contrast, CYP2A13 is expressed at very low levels (Koskela et al., 1999). In addition, CYP2A7 appears to be a nonfunctional CYP (Hoffman et al., 2001) and is termed a pseudogene. There are two CYP2A enzymes present in rat liver; CYP2A1 (Nagata et al., 1987) and CYP2A2 (Matsunaga et al., 1988). Mice express CYP2A4, CYP2A5 and CYP2A12 in liver (Honkakoski et al., 1993; Iwasaki et al., 1993; Su et al., 1996). Hamster liver contains at least two CYP2As designated CYP2A8 and CYP2A9 (Lai and Chiang, 1990). Rabbits also have two CYP2A isoforms, CYP2A10 and CYP2A11 in liver (Hong et al., 1992; Peng et al., 1993). Only one hepatic CYP2A was reported in pigs (CYP2A19; Skaanild and Friis, 2005) and in cows (CYP2A13; Nelson, 2006).

In this study, a low level of CYP2A6 mRNA was expressed in liver obtained from term sheep fetuses compared to adults and newborns. Studies in human and rats also showed the presence of low levels of CYP2A6 or CYP2A1 mRNA in fetal liver (Nagata et al., 2003; Ivan et al., 2007; Nishimura et al., 2003; Borlakoglu et al., 1993), but, Hakkola et al. (1994) failed to detect CYP2A6 mRNA. However, in our study, CYP2A protein was absent in the sheep fetus and this is in agreement with reports in humans (Maenpaa et al., 1993; Shimada et al., 1996; Gu et al., 2000) and rats (Borlakoglu et al., 1993). In contrast, Tateishi et al. (1997) demonstrated low levels of hepatic CYP2A protein during the perinatal period in humans. Expression of CYP2A6 mRNA in newborns compared to that of fetuses in the present study tends to be higher, however, it is not statistically significant and this may be due to the inter-individual variation observed. In newborn sheep, CYP2A protein was absent in liver. The results from the current study are in agreement with a rat study in which microsomal proteins did not cross react with antibody raised against CYP2A1. However, their mRNA transcripts were detectable in Northern blot hybridization experiments in rats (Borlakoglu et al., 1993).

Hepatic CYP2A6 mRNA levels in our study were higher in adult sheep than in fetuses and newborns. Furthermore, Western blot analysis of CYP2A proteins in our study revealed a more intensely stained band and this indicated the presence of high level of CYP2A enzyme in the liver of adult sheep. This was also found in a human study (Shimada et al., 1996). In contrast, there was no specific CYP2A protein (47Kda) in the liver of sheep fetuses and newborns. Therefore, it appears that translation of CYP2A6 mRNA in fetus and newborn does not take place or occurs at an undetectable level and begins to develop during the postnatal period.

However, the postnatal age at which the CYP2A enzyme begins to appear has not yet been investigated in sheep.

4.1.3.2 CYP2C19

In the current study, two CYP2C proteins were identified in Western blots with a polyclonal antibody against rat CYP2C11. This agrees with a previous sheep study, where they used a polyclonal antibody against human CYP2C9 (Machala et al., 2003). Therefore, there might be at least two CYP2C proteins in sheep. In contrast, Szotakova et al. (2004) observed only one very thick CYP2C band with polyclonal antibody against human CYP2C9 and this might be due to the different specificities with different antibodies or to the non separation of two or more proteins on the blot. In cows also, only one CYP2C protein was reported (Szotakova et al., 2004) whereas Machala et al. (2003) showed two proteins. However, in both studies (Szotakova et al., 2004; Machala et al., 2003) the molecular weight of the sheep CYP2C protein appeared to be lower than that of human CYP2C9. Also when compared to the molecular weight of cow CYP2C, that in the sheep appears to be slightly higher. However in neither the Machala et al. (2003) nor Szotakova et al. (2004) papers were molecular weight indicators included in the Western blots, so that determination of the molecular weights is not possible. In our study also, the apparent molecular weights of sheep CYP2C bands (47.5, 47Kda) were below the human CYP2C9 band (52Kda) and slightly below the rat CYP2C11 (49Kda) band. The sheep molecular weights align with human CYP2C8, CYP2C18, CYP2C19 and rat CYP2C13 bands. Murray (1992) reported only one very weak rat CYP2C6 like protein in sheep liver with the molecular weight of 52Kda which is different from the molecular weight in our study (47.5, 47Kda). In the present study, two mRNA sequences of CYP2C isoforms that are orthologous to CYP2C19 and CYP2C18 have been cloned. Therefore, the sheep CYP2C subfamily consists of at least two clearly distinguishable subgroups with a sequence homology of 69%. Sheep CYP2C protein expression and the enzyme activity (7-Methoxy-4-trifluoromethyl-coumarin demethylase) are reported to be higher than that of other farm animals such as pig, goat and cattle (Szotakova et al., 2004).

Several CYP2C isoforms have been reported in the liver in different species: in human CYP2C8, CYP2C9, CYP2C18 and CYP2C19 (Hakkola et al., 1994; Hines, 2007); in pigs CYP2C33 and CYP2C49 (Kojima and Morozumi, 2004); in cattle CYP2C86; in wild goat CYP2C31 (Zeilmaker et al., 1994); in rats CYP2C6, CYP2C7, CYP2C11, CYP2C12, CYP2C13 and CYP2C22 (Borlakoglu et al., 1993; Legraverend et al., 1992; Emi et al., 1990) and in rabbits

CYP2C2, CYP2C3, CYP2C4, CYP2C5, CYP2C15 and CYP2C16 (Leighton et al., 1984; Johnson et al., 1987; Tukey et al., 1985; Imai et al., 1988; Hassett and Omiecinski, 1990).

In term fetal sheep liver, a lower level of CYP2C19 mRNA expression was observed compared to the newborn and adult stages in the present study. This finding is similar to the results of a human study, which reported lower levels of CYP2C19 mRNA in fetuses (Nishimura et al., 2003). However a few other human studies reported that CYP2C19 mRNA was undetectable in the fetus (Hakkola et al., 1994; Ivan et al., 2007). In addition, mRNA of CYP2C7 was not detectable in the fetal rat liver (Borlakoglu et al., 1993). No other CYP2C proteins were detected in the sheep fetuses by Western blot in our study. This agrees with the few studies performed in human fetuses, even at term (Oesterheld, 1998; Treluyer et al., 1997; Cresteil et al., 1985; Ladona et al., 1989; Jacqz-Aigrain et al., 1993; Pons et al., 1991). In contrast, Ratanasavanh et al. (1991) detected very low quantities of CYP2C with immuno-blot and immunohistochemistry in the fetus, but only in some samples. Moreover, a few studies have reported low CYP2C19 protein levels and enzyme activity in fetal samples (Koukouritaki et al., 2004; Hines, 2007). A similar finding was demonstrated in human fetus with antibody against mouse CYP2C (Maenpaa et al., 1993).

In newborn lambs less than 24 hours old, CYP2C19 mRNA was detected and the levels were higher than in the fetus, but lower than in the adult in our study. However, CYP2C7 mRNA was not detectable in perinatal rat liver extracts (Borlakoglu et al., 1993). In the present study, one CYP2C-like protein was detectable in newborn lambs aged less than 24 hours and the level was very low when compared to that of adult. Our findings support previous evidence in human newborns aged less than 24 hours (Treluyer et al., 1997; Koukouritaki et al., 2004; Hines, 2007) and in rat neonates (Borlakoglu et al., 1993). Even though, a lower amount of CYP2C protein was detected in newborns aged less than a day, it does not result in significant drug metabolizing activity. In the human, the development of CYP2C activity occurs over the first few weeks after parturition (Cresteil, 1998; Ratanasavanh et al., 1991; Treluyer et al., 1997). In rats, CYP2C6 and CYP2C7 are transcriptionally active before puberty (Gonzalez et al., 1986).

4.1.3.3 CYP2D6

In adult sheep, even though three proteins cross reacted with polyclonal antibody against human CYP2D6, the darkly stained band with the higher MW value might represent a CYP2D protein (50Kda) and the other two weaker bands might be different CYP2 family enzymes. When the specificity of antibody against human CYP2D6 was analyzed with several similar

human CYP enzymes, this antibody cross-reacted with CYP2A6 and CYP2C8 proteins but they were weak when compared to CYP2D6. This may be the reason for the appearance of the other two non specific bands in the sheep microsomal preparations. The second band (49Kda) might be CYP2C protein, since sheep microsomes gave similar molecular weight proteins with antibody against rat CYP2C11. The third band (47Kda) might be CYP2A because sheep microsomes gave a band with the molecular weight of 47Kda with the antibody against human CYP2A6. In a previous sheep study, polyclonal antibody against human CYP2D6 also cross-reacted with two proteins (Machala et al., 2003). Furthermore, in our study, three bands were observed with human microsomes as well. But, in human CYP2D6 is the only CYP2D subfamily member present in liver while other forms (CYP2D7 and CYP2D8) are pseudogenes (Kimura et al., 1989; Gonzalez et al., 1988). These multiple bands may be due to the high homology between all CYP enzymes. And these two extra bands might be CYP2C8 and CYP2A6, since this antibody cross-reacted with these two proteins in the blots to identify the specificity of the antibody. Several hepatic CYP2D isoforms have been reported in many species such as in rat 2D1, 2D2, 2D3, 2D4 and 2D5 (Matsunaga et al., 1989; 1990; Gonzalez et al., 1987), in mouse CYP2D9 and CYP2D10 (Wong et al., 1989), in cattle CYP2D14 (Tsuneoka et al., 1992), in dog CYP2D15 (Sakamoto et al., 1995) and in pig CYP2D21 and CYP2D25 (Sakuma et al., 2004; Postlind et al., 1997).

In our study, CYP2D6 mRNA and CYP2D protein were detected in the sheep term fetuses but at lower levels compared to the newborn and adult. This is similar to human studies, which have reported a lower level of CYP2D6 mRNA (Hakkola et al., 1994; Ivan et al., 2007; Treluyer et al., 1991) and protein (Treluyer et al., 1991; Jacqz-Aigrain et al., 1993) in the fetus. However, a few other studies have reported that the protein level and activity of enzyme are not detectable until birth in the human (Ladona et al., 1991; Shimada et al., 1996; Cresteil, 1998). CYP2D6 expression increases within hours after birth in human (Treluyer et al., 1991; Cresteil, 1998; Oesterheld, 1998; Hines and McCarver, 2002). In sheep newborns, mRNA and protein levels of CYP2D were higher than those of the fetus but still very low when compared to adult levels.

In addition, in our study, CYP2D6 mRNA levels in the fetus and newborn were slightly lower than that of adult. However, the protein concentration was markedly lower in the fetal (3061 times) and newborn (102 times) liver than adult. Such results suggest that the CYP2D protein synthesis is less efficient in fetal and newborn liver than in adult liver. Further, the regulation of CYP2D6 synthesis might be controlled by transcriptional and post-transcriptional

events (Treluyer et al., 1991). In addition, post translational modifications such as addition of functional groups (e.g., glycosylation, phosphorylation), altered protein structure (e.g., proteolytic cleavage) and protein stabilization due to proteasomal degradation may influence protein levels (Aguiar et al., 2005). In rats, the levels and activity of CYP enzymes such as CYP2B1, CYP2B2, CYP2C6, CYP2C11 and CYP2C12 are altered by phosphorylation (Aguiar et al., 2005). It has been proposed that polymorphic variants may decrease the stability of the CYP mRNA transcript, leading to altered levels and enzymatic activity (Saarikoski et al., 2005). Moreover, human CYP2A6 and mouse CYP2A5 are regulated by similar post-transcriptional mechanisms (Gilmore et al., 2001; Tilloy-Ellul et al., 1999).

Immuno-blot analysis of fetal liver microsomes with CYP2D6 antibody exhibited a slightly lower molecular weight than that found in adult liver samples. This was observed with CYP2E1 protein in a previous human study as well (Carpenter et al., 1996). The sheep microsomes gave a very weak band with antibodies against rat whereas antibodies against human gave darkly stained bands. This was observed in our study with CYP2A and in a previous study (Murray, 1992) with an antibody against rat CYP2C6 in sheep. This suggests a higher homology of sheep CYPs with those in the human compared to rats. Multiple bands for several cytochromes were obtained not only in our Western blots but also in previous studies in farm animals (Machala et al., 2003; Szotakova et al., 2004). This may be due to the use of a polyclonal antibody against human. As there are no antibodies against sheep available commercially, and we had to utilize the antibodies against human. The monoclonal antibody against human might not react with sheep or other species' proteins. A monoclonal antibody against human CYP2C19 was checked with rat microsomes in Dr. Bandiera's lab and it did not give any bands even though human CYP sequences show a higher homology with rat than with sheep. Antibodies against sheep CYP can be raised in the future since the full CYP nucleotide sequences have been identified in this study. In addition, there were no sheep purified or recombinant CYP proteins available commercially. Therefore we could not confirm the bands among multiple bands obtained in our study. Purified sheep CYP proteins could be obtained in the future using the sequences that I have determined.

4.1.3.4 UGT1A

In our study, five UGT1A isoforms were cloned in adult sheep liver. These are orthologs of UGT1A1, UGT1A3, UGT1A4, UGT1A6, and UGT1A9 genes. A previous sheep study reported cloning of UGT1A6 in liver (Kobayashi et al., 1999). Five hepatic UGT1A genes

(UGT1A1, UGT1A3, UGT1A4, UGT1A6, and UGT1A9) have been identified in the human as well (Strassburg et al., 1997a, 1997b and 1998). The rat UGT1 family consists of seven functional members in liver: UGT1A1, UGT1A2, UGT1A3, UGT1A5, UGT1A6, UGT1A7, UGT1A8 and two pseudogenes, UGT1A4 and UGT1A9 (Emi et al., 1995; Shelby et al., 2003; Kobayashi et al., 1998). In mouse, several UGTs including Ugt1a1, Ugt1a5, Ugt1a6, Ugt1a9 (Buckley and Klaassen, 2007; Shiratani et al., 2008) and in cattle, one UGT cDNA named *bovUGT1A6* were cloned from liver tissue (Iwano et al., 2001).

4.1.3.5 UGT1A6 and UGT1A9

In the present sheep study, mRNA levels of UGT1A6 and UGT1A9 were slightly lower in the fetus and newborn compared to the adult. However, only measurement of the protein level or enzyme activity will determine the functional capacity of UGT1A6 and UGT1A9. In this study the UGT1A proteins could not be detected even with a 100µg of protein load and thereby the protein levels were not measured at any of the developmental stages. This could be because the polyclonal antibodies against human UGT1A did not cross-react with sheep liver microsomes. There were no antibodies against sheep UGT1A available commercially and also only a few antibodies against human UGT1A. UGT1A antibody obtained from BD Bioscience is against a peptide specific to human UGT1A. This peptide may be different in sheep, which might explain the lack of response in sheep microsomes. Another antibody against human UGT1A, which is against carboxy terminal (C-19) was obtained from Santa Cruz Biotechnology Inc. There are 3 amino acid differences between C-19 in the carboxy terminus of human and sheep UGT1A and C-19 of the cow is same as sheep. Since this antibody reacts with cow microsomes, it should have reacted with sheep as well. However, it did not work.

There was not much data available on UGT enzymes compared to CYP enzymes. In human, UGT1A6 and UGT1A9 mRNA were not detected in the fetal liver at 20 weeks of gestation whereas it was identified in children and adults (Strassburg et al., 2002). UGT1A6 and UGT1A9 enzyme activities (acetaminophen glucuronidation) were also absent in the fetus and low levels were identified at birth in human (Alam et al., 1977; Rollins et al., 1979; Behm et al., 2003). In contrast, UGT1A6, which glucuronidates 1-naphthol and phenol was detected in the fetal rat at low levels (Akira, 2002; Coughtrie et al., 1988).

UGT1A6 metabolizes acetaminophen, complex phenols and also single phenols (Tukey and Strassburg, 2000) and UGT1A9 glucuronidates phenols (Wooster et al., 1991), anthraquinones, flavones, coumarins (Wooster et al., 1993; Ebner and Burchell, 1993) and

amines (Nowell et al., 1999; Strassburg et al., 1999a). UGT1A6 is likely to be functionally orthologous among several species including humans, rats, mice, and rabbits (Iyanagi et al., 1986; Harding et al., 1988; Lamb et al., 1994). In rats and human UGT1A6 is an important isoform involved in the glucuronidation of small planar phenols (Mackenzie et al., 1997). In addition, the amino acids in the active site for these substrates in the sheep UGT1A6 is similar to human and other species such as rabbit, mice and rats. Therefore, substrate specificity in sheep might be similar to human. However, the substrate specificity remains to be established in sheep in the future.

4.1.3.6 UGT2B7

Only one UGT2B isoform (UGT2B7 like) cDNA was cloned in sheep. However, there are five UGT2B genes (UGT2B4, UGT2B7, UGT2B10, UGT2B11 and UGT2B15) expressed in human liver (Beaulieu et al., 1997; Belanger et al., 1998; Strassburg et al., 1999b; Tukey and Strassburg, 2000). Moreover, the rat UGT2B subfamily consists of six members: UGT2B1, UGT2B2, UGT2B3, UGT2B6, UGT2B8, and UGT2B12 and they all expressed in liver (Green et al., 1995; Shelby et al., 2003). In the mouse, many UGT2B isoforms are expressed in liver (Shelby et al., 2003; Buckley and Klaassen, 2007) including Ugt2b1, Ugt2b5/37/38, Ugt2b34, Ugt2b35, and Ugt2b36 (Buckley and Klaassen, 2007).

In our study, UGT2B7 mRNA levels in the term fetal liver were lower than in adult liver. In human fetal liver at the age of 20 weeks of gestation, UGT2B7 mRNA expression was absent, but it was identified in adults and children (Strassburg et al., 2002). UGT2B7 protein levels were not measured since the polyclonal antibody against human UGT2B7 did not react with the sheep proteins in microsomes. In humans, morphine glucuronidation by UGT2B7 was reported in fetal liver, but at lower levels than in the adults (Pacifci et al., 1982). In newborn sheep in our study, hepatic UGT mRNA levels were higher than in the fetus but lower than in adults. This finding is similar to the results of a few human studies that indicate that UGT2B7 expression increased at birth but it was at a far lower level than in the adult (Choonara et al., 1989; Zaya et al., 2006). Moreover, the glucuronidation activity of UGT2B7 is markedly deficient in most premature infants and some full-term babies due to the delayed onset of UGT2B7 expression (reviewed by McCarver and Hines, 2002). It was further supported by a rat study of UGT2B12 demonstrating that neonates are unable to glucuronidate hexafluoro-2-propanol (general anesthetic agent Sevoflurane) and only 17% of the adult activity was present at day 17 (Payne et al., 1995). Moreover, a few rat studies demonstrated absence of UGT2B1 activity and mRNA in fetal

microsomes and slight activity in prenatal rats just before delivery by analyzing the UGT activity towards bisphenol-A (Yokota, 1999; Akira, 2002). The activity increased after birth, and reached the same level as that of adult rats at postnatal day 21 (Yokota, 1999; Akira, 2002).

In human, UGT2B7 metabolizes several drugs such as chloramphenicol, epirubicin and morphine, therefore these xenobiotics are used to estimate the activity of UGT2B7. Glucuronidation of epirubicin (Innocenti et al., 2001) and morphine (Coffman et al., 1997) are well documented markers of UGT2B7 activity. UGT2B7 is not present in other species (rat, mouse, and rabbit) in contrast to the situation with UGT1A6 or UGT1A9. However, compounds such as testosterone, chloramphenicol, morphine, nalorphine and profen nonsteroidal anti inflammatory drugs are substrates for rat UGT2B1 (Pritchard et al., 1994; King et al., 1996, 1997). Therefore, substrate specificities for UGT2B7 enzymes in sheep should be evaluated even though both humans and sheep share the same important residues necessary for the metabolism of these substrates.

There might be several contributors to the inconsistent results in the detection of mRNA and protein in previous reports (Treluyer et al., 1997; Koukouritaki et al., 2004) including sample size within specific age brackets, sample quality, and assay sensitivity. Since the studies used different techniques to determine the expression levels both for mRNA (Hakkola et al., 1994; Ivan et al., 2007; Nagata et al., 2003) and protein, the detection levels might have been quite different in the various studies.

4.1.4 *In vivo* drug metabolic capacities of fetal, newborn and adult sheep

The results of this study indicate that fetal sheep have no or undetectable amount of CYP2A and CYP2C proteins, low CYP2D protein and low levels of CYP and UGT mRNA expression. This suggests that there would be a limited ability for the fetus to metabolize drugs that are substrate for these enzymes. Also, newborn lambs have very low amounts of the above enzymes when compared to the adult. The capacity of sheep to metabolize several drugs has been studied *in-vivo* at different developmental stages in our laboratory as well as by other researchers. Several xenobiotics including valproic acid, fluoxetine, diphenhydramine, indomethacin, acetaminophen, morphine, ritodrine and labetalol have been studied in pregnant ewes and postnatal lambs and the results give some indication of the ontogeny of the *in vivo* metabolic capacity for these drugs in sheep that can be compared to the data in this thesis on the apparent hepatic expression of these enzymes in the fetus and newborn. Valproic acid (VPA) is

metabolized through three major routes: glucuronidation, mitochondrial β -oxidation and microsomal oxidation. Glucuronidation and renal excretion of unchanged VPA are the major determinants of VPA elimination (77% and 19% respectively) in adult sheep (Dickinson et al., 1979; Kumar et al., 2000a). Both of these routes are significantly underdeveloped (~2 to 3 fold lower) in newborn lambs (Kumar et al., 2000a). Moreover, VPA half life is significantly longer in day old newborn lambs (18.6 ± 2.6 h) than in adults (5.6 ± 1.4 h) (Kumar et al., 2000b) and a smaller percentage of drug is excreted as glucuronide in the urine of newborn lambs ($28.3 \pm 12.0\%$) compared to that in adults ($77.0 \pm 7.8\%$) (Kumar et al., 2000a). These findings indicate that a much slower rate of drug metabolism occurs in newborns compared to adult sheep, a situation similar to that of humans (Ishizaki et al., 1981; Nau et al., 1981). Similar findings have been reported in newborns of other species such as rats and guinea pigs (Yu et al., 1987; Haberer and Pollack, 1994). Therefore, enzymes that participate in the glucuronidation of valproic acid such as UGT1A6, UGT1A9 and UGT2B7 (Ethell et al., 2003) should be present at low concentrations in fetal and newborn lambs. This is consistent with the results of this study, which found lower levels of UGT1A6, UGT1A9 and UGT2B7 mRNA in newborns compared to adults. However, since the UGT proteins were not measured in our study, there is currently no information on the protein concentrations of these enzymes. A similar situation exists for acetaminophen, since the percentage of the acetaminophen dose that was excreted in urine as a glucuronide conjugate was lower in the newborn (46%) compared to the adult (64%) (Wang et al., 1990). Therefore enzymes that participate in the metabolism of acetaminophen such as mainly UGT1A6 and to a lesser extent, UGT1A9 and UGT1A1 (Bock et al., 1993; Curry et al., 1999) may be present at a lower level in newborns than in adults. The fetal lamb appears to lack phase II pathways for fluoxetine since glucuronide conjugates were not detected in the amniotic fluid with maternal dosing of fluoxetine (Kim et al., 2004). Moreover, very small amounts of the glucuronide conjugate have been found in fetal lamb urine for indomethacin (Krishna et al., 1995), acetaminophen (Wang et al., 1985) and VPA (Wong et al., 2001; Kumar et al., 2000a, 2000b). In the fetus, UGT1A6, UGT1A9 and UGT2B7 mRNA expression was lower than in newborns and adults in the current study, and these findings are consistent with the *in vivo* results. In contrast, the ability to glucuronidate alcoholic/phenolic moieties appears to be much more developed in the late gestation fetal lamb as ~63, 22, and 40% of the fetal dose of morphine, ritodrine, and labetalol, respectively, is glucuronidated (Olsen et al., 1988; Wright et al., 1991; Yeleswaram et al., 1993). Since a higher portion of morphine is glucuronidated in fetal

sheep, a UGT2B7, a marker for morphine glucuronidation (Coffman et al., 1997) should be present in the fetus.

In the fetus, some VPA metabolites resulting from β -oxidation or CYP mediated reactions (e.g., 4-OH VPA, 5-OH VPA) are comparable or higher than those in the mother (Kumar et al., 2000a). This may be due to increased activity of these pathways or reduced activity of other metabolic pathways (e.g., glucuronidation), resulting in more of the drug dose utilizing the β -oxidation or CYP-mediated pathways. The renal excretion of several drugs including indomethacin (Krishna et al., 1995), VPA (Kumar, 1998), diphenylmethoxyacetic acid (Kumar et al., 1997) and *para*-aminohippurate (Elbourne et al., 1990) is also limited in the fetus. This could result in the accumulation of intact drug metabolites in the fetus, particularly for polar metabolites, which would not cross the placenta readily. In the fetus, elimination of many drugs (VPA, FX) is mainly the result of transplacental transfer to the mother, followed by ultimate elimination via maternal process (Kim et al., 2004; Kumar et al., 2000a, 2000b). This is consistent with the similar half-life values for these drugs in the fetus and mother. In the current study, the protein levels of CYP2A6, which is involved in the CYP mediated metabolism of VPA (Sadeque et al., 1997) was absent in the fetus in our study. Moreover, in in-vitro metabolic studies with fetal hepatic microsomal preparations and in-vivo studies, norfluoxetine formation was not detected (Kim et al., 2004). Similarly, metabolites of labetalol were also absent in the fetal plasma or amniotic fluid samples following maternal or fetal dosing (Yeleswaram et al., 1993). These results are consistent with the data in the current study, since there were low levels of CYP2D6 protein (3061 fold less) and absence of CYP2C19 protein in the fetus. Mainly these two CYPs and CYP2C9 enzymes participate in the metabolism of fluoxetine (Hamelin et al., 1996; von Moltke et al., 1997; McMillen and Robinson 2005; Fjordside et al., 1999; Steven and Wrighton, 1993) and CYP2D6 also participates in the metabolism of labetalol (Brodde and Kroemer, 2003).

In newborns, levels of some metabolites of β -oxidation and CYP mediated oxidation (4-OH VPA) are higher than that of fetus and perhaps comparable to that of mother (Kumar et al., 2000a). But CYP2A6 mRNA was lower than that of adult while protein was absent in newborns. This may be due to the reduced renal elimination or presence of CYP2C9 enzymes in newborns. However, the longer half life of VPA indicates the reduced metabolism in newborns. Moreover, CYP mediated metabolism increased by 3 fold from 10 day old lambs to adult and the adult level of metabolism of VPA was achieved by 2 months of age (Wong et al., 2000b).

Our combined fetal and newborn *in vivo* and *in vitro* studies provide strong evidence that fetus and newborn have low capacity to metabolize drugs similar to humans. Moreover, the main metabolic pathways and metabolites of these drugs in sheep are similar to those in the human (Kumar, 1998). Therefore, sheep can be used as a model for human drug studies.

4.1.5 Effect of Cortisol

As discussed in the introduction, the levels of several CYP and UGT enzymes rise after birth. In our study also, the levels of all CYPs and UGTs mRNAs except CYP2A6 in newborn liver were significantly higher compared to the levels in the fetus, suggesting that the increased postnatal expression is related to birth. This may relate to factors present during the birth process itself or to mechanisms involved in the initiation of parturition. The maternal and/or fetal concentrations of several hormones change before and during parturition. One such hormone group in the fetus is glucocorticoid, primarily cortisol in the fetal lamb, which exhibits a progressive prepartum rise beginning at about 135 days gestation (Norman et al, 1985). A similar prepartum corticosteroid surge occurs in other species and is involved in the maturation of many organs and tissues including the lung, gastrointestinal tract, liver and bone marrow (Liggins, 1994). Thus it seemed that it also might play a role in the development of drug metabolizing enzymes in the fetus, which could explain the higher levels of many of these enzymes following birth, regardless of the gestational age at birth. Therefore, to determine the effect of glucocorticoids on CYP and UGT enzymes in sheep, liver samples were obtained from fetuses that were treated with cortisol (80hrs of infusion). A significant increase in mRNA and protein levels occurred only with CYP2D6, but CYP2C19, UGT1A9, UGT2B7 and HNF4 α showed a similar trend ($p < 0.1$). Even though the cortisol infusion rate (0.45 mg/h), duration (80 h) and resulting fetal plasma cortisol concentration (25.3ng/ml) were lower than occurs during the normal prepartum cortisol surge (duration ~20d, peak cortisol concentration ~70 ng/ml, Norman et al, 1985), the fetal CYP2D6 mRNA and protein levels achieved were similar to those in the newborn, so a higher rate or longer duration of cortisol infusion may not have resulted in further in CYP2D6 expression. However, if the amount of cortisol has been increased to achieve the levels observed in fetal lambs close to term, it might have led to significant increases in the levels of the other enzymes and HNF4 α as well.

Our findings of a cortisol effect on fetal CYP2D6 expression are consistent with human reports of elevated fetal expression of CYP2C19 in pregnancies with maternal administration of

synthetic glucocorticoid to enhance fetal lung maturation in pregnancies at risk for preterm birth (Chen et al., 2003; Koukouritaki et al., 2004). In addition, dexamethasone and hydrocortisone increase CYP3A4 gene expression in monkey and human hepatocyte cell lines (El Sankary et al., 2000, 2002; Pascussi et al., 2001).

4.1.6 Expression and regulation of HNF4 α

Relative mRNA levels of HNF4 α were studied in all developmental groups to analyze whether HNF4 α plays a role in the regulation of the expression of CYP and UGT enzymes. When compared to adult, the HNF4 α mRNA level in the fetus and newborn was lower and the newborn showed higher expression than the fetus.

A good correlation ($r > 0.8$ and $p < 0.05$) was observed between HNF4 α and all enzymes studied (CYP2A6, CYP2C19, CYP2D6, UGT1A6, UGT1A9 and UGT2B7). Since there is no correlation ($r = 0.50$ and $p > 0.05$) between HNF4 α and 18S, the above correlations only depend on the mRNA level of HNF4 α and all the enzymes analyzed. The present study is in agreement with several human and mouse studies. In the adult human, HNF4 α has been implicated in the activation of several CYP isoforms, including CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2D6, CYP3A4 and CYP3A5 (Cairns et al., 1996; Jover et al., 2001; Tirona et al., 2003; Pitarque et al., 2005; Tirona and Kim, 2005; Kawashima et al., 2006; Martinez-Jimenez et al., 2006). In the case of the CYP2D6 promoter, HNF4 significantly stimulates its activity in COS-7 cells, similar to rabbit CYP2C2 and human CYP2C9 genes (Chen et al., 1994; Ibeanu and Goldstein, 1995). Similarly, HNF4 activates hepatic expression of mouse *Cyp2a4* and *Cyp2a5* genes (Yokomori et al., 1997; Ulvila et al., 2004). In addition, it is a key transcriptional factor in the fetal mouse and involved in the activation of the PXR gene and CYP3A11 (Kamiya et al., 2003). The correlation between HNF4 α and these enzymes indicates a possible regulatory role of HNF4 α in the development of drug metabolizing enzymes in sheep.

Cortisol increased both mRNA and protein levels of CYP2D6 and tended to increase HNF4 α mRNA levels in the fetal lambs. Therefore, the effect of cortisol on CYP2D6 may be through HNF4 α . This has been reported in pregnant rats that glucocorticoids administration results increase in hepatic HNF4 α mRNA in the offspring (Nyirenda et al., 2006).

4.1.7 Inter-individual differences

Inter-individual variation was observed in CYP2A6 mRNA and all CYP protein levels in our study. Previous studies in human also showed large inter-individual fluctuation in several CYPs including CYP2A6, CYP2D6, CYP2C9, CYP2C19, CYP2E1 and CYP3A (Tateishi et al., 1997) at apoprotein and mRNA levels (Pelkonen et al., 1993; Pelkonen and Raunio, 1995). Moreover, the transcript levels for all UGT1A isoforms exhibited inter-individual fluctuation (Strassburg et al., 2002). This inter-individual variation may be due to the polymorphisms or due to differences in regulatory mechanisms. Polymorphisms that occur in the promoter region of the enzymes can affect the expression of mRNA and thereby translation. Even polymorphisms in the 3'UTR region affect the stability of the enzymes and thereby the mRNA and protein expression. In addition, several transcriptional regulatory factors may cause the inter-individual variation. A recent study suggests that HNF4 α is a factor involved in the inter-individual variability of UGT1A6 and UGT1A9 mRNA expression (Aueviriyavit et al., 2007). In addition, some of the inter-individual variability in these enzymes might be due to physiological factors (Ujjin et al., 2002), disease (Pasanen et al., 1997; Raunio et al., 1998) and exposure to other drugs (Dalet-Beluche et al., 1992; Sotaniemi et al., 1995; Donato et al., 2000). Similar factors could have affected the results of the current study, since we do not know the history of the animals. The observed variations in our study could also be in part due to the presence of genetic polymorphism or differences in the regulatory factors.

There is a large inter-individual variability observed for CYP2A6 in liver. More than a 100-fold variability has been reported in CYP2A6 mRNA and protein levels (Yun et al., 1991; Shimada et al., 1994b; Pelkonen et al., 1993, 2000; Rodriguez-Antona et al., 2001). Furthermore, three single nucleotide polymorphisms (SNPs) in the CYP2A6 promoter are described (Pitarque et al., 2005), one of them disrupting a NF-Y binding site (-745A>G), affecting mRNA and protein levels (Pitarque et al., 2001; Pitarque et al., 2004; von Richter et al., 2004). Therefore, SNPs disrupting the binding sites of transcription factors that are critical for CYP2A6 transcription, would likely result in a reduction of CYP2A6 mRNA and protein content. However, transcriptional regulation of human CYP2A6 mRNA and protein are not well understood, even though the regulation of murine *Cyp2a* orthologs has been well studied (Yokomori et al., 1997; Ulvila et al., 2004).

A great inter-individual variation in CYP2C9 and CYP2C19 expression is also observed during development (Shimada et al., 1994b; Hines, 2007). In the human fetus and neonate, a 34

fold inter-individual variation of CYP2C9 protein levels was observed while a 10 and 24 fold variation was detected in CYP2C19 protein levels in the fetus and neonate, respectively (Koukouritaki et al., 2004). Moreover, the protein levels were highly variable during the first five months after birth, suggesting differences in developmental factors that may have influence the expression of these enzymes.

To date, over seventy allelic variants of CYP2D6 have been identified that correspond generally with enzyme function. Mutations within the gene results in the absence or alteration of CYP2D6 protein (Gough et al., 1990) and functional enzyme activity. The presence of two loss-of-function alleles in the CYP2D6 gene constitutes a ‘poor metabolizer’ phenotype that results in inefficient metabolism of over 30 therapeutic drugs. In the adult Caucasian population, approximately 7% of individuals have this mutation (Sachse et al., 1997). This polymorphism was reported to be associated with the incidence of various diseases such as lung cancer, bladder cancer (Gough et al., 1990) and the susceptibility to Parkinson’s disease (Smith et al., 1992).

Inter-individual variability was observed both in terms of immuno-blot content and probe substrate kinetic properties with CYP3A4 (60-and 15 fold, respectively) (Guengerich and Turvy, 1991; Forrester et al., 1992; Shimada et al., 1994b). Polymorphic expression of CYP3A5 and the variability of CYP3A7 expression in fetal liver and suggests that significant inter-individual differences in the metabolism of xenobiotics may already exist at the prenatal stage (Hakkola et al., 2001).

In our study, inter-individual variation may be due to the presence of polymorphisms or a difference in regulatory mechanisms. Since there is a good correlation observed between these drug metabolic enzymes and HNF4 α , HNF4 α may be one of the factors responsible for this inter-individual variation. Moreover, polymorphism in the promoter or 3’ UTR region can be analyzed in the future.

4.2 Conclusions

Maturation of organ systems occurs during fetal, neonatal and childhood periods thereby affecting drug disposition. An effective and safe therapy requires thorough knowledge of ontogeny of drug pharmacokinetics. The current study for the first time in sheep determined the full length sequences of several CYP and UGT drug metabolizing enzymes and their mRNA and protein levels in the fetus, newborn and adult. The determined mRNA and deduced amino acid sequences in sheep exhibited higher homology with those in the cow than in the human. Different expression levels were observed at the different ages, which could thereby affect drug

metabolism. Cytochromes 2A6, 2C19, 2D6 and HNF4a genes are expressed in the fetus, newborn and adult, but the levels are lower in fetus and newborns than in the adult. CYP2D protein is expressed in all three developmental stages, whereas CYP2C is only expressed in newborns and adults; and CYP2A is only expressed in adults. However, the levels are very low in fetuses and newborns compared to adult. Therefore, drug metabolic pathways that are involved by these enzymes should be very low in the fetuses and newborns. These results are in agreement with human studies of hepatic mRNA levels and with sheep in vivo studies of the capacity of fetal and postnatal sheep to metabolize several therapeutic compounds. The expression pattern of these enzymes in sheep follows a similar pattern to that found in humans.

There is a significant inter individual variation present in the level of CYP enzymes observed. This likely occurs mainly due to the allelic variation exhibiting genetic polymorphism and, also due to the differences in regulatory mechanisms of CYP gene expression. One of the regulating factors for the expression of CYPs and UGTs is HNF4 α and there is a possible regulatory role observed in sheep HNF4 α .

Glucocorticoids may play a role in up regulating CYP2D6 and other CYPs and UGTs at term. The duration of cortisol infusion (3d) and the peak fetal plasma cortisol levels (25.3ng/ml) are both less than occurring during the parturition cortisol surge (~20d and ~70ng/ml respectively). However, the cortisol associated rise in CYP2D6 was to the level present in the newborn lambs. In addition, CYP2C19, UGT1A9, UGT2B7 and HNF4 α tended to show a cortisol effect.

4.3 Limitations of this study

There were not any recombinants or purified proteins of CYP or UGT available commercially. Therefore, we could not include the CYPs and UGTs protein standards in the Westernblot study to confirm the bands in the Westernblot. In addition, I was unable to measure the actual amount of protein by using these recombinant enzymes because of the lack of appropriate calibration standards; so instead the relative protein level was measured. Furthermore, there were no antibodies against sheep CYP and UGT available commercially, and attempting to produce them during the current study was not practicable. Therefore I had to use polyclonal antibodies against human, which resulted in multiple bands with the sheep CYP proteins. Moreover, the antibodies against human UGT did not react with sheep microsomes. Another limitation was the relatively low number of animals in each age group (3-5 animals). Although, the sheep used in the study were likely much more genetically homogeneous than

most human populations, the low sample numbers prevented an accurate assessment of intra-individual variability.

4.4 Future directions

The sheep sequences that were cloned can be confirmed in the future. In this study, sheep drug metabolizing enzymes and HNF4 α cDNA sequences were cloned using primers that were designed from nucleotide sequences from the human and several other species. Since there is high homology between different CYP or UGT enzymes, there was a chance that enzymes other than the desired one were in fact cloned. Even though, unique regions for each enzyme were selected when designing the primers to avoid this potential problem, it still might have happened. The substrate specificities and activities of these sheep enzymes remain to be established *in vitro* in the future by transfecting cell lines which are deficient in these CYP and UGT genes. This is particularly important, since it appears some of the amino acid residues in the enzymes that are critical for substrate binding in catalytic activity are different in sheep compared to the human. The availability of the full length sheep cDNA sequences should aid in this task.

Moreover, nucleotide sequences of the CYP, UGT and HNF4 α promoter regions could be cloned and this will be helpful for identifying regulatory sequences and possible polymorphisms. There were no purified or recombinant CYP and UGT sheep proteins available commercially. Therefore, CYP and UGT proteins can be purified in sheep and this will be useful in confirmation of the bands and measurement of actual protein of each enzyme. Moreover, these extracted proteins can be used to produce monoclonal CYP and UGT antibodies specific to sheep. Now that most of the important CYP and UGT enzymes have been cloned in sheep in this study and this process would be facilitated. Subsequently, the UGT protein levels in different developmental stages could be measured in sheep.

REFERENCES

1. Adab N, Smith TC, Vinten J, Williamson PR and Winterbottom JB. 2004: Common antiepileptic drugs in pregnancy in women with epilepsy. *Cochrane Database of Systematic Reviews*. 3:CD004848.
2. Adams EA, Choi HM, Cheung CY and Brace RA. 2005: Comparison of amniotic and intramembranous unidirectional permeabilities in late-gestation sheep. *American Journal of Obstetrics and Gynecology*. 193:247-55.
3. Aguiar M, Masse R and Gibbs BF. 2005: Regulation of cytochrome P450 by post translational modification. *Drug Metab Rev*. 37:379-404.
4. Akira Y. 2002: Developmental increases in rat hepatic microsomal UDP-glucuronosyl transferase activities toward xenoestrogens and decreases during pregnancy. *Environmental Health Perspectives*.
5. Alam SN, Roberts RJ and Fisher LJ. 1977: Age related differences in salicylamide and acetaminophen conjugation in man. *J Pediatr*. 90:130-135.
6. Amri EZ, Bonino F, Ailhaud G, Abumrad NA and Grimaldi PA. 1995: Cloning of a protein that mediates transcriptional effects of fatty acids in preadipocytes. Homology to peroxisome proliferator-activated receptors. *J Biol Chem*. 270:2367-2371.
7. Anderson GD. 2005: Pregnancy-induced changes in pharmacokinetics: a mechanistic-based approach. *Clin Pharmacokinet*. 44:989-1008.
8. Andersson T, Miners JO, Veronese ME, Tassaneeyakul W, Meyer UA and Birkett DJ. 1993: Identification of human liver cytochrome P450 isoforms mediating omeprazole metabolism. *Br J Clin Pharmacol*. 36:521-530.
9. Ariyoshi N, Sawamura Y-I and Kamataki T. 2001: A Novel Single Nucleotide Polymorphism Altering Stability and Activity of CYP2A6. *Biochemical and Biophysical Research Communications*. 281(3):810-814.
10. Aueviriyavit S, Furihata T, Morimoto K, Kobayashi K and Chiba K. 2007: Hepatocyte Nuclear Factor 1 Alpha and 4 Alpha are Factors Involved in Interindividual Variability in the Expression of UGT1A6 and UGT1A9 but not UGT1A1, UGT1A3 and UGT1A4 mRNA in Human Livers. *Drug Metab. Pharmacokinet*. 22(5):391-398.
11. Aukrust P, Liabakk NB, Muller F, Lien E, Espevik T and Froland SS. 1994: Serum levels of tumor necrosis factor-alpha (TNF alpha) and soluble TNF receptors in human immunodeficiency virus type 1 infection-correlation to clinical, immunologic, and virologic parameters. *J Infect Dis*. 169:420-424.
12. Au-Yeung SCS, Riggs KW, Gruber N and Rurak DW. 2007: The Use of Microdialysis for the Study of Drug Kinetics: Central Nervous System Pharmacokinetics of Diphenhydramine in Fetal, Newborn, and Adult Sheep. *Drug Metab Dispos*. 35:1285-1291.

13. Baes M, Gulik T, Choi H-S, Martinoli MG, Simha D and Moore DD. 1994: A new orphan member of the nuclear hormone receptor superfamily that interacts with a subset of retinoic acid response elements. *Mol Cell Biol.* 14:1544-1552.
14. Bajpai M, Roskos LK, Shen DD and Levy RH. 1996: Rules of cytochrome P450C9 and cytochrome P450C19 in the stereoselective metabolism of phenytoin to its major metabolite. *Drug Metab Dispos.* 24:1401-1403.
15. Barker DJP. 1994: The fetal origins of adult disease. *Fetal and Maternal Medicine.* 6:71–80.
16. Batt AM, Magdalou J, Vincent-Viry M, Ouzzine M, Fournel-Gigleux S, Galteau MM and Siest G. 1994: Drug metabolizing enzymes related to laboratory medicine: cytochromes P-450 and UDP-glucuronosyltransferases. *Clin Chim Acta.* 226(2):171-90.
17. Battaglia E, Nowell S, Drake RR, Magdalou J, Fournel-Gigleux S, Senay C and Radominska A. 1997: Photoaffinity labeling studies of the human recombinant UDP glucuronosyl transferase UGT1*6 with 5-azido-UDP-glucuronicacid. *Drug Metab Dispos.* 25:406-411.
18. Battaglia FC and Meschia G. 1986: An introduction to fetal physiology. New York: Academic Press.
19. Battaglia FC. 1978: Principal substrates of fetal metabolism: fuel and growth requirements of the ovine fetus. *Ciba Found Symp.* 63:57-74.
20. Battaglia FC. 1992: New concepts in fetal and placental amino acid metabolism. *J Anim Sci.* 70:3258–3263.
21. Beaulieu M, Levesque E, Tchernof A, et al. 1997: Chromosomal localization, structure, and regulation of the UGT2B17 gene, encoding a C19 steroid metabolizing enzyme. *DNA Cell Biol.* 16:1143–54.
22. Behm MO, Abdel-Rahman SM, Leeder JS and Kearns GL. 2003: Ontogeny of phase II enzymes: UGT and SULT. *Clin Pharmacol Ther.* 73:29.
23. Belanger A, Hum DW and Beaulieu M, et al. 1998: Characterization and regulation of UDP-glucuronosyltransferases in steroid target tissues. *J Steroid Biochem Mol Biol.* 65:301–10.
24. Bellotti M, Pennati G, Gasperi CD, Bozzo M, Battalio FC and Ferrazzi E. 2004: Simultaneous measurements of umbilical venous, fetal hepatic, and ductus venosus blood flow in growth-restricted human fetuses. *American Journal of Obstetrics and Gynecology.* 190(5):1347-1358.
25. Berliner DL and Salhanick HA. 1956: The presence of 6-beta-hydroxylase in human placenta. *Clin Endocrinol Metab.* 16:903-904.
26. Besunder JB, Reed MD and Blumer JL. 1988: Principles of drug biodisposition in the neonate. A critical evaluation of the pharmacokinetic–pharmacodynamic interface (Part II). *Clin. Pharmacokinet.* 14:261-286.

27. Blumberg B, Sabbagh W, Juguilon H, Bolado J, van Meter CM, Ong ES and Evans RM. 1998: SXR, a novel steroid and xenobiotic sensing nuclear receptor. *Genes Dev.* 12:3195-3205.
28. Bock KW, Forster A, Gsaidmeier H, et al. 1993: Paracetamol glucuronidation by recombinant rat and human phenol UDP-glucuronosyltransferases. *Biochem Pharmacol.* 45(9):1809-14.
29. Borlakoglu JT, Scott A, Henderson CJ and Wolf CR. 1993: Expression of P450 isoenzymes during rat liver organogenesis. *Int J Biochem.* 25:1659-1668.
30. Bosma PJ, Seppen J, Goldhoorn B, Bakker C, Elferink RPJO, Chowdhury JR and Chowdhury NR and Jansen PLM. 1994: Bilirubin UDP- glucuronosyltransferase 1. Is the Only Relevant Bilirubin Glucuronidating Isoform in Man. *The Journal of Biol Chem.* 269(27):17960-64.
31. Brace RA, Vermin ML and Huijssoon E. 2004: Regulation of amniotic fluid volume: Intramembranous solute and volume fluxes in late gestation fetal sheep. *American Journal of Obstetrics and Gynecology.* 191(3):837-846.
32. Brace RA. 1995: Progress Toward Understanding the Regulation of Amniotic Fluid Volume: Water and Solute Fluxes in and through the Fetal Membranes. *Placenta.* 16:1-18.
33. Braissant O, Fougelle F, Scotto C, Dauca M and Wahli W. 1996: Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR- α , - β , and - γ in the adult rat. *Endocrinology.* 137:354–366.
34. Bristow J, Rudolph MA and Itskovitz J. 1981: A preparation for studying liver blood flow, oxygen consumption, and metabolism in the fetal lamb in utero. *J Dev Physiol.* 3:255–266.
35. Bristow J, Rudolph MA, Itskovitz J and Barnes R. 1983: Hepatic oxygen and glucose metabolism in the fetal lamb. *J Clin Invest.* 71:1047–1061.
36. Brodde OE and Kroemer HK. 2003: Drug-drug interactions of β -adrenoceptor blockers. *Arzneimittelforschung.* 53:814-22.
37. Brown HL. 2007: Drugs in Pregnancy. The Merck manuals online medical library. Merck Research Laboratories, Merck & Co., Inc. N.J.
38. Buckley DB and Klaassen CD. 2007: Tissue- and gender-specific mRNA expression of UDP-glucuronosyltransferases (UGTs) in mice. *Drug Metab Dispos.* 35:121-127.
39. Burchell B and Coughtrie MWH. 1989: UDP-glucuronosyltransferases. *Pharmacol Ther.* 43:261-289.
40. Burchell B, Coughtrie M, Jackson M, Harding D, Fournel-Gigleux S, Leakey J and Hume R. 1989: Development of human liver UDP-glucuronosyltransferases. *Dev Pharmacol Ther.* 13:70-77.

41. Burchell B, Nebert DW, Nelson DR, Bock KW, Takashi I, Jansen PLM, Lancet D, Mulder GJ, Chowdhury JR, Siest G, Tephly TR and Mackenzie PI. 1991: The UDP glucuronosyl transferase gene superfamily: suggested nomenclature based on evolutionary divergence. *DNA Cell Biol.* 10:487-494.
42. Burchell B. 1974: UDP-Glucuronosyltransferase activity towards estriol in fresh and cultured fetal tissues from man and other species. *Steroid Biochem.* 5:261-267.
43. Burchell B. 1980: Isolation and purification of bilirubin UDP-glucuronyl-transferase from rat liver. *FEBS Lett.* 111(1):131-135.
44. Cairns W, Smith CA, McLaren AW and Wolf CR. 1996: Characterization of the human cytochrome P4502D6 promoter. A potential role for antagonistic interactions between members of the nuclear receptor family. *J Biol Chem.* 271:25269-76.
45. Capdevila JH, Falck JR and Estabrook RW. 1992: Cytochrome P450 and the arachidonate cascade. *FASEB J.* 6:731-736.
46. Carpenter SP, Lasker JM and Raucy JL. 1996: Expression, induction, and catalytic activity of the ethanol-inducible cytochrome P450 (CYP2E1) in human fetal liver and hepatocytes. *Mol Pharmacol.* 49:260-268.
47. Carver LA, Hogenesch JB and Bradfield CA. 1994: Tissue specific expression of the rat Ah receptor and ARNT mRNAs. *Nucleic Acids Res.* 22:3038-44.
48. Casazza J, Felver P and Veech RL. 1984: The metabolism of acetone in rat. *J Bid Clenz.* 259:231-236.
49. Cazeneuve C, Pons G, Rey E, Treluyer J-M, Cresteil T, Thiroux G, D'Athis P and Olive G. 1994: Biotransformation of caffeine in human liver microsomes from foetuses, neonates, infants and adults. *Br J Clin Pharmacol.* 37: 405-412.
50. Cereghini S and Talianidis I. 1996: Isolation and characterization of a third isoform of human hepatocyte nuclear factor 4. *Gene.* 173:275-280.
51. Chartier FL, Bossu JP, Laudet V, Fruchart JC and Laine B. 1994: Cloning and sequencing of cDNAs encoding the human hepatocyte nuclear factor 4 indicate the presence of two isoforms in human liver. *Gene.* 147:269-272.
52. Chauhan DP, Miller MS, Owens IS and Anderson LM. 1991: Gene expression, ontogeny and transplacental induction of hepatic UDP-glucuronosyl transferase activity in mice. *Dev Pharmacol Ther.* 16:139-149.
53. Chen D, Lepar G and Kemper B. 1994: A transcriptional regulatory element common to a large family of hepatic cytochrome P450 genes is a functional binding site of the orphan receptor HNF-4. *J Biol Chem.* 269:5420-27.

54. Chen G, Battalione E, Senay C, Falany CN and Radominska-Pandya A. 1999b: Photoaffinity labeling probe for the substrate binding site of human phenol sulfotransferase (SULT1A1): 7-Azido-4-methylcoumarin. *Protein Science*. 8:2151–2157.
55. Chen GF, Ronis MJ, Ingelman-Sundberg M and Badger TM. 1999a: Hormonal regulation of microsomal cytochrome P4502E1 and P450 reductase in rat liver and kidney. *Xenobiotica* 29:437-451.
56. Chen Y, Ferguson SS, Negishi M and Goldstein JA. 2003: Identification of constitutive androstane receptor and glucocorticoid receptor binding sites in the CYP2C19 promoter. *Mol Pharmacol*. 64:316-324.
57. Chianale J, Dvorak C, Farmer DL, Michaels L and Gumucio JJ. 1988: Cytochrome P-450 gene expression in the functional units of fetal liver. *Hepatology*. 8:318-326.
58. Chiang JY. 2002: Bile acid regulation of gene expression: roles of nuclear hormone receptors. *Endocr Rev*. 23:443-463.
59. Chin RKH and Lao TTH. 1987: Management of Vomiting in Early Pregnancy. 9:6.
60. Choi H-S, Chung M, Tzameli I, Simha D, Lee Y-K, Seol W and Moore DD. 1997: Differential transactivation by two isoforms of the orphan nuclear hormone receptor CAR. *J Biol Chem*. 272:23565-71.
61. Choonara IA, McKay P, Hain R and Rane A. 1989: Morphine metabolism in children. *Br J Clin Pharmacol*. 28:599-604.
62. Christen U, Wolfe T, Mohrle U, Hughes AC, Rodrigo E, Green EA, Flavell RA and von Herrath MG. 2001: A dual role for TNF-alpha in type 1 diabetes: islet-specific expression abrogates the ongoing autoimmune process when induced late but not early during pathogenesis. *J Immunol*. 166:7023-32.
63. Coffman BL, Rios GR, King CD, et al. 1997: Human UGT2B7 catalyzes morphine glucuronidation. *Drug Metab Dispos*. 25(1):1-4.
64. Conney AH. 1982: Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G.H.A. Clowes Memorial Lecture. *Cancer Res*. 42:4875-4917.
65. Cono J, Cragan JD, Jamieson DJ and Rasmussen SA. 2006: Prophylaxis and treatment of pregnant women for emerging infections and bioterrorism emergencies. *Emerg Infect Dis*. 12:1631-37.
66. Coughtrie MW, Burchell B, Leakey JE and Hume R. 1988: The inadequacy of perinatal glucuronidation: immunoblot analysis of the developmental expression of individual UDP-glucuronosyltransferase isoenzymes in rat and human liver microsomes. *Mol. Pharmacol*. 34:729-735.

67. Coughtrie MWH, Bamfoth KJ, Sharp S, Jones AL, Borthwick EB, Barker EV, Roberts RC, Hume R and Burchell B. 1994: Sulfation of endogenous compounds and xenobiotics interactions and function in health and disease. *Chem Biol Interact.* 92:247-256.
68. Court MH et al. 2001: Interindividual variability in acetaminophen glucuronidation by human liver microsomes: identification of relevant acetaminophen UDP-glucuronosyltransferase isoforms. *J. Pharmacol. Exp. Ther.* 299:998-1006.
69. Cresteil T, Beaune P, Kremers P, Celier C, Guengerich FP and Leroux JP. 1985: Immunoquantification of epoxide hydrolase and cytochrome P-450 isozymes in fetal and adult human liver microsomes. *Eur. J. Biochem.* 151:345-350.
70. Cresteil T. 1998: Onset of xenobiotic metabolism in children: toxicological implications. *Food Addit. Contam.* 15(suppl):45-51.
71. Cui W, Aslam S, Fletcher J, Wylie D, Clinton M and Clark AJ. 2002: Stabilization of telomere length and karyotypic stability are directly correlated with the level of hTERT gene expression in primary fibroblasts. *J. Biol Chem.* 277:38531-39.
72. Curry S, Brick P and Franks NP. 1999: Fatty acid binding to human serum albumin: new insights from crystallographic studies. *Biochim Biophys Acta.* 1441:131-140.
73. Dacasto M, Eeckhoutte C, Capolongoa F, Dupuy J, Carletti M, Calleja C, Nebbia, Alvinerie M and Galtier P. 2005: Effect of breed and gender on bovine liver cytochrome P4503A (CYP3A) expression and inter species comparison with other domestic ruminants. *Vet Res.* 36:179-190.
74. Dai D, Blaisdell JA, Chanas B, Ghanayem BI and Goldstein JA. 2000: Genetic polymorphisms of human CYP2C8 and their effects on metabolism of anticancer drug: paclitaxel. *FASEB J.* 14:A1338.
75. Dalet-Beluche I, Boulenc X, Fabre G, Maurel P and Bonfils C. 1992: Purification of two cytochrome P450 isozymes related to CYP2A and CYP3A gene families from monkey (baboon, *Papio papio*) liver microsomes. Cross reactivity with human forms. *Eur J Biochem* 204:641-648.
76. Daly AK, Cholerton S, Gregory W and Idle JR. 1993: *Pharmacol and Ther.* 57:129-160.
77. de Swiet M, et al. 1992: Embryology. In de Swiet M, Chamberlain G (eds): *Basic Science in Obstetrics and Gynaecology*, ed 2. Edinburgh, Churchill Livingstone, pp 27-52.
78. de Wildt SN, Kearns GL, Leeder JS and van den Anker JN. 1997: Cytochrome P450 3A: Ontogeny and drug disposition. *Clin Pharmacokinet.* 37:485-505.
79. de Wildt SN, Kearns GL, Leeder JS and van den Anker JN. 1999: Glucuronidation in human: Pharmacogenetic and developmental aspects. *Clin Pharmacokinet* 36:439-452.

80. deLemos RA and Kuehl TJ. 1987: Animal models for evaluation of drugs for use in the mature and immature newborn. *Pediatrics*. 79:275-280.
81. DeMontellano OPR. 1986: *Cytochrome P-450: Structure, Mechanism and Biochemistry*. Plenum Press, NY.
82. Denison MS, Whitlock JP and Jr. 1995: Xenobiotic-inducible Transcription of Cytochrome P450 Genes. *J Biol Chem*. 270:18175-78.
83. Denisov IG, Markis TM, Sligar SG and Schlichting I. 2005: Structure and chemistry of cytochrome P450. *Chem Rev*. 105:2254-77.
84. Detmer A, Carter AM and Thomas CR. 1992: The metabolism of free fatty acids and triacylglycerols by the fetal liver in a guinea pig model of intrauterine growth retardation. *Pediatr Res*. 32:441-446.
85. Devi BG, Habeebullah CM and Gupta PD. 1992: Glycogen metabolism during human liver development. *Biochem Int*. 28:229-237.
86. Dhe-Paganon S, Duda K, Iwamoto M, Chi YI and Shoelson SE. 2002: Crystal structure of the HNF4 alpha ligand binding domain in complex with endogenous fatty acid ligand. *J Biol Chem*. 277:37973-37976.
87. Dickinson RG, Harland RC, Ilias AM, Rodgers RM, Kaufman SN, Lynn RK and Gerber N. 1979: Disposition of valproic acid in the rat: Dose-dependent metabolism, distribution, enterohepatic recirculation and choleric effect. *J Pharmacol Exp Ther*. 211:583-595.
88. Dickmann LJ, Locuson CW, Jones JP and Rettie AE. 2004: Differential Roles of Arg97, Asp293, and Arg108 in Enzyme Stability and Substrate Specificity of CYP2C9. *Mol. Pharmacol*. 65:842-850.
89. Ding S, Lake BG, Friedberg T and Wolf CR. 1995: Expression and alternative splicing of the cytochrome P-450 CYP2A7. *Biochem J*. 306:161-166.
90. Ding X and Kaminsky S. 2003: Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. *Annu Rev Pharmacol Toxicol*. 43:149-173.
91. Donato MT, Viitala P, Rodriguez-Antona C, Lindfors A, Castell JV, Raunio H, Gomez-Lechon MJ and Pelkonen O. 2000: CYP2A5/CYP2A6 expression in mouse and human hepatocytes treated with various in vivo inducers. *Drug Metab Dispos*. 28:1321-26.
92. Drachenberg DE, Elgamal AA, Rowbotham R, Peterson M and Murphy GP. 1999: Circulating levels of interleukin-6 in patients with hormone refractory prostate cancer. *Prostate*. 41:127-133.

93. Drewes T, Senkel S, Holewa B and Ryffel GU. 1996: Human hepatocyte nuclear factor 4 isoforms are encoded by distinct and differentially expressed genes. *Mol Cell Biol.* 16:925-931.
94. Drukker A and Guignard J-P. 2002: Renal aspects of the term and preterm infants: A selective update. *Curr Opin Pediatr.* 14:175-182.
95. Dutton GJ and Leakey JEA. 1981: The perinatal development of drug-metabolizing enzymes: What factors trigger their onset? *Prog Drug Res.* 25:189-273.
96. Dvorchik BH, Woodward G, Sitar DS and Tweed WA. 1986: Hydroxylation and glucuronidation of various xenobiotics by hepatic microsomes from the fetal lambs, pregnant ewe and human fetus. *Dev Pharmacol Ther.* 9:282-289.
97. Ebner T and Burchell B. 1993: Substrate specificities of two stably expressed human liver UDP-glucuronosyltransferases of the UGT1 gene family. *Drug Metab Dispos.* 21:50-5.
98. Edelstone DI, Rudolph AM and Heymann MA. 1978: Liver and ductus venosus blood flows in fetal lambs in utero. *Circ. Res.* 42:426-433.
99. Edwards PR, Hrycay EG and Bandiera SM. 2007: Differential inhibition of hepatic microsomal alkoxyresorufin O-dealkylation activities by tetrachlorobiphenyls, *Chemico-Biological Interactions.* 169:42-52.
100. Edwards RJ, Adams DA, Watts PS, Davies DS and Boobis AR. 1998: Development of a comprehensive panel of antibodies against the major xenobiotic metabolising forms of cytochrome P450 in humans. *Biochem Pharmacol.* 56:377-87.
101. Eknstrom G, Bahr CV and Ingelman-Sundberg M. 1989: Human liver microsomal cytochrome P4501IE1. Immunological evaluation of its contribution to microsomal ethanol oxidation, carbon tetrachloride reduction and NADPH oxidase activity, *Biochem. Pharmacol.* 38:689-693.
102. El Sheikh HA, Ah BH, Homeida AM, Hassan T and Hapke HJ. 1988: The activities of aminopy-Ndemethylase aniline 4-hydroxylase and UDP-Glucuronyl transferase in tissues of camels, desert sheep and Nubian goats. *Gen Pharmacol.* 19:713-717.
103. Elbourne I, Lumbers ER and Hill KJ. 1990: The secretion of organic acids and bases by the ovine fetal kidney. *Exp Physiol.* 75:211-221.
104. Elliott SL, Morgan DJ, Angus PW, Ghabrial H and Smallwood RA. 1993: Sensitivity of propranolol elimination to hypoxia in the isolated perfused rat liver preparation. *Clin Exp Pharmacol Physiol.* 20:27-33.
105. Ellis SW, Hayhurst GP, Lightfoot T, Smith G, Harlow J, Rowland-Yeo K, Larsson C, Mahling J, Lim CK, Wolf CR, Blackburn MG, Lennard MS and Tucker GT. 1996: Influence of amino acid residue 374 of cytochrome P-450 2D6 (CYP2D6) on the regio- and enantioselective metabolism of metoprolol. *Biochem J.* 316:647-654.

106. El-Sankary W, Bombail V, Gibson GG and Plant N. 2002: Glucocorticoid-mediated induction of CYP3A4 is decreased by disruption of a protein: DNA interaction distinct from the pregnane X receptor response element. *Drug Metab Dispos.* 30:1029-1034.
107. El-Sankary W, Plant NJ, Gibson GG and Moore DJ. 2000: Regulation of the CYP3A4 gene by hydrocortisone and xenobiotics: role of the glucocorticoid and pregnane X receptors. *Drug Metab. Dispos.* 28:493-496.
108. Emi Y, Chijiwa C and Omura TA. 1990: Different cytochrome P450 form is induced in primary cultures of rat hepatocytes. *Proc Natl Acad Sci USA.* 87(24):9746-50.
109. Emi Y, Ikushiro S and Iyanagi T. 1995: Drug-responsive and tissue-specific alternative expression of multiple first exons in rat UDP-glucuronosyltransferase family 1 (UGT1) gene complex. *J Biochem.* 117:392-399.
110. Ethell BT, Anderson GD, Burchell B. 2003: The effect of valproic acid on drug and steroid glucuronidation by expressed human UDP-glucuronosyltransferases. *Biochem Pharmacol.* 65:1441-1449.
111. Fabre G, Crevat-Pisano P, Dragna S, Covo J, Barra Y and Cano J-P. 1988: Involvement of the macrolide antibiotic inducible cytochrome P450 LM3c in the metabolism of midazolam by microsomal fractions prepared from rabbit liver. *Biochem. Pharmacol.* 37:1947-53.
112. Falany CN and Tephly TR. 1983: Separation, purification and characterization of three isoenzymes of UDP-glucuronyltransferase from rat liver microsomes. *Arch Biochem Biophys.* 227(1):248-258.
113. Ferguson RJ, de Morais SM and Benhamou S, et al. 1998: A new genetic defect in human CYP2C19: mutation of the initiation codon is responsible for poor metabolism of S-mephenytoin. *J Pharmacol Exp Ther.* 284:356-361.
114. Ferguson SS, LeCluyse EL, Negishi M and Goldstein JA. 2002: Regulation of human CYP2C9 by the constitutive androstane receptor: discovery of a new distal binding site. *Mol Pharmacol.* 62:737-746.
115. Fernandez-Salguero P and Gonzalez FJ. 1995: The CYP2A gene subfamily: Species differences, regulation, catalytic activities and role in chemical carcinogenesis. *Pharmacogenetics.* 5:S123-S128.
116. Fjordside L, Jeppesen U, Eap CB, Powell K, Baumann P and Brosen K. 1999: The stereoselective metabolism of fluoxetine in poor and extensive metabolizers of sparteine . *Pharmacogenetics.* 9:55-60.
117. Forrester LM, Henderson CJ, Glancey MJ, et al. 1992: Relative expression of cytochrome P450 isoenzymes in human liver and associations with the metabolism of drugs and xenobiotics. *Biochem J.* 281:359-368.

118. Fowden AL, Szemere J, Hughes P, Gilmour RS and Forhead AJ. 1996: The effects of cortisol on the growth rate of the sheep fetus during late gestation. *J Endocrinol.* 151(97): 105.
119. Fuhr U. 2000: Induction of drug metabolising enzymes: pharmacokinetic and toxicological consequences in humans. *Clin Pharmacokinet.* 38:493-504.
120. Fujii-Kuriyama Y and Mimura J. 2005: Molecular mechanisms of AhR functions in the regulation of cytochrome P450 genes. *Biochem Biophys Res Commun.* 338:311-317.
121. Fujisawa-Sehara A, Yamane M and Fujii-Kuriyama Y. 1988: *Proc. Natl. Acad. Sci. USA.* 85:5859-63.
122. Fukuda T, Imal Y, Komori M, Nakamura M, Kusunose E, Kiyoshi Satouchi and Kusunoe M. 1993: Replacement of Thr-303 of P450 2E11 with Serine Modifies the Regioselectivity of Its Fatty Acid Hydroxylase Activity. *J Biochem.* 113:7-12.
123. Gagne J-F, Montminy V, Belanger P, Journault K, Gaucher G and Guillemette C. 2002: Common Human UGT1A Polymorphisms and the Altered Metabolism of Irinotecan Active Metabolite 7-Ethyl-10-hydroxycamptothecin (SN-38). *Mol Pharmacol.* 62(3):608-617.
124. Galtier P and Alvinerie M. 1996: Pharmacologic basis for hepatic drug metabolism in sheep. *Vet Res.* 27:363-72.
125. Gampe RT Jr, Montana VG, Lambert MH, Miller AB, Bledsoe RK, Milburn MV, Kliewer SA, Willson TM and Xu HE. 2000: Asymmetry in the PPAR gamma/RXR alpha crystal structure reveals the molecular basis of heterodimerization among nuclear receptors. *Mol Cell.*5(3):545-55.
126. Ganapathy V and Prasad PD. 2005: Role of transporters in placental transfer of drugs. *Toxicology and applied pharmacology.* 207(2):381-387.
127. Garland MMB. 1998: Pharmacology of drug transfer across the placenta. *Obstetrics and Gynecology Clinics.* 25(1):21-42.
128. Gebhardt R. 1992: Metabolic zonation of the liver: Regulation and implications for liver function. *Pharmac. Ther.* 53:275-354.
129. Gebremedhin D, Lange AR, Lowry TF, Taheri MR, Birks EK, Hudetz AG, Narayanan J, Falck JR, Okamoto H, Roman RJ, Nithipatikom K, Campbell WB and Harder DR. 2000: Production of 20-HETE and Its Role in Autoregulation of Cerebral Blood Flow Circulation Research. 87:60-65.
130. Georgiou M, Perkins LM and Payne AH. 1987: Steroid synthesis-dependent, oxygen-mediated damage of mitochondrial and microsomal cytochrome P-450 enzymes in rat Leydig cell cultures. *Endocrinology.* 121:1390-99.

131. Gerbal-Chaloin S, Daujat M, Pascussi J-M, Pichard-Garcia L, Vilarem M-J, and Maurel P. 2002: Transcriptional regulation of CYP2C9 gene: role of glucocorticoid receptor and constitutive androstane receptor. *J Biol Chem.* 277:209–217.
132. Gerbal-Chaloin S, Pascussi J-M, Pichard-Garcia L, Daujat M, Waechter F, Fabre J-M, Carrère J and Maurel P. 2001: Induction of CYP2C Genes in Human Hepatocytes in Primary Culture. *Drug Metab Dispos.* 29(3):242-251.
133. Germain A, Garrido A, Canas P, Llanos A and Valenzuela A. 1987: Differences in the lipid peroxidative status, cytochrome P-450 content and microsomal oxygen consumption between right and left lobes of the liver in fetal sheep. Comparison with maternal liver. *Biochem Int.* 15:571-577.
134. Ghosal A, Ramanathan R, Kishnani NS, Chowdhury SK and Kevin B. Alton KB. 2005: Cytochrome P450 (CYP) and UDP- Glucuronosyltransferases (UGT) enzymes: Role in drug metabolism, polymorphism and identification of their involvement in drug metabolism, Elsevier, USA, Page 295.
135. Giancarlo GM, Venkatakrisnan K, Granda BW, Von Moltke LL and Greenblatt DJ. 2001: Relative contribution of CYP2C9 and 2C19 to phenytoin 4-hydroxylation in vitro: inhibition by sulfaphenazole, omeprazole and ticlopidine. *Eur J Clin Pharmacol.* 57:31-36.
136. Giguere V. 1999: Orphan Nuclear Receptors: From Gene to Function *Endocrine Reviews.* 20(5):689-725. The Endocrine Society.
137. Gilham DE, Cairns W, Paine MIJ, Modi S, Poulsom R, Roberts GCK and Wolf CR. 1997: Metabolism of MPTP by cytochrome P4502D6 and the demonstration of 2D6 mRNA in human foetal and adult brain by in situ hybridization. *Xenobiotica.* 27:111-125.
138. Gilissen RA, Hume R, Meerman JH and Coughtrie MW. 1994: Sulphation of N-hydroxy-4-aminobiphenyl and N-hydroxy-4- acetylaminobiphenyl by human foetal and neonatal sulphotransferase. *Biochem. Pharmacol.* 48:837-840.
139. Gilmore J, Rotondo F, Pelletier AM, LaMarre J, Alaoui-Jamali M and Kirby GM. 2001: Identification of a 43-kDa protein in human liver cytosol that binds to the 5'-untranslated region of CYP2A6 mRNA. *Biochem Pharmacol.* 62:669-678.
140. Goldstein JA, Faletto MB, Romkes-Sparks M, Sullivan T, Kitareewan S, Raucy JL, Lasker JM and Ghanayem BI. 1994: Evidence that CYP2C19 is the major (S)-mephenytoin 4'-hydroxylase in humans. *Biochemistry.* 33:1743-1752.
141. Goldstein JA. 1996: The Genetic Basis of CYP2C9 and CYP2C19 Polymorphisms. International Business Communications, Inc., Southborough, MA.
142. Goldstein JA. 2001: Clinical relevance of genetic polymorphisms in the human CYP2C subfamily. *Br J Clin Pharmacol.* 52: 349-355.

143. Gonzalez FJ, Matsunaga E, Nagata K, Meyer UA, Nebert DW, Pastewka J, Kozak CA, Gillette JI, Gelboin HV and Hardwick JP. 1987. Debrisoquine 4-hydroxylase: Characterization of a new P450 gene subfamily, regulation, chromosomal mapping, and molecular analysis of the DNA rat polymorphism. *DNA*. 6:149-161.
144. Gonzalez FJ, S Kimura, B J Song, J Pastewka, H V Gelboin and J P Hardwick. 1986: Sequence of two related P-450 mRNAs transcriptionally increased during rat development. An R.dre.1 sequence occupies the complete 3' untranslated region of a liver mRNA. *The Journal of Biological Chemistry*. 261:10667-10672.
145. Gonzalez FJ, Skoda RC, Kimura S, Umeno M, Zanger UM, Nebert DW, Gelboin HV, Hardwick JP and Meyer UA. 1988: Characterization of the common genetic defect in humans deficient in debrisoquine metabolism. *Nature*. 331:442-446.
146. Gonzalez FJ. 1990: *Pharmacol and Ther*. 45:1-38.
147. Gordon JD, Riggs KW, Rurak DW, Kwan E, Hall C and Abbott FS. 1995: The pharmacokinetics of valproic acid in pregnant sheep after maternal and fetal intravenous bolus administration. 23(12):1383-89.
148. Gough AC, John S, Nigel KM, Julie ES, Gaedigk MA, Eichelbaum M and Wolf CR. 1990: Identification of the primary gene defect at the cytochrome P450 CYP2D locus, *Nature*. 347:773-776. doi:10.1038/347773a0.
149. Gray H. 1980. *Splanchnology*. In P. L. Williams and R. Warwick (Eds.), *Gray's Anatomy* (pp. 497–519). Edinburgh: Churchill Livingstone.
150. Green MD, Clarke DJ, Oтуру EM, Styczynski PB, Jackson MR, Burchell B and Tephly TR. 1995: Cloning and expression of a rat liver phenobarbital-inducible UDP-glucuronosyl transferase (2B12) with specificity for monoterpene alcohols. *Arch Biochem Biophys*. 322:460-468.
151. Greenway CV and Stark RD. 1971: The hepatic vascular bed. *Physiol Rev*. 51:23-65.
152. Gu J, Su T, Chen Y, Zhang Q-Y and Ding X. 2000: Expression of biotransformation enzymes in human fetal olfactory mucosa: Potential roles in developmental toxicity. *Toxicol Appl Pharmacol*. 165:158-162.
153. Guengerich FP and Turvy CG. 1991: Comparison of levels of several human microsomal cytochrome P450 enzymes and epoxide hydrolase in normal and disease states using immunochemical analysis of surgical liver samples. *J Pharmacol Exp Ther*. 256:1189-1195.
154. Guengerich FP, Martin MV, Beaune PH, Kremers P, Wolff T and Waxman DJ. 1986b. Characterization of rat and human liver microsomal cytochrome P-450 forms involved in nifedipine oxidation, a prototype for genetic polymorphism in oxidative drug metabolism. *J. Biol. Chem*. 261:5051-5060.

155. Guengerich FP, Muller-Enoch D and Blair IA. 1986a: Oxidation of quinidine by human liver cytochrome P-450. *Mol Pharmacol.* 30:287-295.
156. Guengerich FP, Wu ZL and Bartleson CJ. 2005: Function of human cytochrome P450s: characterization of the orphans. *Biochem Biophys Res Commun.* 338:465-469.
157. Guengerich FP. Human Cytochrome P450 Enzymes. 1995: In *Cytochrome P450*; Oritz de Montellano, P. R., Ed.; Plenum Press: New York, 473-535.
158. Haberler LJ and Pollack GM. 1994: Disposition and protein binding of valproic acid in the developing rat. *Drug Metab Dispos.* 22:113-119.
159. Hakkola J et al. 1998: Developmental expression of cytochrome P450 enzymes in human liver, *Pharmacol. Toxicol.* 82:209-217.
160. Hakkola J, Pasanen M, Purkunen R, Saarikoski S, Pelkonen O, Maenpaa J, Rane A and Raunio H. 1994: Expression of xenobiotic-metabolizing cytochrome P450 forms in human adult and fetal liver. *Biochem Pharmacol.* 48:59-64.
161. Hakkola J, Raunio H, Purkunen R, Saarikoski S, Vahakangas K, Pelkonen O, Edwards RJ, Boobis AR, Pasanen M. 2001: Cytochrome P450 3A Expression in the Human Fetal Liver: Evidence that CYP3A5 Is Expressed in Only a Limited Number of Fetal Livers. *Biol Neonate.* 80:193-201.
162. Hales DB, Sha LL and Payne AH. 1987: Testosterone inhibits cAMP-induced de Novo synthesis of Leydig cell cytochrome P-450(17 alpha) by an androgen receptor-mediated mechanism. *J Biol Chem* 262:11200-06.
163. Hamelin BA, Turguan J, Vallee F and Belanger PM. 1996: The disposition of fluoxetine but not sertraline is altered in poor metabolizers of debrisoquin. *Clin Pharmacol Ther.* 60: 512-521.
164. Hanssens M, Keirse MJ, Vankelecom F and Assche VFA. 1991: Fetal and neonatal effects of treatment with angiotensin-converting enzyme inhibitors in pregnancy. *Obstet Gynecol.* 78:128-35.
165. Harding D, Fournel-Gigleux S, Jackson MR and Burchell B. 1988: Cloning and substrate specificity of a human phenol UDP-glucuronosyltransferase expressed in COS-7 cells. *Proc Natl Acad Sci USA.* 85:8381-8385.
166. Hasler JA, Estabrook R, Murray M, Pikuleva I, Waterman M, Capdevila J, Holla V, Helvig C, Falck JR, Farrell G and et al. 1999: Pharmacogenetics of cytochromes P450. *Mol Aspects Med.* 20:1-137.
167. Hassett C and Omiecinski CJ. 1990: Sequence and gene expression of rabbit cytochrome P450. IIC16: comparison to highly related family members. *Nucleic acid Research.* 18(6):1429-34.

168. Healey JE and Sterling JA. 1963: Segmental anatomy of the newborn liver. *Ann N Y Acad Sci.* 111:25-36.
169. Henderson CJ and Wolf CR. 1992: *Progress in Drug Metabolism* (Gibson, G. G., eds), 13(73). Taylor & Francis, London.
170. Hines RN and McCarver DG. 2002: The ontogeny of human drug metabolizing enzymes: Phase I oxidative enzymes. *J Pharm Exp Ther* 300:355-360.
171. Hines RN. 2007: Ontogeny of human hepatic cytochromes P450. *J Biochem Mol Toxicol* 21(4):169-75.
172. Hiroi T, Chow T, Imaoka S and Funae Y. 2002: *Drug Metab. Dispos.* 30:970-976.
173. Hoffman SM, Nelson DR and Keeney DS. 2001: Organization, structure and evolution of the CYP2 gene cluster on human chromosome 19. *Pharmacogenetics.* 11:687-698.
174. Holewa B, Zapp D, Drewes T, Senkel S and Ryffel GU. 1997: HNF4b, a new gene of the HNF4 family with distinct activation and expression profiles in oogenesis and embryogenesis of *Xenopus laevis*. *Mol Cell Biol.* 17:687-694.
175. Holt DE, Hurley R and Harvey D. 1995: Metabolism of chloramphenicol by glutathione S-transferase in human fetal and neonatal liver. *Biol Neonate.* 67:230-239.
176. Homeida AM, Al-Eknaah MM and Dafalla EA. 1993: Drug-metabolizing Enzymes in the Placenta and Foetus of Camel and Sheep *Reprod. Dom. Anim.* 28:258-262.
177. Hong J-Y, Ding X, Smith TJ, Coon MJ and Yang CS. 1992: Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco-specific carcinogen, by rabbit nasal microsomes and cytochrome P450s NMa and NMb. *Carcinogenesis.* 13(11): 2141-44.
178. Honkakoski P, Pasanen M, Maenpaa J, Leikola J, Juvonen R, Lang MA, Pelkonen O and Raunio H. 1993: Cytochrome P4502A-mediated coumarin 7-hydroxylation and testosterone hydroxylations in the rat lung. *Pharmacol Toxicol.* 72:107-112.
179. Honkakoski P, Zelko I, Sueyoshi T and Negishi M. 1998: The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. *Mol Cell Biol.* 18:5652-5658.
180. Hopkins SJ. 2003: The pathophysiological role of cytokines. *Leg Med (Tokyo)* 5 Suppl 1:S45-57.
181. Hrycay EG and Bandiera SM. 2008: *Cytochrome P450 enzymes. Preclinical Development handbook: ADME and Biopharmaceutical Properties.* John Wiley and Sons, Inc.

182. Hrycay EG and Bandiera SM. 2009: Expression, function and regulation of mouse cytochrome P450 enzymes: comparison with human cytochrome P450 enzymes. *Current Drug Metabolism*. 10:1151-1183.
183. Huang W, Zhang J, Chua SS, Qatanani M, Han Y, Granata R and Moore DD. 2003: Induction of bilirubin clearance by the constitutive androstane receptor (CAR). *Proc Natl Acad Sci USA*. 100:4156-61.
184. Huang X and Miller W. 1991: A time-efficient, linear-space local similarity algorithm. *Adv Appl Math*. 12:337-357.
185. Ibeanu GC and Goldstein JA. 1995: Transcriptional regulation of human CYP2C genes: functional comparison of CYP2C9 and CYP2C18 promoter regions. *Biochem*. 34:8028-36.
186. Ibeanu GC, Blaisdell J, Ghanayem BI, et al. 1998a: An additional defective allele, CYP2C19*5, contributes to the S-mephenytoin poor metabolizer phenotype in Caucasians. *Pharmacogenetics*. 8:129-135.
187. Ibeanu GC, Ghanayem BI, Linko P, Leiping L, Pedersen LG, Goldstein JA. 1996: Identification of residues 99, 220, and 221 of human cytochrome P450 2C19 as key determinants of omeprazole hydroxylase activity. *J Biol Chem*. 271:12496-12501.
188. Ibeanu GC, Goldstein JA, Meyer U, et al. 1998b: Identification of new human CYP2C19 alleles (CYP2C19*6 and CYP2C19*2B) in a Caucasian poor metabolizer of mephenytoin. *J Pharmacol Exp Ther*. 286:1490-1495.
189. Ickenstein LM and Bandiera SM. 2002: Persistent suppression of hepatic CYP2A1 expression and serum triiodothyronine levels by tamoxifen in intact female rats: dose-response analysis and comparison with 4-hydroxytamoxifen, fulvestrant (ICI 182,780), and 17beta-estradiol-3 -benzoate. *J Pharmacol Exp Ther*. 302:584-593.
190. Imai Y, Komori M and Sato R. 1988: Comparison of primary structures deduced from cDNA nucleotide sequences for various forms of liver microsomal cytochrome P-450 from phenobarbital-treated rabbits. *Biochemistry*. 27:80-88.
191. Imaoka S, Yamazoe Y, Kato R and Funae Y. 1992: Hormonal regulation of rat renal cytochrome P450s by androgen and the pituitary. *Arch Biochem Biophys*. 299:179-184.
192. Ingelman-Sundberg M. 2004: Human drug metabolising cytochrome P450 enzymes: properties and polymorphisms. *Naunyn Schmiedebergs Arch Pharmacol*. 369:89-104.
193. Innocenti F, Iyer L, Ramirez J, Green MD and Ratain MJ. 2001: Epirubicin glucuronidation is catalyzed by human UDP-glucuronosyltransferase 2B7. *Drug Metab Dispos*. 29:686-692.
194. Ionescu C and Caira MR. 2005: Drug metabolism current concepts. Springer, Netherlands.

195. Ishizaki T, Yokochi K, Chiba K, Tabuchi T and Wagatsuma T. 1981: Placental transfer of anticonvulsants (phenobarbital, phenytoin and valproic acid) and the elimination from neonates. *Pediatr Pharmacol.* 1:291-303.
196. Issemann I and Green S. 1990: Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature.* 347:645-650.
197. Ivan B, Cèline N, Tarik A, Sophie V, Patrick M, Rosette L, Philippe B and Isabelle de W. 2007: Reverse transcriptase-PCR quantification of mRNA levels from cytochrome (CYP)1, CYP2 and CYP3 families in 22 different human tissues. *Pharmacogenetics and Genomics:* 17(9):731-742 doi: 10.1097/FPC.0b013e32810f2e58.
198. Iwano H, Yotsumoto N, Yokota H and Yuasa A. 2001: cDNA cloning and expression of a bovine phenol UDP-glucuronosyltransferase, BovUGT1A6. *Life Sci.* 68(18):2131-2139.
199. Iwasaki M, Juvonen R, Lindberg R and Negishi MM. 1993: Site-directed mutagenesis of mouse steroid 7 alpha-hydroxylase cytochrome P-450 (7 alpha): Role of residue 209 in determining steroid-cytochrome P-450 interaction. *Biochemical J.* 291:569-573.
200. Iyanagi T, Haniu M, Sogawa K, Fujii-Kuriyama Y, Watanabe S, Shively JE and Anan KF. 1986: Cloning and characterization of cDNA encoding 3-methylcholanthrene inducible rat mRNA for UDP-glucuronosyltransferase. *J Biol Chem.* 261:15607-15614.
201. Jacqz-Aigrain E and Cresteil T. 1992: Cytochrome P450-dependent metabolism of dextromethorphan: Fetal and adult studies. *Dev Pharmacol Ther.* 18:161-168.
202. Jacqz-Aigrain E, Funck-Brentano C and Cresteil T. 1993: CYP2D6- and CYP3A-dependent metabolism of dextromethorphan in humans. *Pharmacogenetics.* 3(4):197-204.
203. Jaiswal AK, Haaparanta T, Luc P-V, Schembri J and Adesnik M. 1990: Glucocorticoid regulation of a phenobarbital-inducible cytochrome P-450 gene: The presence of a functional glucocorticoid response element in the 5'-flanking region of the CYP2B2 gene. *Nucleic Acids Res.* 18:4237-42.
204. Jinno H, Saeki M, Saito Y, Tanaka-Kagawa T, Hanioka N, Sai K, Kaniwa N, Ando M, Shirao K, Minami H, Ohtsu A, Yoshida T, Saijo N, Ozawa S and Jun-Ichi. 2003: Functional Characterization of Human UDP-Glucuronosyltransferase 1A9 Variant, D256N, Found in Japanese Cancer Patients Sawada. *Journal of Pharmacology and Experimental Therapeutics Fast Forward.* DOI: 10.1124/jpet.103.051250.
205. Johnson EF, Barnes HJ, Griffin KJ, Okino S and Tukey RH. 1987: Characterization of a second gene product related to rabbit cytochrome P-4501. *J Biol Chem.* 262:5918-5923.
206. Johnsrud EK, Koukouritaki SB, Divakaran K, Brunengraber LL, Hines RN and McCarver DG. 2003: Human hepatic CYP2E1 expression during development. *J Phar Exp Ther.* 307: 402-407.
207. Jones CT and Rolph TP. 1985: Metabolism during fetal life: a functional assessment of metabolic development. *Physiol Rev.* 65:357-430.

208. Jones DB, Mihaly GW, Smallwood RA, Webster LK, Morgan DJ and Madsen NP. 1984: Differential effects of hypoxia on the disposition of propranolol and sodium taurocholate by the isolated perfused rat liver. *Hepatology*. 4:461-466.
209. Jones SM, Boobis AR, Moore GE and Stanier PM. 1992: Expression of CYP2E1 during human fetal development: methylation of the CYP2E1 gene in human fetal and adult liver samples. *Biochem Pharmacol*. 43:1876-79.
210. Jover R, Bort R, Gomez-Lechon MJ and Castell JV. 2001: Cytochrome P450 regulation by hepatocyte nuclear factor 4 in human hepatocytes: a study using adenovirus-mediated antisense targeting. *Hepatology*. 33:668-675.
211. Juchau MR and Dyer DC. 1972: Pharmacology of the placenta. *Pediatr Clin North Am*. 19:65-79.
212. Juchau MR, Chao ST and Omiecinski CJ. 1980: Drug metabolism by the human fetus. *Clin Pharmacokinet*. 5:320-339.
213. Jung F, Griffin KJ, Song W, Richardson TH, Yang M and Johnson EF. 1998: Identification of amino acid substitutions that confer a high affinity for sulfaphenazole binding and a high catalytic efficiency for warfarin metabolism to P450 2C19. *Biochemistry*. 37:16270-9.
214. Kaddouri M, Brassat N, Alvinerie M, Eeckhoutte C, Bonfils C, Derancourt J and Galtier P. 1992: Ontogenic development of liver progesterone metabolism in sheep: contribution of cytochrome P4502B and P4503A sub families. *J Steroid Biochem Molec Biol*. 42:499-508.
215. Kaddouri M, Larrieu G, Reckhoutte C and Galtier P. 1990: The development of drug metabolizing enzymes in female sheep livers. *J Vet Pharmacol Therap*. 13:340-349.
216. Kaminsky LS and Zhang ZY. 1997: Human P450 metabolism of warfarin. *Pharmacol Ther*. 73:67-74.
217. Kamiya A, Inoue Y and Gonzalez FJ. 2003: Role of the hepatocyte nuclear factor 4a in control of the pregnane X receptor during fetal liver development. *Hepatology*. 37:1375-84.
218. Kanai K, Watanabe J, Asaka Y, Fujimoto S and Kanamura S. 1992: Postnatal changes in sublobular distribution of NADPH-cytochrome P- 450 reductase in rat liver. *Histochem J*. 24:957-63.
219. Kato R and Yamazoe Y. 1992: Sex-specific cytochrome P450 as a cause of sex and species related differences in drug toxicity. *Toxicol. Lett*. 64/65:661-667.
220. Kawade N and Onishi S. 1981: The prenatal and postnatal development of UDP-glucuronyl transferase activity towards bilirubin and the effect of premature birth on this activity in the human liver. *Biochem J*. 196:257-60.

221. Kawashima S, Kobayashi K, Takama K, Higuchi T, Furihata T, Hosokawa M and Chiba K. 2006: Involvement of hepatocyte nuclear factor 4alpha in the different expression level between CYP2C9 and CYP2C19 in the human liver. *Drug Metab Dispos.* 34:1012-1018.
222. Kearns GL, Winter HS. 2003: Proton pump inhibitors in pediatrics: relevant pharmacokinetics and pharmacodynamics. *J Pediatr Gastroenterol Nutr.* 37:S52-9.
223. Kerdpin O, Mackenzie PI, Bowalgaha K, Finel M and Miners JO. 2009: Influence of N-Terminal Domain Histidine and Proline Residues on the Substrate Selectivities of Human UDP-Glucuronosyltransferase 1A1, 1A6, 1A9, 2B7, and 2B10. *Drug metabolism and Disposition.* 37(9):1948-1955.
224. Khanna J, Van look PFA, Griffin PD. 1992: Reproductive health: a key to a brighter future. Geneva: WHO. Pp 170. Sw Fr 35 (18 in developing countries). ISBN 924-1561-53X.
225. Kharasch ED, Hankins DC and Thummel KE. 1995: Human kidney methoxyflurane and sevoflurane metabolism. Intrarenal fluoride production as a possible mechanism of methoxyflurane nephrotoxicity. *Anesthesiology.* 82:689-99.
226. Kidd RS, Straughn AB, Meyer MC, Blaisdell J, Goldstein JA and Dalton JT. 1999: Pharmacokinetics of chlorpheniramine, phenytoin, glipizide and nifedipine in an individual homozygous for the CYP2C9*3 allele. *Pharmacogenetics.* 9:71-90.
227. Kim J, Riggs KW and Rurak DW. 2004: Stereo selective pharmacokinetics of fluoxetine and norfluoxetine enantiomers in pregnant sheep. *Drug Metab Dispos.* 32: 212-221.
228. Kim J, Riggs KW Misri S, Kent N, Oberlander TF, Grunau RE, Fitzgerald C and Rurak DW. 2006: Stereoselective disposition of fluoxetine and norfluoxetine during pregnancy and breast feeding. *British J Clin Pharmacol.* 61(2):155-163.
229. Kimura S, Umeno M, Skoda RC, Meyer UA and Gonzalez FJ. 1989: The human debrisoquine 4-hydroxylase (CYP2D) locus: sequence and identification of the polymorphic CYP2D6 gene, a related gene and a pseudogene. *Am J Hum Genet.* 45:889-904.
230. King CD, Green MD, Rios GR, Coffman BL, Owens IS, Bishop WP and Tephly TR. 1996: The glucuronidation of exogenous and endogenous compounds by stably expressed rat and human UDP-glucuronosyltransferase 1.1. *Arch Biochem Biophys.* 332:92-100.
231. King CD, Rios GR, Green MD, Mackenzie PI and Tephly TR. 1997: Comparison of Stably Expressed Rat UGT1.1 and UGT2B1 in the Glucuronidation of Opioid Compounds. *Drug Metabolism and Disposition.* 25(2):251-5.
232. Kirton SB, Kemp CA, Tomkinson NP, St. Gally S and Sutcliffe MJ. 2002: Impact of incorporating the 2C5 crystal structure into comparative models of cytochrome P4502D6. *Proteins.* 49:216-231.
233. Kitada M and Kamataki T. 1994: Cytochrome P450 in human fetal liver: significance and fetal-specific expression. *Drug Metab Rev.* 26:305-23.

234. Kitada M, Kamataki T, Itahashi K, Rikihisa T and Kanakubo Y. 1987: Significance of cytochrome P-450 (P-450 HFLa) of human fetal livers in the steroid and drug oxidations. *Biochem. Pharmacol.* 36:453-456.
235. Kitada M, Kamataki T, Itahashi K, Rikihisa T, Kato R and Kanabuko Y. 1985: Purification and properties of cytochrome P-450 from homogenates of human foetal livers. *Arch. Biochem. Biophys.* 241:275-280.
236. Kitagawa K, Kunugita N and Kawamoto T. 2001: CYP2A6*6, a novel polymorphism of cytochrome P450 2A6, has a single amino acid substitution (R128Q) that inactivates enzymatic activity. *J Biol Chem.* 16:16.
237. Kliewer SA, Bryan Goodwin, and Willson TM. 2002: The Nuclear Pregnane X Receptor: A Key Regulator of Xenobiotic Metabolism *Endocr. Rev.* 23:687-702.
238. Kliewer SA, Moore JT, Wade L, Staudinger JL, Watson MA, Jones SA, McKee DD, Oliver BB, Willson TM, Zetterstrom RH, Perlmann T and Lehmann JM. 1998: An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell.* 92:73-82.
239. Klose TS, Blaisdell JA and Goldstein JA. 1999: Gene structure of CYP2C8 and extrahepatic distribution of the human CYP2Cs. *J Biochem Mol Toxicol.* 13:289-95.
240. Klose TS, Ibeanu GC, Ghanayem BI, Pedersen LG, Li L, Hall SD and Goldstein JA. 1998: Identification of residues 286 and 289 as critical for conferring substrate specificity of human CYP2C9 for diclofenac and ibuprofen. *Arch Biochem Biophys.* 357:240-248.
241. Kobayashi T, Tatano A, Yokota H, Onaga T, Watanabe T and Yuasa A. 1999: Small intestinal UDP-glucuronosyltransferase sheUGT1A07:Partial purification and cDNA cloning from sheep small intestine. *Arch Biochem Biophys.* 364:143-152.
242. Kobayashi T, Yokota H, Ohgiya S, Iwano H and Yuasa A. 1998. UDP-glucuronosyl transferase UGT1A7 induced in rat small intestinal mucosa by oral administration of 2-naphthoflavone. *Eur J Biochem.* 258(3):948-55.
243. Kojima M and Morozumi T. 2004: Cloning of six full-length cDNAs encoding pig cytochrome P450 enzymes and gene expression of these enzymes in the liver and kidney. *J Health Sci.* 50:518-529.
244. Komai K, Sumida K and Kaneko H, et al. 1996: Identification of a new non-functional CYP2C18 allele in Japanese: substitution of T204 to A in exon2 generates a premature stop codon. *Pharmacogenetics.* 6:117-19.
245. Koop DR and Casazza JP. 1985: Identification of ethanol-inducible P-450 isozyme 30 as the acetone and acetol monooxygenase of rabbit microsomes. *J. Bio2. Chem.* 260:13607-12.
246. Koop DR and Tierney DJ. 1990: *Bio Essays.* 12:429-35.

247. Koren G and Pastuszak A. 1990: Prevention of unnecessary pregnancy termination by counselling women on drug, chemical, and radiation exposure during the first trimester. *Teratology*. 41:657-661.
248. Koren G, Bologna M, Long D, Feldman Y and Shear NH. 1989: Perception of teratogenic risk by pregnant women exposed to drugs and chemicals during the first trimester. *Am J Obstet Gynecol*. 160:1190-94.
249. Koren G, Pastuszak A and Ito S. 1998: Drugs in Pregnancy. *N Engl J Med*. 338:1128-1137.
250. Koskela S, Hakkola J, Hukkanen J, et al. 1999: Expression of CYP2A genes in human liver and extrahepatic tissues. *Biochem Pharmacol*. 57:1407-13.
251. Koukouritaki SB, Manro JR, Marsh SA, Stevens JC, Rettie AE, McCarver DG and Hines RN. 2004: Developmental expression of human hepatic CYP2C9 and CYP2C19. *J Pharmacol Exp Ther*. 308:965-74.
252. Krauer B and Dayer P. 1991: Fetal drug metabolism and its possible clinical implications. *Clin Pharmacokinet*. 21:70-80.
253. Krishna DR and Klotz U. 1994: Extra hepatic metabolism of drugs in humans, *Clin Pharmacokinet*. 26:144-60.
254. Krishna R, Riggs KW, Kwan E, Walker MPR and Rurak DW. 1995: Pharmacokinetics of indomethacin in chronically instrumented ovine fetuses following a 3 day intravenous infusion. *Pharm Res*. 12:S-347.
255. Krishna R, Riggs KW, Kwan E, Wong H, Szeitz A, Walker MPR and Rurak DW. 2002: Clearance and disposition of indomethacin in chronically instrumented fetal lambs following a three day continuous intravenous infusion. *J Pharm Pharmacol*. 54:801-808.
256. Kritis AA, Argyrokastritis A, Moschonas NK, Power S, Katrakili N, Zannis VI, Cereghini S and Talianidis I. 1996: Isolation and characterization of a third isoform of human hepatocyte nuclear factor 4. *Gene*. 173:275-80.
257. Kronbach T, Larabeet TM and Johnson EF. 1989: Hybrid cytochromes P-450 identify a substrate binding domain in P-450IIC5 and P-450IIC4. *Proc. Natl. Acad. Sci. USA*. 86:8262-8265.
258. Ktistaki E, Ktistakis NT, Papadogeorgaki E, Talianidis I. 1995: *Proc Natl Acad Sci*. 92:9876-80.
259. Kubota T, Lewis BC, Elliot DJ, Mackenzie PI and Miners JO. 2007: Critical roles of residues 36 and 40 in the phenol and tertiary amine aglycone substrate selectivities of UDP-glucuronosyltransferases 1A3 and 1A4. *Mol Pharmacol*. 72:1054-1062.

260. Kumar S, Riggs KW and Rurak DW. 1999a: Comparative Formation, Distribution, and Elimination Kinetics of Diphenylmethoxyacetic Acid (a Diphenhydramine Metabolite) in Maternal and Fetal Sheep. *Drug Metab Dispos.* 27(4):463-70.
261. Kumar S, Tonn GR, Kwan E, Hall C, Riggs KW, Axelson JE and Rurak DW. 1997: Estimation of transplacental and non placental diphenhydramine clearances in the fetal lamb: The impact of fetal first pass hepatic uptake. *J Pharm Exp Ther.* 282:617-32.
262. Kumar S, Tonn GR, Riggs KW and Rurak DW. 1999b: Diphenhydramine Disposition in the Sheep Maternal-Placental-Fetal Unit: Determinants of Plasma Drug Concentrations in the Mother and the Fetus. *Journal of Pharmaceutical Sciences.* 88(12):1259-65.
263. Kumar S, Tonn GR, Riggs KW and Rurak DW. 2000c: Diphenhydramine Disposition in the Sheep Maternal-Placental-Fetal Unit: Gestational Age, Plasma Drug Protein Binding, and Umbilical Blood Flow Effects on Clearance. 28(3):279-85.
264. Kumar S, Wong H, Au-Yeung S, Riggs KW, Abbott FS and Rurak DW. 2000a: Disposition of valproic acid in maternal, fetal, and newborn sheep. II: metabolism and renal elimination. *Drug Metab Dispos.* 28:857-64.
265. Kumar S, Wong H, Au-Yeung S, Riggs KW, Abbott FS and Rurak DW. 2000b: Disposition of valproic acid in maternal, fetal, and newborn sheep. I: placental transfer, plasma protein binding, and clearance. *Drug Metab Dispos.* 28:845-56.
266. Kumar S. 1998: Drug disposition in the maternal-fetal unit: Studies with diphenhydramine and valproic acid in pregnant sheep. Ph.D. Thesis, University of British Columbia, Vancouver, B.C., Canada.
267. Lacroix DI, Sonnier M, Moncton A, Cheron G and Cresteil T. 1997: Expression of CYP3A in the human liver: Evidence that the shift between CYP3A7 and CYP3A4 occurs immediately after birth. *Eur J Biochem.* 247:625-34.
268. Ladona MG, Lindstrom B, Thyer C, Dun RP and Rane A. 1991: Differential fetal development of the O- and N-demethylation of codeine and dextromethorphan in man. *Br J Clin Pharmacol.* 32:295-302.
269. Ladona MG, Spalding DJM, Ekman L, Lindstrom B and Rane A. 1989: Human foetal and adult liver metabolism of ethylmorphine. Relation to immunodetected cytochrome P450 PCN and interactions with important foetal corticosteroids. *Biochemical Pharmacology.* 38:3147-3155.
270. Laemmli UK. 1970: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680-85.
271. Lai TS and Chiang JY. 1990: Cloning and characterization of two major 3-methyl cholanthrene-inducible hamster liver CYP P450s. *Arch Biochem Biophys.* 283:429-39.

272. Lake BG, Sauer MJ, Esclangon F, Beaman JA, Price RJ and Walters DG. 1995: Metabolism of coumarin by precision-cut calf liver slices and calf liver microsomes. *Xenobiotica*. 25:133-141.
273. Lamb JG, Straub P and Tukey RH. 1994: Cloning and characterization of cDNAs encoding mouse Ugt1.6 and rabbit UGT1.6: differential induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Biochemistry*. 33:10513-10520.
274. Lankas GR, Wise LD, Cartwright ME, Pippert T and Umbenhauer DR. 1998: Placental P-glycoprotein deficiency enhances susceptibility of chemically induced birth defects in mice. *Reprod Toxicol*. 12:457-63.
275. Leakey JE, Hume R and Burchell B. 1987: Development of multiple activities of UDP-glucuronyltransferase in human liver. *Biochem J*. 243:859-61.
276. Leakey JEA, Althaus ZR, Bailey JR and Slikker WJr. 1983: UDP glucuronyltransferase activity exhibits two developmental groups in liver from foetal rhesus monkeys. *Biochem J*. 214:1007-09.
277. Leemann TD, Transon C, Bonnabry P and Dayer P. 1993: A major role for cytochrome P450TB (CYP2C subfamily) in the actions of non-steroidal antiinflammatory drugs. *Drugs Exp Clin Res*. 19:189-95.
278. Leff T. 2003: AMP-activated protein kinase regulates gene expression by direct phosphorylation of nuclear proteins. *Biochemical Society Transactions*. 31:224-227.
279. Legraverend C, Mode A, Westin S, Strom A, Eguchi H, Zaphiropoulos PG and Gustafsson JA. 1992: Transcriptional regulation of rat P-450 2C gene subfamily members by the sexually dimorphic pattern of growth hormone secretion. *Mol Endocrinology*. 6:259-266.
280. Lehmann JM, McKee DD, Watson MA, Wilson TM, Moore JT and Kliewer SA. 1998: The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest*. 102:1-8.
281. Leighton JK and Kemper B. 1984: Differential induction and tissue-specific expression of closely related members of the phenobarbital-inducible rabbit cytochrome P-450 gene family. *J Biol Chem*. 259(18):11165-8.
282. Lemberger T, Desvergne B and Wahli W. 1996: Peroxisome proliferator-activated receptors: a nuclear receptor signaling pathway in lipid physiology. *Annu Rev Cell Dev Biol*. 12:335-63.
283. Leung GS, Kawai M, Tai JK, Chen J, Bandiera SM and Chang TK. 2009: Developmental expression and endocrine regulation of CYP1B1 in rat testis. *Drug Metab Dispos*. 37:523-28.
284. Levy RH. 1995: CytochromeP450 isozymes and antiepileptic drug interactions. *Epilepsia*. 720:8-13.

285. Lewis BC, Mackenzie PI, Elliot DJ, Burchell B, Bhasker CR and Miners JO. 2007: Amino terminal domains of human UDP-glucuronosyltransferases (UGT) 2B7 and 2B15 associated with substrate selectivity and autoactivation. *Biochemical Pharmacology*. 73(9):1463-1473.
286. Lewis DFV and Lake BG. 1995: Molecular modeling of members of the P4502A subfamily: application to studies of enzyme specificity. *Xenobiotica*. 25:585-598.
287. Lewis DFV and Lake BG. 2002: Species differences in coumarin metabolism: a molecular modelling evaluation of CYP2A interactions. *Xenobiotica*. 32(7):547-561.
288. Lewis DFV, Dickins M, Lake BG, Eddershaw PJ, Tarbit MH and Goldfarb PS. 1999: Molecular modelling of the human cytochrome P450 isoform CYP2A6 and investigations of CYP2A substrate selectivity. *Toxicology*. 133(1):1-33.
289. Lewis DFV, Ito Y and Lake BG. 2006: Metabolism of coumarin by human P450s: A molecular modelling study. *Toxicology in Vitro*. 20(2):256-264.
290. Lewis F. 1996: *Cytochrome P450: Structure, function and mechanism*. Taylor & Francis.
291. Lewis F. 2001: *Guide to cytochrome P450: Structure and Function* Taylor & Francis Inc., New York.
292. Lewis F. 2004: 57 varieties: the human CYP450. *Pharmacogenomics*. 5(3):305-18.
293. Liggins GC. 1994: The role of cortisol in preparing the fetus for birth. *Reprod Fertil Dev*. 6:141-50.
294. Lin Z, Lou Y and Squires EJ. 2004: Molecular cloning, expression and functional characterization of the cytochrome P4502A6 gene in pig liver. *Animal genetics*. 35:314-316.
295. Lipman MM, Katz FJ and Jailer JW. 1962: An alternate pathway for cortisol metabolism: 6-beta-hydroxycortisol production by human tissue slices. *J Clin Endocrinol Metab*. 22:268-72.
296. Little BB. 1999: Pharmacokinetics during pregnancy: evidence-based maternal dose formulation. *Obstet Gynecol*. 93:858-68.
297. Liu C, Zhuo X, Gonzalez FJ and Ding X. 1996: Baculovirus-mediated expression and characterization of rat CYP2A3 and human CYP2A6: role in metabolic activation of nasal toxicants. *Mol Pharmacol*. 50:781-88.
298. Livak KJ and Schmittgen TD. 2001: Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta C_T}$ Method. *Cell*. 25(4):402-08.
299. Loebstein R, Lalkin A and Koren G. 1997: Pregnancy induced pharmacokinetic changes and their clinical relevance. *Clin Pharmacokinet*. 33:328-43.

300. Loughnan PM, Greenwald A, Purton WW, Aranda JV, Watters G and Neims AH. 1977: Pharmacokinetic observations of phenytoin disposition in the newborn and young infant. *Arch Dis Child.* 52:302-9.
301. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. 1951: Protein measurement with the Folin phenol reagent. *J Biol Chem.* 193:265-75.
302. Lu AY and Levin W. 1972: Partial purification of cytochromes P-450 and P-448 from rat liver microsomes. *Biochem Biophys Res Commun.* 46:1334-39.
303. Lumbers ER. 1983: A brief review of fetal renal function. *J Dev Physiol.* 6:1-10.
304. Machala M, Soucek P, Neca J, Ulrich R, Lamka J, Szotakova B and Skalova L. 2003: Inter-species comparisons of hepatic cytochrome P450 enzyme levels in male ruminants. *Arch Toxicol.* 77:555-60.
305. Mackenzie PI, Owens IS and Burchell B et al. 1997: The UDP-glycosyltransferase gene superfamily: Recommended nomenclature update based on evolutionary divergence. *Pharmacogenetics.* 7(4):255–269.
306. Maenpaa J, Rane A, Raunio H, Honkakoski P and Pelkonen O. 1993: Cytochrome P450 isoforms in human fetal tissues related to phenobarbital-inducible forms in the mouse. *Biochem Pharmacol.* 45: 899-907.
307. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P and Evans RM. 1995: The nuclear receptor superfamily: the second decade. *Cell.* 83(6):835-39.
308. Mannervik B, Awasthi YC, Board PG, Hayes JD, Di Ilio C, Ketterer B, Listowsky I, Morgenstern R, Muramatsu M, Pearson WR et al. 1992: Nomenclature for human glutathione transferases (letter). *Biochem J.* 282:305-06.
309. Martinez-Jimenez CP, Gomez-Lechon, Castell JV and Jover R. 2006: Underexpressed Coactivators PGC1 α AND SRC1 Impair Hepatocyte Nuclear Factor 4 α Function and Promote Dedifferentiation in Human Hepatoma Cells. *J Biol Chem.* 281: 29840-9.
310. Matsunaga E, Umeno M and Gonzalez FJ. 1990: The rat P450 IID subfamily: complete sequences of four closely linked genes and evidence that gene conversions maintained sequence homogeneity at the heme-binding region of the cytochrome P450 active site. *J Mol Evol.* 30:155-169.
311. Matsunaga E, Zanger UM, Hardwick JP, Gelboin HV, Meyer UA and Gonzalez FJ. 1989: The CYPZD gene subfamily: analysis of the molecular basis of the debrisoquine 4-hydroxylase deficiency in DA rats. *Biochemistry.* 28:7349-55.
312. Matsunaga T, Nagata K, Holsztynska EJ, Lapenson DP, Smith R, Kato R, Gelboin HV, Waxman DJ and Gonzalez FJ. 1988: Gene conversion and differential regulation in the rat

- P450 IIA gene subfamily: purification, catalytic activity, cDNA and deduced amino acid sequence, and regulation of an adult male-specific hepatic testosterone 15 α -hydroxylase. *J Boil Chem.* 263:17995-18002.
313. May Au, Dan Rurak, Geoff Hammond, Caroline Underhill, Steve Lye, William Gibb, John Challis. Expression of cytochrome CYP2D6 mRNA in fetal sheep liver and the effect of exogenous cortisol. 33rd Annual meeting of the Fetal and Neonatal Physiological Society 2006, Sep 17-20. Cambridge, UK.
314. McCarver DG and Hines RN. 2002: Ontogeny of human drug metabolizing enzymes: Phase II conjugation enzymes and regulatory mechanisms. *J Pharm Exp Ther.* 300:361-66.
315. McMillen IC and Robinson JS. 2005: Developmental origins of the metabolic syndrome: Prediction, plasticity and programming. *Physiol Rev.* 85:571-633.
316. McRorie TI, Lynn AM, Nespeca MK, Opheim KE and Slattery JT. 1992: The maturation of morphine clearance and metabolism. *Am J Dis Child.* 146:972-976.
317. Meech R and Mackenzie PI. 1997: Structure and function of uridine diphosphate glucuronosyltransferases. *Clin Exp Pharmacol Physiol.* 24:907-915.
318. Messina A, Nencioni S, Gervasi PG, Gotlinger KH, Schwartzman ML and Longo V. 2010: Molecular cloning and enzymatic characterization of sheep CYP2J. *Xenobiotica.* 40(2):109-118.
319. Mihaly GW, Morgan DJ, Smallwood R and Hardy KJ. 1982: The developing liver: The steady-state disposition of propranolol in pregnant sheep. *Hepatology.* 2:344-349.
320. Mikkelsen S, Feilberg VL, Christensen CB and Lundstrom KE. 1994: Morphine pharmacokinetics in premature and mature newborn infants. *Acta Paediatr.* 83:1025-1028.
321. Miles JS, McLaren AW, Forrester LM, Glancey MJ, Lang MA and Wolf CR. 1990: Identification of the human liver cytochrome P450 responsible for the coumarin 7-hydroxylase activity. *Biochem J.* 267:365-371.
322. Miley MJ, Zielinska AK, Keenan JE, Bratton SM, Radomska-Pandya A and Redinbo MR. 2007: Crystal structure of the cofactor binding domain of the human phase II drug metabolism enzyme UDP-glucuronosyltransferase 2B7. *J Mol Biol.* 369:498-511.
323. Miners JO and Mackenzie PI. 1991: Drug glucuronidation in humans. *Pharmacol Ther.* 51:347-369.
324. Miners JO et al. 1988: Comparison of paracetamol metabolism in young adult and elderly males. *Eur J Clin Pharmacol.* 35:157-160.
325. Miquerol L, Lopez S, Cartier N, Tulliez M, Raymondjean M and Kahn A. 1994: Expression of the L-type pyruvate kinase gene and the hepatocyte nuclear factor 4 transcription factor in exocrine and endocrine pancreas. *J Biol Chem.* 269:8944-8951.

326. Mode A. 1993: Sexually differentiated expression of genes encoding the P450C2 cytochromes in rat liver. A model system for studying the action of growth hormone. *J Reprod Fert Suppl.* 46:77-86.
327. Moore JT and Kliewer SA. 2000: Use of the nuclear receptor PXR to predict drug interactions. *Toxicology.* 153:1-10.
328. Moore KL. 1982: *The Developing Human: Clinical Orientated Embryology.* Philadelphia: W. B. Saunders.
329. Morgan ET. 1997: Regulation of cytochromes P450 during inflammation and infection. *Drug Metab Rev.* 29:1129-1188.
330. Morita J, Kobayashi K, Wanibuchi A, Kimura M, Irie S, Ishizaki T and Chiba K. 2004: A novel single nucleotide polymorphism (SNP) of the CYP2C19 gene in a Japanese subject with lowered capacity of mephobarbital 4'-hydroxylation. *Drug Metab Pharmacokinet.* 19:236-238.
331. Morrison JL, Rurak DW, Chien C, Kennaway DJ, Gruber N, McMillen IC and Riggs KW. 2005: Maternal fluoxetine infusion does not alter fetal endocrine and biophysical circadian rhythms in pregnant sheep. *J Soc Gynecol Investig.* 12:356-364.
332. Morselli PL. 1989: Clinical pharmacology of the perinatal period and early infancy. *Clinical Pharmacokinetics* 17:13-28.
333. Moses-Kolko EL, Bogen D, Perel J; et al. 2005: Neonatal signs after late in utero exposure to serotonin reuptake inhibitors: literature review and implications for clinical applications. *JAMA.* 293:2372-2383.
334. Mugford C and Kedderis G. 1998: Sex dependent metabolism of xenobiotics. *Drug Metabolism Reviews.* 30:441-498.
335. Murray GI, Foster CO, Barnes TS, Weaver RJ, Snyder CP, Ewen SW, Melvin WT and Burke MD. 1992: Cytochrome P450IA expression in adult and fetal human liver. *Carcinogenesis.* 13:165-169.
336. Murray M. 1991: Microsomal cytochrome P450-dependent steroid metabolism in male sheep liver. Quantitative importance of 6 beta-hydroxylation and evidence for the involvement of a P450 from the IIIA subfamily in the pathway. *J Steroid Biochem Mol Biol.* 38:611-619.
337. Murray M. 1992. Participation of a cytochrome P450 enzyme from the 2C subfamily in progesterone 21-hydroxylation in sheep liver. *J Steroid Biochem Mol Biol* 43(6):591-593.
338. Nagata K, Matsunaga T, Gillette J, Gelboin HV and Gonzalez FJ. 1987: Rat testosterone 7 α -hydroxylase: isolation, sequence and expression of cDNA, and its developmental regulation and induction by 3-methylcholanthrene. *J Biol Chem.* 262:2787-93.

339. Nagata T, Takahashi Y, Ishii Y, Asai S, Sugahara M, Nishida Y, Murata A, Chin M, Schichino H and Koshinaga T, et al. 2003: Profiling of genes differentially expressed between fetal liver and postnatal liver using high-density oligonucleotide DNA array. *Int J Mol Med*. 11:713-721.
340. Nakagawa M and Setchell KD. 1990: Bile acid metabolism in early life: studies of amniotic fluid. *J Lipid Res*. 31:1089-1098.
341. Nakajima M, Yamamoto T, Nunoya K-I, Yokoi T, Nagashima K, Inoue K, Funae Y, Shimada N, Kamataki T and Kuroiwa Y. 1996b: Characterization of CYP2A6 involved in 3'-hydroxylation of cotinine in human liver microsomes. *J Pharmacol Exp Ther*. 277:1010-15.
342. Nakajima M, Yamamoto T, Nunoya K-I, Yokoi T, Nagashima K, Inoue K, Funae Y, Shimada N and Kuroiwa Y. 1996a: Role of human cytochrome P4502A6 in C-oxidation of nicotine. *Drug Metab Dispos*. 24:1212-1217.
343. National Center for Biotechnology Information. <http://www.ncbi.nlm.nih.gov/>.
344. Nau H, Rating D, Koch I, Hauser I and Helge H. 1981: Valproic acid and its metabolites: Placental transfer, neonatal pharmacokinetics, transfer via mother's milk and clinical status in neonates of epileptic mothers. *J Pharmacol Exp Ther*. 219:768-777.
345. Navas MA, Munoz-Elias EJ, Kim J, Shih D and Stoffel M. 1999: Functional characterization of the MODY1 gene mutations HNF4(R127W), HNF4(V255M), and HNF4(E276Q). *Diabetes*. 48(7):1459-1465.
346. Nawata Y, Eugui EM, Lee SW and Allison AC. 1989: IL-6 is the principal factor produced by synovia of patients with rheumatoid arthritis that induces B-lymphocytes to secrete immunoglobulins. *Ann N Y Acad Sci*. 557:230-238.
347. Nebert DW, Adesnik M, Coon MJ, Estabrook RW, Gonzalez FJ, Guengerich FP, Gunsaius IC, Johnson EF, Kemper B, Levin W, Phillips IR, Sato R and Waterman MR. 1987: The P450 gene superfamily: recommended nomenclature. *DNA* 6:1-11.
348. Nebert DW. 1991: *Mol Endocrinol*. 5:1203-1214.
349. Nelson D. 2006: Cytochrome P450 homepage. <http://drnelson.utmem.edu/CytochromeP450.html>.
350. Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, Feyereisen R, Gonzalez FJ, Coon MJ, Gunsalus IC, Gotoh O, Okuda K and Nebert DW. 1993: The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes and nomenclature. *DNA Cell Biol*. 12:1-51.
351. Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC and Nebert DW. 1996: P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics*. 6(1):1-42.

352. Nelson DR, Zeldin DC, Hoffman SM, Maltais U, Wain HM and Nebert DW. 2004: Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. *Pharmacogenetics*. 14:1-18.
353. Newton ER, et al. 1989: Medical problems in pregnancy. *Med Clin North Am*. 73:517-752.
354. Nishimura M, Yaguti H, Yoshitsugu H, Naito S, Satoh T. 2003: Tissue distribution of mRNA expression of human cytochrome P450 isoforms assessed by high-sensitivity real-time reverse transcription PCR. *Yakugaku Zasshi*. 123(5):369-375.
355. Niwa T, Kageyama A, Kishimoto K, Yabusaki Y, Ishibashi F and Katagiri M. 2002: Amino acid residues affecting the activities of human cytochrome P450 2C9 and 2C19. *Drug Metab Dispos Biol Fate Chem*. 30:931-936.
356. Norman LJ, Lye SJ, Wlodek ME and Challis JRG. 1985: Changes in pituitary responses to synthetic ovine corticotrophin releasing factor in fetal sheep. *Can J Physiol Pharmacol*. 63:1398-403.
357. Nosten F, McGready R, d'Alessandro U, Bonell A, Verhoeff F, Menendez C, Mutabingwa T and Brabin B. 2006: Antimalarial Drugs in Pregnancy: A Review Current Drug Safety. 1:1-15.
358. Nowell SA, Massengill JS, Williams S, et al. 1999: Glucuronidation of 2-hydroxyamino-1-methyl-6-phenylimidazo [4,5-b] pyridine by human microsomal UDP-glucuronosyl transferases: identification of specific UGT1A family isoforms involved. *Carcinogenesis*. 20:1107-14.
359. Nyirenda MJ, Dean S, Lyons V, Chapman KE and Seckl JR. 2006: Prenatal programming of hepatocyte nuclear factor 4alpha in the rat: a key mechanism in the 'foetal origins of hyperglycaemia'. *Diabetologia*. 49:1412-1420.
360. Oberlander TF, Eckstein GR, Fitzgerald C et al. 2002: Prolonged prenatal psychotropic medication exposure alters neonatal acute pain response. *Pediatr Res*. 51:443-453.
361. Oberlander TF, Grunau RE, Fitzgerald C, Papsdorf M, Rurak D and Riggs W. 2005: Pain reactivity in 2-month-old infants after prenatal and postnatal serotonin reuptake inhibitor medication exposure. *Pediatrics*. 115(2):411-25.
362. Oberlander TF, Misri S, Fitzgerald CE, Kostaras X, Rurak D and Riggs W. 2004: Pharmacologic factors associated with transient neonatal symptoms following prenatal psychotropic medication exposure. *J Clin Psychiatry*. 65:230-237.
363. Oberlander TF, Warburton W, Misri S, Aghajanian J and Hertzman C. 2006: Neonatal outcomes after prenatal exposure to selective serotonin reuptake inhibitor antidepressants and maternal depression using population-based linked health data. *Arch Gen Psychiatry*. 63(8):898-906.

364. Oberlander TF, Warburton W, Misri S, Aghajanian J and Hertzman C. 2007: Neonatal Risks of Maternal Treatment with Mood Stabilizers-Reply. *Arch Gen Psychiatry*. 64(7):867-868.
365. Odom DT, Dowell RD, Jacobsen ES, Nekludova L, Rolfe PA, Danford TW, Gifford DK, Fraenkel E, Bell GI and Young RA. 2006: Core transcriptional regulatory circuitry in human hepatocytes. *Mol Syst Biol*. 2:0017.
366. Odom DT, Zizlsperger N, Gordon DB, Bell GW, Rinaldi NJ, Murray HL, Volkert TL, Schreiber J, Rolfe PA, Gifford DK, Fraenkel E, Bell GI and Young RA. 2004: Control of pancreas and liver gene expression by HNF transcription factors. *Science*. 303:1378-1381.
367. Oesterheld JR. 1998: A review of developmental aspects of cytochrome P450. *J Child Adolesc Psycho pharmacol*. 8(3):161-74.
368. Okajima Y, Inaba N, Fukazawa I, Ota Y, Hirai Y, Sato N, Yamamoto G, Itahashi K, Kitada M and Kamataki T. 1993: Immunohistochemical and immuno electron microscopic study of cytochrome P-450 of human fetal livers (P-450HFLa): implications for an onco-fetoplacental enzyme. *Asia Oceania J Obstet Gynaecol*. 19:329-341.
369. Okey AB. 1990: Enzyme induction in the cytochrome P-450 system. *Pharmacol Ther*. 45:241-298.
370. Okuyama E, Tomita S, Takeuchi H and Ichikawa Y. 1996. A novel mutation in the cytochrome P450 (27) (CYP27) gene caused cerebrotendinous xanthomatosis in a Japanese family. *Journal of Lipid Research*. 37:631-639.
371. Olsen GD, Sommer KM, Wheeler PL, Boyea SR, Michelson SP and Cheek DBC. 1988: Accumulation and clearance of morphine-3- β -D-glucuronide in fetal lambs. *J Pharmacol Exp Ther*. 247:576-584.
372. Omiecinski CJ, Redlich CA and Costa P. 1990: Induction and developmental expression of cytochrome P4501A1 messenger RNA in rat and human tissue: detection by the polymerase chain reaction. *Cancer Res*. 50:4315-4321.
373. Omura T. 1999: Forty years of Cytochrome P450. *Biochem Biophys Res Commun*. 266:690-698.
374. Onishi S, Kawade N, Itoh S et al. 1979: Postnatal development of uridine diphosphate glucuronyltransferase activity towards bilirubin and 2-aminophenol in human liver. *Biochem J*. 184:705-7.
375. O'Shea D, Davis SN, Kim RB and Wilkinson GR. 1994: Effect of fasting and obesity in humans on the 6-hydroxylation of chlorzoxazone: a putative probe of CYP2E1 activity. *Clin Pharmacol Ther*. 56:359-367.

376. Ostrea EM, Jr MD, Mantaring JB and Silvestre MA. 2004: Drugs that affect the fetus and newborn infant via the placenta or breast milk. *Pediatric Clinics of North America*. 51(3):539-579.
377. Pacifici GM, Bencini C and Rane A. 1986: Acetyltransferase in humans: development and tissue distribution. *Pharmacology*. 32:283-291.
378. Pacifici GM, Franchi M, Colizzi C, Giuliani L and Rane A. 1988: Sulfotransferase in humans: development and tissue distribution. *Pharmacology*. 36:411-419.
379. Pacifici GM, Franchi M, Giuliani L and Rane A. 1989: Development of the glucuronyltransferase and sulphotransferase towards 2-naphthol in human fetus. *Dev Pharmacol Ther*. 14:108-114.
380. Pacifici GM, Kubrich M, Giuliani L, deVries M and Rane A. 1993: Sulphation and glucuronidation of ritodrine in human foetal and adult tissues. *Eur J Clin Pharmacol*. 44:259-264.
381. Pacifici GM, Norlin A and Rane A. 1981: Glutathione-S-transferase in human fetal liver. *Biochem Pharmacol*. 30:3367-3371.
382. Pacifici GM, Sawe J, Karger L and Rane A. 1982: Morphine glucuronidation in human fetal and adult liver. *Eur J Clin Pharmacol*. 22:553-558.
383. Pampori NA and Shapiro BH. 1999: Gender differences in the responsiveness of the sex-dependent isoforms of hepatic P450 to the feminine plasma growth hormone profile. *Endocrinology*. 140:1245-1254.
384. Pariente-Khayat A, Pons G, Rey E, Richard MO, D'Athis P, Moran C, Badoual J and Olive G. 1991: Caffeine acetylator phenotyping during maturation in infants. *Pediatr Res*. 29:492-495.
385. Pariente-Khayat A, Rey E, Gendrel D, Vauzelle-Kervroedan F, Cremier O, d'Athis P, Badoual J, Olive G and Pons G. 1997: Isoniazid acetylation metabolic ratio during maturation in children. *Clin Pharmacol Ther*. 62:377-383.
386. Park Y and Harris D. 2003: Construction and Assessment of Models of CYP2E1: Predictions of Metabolism from Docking, Molecular Dynamics, and Density Functional Theoretical Calculations. *J Med Chem*. 46(9):1645-60.
387. Parkinson A. 1996: Biotransformation of xenobiotics, in Casarett & Doull's Toxicology. *The Basic Science of Poisons* (Klaassen CD ed) pp 113-186, McGraw-Hill, New York.
388. Pasanen M, Pelkonen O, Kauppila A, Park SS, Friedman FK and Gelboin HV. 1987: Characterization of human fetal hepatic cytochrome P-450-associated 7-ethoxyresorufin O-deethylase and aryl hydrocarbon hydroxylase activities by monoclonal antibodies. *Dev Pharmacol Ther*. 10:125-132.

389. Pasanen M, Rannala Z, Tooming A, Sotaniemi EA, Pelkonen O and Rautio A. 1997: Hepatitis A impairs the function of human hepatic CYP2A6 in vivo. *Toxicology*. 123:177-184.
390. Pascussi JM, Drocourt L, Gerbal-Chaloin S, Fabre JM, Maurel P and Vilarem MJ. 2001: Dual effect of dexamethasone on CYP3A4 gene expression in human hepatocytes. Sequential role of glucocorticoid receptor and pregnane X receptor. *Eur J Biochem*. 268:6346-6358.
391. Patana AS, Kurkela M, Finel M and Goldman A. 2008: Mutation analysis in UGT1A9 suggests a relationship between substrate and catalytic residues in UDP-glucuronosyl transferases. *Protein Eng Des Sel*. 21:537-543.
392. Pavek P and Dvorak Z. 2008: Xenobiotic-induced transcriptional regulation of xenobiotic metabolizing enzymes of the cytochrome P450 superfamily in human extrahepatic tissues. *Curr Drug Metab*. 9:129-143.
393. Payne AH and Hales DB. 2004: Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocr Rev*. 25:947-970.
394. Payne AH and Youngblood GL. 1995: Regulation of expression of steroidogenic enzymes in Leydig cells. *Biol Reprod* 52:217-225.
395. Payne AH. 1990: Hormonal regulation of cytochrome P450 enzymes, cholesterol side-chain cleavage and 17 alpha-hydroxylase/C17-20 lyase in Leydig cells. *Biol Reprod*. 42:399-404.
396. Payne AK, Morgan SE, Gandolfi AJ and Brendel K. 1995: Biotransformation of sevoflurane by rat neonates liver slices. *Drug Metab Dispos*. 23:497-500.
397. Pelkonen O and Raunio H. 1995: Individual expression of carcinogen-metabolizing enzymes: cytochrome P4502A. *J Occup Environ Med*. 37:19-24.
398. Pelkonen O et al. 1997: The metabolism of coumarin. In: R. O'Kennedy and R.D. Thornes, Editors, *Coumarins: Biology, Applications and Mode of Action*, John Wiley & Sons Ltd pp. 67-92.
399. Pelkonen O, Raunio H, Rautio A, Maenpaa J and Lang MA. 1993: Coumarin 7-hydroxylase: characteristics and regulation in mouse and man. *J Irish Coll Phys Surg*. 22:24-28.
400. Pelkonen O, Rautio A, Raunio H and Pasanen M. 2000: CYP2A6: a human coumarin 7-hydroxylase. *Toxicology*. 144:139-147.
401. Pelkonen O. 1980: Biotransformation of xenobiotics in the fetus. *Pharmacol Ther*. 10:261-281.

402. Peng DR, Birgersson C, von Bahr C and Rane A. 1984: Polymorphic acetylation of 7-amino-clonazepam in human liver cytosol. *Pediatr Pharmacol.* 4:155-159.
403. Peng HM, Ding X and Coon MJ. 1993: Isolation and heterologous expression of cloned cDNAs for two rabbit nasal microsomal proteins, CYP2A10 and CYP2A11, that are related to nasal microsomal cytochrome P405 form a. *J Boil Chem.* 268:17253-60.
404. Perrot N, Nalpas B, Yang CS and Beaune PH. 1989: Modulation of cytochrome P450 isozymes in human liver by ethanol and drug intake. *Eur J Clin Invest.* 19:549-555.
405. Perry JS. 1981: The mammalian fetal membranes. *J Reprod Fertil.* 62:321-335.
406. Perucca E. 1987: Drug metabolism in pregnancy, infancy and childhood. *Pharmacol Ther.* 34:129-143.
407. Pichard L, Fabre I, Fabre G, Domergue J, Aubert BS, Mourad G and Maurel P. 1990. Cyclosporin A drug interactions: screening for inducers and inhibitors of cytochrome P-450 (cyclosporin A oxidase) in primary cultures of human hepatocytes and in liver microsomes. *Drug Metab Dispos.* 18:595-606.
408. Pineau T, Galtier P, Bonfils C, Derancourt J and Maurel P. 1990: Purification of a sheep liver cytochrome P-450 from the P450III_A gene subfamily. Its contribution to the N-dealkylation of veterinary drugs. *Biochem Pharmacol.* 39:901-909.
409. Pitarque M, Rodriguez-Antona C, Oscarson M and Ingelman-Sundberg M. 2005: Transcriptional Regulation of the Human CYP2A6 Gene. *Pharmacol and Exp Ther.* 313(2):814-822.
410. Pitarque M, von Richter O, Oke B, Berkkkan H, Oscarson M and Ingelman-Sundberg M. 2001: Identification of a single nucleotide polymorphism in the TATA box of the CYP2A6 gene: impairment of its promoter activity. *Biochem Biophys Res Commun.* 284:455-460.
411. Pitarque M, von Richter O, Rodriguez-Antona C, Wang J, Oscarson M and Ingelman-Sundberg M. 2004: A nicotine C-oxidase gene (CYP2A6) polymorphism important for promoter activity. *Hum Mutat.* 23:258-266.
412. Plessinger MA and Woods JR. 1993: Maternal, placental, and fetal pathophysiology of cocaine exposure during pregnancy. *Clin Obstet Gynecol.* 36:267-278.
413. Poland A and Knutson JC. 1982: *Annu Rev Pharmacol Toxicol.* 22:517-554.
414. Pons C, Dansette PM, Amar C, Jaouen M, Wolf CR, Gregeois J, Homberg JC and Mansuy D. 1991: *J Pharmacol Exp Ther.* 259:1328-34.
415. Porter TD and Coon MJ. 1991: *J Biol Chem.* 266:13469-13472.
416. Posner J et al. 1991: Comparison of lamotrigine pharmacokinetics in young and elderly healthy volunteers. *J Pharm Med* 1:121-128.

417. Postlind H, Axen E, Bergman T and Wikvall K. 1997: Cloning, structure and expression of a cDNA encoding vitamin D3 25-hydroxylase. *Biochem Biophys Res Commun.* 241:491-7.
418. Pritchard M, Fournel-Gigleux S, Siest G, Mackenzie P and Magdalou J. 1994: A recombinant phenobarbital-inducible rat liver UDP-glucuronosyltransferase (UDP-glucuronosyltransferase 2B1) stably expressed in V79 cells catalyzes the glucuronidation of morphine, phenols, and carboxylic acids. *Mol Pharmacol.* 45:42-50.
419. Prowler M and Kim DR. 2009: Perinatal Akathisia: Implications for Pharmacokinetic Changes During Pregnancy. *Am J Psychiatry.* 166:1296-1297.
420. Quattrochi LC, Vu T and Tukey RH. 1994: The Human CYP1A2 Gene and Induction by 3-ethylcholanthrene. *J Biol Chem.* 269:6949-6954.
421. Radominsky-Pandya AR, Czernik PJ, Little JM, Battaglia E and Mackenzie PI. 1999: Structural and functional studies of UDP-glucuronosyltransferase. *Drug Metab Rev.* 31:817-899.
422. Raija L, Lindberg P and Negishi M. 1989: Alteration of mouse cytochrome P450co substrate specificity by mutation of a single amino-acid residue. *Nature.* 339:632-634.
423. Ratanasavanh D, Beaune P, Morel F, Flinois JP, Guengerich FP and Guillouzo A. 1991: Intralobular distribution and quantitation of cytochrome P-450 enzymes in human liver as a function of age. *Hepatology.* 13:1142-1151.
424. Raucy JL and Carpenter SJ. 1993: The expression of xenobiotic-metabolizing cytochromes P450 in fetal tissues. *J Pharm Toxicol Methods.* 29:121-128.
425. Raucy JL, Lasker JM, Lieber CS and Black M. 1989: Acetaminophen activation by human liver cytochromes P450IIE1 and P450IA2. *Arch Biochem Biophys.* 271:270-283.
426. Raucy JL, Mueller L, Duan K, Allen SW, Strom S and Lasker JM. 2002: Expression and Induction of CYP2C P450 Enzymes in Primary Cultures of Human Hepatocytes. *Pharm Exp Ther.* 302(2):475-482.
427. Raunio H, Juvonen R, Pasanen M, Pelkonen O, Paakko P and Soini Y. 1997: Cytochrome P4502A6 expression in human hepatocellular carcinoma. *Hepatology.* 27(2):427-432.
428. Raunio H, Juvonen R, Pasanen M, Pelkonen O, Paakko P and Soini Y. 1998: Cytochrome P4502A6 (CYP2A6) expression in human hepatocellular carcinoma. *Hepatology.* 27:427-32.
429. Rendic S and Di Carlo FJ. 1997: Human cytochrome P450 enzymes: a status report summarizing their reactions, substrates, inducers, and inhibitors. *Drug Metab Rev.* 29:413-580.
430. Rendic S. 2000: Human P450 Metabolism Database. Gentest Corporation, http://www.gentest.com/human_p450_database.

431. Renton KW. 2001: Alteration of drug biotransformation and elimination during infection and inflammation. *Pharmacol Ther.* 92:147-163.
432. Renton KW. 2004: Cytochrome P450 regulation and drug biotransformation during inflammation and infection. *Curr Drug Metab.* 5:235-243.
433. Rettie AE, Wienkers LC, Gonzalez FJ, et al. 1994: Impaired (S)-warfarin metabolism catalysed by the R144C allelic variant of CYP2C9. *Pharmacogenetics.* 4:39-42.
434. Reynolds F and Knott C. 1989: Pharmacokinetics in pregnancy and placental drug transfer. *Oxf Rev Reprod Biol.* 11:389-449.
435. Richard K, Hume R, Kaptein E, Stanley EL, Visser TJ and Coughtrie MW. 2001: Sulfation of thyroid hormone and dopamine during human development: ontogeny of phenol sulfotransferases and arylsulfatase in liver, lung, and brain. *J Clin Endocrinol Metab.* 86:2734-42.
436. Ridderstrom M, Masimirembwa C, Trump-Kallmeyer S, Ahlefeldt M, Otter C and Andersson TB. 2000: Arginines 97 and 108 in CYP2C9 Are Important Determinants of the Catalytic Function. *Biochemical and Biophysical Research Communications.* 270(3):983-987.
437. Riddick DS, Lee C, Bhatena A and Timsit YE. 2003: The 2001 Veylien Henderson Award of the Society of Toxicology of Canada. Positive and negative transcriptional regulation of cytochromes P450 by polycyclic aromatic hydrocarbons. *Can J Physiol Pharmacol.* 81:59-77.
438. Riggs KW, Rurak DW and Taylor SM, et al. 1990: Fetal and maternal placental and nonplacental clearances of metoclopramide in chronically instrumented pregnant sheep. *J Pharm Sci.* 79:1056-1061.
439. Riggs KW, Rurak DW, Yoo SD, McErlane BA, Taylor SM, McMorland GH and Axelson JE. 1987: Drug accumulation in lung fluid of the fetal lamb after maternal or fetal administration. *Am J Obstet Gynecol.* 157(5):1286-91.
440. Ring JA, Ghabrial H, Ching MS, Shulkes A, Smallwood RA and Morgan DJ. 1998: Propranolol Elimination by Right and Left Fetal Liver: Studies in the Intact Isolated Perfused Fetal Sheep Liver. 284(2):535-541.
441. Ritter JK, Chen F, Sheen YY, Tran HM and Kimura S, et al. 1992: A novel complex locus UGT1 encodes human bilirubin, phenol, and other UDP-glucuronosyltransferase isozymes with identical carboxyl termini. *J Biol Chem.* 267:3257-61.
442. Rodriguez-Antona C, Donato MT, Pareja E, Gomez-Lechon MJ and Castell JV. 2001: Cytochrome P-450 mRNA expression in human liver and its relationship with enzyme activity. *Arch Biochem Biophys.* 393:308-315.

443. Rogers AS. 1994: The role of cytochrome P450 in developmental pharmacology. *J Adolesc Health*. 15:635-640.
444. Rollins DE, Glaumann H and Rane A. 1979: Acetaminophen: potentially toxic metabolite formed by human fetal and adult liver microsomes and isolated fetal liver cells. *Science*. 205:1414-16.
445. Rosenfeld CR. 1977: Distribution of cardiac output in ovine pregnancy. *Am J Physiol*. 1:H231-H235.
446. Rowland A et al. 2006: In vitro characterization of lamotrigine N2-glucuronidation and the lamotrigine-valproic acid interaction. *Drug Metab Dispos*. 34:1055-1062.
447. Rudolph AM. 1983: Hepatic and ductus venosus blood flows during fetal life. *Hepatology*. 3:163-174.
448. Runge D, Kohler C, Kostrubsky VE, Jager D, Lehmann T, Runge DM, May U, Stolz DB, Strom SC, Fleig WE, et al. 2000: Induction of cytochrome P450 (CYP) 1A1, CYP1A2 and CYP3A4 but not of CYP2C9, CYP2C19, multidrug resistance (MDR-1) and multidrug resistance associated protein (MRP-1) by prototypical inducers in human hepatocytes. *Biochem Biophys Res Commun*. 273:333-341.
449. Rurak DW, Wright MR and Axelson JE. 1991: Drug disposition and effects in the fetus. *J Dev Physiol*. 15:33-44.
450. Rurak DW, Yoo SD, Kwan E, Taylor SM, Riggs KW and Axelson JE. 1988: Effects of diphenhydramine in the fetal lamb after maternal or fetal administration. *J Pharmacol Exp Ther*. 247:271-278.
451. Saarikoski ST, Rivera SP, Hankinson O and Husgafvel-Pursiainen K. 2005: CYP2S1: A short review. *Toxicol Appl Pharmacol*. 207:62-69.
452. Sachdeva P, Patel BG and Patel BK. 2009: Drug use in pregnancy; a point to ponder. *Indian Journal of pharmaceutical sciences*. 71(1):1-7.
453. Sachse C, Brockmoller J, Bauer S and Roots I. 1997: Cytochrome P450 2D6 variants in a Caucasian population: allele frequencies and phenotypic consequences. *Am J Hum Genet*. 60:284-295.
454. Sadeque AJ, Fisher MB, Korzekwa KR, Gonzalez FJ, Rettie AE. 1997: Human CYP2C9 and CYP2A6 mediate formation of the hepatotoxin 4-ene-valproic acid. *J Pharmacol Exp Ther*. 283:698-703.
455. Sakamoto K, Kirita S, Baba T, Nakamura Y, Yamazoe Y, Kato R, Takanaka A and Matsubara T. 1995: A new CYP450 form belonging to the CYP2D in dog liver microsomes: purification, cDNA cloning, and enzyme characterization. *Arch Biochem Biophys*. 319(2):372-82.

456. Sakuma T, Shimojima T, Miwa K and Kamataki T. 2004: Cloning CYP2D21 and CYP3A22 cDNAs from liver of miniature pigs. *Drug Metab Disp.* 32:376-378.
457. Savas U, Bhattacharyya KK, Christou M, Alexander DL and Jefcoate CR. 1994: Mouse cytochrome P-450EF, representative of a new 1B subfamily of cytochrome P-450s. Cloning, sequence determination, and tissue expression. *J Biol Chem.* 269:14905-11.
458. Schmidt JV and Bradfield CA. 1996: Ah receptor signaling pathways. *Annu Rev Cell Dev Biol.* 12:55-89.
459. Schneider H, Sodha RJ, Progler M and Young MPA. 1985: Permeability of the human placenta for hydrophilic substances studied in the isolated dually in vitro perfused lobe. *Contrib Gynecol Obstet.* 13:98-103.
460. Schuetz EG and Guzelian PS. 1984: Induction of cytochrome P-450 by glucocorticoids in rat liver. II. Evidence that glucocorticoids regulate induction of cytochrome P-450 by a nonclassical receptor mechanism. *J Biol Chem.* 259:2007-2012.
461. Schuetz EG, Brimer C and Schuetz JD. 1998: Environmental xenobiotics and the antihormones cyproterone acetate and spironolactone use the nuclear hormone pregnanolone X receptor to activate the CYP3A23 hormone response element. *Mol pharmacol.* 54:1113-1117.
462. Schuetz JD, Beach DL and Guzelian PS. 1994: Selective expression of cytochrome P450 CYP3A mRNAs in embryonic and adult human liver. *Pharmacogenetics.* 4:11-20.
463. Senay C, Ouzzine M, Battaglia E, Pless D, Cano V, Burchell B, Radominska A, Magdalou J and Fournel-Gigleux S. 1997: Arginine 52 and histidine 54 located in a conserved amino-terminal hydrophobic region (LX2-R52-G-H54-X3-V-L) are important amino acids for the functional and structural integrity of the human liver UDP-glucuronosyltransferase UGT1*6. *Mol. Pharmacol.* 51:406-413.
464. Sharief MK and Hentges R. 1991: Association between tumor necrosis factor- α and disease progression in patients with multiple sclerosis. *N Engl J Med.* 325:467-472.
465. Shelby MK, Cherrington NJ, Vansell NR and Klaassen CD. 2003: Tissue mRNA Expression of the Rat UDP-Glucuronosyltransferase Gene Family. *Drug metabolism and Disposition.* 31(3):326-333.
466. Shimada T, Gillam EMJ, Sandhu P, Guo Z, Tukey RH and Guengerich FP. 1994a: Activation of procarcinogens by human cytochrome P450 enzymes expressed in *Escherichia coli*. Simplified bacterial systems for genotoxicity assays. *Carcinogenesis.* 15(II):2523-29.
467. Shimada T, Inoue K, Suzuki Y, Kawai T, Azuma E, Nakajima T, Shindo M, Kurose K, Sugie A, Yamagishi Y, Fujii-Kuriyama Y and Hashimoto M. 2002: Aryl hydrocarbon receptor dependent induction of liver and lung cytochromes P450 1A1, 1A2, and 1B1 by polycyclic aromatic hydrocarbons and poly chlorinated biphenyls in genetically engineered C57BL/6J mice. *Carcinogenesis.* 23:1199-1207.

468. Shimada T, Mimura M, Nakamura KIS-I, Oda H and Yamazaki SOH. 1997: Cytochrome P450-dependent drug oxidation activities in liver microsomes of various animal species including rats, guinea pigs, dogs, monkeys, and humans. *Arch Toxicol.* 71:401-408.
469. Shimada T, Sugie A, Shindo M, Nakajima T, Azuma E, Hashimoto M and Inoue K. 2003: Tissue-specific induction of cytochromes P450 1A1 and 1B1 by polycyclic aromatic hydrocarbons and polychlorinated biphenyls in engineered C57BL/6J mice of arylhydrocarbon receptor gene. *Toxicol Appl Pharmacol.* 187:1-10.
470. Shimada T, Yamazaki H, Mimura M, Inui Y and Guengerich FP. 1994b: Interindividual variations in human liver cytochrome P450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J Pharmacol Exp Ther.* 270:414-423.
471. Shimada T, Yamazaki H, Mimura M, Wakamiya N, Ueng Y-F, Guengerich FP and Inui Y. 1996: Characterization of microsomal cytochrome P450 enzymes involved in the oxidation of xenobiotic chemicals in human fetal liver and adult lungs. *Drug Metab Disp.* 25:512-22.
472. Shiratani H, Katoh M, Nakajima M and Yokoi T. 2008: Species Differences in UDP-Glucuronosyltransferase Activities in Mice and Rats. *Drug Metab. Dispos.* 36:1745-1752.
473. Sivapathasundaram S, Magnisali P, Coldham NG, Howells LC, Sauer MJ and Ioannides C. 2001: A study of the expression of the xenobiotic-metabolising cytochrome P450 proteins and of testosterone metabolism in bovine liver. *Biochem Pharmacol.* 62(5):635-645.
474. Skaanild MT and Friis C. 2005: Porcine CYP2A polymorphisms and activity. *Basic Clin Pharmacol Toxicol.* 97:115-121.
475. Sladek FM, Zhong W, Lai E and Jr JED. 1990: Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. *Genes Dev.* 4:2353-65.
476. Smith CAD, Gough AC, Leigh PN, Summers BA, Harding AE, Maranganore DM, Sturman SG, Schapira AHV, Williams AC, Spurr NK and Wolf CR. 1992: Debrisoquine hydroxylase gene polymorphism and susceptibility to Parkinson's disease. *Lancet.* 339:1375-1377.
477. Smith FG and Lumbers ER. 1989: Comparison of renal function in term fetal sheep and newborn lambs. *Biol Neonate.* 55:309-316.
478. Sonnier M and Cresteil T. 1998: Delayed ontogenesis of CYP1A2 in the human liver. *Eur J Biochem.* 251:893-898.
479. Sotaniemi EA, Rautio A, Backstrom M, Arvela P and Pelkonen O. 1995: CYP3A4 and CYP2A6 activities marked by the metabolism of lignocaine and coumarin in patients with liver and kidney diseases and epileptic patients. *Br J Clin Pharmacol.* 39:71-76.
480. Steven JC and Wrighton SA. 1993: Interaction of the enantiomers of fluoxetine and norfluoxetine with human liver cytochrom P450. *J Pharmacol Exp Ther.* 226:964-971.

481. Stevens JC, Hines RN and Gu C. 2003: Developmental expression of the major human hepatic CYP3 enzymes. *J Pharmacol Exp Ther.* 307:573-582.
482. Stone AN, Mackenzie PI, Galetin A, Houston JB and Miners JO. 2003: Isoform selectivity and kinetics of morphine and 3- and 6-glucuronidation by human and UDP glucuronosyl transferases: evidence for atypical glucuronidation kinetics by UGT2B7. *Drug Metab Dispos.* 31:1086-9.
483. Strange RC, Howie AF, Hume R, Matharoo B, Bell J, Hiley C, Jones P and Beckett GJ. 1989: The development expression of alpha-, mu- and pi-class glutathione S-transferases in human liver. *Biochim Biophys Acta.* 993:186-190.
484. Strassburg CP, Kneip S, Topp J, et al. 2000: Polymorphic gene expression and inter individual variation of UDP-glucuronosyltransferase activity in human small intestine. *J Biol Chem.* 275:36164-71.
485. Strassburg CP, Manns MP and Tukey RH. 1997a: Differential down-regulation of the UDP-glucuronosyltransferase 1A locus is an early event in human liver and biliary cancer. *Cancer Res.* 57:2979-85.
486. Strassburg CP, Manns MP and Tukey RH. 1998: Expression of the UDP-glucuronosyltransferase 1A locus in human colon. Identification and characterization of the novel extrahepatic UGT1A8. *J Biol Chem.* 273:8719-26.
487. Strassburg CP, Nguyen N, Manns MP, et al. 1999a: UDP-glucuronosyltransferase activity in human liver and colon. *Gastroenterology.* 116:149-60.
488. Strassburg CP, Oldhafer K and Manns MP, et al. 1997b: Differential expression of the UGT1A locus in human liver, biliary, and gastric tissue: identification of UGT1A7 and UGT1A10 transcripts in extrahepatic tissue. *Mol Pharmacol.* 52:212-20.
489. Strassburg CP, Strassburg A, Kneip S, Barut A, Tukey RH, Rodeck B and Manns MP. 2002: Developmental aspects of human hepatic drug glucuronidation in young children and adults. *Gut.* 50(2):259-265.
490. Strassburg CP, Strassburg A, Nguyen N, et al. 1999b: Regulation and function of family 1 and family 2 UDP-glucuronosyltransferase genes (UGT1A, UGT2B) in human oesophagus. *Biochem J.* 338:489-98.
491. Strom SC, Pizarov LA, Dorko K, Thompson MT, Schuetz JD and Schuetz EG. 1996: Use of human hepatocytes to study P450 gene induction. *Methods Enzymol.* 272:388-401.
492. Stulc J. 1996: Letter to the editor: Transfer pathways across the sheep placenta. *Placenta.* 17:377-379.
493. Su T, Sheng JJ, Lipinskas TW and Ding X. 1996: Expression of CYP2A genes in rodent and human nasal mucosa. *Drug Metab. Dispos.* 24:884-890.

494. Sueyoshi T, Kawamoto T, Zelko I, Honkakoski P and Negishi M. 1999: The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. *J Biol Chem.* 274:6043-6046.
495. Sullivan-Klose TH, Ghanayem BI, Bell DA, et al. 1996: The role of the CYP2C9-Leu359 allelic variant in the tolbutamide polymorphism. *Pharmacogenetics.* 6:341-349.
496. Sutter TR, Tang YM, Hayes CL, Wo Y-Y, Jabs EW, Li X, Yin H, Cody CW and Greenlee WF. 1994: Complete cDNA sequence of a human dioxin-inducible mRNA identifies a new gene subfamily of cytochrome P450 that maps to chromosome 2. *J Biol Chem.* 269:13092-99.
497. Szeto HH, Umans JG and McFarland J. 1982: A comparison of morphine and methadone disposition in the maternal-fetal unit. *Am J Obstet Gynecol.* 143(6):700-6.
498. Szeto HH: Pharmacokinetic studies in experimental animals. 1993: In Reynolds F (ed): *Effects on the Baby of Maternal Analgesia and Anaesthesia*, ed 2. London, WB Saunders. 29-45.
499. Szorady I, Santa A and Veress I. 1987: Drug acetylator phenotypes in newborn infants. *Biol Res Pregnancy Perinatol.* 8:23-25.
500. Szotakova B, Baliharova V, Lamka J, Nozinova E, Wsol V, Velik J, Machala M, Neca J, Soucek P, Susova S and Skalova L. 2004: Comparison of in vitro activities of biotransformation enzymes in pig, cattle, goat and sheep. *Research in Veterinary Science,* 76(1):43-51.
501. Takahashi H, Ishikawa S, Nomoto S et al. 2000: Developmental changes in pharmacokinetics and pharmacodynamics of warfarin enantiomers in Japanese children. *Clin Pharmacol Ther.* 68:541-555.
502. Takahashi T, Lasker JM, Rosman AS and Lieber CS. 1993: Induction of cytochrome P-4502E1 in the human liver by ethanol is caused by a corresponding increase in encoding messenger RNA. *Hepatology.* 17:236-245.
503. Tateishi T, Nakura H, Asoh M, Watanabe M, Tanaka M, Kumai T, Takashima S, Imaoka S, Funae Y, Yabusaki Y, Kamataki T and Kobayashi S. 1997: A comparison of hepatic cytochrome P450 protein expression between infancy and postinfancy. *Life Sci.* 61:2567-74.
504. Tephly TR and Burchell B. 1990: UDP-glucuronosyltransferases: a family of detoxifying enzymes. *TIPS.* 11:276-280.
505. Thangavel C, Dhir RN, Volgin DV and Shapiro BH. 2007: Sex-dependent expression of CYP2C11 in spleen, thymus and bone marrow regulated by growth hormone. *Biochem Pharmacol.* 74:1476-1484.
506. Thummel KE, Kharasch ED, Podoll T and Kunze K. 1993: Human liver microsomal enflurane defluorination catalyzed by cytochrome P4502E1. *Drug Met Dispos.* 21:350-357.

507. Tilloy-Ellul A, Raffalli-Mathieu F and Lang MA. 1999: Analysis of RNA-protein interactions of mouse liver cytochrome P450A5 mRNA. *Biochem J.* 339:695-703.
508. Tirone RG and Kim RB. 2005: Nuclear Receptors and Drug Disposition Gene Regulation. *J of Pharm Sc.* 94(6):1169-86.
509. Tirone RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, Parviz F, Duncan SA, Inoue Y, Gonzalez F J, Schuetz EG and Kim RB. 2003: The orphan nuclear receptor HNF4alpha determines PXR and CAR mediated xenobiotic induction of CYP3A4. *Nat Med.* 9:220-224.
510. Toide K, Itoh S, Nagasaka Y, Yanagimoto T and Kamataki T. 1997: Gene structure of mouse Cyp3a11: Evidence for an enhancer element within its 5' flanking sequences. *Arch Biochem Biophys.* 338:43-49.
511. Topcu Z, Chiba I, Fujieda M, et al. 2002: CYP2A6 gene deletion reduces oral cancer risk in betel quid chewers in Sri Lanka. *Carcinogenesis.* 23:595-598.
512. Towbin H, Staehelin T and Gordon J. 1979: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl AcadSci USA.* 76:4350-4354.
513. Treluyer J-M, Benech H, Colin I, Pruvost A, Cheron G and Cresteil T. 2000: Ontogenesis of CYP2C dependant arachidonic acid metabolism in the human liver: Relationship with sudden infant death syndrome. *Pediatr Res.* 47:677-683.
514. Treluyer J-M, Cheron G, Sonnier M and Cresteil T. 1996: Cytochrome P450 expression in sudden infant death syndrome. *Biochem Pharmacol.* 52:497-504.
515. Treluyer J-M, Gueret G, Cheron G, Sonnier M and Cresteil T. 1997: Developmental expression of CYP2C and CYP2C dependant activities in the human liver: Invivo/invitro correlation and inducibility. *Pharmacogenetics.* 7:441-452.
516. Treluyer J-M, Jacqz-Augrain E, Alvarez F and Cresteil T. 1991: Expression of CYP2D6 in developing human liver. *Eur J Biochem.* 200:583-588.
517. Tsao C-C, Wester MR, Ghanayem B, Coulter SJ, Chanas B, Johnson EF and Goldstein JA. 2001: Identification of Human CYP2C19 Residues that Confer S-Mephenytoin 4'-Hydroxylation Activity to CYP2C9. *Biochemistry.* 40(7):1937-1944.
518. Tsuneoka Y, Matsuo Y, Higuchi R and Ichikawa Y. 1992: Characterization of the cytochrome P-450IID subfamily in bovine liver. Nucleotide sequences and microheterogeneity. *Eur J Biochem.* 208(3):739-746.
519. Tsuneoka Y, Matsuo Y, Okuyama E, et al. 1996: Genetic analysis of the cytochrome P-450IIC18 (CYP2C18) gene and a novel member of the CYP2C subfamily. *FEBS Lett.* 384: 281-284.

520. Tukey RH and Strassburg CP. 2000: Human UDP-glucuronosyltransferases: metabolism, expression, and disease. *Annu Rev Pharmacol Toxicol.* 40:581-616.
521. Tukey RH, Okino S, Barnes H, Griffin KJ and Johnson EF. 1985: Multiple gene-like sequences related to the rabbit hepatic progesterone 21-hydroxylase cytochrome P-4501. *J Biol Chem.* 260:13347-54.
522. Ujjin P, Satarug S, Vanavanitkun Y, Daigo S, Ariyoshi N, Yamazaki H, Reilly PE, Moore MR and Kamataki T. 2002: Variation in coumarin 7-hydroxylase activity associated with genetic polymorphism of cytochrome P450 2A6 and the body status of iron stores in adult Thai males and females. *Pharmacogenetics.* 12:241-249.
523. Ulvila J, Arpiainen S, Pelkonen O, Aida K, Sueyoshi T, Negishi M and Hakkola J. 2004: Regulation of Cyp2a5 transcription in mouse primary hepatocytes: roles of hepatocyte nuclear factor 4 and nuclear factor I. *J Pharmacol Exp Ther.* 381:887-894.
524. Unadkat JD, Dahlin A and Vijay S. 2004: Placental Drug Transporters. *Current Drug Metabolism,* 5(1):125-131(7).
525. Urquhart BL, Tirona RG and Kim RB. 2007: Nuclear receptors and the regulation of drug metabolizing enzymes and drug transporters: implications for inter-individual variability in response to drugs. *J Clin Pharmacol.* 47:566-578.
526. US Food and Drug Administration. 2006: Questions and answers on antidepressant use in children, adolescents, and adults. Available at: www.fda.gov/cder/drug/antidepressants/Q&A_antidepressants.htm.
527. van den Anker JN. 1996: Pharmacokinetics and renal function in preterm infants. *Acta Paediatr.* 85:1383-1389.
528. Vieira I, Sonnier M and Cresteil T. 1996: Development of expression of CYP2E1 in the human liver. Hypermethylation control of gene expression during the neonatal period. *Eur J Biochem.* 238:476-483.
529. Vitale N, De Feo M, De Santo LS, Pollice A, Tedesco N and Cotrufo M. 1999: Dose-dependent fetal complications of warfarin in pregnant women with mechanical heart valves. *J Am Coll Cardiol.* 33:1637-41.
530. von Meyerinck L, Coffman BL, Green MD, Kirkpatrick RB, Schmoldt A and Tephly TR. 1985: Separation, purification, and characterization of digitoxigenin-monodigitoxoside UDP-glucuronosyl transferase activity. *Drug Metab Dispos.* 13(6):700-704.
531. von Moltke LL, Greenblatt DJ, Duan SX, Schmider J, Wright CE, Harmatz JS and Shader RI. 1997: Human cytochromes mediating *N*-demethylation of fluoxetine in vitro. *Psychopharmacol.* 132:402-407.

532. von Richter O, Pitarque M, Rodriguez-Antona C, Testa A, Mantovani R, Oscarson M and Ingelman-Sundberg M. 2004: Polymorphic NF-Y dependent regulation of human nicotine C-oxidase (CYP2A6). *Pharmacogenetics*. 14:369-379.
533. Vos RM and Van Bladeren PJ. 1990: Glutathione S-transferases in relation to their role in the biotransformation of xenobiotics. *Chem Biol Interact*. 75:241-265.
534. Wada Y, Mitsuda M, Ishihara Y, Watanabe M, Iwasaki M and Asahi S. 2008: Important Amino Acid Residues that Confer CYP2C19 Selective Activity to CYP2C9. *Journal of Biochemistry*. 144(3):323-333.
535. Waehre T, Yndestad A, Smith C, Haug T, Tunheim SH, Gullestad L, Froland SS, Semb AG, Aukrust P and Damas JK. 2004: Increased expression of interleukin-1 in coronary artery disease with downregulatory effects of HMG-CoA reductase inhibitors. *Circulation*. 109:1966-1972.
536. Walters E, Buchheit K and Maruniak JA. 1993: Olfactory cytochrome P450 immuno reactivity in mice is altered by dichlobenil but preserved by metyrapone. *Toxicology*. 81:113-122.
537. Wang B, Yang L-P, Zhang X-Z, Huang S-Q, Bartlam M and Zhou S-F. 2009: New insights into the structural characteristics and functional relevance of the human cytochrome P450 2D6 enzyme. *Drug Metabolism Reviews*. 41(4):573-643.
538. Wang H and Strobel HW. 1997: Regulation of CYP3A9 gene expression by estrogen and catalytic studies using cytochrome P450 3A9 expressed in *Escherichia coli*. *Arch Biochem Biophys*. 344:365-372.
539. Wang LH, Rudolph AM and Benet LZ. 1985: Distribution and fate of acetaminophen conjugates in fetal lambs in utero. *J Pharmacol Exp Ther*. 235:302-307.
540. Wang LH, Rudolph AM and Benet LZ. 1986b: Pharmacokinetic studies of the disposition of acetaminophen in the sheep maternal-placental- fetal unit. *J Pharmacol Exp Ther*. 238:198-205.
541. Wang LH, Rudolph AM and Benet LZ. 1990: Comparative study of acetaminophen disposition in sheep at three developmental stages: The fetal, neonatal and adult periods. *Dev Pharmacol Ther*. 14:161-179.
542. Wang LH, Zakim D, Rudolph AM and Benet LZ. 1986a: Developmental alterations in hepatic UDP-glucuronosyltransferase. A comparison of the kinetic properties of enzymes from adult sheep and fetal lambs. *Biochem Pharmacol*. 35:3065-3070.
543. Wang Z, Hall SD, Maya JF, Li L, Asghar A and Gorski JC. 2003: Diabetes mellitus increases the in vivo activity of cytochrome P450 2E1 in humans. *Br J Clin Pharmacol*. 55: 77-85.

544. Warner A. 1986: Drug used in the neonate: Interrelationship of pharmacokinetics, toxicity and biochemical maturity. *Clin Chem.* 32:721-727.
545. Watanabe J, Asaka Y and Kanamura S. 1993: Postnatal development and sublobular distribution of cytochrome P-450 in rat liver: a microphotometric study. *J Histochem Cytochem.* 41:397-400.
546. Watkins PB, Wrighton SA, Maurel P, Schuetz EG, Mendez-Picon G, Parker GA and Guzelian PS. 1985: Identification of an inducible form of cytochrome P-450 in human liver. *Proc Natl Acad Sci USA.* 82:6310-14.
547. Watt AJ, Garrison WD and Duncan SA. 2003: A central regulator of hepatocyte differentiation and function. *Hepatology.* 37:1249-1253.
548. Waxman DJ, Dannan GA and Guengerich FP. 1985: Regulation of rat hepatic cytochrome P450: age dependant expression, hormonal imprinting, and xenobiotic inducibility of sex specific isoenzymes. *Biochemistry.* 24:4409-13.
549. Weatherill PJ and Burchell B. 1980: The separation and purification of rat liver UDP-glucuronyltransferase activities towards testosterone and oestrone. *Biochem J.* 189(2):377-380.
550. Wei P, Zhang J, Dowhan DH, Han Y and Moore DD. 2002: Specific and overlapping functions of the nuclear hormone receptors CAR and PXR in xenobiotic response. *Pharmacogenomics J.* 2:117-126.
551. Wester MR, Lasker JM, Johnson EF and Raucy JL. 2000: CYP2C19 participates in tolbutamide hydroxylation by human liver microsomes. *Drug Metab Dispos.* 28:354-359.
552. Wester MR, Yano JK, Schoch GA, Yang C, Griffin KJ, Stout CD and Johnson EF. 2004: The structure of human cytochrome P450 2C9 complexed with flurbiprofen at 2.0-Å resolution. *J Biol Chem.* 279:35630-35637.
553. Whitlock Jr JP. 1993: Mechanistic aspects of dioxin action. *Chem Res Toxicol.* 6:754-763.
554. Whitlock Jr JP. 1999: Induction of cytochrome P4501A1. *Annu Rev Pharmacol Toxicol.* 39:103-125.
555. Widdowson EM. 1974: Changes in body proportions and composition during growth. In: JA Davies and J. Dobbing, Editors, *Scientific Foundations of Pediatrics*, Heinemann Medical Books Ltd, London pp. 153-163.
556. Wilson JW, Groat CS and Leduc EH. 1963: Histogenesis of the liver. *Ann N Y Acad Sci.* 111:8-44.
557. Wishart GJ. 1978: Functional heterogeneity of UDP-glucuronosyltransferase as indicated by its differential development and inducibility by glucocorticoids. Demonstration of two groups within the enzyme's activity towards twelve substrates. *Biochem J.* 174:485-489.

558. Wong G, Itakura T, Kawajiri K, Skow L and Negishi M. 1989: Gene family of male-specific testosterone 16 alpha-hydroxylase (C-P-450(16 alpha)) in mice. Organization, differential regulation, and chromosome localization. *J Biol Chem.* 264(5):2920-7.
559. Wong H, Kumar S, Riggs KW and Rurak DW. 2000a Pharmacokinetics and renal excretion of diphenhydramine and its metabolites, diphenhymethoxy acetic acid and diphenhydramine-N-oxyde, in developing lambs. *J Pharm Sci.* 89:1362-1370.
560. Wong H, Kumar S, Rurak DW, Kwan E, Abbott FS and Riggs KW. 2000b Ontogeny of valproic acid disposition and metabolism: A developmental study in postnatal lambs and adult sheep. *Drug Metab Dispos.* 28:912-919.
561. Wong H, Rurak DW, Kumar S, Kwan E, Abbott FS and Riggs KW. 2001: Dose dependent pharmacokinetics and metabolism of valproic acid in newborn lambs and adult sheep. *Drug Metab Dispos.* 29:664-675.
562. Wooster R, Ebner T, Sutherland L, et al. 1993: Drug and xenobiotic glucuronidation catalysed by cloned human liver UDP-glucuronosyltransferases stably expressed in tissue culture cell lines. *Toxicology.* 82:119-29.
563. Wooster R, Sutherland L, Ebner T, Clarke D, Da Cruze Silva O and Burchell B. 1991. Cloning and stable expression of a new member of the human liver phenol/bilirubin: UDP-glucuronosyltransferase cDNA family. *Biochem J.* 278:465-469.
564. Wortham M, Czerwinski M, He L, Parkinson A and Wan YY. 2007: Expression of Constitutive Androstane Receptor, Hepatic Nuclear Factor 4 α , and P450 Oxidoreductase Genes Determines Interindividual Variability in Basal Expression and Activity of a Broad Scope of Xenobiotic Metabolism Genes in the Human Liver. *Drug Metab Dispos.* 35(9):1700-10.
565. Wright MR, Rurak DW, van der Wayde MP, Taylor SM and Axelson JE. 1991: Clearance and disposition of ritodrine in the fluid compartments of the fetal lamb during and after constant rate fetal intravenous infusion. *J Pharmacol Exp Ther.* 258:897-902.
566. Wrighton SA and Stevens JC. 1992: The human hepatic Cytochromes P450 involved in drug metabolism. *Crit Rev Toxicol.* 22:1-21.
567. Wrighton SA and Vandenbranden M. 1989: Isolation and characterization of human fetal liver cytochrome P450HLp2: a third member of the P450III gene family. *Arch Biochem Biophys.* 268:144-151.
568. Wrighton SA, Brian WR, Sari M-A, Iwasaki M, Guengerich FP, Raucy JL, Molowa DT, and VandenBranden M. 1990: Studies on the expression and metabolic capabilities of human liver cytochrome P450IIIA5 (HLp3). *Mol Pharmacol.* 38:207-213.
569. Wrighton SA, Schuetz EG, Watkins PB, Maurel P, Barwick J, Bailey BS, Hartle HT, Young B and Guzelian P. 1985: Demonstration in multiple species of inducible hepatic

- cytochromes P-450 and their mRNAs related to the glucocorticoid-inducible cytochrome P-450 of the rat. *Mol Pharmacol.* 28:312-321.
570. Yamazaki H, Inui Y, Wrighton SA, Guengerich FP, Shimada T. 1995: Procarcinogen activation by cytochrome P450 3A4 and 3A5 expressed in *Escherichia coli* and by human liver microsomes. *Carcinogenesis.* 16(9):2167-70.
571. Yamazoe Y, Murayama N, Shimada M and Kato R. 1989: Thyroid hormone suppression of hepatic levels of phenobarbital-inducible P-450b and P-450e and other neonatal P-450s in hypophysectomized rats. *Biochem Biophys Res Commun.* 160:609-614.
572. Yanagimoto T, Itoh S, Sawada M and Kamataki T. 1997: Mouse cytochrome P450 (Cyp3a11): Predominant expression in liver and capacity to activate aflatoxin B1. *Arch Biochem Biophys.* 340:215-218.
573. Yang H-YL, Namkung MJ and Juchau MR. 1995: Expression of functional cytochrome P4501A1 in human embryonic hepatic tissues during organogenesis. *Biochem Pharmacol.* 49:717-726.
574. Yano JK, Hsu M-H, Griffin KJ, Stout CD and Johnson EF. 2005: Structures of human microsomal cytochrome P450 2A6 complexed with coumarin and methoxsalen. *Nature Structural and Molecular Biology.* 12:822-823.
575. Yasar U, Eliasson E, Forslund-Bergengren C, Tybring G, Gadd M, Sjöqvist F and Dahl M-L. 2001: The role of CYP2C9 genotype in the metabolism of diclofenac in vivo and in vitro. *Eur J Clin Pharmacol.* 57:729-735.
576. Yeleswaram K, Rurak DW, Kwan E, Hall C, Doroudian A, Wright MR, Abbott FS and Axelson JE. 1993: Trans-placental and non-placental clearances, metabolism and pharmacodynamics of labetalol in the fetal lamb after direct intravenous administration. *J Pharmacol Exp Ther.* 267:425-431.
577. Yokomori N, Nishio K, Aida K and Negishi M. 1997: Transcriptional regulation by HNF-4 of the steroid 15alpha-hydroxylase P450 (Cyp2a-4) gene in mouse liver. *J Steroid Biochem Mol Biol.* 62:307-314.
578. Yokota H, Iwano H, Endo M, Kobayashi T, Inoue H, Ikushiro S and Yuasa A. 1999: Glucuronidation of the environmental bisphenol-A by an isoform of UDP-glucuronosyl transferase, UGT2B1, in the rat liver. *Biochem J.* 340:405-409.
579. Young AM, Allen CE and Audus KL. 2003: Efflux transporters of the human placenta. *Adv Drug Deliv Rev.* 55:125-132.
580. Yu D, Gordon JD, Zheng J, Panesar SK, Riggs KW, Rurak DW and Abbott FS. 1995: Determination of valproic acid and its metabolites using gas chromatography with mass-selective detection: application to serum and urine samples from sheep. *J Chromatogr B Biomed Appl.* 666:269-281.

581. Yu H-Y, Shen Y-Z, Sugiyama Y and Hanano M. 1987: Dose-dependent pharmacokinetics of valproate in guinea pigs of different ages. *Epilepsia*. 28:680-687.
582. Yuan X, Ta TC, Lin M, Evans JR, Dong Y, Bolotin E, Sherman MA, Forman BM and Sladek FM. 2009: Identification of an Endogenous Ligand Bound to a Native Orphan Nuclear Receptor. *PLoS ONE*. 4(5):e5609. doi: 10.1371/journal.pone.0005609.
583. Yun CH, Shimada T and Guengerich FP. 1991: Purification and characterization of human liver microsomal cytochrome P-450 2A6. *Mol Pharmacol*. 40:679-685.
584. Zaya MJ, Hines RN, and Stevens JC. 2006: Epirubicin glucuronidation and UGT2B7 developmental expression, *Drug Metab. Dispos*. 34:2097-2101.
585. Zeilmaker WM, van 't Klooster GA, Gremmels-Gehrmann JF, van Miert AS and Horbach GJ. 1994: cDNA and deduced amino acid sequence of a dwarf goat liver cytochrome P450 fragment belonging to the CYP2C gene subfamily. *Biochem Biophys Res Commun*. 200(1):120-125.
586. Zhou HH, Anthony LB, Wood AJ and Wilkinson GR. 1990: Induction of polymorphic 4-hydroxylation of S-mephenytoin by rifampicin. *Br J Clin Pharmacol*. 30:471-475.
587. Zink J and Van Petten GR. 1980: Time course of closure of the ductus venosus in the newborn lamb. *Pediatr Res*. 14(1):1-3.

APPENDICES

Appendix A: Animal care certificate

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THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A07-0302	
Investigator or Course Director: <u>Dan W. Rurak</u>	
Department: Obstetrics & Gynaecology	
Animals:	
<div style="border: 1px solid black; display: inline-block; padding: 2px;">Sheep 10</div>	
Start Date:	September 1, 2007
Approval Date:	August 19, 2009
Funding Sources:	
Funding Agency:	Canadian Institutes of Health Research (CIHR)
Funding Title:	Fetal Programming of Postnatal Drug Disposition
Unfunded title:	Fetal Programming of Postnatal Drug Disposition

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

Office of Research Services and Administration
102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093

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