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This thesis is dedicated to my father and my late grandmother,

Harun Aliwarga (Papi) and Kuiheni Wongswarga (Ama)
ABSTRACT

The determination of pharmacokinetic parameters is crucial both for clinical studies and early in the drug discovery and development. This thesis describes a clinical pharmacokinetics study of one anticonvulsant drug, i.e. phenytoin (PHT) and the early screening study of potential chemopreventive agent for prostate cancer.

PHT is extensively bound to plasma proteins, is excreted from the body as oxidative metabolites in the urine, and exhibits a non-linear pharmacokinetics profile. In this study, stable-labeled PHT was given either intravenously or intramuscularly. The simultaneous administration of oral and IV PHT enables a direct determination of the pharmacokinetic parameters of clearance, volume, half-life, and absolute oral bioavailability. Urine samples from epilepsy patients who were on maintenance therapy of PHT were collected from 0-12 hours and 12-24 hours after a single daily dose to measure the two principal PHT urinary metabolites, 5-(4-hydroxyphenyl)-5-phenylhydantoin (p-HPPH) and 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin (DHD). An isocratic HPLC-NI-APCI-MS method was used to quantify metabolites in urine. A weak, but significant, Spearman Rank Correlation was observed between the total urinary metabolites recovered and the oral bioavailability (p-value = 0.00924, \( r^2 = 0.166 \)). The percent of dose recovered in urine ranged from 35.4% ± 15.7% in young adult patients (age 21-49 years old) and 32.9% ± 15.0% in patients of age 65-93 years indicating highly variable absorption. In contrast, absolute bioavailability was 0.864 ± 0.194 and 0.925 ± 0.252 for the two groups, as determined by the stable-isotope technique. Unaccounted biliary-fecal excretion of p-HPPH
glucuronide, subjects’ noncompliance, and incomplete urine collection are possible explanations. Consequently, bioavailability is best determined by stable-isotope method.

Chapter 2 of this thesis illustrates the early screening study of chemopreventive agent for prostate cancer. The rationale of this study was attempting to inhibit the metabolism of $5\alpha$-androstan-3$\beta$,17$\beta$-diol (3$\beta$-Adiol). A metabolite of dihydrotestosterone, 3$\beta$-Adiol, inhibits LNCaP prostate cell proliferation in the presence of Estrogen Receptor $\beta$. CYP7B1 is the enzyme responsible for catalyzing the 6$\alpha$ and 7$\alpha$ hydroxylations of 3$\beta$-Adiol in prostate. In this study, expression and purification of human CYP7B1 in \textit{E.coli} was attempted. Despite spectroscopic evidence of P450 expression, the enzyme failed to turn over its endogenous substrate, DHEA to 7$\alpha$-hydroxy DHEA.
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CHAPTER 1

Clinical Pharmacokinetics of Phenytoin in Epilepsy Patients

INTRODUCTION

Epilepsy and Available Antiepileptic Drugs (AEDs)

Epilepsy is a chronic neurological disorder that affects about 2.1 million Americans with more than 140,000 new cases reported in 2005 (Hirtz et al., 2007). The causes of most identified epilepsy cases are not known. However, some factors that may lead to development of epilepsy include asphyxia during childbirth, low birth weight, head trauma, brain tumor, central nervous system infections, cerebral palsy and mental retardation, stroke, complex febrile seizures, cerebrovascular diseases, alcohol, drug abuse, family history of epilepsy, multiple sclerosis, and certain genetic disorders (Hauser and Hesdorffer, 1990). The International League Against Epilepsy classifies seizures as follows (1981):

1. Generalized seizures (convulsive or nonconvulsive) involve both sides of the brain. These seizures include absence (petit mal seizure), tonic, clonic, tonic-clonic (grand mal seizures), myoclonic, and atonic seizures.

2. Partial seizures (focal seizures) involve one side of the brain. These seizures are classified into simple and complex partial seizures.

3. Unclassified epileptic seizures are caused by electrical perturbation in the brain, whereas, epileptic seizures are caused by anomalous electrical perturbation in the
4. Status epilepticus is a condition in which the patient experiences extended and continuous seizures.

Depending on their molecular targets, AEDs can act as voltage-gated sodium channel blockers such as phenytoin (PHT) and carbamazepine, voltage-gated calcium channel blockers such as ethosuximide (t-type calcium channel), gabapentin (l-type calcium channel), γ-aminobutyric acid (GABA) transaminase inhibitors such as vigabatrin, GABA reuptake inhibitors such as tiagabine, and GABA agonists such as progabide. Other AEDs that may have multiple molecular targets include felbamate, lamotrigine, and topiramate. The structures of the AEDs employed in the current study are illustrated in Figure 1.1.

**Figure 1.1.** Structures of the AEDs used in the current study.
Phenytoin Therapy

Phenytoin (Dilantin®) was first introduced as an anticonvulsant in 1938. Merritt and Putnam found PHT had a greater anticonvulsant effect compared to phenobarbital, which had been previously used to treat epilepsy (Merritt and Putnam, 1938). In the United States, PHT has been shown to effectively treat generalized tonic-clonic seizures (DeLorenzo and Dashefsky, 1985), complex and simple partial seizure (Leppik, 2001), and status epilepticus (DeLorenzo, 1990) without the sedative effects associated with phenobarbital.

The mean conventional PHT maintenance dose for epilepsy patients is 5 mg/kg/day (Leppik, 2001). It usually takes about 5 to 7 days (5 half-lives) for PHT maintenance dose to reach steady-state, with typical therapeuitic PHT levels in plasma ranging from 10 to 20 µg/mL. These therapeutic concentrations usually exceed the median $K_m$ of PHT, which is 6.2 µg/mL (range = 1.5 – 30.7 µg/mL). The mean apparent PHT $V_{max}$ is 0.45 µg/mL/hr (range = 0.14 – 1.36 µg/mL/hr) assuming a volume of distribution of ~0.7L/kg (Browne and LeDuc, 2002). Consequently, PHT displays concentration-dependent pharmacokinetics (Michaelis-Menten kinetics), in which small changes in dose can produce large changes in concentrations in plasma.

Phenytoin Formulations

In the United States, parenteral and oral PHT are formulated as both free acid and
sodium salt forms. The free acid form is less soluble than the sodium salt form. This poorer solubility may lead to delayed absorption (Glazko et al., 1969). The aqueous suspension and chewable tablet formulation of PHT are available as free acids. The aqueous suspension comes in 30 mg or 125 mg of PHT acid per 5 mL. The chewable tablet comes as 50 mg of PHT acid per tablet. PHT formulations in the sodium salt form only contain 92% of PHT acid (Browne and LeDuc, 2002). In order to improve PHT water solubility for intravenous administration, PHT is dissolved in 40% propylene glycol and 10% ethanol in water adjusted to pH 12 with sodium hydroxide (Pryor and Ramsay, 2002). Donovan and Cline reported that the presence of propylene glycol in the formulation induced hypotension and cardiac arrhythmias in patients receiving rapid infusions of PHT (Donovan and Cline, 1991). Infusion rate also plays an important role in order to reduce the precipitation at the administration site, phlebitis, and other cardiovascular complications. The infusion rate typically ranges between 20 to 40 mg/min, however, it can go up to 50 mg/min in an emergency situation, such as status epilepticus. The solubility of PHT in plasma is 75 μg/mL at 37°C. If the infusion rate exceeds the solubility of PHT in plasma, then the rest of infused PHT would precipitate.

A parenteral prodrug formulation of PHT that possesses better water solubility is Fosphenytoin sodium (Cerebyx®). As depicted in Figure 1.1, FOS is a phosphate ester prodrug of PHT. Upon entering the blood and vascularized tissue, the phosphate moiety will be hydrolyzed by phosphatase enzymes. The half-life of this rapid conversion is about 8 to 12 minutes (Leppik, 2001). FOS is available as 75 mg/mL of
FOS which is equivalent to 50 mg/mL PHT (Pryor and Ramsay, 2002). The FOS preparation is buffered at pH 8.8 without requiring the addition of an organic solvent to improve its solubility (Fischer et al., 2003).

Differences in the various PHT formulations available can lead to alterations in the rate and extent of PHT absorption, which in turn, can further lead to an alteration of PHT bioavailability. In 1968, a study in Australia reported a PHT overdose due to an excipient switch from calcium sulfate dihydrate to lactose in the 100 mg PHT tablets (Eadie et al., 1968; Tyrer et al., 1970). Content of PHT from these two different capsules showed similar amounts of PHT. Figure 1.2 demonstrated the fluctuations in PHT blood levels in a patient receiving PHT with two different excipients. Bochner et al. corroborated this finding and also stated that a portion of PHT cannot be absorbed due to the presence of calcium sulfate as an excipient (Bochner et al., 1972). Other studies have suggested a considerable difference in the plasma levels of patients receiving single doses of PHT from different manufacturers (Martin et al., 1968; Arnold et al., 1970).
**Figure 1.2.** PHT blood levels in a patient receiving 400 mg of PHT with different excipients. The patient’s PHT blood level decreases approximately four-fold upon receiving PHT containing calcium sulfate dehydrate excipient, then the level rises upon reintroduction of PHT containing lactose excipient (Bochner et al., 1972).

Particle size of the PHT being administered is also another factor that can have a great impact on the dissolution of PHT, which can further change the absorption properties of the drug. A study by Doluisio et al. compared in dogs the absorption of sodium PHT with a 5 – 25 μm particle size, free acid form of PHT with a 10 – 50 μm particle size, and flat plates of free acid form of PHT with 0.5 x 3 x 3 mm dimension. Animals administered the sodium PHT formulation with the smallest particle size had the highest plasma levels (Figure 1.3).
**Figure 1.3.** PHT plasma levels in dogs receiving sodium PHT with 5 – 25 μm particle size (squares), free acid form of PHT with 10 – 50 μm particle size (triangles), and flat plates of free acid form of PHT with 0.5 x 3 x 3 mm dimension (circles) (Doluisio, 1972).

Phenytoin Side Effects

Besides occasional cases of hypotension and cardiac arrhythmia, the most common adverse effects associated with infusion of PHT and FOS are nystagmus, dizziness, drowsiness, and lack of coordination (Fischer et al., 2003), especially at concentrations greater than 20 μg/mL. Intramuscular (IM) and IV administration are two common parenteral routes for PHT administration. In order to obviate potential hypotension or cardiac arrhythmia, PHT should be diluted and must be administered slowly when IV administration is adopted (Leppik, 2001; Fischer et al., 2003). Leakage
from the infusion site can cause premature tissue death (Meek et al., 1999; Leppik, 2001). Additionally, the presence of propylene glycol in the formulation of PHT causes pain and irritates vascularized tissue (Meek et al., 1999). Intramuscular (IM) administration of PHT is not recommended due to erratic absorption, pain caused by insoluble precipitation of the drug at the injection site, premature tissue death, and abscess formation (Meek et al., 1999). The use of IM and IV FOS eliminates the adverse effects associated with the presence of propylene glycol and high pH in the PHT formulation. However, patients also experience minor adverse effects such as localized paraesthesias and pruritus of the groin, buttocks, and face, which subsides 5 to 10 minutes after injection of FOS (Meek et al., 1999; Fischer et al., 2003). Another side effect associated with chronic PHT use is gingival hyperplasia. Soga et al. demonstrated that patients with severe gingival hyperplasia also had high serum PHT concentration (Soga et al., 2004).

The covalent binding of PHT arene-oxide to the constituent of gestational tissue has been shown by Martz et al. to be associated with orofacial anomalies observed in fetuses of Swiss mice (Martz et al., 1977). This finding suggested that susceptibility of tissues to arene-oxide was the determinant factor that led to the observed anomalies. Maguire and McClanahan showed that the patients in their study who had been on PHT for at least 6 months did not show any idiosyncratic hypersensitivity reactions (Maguire and McClanahan, 1986).
Phenytoin Pharmacokinetics Profile

Four key features of PHT are its poor solubility, its extensive protein binding, its erratic absorption, and its Michaelis-Menten kinetics profile. PHT is a weak organic acid that has a pKa of 8.31. It has a poor solubility at low pH. Solubility at pH 5.4 at 25°C is 19.4 µg/mL and at pH 7.4, the solubility is 20.5 µg/mL. Under basic conditions, PHT is ionized and solubility increases dramatically (1,520 µg/mL at pH 10). PHT is very slowly cleared from the body; hence, the fractions of PHT metabolized during first-pass by the gut wall and by the liver are negligible. Dill et al. successfully illustrated increased plasma levels of PHT in isolated segments of rats gastrointestinal tract when delivered to the stomach (0.6 µg/mL), small intestine (1.5 µg/mL) before the plasma PHT level declined or large intestine (0.7 µg/mL) (Dill et al., 1956). The low pH environment in the stomach favors the rapid absorption of PHT. However, despite the less favorable pH, the small intestine is the major absorption site due to its greater absorptive surface area.

As PHT enters the systemic circulation, about 75 to 90% of it binds extensively and reversibly to plasma protein (Peterson et al., 1982). By radioimmunoelectrophoresis of human serum, Lightfood and Christian were able to identify that PHT bound to albumin and two thyroxine α-globulin proteins (Lightfoot and Christian, 1966). Lai et al. also reported that FOS binds primarily to albumin at the same site as PHT (Lai et al., 1995).
Because of PHT’s poor water solubility, the absorption rate of PHT is expected to be slow and erratic. However, in a comparative excretion study with radiolabeled PHT, Glazko et al. reported 81.9% and 81% mean urinary radioactivity recoveries from healthy volunteers receiving the same PHT dose intravenously and orally (Glazko, 1987). This data suggests that PHT has high absorption regardless of its route of administration. Food intake had been shown to affect PHT absorption that consequentially influenced its bioavailability. Sekikawa et al. reported that the average recovery of urinary metabolites was 63.7% with fasting, 77.9% after a balanced meal, and 69.3% after a high lipid meal (Sekikawa et al., 1980). These results clearly suggest that food intake, especially a high lipid meal, increases PHT bioavailability. It is also known that concomitant administration of PHT and calcium-containing antacids reduces PHT absorption, leading to a decrease in its bioavailability (Cloyd and Remmel, 2000).

PHT exhibits nonlinear (dose-dependent, Michaelis-Menten) pharmacokinetics. The contributing factors that may cause a nonlinear pharmacokinetics profile of drugs are carrier-mediated gastrointestinal absorption, saturability of plasma protein and tissue binding, concentration-dependent renal secretion, and saturable hepatic metabolism or uptake (Rowland and Tozer, 1995). Jusko et al. found that absorption of PHT was slow (4-7 hours) with a secondary peak of 8-15 hours (absorption half-life is approximately 8 hours) (Jusko et al., 1976). Capacity-limited hepatic metabolism is responsible for the observed dose-dependent kinetics of PHT ($K_m$ of (S)-p-HPPH with purified human CYP2C9 as enzyme source = 5.4 μM). Autoinduction of the drug metabolizing
enzymes catalyzing PHT hydroxylation could be responsible for the observed PHT dose-dependent kinetics. However, Martin et al. found that the $V_{\text{max}}$ values of nine normal volunteers remained relatively constant ($\sim 10.3 \text{ mg/kg/day}$) over 6 to 14 day study period (Martin et al., 1977).

The mechanism of PHT dose-dependent kinetics was hypothesized to be due to product inhibition by p-HPPH. Borondy et al. reported that the rate of glucuronidation of p-HPPH did not change significantly with increasing concentration in the therapeutic range in in vitro glucuronidation experiments (Borondy et al., 1972). The conjugation of p-HPPH did not appear to contribute to the dose dependent kinetics observed in vitro. However, an in vitro inhibition study with PHT as the substrate and p-HPPH as the inhibitor showed that p-HPPH was a competitive inhibitor of PHT hydroxylation (Figure 1.4) (Borondy et al., 1972). Furthermore, a diagnostic Dixon plot determined that the observed inhibition was a mixed type (Figure 1.5) with a $K_i$ of 64 $\mu$M. Doluisio suggested that the observed PHT hydroxylation inhibition by p-HPPH was due to binding site competition on the enzyme complex between PHT and p-HPPH (Doluisio, 1972). Additionally, another phenomenon that may be explained by the observed product inhibition was the 6 to 8-hour delay before the maximum amount of p-HPPH was excreted in the urine following 250 milligrams IV doses of PHT sodium (Figure 1.6) (Glazko et al., 1969), although slow or delayed absorption would also result in the same profile. Alternatively, the delay in p-HPPH glucuronide in the urine may be due to the need for two metabolic steps (oxidation and conjugation) prior to excretion. A comparison of PHT elimination half-life between rats that were treated with only PHT
and rats that were treated concomitantly with p-HPPH showed a four-fold elimination half-life prolongation in rats receiving both PHT and p-HPPH (Ashley and Levy, 1972). This product inhibition phenomenon was also supported by a study in rhesus monkeys administered a loading dose of p-HPPH prior to the administration of radiolabeled PHT, which was followed by a second dose of p-HPPH. As observed on Figure 1.7, the half-life of PHT was extended in the presence of p-HPPH (Glazko et al., 1977). Consequently, the exposure of PHT was also protracted, which could lead to toxicity. However, circulating concentrations of free p-HPPH in plasma are very low (< 0.1 µM), compared to an in vitro Kᵢ of 64 µM, thus product inhibition is unlikely to play a major role in dose-dependency, unless there is high sequestration of p-HPPH within the liver cytosol (location of CYP2C active site).

**Figure 1.4.** A competitive inhibition of PHT hydroxylation by p-HPPH in S9 rat liver homogenate obtained from a Lineweaver-Burk plot with Kᵢ value of 24.7 µM and Vₘₐₓ value of 25 nmoles/mg protein/20 minutes (Doluisio, 1972).
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Figure 1.6. A lag time of p-HPPH urinary excretion following 250 mg IV doses of PHT sodium. The maximum p-HPPH excretion rate reached after six to eight hours after PHT administration in five male subjects (Glazko et al., 1969).
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**Phenytoin Metabolism**

Hepatic metabolism is responsible for PHT elimination. Figure 1.8 shows the metabolic pathway of PHT including the distribution of urinary metabolites. The principal urinary metabolites, which reportedly account for 65% to 90% of administered PHT, are 5-(4-hydroxyphenyl)-5-phenylhydantoin (p-HPPH) and 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin (DHD) (Kadar et al., 1983; Browne and LeDuc, 2002). In spite of PHT extensively binding to plasma protein, PHT is almost completely metabolized, with metabolites eliminated in both urine and feces. The
fraction of unbound PHT determines the actual fraction of PHT available for exerting its anticonvulsant effect in the brain and for metabolism. Less than 5% of administered dose is eliminated renally unchanged (Dill et al., 1956; Browne and LeDuc, 2002). When the protein is not saturated, the extensive protein binding would affect the rate of PHT elimination and metabolic clearance by the liver.

The oxidative metabolism of PHT is mediated by cytochrome P450 enzymes via the formation of an arene-oxide intermediate that spontaneously opens to form p-HPPH or reacts with water to form DHD. Both CYP2C and CYP3A family enzymes are involved in the biotransformation of PHT. CYP2C enzymes are responsible for the first PHT hydroxylation to form p-HPPH, whereas the CYP3A family is likely responsible for the subsequent hydroxylation processes (Browne and LeDuc, 2002).

Specifically, CYP2C9, which has the highest predominant expression among the CYP2C enzymes expressed in the liver (Goldstein and de Morais, 1994), is the primary catalyst of PHT hydroxylation to form (S) p-HPPH. This isoform stereoselectively hydroxylates PHT in order to form the optically active p-HPPH. Bajpai et al. reported that human recombinant CYP2C9 was about 43 times more selective towards the formation of (S)-p-HPPH ($K_m = 5.4 \mu M$, $V_{max} = 388 \text{ pmol/min/ nmol P450}$) compared to the R enantiomer ($K_m = 5.5 \mu M$, $V_{max} = 9.0 \text{ pmol/min/ nmol P450}$) (Bajpai et al., 1996). Argikar et al. also reported the urinary ratio of (S)/(R) p-HPPH for a homozygous CYP2C9 wild-type was about 24 (Argikar et al., 2006). Another study demonstrated that (S)-p-HPPH was the dominant metabolic product of PHT by human
recombinant CYP2C9 with $K_m$ value of 25 $\mu$M and $V_{\text{max}}$ value of 180 pmol/min/(nmol P450) (Mosher et al., 2009).

**Figure 1.8.** PHT metabolic pathway including its urinary metabolites distribution in human.

CYP2C19 is also known to make a small contribution in the metabolism of PHT in human liver microsomes (Giancarlo et al., 2001). However, CYP2C19 does not possess the same stereoselective preference compared to CYP2C9. Human recombinant CYP2C19 afforded only about 1.2 times more selectivity towards the
formation of (S)-p-HPPH ($K_m = 70 \mu M$, $V_{max} = 74$ pmol/min/ nmol P450) compared to the (R)-p-HPPH ($K_m = 71 \mu M$, $V_{max} = 61$ pmol/min/ nmol P450) (Bajpai et al., 1996). Although different source of reductase were used with the two enzymes, a direct comparison of $V_{max}$ between human recombinant CYP2C9 and CYP2C19 in the conducted study was made (Bajpai et al., 1996). From the aforementioned study, therapeutic concentrations of PHT are well below the $K_m$ of CYP2C19 for (R)-p-HPPH indicating that CYP2C19 mainly contributes to the formation of the R enantiomer of p-HPPH. Since the intrinsic clearance of CYP2C9 is much greater than that for CYP2C19, the urinary ratios reflect the dominant contribution of CYP2C9 activity in persons who have the CYP2C9*1/*1 genotype (wild type). This hypothesis was in agreement with a previous study demonstrating lower S/R ratio of p-HPPH in the urine of extensive metabolizers (EMs) compared to poor metabolizers (PMs) of mephenytoin, a substrate for CYP2C19 (Fritz et al., 1987). Another study in Japanese subjects corroborated this hypothesis with the observation of higher urinary excretion of (R)-p-HPPH in the CYP2C19 EMs compared to CYP2C19 PMs (Ieiri et al., 1997).

Both CYP2C9 and CYP2C19 have been shown to be inducible by both constitutive androstane receptor (CAR) (Giancarlo et al., 2001) and pregnane X receptor (PXR) (Ferguson et al., 2002; Chen et al., 2003; Chen et al., 2004). PHT is a CAR activator (Wang et al., 2004) and induces its own metabolism. The CAR response element (phenobarbital response element or PBREM) on these two CYP2C genes possesses ability to bind with its ligand as well as PXR as a dimer with RXR$\alpha$ (Chen et al., 2003). PHT is also known as a PXR activator. Additionally, glucocorticoids have
been reported to enhance the expression of CYP2C19 in HepG2 cells (Chen et al.,
2003).

The most common single nucleotide polymorphisms (SNPs) in CYP2C9 are a
transition of 430C>T in exon 3, that leads to an amino acid change of R144C
(CYP2C9\(^*2\)) and a mutation of 1075A>C in exon 7, which encodes for the I359L
variant (CYP2C9\(^*3\)) (Stubbins et al., 1996; Xie et al., 2002). The allele frequencies of
CYP2C9\(^*1\) (wild-type), CYP2C9\(^*2\), and CYP2C9\(^*3\) in the Caucasian population are
reported to be 0.79, 0.125, and 0.085, respectively (Stubbins et al., 1996). Additionally,
the allele frequency percentage for CYP2C9\(^*2\) is between 8% to 19% in the Caucasian
population, 12% in the Hispanic population, 3.2% in the African-American and
Ethiopian populations, and 0% in East Asian (Chinese, Japanese, Korean) populations,
whereas the percentage for CYP2C9\(^*3\) is between 3.3% to 16.2 % in the Caucasian
population, 3.4 % in the Hispanic population, 1.3% in the African-American population,
0% in West African population and 6% Ethiopian populations, 3.3% in the Chinese
population, 2.2% in Japanese, and 1.1% in Koreans (Scordo et al., 2001; Xie et al.,
2002; Allabi et al., 2003). With human liver microsomes as the enzyme source,
Yamazaki et al. showed about 2.2-fold and about 10-fold decreases in the intrinsic
clearances of warfarin and diclofenac in yeast microsomes expressing CYP2C9.2 and
CYP2C9.3 variants, respectively (Yamazaki et al., 1998). These SNPs also have been
shown to exhibit reduced enzyme activities \textit{in vivo} (Higashi et al., 2002; Xie et al.,
2002; Vormfelde et al., 2009). CYP2C9.2 and CYP2C9.3 expressed in insect cells had
been shown to reduce the catalytic efficiency relative to CYP2C9.1 by about 1.5-fold
and 20-fold, respectively for both (S)-Warfarin-7-hydroxylation and (S)-p-HPPH formation (Rettie et al., 1999). CYP2C9.2 expressed in HepG2 cells and CYP2C9.3 expressed and purified from baculovirus -transfected insect cells had been shown to have about 18% and less than 5% of wild-type enzyme activity for (S)-warfarin-7-hydroxylation, respectively (Rettie et al., 1994; Haining et al., 1996). The CYP2C9.3 variant has been shown to have much more profound effects on (S)-Warfarin-7-hydroxylation and PHT-4-hydroxylation compared to CYP2C9.2 variant (Rettie et al., 1999). Due to these altered enzyme activities, the mean PHT maintenance dose for patients who possessed one or more CYP2C9 allelic variant is about 30% lower than subjects homozygous for CYP2C9*1 (van der Weide et al., 2001). In addition, because CYP2C9.3 has a diminished level of enzyme activity, patients with this mutation would have a higher risk of developing gingival overgrowth due to potentially higher serum PHT concentration (Soga et al., 2004).

Polymorphisms of the CYP2C19 gene will affect subsidiary PHT metabolism as well. There are 8 allelic variants reported for CYP2C19 (Wedlund, 2000). However, only 2 allelic variants, CYP2C19*2 and CYP2C19*3, have been studied in relationship to PHT metabolism (Odani et al., 1997; Mamiya et al., 1998). CYP2C19*2 is associated with a point mutation of 681G>A within the coding sequence of exon 5 of the CYP2C19 gene that leads to the creation of a cryptic splice site (de Morais et al., 1994b). CYP2C19*3 is associated with a point mutation of 636G>A within exon 4 that leads to the creation of a premature stop codon (De Morais et al., 1994a). The allele frequency for CYP2C19*2 was 0.022 in the Caucasian population, 0.03 in the Black
population, and 0.09 in the Chinese population, whereas the allele frequencies for
CYP2C19*3 were 0.0004, 0.004, and 0.05, respectively (Wedlund, 2000). It has been
shown that $V_{\text{max}}$ values of PHT were decreased approximately 8 to 14% in Japanese
patients who were CYP2C19 PMs (Odani et al., 1997). Based on the NONMEM
analysis, the mean $K_m$ value in CYP2C19 poor metabolizers was 54% higher than the
mean $K_m$ values of homozygous EM and 22% higher than heterozygous EM, suggesting
a gene-dose effect (Mamiya et al., 1998). Recent evidence has demonstrated that there
is a linkage disequilibrium between CYP2C19*2 and a mutation in the regulatory
portion of CYP2C9 (CYP2C9*1B), causing reduced response to CAR/PXR inducers,
resulting in lower clearance of PHT (Chaudhry et al., 2010).

In order to increase the solubility of p-HPPH and DHD prior to urinary excretion,
these metabolites are conjugated with a glucuronic acid. Uridine 5’-diphosphate-
glucuronosyltransferases (UGTs) are enzymes responsible for catalyzing the attachment
of glucuronic acid to the phenolic hydroxyl of p-HPPH. Maynert et al. demonstrated
that p-HPPH is extensively glucuronidated before excretion in the urine (Maynert,
1960). Like cytochrome P450, UGT also has several isoforms. To date, UGT1, UGT2,
UGT3, and UGT8 families have been identified in humans (Mackenzie et al., 2005).
Nakajima et al. illustrated that multiple UGT1A isoforms, namely UGT1A1, UGT1A4,
UGT1A6, and UGT1A9, were responsible for glucuronidation of p-HPPH (Nakajima et
al., 2002). The dominant formation of (R)-p-HPPH-O-glucuronides are catalyzed by
UGT1A1, whereas (S)-p-HPPH-O-glucuronides are dominantly formed by UGT1A9
and UGT2B15 Supersomes (Nakajima et al., 2007). UGT1A1 and UGT1A9 expressed
in HEK293 cells were found to selectively mediate the glucuronidation of (R) and (S)-p-HPPH, respectively (Nakajima et al., 2007). In the case of DHD, besides the absence of DHD glucuronide in the urine, Chang et al. reported that the level of DHD in the animals’ urine remained the same before and after β-glucuronidase treatment and the conversion of DHD to any phenolic products was not observed (Chang et al., 1970). However, Maguire et al. reported that urinary DHD was partially conjugated and was present substantially as the S enantiomer based on the optical rotary dispersion spectrum (Maguire et al., 1979; Maguire et al., 1980; Maguire and McClanahan, 1986).

Principal Urinary Metabolites of Phenytoin

In 1957, Butler reported p-HPPH as the major metabolite of PHT in humans. This pharmacologically inactive metabolite exists largely as the (S) - levorotatory enantiomer (Butler, 1957). In seven pediatric patients, (S)-p-HPPH accounted for 89.3 ± 4.0 % of total p-HPPH. In nine healthy males, average (S)-p-HPPH accounted for 94 % of total p-HPPH (Maguire and McClanahan, 1986). In addition, Glazko reported that p-HPPH accounted for 65.9% of the urinary excretion of oral administered PHT (Glazko, 1987).

The identification of DHD as one of PHT metabolites was performed in rat and monkey urine by observing a loss of aromaticity (reduced absorbance between 1400 – 1600 cm\(^{-1}\)) and the potential presence of two aliphatic alcohols (two distinct peaks between 1000 – 1100 cm\(^{-1}\)) by infrared spectrophotometry (Chang et al., 1970). By a gas chromatographic- mass spectrometric method, Horning et al. found DHD in the
urine of a newborn who received PHT by placental transfer and an eight-day-old infant who was on PHT therapy (Horning et al., 1971). A negative color test with osmium tetraoxide salt in acidic solution, which usually forms a complex only with cis-diols, elucidated that hydroxyl groups on DHD were in the trans configuration (Chang and Glazko, 1972). Glazko et al. reported that DHD accounts for 9.3% of the urinary excretion of orally administered PHT (Glazko, 1987). The detection of DHD in human urine implies that PHT arene-oxide is the precursor to DHD after its reaction with water. This reaction may be catalyzed by microsomal epoxide hydrolase, since addition of epoxide hydrolase inhibitors to a microsomal incubation increased covalent binding of $^{14}$C-PHT (Pantarotto et al., 1982). Due to the unstable nature of the PHT arene-oxide intermediate, the characterization of its stereochemistry was established by the characterization of both p-HPPH and DHD stereochemistry optical rotations.

In seven pediatric patients, average (S)-DHD accounted for 74% of the total DHD. In nine healthy males, average (S)-DHD accounted for 78% of the total DHD. The presence of both diastereomers of DHD infers that there are two isomeric forms of PHT arene-oxide. The proposed metabolic pathway of the stereoselective formation of both p-HPPH and DHD is shown Figure 1.9 (Maguire and McClanahan, 1986).

The 5-(3-hydroxyphenyl)-5-phenylhydantoin (m-HPPH) used as an internal standard was reported to be the major metabolite in dogs (Atkinson et al., 1970). In a cross-over study with six adult males, m-HPPH was not detected in the subjects’ urine samples (Chang et al., 1977). Equal amounts of m-HPPH and p-HPPH could be formed
by heating DHD in 3 N hydrochloric acid at 90°C or by heating the dry powder at 250°C (Chang et al., 1970).

**Figure 1.9.** The proposed metabolic pathway of the stereoselective formation of both p-HPPH and DHD

*Stable-Labeled Isotope Approach*

For pharmacokinetics studies, stable-labeled compounds have been progressively substituted for radiolabeled compounds, whereas radiolabeling is still widely used to
detect and quantify metabolites (Mutlib, 2008). The stable-labeled compounds are typically used to elucidate metabolic pathways, structure of metabolites, metabolic disposition, and potential metabolic activation leading to toxic effects as well as for determination of pharmacokinetic parameters in clinical studies (Mutlib, 2008; Yamazaki et al., 2009). Additionally, stable-labeled analogs are often employed as internal standards in quantitative LC/MS or GC-MS assays. For absolute bioavailability determinations, the advantage of simultaneous stable isotope administration is the elimination of intrasubject variability in drug ADME studies compared to classical IV/oral cross-over designs.

In the clinical study reported for this thesis, SL-PHT was used to determine PHT pharmacokinetic parameters and absolute bioavailability. This process involved replacing a fraction of the PHT sodium maintenance dose with a 100 mg dose of SL-PHT, $[^{13}\text{C}_3]$-PHT or 100 mg PHT equivalent dose of $[^{13}\text{C}_3,^{15}\text{N}_2]$-FOS. The parenteral routes of administration of the PHT were through intravenous (M+3) for SL-PHT or intramuscular (M+5) injections for SL-FOS. The remaining maintenance dose (less 100 mg) was given as an oral dose of non-labeled PHT sodium administered immediately after the administration of the tracer dose. This method enabled the direct determination of pharmacokinetic parameters of clearance, volume of distribution, and half-life via WinNonLin software. Bioavailability for each subject was obtained by Non-Linear Mixed Effects Modeling (NONMEM). Absolute bioavailability was determined by dividing the oral AUC by the intravenous AUC after dose correction following Equation 1.1. As previously mentioned, the principal urinary metabolites of
PHT account for 65% to 90% of the dose (Kadar et al., 1983; Browne and LeDuc, 2002). Therefore, quantifying those metabolites should be a good representation of PHT dose recovered at steady state. The aim of this study was to determine whether the excretion of urinary metabolites is a good indicator of the absolute bioavailability of PHT in epilepsy patients on maintenance therapy.

\[
F = \frac{[AUC]_{po}}{[AUC]_{iv}} \times \frac{Dose_{iv}}{Dose_{po}} \tag{Equation 1.1}
\]
MATERIALS AND METHODS

Chemicals

HPLC grade ethyl acetate, ammonium acetate, methanol, acetonitrile, ACS certified sodium acetate, and sodium chloride were obtained from Fisher Scientific, Co. (Fair Lawn, NJ). HPLC grade glacial acetic acid, β-glucuronidase (type H-3AF isolated from Helix pomatia, G0762-100KU), 5,5-diphenylhydantoin sodium salt, p-HPPH, m-HPPH, and Filter agent Celite® 545 were obtained from Sigma-Aldrich, Inc. (St. Louis, MO). SL-PHT and p-HPPH were purchased from Cambridge Isotope Laboratories, Inc (Andover, MA).

Materials

Luknova Flash columns, preloaded with 12 grams of normal phase silica gel were obtained from Luknova, Inc. (Mansfield, MA). Acrodisc® CR (13 mm, 0.45 µm) and syringe filters with 0.2 µm PTFE membranes were purchased from Fisher Scientific, Co. (Fair Lawn, NJ). Solid phase extraction Oasis® HLB cartridges (3cc/60mg, 30µm) were obtained from Waters, Corp. (Milford, MA). Polypropylene vials and 0.45” PTFE/Silicone caps were purchased from Chrom Tech, Inc. (Apple Valley, MN).
Subject Characteristics

The subjects were recruited from nursing homes and epilepsy clinics in the Minneapolis metro area. All subjects were on maintenance monotherapy of PHT or PHT in combination with non-interacting drugs. The study was approved by the University of Minnesota Human Subjects Committee. Consent from all the subjects was obtained prior to the beginning of the study. Forty healthy patients (17 women, 23 men) with epilepsy, ranging from 21 to 93 years old, participated in the study. However, two subjects only provided 12-24 hour urine samples and one subject only provided 0-12 hour urine sample.

Dosing of Phenytoin

On the morning of the study, the subjects arrived at the University of Minnesota General Clinical Research Center (GCRC) after taking their regular evening dose of PHT the night before admission. Prior to the study drug administration, the subjects voided. The study drugs were 100 mg of IV stable labeled PHT $[^{13}\text{C}_3]$ or 150 mg of intramuscular FOS $[^{13}\text{C}_3,^{15}\text{N}_2]$, which were equivalent to 100 mg of PHT sodium. The remainder of the PHT morning maintenance dose was administered immediately following the administration of the tracer dose. Urine samples were collected from 0-12 hours and 12-24 hours.
Preparation of Solutions

Preparation of 100 mM Sodium Acetate

2.72 grams of sodium acetate were dissolved in 200 mL of distilled water.

Preparation of 100 mM Acetic acid

1.143 mL of glacial acetic acid were brought to a final volume of 200 mL with distilled water.

Preparation of 100 mM Acetate Buffer, pH 5

A solution of 100 mM of acetic acid was added to 100 mM of sodium acetate solution until pH 5 was reached on a pH meter while the mixed solution was constantly stirred.

Preparation of 10% Acetonitrile (v/v)

100 mL of acetonitrile were added to 900 mL of deionized water

Preparation of 1 M Ammonium Acetate

38.54 grams of ammonium acetate were dissolved in 500 mL of distilled water.

Preparation of 1 M Acetic acid

28.56 mL of glacial acetic acid were brought to a final volume of 500 mL with distilled water.
Preparation of 10 mM Ammonium Acetate

10 mL of 1 M ammonium acetate were brought up to a final volume of 1 L with distilled water.

Preparation of 10 mM Acetic acid

Five mL of 1 M acetic acid were brought up to a final volume of 500 mL with distilled water.

Preparation of 10 mM Ammonium Acetate Buffer, pH 5.0

A 10 mM solution of acetic acid was added into 10 mM of ammonium acetate solution until pH 5.0 was reached while the mixed solution was stirred constantly.

Extraction and Purification of DHD

An overnight urine sample was obtained from a patient on a 300 mg PHT maintenance dose. An equal volume of 100 mM of sodium acetate buffer, pH 5 was added to the patient urine. For each liter of urine, 2.78 mL of β-glucuronidase were added (approximately 250 unit/mL final). The urine was incubated at 37°C overnight at a pH of approximately 5.26. Two hundred grams of sodium chloride were added into each liter of urine mixture until the salt reached its saturation point. The urine mixture was centrifuged at 10,000 x gravity, 4°C for 15 minutes in an Avanti J-E centrifuge (Beckman Coulter, Fullerton, CA) in order to precipitate out any impurities in the urine. The urine mixture was decanted through a fluted filter paper to sift out any remaining
precipitated impurities. The urine mixture was then extracted three times with equal volumes of ethyl acetate. The collective organic layers were evaporated on a Buchi Rotary Evaporator R-200 (Buchi Corp., New Castle, DE). The resulting oil was then dissolved in 20 mL ethyl acetate and approximately 1 mL of methanol. The solution was divided into three separate round bottom flasks and approximately two spoonfuls of celite were added into each flask. The mixture was then dried under vacuum by both rotary evaporation and high vacuum. The solid mixture was then packed into a Luknova flash column with 12 g preloaded normal phase silica gel. Based on the solvent system developed on thin layer chromatography (TLC), a mobile phase composition of 100% ethyl acetate was utilized. After the cartridge had been equilibrated, the separation of DHD from other PHT urinary metabolites on CombiFlash Companion T/S (Teledyne Isco, Inc., Lincoln, NE) was performed for 30 column volumes with a 13 mL/min flow rate. Due to the absence of any chromophore on PHT and its metabolites, all the output flow from the CombiFlash Companion T/S was collected into separate test tubes. Each of these collected fractions were then subjected to direct injections into Agilent 1100 series LC/MSD TOF (Agilent Technologies, Inc., Santa Clara, CA) in APCI negative ion mode and were analyzed with the Analyst QS software. Fractions from test tubes that had DHD (m/z 285.111) were pooled together and dried under vacuum. A sample of MS trace can be found in Figure 1.10.
**Figure 1.10.** An example of MS trace of DHD in a negative ion mode APCI at m/z 285 obtained after the ethyl acetate extraction and separation from other nonpolar compounds.

**Preparative HPLC-UV Method for Isolation of DHD**

An analytical isocratic HPLC-NI-APCI-MS-TOF method with simultaneous diode array detection was developed on a Varian Microsorb-MV 100-5 C18 (250x 4.6 mm) column with an Agilent 1100 series LC/MSD TOF (Agilent Technologies, Inc., Santa Clara, CA) in order to scale up the purification of the crude DHD on the preparative HPLC. Varian Dynamax Preparative HPLC column Microsorb 100-8 C18
(250 x 41.4 mm inner diameter) with 40 mL/min flow of water: acetonitrile (9:1) was accomplished on a Varian ProStar 410 Prep HPLC system (Varian, Inc., Palo Alto, CA) that was equipped with an autosampler, diode array detector, and a Galaxie™ Workstation. The crude DHD oil was dissolved in 8 mL of 10% acetonitrile. About 49.5 mg of crude DHD were injected per one preparative HPLC run. A Varian fraction collector model 701 was used to collect the fractions. The DHD peak eluted at about 47 minutes. The fractions with DHD were pooled together and evaporated under high vacuum until the volume was about 5 mL. The solution was then filtered through fluted filter paper and evaporated under high vacuum until the volume reduced to about 2 mL. Deionized water was added to rinse any residual DHD remaining in the flask. The solution was then frozen in an acetone/dry ice bath, followed by an overnight lyophilization. One milligram of lyophilized sample was dissolved in deuterated methanol to be subsequently analyzed by proton NMR on a Varian Oxford 600 NMR, 600 MHz (Varian, Inc., Palo Alto, CA). A sample of chromatogram for DHD purification obtained by preparative HPLC is illustrated in Figure 1.11. The $^1$H NMR of the crude DHD trace is illustrated in Figure 1.12.
**Figure 1.11.** A chromatogram obtained from one of preparative HPLC-UV runs in the purification of DHD at a wavelength of 210 nm. The x-axis shows the absorbance of the molecules present in the mixture at the specified wavelength and the y-axis is the retention time in minutes.

![Chromatogram](image1.png)

**Figure 1.12.** The $^1$H NMR traces of DHD obtained from Varian Oxford 600 NMR, 600 MHz on crude DHD. The x-axis is the chemical shift in ppm.

![NMR traces](image2.png)
Due to the presence of a large number of impurities, the lyophilized sample obtained from the preparative HPLC was subjected to another purification step by semi-preparative HPLC on a Varian Dynamax Semi-prep HPLC column Microsorb 100-5 C18 (250 x 10 mm) with 4 mL/min flow of isocratic water: acetonitrile (9:1) on the Varian ProStar 410 HPLC system and diode array detection. The lyophilized sample was dissolved in 8 mL of 10% of acetonitrile. Approximately 13.4 mg of crude DHD were injected per one semi-preparative HPLC run. The Varian fraction collector model 701 was used to collect the fractions in separate test tubes. The DHD peak eluted at about 32 minutes. A direct injection into the Agilent 1100 series LC/MSD TOF was employed to check the presence of DHD in each collected fractions. The fractions with DHD were then pooled and lyophilized overnight. The purity of DHD was then analyzed by analytical HPLC on a Varian ProStar 410 instrument with 1 mL/min flow rate of 10% acetonitrile/water on Varian Microsorb-MV 100-5 C18 (250x 4.6 mm column) at 210 nm. The DHD peak eluted at about 17 min. Sample of chromatograms from final separation of DHD is illustrated in Figures 1.13A-C.
Figure 1.13. Chromatograms obtained from

(A) Semi-preparative HPLC on crude DHD showing the DHD’s retention time is around 32 minutes. Collections were made from 31-34 minutes. The peak at 37 minutes is likely the diastereomer of the peak at 32 minutes.
(B)  MS-TOF of a pure DHD at m/z of 285.11

(C)  An analytical HPLC-UV run (retention time = 17.6 minutes) to show the purity of the extracted DHD
Enzymatic Hydrolysis and Extraction of Both DHD and p-HPPH

The urine samples that were stored in -80°C were thawed at room temperature. A sample of urine (0.5 mL) from the 0-12 hour and the 12-24 hour urine collected from each subject was transferred into separate 1.5 mL centrifuge tubes. 0.5 mL of 100 mM of sodium acetate buffer, pH 5 was added, followed by addition of 504 units of β-glucuronidase. 49.8 µg of the internal standard, m-HPPH, were also added. The samples were then incubated overnight at 37°C with shaking at 85 rpm. Waters solid phase extraction Oasis® HLB cartridges (3cc/60mg, 30µm) were activated with methanol (2 mL) and followed by 2 mL of water rinse. The hydrolyzed urine samples were then applied onto the cartridges and were allowed to flow through the cartridges by gravity. The sorbent of the cartridges were next washed with 10% methanol. The samples were centrifuged on a Hettich Rotanta 460 RS Centrifuge (Hettich Laborapparate, Switzerland) at 80 rpm for approximately 10 minutes. The elutions of p-HPPH, DHD, and m-HPPH from the SPE cartridge were accomplished with methanol, followed by a centrifugation step at 80 rpm. The eluents were dried under nitrogen for 45 minutes using Zymark Nitrogen Dryer (Zymark, Hopkinton, MA). After drying, the samples were reconstituted in 2 mL of mobile phase followed by a 10-fold dilution prior to transfer into autosampler vials.

HPLC-MS Method for Separation and Quantification of DHD, p-HPPH and m-HPPH

Separation and quantification of both p-HPPH and DHD were achieved on a
Shimadzu LC/MS system (Shimadzu, Corp., Columbia, MD) equipped with LC-10 AD binary pumps, a SIL-10 AD VP autosampler, and a Shimadzu LCMS-2010 A Liquid Chromatograph Mass Spectrometer. The mobile phase contained 80:20 of 10mM ammonium acetate buffer, pH 5 and acetonitrile with a 1 mL/min flow rate. The column used for separation was an Agilent Eclipse XDB C18 (250x4.6 mm, 5 μm) column at ambient temperature. The detection was performed in APCI negative ion mode with selective ion monitoring (SIM) mode at m/z 285 (DHD), 267 (p-HPPH and m-HPPH), 272 (stable labeled p-HPPH), and 290 (stable labeled DHD). Five μL of each of the samples were injected to be analyzed into the LC/MS system. The DHD peak eluted at about 3.0 minutes, the p-HPPH peak eluted at about 10.6 minutes, and the m-HPPH peak eluted at about 13.5 minutes. Samples of chromatograms for the separation of DHD, p-HPPH, and m-HPPH on LC/MS system are depicted in Figures 1.14A and 1.14B. The peak areas of each metabolite of interest and the internal standard were calculated with Shimadzu LCMS Solution software (Shimadzu, Corp., Columbia, MD).
**Figure 1.14.** Chromatograms acquired from a patient’s urine sample

(A) Top: Blank urine, Bottom: a patient’s urine sample showing DHD with retention time of 3.056 minutes.
(B) Top: Blank urine, bottom: a patient’s urine sample showing p-HPPH with retention time of 10.759 minutes and internal standard, m-HPPH, with retention time of 13.686 minutes.
**Extraction Efficiencies of DHD, p-HPPH, and m-HPPH**

Five replicates of blank urine with the same volume as the patient sample were spiked with the middle concentration of stock standards (20 µg/mL of DHD and 120 µg/mL of p-HPPH) and 99.6 µg/mL of m-HPPH. The spiked urine samples were then subjected to the same extraction procedure as the patient urine samples. The peak areas obtained from these extracted standards were compared to their corresponding unextracted standards. The extraction efficiencies of each analytes can be found on Table 1.1.

**Table 1.1.** Extraction efficiencies of PHT principal metabolites and internal standard

<table>
<thead>
<tr>
<th>Extraction Efficiency ± Standard deviation</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHD</td>
<td>0.518 ± 0.011</td>
</tr>
<tr>
<td>p-HPPH</td>
<td>0.895 ± 0.028</td>
</tr>
<tr>
<td>m-HPPH</td>
<td>0.771 ± 0.005</td>
</tr>
</tbody>
</table>

Two concentrations of DHD (8 µg/mL and 45 µg/mL) and three concentrations of p-HPPH (50, 150, and 250 µg/mL) were used to assess intraday accuracy and precision (Table 1.2) as well as validation of the LC/MS assay. The assay was validated by running triplicates of the standard curve over a period of five days. The accuracy of the assay was determined by comparing the concentration of each metabolite recovered in quality control samples after subjected to the same extraction procedure to its actual
concentration. The precision of the assay was determined by calculating the coefficient of variation of each metabolite at different concentrations. The FDA guidelines dictate that the percent of coefficient of variation should be less than 15%. The limit of detection (LOD) of p-HPPH was 0.13 ng and 0.20 ng for DHD at a signal to noise ratio of 10.

**Table 1.2.** Inter-day accuracy and precision of the quantitation of principal urinary metabolites of phenytoin in human urine

<table>
<thead>
<tr>
<th>Concentration in µg/mL</th>
<th>% Accuracy</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>100.3</td>
<td>13.3</td>
</tr>
<tr>
<td>45</td>
<td>98.7</td>
<td>11.7</td>
</tr>
<tr>
<td>p-HPPH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>89.1</td>
<td>8.98</td>
</tr>
<tr>
<td>150</td>
<td>107.9</td>
<td>10.6</td>
</tr>
<tr>
<td>250</td>
<td>102.1</td>
<td>5.4</td>
</tr>
</tbody>
</table>

*Data and Statistical Analysis*

The ratio of peak areas between the metabolite of interest and the internal standard were exported to MS Excel and linear regression for each standard curve was done with SigmaPlot 10.0. The reciprocal of y (ratio of peak areas) was utilized as the weighting factor. The linear regression equation obtained was then used to calculate the concentration of each metabolite of interest in patient samples. Assuming that both DHD and p-HPPH represent greater than 90% of all absorbed drug metabolites excreted in the urine (Kadar et al., 1983; Browne and LeDuc, 2002), the percent of dose
recovered in the urine was calculated based on the total metabolites recovered within a
dosing interval (at steady-state) and the dose received. In order to account for the actual
PHT dose that entered the body, the 24-hour dose was adjusted based on the
formulation of PHT administered to each subject. For instance, if patient received a
PHT sodium as a Kapseal formulation, the PHT dose was normalized by multiplying
the dose with the actual amount of PHT in Kapseal formulation, i.e. there is 27.6 mg of
PHT in 30 mg PHT sodium Kapseal. Then the plot of bioavailable dose (F*dose) using
the bioavailability obtained from NONMEM versus the total metabolites recovered in
the urine was generated with MS Excel. Bioavailable dose is the actual amount of the
administered dose that reaches the systemic circulation. The normality tests obtained
from SAS®9.2 (SAS Institute, Inc., Cary, NC) showed both the total major urinary
metabolites recovered and bioavailable dose variables were not normally distributed.
Therefore, the Spearman Rank Correlation test was used to examine the correlation
between these two variables.
RESULTS

Dosing of PHT

The average PHT dose received by the subjects was 4.79 mg/kg/day with a range of 1.88 mg/kg/day to 8.73 mg/kg/day. Majority of the patients took PHT in Dilantin® Kapseal formulations, however, three patients took PHT as an Infatabs® formulation, and one took Mylan (a generic brand of PHT).

Extraction and Purification of DHD

One subject on 300 mg of PHT daily dose provided overnight urine. Three mg of DHD and 5.9 mg of diastereomeric DHD were obtained from the purification.

Quantification of DHD, p-HPPH, and m-HPPH

Because the stable labeled dose of PHT only accounted for about 33% of the total daily dose, the amount of both stable labeled metabolites in the urine did not make any noticeable change in the dose recovery. The distribution of percent dose recovered in the patients’ 24-hour urine samples and absolute bioavailability by age is presented in Table 1.3. The distribution of the percent 24-hour recovery of total principal urinary metabolites recovered in the 24-hour urine relative to bioavailable dose, is illustrated in Figure 1.15. A positive correlation between the two parameters was significant (p-value
= 0.00924) based on the Spearman Rank Correlation test. However, only 16.6% (R²) of the variability in the total metabolites recovered in the 24 hour urine was explained by the linear regression of the total urinary metabolites recovered on the bioavailable dose as illustrated in Figure 1.16.

Table 1.3. Distribution by age group of percent PHT dose recovered in 24 hour patient urine samples and absolute bioavailability determined from plasma PHT concentration.

SL = stable isotope label.

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Sample size</th>
<th>Range of percent dose recovered</th>
<th>Percent dose recovered (mean ± s.d)</th>
<th>Absolute Bioavailability from SL-data (mean ± s.d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>21 – 49</td>
<td>12</td>
<td>13.5% - 62.2%</td>
<td>35.4% ± 15.7%</td>
<td>0.864 ± 0.194</td>
</tr>
<tr>
<td>65 – 93</td>
<td>28</td>
<td>11.8% - 85.3%</td>
<td>32.9% ± 15.0%</td>
<td>0.925 ± 0.252</td>
</tr>
</tbody>
</table>

Figure 1.15. A histogram depicting distribution of the percent recovery of total principal urinary metabolites.
Figure 1.16. The weak correlation between total urinary metabolites recovered and bioavailable dose. However, Spearman Rank Correlation test showed a significant correlation ($r^2 = 0.280$ and slope = 0.267).
DISCUSSION

Despite dosing challenges due to PHT’s narrow therapeutic range and absorption, distribution, metabolism, and excretion (ADME) characteristics, PHT is still widely prescribed for treatment of seizure disorders. Moreover, highly protein bound PHT is known to be almost completely excreted as metabolites (Kadar et al., 1983; Browne and LeDuc, 2002). Therefore, investigating PHT absolute oral bioavailability at steady state is helpful in providing specific information about PHT’s ADME characteristic in each subject. In the current study, both stable labeled and nonlabeled PHT were utilized to directly determine the pharmacokinetic parameters.

Glazko et al. reported means of 81.9% and 81% for urinary dose recoveries (total radioactivity) from a 250 mg intravenous (IV) dose and a 250 mg oral dose of $^{14}$C-labeled PHT, respectively in six healthy male subjects (Glazko, 1987). This study suggested that oral dosing of PHT result in 100% absorption. Therefore, the urinary dose recoveries in this study were expected to correlate strongly with their corresponding absolute oral bioavailability. However, Figure 1.16 shows a very weak correlation with dose recovery and underestimates the bioavailable dose. The possible explanations for this phenomenon are related to the variability in the gastrointestinal (GI) absorption, subjects’ compliance, unaccounted biliary-fecal excretion of PHT, unaccounted biliary-fecal excretion of p-HPPH glucuronide, and incomplete urine collection.
The absorption of PHT depends on its pKa, lipophilicity, concentration, pH of the absorption site and the solubility of PHT in the intestinal fluid. The formulation of PHT, subjects’ diet or other co-medications can influence the aforementioned factors (Woodbury, 1982). Concentrations of PHT were normalized during data analysis based on the PHT formulation received by each subjects. Dill et al. also showed variability of PHT absorption in different parts of isolated segments of rat GI tract. They showed that PHT had a low level of absorption in the stomach. The extent of absorption gradually increased and reached a maximum in the upper small intestine, thereafter, absorption decreased as PHT entered the large intestine (Dill et al., 1956). Melander et al. found an increase in PHT absorption after a meal in eight healthy volunteers who received single doses of 300 mg PHT. There was an increase in PHT levels in serum; however, the elimination process remained relatively unaffected (Melander et al., 1979). This finding was also corroborated by the work of Sekikawa et al., who demonstrated a significant increase in the urinary excretion rate of p-HPPH in five healthy volunteers who received balanced meals (Sekikawa et al., 1980). The boost in the PHT absorption observed with the presence of food was postulated due to the enhancement of PHT dissolution in the GI tract as well as the retention of PHT in the region of upper small intestine where most of the PHT absorption occurred (Sekikawa et al., 1980).

Patient noncompliance is the most common cause of epilepsy treatment failure (Leppik and Schmidt, 1988). In this study, all subjects were assumed to be on PHT steady state. Depending on the subjects’ compliance, they might not have been at steady-state at the beginning of the study, which could partially explain the low dose
recoveries observed. However, patient compliance in this study was likely good. On the study day, patients were directly observed to take their oral dose by nursing staff and maintenance concentrations in plasma were similar to prior measurements. Poor compliance just prior to admission into the GCRC is a possible explanation, but is unlikely in this group of motivated patients. Every attempt was made to get complete urine collections within the GCRC and patients were instructed by nursing staff to collect all of urine. In a study of intravenous PHT disposition in normal human subjects by Glazko et al., there was a lag time of between 6 to 8 hours of p-HPPH excretion after a single dose, which was hypothesized to be due to the slow hydroxylation or conjugation of PHT metabolites prior the excretion (Glazko et al., 1969). In addition, they found that the percentage of excreted PHT dose kept increasing over a period of 3 days from 36% to 60% and to 71% (Glazko et al., 1969). This finding implies that a 24-hour urine collection would not be adequate after a single dose in order to study the metabolite recovery of PHT because it does not capture all the p-HPPH excretion from the study dose. Furthermore, it explains the scarcely detectable level of stable labeled metabolites in the patient urine samples in this study. However, all our patients were at steady-state. The steady-state assumption is supported by the finding that PHT trough concentrations were relatively constant over a period of 196 hours (Figure 1.17). Depending on the time interval between the evening dose and the first study dose, this factor might also contribute to the variability in the excreted metabolites levels that we observed in this subject population. If subjects took their second PHT maintenance dose later in the evening, the amount of excreted dose might be delayed.
**Figure 1.17.** PHT levels for all subjects within 196 hours after receiving the tracer dose along with the remainder oral PHT maintenance dose.

The distribution of percent dose recovered in 24 hour patients’ urine samples classified by age showed no apparent trend. The highest percent of dose recovered was 85.3% and the lowest percent of dose recovered was 11.8%. These results clearly contradict the aforementioned reported dose recovery by Browne and LeDuc and Kadar et al. (Kadar et al., 1983; Browne and LeDuc, 2002). One might think other PHT metabolites contribute substantially to the dose recovery. However, the other known metabolites such as (3, 4-dihydroxyphenyl)-5-phenylhydantoin and 5-(4-hydroxy-3-
methoxyphenyl)-5-phenylhydantoin accounted for less than 3% of total dose (Glazko, 1987). Unfortunately, authentic standards of these metabolites were not available to us, but combined, both of these metabolites would not alter the dose recovery dramatically. The variable results were very surprising because of the nature of the study environment. All of the subjects were admitted to the GCRC at least a few hours prior to the start of the study and a nurse practitioner was always present when subjects took the study dose.

Unaccounted fecal excretion of PHT could also explain the low urinary metabolites recovery in this study. Glazko et al. reported 4.6% and 7.7% mean fecal dose recoveries from a 250 mg intravenous (IV) dose and a 250 mg oral dose of $^{14}$C-labeled PHT, respectively in six healthy male subjects (Glazko, 1987), suggesting that some biliary-fecal excretion may be occurring after IV dosing. Kadar et al. reported 30.6% and 36.4% fecal radioactivity recoveries from two healthy male volunteers each receiving a 200 mg oral dose of $^{14}$C-labeled PHT (Kadar et al., 1983) that was presumed to be from unabsorbed PHT. The portion of the dose recovered in the feces that are usually neglected in accounting for PHT dose recovery might be significant. The absolute bioavailability of PHT as determined by the stable-labeled isotope technique in this study was $0.91 \pm 0.24$. In five young healthy male volunteers, bioavailability of single dose PHT was estimated as $0.87 \pm 0.14$ (Lund et al., 1974). With the same pharmacokinetic data set, Jusko et al reanalyzed the concentration-time profile by a nonlinear method and obtained a mean bioavailability of $0.98 \pm 0.07$ (Jusko et al., 1976).
Another possible explanation for variable or poor urinary recovery of metabolites is biliary-fecal excretion of p-HPPH glucuronide. In rats, p-HPPH glucuronide is excreted into bile via MRP2 (Auansakul and Vore, 1982). It is unknown whether the glucuronide is a substrate for human MRP2, but if this were the case, fecal excretion of intact p-HPPH glucuronide or deconjugated p-HPPH (from bacterial β-glucuronidase in the colon) may account for incomplete recovery. Kadar et al. determined the fecal excretion in two subjects receiving ^14^C-PHT. Thirty-five percent of the dose was recovered in feces, mainly as intact PHT (Kadar et al., 1983). Glazko et al. in a similar design reported that fecal excretion of oral radiolabeled PHT in six young male volunteers ranged from 5-8%, whereas p-HPPH accounted for 50-80% of the fecal radioactivity, the DHD was 15-35%, and 5-10% of free catechol (Glazko et al., 1977).

Our study is the only study to be done in patients under steady-state. The dose recovery studies conducted by Glazko et al. in six healthy volunteers and Kadar et al. in two healthy volunteers used single-dose radiolabeled PHT, however, the subjects in our study were epileptic and on steady-state PHT. The difference in our findings with these two studies may be due to difference in the subjects’ health status and their PHT’s steady-state status.

In summary, an underestimation of absolute bioavailability of PHT in epilepsy patients was observed when urinary recovery of metabolites is used. The findings indicate that stable-isotope technology was a more appropriate approach to determine the absolute bioavailability in patients on maintenance therapy. PHT has a long
variable elimination half-life, ranging from 10 to 69 hours, with slowly increasing plasma concentrations until steady-state is reached (Browne and LeDuc, 2002). With a single dose and cross-over design, urine collections for a minimum of five half-lives after administration of a stable isotope dose could be conducted even though the process would be impractical and in epilepsy patients it is not possible to stop therapy. The large variability in PHT absolute bioavailability observed in this study suggests that this drug is not as well absorbed as previously thought. This may be especially true in elderly patients. A previous study conducted by our group in nursing home patients found high intrasubject variability in “steady state” therapeutic concentrations (Birnbaum et al., 2003). It is likely that much of this variability can be attributed to variable absorption. Finally, the confounding factor of patients’ compliance would influence the underestimation of absolute bioavailability observed in the current study. Compliance is crucial to maintain therapeutic concentration of PHT because it is the most common factor for the failure of epilepsy treatment (Leppik and Schmidt, 1988).
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CHAPTER 2

Expression of Oxysterol 7 Alpha - Hydroxylase (hCYP7B1) in *E.coli*

INTRODUCTION

*Prostate Cancer and Available Treatments*

Prostate cancer is a cancer that initially often grows in the prostate gland. The occurrence of prostate cancer is prevalent in older men. Prostate cancer is the second most common cause of cancer deaths in males. In 2009, the National Cancer Institute estimates about 192,000 new cases and 27,000 deaths caused by prostate cancer in the United States (Institute, 2008). The American Cancer Society states that about 5-10% of prostate cancer cases are linked to genetic inheritance (Society, 2009). However, the causes of prostate cancer are not yet known. Some risk factors that would increase a man’s chance to develop prostate cancer have been identified. They include age, race, nationality, family history, genes, diet, obesity, exercise, and infection and inflammation of the prostate.

Prostate-Specific-Antigen (PSA) test and the Digital Rectal Exam are the common tests utilized for prostate cancer screening. Biopsy and Gleason score assignment are then used to predict the stage of cancer and to help guide therapy. Active surveillance, surgery (prostatectomy), radiation therapy, cryosurgery, hormone therapy (androgen deprivation), and chemotherapy are the currently available treatments for prostate
cancer (Institute, 2008).

**Rationale of Prostate Cancer Linkage to CYP7B1**

The rationale for the study is best depicted in Figure 2.1 (Weihua et al., 2002). When CYP7B1 is inhibited, there should be an accumulation of the estrogenic compound, 5α-androstane-3β,17β-diol (3β-Adiol), which has been shown to have an anti-proliferative effect in the presence of Estrogen Receptor Beta (ERβ). 5α-reductase inhibitors such as finasteride are currently used to prevent prostate cancer with mixed results. This may be due to a decrease in both DHT (AR agonist) and 3β-Adiol (ERβ agonist).

ERβ can be found in both normal and malignant human prostate. Unlike ERα that is selectively expressed in the stroma, ERβ is present in the stroma (35%) and epithelium (>95%) (Horvath et al., 2001). The highest expressions of ERβ in human tissue were found in the nuclei of granulose cells of ovarian follicles in females and prostate in males (Taylor and Al-Azzawi, 2000). By an in situ hybridization technique, Kuiper et al. showed that ERβ expression was present highly in rat prostate epithelium and selectively in the ovaries (Kuiper et al., 1996). In addition to estradiol and 3βAdiol, 7α-hydroxy DHEA had been shown to act as ERβ agonist in CYP7B1-transfected COS-1 cells and HepG2 cells (Martin et al., 2004). In an ER-responsive luciferase reporter assay in HEK293 cells overexpressing ERβ, Pettersson et al. showed that 3β-Adiol
efficiently activated ERβ compared to 7α-hydroxy DHEA (Pettersson et al., 2008).

**Figure 2.1.** The proposed mechanism of 3β-Adiol and ER β in the prostate growth.

3β-Adiol, a metabolite of dihydrotestosterone (DHT), is metabolized to inactive 6α, 7α, and 7β triols in rat, cynomolgus monkey, and human prostate microsomes (Gemzik et al., 1992). Inhibition of 7α and 7β hydroxylation of 3β-Adiol, but not 6α-hydroxylation, was observed in human prostate microsomes in the presence of rat liver NADPH-cytochrome P450 reductase antibody or with imidazole-containing antifungal drugs. These results led them to believe that 7α and 7β hydroxylation of 3β-Adiol was mediated by cytochrome P450 enzymes, but not 6α-hydroxylation (Gemzik et al., 1992). Weihua et al. hypothesized that CYP7B1 promoted cell proliferation by inactivating 3β-Adiol because of their observations of a high expression...
of CYP7B1 and a low expression of ER β in proliferating epithelial cells in the rat ventral prostate compared to the opposite phenomenon was observed in non-proliferating cells. In the same study, the authors successfully showed that in proliferating cells in rat ventral prostate possessed a higher level of Androgen Receptor (AR) compared to the surrounding cells and the required presence of ERβ for 3β-Adiol to exert its anti-proliferative effects in ER β knockout (BERKO) mice model (Weihua et al., 2002). Previously, Weihua et al. had observed an increase in AR level in BERKO mouse prostate and a reduction in AR levels in wild-type mice that received 3 β-Adiol treatments, but this effect was not observed in the BERKO mice (Weihua et al., 2001).

**Steroid-Specific Cytochrome P450**

Cytochrome P450 (CYP) is a member of a superfamily of heme-containing monooxygenases involved in xenobiotic metabolism, cholesterol biosynthesis, bile acid synthesis, and steroid biosynthesis, is mainly found in the endoplasmic reticulum. Ten out of 18 mammalian families of P450s are involved in the steroid biosynthesis. CYP17 and CYP19 (aromatase) are responsible for estrogen biosynthesis (Guo et al., 2006) from androgens. CYP7A, CYP8B, CYP27 are involved in bile acid biosynthesis (Javitt, 1994; Ishida et al., 1999; Hall et al., 2001). CYP11B and CYP21 are responsible for glucocorticoid (cortisol) and mineral corticoid (aldosterone) production (White et al., 1986; Zhang and Miller, 1996). CYP46 is involved in elimination of excess cholesterol (Wolozin, 2003). CYP51 is involved in sterol biosynthesis (Lepesheva and Waterman, 2004). CYP7B1 and CYP39 are oxysterol 7 α-
hydroxylases. CYP7B1 possesses broad substrate specificity for androgens (Li-Hawkins et al., 2000).

**Oxysterol 7α-Hydroxylase (CYP7B1)**

Human oxysterol 7 α-hydroxylase (CYP7B1), located on chromosome 8q21.3, consists of six exons and five introns that are located at the same position as the introns of human cholesterol 7α-hydroxylase (CYP7A1) (Setchell et al., 1998). Setchell et al. also showed 65% and 66% of identical amino acid sequence in the encoded human protein to the rat and mouse enzymes, respectively. Stapleton et al. observed mouse and rat hct-1 genes (CYP7B) shared 39% amino acid sequence identity with human CYP7A polypeptides (Stapleton et al., 1995). Hepatic CYP7B1 expression and mRNA levels in mice show a sexual dimorphism, as male mice express more CYP7B1 and possess more mRNA than female mice (Li-Hawkins et al., 2000). In human, the mRNA level of CYP7B1 has been shown to be age-dependent and tissue-specific. Fetal extrahepatic tissues had been shown to possess higher mRNA levels compared to adult tissues (varies by 2-20 fold) (Tang et al., 2006). The age-dependent increase in hepatic CYP7B1 level and decrease in renal CYP7B1 level in human multiple tissue cDNA panel were in agreement with a subsequent pig study (Pettersson et al., 2008).

Both CYP7A1 and CYP7B1 involve in bile acid synthesis pathway. There are two pathways involved in bile acid synthesis. The classic pathway is initiated by 7α-hydroxylation of cholesterol in the liver. The alternate pathways is initiated by sterol-
27-hydroxylase in the mitochondria, followed by 7α-hydroxylation by CYP7B1 in the liver, and finally converted to bile acids (Wu et al., 1999). CYP7A1 is selectively expressed in the liver for bile acid synthesis. Based on CYP7B1 mRNA levels, Wu et al. reported that CYP7B1 is expressed in tissues involved in steroid production and neurosteroid metabolism (brain and reproductive organs), in bile acid synthesis (liver), and in absorption (colon, kidney, small intestine) (Wu et al., 1999).

CYP7B1 catalyzes the 7α-hydroxylation of sterol molecules. With a thin layer chromatography method, Kim et al. confirmed that CYP7B1 has the preference of 7α-hydroxylation compared to 7β-hydroxylation for both DHEA and pregnenolone (Kim et al., 2004). They found the Rf values of DHEA, 7α-hydroxy-DHEA, and 7β-hydroxy DHEA were 0.72, 0.30, and 0.20, respectively, based on co-chromatography with authentic standards in an ethyl acetate solvent system on silica gel plates. Some of endogenous substrates of CYP7B1 include dehydroepiandrosterone (DHEA), pregnenolone (Rose et al., 1997; Kim et al., 2004), 25-hydroxycholesterol (Toll et al., 1994), 27-hydroxycholesterol (Martin et al., 1993), 5α-androstane-3β,17β-diol (3β-Adiol) (Kim et al., 2004), 5α-dihydrotestosterone (DHT) (Warner et al., 1989), and epiandrosterone (Kim et al., 2004).

Polymorphisms of CYP7B1

Two single nucleotide polymorphisms (SNPs) have been identified in CYP7B1. The first SNP is a transition of cytosine to thymidine in exon 5, which leads to the
creation of premature stop codon at position 388 (Setchell et al., 1998). This inactive enzyme perturbs bile acid synthesis and leads to increased urinary bile acid excretion and an accumulation of hepatotoxic unsaturated monohydroxy bile acids in a newborn. This neonate showed a severe cholestasis and cirrhosis. The second SNP is a transversion of -104C>G in the promoter region that leads to an enhanced affinity of the transcription factor, C/EBPα, to the CCAAT-box element. The allele frequency of this variant is 4.04% in Swedish men and 0.33% in Korean men (Jakobsson et al., 2004).

**Regulation of CYP7B1 Metabolism**

In normal human prostate, DHEA is present of 10-fold higher concentrations than βAdiol. In benign prostate hyperplasia, the level of both DHEA and 3βAdiol are reduced by approximately 40% (Voigt and Bartsch, 1986). Pettersson et al. showed the rate of hydroxylation of 3βAdiol was decreased by 70% in HEK293 cells and 90% in pig liver microsomes in the presence of 10-fold higher concentration of DHEA (Pettersson et al., 2008). They then proposed the regulation of 3βAdiol metabolism by CYP7B1 was inhibited in the presence of high DHEA level (Figure 2.2). The subsequent accumulation of 3βAdiol would further activate the ERβ.

Moreover, Pettersson et al. showed there were tissue- and age-specific differences, but no gender difference in the metabolism of DHEA and 3βAdiol in pigs. The rate of 7α-hydroxylation in the liver increased with age in both male piglets and adult pigs, whereas, the rate in the kidney decreased with age (Pettersson et al., 2008).
These authors also showed similar affinity of CYP7B1 towards DHEA ($K_m$ value of 5 $\mu$M) and 3βAdiol ($K_m$ value of 3 $\mu$M) (Pettersson et al., 2008).

**Figure 2.2.** The proposed regulation of the 3βAdiol metabolism in the presence of high DHEA level

Despite the selective location of ERβ in the stroma, the level of CYP7B1 mRNA has been shown to increase in the presence of ERβ and estradiol. ERβ had been shown to stimulate CYP7B1 promoter activity in HEK293 cells. Tang et al. proposed another pathway of how ERβ regulates DHEA level in the cells via upregulation of CYP7B1 expression (Figure 2.3) (Tang et al., 2006). In this pathway, estrogens increase DHEA
7α-hydroxylation, resulting in a lower synthesis of estrogens from DHT.

**Figure 2.3.** The proposed mechanism of regulation of DHEA level via upregulation of CYP7B1 by ERβ

![Diagram of steroid metabolism involving DHEA, CYP7B1, and ERα](image)

**CYP7B1 Gene Transcription Regulation**

With CYP7B1 clones obtained from cDNA libraries of human hippocampus, Wu et al. found CAAT and three GC clusters between nucleotide -105 and nucleotide -26, respectively, instead of the classical TATA box between nucleotide -709 and nucleotide -703 upstream of the transcription start site (Wu et al., 1999). Mutations of the first GC cluster decreased the promoter activity by 60% and only 20% promoter activity remained when all three GC clusters were mutated (Wu and Chiang, 2001). This
clearly indicated that the importance of the presence of GC clusters (nucleotide -25 to nucleotide +10) in the CYP7B1 promoter region for basal transcriptional activity. Furthermore, based on electrophoretic mobility shift assay, Wu and Chiang observed the presence of a complex of Sp (Specificity protein) 1 and the GC cluster in the HEK293 nuclear extract (Wu and Chiang, 2001). Given that Sp1 is expressed in many tissues and responsible for transcription of housekeeping genes and many other genes, Wu and Chiang hypothesized that Sp1 would interact with Sterol Response Element Binding Protein (SREBP) in the nucleus (Wu and Chiang, 2001). Cotransfection of CYP7B1 promoter-luciferase reporter gene with SREBP-1a and SREBP-1c in rat hepatoma cells (McA-RH7777) showed a suppression of CYP7B1 promoter activity (Norlin and Chiang, 2004). Norlin and Chiang also showed CYP7B1 promoter activity was lost when all GC clusters, which had been previously shown to form a complex with Sp1, were deleted in the presence of SREBP-1. In addition, CYP7B1 promoter activity was shown to be suppressed in the presence of both Sp1 and SREBP-1c. Due to the absence of direct binding of SREBP to CYP7B gene, the suppression mechanism of CYP7B1 transcription by SREBP was postulated by inhibition of Sp1 by SREBP to the GC clusters (Norlin and Chiang, 2004).

Oxysterols play an important gene regulatory role in the body; however, they can be toxic at higher level. The balance of these oxysterols has to be tightly controlled. Norlin and Chiang proposed a regulation of oxysterols level pathway by CYP7B1. When the level of oxysterols was high due to cholesterol metabolism, these oxysterols will suppress SREBP. The result of this inactivation was an increase in the CYP7B1
gene transcription. The presence of more CYP7B1 protein will then catalyze the metabolism of oxysterols (Figure 2.4) (Norlin and Chiang, 2004).

**Figure 2.4.** The proposed mechanism of oxysterol level regulation pathway by CYP7B1

![Diagram of oxysterol level regulation pathway](attachment:image.png)

Retinoid-Related Orphan Receptor α (RORα) (NR1F1) had been shown to be an activator of Cyp7b1 expression and LXRα had been shown as an inactivator of activation of Cyp7b1 promoter by RORα (Wada et al., 2008).

DHT has been identified to act as a controller of intraprostatic androgen and estrogen level (Figure 2.5). In the presence of DHT, CYP7B1 promoter activity was inhibited in a dose-dependent manner (Tang and Norlin, 2006). Tang and Norlin also showed that the DHT response element is located upstream in the promoter region within the first 100 bases and when DHT binds, it suppresses CYP7B1 expression. Overexpression of ERβ and treatment with DPN (ER agonist) increased CYP7B1 promoter activity, whereas, treatment with ICI 182,780 (ER antagonist) abolished the promoter activity (Tang and Norlin, 2006).
**Figure 2.5.** The proposed intraprostatic androgen and estrogen balance mechanism by suppression of CYP7B1
MATERIALS AND METHODS

Chemicals

Difco™ Luria-Bertani Broth, LB Agar, and Terrific Broth (TB) were obtained from BD Biosciences (San Jose, CA). Basal Medium Eagle (BME) vitamins 100x solution, ethylenediaminetetraacetic acid (EDTA), γ-aminolevulinic acid hydrochloride (Allabi et al.), dioxane free isopropyl-β-D-thiogalactopyranoside (IPTG), thiamine hydrochloride, tetrasodium salt of β-Nicotinamide Adenine Dinucleotide Phosphate (NADPH), 1,2-didodecanoyl-rac-glycero-3-phosphocholine (DLPC), ferric chloride hexahydrate, sodium molybdate dihydrate, copper (II) chloride, phenylmethylsulfonyl fluoride (PMSF), and sodium dithionite were purchased from Sigma-Aldrich (St. Louis, MO). Low EEO/multipurpose molecular biology grade agarose, electrophoresis grade 50x solution of Tris-Acetate-EDTA, 1% solution of Ethidium bromide, glycerol, crystallized free base Tris, potassium phosphate dibasic, zinc chloride, cobaltous chloride hexahydrate, boric acid, hydrochloric acid, ACS grade methyl tert-butyl ether, sucrose were obtained from Fisher Scientific, Co. (Fairlawn, NJ). Regisil® RC-2 (N,O-Bis(Trimethylsilyl) trifluoroacetamide + 1% Trimethylchlorosilane; BSTFA + 1% of TMCS) was purchased from Regis Technologies, Inc. (Morton Grove, IL). 3β-hydroxy-5-androsten-17-one (DHEA) and 3β,7α-dihydroxy-5-androsten-17-one (7-OH DHEA) were obtained from Steraloids, Inc (Newport, RI). Calcium chloride hexahydrate and ampicillin sodium salt were purchased from Acros Organics (Geel, Belgium).
Materials

1 μg of dried cDNA of *Homo sapiens* cytochrome P450, family 7, subfamily B, polypeptide 1 (NM_004820.2) in pCMV6-XL4, 100 pmol of dried 5’(VP1.5) and 3’ (XL39) vector primers were obtained from OriGene Tech, Inc. (Rockville, MD). One Shot® Stbl3™ Chemically Competent *E.coli*, MAX Efficiency® DH5αF’IQ™ Competent cells, NADPH-Cytochrome P450 Reductase, Human cytochrome b5, Primers FTA1 (5’-CTCTTGAAAGCATGATG-3’), FTA2 (5’-ATGGCAGCAGTGCGTGACG-3’), FTA3 (5’-ATGATAAGCCCATAGGAC-3’), FTA4 (5’-AGCCAAGCTGCAAGGCTG-3’), 10 mM dNTP mix, and restriction endonucleases such as Sall, NdeI, and EcoRI, Pfx50™ DNA Polymerase were purchased from Invitrogen (Carlsbad, CA). QIAprep Spin Miniprep Kit, QIAquick Gel Extraction Kit, and QIAGEN Plasmid Midi Kit were obtained from Qiagen, Inc. (Valencia, CA). Sterile Corning® petri dishes, protease inhibitor cocktail (Product # P 8849) for use in purification of 6-histidine-tagged proteins were obtained from Sigma-Aldrich (St. Louis, MO). FMTA (5’-CATATGGCATTATTATGCGTGGCCACCGGGCGCTTTTCGCT-3’), RMTA (5’-GTCGACAGATTTCACTTTGTATCTAAATAAAACATCG-3’), and SpCWTA (5’-CGGCTCGTATAATGTGTGCGTATAATGTGTGG-3’) were ordered through Integrated DNA Technologies (Coralville, IA). TaKaRa PrimeSTAR® HS DNA Polymerase was purchased through Clontech Laboratories, Inc. (Madison, WI). Quick Ligation™ Kit was obtained from New England BioLabs (Ipswich, MA). Pfu Ultra II Fusion HS DNA Polymerase was a gift from Stratagene (Agilent Technologies, Inc., La Jolla, CA). Fisher BioReagents exACTene 1 kb and 1 kb Plus DNA ladders were bought from Fisher Scientific, Co
Preparation of Solutions

Preparation of 10 mM Tris, 1 mM EDTA buffer pH 7.5
Dissolve 0.61 g of crystallized free base Tris and 0.15 g of EDTA in 500 mL of deionized water. A few drops of concentrated HCl were added into the constantly stirred solution until pH 7.5 was reached.

Preparation of 50 mg/mL Ampicillin solution
Five g of ampicillin sodium salt were dissolved in 100 mL of deionized water, followed by aliquoting the solution into 1.5 mL centrifuge tubes.

Preparation of trace element stock
A mixture consisted 13.5 g of ferric chloride hexahydrate, 1.0 g of zinc chloride, 1.0 g of cobaltous chloride hexahydrate, 1.0 g of sodium molybdate dihydrate, 0.5 g of calcium chloride hexahydrate, 0.5 g of copper (II) chloride, 0.25 g of boric acid, and 50 mL of hydrochloric acid were dissolved in 500 mL distilled water followed by a 30-minute autoclaving.

Preparation of 1M Thiamine
Dissolve 5.06 g of thiamine hydrochloride in 15 mL of distilled water.
**Preparation of LB-Ampicillin agar plates**

Forty g of Difco™ LB agar, Miller were dissolved in 1 L of distilled water followed by autoclaving for 30 minutes and 2.0 mL of 50 mg/mL Ampicillin solution added into a lukewarm LB Agar solution before letting the agar solidify in the petri dishes.

**Preparation of LB broth**

Twenty-five g of Difco™ LB broth, Miller were dissolved in 1 L of distilled water followed sterilization for 30 minutes in an autoclave.

**Preparation of Terrific Broth (TB)**

Dissolve 23.8 g of Difco™ TB broth in 500 mL of distilled water in a round bottom Fernback flask. Two mL of glycerol were added before autoclaving the broth mix for 30 minutes.

**Preparation of 1M IPTG**

Dissolve 3.57 g of IPTG into 15 mL of distilled water.

**Preparation of 500 mM ALA**

Dissolve 1.26 g of ALA into 15 mL of distilled water.

**Preparation of Storage buffer (50 mM potassium phosphate, 0.5 mM EDTA, and 20% glycerol, pH 7.4)**

Potassium phosphate dibasic (8.71 g), 0.15 g of EDTA, and 200 mL of glycerol (20%)
were brought up to 1 L with distilled water. This solution was autoclaved for 30 min. A few drops of concentrated HCl were added into the constantly stirred solution until pH 7.4 was reached.

*Preparation of Buffer A (100 mM potassium phosphate, 500 mM sucrose, 0.5 mM EDTA, pH 7.6)*

Mix 8.71 grams of potassium phosphate dibasic, 85.58 grams of sucrose, 0.07 gram of EDTA, and 100 mL of glycerol in distilled water and then take up to a total volume of 500 mL. The pH was adjusted to 7.6 with concentrated HCl.

*Preparation of Buffer B (100 mM potassium phosphate, 1 mM PMSF, 20% glycerol, pH 7.6)*

Mix 8.71 grams of potassium phosphate dibasic, 0.09 gram of PMSF, and 100 mL of glycerol in distilled water to a total volume of 500 mL.

*Preparation of Buffer C (500 mM potassium phosphate, 250 mM sucrose, 0.25 mM EDTA)*

Dissolve 43.55 grams of potassium phosphate dibasic, 42.79 grams of sucrose, and 0.04 gram of EDTA in 500 mL of distilled water.

*Preparation of 100 mM potassium phosphate buffer with 20% glycerol*

Mix 8.71 grams of potassium phosphate dibasic and 100 mL of glycerol in distilled water to a total volume of 500 mL.
**Cloning and Transformation of CYP7B1 in pCMV6-XL4**

Fifteen µL of sterile distilled water were added to a vial containing 1 µg of dried CYP7B1 cDNA. A vial containing 50 µL of One Shot Stbl3 cells (Invitrogen, Inc., Carlsbad, CA) was thawed on ice. About three ng of human CYP7B1 cDNA were added to the thawed cells, followed by 30-min incubation on ice. The cell mixture was heat-shocked for 45 sec at 42°C without shaking in Isotemp 202S Fisher Scientific Incubator (Fisher Scientific, Fairlawn, NJ). The cell mixture was then cooled on ice for 2 min. Two hundred and fifty µL of warmed LB broth were added before the cell mixture was shaken on a New Brunswick Scientific C25K Incubator Shaker Classic series (New Brunswick Scientific, New Brunswick, NJ) at 37°C for 1 hour at 225 rpm. Following the incubation, different volumes (5, 10, 15, 20 µL) of the cell mixture were spread onto the LB-Amp plates in the sterile fume hood. The plates were then inverted and incubated overnight at 37°C. The following morning, the plates were taken out from the incubator and kept in the refrigerator until the preparation of plasmid purification was ready.

**Plasmid Purification of pCMV6-XL4 Containing CYP7B1 cDNA**

A pre-culture that consisted of 5 mL LB broth and 10 µL of 50 mg/mL ampicillin were inoculated with one isolated colony from the transformation that was done on the plate. The following day, the pre-cultures were centrifuged at 4°C for 10 min at 3500 rpm in an Eppendorf Centrifuge 5180R (Eppendorf AG, Hamburg, Germany). The cell
pellets were then subjected to plasmid purification treatment by a Qiaprep Spin Miniprep Kit protocol using microcentrifuge. The elution step was done with 10 mM Tris, 1 mM EDTA buffer pH 7.5 instead of Qiagen’s buffer EB.

**Preparation of Sequencing Reaction Samples**

In order to check the complete sequence of the human CYP7B1 cDNA obtained from Origene, the purified plasmid was subjected to DNA sequencing and analysis on an ABI PRISM™ 3730xl DNA Analyzer through University of Minnesota’s BioMedical Genomic Center (BMGC). Four forward primers that would elongate middle regions of the cDNA were ordered through Invitrogen, Inc (Carlsbad, CA) to ensure the resolution of signal reading from the sequencing analyzer. The four forward primers are FTA1, FTA2, FTA3, and FTA4. The human CYP7B1 cDNA with serial number FB2675_E07 had the perfect sequence that matches with GenBank NM_004820.2.

**Barnes Modification and Creation of NdeI and SalI Restriction Sites**

To enable the expression of recombinant Cytochrome P450 in *E.coli*, the first seven codons of the cDNA have to be modified according to the study by Barnes et al. According to Barnes et al, the first seven codons should be Methionine-Alanine-Leucine-Leucine-Leucine-Alanine-Valine to properly insert into the bacterial inner membrane (Figure 2.6). The third and fourth codons should be rich in Adenine and Uracil (Barnes et al., 1991). The creation of NdeI restriction site on 5’ end and SalI on
3’ end were performed to ensure the human CYP7B1 cDNA will be successfully sit on the expression vector pcWORi that has both sites available. pcWORi expression vector was obtained from Dr. Tim Tracy’s laboratory (Cheesman et al., 2003; Locuson et al., 2006). A forward primer, FMTA ($T_m = 71.2^\circ C$) and a reverse primer, RMTA ($T_m = 57.8^\circ C$) that introduce the Barnes modification and create NdeI and SalI restriction sites were ordered through Integrated DNA Technologies (Coralville, IA).

**Figure 2.6.** Nucleotide and amino acid sequences at the 5’ end of the human CYP7B1 cDNA before and after Barnes modification.

<table>
<thead>
<tr>
<th>Origene human CYP7B1 cDNA</th>
<th>5’- ATG GCA GGA GAA GTG TCC GCG ... -3’</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5’- Met Ala Gly Glu Val Ser Ala ... -3’</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Modified human CYP7B1 cDNA</th>
<th>5’- ATG GCA <strong>TTA TTA TTG GCC GTG</strong> ... -3’</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5’- Met Ala Leu Leu Leu Ala Val ... -3’</td>
</tr>
</tbody>
</table>

High fidelity PCR reactions with TaKaRa PrimeSTAR® HS DNA Polymerase were run to ensure the modifications would be incorporated. The amplifications were performed with a PCR program of 98°C for two min, 30 cycles of 98°C for 10 sec, 30 cycles of 61°C for 5 sec, and 30 cycles of 72°C for 1 min 50 sec. The PCR products were run on 0.7% agarose gel containing a drop of 0.625 mg/mL ethidium bromide followed by gel extraction of 1.5 kb band with QIAquick gel extraction kit (Qiagen, Inc, Valencia, CA). The gel extraction products were subjected to another PCR reaction to add poly Adenine on both 5’ and 3’ ends with Taq Polymerase (Invitrogen, Inc,
These PCR products were then cloned and transformed with Invitrogen’s TOPO TA Cloning® Kit (with pCR®2.1-TOPO® vector) with One Shot® Mach1™ T1-Phage-Resistant Chemically Competent E.coli (Invitrogen, Inc, Carlsbad, CA). The cell mixture was then plated on LB-Kanamycin agar plate and a drop of X-gal was added for screening after an overnight incubation at 37°C. Four of the successful transformed colonies (white in color) were subjected to plasmid purification treatment by the Qiaprep Spin Miniprep Kit protocol in a microcentrifuge (Qiagen, Inc, Valencia, CA). The purified plasmids were linearized with EcoRI followed by running a 0.7% agarose gel to further ensure EcoRI did not cut anywhere else on the plasmid. The purified plasmid was then sent out to University of Minnesota BMGC for sequencing with M13 forward and M13 reverse primers. The results were confirmed that both Barnes modification and the creation of NdeI and SalI restriction sites were successful.

*Double Digestion of Modified CYP7B1 Gene And Its Ligation into pCWOri Expression Vector*

The purified plasmid was subjected to double digestion with NdeI and SalI for 2 hr 30 minutes at 37°C. The reaction buffer used was RE® act 6 buffer that came with NdeI endonuclease instead of RE® act 10 buffer that came with SalI endonuclease (Invitrogen, Inc, Carlsbad, CA). Quick Ligation™ kit (New England BioLabs, Inc, Ipswich, MA) was used to perform the ligation reaction for 1 hr.
**Transformation of Modified CYP7B1 in pCWOri into DH5αF’IQ Competent Cells**

The ligation products were transformed into Maximum efficiency DH5αF’IQ competent cells that had prokaryotic molecular chaperone plasmid, pG-KJE8 (Takara Bio Inc, Japan) following the transformation procedure on page 83. One hundred μL of the cell mixture were spread on LB-Ampicillin agar plate. The plate was inverted and incubated overnight at 37°C. Ten mL pre-cultures from isolated colonies were set up to start the expression of the protein on the next day.

**Expression of CYP7B1 Protein**

One M Thiamine (500 μL), 125 μL of trace element stock solution, and 1 mL of 50 mg/mL ampicillin were added to each of the autoclaved TB broths before inoculating the broth. The inoculated broths were shaken in a New Brunswick Scientific C25K Incubator Shaker Classic series (New Brunswick Scientific, New Brunswick, NJ) at 37°C, 225 rpm. After 4 hr of shaking, the cell density was checked using Beckman Coulter DU530 Life Sciences UV/Vis Spectrophotometer (Beckman Coulter, Inc, Brea,CA) at 600 nm wavelength. The targeted reading from the UV/Vis Spectrophotometer was between 0.4-0.6. Once the targeted readings were reached, the induction for heme formation was started with 500 μL of 1 M IPTG, 500 μL of 500 mM ALA, and 500 μL of 100X BME vitamin stock. The broth mixtures were then shaken at 28°C, 160 rpm for 16 hr. After 16 hr had passed, the P450 content was checked on an Olis DW-2000 UV/Vis Spectrophotometer equipped with Olis Spectral Works software.
version 4.3 (Olis, Inc, Bogart, GA). One mL aliquot from each broth mixtures was spun at 4000 rpm for 4 minutes on an Eppendorf MiniSpin Plus® centrifuge (Eppendorf AG, Hamburg, Germany). The cell pellet was resuspended in 2.0 mL of storage buffer. A small spatula full of sodium dithionite was added to the suspension in order to reduce the heme on the protein. One mL of the mixture was transferred into two separate disposable cuvettes. One cuvette was served as a blank and the other was designated carbon monoxide bubbling. After obtaining a stable baseline on the Olis UV/Vis Spectrophotometer, the sample cuvette was bubbled with carbon monoxide for one minute followed by taking the reading on the spectrophotometer (Figure 2.7). The P450 content in nanomoles was obtained by equation 2.1. The maximal absorbance of the cytochrome was always between 452 nm to 454 nm. The cells that expressed CYP7B1 were harvested by centrifuging them at 5000 x g for 15 minutes. The supernatant was discarded and the cell pellets were stored at -80°C freezer for further purification step.

\[
\text{Yield (nmol)} = \Delta_{450-490} \times \text{dilution factor} \times 10.99 \times \text{total volume of TB broth (Equation 2.1)}
\]
Figure 2.7. A P450 absorbance spectrum after reduction with sodium dithionate and carbon monoxide bubbling obtained from Olis DW-2000 UV/Vis Spectrophotometer.

CYP7B1-Expressed Cell Lysate Preparation

The cell pellets were solubilized in 1 mL of storage buffer on ice. The sonication cycle was 20 sec on and 30 sec off cycle. The cycle was repeated twice on ice.

Purification of Membrane-Intact CYP7B1 Protein

The purification of membrane-intact CYP7B1 from E.coli was adapted from protocol established by Locuson et al (Locuson et al., 2009). Two cell pellets were used from this purification. The solubilization of cell pellets were achieved by adding 25 mL of Buffer A, that contained 100 mM potassium phosphate dibasic, pH 7.6, 500 mM
sucrose, and 0.5 mM EDTA, to each cell pellet on ice. All the solubilized cell pellets were combined in a flask and stirred at 4°C. Fifty mg of lysozyme and 1 mL of protease inhibitor cocktail for use in purification of Histidine-tagged proteins (Sigma-Aldrich, St. Louis, MO) were added to the mixture and stirred at 4°C for 1 hour. The mixture were transferred into 50-mL Falcon tubes and centrifuged at 4°C for 10 min at 4000 rpm. After decanting the supernatant, the lysozyme-treated pellets (sphaeroplast) were solubilized in Buffer B that contained 100 mM potassium phosphate dibasic, pH 7.6, 20% Glycerol, 1 mM PMSF. One mL of protease inhibitor cocktail was added to every 50 mL of Buffer B. For every 20 mL of sphaeroplasts, two cycles in a French press at 1000 psi were run. The sphaeroplasts were centrifuged at 4°C, for 20 min, at 4000 rpm followed by another centrifugation at 4°C, for 1 hr, at 40,000 rpm on a Beckman Coulter Optima L-90K Ultracentrifuge (Beckman Coulter, Inc., Brea, CA). After decanting the supernatant, the brownish grey pellet with red streaks were resuspended in Buffer C, that contained 500 mM potassium phosphate dibasic, pH 7.6, 250 mM sucrose, and 0.25 mM EDTA, followed by sonication on ice for 15 min with a Branson 1510 sonicator (Branson, Inc., Danbury, CT). The bacterial membrane fractions were filtered through a Millipore Millex –SV 5.0 μm, PVDF membrane (25 mm, ethylene oxide sterilized) into cryovials followed by checking the cytochrome P450 content as described in the expression of CYP7B1 method section.
Incubation and Extraction of DHEA and 7-OH DHEA Standards

Each incubation tube consisted of 10 pmol of CYP7B1, 20 pmol of cytochrome P450- NADPH reductase, 10 pmol of cytochrome b5, and 10 mg of DLPC vesicle, 46 μM of DHEA. The incubation mixtures were equilibrated in a 37°C water bath for 3 min. The reactions were started by adding 50 μL of 1 mM NADPH dissolved in 50 mM potassium phosphate buffer, pH 7.4. The controls were terminated immediately after NADPH addition. The other mixtures were terminated at 10, 20, and 30 min. All time points were done in three replicates. The same volume of HPLC grade acetonitrile was added to terminate the reactions. For the authentic standards, 2 μL of each methanol stocks of 346.7 μM of DHEA and 62.5 μM of 7-OH DHEA were mixed with 10 pmol of CYP7B1 and 50 mM potassium phosphate buffer, pH 7.4 in separate tubes for a total volume of 200 μL. The same volume of HPLC grade acetonitrile was also added to these standards.

All the mixture was then centrifuged at 4°C, 10,000 rpm, for 10 min. After transferring the mixture into a glass test tube, 500 μL of MTBE were added and vortexed for about 1 min followed by centrifugation on Forma Scientific Benchtop centrifuge equipped with rotor # 284 (Forma Scientific, Inc., Marietta, OH) at 2500 rpm, for 10 min, at room temperature. The analytes extract would both go into the organic layer. The extraction was repeated twice to ensure higher recovery. The organic layers were then dried under nitrogen for 45 min on a Zymark Nitrogen Dryer (Zymark, Hopkinton, MA). After drying, the samples were derivatized in 100 μL of
derivatizing agent containing 39.6% of BSTFA and 0.4% TMCS in toluene. The derivatized samples were transferred into vials followed by heating the samples at 60°C for 1 hr on a Lab Line Multi-Blok Heater model 2052 (Lab-Line Instruments, Inc., Melrose Park, IL).

**GC-MS Method for Separation of DHEA and 7-OH DHEA**

Separation of DHEA and its major metabolite, 7-OH DHEA, was achieved on an Agilent® GC/MS system (Agilent Technologies, Inc., Santa Clara, CA) equipped with an Agilent® 6890 series Plus Gas Chromatograph with a built-in Agilent® 7683 automatic liquid sampler coupled with an Agilent® 5973 series Mass Selective Detector. A 27 meter SolGel-1ms capillary column (SGE, Inc., Austin, TX) with inner diameter of 0.25 mm and film thickness of 0.25 μm was utilized for the separation. The carrier gas was ultra pure (5.0 grade) helium flowing at a rate of 1 mL/min. The injection size was 2 μl and a splitless injection mode was utilized. In each analytical run the column temperature was initially maintained at 140°C for 1 min, raised to 220°C in 2 min, raised to 300°C for 10 min and kept at 300 °C for 2 min (total run time 15 min). The injection port, electron impact source, and MS analyzer were set at temperatures of 250, 230, and 250 °C, respectively. The retention times for the TMS-derivatives of DHEA and 7-OH DHEA were 7.29 and 7.75 min, respectively. DHEA had two major ions at \( m/z \) 270 and 360, while 7-OH DHEA had only one major ion at \( m/z \) 358. Samples of chromatograms for the separation of DHEA and 7-OH DHEA on GC/MS system are depicted in Figures 2.8A and 2.8B. The peak areas of each metabolite of DHEA and 7-
OH DHEA were integrated with Agilent® ChemStation software (Agilent Technologies, Inc., Santa Clara, CA).

**Figure 2.8.** Chromatograms acquired after liquid-liquid extraction treatment of authentic standards of

(A) DHEA

(B) 7-OH DHEA
Binding Study of CYP7B1 Using DHEA as Substrate

At the beginning of the study, the co-binding spectrum reduced P450 was determined by the procedure described on Expression of CYP7B1 method section. Seven concentrations of DHEA stocks ranging from 200 to 9600 μM in methanol were made. Eight hundred μL of 0.4 μM of membrane-intact CYP7B1 were prepared by diluting the protein stock with 100 mM potassium phosphate buffer, pH 7.4 containing 20% glycerol. The protein solution was then transferred into two separate quartz cuvette equally. After obtaining a stable baseline on the Olis UV/Vis Spectrophotometer, DHEA was titrated into the sample cuvette and methanol was titrated into the reference cuvette simultaneously. After each addition, both cuvettes were shaken and equilibrated for 2 min. The DHEA concentrations used for this study were 0.5, 1.0, 2.0, 2.97, 4.94, 6.9, 9.8, 12.8, 18.6, 30.2, 41.9, 65.1, and 156μM.

PCR Reactions

One of the attempts to confirm the sequence cDNA of CYP7B1 in pCWOri vector was to do PCR reactions with two different high-fidelity DNA Polymerases. Each reaction tube contained 2 μL of PCR mix that came with each DNA Polymerase, 0.5 μL of 10 mM dNTP mix, 1 μL of 100 pmol FMTA, 1 μL of 100 pmol RMTA, 0.2 μL of 199 ng/mL purified pCWOri that contained CY7B1 gene, 0.4 μL of DNA Polymerase, and 14.9 μL of RNA-free water. The thermocyler used was a PCR GeneMate Genius (ISC BioExpress, Kaysville, UT). The PCR cycling conditions, when Pfx50™ DNA
Polymerase was used, were 1 cycle of 94°C for 2 min, 35 cycles of 94°C for 15 sec, 35 cycles of 68°C for 30 sec, 35 cycles of 68°C for 1 min 30 sec, and 1 cycle of 68°C for 5 min. When Pfu Ultra II Fusion HS DNA Polymerase was used, the cycling conditions were 1 cycle of 95°C for 2 min, 30 cycles of 95°C for 20 sec, 30 cycles of 66°C for 20 sec, 20 cycles of 72°C for 15 sec, and 1 cycle of 72°C for 3 min. Each PCR product was run on 0.5 % Agarose gel in 1X TAE buffer along with exACTene 1 kB Plus DNA ladder (Fisher Scientific, Co., Fairlawn, NJ).
RESULTS

The sequencing signal trace from the BMGC confirmed that the NdeI and SalI restriction sites as well as the Barnes modifications were successfully incorporated as depicted on Figures 2.9A and 2.9B. In these sequencing reactions, the modified CYP7B1 cDNAs were in pCR®2.1-TOPO® vector. However, the sequencing reaction with FMTA, FTA1, FTA2, FTA3, and FTA4 primers failed repeatedly after the modified CYP7B1 cDNA was in pCWOri expression vector. In an attempt to successfully sequence the modified gene, a sequencing reaction mixtures containing 500 ng of DNA and a separate mixture containing 2 ng of DNA and 5% v/v DMSO as the secondary structure denaturant were submitted. Both of these mixtures gave weak signals on the sequencer.

Figure 2.9. Sequencing signal traces received from University of Minnesota BMGC successfully confirmed

(A) the creation of NdeI restriction site (box) and the introduction of Barnes modification (underline)
Based on the P450 absorbance, the CYP7B1 was present in transformed *E.coli* lysates as illustrated on Figure 2. However, the incubation reactions with DHEA on two different enzyme preparations, namely the sonication preparation and membrane-intact CYP7B1 preparation, did not show any substrate turnover as illustrated on samples of chromatograms from different time point incubation (Figures 2.10A-2.10C and Figures 2.11A-2.11C). The assay had sufficient sensitivity to detect 0.0624 μM
Figure 2.10. GC traces from different time point incubations with DHEA as the substrate and CYP7B1 cell lysate as the enzyme source. 7-OH-DHEA elutes at 7.6 min and DHEA elutes at 7.8 min.

(A) 0 minute

(B) 30 minutes
(C) Negative control

Figure 2.11. GC traces from different time point incubations with DHEA as the substrate and membrane-intact CYP7B1 preparation as the enzyme source.

(A) 0 minute
The binding study of CYP7B1 with its endogeneous substrate, DHEA, did not show the typical Type I or Type II binding of Cytochrome P450 substrates as depicted.
on Figure 2.12.

**Figure 2.12.** Difference of binding spectra of CYP7B1 with DHEA that was normalized at 400 nm

PCR reactions with both DNA Polymerases failed to amplify the modified CYP7B1 cDNA in pCWOri as can be seen on Figure 2.13. The length of the modified cDNA remains the same as the native CYP7B1 cDNA, 1.5 kb.
Figure 2.13. A gel electrophoresis result from the PCR products. Lane 1 was modified CYP7B1 cDNA in pCR®2.1-TOPO® vector, lane 2 and 5 were PCR products of modified CYP7B1 cDNA in pCR®2.1-TOPO® vector, lane 4 was the DNA ladder, and lane 3 and 6 were PCR products of modified CYP7B1 cDNA in pCWOri. Pfx50 is a high fidelity DNA polymerase from Invitrogen (Carlsbad, CA) and Pfu Ultra is a high fidelity DNA Polymerase from Stratagene (Agilent Technologies, Inc., La Jolla, CA).
DISCUSSION

The initial sequencing indicated that Barnes modification to the CYP7B1 gene was successful. The typical yield of P450 obtained in this study from sonication preparation and membrane-intact purification were 50 nmoles/ L of culture and 10 nmoles/ L of culture, respectively. Locuson et al. reported the yield of different canine rP450s from membrane-intact purification ranged from 4.8 to 108 nmol/ L of culture (Locuson et al., 2009). Based on personal communication with Dr. John Y. L. Chiang, the expression of recombinant CYP7B1 in *E.coli* is low. Although there appeared to be expressed cytochrome based on the co-binding spectrum at around 452 nm, the subsequent experiments did not show any activity of the enzyme. The peak at 452 nm absorbance for the reduced enzyme indicated that the presence of heme incorporation in the protein. A significant peak at 420 nm indicates that there also was inactive P450, suggesting instability of the protein in *E.coli*. *E.coli* is not known to express endogenous P450, however, it possesses cytochrome o, a membrane-bound heme protein complex that absorbs at 416 nm and displays a trough between 428-436 nm in its reduced state (Jenkins et al., 1998). The plausible explanations of the absence of enzyme activity were incorrect protein folding resulting in lack of substrate binding or loss of an interaction with reductase. Alternatively, the potential frameshift mutation at the 3’ end of the CYP7B1 gene may have resulted in overextended translation with incorporation of additional non-intended amino acids at the amino terminal end of the protein.
Although the heme was successfully incorporated into the protein, it did not mean that the protein was folded correctly. When a protein is not folded properly, it would affect substrate binding and possibly NADPH-cytochrome P450-reductase binding as well. When the protein was not in a correct conformation, the substrate may still be able to bind. However, conversion of the P450 into its reactive iron-oxo intermediate state may not have occurred in order to trigger metabolism. Reductase is an important electron carrier in the first and subsequent steps of P450 catalytic cycle. If the reductase was unable to bind, there would be no electron flow. This would lead to an absent in substrate turnover.

Besides introducing the Barnes modification, the creations of NdeI and SalI sites were also introduced on the CYP7B1 gene. Based on the sequencing data, the SalI site had an extra T (Figure 2.14) which might affect the enzyme activity or express an inactive enzyme. Six histine tags are present in pCWOri expression vector for a purification purpose. The extra T at the end of the gene would add an extra base to be translated that would shift the reading frame of the gene. Therefore, the protein that was expressed may not be the desired CYP7B1 product. Alternatively, an unintended PCR-mediated mutation may have occurred in the active site resulting in an inactive enzyme.
Amplification by PCR for resequencing of the CYP7B1 in pCWOri was attempted, however, Figure 2.13 clearly showed that the amplification failed when CYP7B1 gene was in pCWOri. Another attempt to sequence the CYP7B1 was attempted with a primer (SpCWTA) that starts upstream of the start codon (ATG). The sequencing result was mixed and inconclusive.

Dr. John Y.L. Chiang from the Northeastern Ohio University had succeeded in expressing an active CYP7B1 protein transiently in HEK 293/T cells. Chiang’s CYP7B1 gene was inserted in pcDNA3 (Invitrogen, Carlsbad, CA) plasmid. The next attempt to obtain active enzyme for screening would be expressing CYP7B1 in mammalian cells (see Future Directions).
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Institute NC (2008) What you need to know™ Prostate Cancer.


CHAPTER 1

PHT arene-oxide is the intermediate for p-HPPH formation and the precursor for DHD formation. This reactive epoxide potentially attracts Gluthathione-S-Transferases (GSTs) that are present as membrane-bound or cytosolic enzymes. Upon binding to the PHT arene-oxide, GST will undergo three catalytic steps and end up as a mercapturic acid derivative. The quantitation of this mercapturate in the urine samples may be beneficial to account for the low recoveries that were obtained for the current study. By tandem mass spectrometric detection in the negative ion mode, the neutral loss of 129 Da should be observed as the signature of pyroglutamate falling off of the mercapturate. Figure F1 illustrates the expected structures of PHT mercapturic acid derivatives.

**Figure F1.** The expected structures of PHT mercapturates

MRP2 is an important efflux transporter on the canalicular (apical) membrane of hepatocyte. Expression of MRP2 is regulated by PXR and CAR. In a primary culture
of rat hepatocytes, Kast et al. showed PXR activators (pregnenolone 16α-carbonitrile and dexamethasone), CAR activator (phenobarbital), and farnesoid X-activated Receptor (FXR) activator (3-(2,6-dichlorophenyl)-4-(3’-carboxy-2-chloro-stilben-4-yl)-oxymethyl-5-isopropyl-isoxazole (GW 4064)) significantly increase the MRP2 mRNA levels (Kast et al., 2002). Moreover, these ligands activated the expression of MRP2 of reporter genes regulated by rat MRP2 proximal promoter or two copies of novel hormone response elements in rat hepatocytes reported by this group (Kast et al., 2002). PHT has been shown to be a CAR activator. It would be interesting to see if PHT induced MRP2 expression. This would involve obtaining plasmid containing MRP2 gene and stably transfecting the plasmid into HepG2 cells. As mentioned in the introduction in Chapter 1 of this thesis, p-HPPH glucuronide in rats is transported by MRP2 (Auansakul and Vore, 1982), however, it is unknown if it is a human MRP2 substrate in humans. It might be worthwhile to check and confirm if p-HPPH glucuronide is a human MRP2 ligand. In order to confirm p-HPPH glucuronide as a MRP2 ligand, an in vitro experiment involving growing monolayer confluent cells expressing MRP2 in trans-well plates, incubating the cells with p-HPPH glucuronides, followed by measurement of the flux of p-HPPH glucuronide from both apical and basolateral sides, should be done. If p-HPPH glucuronide is indeed a MRP2 ligand in the cellular model, biliary excretion experiments with a cynomolgous monkey liver perfusion model or in human hepatocyte spheroids would be ideal. An in vivo experiment to collect bile from patients would also be beneficial. Ghibellini et al. developed a novel multilumen oroenteric tube that was temporarily positioned (3 hr) in the duodenum (Ghibellini et al., 2004). If PHT upregulated MRP2 expression and p-
HPPH glucuronide was a MRP2 ligand in human, the low recoveries in the current study could be accounted for by this route of elimination.

If another clinical study could be conducted, it would be interesting to conduct a study in healthy volunteers who are on steady-state. After recruitment, the healthy volunteers would be housed in a research center where their conditions could be monitored closely. After steady-state was reached, the SL-PHT would be administered and the urine would be collected. The urine extraction and quantitation procedure would follow the same procedure in the study reported in this thesis. If the urinary recoveries showed similar trend, the stable-labeled technology is still the appropriate approach to determine absolute bioavailability in epilepsy patients on PHT maintenance therapy. If the urinary recoveries showed a completely different trend, the subjects’ health status and steady-state status had an important role. This role might impact PHT’s ADME.

CHAPTER 2

Dr. John Y.L. Chiang from the Northeastern Ohio University sent pcDNA3 hCYP7B1 plasmid to our laboratory. The plasmid was then cloned into One Shot® Stbl3™ Chemically Competent E.coli. Glycerol stocks of the plasmid were generated and stored in -80°C freezer. In order to check whether the plasmid has the correct CYP7B1 gene, the plasmid has to be resequenced. In addition, running a diagnostic agarose gel on the double digested plasmid with KpnI and XbaI endonucleases would
be necessary. If the sequence of the plasmid was correct, transfection into HEK293 cells should follow. Wu et al. already established the condition of the transfection of pcDNA3.hCYP7B1 into HEK 293 cells (Wu et al., 1999). Once the transfection was successful, there would be two possible ways to conduct the incubations. The first one would be to incubate the cells with the substrate in the media for 0.5, 1, 2, 4, and 8 h once the cells were confluent. Initially, the cells should be incubated with DHEA around its $K_m$ (13.6 μM) (Rose KA, 1997) to check the activity of the expressed CYP7B1. The media will then be used for the GC-MS analysis of 7-OH DHEA after MTBE liquid-liquid extraction. The addition of sodium sulfate might be needed because MTBE’s tendency to retain water. If the expressed CYP7B1 was active, the incubation of its potential mechanism-based inhibitors should follow. Western blot of the expressed CYP7B1 should also be performed to quantify the amount of expressed CYP7B1. Then the cell lysate or microsomes prepared from the cells can be used as the enzyme source in the incubations. The incubations should be supplemented with NADPH-Cytochrome P450- reductase (or co-transfected) along with NADPH to start the reactions. Upon the termination of the incubation reactions, the incubation samples will be centrifuged at 4°C, 10,000 rpm, for 10 minutes. The same liquid-liquid extraction procedure with MTBE would be applied followed by GC-MS analysis. If the activity of expressed CYP7B1 was positively confirmed, the incubations with potential inhibitors of CYP7B1 previously synthesized by Ranganathan Balasubramanian in Dr. Fecik’s laboratory should follow. The incubations would be conducted with 10 μM of DHEA to determine time linearity and linearity with protein concentration. Incubations using several concentrations of inhibitors within the linear time range should follow to
determine the IC$_{50}$ of the inhibitors.

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APPENDICES

A1. Detailed results of patients’ urinary dose recovery of PHT

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<th>p-HPHP (mmol)</th>
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<th>Stable labeled p-HPHP (mmol)</th>
<th>Total urinary metabolites (mmol)</th>
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<th>24 hr dose (Horning MG)</th>
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A2. Nucleotide sequences of full-length wild type human CYP7B1 obtained from GenBank (NM_004820.2) that was in agreement with the sequencing result of modified CYP7B1 in this study. The start codon (ATG) and stop codon (TAG) are shown in bold letters.

**ORIGIN**

1 GGCGGGGAGG GGCCAGTCCA CGAACCTTTTG TCATTCAGCC TGAGAGACCA CGGTGACATT
61 CCTGTCTGCG CTGCAGCGAC GGGAGCCTCT CCGGCCCTGG ACTAGGGGAGC GCCGCCGAGA
121 GAAGCCCGGC CAGGGTGCAG AGTGCAGCGAC GGCAGAGGCG TCAGAGGCCGAGCCGACTGC
181 CCACCGCAGG CTAAGTCCGC CCGGATGCA GGAGAAGTGT CCGGAGCCAC GGGCGGCTTT
241 TGAGTGGACG GTTGGGCTCT GCCGCCGAGA GGGCCTGGGC CATCAATTTTAACTCCATC
301 CTTCTACGCT TTGGCAGGCT GGGGGGACAG GGAGAGGAGA AAAAGTTTGG
361 CTCTGAGGATG CAGTTCTCTA GAAAAAGGAG CCAAAAGGAC GTTGGAGGTATG CATAACATTT
421 GTTGGTTGCTT AATTTGTTT TGAAAGGAGCA AGTGGTGGTATG CAAATTTTGC AAGGAAATCT TTTGGACATA
481 CTAAGGAGAG CCCACAGGCG CGCAGGCGAG CAGGCGGTGC GCGCTGGTGGC AGGCCCTGG
541 GTATTTTTCA ATAGATTTATG AGAGAAGACG TTTAGCAGG CATCGTGGCA AAAAAATCAT
601 GACATGAATG ATGAGCTTCA CCTCTGCTAT CAATTTTTGC AAGGCAATCT TTTGGACATA
661 CTTTTGGAAGA CTGAGGAGCA GAATCTTTTTA AAGTTTTTGG AAAAGGACAGCA GTTGGAGGTATG
721 ACAAGTGCC ACACCGGACA ACTGATTTCCA TCTGGCATCT CAATAATATT TGAAGATCACA
781 TTTGAGGACA TTTTTTTATG TGTGGAACTCT TAAAGATATG TGTGGAGGCTT AAGTGGGAGG
841 AGAGATGATT TTTTTAATATG AGTGGCAGATT TGTGGTATTG TGAAGATCACA
901 CGACGTAGTGA CTTCCAGCTA CGAATGCTTGA GAAATATTAT GAAAGTCCGT TTTGGGACATA
961 AAAGGTGCA AGAGCAGGCA ATGGGCTGAGA TTTTCTAAA GAGAGCAGGA TGGTGGAGGA
1021 AAATATTATT TGGCTCAGAGA GGGAGAAGA TGGTGACTCTA AGTGGGAGG CTGAGGCTATG
1081 TCTGGCGCAA CATATATATCC AACTAGTCTC TGGGGCAATGT ATTATCTTCC GGGCAGCCCA
1141 GAAGGCTAGTG CAGCAAGAGA AGAGAAGGGA TGGGAGGCTG TCCCGTTTGG ACAGGCTATG
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1261 AAGAGGCACTCC TTTTGAAGACG TTTACGAGCT TGTCTGATTT CAAAGCCACAT TTCTGTTTCC
1321 GAGGAGATT TGACTCTCGA TCCAGAGGAA GGGGACTACAG GTGAGAGGAGA TGGGAGGCTT
1381 GTAGCCATCT TTCCCTCAGT CCTACATGGT GACCCTGAAA TCTTGAAGC TCCAGAGGAG
1441 TTTAGATATG ATCGTTTTAT AGAAGATGGT AAGAAGAAAA CCACCTTTTT CAAAAGAGGG
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1561 TTTTTTGAC TTATGGAAT AAAACAATTG TTGGTTATAC TTTAACTTA TTGTGATTTTA
1621 GAAATAATTTG ATGATAAGCC CATAAGACTA AACTACAGCC GCTTGTTTGG TGGTATTCAG
1681 TATCCAGATT CTGATGTTTT ATTTAGATAC AAAGTGAAAAT CT\text{TAGAGAAG CTAAAAGGAA
1741 AGAAAAATAA TCTATCCTAA TTACCTAAAA CATCCTAAGC TCTATATT TTATTTAATT
1801 TCTGCAAAGT AATTGATTTA TTGTTTAA TGGCGCTAAT TTCTATTTGA TCTGATATCA
1861 GTCCAGTTTG TCCTTACCA CAAAGCCCAT ATAATAAAA ACAGGATGGT GGCAGGAAAA
1921 TGGACATCAA AATCAAATTA AGGTAGGGT CAAACAGGG TTGGTGTGT TTTTTAGTT
1981 TCTCTGGTT GTGATTTTCA CCTGTTATAA TAAATGTACC TTCACACCAA TAGATTTGAG
2041 ACTGTGAAT TTAAATCCT CAAATTTTC TCTAATATGT AAAATTACAC TTTGAACCAT
2101 TAGAAAAATGG TGCTGGAATT GCAGCAAGTCA AAAAAATTCA CAAATTCTCA CAGAAGAAGG
2161 AAAATTGAGTT TCATGAGGT ACACCTGGATG AAAATGTTC TCTTAAGC ATATAAATAA
2221 TACCTATATC
A3. Nucleotide sequences of full-length pCWOr expression vector (Cirino, 2004). The bold underlined sequence is the nucleotide sequence for SpCWTA primer.

1 AGCGGGCAGT GAGCGCAACG CAATTAATGT GAGTTAGCTC ACTCATTAGG  
51 CACCCCCAGGC TTACACCTTT ATGCTTCCGG CTCGTATAAT GTGTTGAATT  
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151 CTTACTCCCC ATCCCCCTGT TGACAATTAA TCATCGGCTC GTATAATGTG  
201 TGGAATTTGTC AGCGGATAAC AATTTCAACAC AGGAAACAGG ATCAGCTTAC  
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301 TTGTGAAGCGG ATAAACATTTC ACACAGGGA ACAGATCTCC TCGAGCTTAC  
351 GGAGGTCTATA TGTAATTCA TCGTAATTAA GCTGTCAAAC ATGACGAGAT  
401 CTTACTGTATG TTACGCGGCT CAGGACGAGG AACGATTGCG GCGCTTGCAG  
451 AAGGGCCTAGC TGATACCGCT ATTTTTTAGG TGAATGCTCA TGATAATAAT  
501 GGGACCTTAGC CTTCAAGGCAA ACCATAGTAC GCGCCCTGTA GCCGCCGATT  
551 AAGCGCCGCGG GGTTGCGTGGT TTACGCCGAC CTTGACCCGCT ACACTTGGCA  
601 GCGCCCTAGC GCCCCCTCTCT TCGCTTTTCT TCCCTTCCCT TCTGCCCAGC  
651 TCCCTCCCCTC TTTCCCTTCT CCCTTCTTCT CCGCTGCGAT CTGAGTACTTC  
701 CCGATTTTAG GTCTTAGGCG ACCTCAGCCC AAAAATGAGTT GATTTGGGTG  
751 ATGGTTTGCT CAGTTGCGCA CCGGTGTGTTTT TGGAGGTCAGG GGGATTTTGC  
801 AGATTTTTTC ATGTGGCGTAC ATCCCTGCTGCTGTTTTTTT TCGGCGCTTTT  
851 CGATTTTCGC TATTTGCTGTA AAAACTTTGAG TTCCCTGCTG AAATTTAAAC  
901 GCCAACCTTC TAAACTTGGTTA AAAATGAGC TGATTTAACA AAAATTTAAC  
951 GCCGAATTTTA ACAAAATATT AACGTTTACA ATTTCATGTTG GCACCTTTGC  
1001 GGGAAATGTTG CGCGGAAACC CTATTGTTTT ATTTTTCTAA ATACATTCAA  
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1101 TGAAAAAGGA AGAGTATGAG TATTTCAACAT TTCCGCTGTCG CCCTTATTCC
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1351 TATCCGCGGTG CAACTGAGCAATGTCGCTGGCCTCAGCTACG CACGTCGAGCGAC
1401 TCTCAGAATGGGCGATAGCCGGAAGGAGTG GGGTGACATC
1451 GCATTTTGCC CAAGAGCAAC TCGGTCGCCG CATACACTAT
1501 TCTCAGAATGGGCGATAGCCGGAAGGAGTG GGGTGACATC
1551 CTAACCTGCC TTTTGACAA CATGGGGGAT CATGTAACCTG TTCTTCTG
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2201 GCTGCCGCGG TTTGTTTGT GGGCTCCGCA ACAGTCAAGC TACTTCTTTC
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2351 ATACCTGCTCT GCTGCAAATTT CTGGTACGAG GGGTCGTGGCGG ATGCGAGATA
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2951 AGCGAGGAGA GCTCCCTGAG CAGCGAGATG GGTGCGGCGC TCTTCGACCT
3001 GCACTGACGC ATGCGAGCAG ATTTTGGTAC AGCATGGGAA CATCTGAGTC
3051 GGTCATGGCT CGGCCCCGAC ACCCGCAACA ACCGCTGAC GCAGCCCTGAC
3101 GGGCTTGTCTC TCCTCCGGCA CAGCTTTTAC TCTTCTTCCC TTACGCATCT
3151 GGGGAGCTGCA TGTTAGAGCT GGTTTTACAG TCAACGCGCA TATATTGGTG ACTTCTGACTG
3201 CGCAAGGCCC ATCAAAAAATA ATGGCGCCTG GCGTTCCTGG TACGCTGACTG
3251 CATACACAT TAAATAGTGC CGAGTAACAA CGCGCGAGAC TGCACTGACG
3301 AACGACGCCG GTATGGGAGC TATGAGGCGC GGTGACACAG AAGGGACGAC
3351 GCCATAGGCA CTTAGAGGCTT CAGGCTTGAG GAGGTGCGGA AAGTTGGGTA
3401 GCCTCAGGAA GATTCGACCT CAGCGGAGCT TCCGCGACC CTTCTTGATG
3451 GGGAAAGCCG CCAAAAGGCC ATGGCGGAGC AACTGTGGGG
3501 AAGGGCCGATC GGTGGCGGCC CTTGCGGCATT CAGGTGAGGC AACACGACG
3551 GATGTCGCTG CAGGGCGGAT TGGTGGCCGT GGGAGGTCG GATGACACAG
3601 ACGCAGTGTG CAAAACCGAG CCGTGAATC CAGAATCATG GTGATGCTG
3651 TCTCCGTGGG TACAGAAGGC TGTTAGAGTGC TACGCTGACTG
3701 CAGGCGTGC TAAATTGAG GCTTCTGAG GC TGTAGTAACG AGCTAACTG
3751 CATTAATGC GTTGCCGCTCA TGGCGGCGCT TCCAATCGTTA AAACCTGTCG
3801 TGCAGGTGCTG CTGCGGCGCC CAGCAGAGAG GCGTGTGGCG
3851 TATGTCGCTG TTTTCCCACT CACAGTGCAGC CGGCAAGCCG
3901 TGATGCGCT GTGACGGCA CAGGAATCCT TGTTAGGCTG GTTAAGCGGCG
3951 GCTGGTGTCTG CCCGGCAGG GC AAATCCTTG TTTGATGGTG GTTAAAGCGG
4001 GGATATAAAG TGAGCTGGCT CTTCTGACCT CTTACGCCCA TCAGCAGGAT
REFERENCES