Effect of Phenytoin on the Pharmacokinetics of Efavirenz in Rabbits

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ชื่อวิทยานิพนธ์ ผลของยาเฟนิโตอินต่อเภสัชจลนศาสตร์ของยาอีฟาวิเร็นซ์ในกระต่าย

ผู้เขียน นางสาวกรกนก พิบูลย์ผล

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บทคัดย่อ

เฟนิโตอินมีฤทธิ์เหนี่ยวนำการทำงานของเอนไซม์ CYP3A4 และ CYP2B6 และอีฟาวิเร็นซ์ถูกแปรรูปผ่านทางเอนไซม์ CYP3A4 และ CYP2B6 ดังนั้นเมื่อมีการบริหารยาทั้งสองร่วมกันอาจส่งผลให้มีการเปลี่ยนแปลงทางเภสัชจลนศาสตร์ของยาอีฟาวิเร็นซ์ วัตถุประสงค์ ของการศึกษาคือ เพื่อศึกษาผลของยาเฟนิโตอินต่อเภสัชจลนศาสตร์ของยาอีฟาวิเร็นซ์ในกระต่าย โดยทำการศึกษาในกระต่าย 10 ตัวแบบสุ่มไขว้สลับ ซึ่งเว้นระยะห่างของการให้ยาเป็นเวลา 2 สัปดาห์ ในระยะแรกกระต่าย 5 ตัวในกลุ่มที่ 1 ได้รับยาอีฟาวิเร็นซ์ขนาด 70 มิลลิกรัมต่อกิโลกรัมครั้งเดียวทางปาก ในกลุ่มที่ 2 กระต่ายอีก 5 ตัวจะได้รับยาเฟนิโตอินขนาด 30 มิลลิกรัมต่อกิโลกรัมทางปากวันละครั้งเป็นเวลา 7 วัน โดยในวันที่ 7 กระต่ายจะได้รับยาอีฟาวิเร็นซ์ขนาด 70 มิลลิกรัมต่อกิโลกรัมครั้งเดียวทางปากร่วมด้วย ในระยะที่ 2 กระต่ายทั้ง 2 กลุ่มจะได้รับยาแบบไขว้สลับกัน เก็บตัวอย่างเลือดของกระต่ายก่อนได้รับยาอีฟาวิเร็นซ์และหลังได้รับยาอีฟาวิเร็นซ์ที่เวลา 0.5, 1, 2, 4, 8, 12, 24, 48, 72 และ 96 ชั่วโมง ผลการทดลองพบว่ายาเฟนิโตอินทำให้พื้นที่ใต้กราฟระหว่างความเข้มข้นของยากับเวลาในช่วง 96 ชั่วโมงและที่เวลาอนันต์, ความเข้มข้นสูงสุดของยาและเวลาที่มีความเข้มข้นของยาสูงสุดของยาอีฟาวิเร็นซ์ลดลงอย่างมีนัยสำคัญ 55.99% , 48.84% , 48.88% และ 71.43% ตามลำดับ ส่วนค่าอัตราการกำจัดยาเพิ่มขึ้นอย่างมีนัยสำคัญ 101.47% ขณะที่ค่าครึ่งชีวิตในการกำจัดยาลดลงแต่ไม่มีความแตกต่างอย่างมีนัยสำคัญ จากการศึกษาอาจจะสรุปได้ว่าเฟนิโตอินเพิ่มอัตราการกำจัดยาอีฟาวิเร็นซ์ผ่านทางการเหนี่ยวนำเอนไซม์ CYP3A4 และ CYP2B6

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ABSTRACT

Phenytoin is an inducer of the CYP3A4 and CYP2B6. Efavirenz is a substrate of the CYP3A4 and CYP2B6. Co-adminstration of both drugs may result in changes of efavirenz pharmacokinetics. The aim of this study was to investigate the effect of phenytoin on the pharmacokinetic of efavirenz in rabbits. Ten male rabbits were randomly assigned into a 2-period pharmacokinetic study with a 14 days wash-out period in a crossover design. In period 1, 5 rabbits in group 1 received efavirenz 70 mg/kg orally. In group 2, another 5 rabbits received phenytoin 30 mg/kg once daily orally for 7 days, followed by efavirenz 70 mg/kg on day 7. In period 2, the 2 groups were treated in reverse. Blood samples were collected at 0, 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96 h after each treatment. Phenytoin significantly decreased the AUC_{0-96} , $AUC_{0-\infty}$, C_{max} and T_{max} of efavirenz by 55.99% , 48.84% , 48.88% and 71.43%, respectively. The clearance of efavirenz was significantly increased by 101.47% whereas the $t_{1/2}$ was not significantly decreased. Phenytoin increase efavirenz clearance probably via CYP3A4 and CYP2B6 induction.

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LIST OF ABBREVIATIONS AND SYMBOLS

AIDS	=	Acquired immunodeficiency syndrome
AUC	=	Area under the concentration-time curve
ºC	=	Degree Celsius
CCR5	=	chemokine coreceptor-5
CD4	=	Antigenic marker of the helper/inducer T cells
Cl/f	=	Oral clearance
C_{max}	=	Maximum plasma concentration
C.V.	=	Coefficient of variation
CYP	=	Cytochrome P450
λ_z	=	Elimination rate constant
h	=	Hour
HIV	=	Human immunodeficiency virus
HPLC	=	High performance liquid chromatography
Kg	=	Kilogram
L	=	Liter
µg	=	Microgram
µl	=	Microliter
µm	=	Micrometer
mg	=	Milligram
min	=	Minute
ml	=	Milliliter
mm^3	=	Cubic milliliter
MW	=	Molecular weight
NNRTI	=	non-nucleoside reverse transcriptase inhibitors
NRTI	=	nucleoside reverse transcriptase inhibitors
PI	=	protease inhibitors
P	=	P value
r	=	Correlation coefficient

LIST OF ABBREVIATIONS AND SYMBOLS (Continued)

SD	=	Standard deviation
$t_{½\ el}$	=	Elimination half-life
t_{max}	=	Time to maximum serum concentration
V_z/f	=	Volume of distribution
∞	=	Infinity
%	=	Percent
®	=	Trade name

CHAPTER 1

INTRODUCTION

Acquired immune deficiency syndrome (AIDS) is a set of symptoms and infections resulting from the damage to the human immune system caused by the human immunodeficiency virus (HIV). HIV is a pandemic infection which affects every part of the globe. According to the AIDS epidemic update 2009 by UNAID/WHO, released on December 2009, an estimated 33.3 million people lived with the disease worldwide, and it killed an estimated 1.8 million people, including 260,000 children (UNAID/WHO, 2009). An estimated number of 372,202 were infected in Thailand at the end of 2010 and 526 have died from the infection in 2010.

Starting in 1996, the mortality rate of HIV-infected patients has dramatically decreased in western countries, when highly active antiretroviral combination therapy (HAART) became available (Pallela *et al*., 1998). HAART decreased HIV viral load and leads to the increased of CD4 lymphocytes counts, causing an improvement of immunity and a decrease in the incidence of opportunistic infection (Powderly *et al*., 1998). There are more than 20 approved antiretroviral drugs across six mechanistic classes, with which to design combination regimens. These six classes include the nucleoside/nucleotide reverse transcriptase inhibitors (NRTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI), fusion inhibitors, chemokine coreceptor-5 (CCR5) antagonists, and integrase inhibitors. The most extensively studied combination antiretroviral regimens for treatment-naive patients generally consist of one NNRTI with two NRTIs, or a PI with two NRTIs. NNRTI-base regimens are commonly prescribed as initial therapy for treatment-naive patients. The advantage of NNRTI-based regimens is lower pill burden as compared to most of PI- based regimens. Use of NNRTI-based regimens can preserve PIs for later use, thus reducing or delaying patient exposure to some of the adverse effects more commonly associated with PIs. The major disadvantages of currently available NNRTIs involve prevalence of NNRTI-resistant viral strains in

treatment-naive patients and the low genetic barrier of NNRTIs for development of resistance (DHHS, 2008).

To date, recommendations are designated as the initial regimen in treatment naive patients, who have CD4 fall below 350 cell/mm^3 in all asymptomatic individuals. The preferred regimen of NNRTI-based has been based on a combination of efavirenz plus 2NRTIs (DHHS, 2008). The government of Thailand launched the programme aims to provide GPO-Vir, a generic fixed dose combination of stavudine, lamivudine and nevirapine, as a firstline HAART regimen. Since nevirapine is more hepatobiliary toxic than efavirenz (van Leth *et al*., 2004) and nevirapine can causes severe rash (van Leth *et al*., 2005). Moreover the availability of efavirenz, the first antiretroviral drug to be administered as a single daily dose, enhanced adherence and improved quality of life (Maggiolo, 2007). These factors make efavirenz an important component of NNRTI-based instead of nevirapine.

Efavirenz is metabolized by cytochrome P450, mainly by CYP2B6 and, to a lesser degree, by CYP3A4 (Ward *et al*., 2003). Patients with HIV infection carries a risk for seizures through opportunistic infections of the central nervous system such as toxoplasmosis, central nervous system lymphoma, cryptococcal or tuberculous meningitis and progressive multifocal leukencephalopathy (PML) (Pascual-Sedano *et al*., 1999; Price, 1996; Weisberg, 2001; Wong *et al*., 1990). In addition, HIV is associated with neurological defecits due to direct effects of the neurotropic virus (Di Stefano M, *et al*., 1998). Seizures is not rare among HIV-infected patients. The incidence of seizures may be as high as 11 % in patients with HIV, compared with 1-2 % of the general population (Wong, 1990). Seizures in HIV-infected patients seem to have a high chance of recurrence (Chadha *et al*., 2000), therefore, it has been recommended to start anticonvulsant therapy even after the first seizure (Mullin *et al*., 2004).

Phenytoin is an effective and a major first-line antiepileptic drug widely used for the treatment of partial and generalized epilepsy in Thailand. Phenytoin is useful for controlling seizures, without the sedative effects. It is known as an inducer of CYP3A4 and CYP2B6 (Patsalos *et al*., 2002). There has been a case report of the lower-than-expected concentration (12-h concentration, < 1 μg/ml.) after initiation of efavirenz in a patient who was receiving phenytoin. Therapeutic drug

monitoring was used in this case to ensure adequate efavirenz exposure (Robertson *et al*., 2005).

Since, coadministration of antiviral drugs and antiepileptic drugs is very often in HIV-infected patients, clinically important interaction can occur and may lead to failure of treatment with efavirenz. To date, there has been no study aimed at determining the pharmacokinetic drug interaction of efavirenz and phenytoin. Since the nervous system symptoms such as dizziness, abnormal dreams and hallucinations usually occur during efavirenz therapy, the volunteers may ask to withdraw from the study. Then we choose the rabbits as animal model, because they can be easily restrained in stocks and are generally docile and cheap to maintain. In addition, the rabbit CYP3A6 and the human CYP3A4 have similar P-450 predominance and substrate specificity and are both induced by rifampicin as well as by RU486, clotrimazole, trans-nonachlor, and phenobarbital (Kocarek *et al*., 1995; Barwick *et al*., 1996). Moreover the ligand activation profile of rabbit PXR is distinct from rat PXR and more closely resembles that of human PXR (Savas *et al*., 2000). Therefore, in this study, we carried out a preclinical investigation of a possible pharmacokinetic interaction between efavirenz and phenytoin in rabbits.

CHAPTER 2

LITERATURE REVIEWS

2.1 HIV infection and seizure

Disorders of the central nervous system (CNS) in patients with human immunodeficiency virus (HIV) type-1 infection is often associated with severe morbidity and mortality. Seizures are relatively common manifestations of HIV infection itself and of its several complications involving the brain (Dore *et al*., 1996).

2.1.1 Incidence

Available data on the incidence of new-onset seizures in HIV-infected persons are derived from hospital-based studies. Wong et al observed an incidence of 11% among 630 HIV-infected patients (Wong *et al*., 1990). In a more recent study, van Paesschen et al. (1995) observed that 4% of AIDS patients had new-onset seizures. In their study, the incidence of seizures was much lower than in the former study, presumably because strict inclusion criteria were applied. All patients were admitted, or were already in-patients, on the day of first seizure. Out of 68 selected patients, 62 had acquired immunodeficiency syndrome (AIDS), only six (9%) patients had AIDS-related complex or were asymptomatic HIV-seropositive (van Paesschen *et al*., 1995). In the study by Wong et al, 28% of the patients had AIDS-related complex or were asymptomatic HIV-seropositive (Wong *et al*., 1990).

The epidemiology of epilepsy in Thailand is about 1 % of the population. Approximately 700,000 of epilepsy cases were reported in Thailand and about 40,000 cases who were refractory to the conventional antiepileptic drugs (Chinvarun, 2009).

2.1.2 Associated seizure disorders

In most AIDS patients, seizures are seen in advanced stages of the disease (Wong *et al*., 1990; van Paesschen *et al*., 1995). Seizures may be the presenting clinical symptom of HIV disease. In a few patients, seizures can occur early in the course of HIV-infection. The majority of patients have generalised seizures; partial seizures are less frequently observed and do not necessarily imply the presence of focal mass lesions (Wong *et al*., 1990; Dore *et al*., 1996; Labar, 1992). Both simple and complex partial seizures are seen in patients with diffuse brain disease, such as HIV encephalopathy and meningitis (Wong *et al*., 1990). The incidence of convulsive status epilepticus has been reported as between 8% and 18% in different studies (Wong *et al*., 1990; van Paesschen *et al*., 1995) and is often associated with poor prognosis.

2.1.3 Etiologies of Seizures

The majority of seizure patients with HIV infection have a brain lesion or AIDS-defining illness. Most commonly identified are cerebral toxoplasmosis, cryptococcal meningitis, tuberculoma, the AIDS dementia complex, syphilitic meningovasculitis, and primary central nervous system lymphoma. Although epileptic seizures are traditionally associated with disease processes affecting the cerebral cortex (gray matter), progressive multifocal leukoencephalitis is a disease that predominantly involves the white matter and is frequently complicated by seizures. In a 5-year retrospective study of 49 HIV-infected patients with progressive multifocal leukoencephalopathy (PML) who did not meet criteria of the AIDS dementia complex and who did not have a concomitant opportunistic infection, 20% had presented with new-onset seizures (Moulignier *et al*., 1995). Lastly, seizures may be the only clinical manifestation of HIV infection in central nervous system, as no other causes can be identified in as many as 30% of patients (Chada *et al*., 2000).

Mass lesions

Intracranial mass lesions account for nearly half the neurological disorders in AIDS patients. The nature of these mass lesions can be broadly divided into three distinct groups: opportunistic infections, neoplasms, and cerebrovascular diseases. Seizures are dominant manifestations of most of these disorders

Toxoplasmosis is the most common cause of intracranial mass lesions in AIDS and occurs in 3–10% of patients in the USA and in up to 50% of patients in Europe and Africa. Seizures have been reported as an early manifestation in 15–40% of patients with cerebral toxoplasmosis (Wong *et al*., 1989). Various studies have described different incidences (12–28%) of toxoplasmosis in patients with new-onset seizures among HIV infected persons (Holtzman *et al*., 1989; van Paesschen *et al*., 1995; Dore *et al*., 1996).

Primary CNS lymphoma, the second most common cause of AIDS-related intracranial mass lesions, occurs in up to 2% of patients with AIDS. It is also the second most common mass lesion producing seizures in HIV-infected persons.

Other focal lesions

Other focal lesions without significant mass effect, such as progressive multifocal leukoencephalopathy (PML) may also be responsible for new-onset seizures in several AIDS patients. Moulignier et al. (1995) reported on 10 HIV-infected patients with PML in whom partial or generalised seizures were the presenting neurological manifestations. They suggested that demyelinated lesions adjacent to the cerebral cortex acting as irritative foci, axonal conduction abnormalities, or disturbance of the neuron-glia balance are the possible reasons for a pure white matter disease producing seizures.

Meningitis and encephalitis

In patients without mass lesions, meningo-encephalitis caused by some opportunistic infections is a frequent source of seizures. The incidence of meningitis and encephalitis in HIV-infected patients with new onset seizures varies from 12% to

16%. Cryptococcal meningitis is the most frequent meningoencephalitis producing seizures (Labar *et al*., 1992). Infrequent causes include aseptic meningitis, neurosyphilis, Herpes zoster leukoencephalitis, toxoplasma and cytomegalovirus encephalitis (Dore *et al*., 1996). In the developed countries, subacute sclerosing panencephalitis has re-emerged in children infected with HIV and can present with seizures and encephalopathy.

HIV infection

Approximately half of HIV-infected patients with seizures have no definite identifiable disease of the brain, and cerebral HIV infection seems to be the most likely cause of the seizures (Dore *et al*., 1996; van Paesschen *et al*., 1995).

2.1.4 Treatment

The seizure recurrence rate for non–HIV-infected patients with idiopathic unprovoked seizures is less than 50%, thus most practitioners wait until a second seizure occurs before initiating anticonvulsants. In patients who are HIV positive, the rate of reoccurrence is much higher and approaches 70%; thus it is reasonable to begin treatment after a first seizure (Chada *et al*., 2000). The choice of an appropriate anticonvulsant can be challenging. Phenytoin, carbamazepine, and valproic acid, the three most commonly prescribed antiepileptic drugs in the United States, are relatively contraindicated. Both phenytoin and carbamazepine are strong inducers of the hepatic cytochrome P450 system. HIV-protease inhibitors are substrates and inhibitors of this system, particularly CYP3A. Not only an interaction between these medications is expected, but the literature contains numerous reports where the addition of anticonvulsant led to failure of antiretroviral therapy (Hugen *et al*., 2000; Kato *et al*., 2000).

When absolutely necessary, phenytoin may be initiated. However antiretroviral medications need to be increased to compensate for the liver-inducing effects, and the plasma levels of both antiepileptic drugs and antiretroviral drugs must be measured and monitored (Leppik *et al*., 2003).

Valproic acid has been studied in cell culture and actually stimulates replication of HIV via a dose-dependent increase in reverse trancsriptase activity. This has been observed in both acute and chronically HIV-infected cell lines. Valproate has been associated with hepatic and multiorgan system failure when used with antiretroviral drugs (Cozza *et al*., 2000). Thus, Valproic acid should not be recommended for the treatment of seizures or other illnesses in HIV-positive patients.

There have been eight new antiepileptic drugs released since 1992. None have been studied specifically for the treatment of seizures in HIV-positive patients; however, some have pharmacokinetic properties, routes of metabolism, and a lack of drug-drug interactions that make them potentially good choices. The newer aniticonvulsant agents like gabapentin, lamotrigine and levitiracetam are not significantly metabolized and do not appear to alter CYP450 enzyme function. Therefore they are recommended for use in HIV-associate seizures (Mullin *et al*., 2004).

2.2 Efavirenz

Efavirenz is a human immunodeficiency virus type 1 (HIV-1) specific, non-nucleoside reverse transcriptase inhibitor (NNRTI). Efavirenz is chemically described as (S)-6-chloro-4(cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one. Its empirical formula is $C_{14}H_9ClF_3NO_2$ and its structural formula is shown in figure 1.

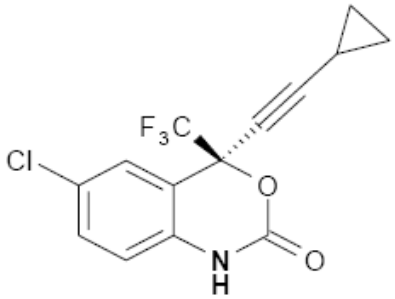


Figure 1: Chemical structure of efavirenz

Efavirenz is a white to slightly pink crystalline powder with a molecular mass of 315.68. It is practically insoluble in water (<10 μg/mL). The melting point is 137.2 ± 1.4ºC. The pKa is 10.2. Sustiva® is available as capsules containing either 50 mg or 200 mg and film-coated tablets containing 600 mg of efavirenz. The inactive ingredients in capsules are lactose monohydrate, magnesium stearate, sodium lauryl sulfate and sodium starch glycolate. The inactive ingredients in tablet form are croscarmellose sodium, hydroxypropyl cellulose, lactose monohydrate, magnesium stearate, microcrystalline cellulose and sodium lauryl sulfate.

2.2.1 Mechanism of Action

Efavirenz activity is mediated predominantly by noncompetitive inhibition of HIV-1 reverse transcriptase. HIV-2 reverse transcriptase and human cellular DNA polymerases α, β, γ, and δ are not inhibited by efavirenz (sustiva, product monograph). Efavirenz acts by attaching to HIV-1 reverse transcriptase in the NNRTI-binding pocket, a hydrophobic nonsubstrate-binding region of the reverse transcriptase p66 unit. Inhibition of reverse transcriptase activity occurs by disrupting the orientation of the conserved aspartic acid side chains necessary for catalytic activity. Efavirenz is the most potent of the available NNRTIs, with 90 – 95% inhibitory concentrations (IC90–95) for clinical isolates and wild-type HIV-1 ranging from 1.7-2.5 nM (0.53-7.88 ng/ml) (Maggiolo, 2007).

2.2.2 Pharmacokinetics

Absorption

Efavirenz is well absorbed after oral administration. At steady-state, after a dose of 600 mg, the C_{max} is 14.3 μM. Regular meals increase the AUC and C_{max} by 28 and 79%, respectively, compare to fasted conditions. A high-fat meal increases AUC by 50% and should be avoided. For the 600 mg dose at 24 h, the trough plasma concentration ranges from 4.5 to 5.5 μM, well above the IC90 of HIV-1 wild type (Maggiolo, 2007). The time to peak plasma concentration is 3-5 h and

steady state plasma concentrations of efavirenz are reached in 6-7 days. The bioavailability of oral efavirenz was 42% in animal studies after 2 mg/kg doses (Balani *et al*., 1996). Treatment failure has been discussed as being associated with low efavirenz level (<1 μg/ml) and central nervous system toxicity may be associated with high efavirenz level (> 4 μg/ml) (Marzolini *et al*., 2001).

Distribution

Efavirenz is highly bound (95 %) to albumin (Dicenzo *et al*., 2004). In HIV-1 infected patients who received efavirenz 200 to 600 mg once daily for at least one month, cerebrospinal fluid concentrations ranged from 0.26 to 1.19% (mean 0.69%) of the corresponding plasma concentration.

Metabolism

Efavirenz is metabolized by cytochrome P450 (CYP), mainly by CYP 2B6 and, to a lesser degree, by CYP3A4 (Ward *et al*, 2003). It is extensively metabolized to inactive hydroxylated metabolites that include 8- hydroxyefavirenz (major metabolite) and 7-hydroxyefavirenz, with subsequent urinary and biliary excretion of these metabolites after conjugation, mainly glucuronidation (Mutlib *et al*., 1999). Efavirenz 8-hydroxylation and the subsequent oxidation to 8,14-dihydroxyefavirenz is predominantly catalyzed by CYP2B6 (Bryan *et al*.,2003). Efavirenz has been shown to induce CYP enzymes, resulting in the induction of its own metabolism. In vivo and in vitro metabolism studies have shown that all the efavirenz metabolites identified in humans are also found in rats and cynomolgus monkeys. The major inactive metabolites identified in the three species are the 8-hydroxyefavirenz and its glucuronide conjugate (Mutlib *et al*., 1999).

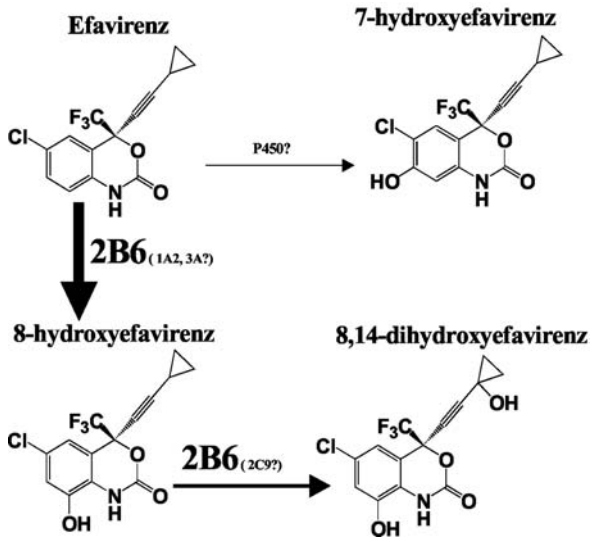


Figure 2: Proposed human metabolism of efavirenz (Ward *et al*., 2003)

Excretion

Efavirenz has a terminal half-life of 52-76 hours after single doses and 40-55 hours after multiple doses (Maggiolo, 2007). The majority of excretion efavirenz was excreted in the form of feces (16-61%). Metabolites were excreted in urine (14-34%). Less than 1 % of an administered dose of efavirenz is excreted unchanged in the urine (Adkins *et al*., 1998). Clearance is even slower in recipients with the 516G→T and 983C→T CYP 2B6 genotype (Haas *et al*., 2009), a common polymorphism in those of Japanese and African ancestry.

2.2.3 Clinical Use

Efavirenz is indicated for the treatment of HIV-1 infection in combination with other antiretroviral agents.

2.2.4 Dosage and administration

The recommended dosage of efavirenz is 600 mg orally, once daily in adults in combination with a protease inhibitor and/or nucleoside analogue reverse transcriptase inhibitors (NRTIs). It is recommended that efavirenz be taken on an empty stomach, preferably at bedtime. The increased efavirenz concentrations

observed following administration of efavirenz with food may lead to an increase in frequency of adverse reactions. Dosing at bedtime may improve the tolerability of nervous system symptoms.

2.2.5 Contraindications

efavirenz is contraindicated in patients with clinically significant hypersensitivity to any of its components. Efavirenz should not be administered concurrently with cisapride, midazolam, triazolam, pimozide or ergot derivatives because competition for CYP3A4 by efavirenz could result in inhibition of metabolism of these drugs and create the potential for serious and/or life threatening adverse events such as cardiac arrhythmias, prolonged sedation or respiratory depression (sustiva®, product monograph).

2.2.6 Adverse drug reaction

Nervous system symptom

These include dizziness, insomnia, impaired concentration, somnolence, abnormal dreams and hallucinations. The symptoms usually occur during the first 2 days of efavirenz therapy and last for several hours after each dose. They usually resolve after the first 2 to 4 weeks of continued efavirenz intake, but may persist as mild symptoms for a longer period of time. Taking efavirenz prior to bedtime may reduce the discomfort of these symptoms (Maggiolo, 2007).

Psychiatric symptoms

Serious psychiatric symptoms were reported in clinical trials in < 2% of patients. They include severe depression, suicidal ideation, aggressive behavior and paranoid reactions. Patients with a history of psychiatric disorders seem to be at greater risk of such adverse events (Haas, *et al*., 2004).

Skin rash

Rash can be associated with efavirenz. Most episodes are mild-to-moderate, occur during the first 2 weeks of therapy and resolve within 1 month without discontinuation of the drug. Approximately 8 – 18% of patients develop a

maculopapular rash of mild-to-moderate severity. Although rare, if a patient experiences a more severe (grade 3 or 4) skin rash during efavirenz treatment, the drug should be discontinued (Maggiolo, 2007).

Other adverse effect

Efavirenz has produced fetal malformations in fetuses/infants of treated cynomolgus monkeys. It should be used during pregnancy only if the potential benefits justify the potential fetal risk and no other therapeutic option is available (Chersich, *et al*., 2006).

2.2.7 Special populations (Sustiva®, 2004)

Liver disease

Efavirenz is contraindicated in patients with severe hepatic impairment and not recommended in patients with moderate hepatic impairment because of insufficient data to determine whether dose adjustment is necessary. Because of the extensive cytochrome P450-mediated metabolism of efavirenz and limited clinical experience in patients with chronic liver disease, caution must be exercised in administering efavirenz to patients with mild hepatic impairment. Patients should be monitored carefully for dose-related adverse reactions, especially nervous system symptoms.

Renal insufficiency

The pharmacokinetics of efavirenz have not been studied in patients with renal insufficiency; however, less than 1% of an efavirenz dose is excreted unchanged in the urine, so the impact of renal impairment on efavirenz elimination should be minimal. There is no experience in patients with severe renal failure and close safety monitoring is recommended in this population.

Elderly patients

Insufficient numbers of elderly patients have been evaluated in clinical studies to determine whether they respond differently than younger patients.

Paediatric population

Efavirenz has not been evaluated in children below 3 years of age or whose weight is less than 13 kg. Therefore, efavirenz should not be given to children

less than 3 years of age. Rash was reported in 26 of 57 children (46%) treated with efavirenz during a 48-week period and was severe in three patients. Prophylaxis with appropriate antihistamines prior to initiating therapy with efavirenz in children may be considered.

Pregnancy

Efavirenz may cause fetal harm when administered during the first trimester to a pregnant woman. Pregnancy should be avoided in women receiving efavirenz. There are no adequate and well-controlled studies in pregnant women. Efavirenz should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus, such as in pregnant women without other therapeutic options (Chersich, *et al*., 2006).

2.2.8 Drug interactions

Efavirenz has been shown *in vitro* and *in vivo* to induce CYP3A4 activity in a concentration-dependent and time-dependent manner (Hariparsad *et al*.,2004; Mouly *et al*.,2002). Other compounds that are substrates of CYP3A4 may have decreased plasma concentrations when coadministered with efavirenz. *In vitro* studies have shown that efavirenz inhibits CYP2C9, CYP2C19 and CYP3A4 isozymes with inhibition constant (K_i) values (8.5-17 μM) in the range of observed efavirenz plasma concentrations (Von *et al*.,2001). Coadministration of efavirenz with drugs primarily metabolized by these isozymes may result in altered plasma concentrations of the coadministered drug. Therefore, appropriate dose adjustments may be necessary for these drugs.

Drugs which induce CYP3A4 activity (e.g., phenobarbital, rifampin, rifabutin) would be expected to increase the clearance of efavirenz resulting in lowered plasma concentrations.

Rifampicin

Eight HIV infection and tuberculosis patients received rifampicin for 7 days then efavirenz 800 mg once daily was added to the regimen after 7 days. Efavirenz, mean peak concentration, trough concentration and area under the concentration-time curve over the administration interval decreased 24%, 25% and 22%, respectively, in the presence of rifampicin, suggesting that plasma concentration monitoring of efavirenz may be advisable. Overall, the pharmacokinetics of efavirenz 800 mg plus rifampicin were similar to those of efavirenz 600 mg without rifampicin (Lopez-Cortes *et al*., 2002). In subjects weighing more than 50 kg, an increase of efavirenz dose from 600 mg to 800 mg once daily has been advised (Manosuthi *et al*., 2006).

Nevirapine

Concomitant use of efavirenz 600 mg once daily and nevirapine 400 mg once daily in HIV infected patients decreased area under the time-curve, minimum plasma concentration and maximum plasma concentration of efavirenz by 22%, 36% and 17%, respectively. The efavirenz dose may need modification when used with nevirapine (Veldkamp *et al*., 2001).

Amprenavir

HIV-infected patients were treated initially with amprenavir, 1200 mg twice daily, and abacavir, 300 mg twice daily for 1 week and then efavirenz 600 mg once daily was added to the regimen for 1 week. Efavirenz decreased the steady-state area under the curve, maximum plasma concentration, and minimum plasma concentration of amprenavir by 24%, 33%, and 43%, respectively. Amprenavir doses should be adjusted when concurrent efavirenz is given (Falloon *et al*., 2000).

Indinavir

The addition of efavirenz to a combination of 800 mg indinavir and 100 mg ritonavir twice daily results in significant decreases in indinavir area under the curve (AUC,–25%), trough concentration (C_{min},–50%), and maximum

concentration (C_{max}, –17%). The dose of indinavir or ritonavir should be increased to maintain similar indinavir drug levels after addition of efavirenz to the indinavir-ritonavir combination. Dose modifications may not be needed in antiretroviral-naive human immunodeficiency virus–infected patients if the reference C_{min} of the regimen of 800 mg indinavir 3 times a day is considered to be adequate (Aurnoutse *et al*., 2002).

Lopinavir

Hepatic induction by efavirenz lowers lopinavir pharmacokinetic parameters. $C_{predose}$ and mean area under the curve after of lopinavir were decresed by 33 % and 25 %, respectively. Increasing the lopinavir/ ritonavir dose to 533/133 mg twice daily during coadministration with efavirenz would likely compensate for the enzyme inductive effect of efavirenz that results in reduced lopinavir levels with the standard lopinavir/ritonavir dose of 400/100 mg twice daily (Hsu *et al*., 2003).

Atazanavir

The means of atazanavir clearance are significantly increased when co-administered with efavirenz. The mean trough plasma concentrations of atazanavir were 32% lower in the groups of patients treated with atazanavir/ritonavir associated with efavirenz , the atazanavir/ritonavir dosage was increased to 400 mg/100 mg once daily for six patients in co-administered with efavirenz. Therapeutic drug monitoring of atazanavir when ritonavir-boosted atazanavir is used in combination with efavirenz (Dailly *et al*., 2006).

Ritonavir

Twenty four healthy subjects received 1875 mg nelfinavir plus 200 mg ritonavir once daily with a 300-kcal snack for 10 days. During days 11–20 efavirenz 600 mg once daily was added to the regimen. Decreases were observed in ritonavir AUC_{0-24} (- 20%), C_{max} (- 24%) and C_{min} (- 12%) after the addition of efavirenz to the regimen (Porte *et al*., 2004).

Saquinavir

Concomitant of efavirenz 600 mg once daily for 10 days and saquinavir 1200 mg 3 times daily as soft gelatin capsule in healthy volunteers decreased the C_{max} and AUC by 50 % and 60 %, respectively. Use of efavirenz in combination with saquinavir is not recommended (Adkins *et al*., 1998).

Methadone

In a study of 11 patients receiving methadone therapy, efavirenz resulted in an over 50 % decrease in methadone area under the curve after only 24 hours of efavirenz administration. This led to nine patients complaining of withdrawal symptoms beginning on day 8 of efavirenz treatment. The dose of methadone was adjusted by increments of 10 mg to counteract the efavirenz inducing effect (Clarke *et al*., 2000).

Voriconazole

In a study of 34 healthy volunteers received efavirenz 400 mg once a day plus voriconazole 200 mg twice daily for 9 days. Efavirenz decreased steady-state mean area under the curve after only 12 hours and C_{max} of voriconazole by 80 % and 66 %, respectively. Voriconazole moderately increased steady-state mean area under the curve after only 24 hours and C_{max} of efavirenz by 43 % and 37 %, respectively. Coadministration of 200 mg twice daily with 400 mg (or higher) once daily efavirenz is contraindicated due to the clinically significant effect of efavirenz on voriconazole pharmacokinetics (Liu *et al*., 2008).

Ketoconazole

In a study of twelve HIV-infected patients receiving efavirenz 600 mg once daily in combination with 150 mg of lamivudine and 30 or 40 mg of stavudine twice daily for 2 weeks. Ketoconazole 400 mg was added to the regimen as a single oral dose after 2 weeks. Efavirenz increased the clearance of ketoconazole by 201 %. Cmax and mean area under the curve after only 24 hours of ketoconazole were

decreased by 44 % and 72 %, respectively. The half-life was significantly shorter by 58%. Therefore, concomitant administration of ketoconazole and efavirenz should be avoided (Sriwiriyajan *et al*., 2007).

Simvastatin, Atorvastatin, and Pravastatin

Fifty two healthy adult HIV-seronegative subjects received 40 mg of simvastatin, 10 mg of atorvastatin, or 40 mg of pravastatin daily co-administration of efavirenz 600 mg daily for 3 days. Efavirenz reduced area under the curve at 0 to 24 hours of simvastatin, atorvastatin and pravastatin by 58%, 43% and 34%, respectively (Gerber *et al*.,2005).

2.3 Phenytoin

Phenytoin is the oldest nonsedative antiseizure drug, introduced in 1938. It was known as diphenylhydantoin. It is related to barbiturates in chemical structure, but has a five-membered ring. The chemical name is 5,5-diphenyl-2,4-imidazolidinedione. Its molecular formula is $C_{15}H_{12}N_2O_2$ and its molecular weight is 252.3. The structure of phenytoin is shown in figure 3.

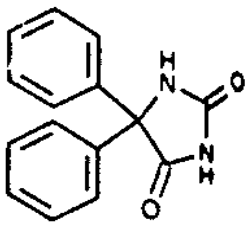


Figure 3: Chemical structure of phenytoin

Phenytoin is a white, odorless powder at room temperature. It is practically insoluble in water, but it is soluble in acetone, ethanol, and alkali hydroxides. The melting point is 295-298°C. The relatively high negative logarithm of the association constant (p*K*a) is 8.3.

2.3.1 Pharmacological effects

Central nervous system

Phenytoin exerts anti-seizure activity without causing general depression of the CNS. In toxic doses, it may produce excitatory signs and at lethal levels a type of decerebrate rigidity. The most significant effect of phenytoin is its ability to modify the pattern of maximal electroshock seizures. The characteristic tonic phase can be abolished completely, but the residual clonic seizure may be exaggerated and prolonged. This seizure-modifying action is observed with many other anti-seizure drugs that are effective against generalized tonic-clonic seizures. By contrast, phenytoin does not inhibit clonic seizures evoked by pentylenetetrazol.

2.3.2 Mechanism of action

Phenytoin binds to specific site on voltage-dependent sodium channels and is thought to exert its anticonvulsant effect by suppressing the sustained repetitive firing of neurons by inhibiting sodium flux through these voltage dependent channels (Francis and Burnham, 1992). Phenytoin stabilises membranes, protecting the sodium pump in the brain and in the heart. It limits the development of maximal convulsive activity from a discharging focus without influencing the focus itselt (Reynolds *et al*., 1996). These effects of phenytoin are evident at concentrations in the range of therapeutic drug levels in cerebrospinal fluid in humans, which correlate with the free (or unbound) concentration of phenytoin in the serum. At these concentrations, the effects on Na^+ channels are selective, and no changes of spontaneous activity or responses to iontophoretically applied GABA or glutamate are detected. At concentrations 5- to 10-fold higher, multiple effects of phenytoin are evident, including reduction of spontaneous activity and enhancement of responses to GABA; these effects may underlie some of the unwanted toxicity associated with high levels of phenytoin.

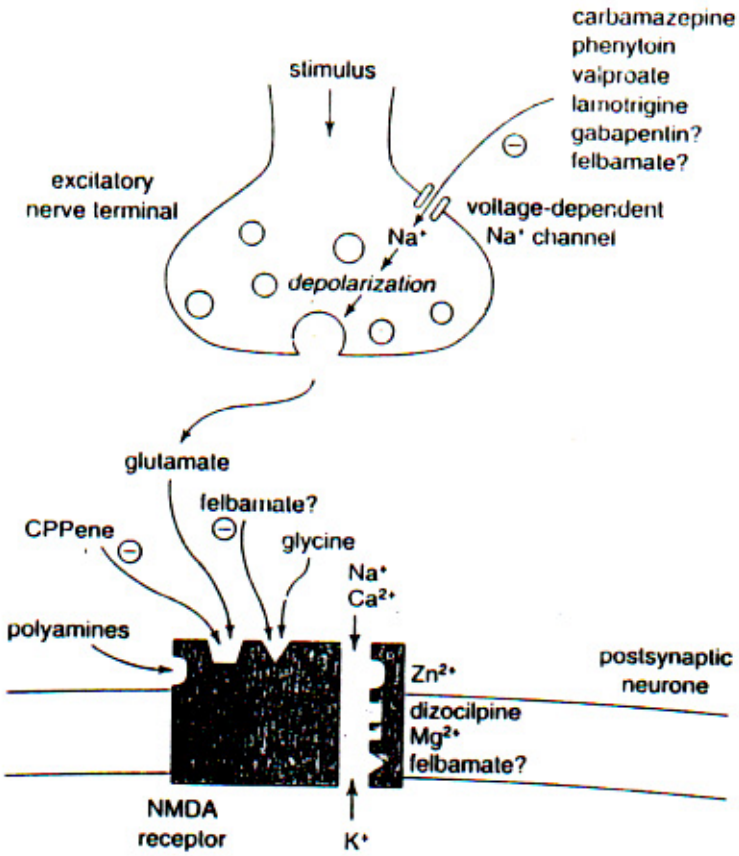


Figure 4: Some antiepileptic drugs stabilize inactive configuration of sodium (Na+) channel, preventing high-frequency neuronal firing.

2.3.3 Pharmacokinetics

Absorption

Phenytoin, a weak acid with a pK_a of 8.3 is practically insoluble in water. A parenteral preparation containing the sodium salt of phenytoin is available, although in most instances the drug is administered orally. The rate of absorption of phenytoin varies among dosage form. Absorption of phenytoin sodium from the gastrointestinal tract is nearly complete in most patients. The time at which the concentration peaks is 3 to 12 hours after a single oral dose of a capsule or tablet (Gugler *et al*., 1976). The intramuscular route of administration should be avoided with phenytoin sodium injection because phenytoin precipitates at the site of

injection. In contrast, fosphenytoin, a more soluble phosphate prodrug of phenytoin, is well absorbed after intramuscular administration.

Distribution

Phenytoin is extensively (90 percent) bound to plasma proteins, especially albumin (Tozer and Winter, 1992). The apparent volume of distribution is approximately 0.5 to 0.8 L/kg (Gugler *et al*., 1976; Hvidberg & Dam, 1976). Phenytoin rapidly distributes to the brain (Vajda *et al*., 1974). The free phenytoin fraction normally constitutes 10 percent of the plasma level.

Metabolism

Phenytoin is extensively metabolised in the liver by CYP 2C9 and CYP 2C19 (Bajpai *et al*, 1996) to several inactive hydroxylated metabolites. Some of these metabolites, notably 5-(p-hydroxyphenyl)-5 phenylhydantoin (p-HPPH) are further metabolized by conjugation with glucuronic acid. Less amounts appear as free and conjugated meta-hydroxylated and dihydrodiol metabolites (Chang and Glazko, 1982). It would appear that the majority of the metabolites are formed via an epoxide intermediate (Tozer and Winter, 1992). Phenytoin hydroxylation is capacity-limited (dose dependent) because of the saturable enzyme systems in the liver. At very low blood levels, phenytoin metabolism follows first-order kinetics. However, as blood levels rise within the therapeutic range, the maximum capacity of the liver to metabolize phenytoin is approached (Reynolds, 1996). Further increases in dosage, though relatively small, may produce very large changes in phenytoin concentrations. The rate of metabolism appears to be subject to genetic polymorphism (Reynolds, 1996).

Excretion

The urinary recovery of p-HPPH and its glucuronide account for 60-90 % of an oral dose of phenytoin (Gugler *et al*., 1976). Only 1 to 5 percent of phenytoin is excreted unchanged in the urine. Importantly, the clearance of phenytoin is

concentration-dependent within the usual therapeutic plasma concentration range, as a result of saturable biotransformation. Following oral administration of therapeutic doses, phenytoin has a very variable, dose-dependent half-life. The range for a therapeutic dose is from 8 to 60 hours with an average of from 20 to 30 hours (Robinson *et al*, 1975; Hvidberg and Dam, 1976). In overdose in adults the range is from 24 to 230 hours (Gill *et al*., 1978; Albertson *et al*., 1981).

2.3.4 Clinical use

Phenytoin is an effective anticonvulsant for the chronic treatment of tonic-clonic (grand mal) or partial seizures and the acute treatment of generalized status epilepticus. It can be used to control seizures occurring during neurosurgery and to reverse digitalis-induced arrhythmia.

2.3.5 Dosage and administration

Serum concentrations should be monitored in changing from Extended Phenytoin Sodium Capsules, USP (Dilantin) to Prompt Phenytoin Sodium Capsules, USP, and from the sodium salt to the free acid form.

Dilantin® Kapseals® is formulated with the sodium salt of phenytoin. The free acid form of phenytoin is used in Dilantin-125 Suspension and Dilantin Infatabs. Because there is approximately an 8% increase in drug content with the free acid form over that of the sodium salt, dosage adjustments and serum level monitoring may be necessary when switching from a product formulated with the free acid to a product formulated with the sodium salt and vice versa.

Dosage should be individualized to provide maximum benefit. In some cases, serum blood level determinations may be necessary for optimal dosage adjustments. The clinically effective serum level is usually 10–20 μg/ml. With recommended dosage, a period of seven to ten days may be required to achieve steady-state blood levels with phenytoin and changes in dosage (increase or decrease) should not be carried out at intervals shorter than seven to ten days.

2.3.6 Adverse effects

Phenytoin side effects, such as hypertrichosis, gingival hypertrophy, thickening of facial features, carbohydrate intolerance, folic acid deficiency, peripheral neuropathy, vitamin D deficiency, osteomalacia and systemic lupus erythematosus do not appear to be readily related to the plasma phenytoin concentration.

Central nervous system side effects, such as nystagmus, ataxia and decreased mentation have been associated with elevated phenytoin concentrations. Nystagmus is usually the first sign to appear, often within the therapeutic range and as the serum concentration increases because it is probably the most frequent objective symptom that can be documented. Far-lateral nystagmus occurs in some patients with phenytoin concentrations below 15 mg/L but is usually observed in the majority of patients at concentrations exceeding 20 mg/L. Nystagmus at a 45° lateral gaze, as well as ataxia, occurs frequenly at concentrations exceeding 30 mg/L. Diminished mental capacity and ataxia are usually marked when phenytoin concentrations are above 40 mg/L. There may be a significant variance for any individual patient as to which of the side effects is observed first and as to the concentration at which the side effect is first noted.

The other toxic effect occur with increasing frequency and severity (Reynolds and Trimble, 1985; Kutt and Penry, 1974). Seizure activity and the induction of involuntary movements have also been described in patients with phenytoin concentration greater than 20 μg/ml (Levy and Fenichel, 1969; Shuttleworth *et al*., 1974).

Elderly patients appear to have greater mental changes than do younger patients at the same concentration (Kutt *et al*., 1964). Decreased plasma protein binding with age may partially account for this observation.

Additional precautions must be taken when phenytoin is given intravenously. Phenytoin sodium injection and fosphenytoin, when administered by the intravenous route can produce bradycardia, hypotension and widening of the QRS and QT intervals on an electrocardiogram (Browne, 1997). Some of the

cardiovascular side effects of phenytoin sodium injection are caused by the propylene glycol diluents (Louis *et al*., 1967). The cardiovascular symptoms can be diminished or avoided by injecting the intravenous solution slowly.

2.3.7 Special populations

Effects of liver disease

Changes in hepatic blood flow do not alter phenytoin clearance because it is a low-extraction drug (Tozer and Winter, 1992). However, loss of functional hepatocytes decreases phenytoin metabolism. Phenytoin accumulates as hepatic dysfunction increases (Kutt *et al*., 1964). Because of decreased albumin production, liver disease is associated with decreased protein binding capacity for phenytoin. Further, bilirubin may compete for binding sites of the albumin molecule, further increasing the unbound concentration (Blaschke *et al*., 1975).

The effect of liver diseases on fosphenytoin was evaluated in four patients with liver dysfunction, four patients with renal dysfunction, and four control subjects. There was no difference in the time to peak fosphenytoin concentrations, but the time to achieve peak plasma concentrations of phenytoin was faster in the patients with liver and renal impairment because of decreased protein binding of fosphenytoin and phenytoin (Aweeka *et al*., 1999).

Renal impairment

Less than 5% of phenytoin is cleared by renal clearance. Nevertheless, renal impairment has significant effects on phenytoin pharmacokinetics. Because of decreased protein binding in renal failure increase in the unbound phenytoin concentration. As a result the plasma phenytoin concentration falls since an increase in the unbound phenytoin fraction results in an increased rate of metabolism of phenytoin. These changes can lead to overdosing of phenytoin if the dosing rate is based upon the total phenytoin plasma concentration. Monitoring of the free phenytoin plasma concentration is preferred in patients with renal insufficiency.

Geriatric Considerations

Elderly may have reduced hepatic clearance due to age decline in phase I metabolism. Elderly may have low albumin which will increase free fraction of phenytoin and, therefore, pharmacologic response. Monitor closely in those who are hypoalbuminemic. Free fraction measurements advised, also elderly may display a higher incidence of adverse effects (cardiovascular) when using the I.V. loading regimen; therefore, recommended to decrease loading I.V. dose to 25 mg/minute.

Pregnancy Considerations

Phenytoin crosses the placenta. Congenital malformations (including a pattern of malformations termed the "fetal hydantoin syndrome" or "fetal anticonvulsant syndrome" have been reported in infants. Isolated cases of malignancies (including neuroblastoma) and coagulation defects in the neonate following delivery have also been reported. Total plasma concentrations of phenytoin are decreased by 56% in the mother during pregnancy; unbound plasma (free) concentrations are decreased by 31%. Because protein binding is decreased, monitoring of unbound plasma concentrations is recommended. Concentrations should be monitored through the 8th week postpartum. The use of folic acid throughout pregnancy and vitamin K during the last month of pregnancy is recommended.

2.3.8 Phenytoin drug interactions

Phenytoin has been shown to induce CYP3A4, CYP2C9, CYP1A2, CYP2B6 (LeCluyse *et al*., 1996; Spatzenegger and Jaeger, 1995; Ducharme *et al*., 1997; Patsalos *et al*., 2002). In rat, phenytoin induces CYP3A and CYP2B1 (Nims *et al*., 1994). Coadministration of phenytoin with drugs primarily metabolized by these isozymes may result in altered plasma concentrations of coadministered drug. Therefore, appropriate dose adjustment may be necessary for these drugs.

Lopinavir/ritonavir

Lim et al (2004) studied the pharmacokinetic of lopinavir/ritonavir 400/100 mg twice daily combined with phenytoin 300 mg once daily in 24 healthy subjects. The results revealed a 2-way interaction in which both phenytoin and lopinavir/ritonavir AUC were decreased by approximately 30% (Lim *et al*., 2004).

Efavirenz

There has been a case report of a patient with HIV infection, CMV retinitis and probable toxoplasmic encephalitis who was received 300 mg phenytoin twice daily combination with efavirenz 600 mg once daily. Ten days later, efavirenz concentration was 0.34 μg/ml that lower than expected concentration (12 h concentration < 1 μg/ml) Phenytoin may induce the metabolism of efavirenz. The efavirenz dosage was increased to 800 mg/day, after increase dosage of efavirenz, 12 day later, the efavirenz concentration was 0.58 μg/ml. Thus phenytoin therapy was discontinued and to commence therapy with levitiracetam, an anticonvulsant without CYP modulation potential. Two weeks later after phenytoin discontinuation, efavirenz concentration was 2.5 μg/ml. Then efavirenz dosage could be reduced to 600 mg/day. The pateint's lastest measured efavirenz concentration was 1.96 μg/ml after 3 weeks at a dosage of 600 mg/day. Coinduction of CYP2B6 and CYP3A4 is expected to result in a reduction in efavirenz exposure during concurrent use of phenytoin. In addition, Phenytoin levels measured after efavirenz therapy initiation showed a gradual increase, despite a stable phenytoin dosage and no additional changes in medications. After a steady state was achieved, phenytoin plasma concentrations continued to increase. Both phenytoin and efavirenz levels should be monitored closely when the drugs are given concurrently, to avoid potential toxicity or treatment failure (Robertson *et al*., 2005).

Quetiapine

In a treatment phase in patients, phenytoin decreases the plasma concentration of quetiapine (substrate of CYP3A4). The quetiapine geometric mean $AUC_{0–8}$, C_{max} and C_{min} were reduced to 19%, 27%, and 12% respectively, after the administration of phenytoin. Quetiapine CL/f increased more than 5-fold after phenytoin coadministration. Dosage adjustment of quetiapine may be necessary when the two drugs are given concurrently (Wong *et al*., 2001).

Cyclophosphamide

William et al (1999) studied the effect of phenytoin (PHE) on cyclophosphamide (CP) disposition in 6 adult patients in bone marrow transplantation. Phenytoin pretreatment increased the clearance of (*R*)- and (*S*)-CP via 4-hydroxylation, relative to control patients, by inducing CYB2B (William *et al*.,1999).

Ifosfamide

There has been a case report of a pediatric patient receiving phenytoin combination with ifosfamide (IFF). The metabolic formation of IFF enantiomers was increased and the metabolic pattern of the *N*-dechloroethylation altered from nonphenytoin-treated patients. Phenytoin treatment of a patient lead to a larger increase in N2- dechloroethylation of (*S*)-IFF and N3-dechloroethylation of (*R*)-IFF (both CYP 2B-medaited) than N2- dechloroethylation of (*R*)-IFF and N3-dechloroethylation of (*S*)-IFF (both CYP 3A-medaited) (Ducharme *et al*., 1996).

Tirilazad

Fleishaker et al (1998) studied the induction of tirilazad clearance by phenytoin in 12 volunteers. Tirilazad clearance was increased by 91.8% and AUC of U-89678 (an active metabolize) was reduced by 93.1% during phenytoin coadministration (Fleishaker *et al*., 1998).

Itraconazole

Ducharme et al (1995) studied the disposition of single doses of 300 mg phenytoin and 200 mg itraconazole when administered alone and after chronic treatment with the other drug in healthy male volunteer. Phenytoin decreased the AUC of itraconazole by more than 90% and decreased half-life. Similar changes were observed for hydroxyitraconazole AUC and half-life. Itraconazole increased the AUC of phenytoin with no change in any other pharmacokinetic parameter. The striking decrease in itraconazole concentrations with phenytoin is due to induction of metabolism combined with a reduction in the degree of saturable metabolism normally exhibited by itraconazole at this dose. The magnitude of interaction likely accounts for reports of therapeutic failures in patients with fungal infections who are receiving both itraconazole and phenytoin (Ducharme *et al*., 1995).

Ketoconazole

Concomitant administration of phenytoin 300 mg/d and ketocanazole 600 mg/d, the area under the steady-state concentration of ketoconazole were 0.16 µg/ml during concurrent and 4.6 µg/ml after discontinue phenytoin. Patient failed to respond during concurrent therapy. The coadministration of phenytoin with ketoconazole leads to a profound reduction in serum ketoconazole concentration. This decrease has an effect on the clinical response to therapy that appears to correlate with *in vitro* susceptibility result for the relevant fungal pathogen (Tucker *et al*., 1992).

Voriconazole

Phenytoin reduced voriconazole C_{max} and AUC by 49% and 69%, respectively, compared with steady-state voriconazole levels achieved with 200 mg twice-daily dosing. However, when the voriconazole dose was increased to 400 mg twice daily, C_{max} and AUC were increased by 34% and 39%, respectively. In addition, administration of voriconazole at the higher dose regimen of 400 mg twice daily resulted in increases phenytoin C_{max} and AUC_t by 67% and 81%, respectively, compared with placebo. It is recommended that phenytoin levels should be routinely

monitored and its dose adjusted to ensure the maintenance of therapeutic plasma levels when phenytoin is coadministered with voriconazole (Purkins *et al*., 2003).

Digoxin

Rameis (1985) reported that coadmonistration of phenytoin to healthy volunteers caused a significant reduction in the elimination half-life of digoxin and a significant increase in its total clearance. Because the renal clearance of digoxin was not affected. It was concluded that phenytoin increased its hepatic clearance (Rameis, 1985).

Quinidine

Data et al (1976) reported that both the half-life and AUC of quinidine, following a single oral dose, were decreased substantially during phenytoin treatment, an effect attributed to increased metabolic clearance of quinidine. The magnitude of the interaction led Data and co-workers (1976) to caution that a patient who is well controlle don quinidine may become quinidine toxic if phenytoin is discontinued, or serious arrhythmias may emerge if phenytoin is started (Data *et al*., 1976).

Mexiletine

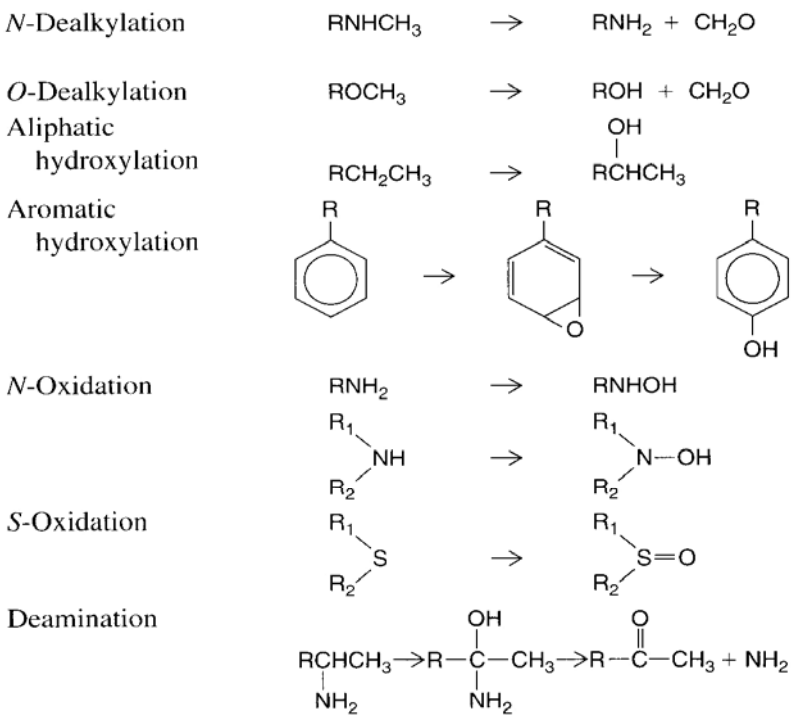
Begg et al (1982) conducted a pharmacokinetic drug interaction study in 6 healthy volunteers who were given a single oral dose of mexiletine before and after 1 week's administration of phenytoin 300 mg daily. Pretreatment with phenytoin resulted in a significant reduction in the half-life and AUC of mexiletine; the mean value of each was approximately halved. Because mexiletine is extensively metabolized, it was suggested that the most likely explanation for the interaction was induction of the hepatic mixed-function oxidase system (Begg *et al*., 1982).

2.4 Cytochrome P450 Enzymes

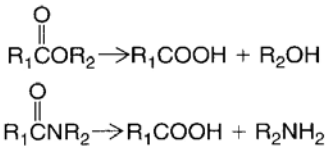
The cytochrome P450 (P450 or CYP) are a group of heme-containing enzymes that take part in the metabolism of many drugs, steroids and carcinogens (Guengerich, 1992) They are present in the endoplasmic reticulum of many types of cells but are at highest concentration in hepatocytes. In the intestinal tract, P450s are present in the crypt cells, but the highest concentration is in the enterocytes at the tips of the villi and can account for first-pass metabolism of many drugs. The name cytochrome P450 is derived from the spectral properties of this hemoprotein. In its reduced (ferrous) form, it binds carbon monoxide to give a complex that absorbs light maximally at 450 nm. The relative abundance of P450s, compared with that of the reductase in the liver, contributes to making P450 heme reduction a rate-limiting step in hepatic drug oxidations.

When drugs are administered they are metabolized through a series of reactions to enhance drug hydrophilicity and facilitate drug excretion. These drug biotransformation reactions are grouped into two phases, phase I and phase II. Phase I reactions involve intramolecular changes such as oxidation, reduction, and hydrolysis. These reactions expose or induce a functional group (-OH, $-NH_2$, -SH, $-CO_2H$) and usually result in only a small increase in the hydrophilicity of the drug. The CYP enzyme family plays an important role in phase-1 metabolism of many drug. Some examples of phase I reactions are presented in figure 5. Phase II reactions are conjugation reactions in which an endogenous substance combines the functional group derived from phase I reactions to produce a highly polar drug conjugate that can be readily eliminated. These reactions involve glucuronidation, sulfation, acetylation, methylation, amino acid conjugation and glutathione conjugation. Phase 2 preceding phase 1 reaction, although less common, can also occur. These reactions, either alone or in concert are responsible for the generation of readily excretable metabolites (Yan *et al*., 2001). Some examples of phase II reactions are presented in figure 6.

Oxidation reactions



Hydrolysis reactions



Conjugation reactions

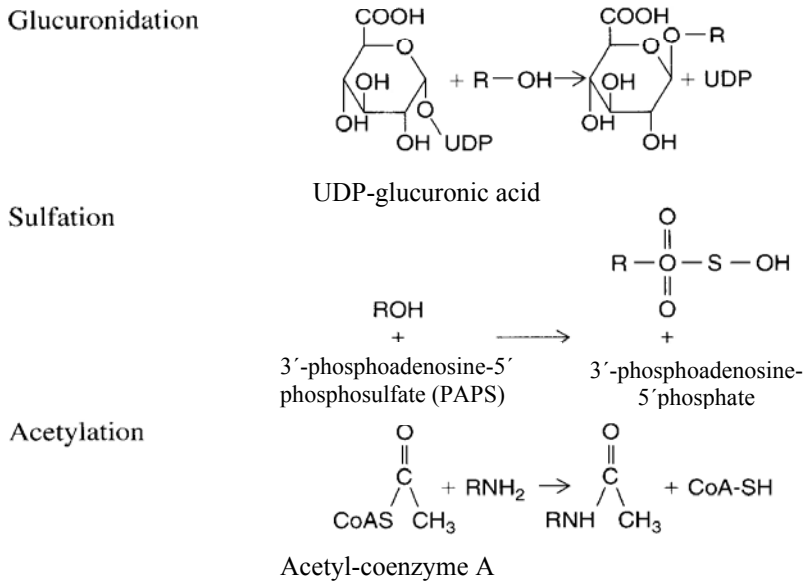


Figure 5 : Major reactions involved in drug metabolism (Wilkinson, 2001)

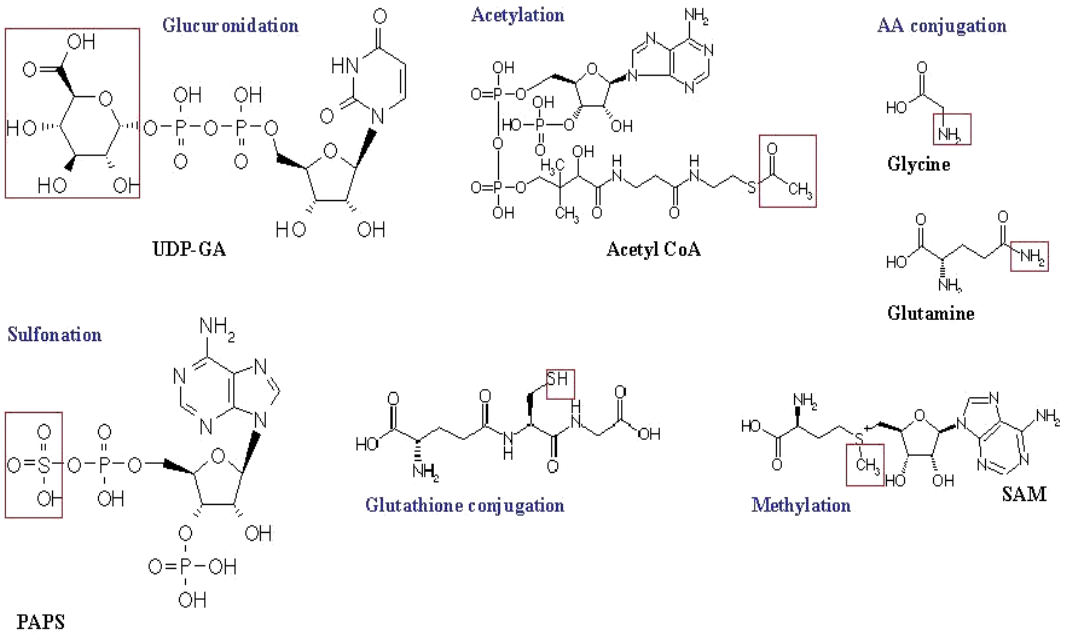


Figure 6 : Structures of cofactors for phase II biotransformation (Jassal, 2004)

At the present time, a number of CYP isoenzymes are expressed in each mammalian species including humans (Nelson *et al*., 1993), many of these have specific role involving anabolic steroids and are localized in the liver. The present system of nomenclature for the various CYP isozymes employs a three-tiered classification based on the conventions of molecular biology: the family (members of the same family display > 40% homology in their amino acid sequences), subfamily (> 55% homology), and individual gene (Nebert *et al*., 1991). This pedigree is indicated by, respectively, an arabic numeral (family), a capital letter (subfamily) and another arabic numeral (gene), e.g. CYP1A2. Of the 74 gene families so far described, 14 exist in all mammals. These 14 families comprise of 26 mammalian subfamilies (Nelson *et al*., 1996).

The human genome has 57 CYP genes, and the function for most of the corresponding enzymes is known at least to some degree. Fifteen individual CYP enzymes in families1, 2 and 3 metabolize xenobiotics, including the majority of small molecule drugs currently in use (Pelkonen *et al*., 2008). Three main CYP gene families, CYP1, CYP2, CYP3, are responsible for most hepatic drug metabolism.

These isoforms have the same oxidising centre (the haem iron), but differ by their protein structures.

Approximately 70 % of human liver CYP is accounted for by CYP1A2, CYP2A6, CYP2B6, CYP2C, CYP2D6, CYP2E1 and CYP3A enzymes. Among these, CYP3A (CYP3A4, CYP3A5) and CYP2C (CYP2C8, CYP2C9, CYP2C18 and CYP2C19) are the most abundant subfamilies, accounting for 30% and 20 % of the total CYP, respectively. Other isoforms are minor contributors to the total CYP: CYP1A2 at 13%, CYP2E1 at 7%, CYP2A6 at 4%, CYP2D6 at 2% and CYP2B6 at 0.2 % (Shimada *et al*., 1994).

CYP3A4 has a pivotal role in xenobiotic metabolism and it has been estimated to be involved in the metabolism of approximately 50% of the drugs in clinical use. There is considerable interindividual variability in hepatic and intestinal CYP3A activity (about 5-10 fold) (Shimada *et al*., 1994). Since 40-50% of drugs used in humans involve 3A mediated oxidation to some extent, the members of this subfamily are involved in many clinically important drug interactions (Thummel and Wilkinson, 1998).

The evidence now indicates that there are three P450 3A subfamily proteins in humans, 3A4, 3A5, and 3A7, with the latter apparently being expressed only in fetal tissue, placenta, and possibly tumors (Guengerich *et al*., 1995; Komori *et al*., 1990; Schuetz *et al*., 1993). In rats, at least four or five P450 3A enzymes are found, 3A1, 3A2, 3A9, 3A18, and 3A23 (Nagata *et al*., 1990; Mahnke *et al*., 1997). To date only one rabbit P450 3A enzyme, 3A6, has been reported but it is not clear if searches for more members have been made (Guengerich *et al*., 1997).

CYP2B6 plays important roles in the metabolism of a number of therapeutic drugs such as the antineoplastic agent cyclophosphamide, antiestrogen tamoxifen, anticonvulsant *S*-mephenytoin, benzodiazepine diazepam and efavirenz (Chang *et al*., 1993; White *et al*., 1995; Heyn *et al*., 1996; Ono *et al*., 1996; Ward *et al*., 2003). CYP2B is well conserved between rodents and rabbits, but is poorly expressed in human liver. In humans CYP2B6 has 76% amino acid sequence identity with rat CYP2Bl (Yamano *et al*., 1989). Rabbit CYP2B4 appears to have a catalytic

specificity similar to that of rat CYP2B1, in so far as rough comparisons are possible from the literature (Guengerich *et al*., 1997).

2.4.1 Mechanisms of induction of CYP enzymes

Many of the CYPs are induced in humans including CYP1A, CYP2A, CYP2B, CYP2C, CYP2E1, and CYP3A by a diverse array of compounds including drugs, industrial chemicals, natural products, and ethanol. The inducible CYPs make up a large percentage of the CYPs in the human liver and are responsible for the metabolism of a large proportion of pharmaceutical drugs. In most cases, induction of CYPs occurs by a process involving de novo RNA and protein synthesis that has been demonstrated in studies using transcription and translation inhibitors (Daujat *et al*., 1991). The induction of CYPs is highly conserved and is found not only in humans but also in many other species including rodent models.

The induction of CYP enzymes can be caused by at least 5 different mechanisms. Ethanol selectively induces CYP2E1 primarily by stabilizing the enzyme protein (Fuhr 2000). Other types of induction of CYP enzymes seem to be mediated by intracellular receptors, namely the aryl hydrocarbon receptor (AhR) receptor, the constitutive androstane receptor (CAR), the pregnane X receptor (PXR), and the peroxisome proliferator-activated receptor (PPAR) (Fuhr 2000). The AhR receptor is a transcription factor that belongs to the basic-helix-loop-helix-PAS (bHLH-PAS) family, whereas CAR, PXR, and PPAR are orphan nuclear receptors (Waxman, 1999).

The mechanism of the induction of protein synthesis by the nuclear receptors CAR, PXR, and PPAR is essentially similar. An inducer binds to CAR, PXR, or PPAR, and the inducer-receptor complex forms a heterodimer with the retinoid X receptor (RXR). This heterodimer binds to a DNA response element and enhances DNA transcription and eventually protein synthesis (Waxman, 1999). The mechanism of the induction of protein synthesis by the nuclear receptors PXR is shown in figure 7.

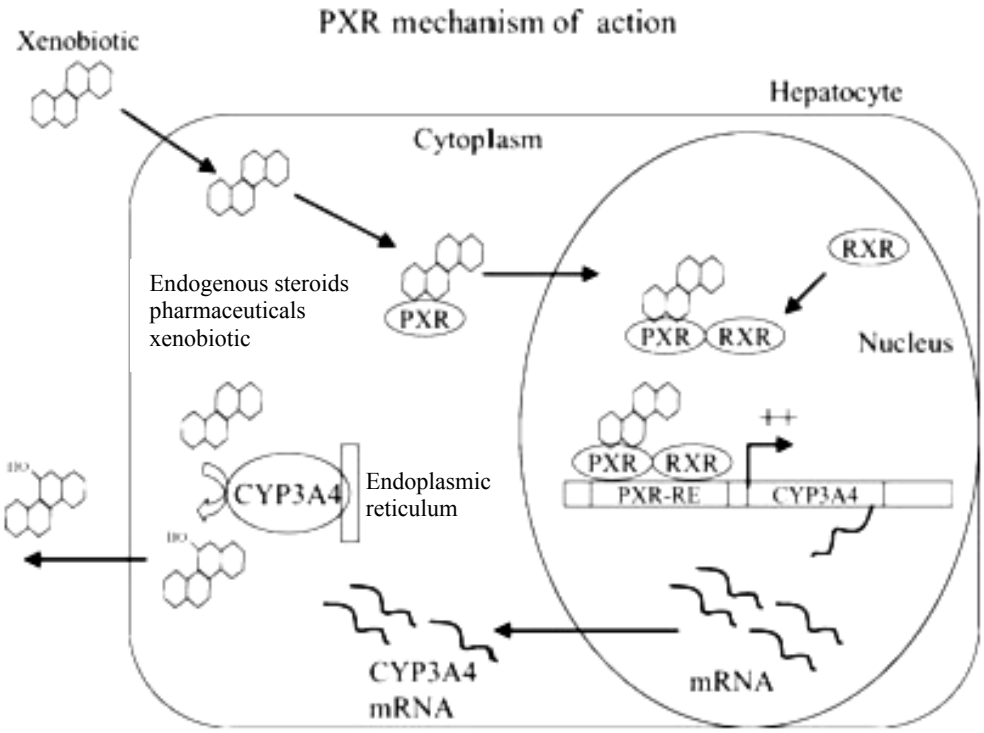


Figure 7 : Schematic model of a xenobiotic binding and activating PXR leading to CYP3A4 induction (Tompkins and Wallace, 2007).

Several clinical studies have reported a reduction in the plasma levels of other CYP3A4 and CYP2B6 substrates such as lopinavir/ritonavir, quetiapine, ifosfamide and cyclophosphamide when they are coadministered with phenytoin (Lim *et al*., 2004; Wong *et al*., 2001; Ducharme *et al*., 1996; William *et al*.,1999). The mechanism of CYP3A4 induction of phenytoin is mediated by PXR (Luo *et al*., 2002). Phenytoin induction of CYP2B6 gene expression in human primary hepatocytes is mediated predominantly through activation of human CAR (Wang *et al*., 2004).

PXR and induction of CYP3A

PXR is capable of binding to and activating transcription from specific response elements found in the CYP3A gene promoter from multiple species. Notably, compounds that are known to induce CYP3A selectively in human, mouse, rat, or rabbit also activate the corresponding PXR (LeCluyse, 2001). Orthologous receptors from human, mouse, rat, and rabbit have been cloned and characterized and share approx. 95% identity in their DNA binding domains. By contrast, they share only 75–80% identity in their amino acid sequences in the ligand-binding domain. Together, these data suggest that PXR is a critical regulator of CYP3A gene expression and activation of PXR is predictive of CYP3A induction. Furthermore, sequence differences in the ligand-binding domain, and not the DNA binding domain, appear to serve as the molecular basis for the species differences in CYP3A induction observed in vivo. PXR is most closely related to the vitamin D receptor (VDR) and, like VDR, binds to DNA elements as a heterodimer with a retinoid X receptor alpha (RXRα). PXR response elements have been well characterized, with the receptor binding to direct repeat with a 3-nucleotide (nt) spacer (DR3), everted repeat with a 6-nt spacer (ER6), and a direct repeat with a 4-nt spacer (DR4). X-ray crystallography of the ligand-binding domain of PXR determined that PXR has a much larger ligand-binding pocket when compared to other receptors; enabling PXR to bind such a wide variety of ligands (Watkins *et al*., 2001).

CAR and induction of CYP2B6

In humans, CYP2B6 is responsible for the metabolism of large number of drugs including chemotherapeutics, opioids, and the HIV-1 reverse transcriptase inhibitor efavirenz (Ekins *et al*., 1999; Lamba *et al*., 2003; Hodgson and Rose, 2007). CYP2B6 is highly inducible not only by phenobarbital but like CYP3A4 is also induced by a large number of compounds with diverse structures (Kretschmer *et al*., 2005). Studies of CYP2B promoters in rat and mouse identified critical elements necessary for phenobarbital induction, which were termed as a phenobarbital-responsive enhancer modules (PBREMs) (Honkakoski and Negishi, 1997; Trottier *et*

al., 1995). Use of the PBREM DNA element in affinity chromatography studies led to the identification of a liver-specific transcription factor that bound to this element (Honkakoski and Negishi, 1998). This transcription factor was named the constitutive androstane receptor or constitutively active receptor (CAR, NR1I3) for its ability to interact with androstanol and adrostenol and its in vitro ability to be constitutively active. In vivo, CAR is not constitutively active, but translocates from the cytoplasm to the nucleus following activation by phenobarbital. CAR is a member of the orphan receptor subfamily and as such contains modular functional domains, including a DNA-binding domain and a ligand-binding domain. Like PXR, CAR binds to DNA elements as a heterodimer with RXRα. CAR DNA-binding sites have been well characterized, with the receptor binding to a direct repeat with a 4-nt spacer (DR4). These elements are located in the human CYP2B6 and mouse Cyp2b10 promoters, and sites are bound by CAR in the presence of phenobarbital and xenobiotics. In X-ray crystallography studies, CAR was shown to have much smaller ligand-binding domain than PXR. In addition, unique structural conformations were identified that may explain CAR's ligand independent activities (Xu *et al*., 2004; Shan *et al*., 2004). The induction of CYP2B family members by phenobarbital via CAR activation is just one of the groups of metabolizing enzymes regulated by this mechanism. Along with CYP2B induction, upregulation of other genes including CYP2C, CYP3A, sulfotransferases, glucuronosyltransferases, glutathione S-transferases, and transporters occurs with CAR activation (Assem *et al*., 2004; Ueda *et al*., 2002).

Cross-Talk Between CAR and PXR

CAR is the closest relative of PXR on the branch of the orphan nuclear receptor tree. Although they were originally recognised as the regulators of CYP3A and CYP2B, respectively, there is significant overlap in the inducers of these two gene subfamilies. Several studies have found a large degree of cross-regulation between CYP2B6 and CYP3A4 expression, and increases in CYP2B6 activity have been demonstrated with the use of several drugs that are known inducers of CYP3A4 such as phenobarbital, rifampicin, phenytoin, dexamethasone (Sueyoshi *et al*., 1999;

Goodwin *et al*., 2001; Faucette *et al*., 2004). This 'cross-talk' may occur because both CAR and PXR recognise the other's response elements (i.e. DR4, DR3 and ER6) and trigger gene expression (CYP3A or CYP2B) upon activation by either common or selective ligands. Recently, several groups have shown evidence for cross-talk from several different perspectives. Using CV-1 cells and primary rat hepatocytes, Xie et al. (2000) demonstrated that both CAR and PXR could regulate CYP3A and CYP2B gene expression upon activation by their specific ligands. Smirlis et al. (2001) reported that when equimolar amounts of CAR and PXR expression vectors are cotransfected with a PBREM-reporter construct, a 60% decrease of the reporter gene expression was observed, suggested that the receptors could compete with each other for binding to the same response element.

AhR and induction of CYP1A

CYP1A induction has been shown to be important in the metabolism of polycyclic aromatic hydrocarbons (PAHs) and the production of reactive genotoxic metabolites important in cancer formation (Whitlock, 1999). Similarly, induction of CYP1A by cigarette smoke is associated with toxicity and carcinogenesis. When AhR is bound by polycyclic aromatic hydrocarbon (PAH), such as dioxin and 3-methylchoranthrene (3-MC), AhR translocates from the cytoplasm to the nucleus, heterodimerizes with Arnt, and activates transcription through the XRE located in the promoters of CYP1 family genes (Li *et al*., 1998; Nakajima *et al*., 2003). The requirement of AhR in CYP1 expression induction was demonstrated in AhR-null mutant mice (Gonzalez and Fernandez-Salguero, 1998; Shimizu *et al*., 2000). The expression of CYP1 genes induced by the AhR, in response to PAHs or halogenated aromatic hydrocarbon ligands such as benzo[a]pyrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or dioxin is well established (Gonzalez and Fernandez-Salguero, 1998; Levine and Perdew, 2001; Li *et al*., 1998; Nakajima *et al*., 2003; Shimizu *et al*., 2000).

CYP induction and other receptors

The mechanisms of CYP enzyme induction involve not only the AhR, PXR, and CAR receptors but also a number of other transcription factors including peroxisome proliferator-activated receptor (PPAR), farnesoid X receptor (FXR), liver X receptor (LXR), hepatic nuclear f actor (HNF) family members, glucocorticoid receptor (GR), and CCAAT /enhancer-binding proteins (C/EBPs). Many of these transcription factors also play roles in the previously discussed inductions of CYP1A, CYP2B, and CYP3A family of enzymes, as there is significant overlap of regulatory pathways and multiple DNA-binding elements in each CYP promoter. CYP4A induction is dependent on PPAR activation by a diverse range of drugs and environmental contaminants (Waxman, 1999). CYP7A1 regulation involves the FXR and LXR transcription factors (Kalaany and Mangelsdorf, 2006).

Nonreceptor-mediated induction of CYP2E1

In the human liver, CYP2E1 is highly expressed and is inducible by ethanol, acetone, and the drug isoniazid. The induction of CYP2E1, unlike that of other CYP isoforms discussed above, involves posttranscriptional stabilization of CYP2E1 (Song *et al*., 1989; Koop *et al*., 1985). Induction of CYP2E1 has been shown to be responsible for bioactivation of a number of substrates to reactive metabolites, causing oxidative stress and hepatotoxicity. CYP2E1 metabolizes organic solvents, nitrosamines, and drugs such as acetaminophen. Alcohol induction of CYP2E1 alters acetaminophen metabolism leading to an increase in the formation of the reactive metabolite N -acetyl- p-benzoquinone imine (NAPQI) (Hinson *et al*., 2004). The NAPQI metabolite is highly reactive and damages DNA, proteins, and lipid membranes. The mechanism of CYP2E1 induction involves both enhanced translation of CYP2E1 and inhibition of the proteasome ubiquitin degradation pathway (Kocarek *et al*., 2000; Kim *et al*., 1990; Roberts *et al*., 1995). The importance of CYP2E1 in acetaminophen metabolism and toxicity has been demonstrated in CYP2E knockout mice, as these mice are resistant t o acetaminophen toxicity.

Species differences in CYP3A induction

A novel orphan nuclear receptor, termed pregnane X receptor (PXR), has been implicated to play a key role in the regulation of CYP3A genes by xenobiotics. Jones et al. examined a variety of different xenobiotics for their ability to activate the rabbit and rat PXR (Jones *et al*., 2000). Many of these compounds, including dexamethasone, phenobarbital, RU486, spiranolactone, clotrimazole, and trans-nonachlor, induced CYP3A expression in hepatocytes from both species (Kocarek *et al*., 1995). However, there were notable differences. For example, rifampicin was a much more efficacious inducer of CYP3A expression in rabbit hepatocytes than rat hepatocytes. Conversely, pregnenolone 16α-carbonitrile (PCN) induced CYP3A expression in rat hepatocytes but not rabbit hepatocytes. In general, activation of the rat and rabbit PXR agreed with the reported induction of CYP3A expression in primary hepatocytes from these same species (Kocarek *et al*., 1995). Rifampicin was an efficacious activator of rabbit PXR but had no effect on rat PXR. Although both receptors were activated by PCN, full dose response analysis revealed that PCN was roughly an order of magnitude more potent on rat PXR than rabbit PXR.

When the same panel of CYP3A inducers was also tested on the human and mouse PXR, the human and rabbit PXR were both efficiently activated by rifampicin as well as by RU486, clotrimazole, trans-nonachlor, and phenobarbital. However, the rabbit PXR was much more sensitive than its human ortholog to activation by the synthetic steroids dexamethasone, PCN, CPA, and spiranolactone. These findings are in good agreement with the finding that rifampicin is a much more efficacious activator of CYP3A expression in hepatocytes from rabbits and humans than from rats (Kocarek *et al*., 1995; Barwick *et al*., 1996) together with the observation that rifampicin is an efficacious activator of human PXR (Lehmann *et al*., 1998; Bertilsson *et al*., 1998; Blumberg *et al*., 1998). However, there still remain clear differences in responsiveness of rabbit and human PXR to xenobiotics.

Structurally, PXR is similar to other nuclear receptors, including a N-terminal transactivating domain (TAD), followed by a DNA binding domain (DBD),

and a C-terminal ligand-binding domain (LBD). Cloning and characterization of PXR from human, rabbit, rat and mouse have shown that there is > 95% sequence identity in the DBD regions (Fig. 4). However, the LBDs of the PXRs are much less identical and share only 75–80% amino acid identity and display markedly different activation profiles in response to xenobiotics (Jones *et al*., 2000; Zhang *et al*., 1999).Therefore, the species differences in CYP3A regulation by xenobiotics is likely due to the differences in the LBD, in that the DBD is highly conserved among the PXRs from multiple species.

Species differences in activation of CAR

In humans the only P450 2B subfamily enzyme expressed in liver is P450 2B6, which has 76% amino acid sequence identity with rat P450 2Bl (Yamano *et al*., 1989). The level of expression appears to be very low, and even in the individuals expressing the highest levels only 1% of the total P450 can be accounted for as P450 2B6 (Mimura *et al*., 1993). In rabbits, P450 2B4 (van der Hoeven *et al*., 1974; Imai and Sato, 1974) and 2B5 differ in 11 amino acids and P450 2B4 is usually more active with most substrates (Grimm *et al*., 1994). Evidence for the existence of other rabbit P450 2B subfamily members has been presented (Nelson *et al*., 1993; Imai *et al*., 1988). Rabbit P450 2B4 appears to have a catalytic specificity similar to that of rat P450 2B1, in so far as rough comparisons are possible from the literature. Of the 11 amino acid differences, four (114, 294, 363, 367) were found to be critical in causing alterations in catalytic activity toward a variety of substrates (Szklarz *et al*., 1996).

The constitutively active receptor (CAR) is a novel nuclear receptor that can activate or repress CYP2B genes in response to both endogenous and exogenous compounds in the rodent (Negishi and Honkakoski, 2000; Zelko and Negishi, 2000).In mouse, rat, and human cells, CYP2B genes are known to be induced by phenobarbital (PB) and a large number of structurally diverse xenochemicals. The molecular details of CYP2B regulation in mammalian cells have been largely unknown.Rapidly emerging studies revealed that CAR dimerized with

retinoid X receptor (RXR) is involved in the induction of Cyp2b10 mRNA in PB-treated mice (Honkakoski and Negishi, 1997; Honkakoski *et al*., 1998).

Although human CAR exhibits some common characteristics with its rodent counterparts, such as undergoing nuclear translocation after phenobarbital treatment and binding to the PBREM, there are distinct differences between rodent and human CAR. For example, TCPOBOP, the most potent mCAR ligand identified to date, cannot bind or activate either rat or human CAR. Notably, no compounds have been reported thus far that effectively bind and activate human CAR. All known mCAR inhibitors, such as androstenol, progesterone, androgens and CaMK inhibitors, do not inhibit hCAR activation. For these reasons, regulation of hCAR by drugs and other xenobiotics has become a more complex, and urgent, issue that has yet to be resolved. Recent studies have shown that the interspecies differences in CAR and PXR regulation of CYPs also happen at the level of cross-regulation (Xie *et al*., 2000).

2.4.2 Mechanisms of inhibition of CYP enzymes

The inhibition of drug metabolism is the most important mechanism for drug interactions because it can lead to an increase in plasma drug concentration, increased drug response, and toxicity.

The catalytic cycle of CYP consist of at least 7 discrete steps (Figure 8) :

(i) binding of the substrate to the ferric form of the enzyme

(ii) reduction of the haem group from the ferric to the ferrous state by an electron provided by NADPH via CYP reductase

(iii) binding of molecular oxygen

(iv) transfers of a second electron from CYP reductase and/or cytochrome b5

(v) cleavage of the O-O bond

(vi) substrate oxygenation

(vii) product release.

Although impairment of anyone of these steps can lead to inhibition of CYP enzyme activity, step (i), (iii) and (iv) are particularly vulnerable to inhibition.

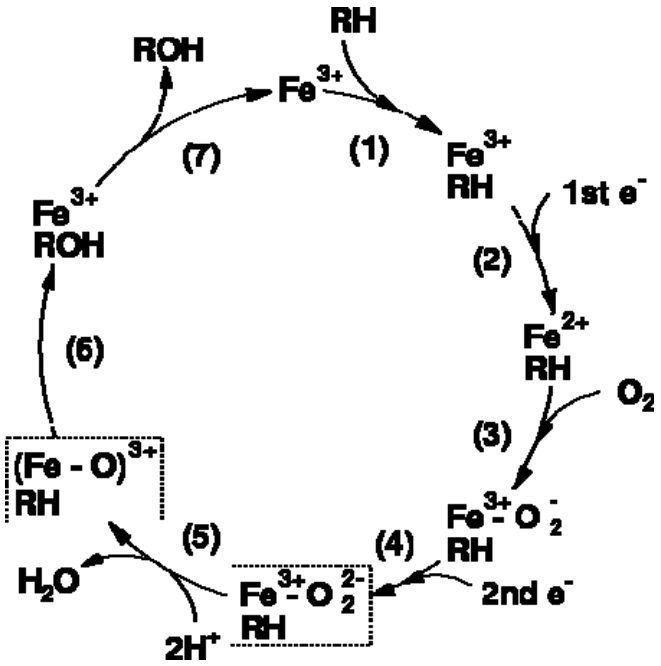


Figure 8 : The catalytic cycle of cytochrome P450 (Shaik *et al*., 2004)

The mechanisms of CYP inhibition can be divided grossly into 2 categories: reversible inhibition, quasi-irreversible inhibition and irreversible inhibition (Lin and Lu, 1998).

1. Reversible inhibition

In mechanistic terms, reversible interactions arise as a result of competition at the CYP active site and probably involve only the first step of the CYP catalytic cycle. Reversible inhibition can be divided, on a kinetic basis, into competitive, noncompetitive, and uncompetitive inhibition. In competitive inhibition, the inhibitor competes with the substrate for the same binding site within a CYP enzyme. In noncompetitive inhibition, the inhibitor binds to the same enzyme as does the substrate, but the binding site differs. In uncompetitive inhibition, the inhibitor binds only to the complex formed between the enzyme and the substrate. Many of the potent reversible CYP inhibitors are nitrogen-containing drugs, including imidazole,

pyridines and quinolones. These compound can not only bind to the prosthetic haem iron, but also to the lipophilic region of the protein. (Lin and Lu 1998). The potency of an inhibitor is determined both by its lipoohilicity and by the strength of the bond between its nitrogen lone electron pair and the prosthetic haem iron. Potent reversible inhibitors of CYP enzymes include, for example, itraconazole (CYP3A4), fluoxetine (CYP2D6), miconazole (CYP2C9), and ciprofloxacin (CYP1A2) (von Moltke *et al*., 1996; Stevens and Wrighton, 1993; O'Reilly *et al*., 1992; Fuhr *et al*., 1992).

2. Quasi-irreversible inhibition

In quasi-irreversible inhibition, the inhibitor undergoes metabolic activation by the CYP enzymes to form inhibitory intermediate metabolites. These metabolites form stable inactive complexes with the prosthetic heme of CYP. In *in vitro*, the metabolic activity of the inactive CYP can be reversed during incubation with highly lipophilic compounds that displace the metabolic intermediate from the active site, or by irradiation, or by oxidation to the ferric state by the addition of potassium ferricyanide. In *in vivo*, these complexes are, however, so stable that the CYP enzymes involved are unavailable for drug metabolism, and synthesis of new enzymes is required to overcome the inhibition; hence the name quasi-irreversible inhibition (Lin and Lu, 1998).

3. Irreversible inhibition

In irreversible inhibition, functional groups of certain drugs are oxidized by the CYP enzymes to form reactive metabolites, then covalently bind and irreversibly inactivate CYP. Because metabolic activation is required for enzyme inactivation, the inhibitors are often called mechanism-based inactivators or suicide substrates. The mechanism-based inactivation of CYP may result from irreversible alteration of haem or protein, or a combination of both. In general, modification of the haem group invariably inactivates the CYP, whereas protein alteration will result in loss of catalytic activity only if essential amino acid, which are vital for substrate binding, electron transfer and oxygen activation, are modified (Lin and Lu, 1998).

2.5 Human immunodeficiency virus (HIV)

Human immunodeficiency virus (HIV) is a retrovirus that can lead to acquired immunodeficiency syndrome (AIDS), a condition in humans in which the immune system begins to fail, leading to life-threatening opportunistic infections.

HIV stands for Human Immunodeficiency Virus. Like all viruses, HIV cannot grow or reproduce on its own. In order to make new copies of itself it must infect the cells of a living organism. HIV belongs to a special class of viruses called retroviruses. Within this class, HIV is placed in the subgroup of lentiviruses.

There are two types of HIV: HIV-1 and HIV-2. Both types are transmitted by sexual contact, through blood, and from mother to child, and they appear to cause clinically indistinguishable AIDS. However, it seems that HIV-2 is less easily transmitted, and the period between initial infection and illness is longer in the case of HIV-2. Worldwide, the predominant virus is HIV-1, and generally when people refer to HIV without specifying the type of virus they will be referring to HIV-1. The relatively uncommon HIV-2 type is concentrated in West Africa and is rarely found elsewhere. The strains of HIV-1 can be classified into three groups: the "major" group M, the "outlier" group O and the "new" group N.

Infection with HIV occurs by the transfer of blood, semen, vaginal fluid, pre-ejaculate, or breast milk. Within these bodily fluids, HIV is present as both free virus particles and virus within infected immune cells. The four major routes of transmission are unprotected sexual intercourse, contaminated needles, breast milk, and transmission from an infected mother to her baby at birth. Screening of blood products for HIV has largely eliminated transmission through blood transfusions or infected blood products in the developed world.

2.5.1 The structure of HIV

HIV particles (Figure 9) surround themselves with a coat of fatty material known as the viral envelope (or membrane). Projecting from this are around 72 little spikes, which are formed from the proteins gp 120 and gp 41. Just below the

viral envelope is a layer called the matrix, which is made from the protein p17. The viral core (or capsid) is usually bullet-shaped and is made from the protein p24. Inside the core are three enzymes required for HIV replication called reverse transcriptase, integrase and protease. Also held within the core is HIV's genetic material, which consists of two identical strands of RNA.

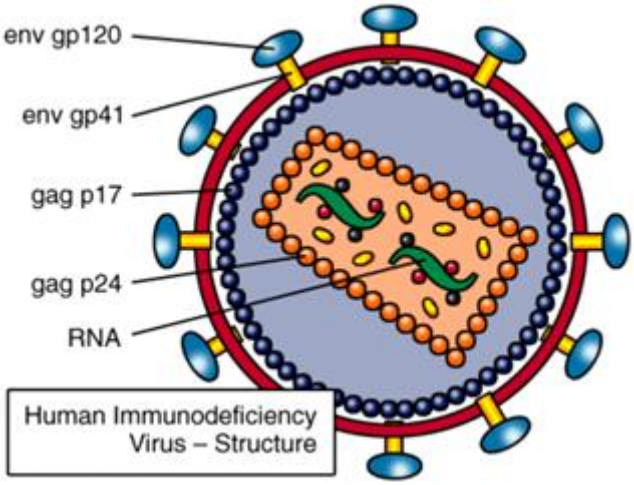


Figure 9 : Human immunodeficiency virus structure (www.avert.org/hivvirus.htm)

2.5.2 The HIV Life Cycle

1. Binding and Fusion: HIV begins its life cycle when it binds to a CD4 receptor and one of two co-receptors on the surface of a $CD4^+$ T- lymphocyte. The virus then fuses with the host cell. After fusion, the virus releases RNA, its genetic material, into the host cell.

2. Reverse Transcription: An HIV enzyme called reverse transcriptase converts the single- stranded HIV RNA to double-stranded HIV DNA.

3. Integration: The newly formed HIV DNA enters the host cell's nucleus, where an HIV enzyme called integrase "hides" the HIV DNA within the host cell's own DNA. The integrated HIV DNA is called provirus. The provirus may remain inactive for several years, producing few or no new copies of HIV.

4. Transcription: When the host cell receives a signal to become active, the provirus uses a host enzyme called RNA polymerase to create copies of the HIV

genomic material, as well as shorter strands of RNA called messenger RNA (mRNA). The mRNA is used as a blueprint to make long chains of HIV proteins.

5. Assembly: An HIV enzyme called protease cuts the long chains of HIV proteins into smaller individual proteins. As the smaller HIV proteins come together with copies of HIV's RNA genetic material, a new virus particle is assembled.

6. Budding: The newly assembled virus pushes out ("buds") from the host cell. During budding, the new virus steals part of the cell's outer envelope. This envelope, which acts as a covering, is studded with protein/sugar combinations called HIV glycoproteins. These HIV glycoproteins are necessary for the virus to bind CD4 and co- receptors. The new copies of HIV can now move on to infect other cells.

CHAPTER 3

METERIALS AND METHODS

3.1 Meterials

3.1.1 Drugs

Phenytoin (Dilantin ®) was purchased from Pfizer Inc NY, USA.

Efavirenz (Storcrin ®) was purchased from B.L.H Trading Co., LTD., Bangkok, Thailand, under the contract with Bristol-Mayers Squibb Holding Pharma limited, Puerto Rico, USA.

3.1.2 Reagents and chemical substances

Acetonitrile HPLC grade was purchased from Mallinckrodt Baker NJ, USA.

Methanol HPLC grade was purchased from J.T. Baker NJ, USA.

Ethyl acetate was purchased from QReC, New Zealand

Hydrochloric acid was purchased from Merck Darmstadt, Germany

Potassium dihydrogen orthophosphate (KH_2PO_4) was purchased from Merck Darmstadt, Germany

Water was purified for HPLC by the Milli Q Water Purification System, Millipore, Milford, USA.

3.2 Equipments and columns

3.2.1 HPLC model

- Waters 2487 Dual λ Absorbance detector (Waters Associates, Milliford, USA.)

- Empower software (Waters Associates, Milliford, USA.)

- µ-Bondapak® C_{18} column : a reverse phase column C_{18}, 5µm particle, 150 mm length × 4.6 mm internal diameter. (Waters Associates, Milliford, USA.)

- µ-Bondapak® C_{18} guard column : packed with resolved C_{18}. (Waters Associates, Milliford, USA.

3.2.2 Instruments

- Vortex mixer
- Centrifuge machine
- pH meter
- Centrivap concentrator
- Micropipette (200 and 1,000 µl)
- Pipett tip
- Disposable needle (22G)
- Heparin lock
- Test tube with cap
- Disposable syringe (3 ml and 5 ml)
- Eppendorf microcentrifuge tube (1.5 ml)
- PTFE filter, pore size 0.45 µm

3.3. Methodology

3.3.1 Determination of efavirenz in plasma

3.3.1.1 Sample preparation

Serum samples were extracted following the technique described by Ramachandran and colleaques with some modifications (Ramachandran *et al*., 2006). The 250 µl of plasma sample was transferred into an eppendorf microcentrifuge. One milliliter of ethyl acetate was added and the contents were shaked vigorously, centrifuged at 13,000 rpm for 15 minutes and 750 µl of organic phase was evaporated to dryness. The dried residue was reconstituted in 75 µl of mobile phase and 20 µl was injected into the HPLC column.

3.3.1.2 Chromatographic conditions

Plasma efavirenz concentrations were determined by high-performance liquid chromatography (HPLC) (Ramachandran *et al*., 2006)

Column : reverse phase column (µ-Bondapak® C_{18}, 5µm particle, 150 mm length × 4.6 mm internal diameter)

Mobile phase : Mixture of 10 mM phosphate buffer pH 2.4 (adjusted with 1N HCl) : acetronitrile (55 : 45 % v/v)

Flow rate : 1.5 ml / min

Injection volume : 20 µl

Detector : UV detector, wavelength of 245 nm

Temperature : room temperature (25 ºC)

3.3.1.3 Mobile phase preparation

Mobile phase consisted of 10 mM phosphate buffer pH 2.4 (adjusted with 1N HCl) and acetronitrile (55 : 45 % by volume). The mobile phase was freshly prepared each day and was filtered through 0.45 µm nylon filtered paper and degassed before using (30 min).

3.3.1.4 Standard curve

Standard curve was prepared by diluted stock solution to serial concentrations of 4, 2, 1, 0.5, 0.25 µg/ml with drug-free plasma. All samples were proceeded following the procedures as described in 3.3.1.1 and 3.3.1.2. Standard calibration curves were conducted by the least-square linear regression of the efavirenz concentration and peak area efavirenz. Unknown concentration of efavirenz in rabbits's plasma were calculated from the standard curves by reverse prediction.

3.3.2 Method validation

The method of analysis was validated in accordance with the Guidance for Industry: Bioanalytical Method Validation (FDA, 2001).

3.3.2.1 Linearity and range

Linearity was evaluated by preparing five standard samples with different concentrations of efavirenz; 0.25, 0.5, 1, 2, 4 µg/ml. Calibration curve was constructed by plotting peak area (Y) of efavirenz versus the concentrations of efavirenz (X). Regression analysis was performed to obtain the calibration equation and correlation coefficient (r).

3.3.2.2 Recovery study

Three concentration of efavirenz (0.25, 1.5, 3.0 µg/ml) were prepared in drug –free plasma and extracted as described above. The percentage of drug recovered from the plasma samples was determined by comparing the peak area after extraction with those of unextracted methanolic solutions containing the same concentrations of efavirenz in plasma. An acceptable recovery should be more than 90 % and percentage of coefficient of variation (% CV) should be less than 5%.

3.3.2.3 Lower limit of quantification

Lower limit of quantification was obtained by adding known amount of efavirenz to drug-free plasma (0.25-4 µg/ml) and deproteinized as described above. The peak areas of efavirenz were calculated and plotted for the correlation between the concentration of efavirenz and peak area. The lowest concentration of efavirenz which was still linearly correlated was regarded as lower limit of quantification (LLOQ) that could be determined with a precision of 20% and accuracy of 80-120% .

3.3.2.4 Precision

Analytical intraday and interday precision were determined by adding a series of known amount of efavirenz to drug-free plasma (0.25, 1.5, 3 µg/ml). To determine intraday precision, five replications of each concentration were carried out in one day. To determine interday precision, each concentration were carried out in 5 days. The precision was calculated as percentage of coefficient of variation (% CV). It should be less than 15 % for intraday and interday except at the LLOQ where 20% CV is accepted.

$$\text{CV (\%)} = \frac{\text{Standard deviation (SD)}}{\text{Mean value}} \times 100$$

3.3.2.5 Accuracy

Accuracy of analysis was determined by using three quality controls (QC) samples (0.25, 1.5, 3 µg/ml). To determine intraday accuracy, five replications of each concentration were carried out in one day. To determine interday accuracy, each concentration were carried out in 5 days. The accuracy of intraday and interday

determination for each concentration should be less than 15 % except for the LLOQ where less than 20% is accepted.

$$\text{Accuracy}\,(\%) = \frac{\text{Calculated concentration}}{\text{Actual concentration}} \times 100$$

3.3.3 Pharmacokinetic study

3.3.3.1 Animal

Ten male New Zealand white rabbits weighing 3-4 kg were obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The experimental protocol was approved by the Animal Ethic Committee, Prince of Songkla University. During the experimental period, all animals were housed in individual stainless cages which allowed the isolation of feces in a lower container to avoid coprophagy. The environmental conditions were: constant humidity (55 ± l0%), temperature (19±2°C) and a 12 h light- dark cycle. The animals were maintained on laboratory chow and water ad libitum. Before the experiment, the animals were fasted 12 h but were allowed free access to food and water after the 4th hour of blood sample collection.

3.3.3.2 Study design

The study was a 2-period pharmacokinetic study with 14 days washout period in a crossover design. The rabbits were fasted 12 hours before the experiment.

In period 1, 5 rabbits in group 1 were given 70 mg/kg of efavirenz suspended in 20% gum acacia orally. In group 2, another 5 rabbits were given 30 mg/kg of phenytoin suspended in 20% gum acacia once daily orally for 7 days, followed by efavirenz 70 mg/kg on day 7. In period 2, the 2 groups were treated in reverse.

Blood samples (approximately 2 ml) were obtained from an indwelling venous catheter before efavirenz administration and at 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96 hours after each treatment. Each sample was added to the heparinzed tube and centrifuged at 12,470 g for 15 min, and plasma was collected and stored at -80 ºC until the time of analysis.

3.3.3.3 Sample size calculation

The study was conducted to determine the effect of phenytoin on the pharmacokinetics of efavirenz in rabbits. However, there were no reports on the pharmacokinetic interaction between phenytoin and efavirenz. The study of Ducharme et al (1995) on the interaction of phenytoin and itraconazole in human was therefore used to calculate the sample size. They found that the AUC of itraconazole is significantly decrease from 3.20 ± 1.86 to 0.22 ± 0.19 μg × h/ml when co-administered with phenytoin.

The AUC difference of itraconazole (d) = 3.20 – 0.22 = 2.98 μg × h/ml, Type I error 5 % (α=0.05), Z_α = 1.645, Type II error 10 % (β=0.10), Z_β = 1.282 and pooled variance $(Sp)^2$ = 1.75

$$N = \frac{2(Z_\alpha + Z_\beta)^2 (Sp)^2}{d^2}$$

$$= \frac{2\,(\,1.645+1.282\,)^2\,(1.75)}{(2.98)^2}$$

$$= 3.34$$

$$\approx 4$$

A total sample size of four rabbits were sufficient to detect a significant pharmacokinetic difference in AUC of itraconazole when co-administered with phenytoin. This study was adjusted the sample size of rabbits upward to ten because the four rabbits were too small and would create potential for an error in statistical analysis.

3.3.3.4 Pharmacokinetic analysis

Pharmacokinetic analysis was performed with WinNonlin by non-compartment model. The maximum plasma concentration (C_{max}), the time to maximum plasma concentration (t_{max}) were determined by direct inspection from the individual plasma concentration-time profiles. The terminal elimination rate constant (λ_z) was determined by linear regression of at least the three last data points from the log-linear concentration–time curve. The area under the concentration–time curve from 0 h to the last measurable concentration (AUC_{0-t}) was determined by the linear trapezoidal method. The area under the concentration–time curve from 0 h to infinity

($AUC_{0-\infty}$), half life ($T_{1/2}$), clearance (CL/F), and volume of distribution (Vd/F) were calculated using the formular:

AUC_{0-t} ; $AUC_{0-last} = \sum \frac{(C_{n-1} + C_n)}{2} (t_n - t_{n-1})$; n = 1, 2, 3,…

$AUC_{0-\infty} = AUC_{0-t} + C_{last}/ \lambda_z$ (the ratio of the last quantifiable concentration over the elimination rate constant (C_t/ λ_z))

$$\lambda_z = \frac{\ln C_1 - \ln C_2}{t_2 - t_1}$$

$T_{1/2} = 0.693/ \lambda_z$

$CL/F = dose/ AUC_{0-\infty}$

$Vd/F = CL/F / \lambda_z$

3.3.3.5 Statistical analysis

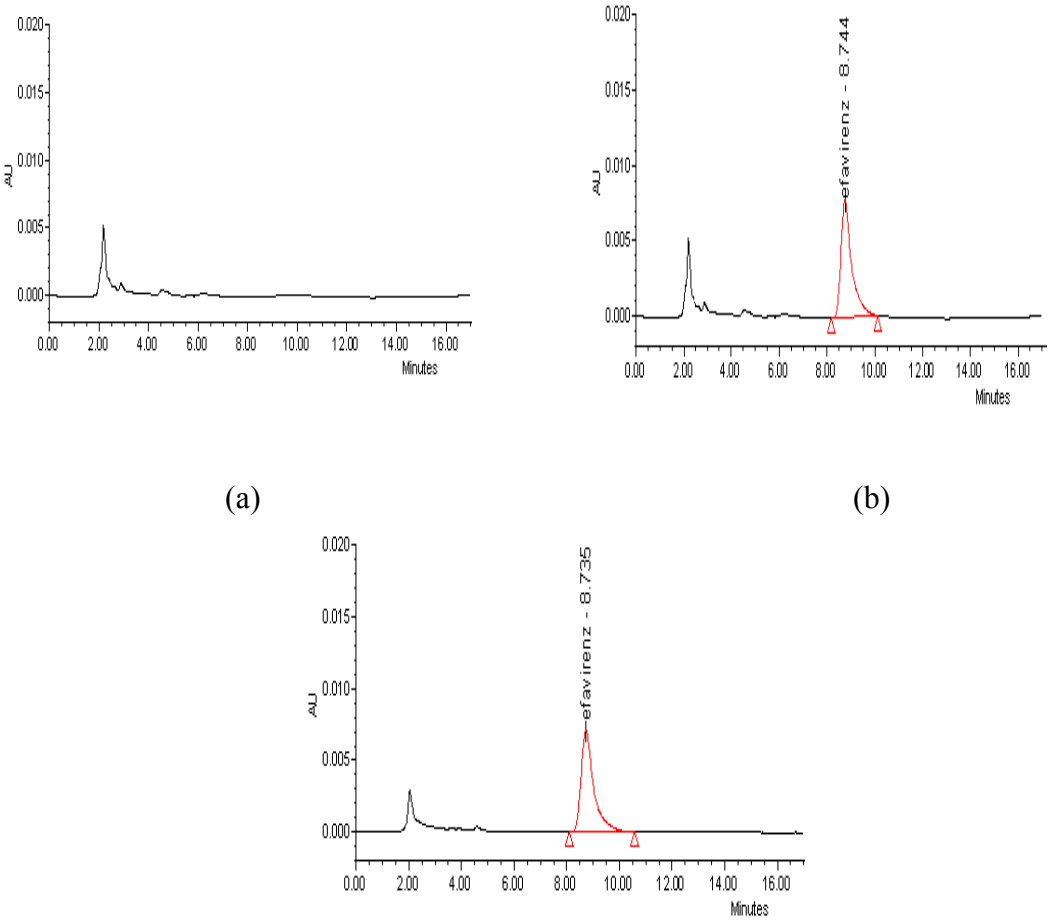
Results were expressed as mean ± standard deviation (SD), and statistical comparisons were made using the student's paired t-test. P values of < 0.05 were considered significant.

CHAPTER 4

RESULTS

4.1 Chromatograms

The chromatograms showed that the peak of efavirenz was clearly seperated from plasma. Chromatograms of drug free plasma (a); efavirenz 4 µg/ml in plasma (b); efavirenz 4 µg/ml in methanol (c) are shown in figure 11. Efavirenz was eluted at 8 minutes as sharp and symmetrical peak.



(a) (b)

(c)

Figure 10 Representative HPLC chromatograms of drug free plasma (a); efavirenz 4 µg/ml in plasma (b); efavirenz 4 µg/ml in methanol (c)

4.2 Assay validation

4.2.1 Linearity

The standard calibration curve of efavirenz was linear over the range of 0.25, 0.5, 1, 2 and 4 µg/ml. Calibration curves were plotted between the peak area of efavirenz versus plasma efavirenz concentration, as shown in figure 11. Using the least-square linear regression analysis, the correlation coefficient of efavirenz was 0.9997 and the linear regression was Y = 87655x - 8087.5.

Where:

X = plasma efavirenz concentration (µg/ml)

Y = the peak area of efavirenz

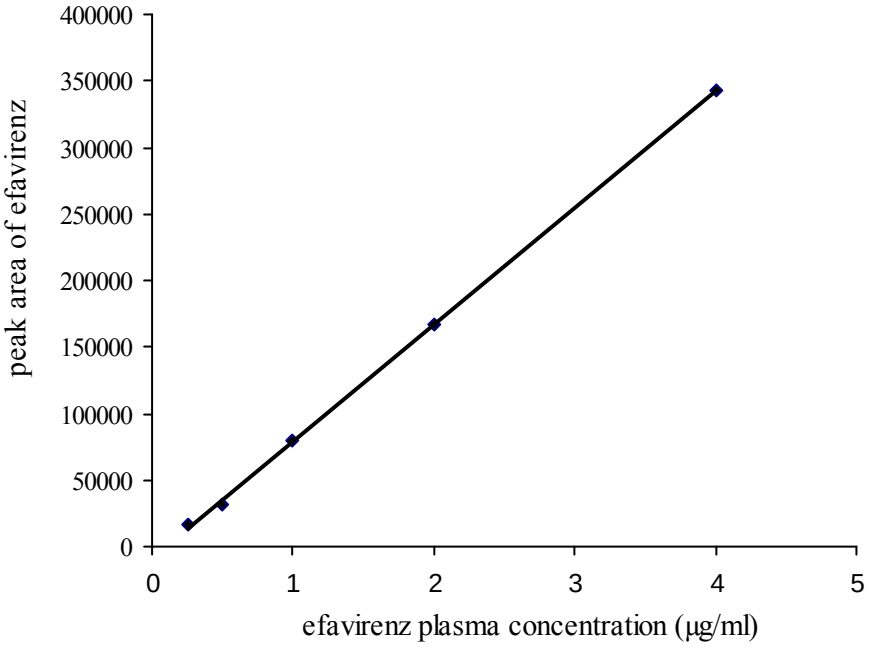


Figure 11 Calibration curve of efavirenz in plasma (Y = 87655x - 8087.5, r^2 = 0.997)

4.2.2 Precision

The precision of the assay procedure was assessed from % CV of the area of efavirenz and their concentrations from intraday and interday, as shown in table 1. The % CV of intraday and interday precision was found in the range of 2.77 – 3.56 % and 1.99 – 4.28 %, respectively.

4.2.3 Accuracy

The accuracy of efavirenz in plasma was controlled by calculating the intraday and interday at three concentrations (0.25, 1.5 and 3 µg/ml) for efavirenz in five replication for intraday and interday as shown in table 1. The intraday and interday accuracy of efavirenz ranged between 94.85 – 107.84 % and 100.68 – 109.79 %, respectively.

Table 1 The precision of the analytical method of efavirenz, intraday and interday precision

Concentration (µg/ml)	Mean ± SD of area under efavirenz peak	% CV	Accuracy
Intraday (n = 5)			
0.25	11057 ± 341	3.09	94.85
1.5	118187 ± 3277	2.77	107.84
3	261575 ± 9305	3.56	107.09
Interday (n = 5)			
0.25	16547 ± 708	4.28	109.79
1.5	121267 ± 2455	2.02	100.68
3	267762 ± 5322	1.99	107.99

4.2.4 Lower limit of quantification (LLOQ)

The lower limit of quantification (LLOQ) obtained in this chromagraphic condition of efavirenz was found to be 0.25 μg/ml.

4.2.5 Recovery

Efficacy of deproteinization procedure was assessed from the percentage recovery, as shown in table 2. The mean percentage recoveries of efavirenz at the concentration of 0.25, 1.5 and 3 μg/ml were 76.89, 104.56 and 88.63 %, respectively.

Table 2 Relative percent recovery of efavirenz in plasma

Concentration (μg/ml)	% Mean recovery	% CV
0.25	76.89	1.75
1.5	104.56	1.86
3	88.63	2.78

4.3 Plasma efavirenz concentrations

The plasma efavirenz concentration of each rabbit after a single oral dose of 70 mg/kg efavirenz and after pretreatment with 30 mg/kg of phenytoin once daily for 7 days were shown in table 3 and 4, respectively. The mean ± SD and the individual pharmacokinetic parameters of efavirenz between efavirenz alone and after pretreatment with phenytoin was shown in table 5 and 6, respectively. The mean ± SD of efavirenz C_{max}, AUC_{0-96} , $AUC_{0-\infty}$, Cl/f , λ_Z and T_{max} of rabbits receiving efavirenz alone were 2.23 ± 1.70 μg/ml, 26.86 ± 12.07 μg/ml·h, 32.86 ± 14.42 μg/ml·h, 2.73 ± 1.61 L/h/kg, 0.09 ± 0.59 h^{-1} and 5.95 ± 3.32 h, respectively. After phenytoin pretreatment, the mean ± SD of efavirenz C_{max}, AUC_{0-96} , $AUC_{0-\infty}$, Cl/f , λ_Z and T_{max} of rabbits receiving efavirenz were 1.14 ± 0.85 μg/ml, 11.82 ± 6.86 μg/ml·h, 16.81 ± 11.03 μg/ml·h, 5.50 ± 2.66 L/h/kg, 0.06 ± 0.44 h^{-1} and 1.70 ± 1.32 h, respectively. These parameters showed statistically significant difference ($p < 0.05$), whereas $t_{1/2,z}$ (27.00 ± 51.46 vs 22.96 ± 26.34 h) and $V_{z/f}$ (66.40 ± 92.19 VS

138.26 ± 105.26 L/kg) were not significant different when compared with efavirenz alone as the data shown in table 5 and table 6. The mean plasma efavirenz concentration-time curves of efavirenz alone and after pretreatment with phenytoin were shown in figure 12, and individual rabbit profile are shown in appendix A.

Table 3 Plasma efavirenz concentration of each rabbit after a single oral dose of 70 mg/kg efavirenz alone

Rabbits	plasma efavirenz concentrations (μg/ml) at each time of blood drawn (h)										
No.	0	0.5	1	2	4	8	12	24	48	72	96
1	0.00	3.77	6.63	6.07	4.25	2.34	1.19	0.12	0.00	0.00	0.00
2	0.00	0.17	0.18	0.19	0.43	1.28	0.66	0.50	0.21	0.19	0.00
3	0.00	0.17	0.25	0.28	1.35	2.37	1.29	0.36	0.14	0.14	0.00
4	0.00	1.00	1.24	0.60	0.36	2.34	2.04	0.31	0.00	0.00	0.00
5	0.00	0.79	1.03	0.71	0.72	1.07	0.28	0.17	0.15	0.14	0.00
6	0.00	2.17	1.04	0.60	0.32	1.02	0.89	0.17	0.00	0.00	0.00
7	0.00	0.17	0.17	0.26	0.23	1.46	1.16	0.84	0.14	0.17	0.00
8	0.00	0.56	0.42	0.38	0.34	0.88	0.53	0.16	0.00	0.00	0.00
9	0.00	0.38	0.58	0.65	0.53	1.05	0.53	0.13	0.00	0.00	0.00
10	0.00	2.10	2.02	3.00	2.27	1.59	0.96	0.24	0.00	0.00	0.00
Mean	0.00	1.13	1.36	1.27	1.08	1.54	0.95	0.30	0.06	0.06	0.00
SD	0.00	1.19	1.94	1.87	1.28	0.60	0.50	0.22	0.08	0.08	0.00

LLOQ = 0.25 μg/ml

Table 4 Plasma efavirenz concentration of each rabbit after pretreatment with phenytoin 30 mg/kg once daily for 7 days

Rabbits	plasma efavirenz concentrations (µg/ml) at each time of blood drawn (h)										
No.	0	0.5	1	2	4	8	12	24	48	72	96
1	0.00	0.22	0.73	2.99	3.31	1.09	0.58	0.12	0.00	0.00	0.00
2	0.00	0.41	0.54	1.39	0.81	0.51	0.30	0.21	0.18	0.18	0.00
3	0.00	0.48	0.76	0.44	0.36	0.33	0.24	0.12	0.12	0.12	0.00
4	0.00	0.67	1.38	1.43	0.71	0.53	0.30	0.12	0.00	0.00	0.00
5	0.00	0.62	0.42	0.31	0.27	0.41	0.54	0.34	0.15	0.00	0.00
6	0.00	0.63	0.67	0.55	0.19	0.37	0.22	0.08	0.00	0.00	0.00
7	0.00	0.55	0.85	0.58	0.26	0.20	0.19	0.00	0.00	0.00	0.00
8	0.00	0.18	0.19	0.17	0.14	0.16	0.16	0.12	0.00	0.00	0.00
9	0.00	0.37	0.37	0.71	0.89	0.52	0.30	0.11	0.00	0.00	0.00
10	0.00	1.32	1.00	0.67	1.06	0.67	0.37	0.14	0.00	0.00	0.00
Mean	0.00	0.55	0.69	0.92	0.80	0.48	0.32	0.14	0.05	0.03	0.00
SD	0.00	0.32	0.34	0.84	0.94	0.27	0.14	0.09	0.07	0.06	0.00

LLOQ = 0.25 µg/ml

Table 5 Pharmacokinetic parameters (mean ± SD) of efavirenz in rabbits after receiving oral single dose 70 mg/kg efavirenz alone and after pretreatment with phenytoin 30 mg/kg for 7 days.

Parameters	Efavirenz alone	Efavirenz plus phenytoin	*P*-value
C_{max} (μg/ml)	2.23 ± 1.70	1.14 ± 0.85 *	*P*= 0.007
AUC_{0-96} (μg/ml·h)	26.86 ± 12.07	11.82 ± 6.86 *	*P*= 0.001
$AUC_{0-\infty}$ (μg/ml·h)	32.86 ± 14.42	16.81 ± 11.03 *	*P*= 0.004
λ_Z (h^{-1})	0.09 ± 0.06	0.06 ± 0.04	*P*= 0.029
$t_{1/2,z}$ (h)	27.00 ± 51.46	22.96 ± 26.34	*P*= 0.823
T_{max} (h)	5.95 ± 3.32	1.70 ± 1.32 *	*P*= 0.005
Cl/f (L/h/kg)	2.73 ± 1.61	5.50 ± 2.66 *	*P*= 0.003
$V_{z/f}$ (L/kg)	66.40 ± 92.19	138.26 ± 105.26	*P*= 0.132

P value less than 0.05 is considered significantly different

Table 6 Effect of phenytoin on efavirenz pharmacokinetic parameters in each of ten rabbits

	Efavirenz							
Rabbit	C_{max}(μg/ml)		AUC_{0-96}(μg/ml·h)		$AUC_{0-\infty}$(μg/ml·h)		λ_z (h^{-1})	
No	efavirenz	efavirenz + phenytoin	efavirenz	efavirenz + phenytoin	efavirenz	efavirenz + phenytoin	efavirenz	efavirenz + phenytoin
1	6.63	3.31	48.31	24.79	48.95	25.67	0.19	0.14
2	1.28	1.39	28.52	19.83	37.06	43.56	0.02	0.01
3	2.37	0.76	36.06	12.27	39.40	17.57	0.04	0.02
4	2.34	1.43	30.95	10.89	33.27	12.22	0.13	0.09
5	1.07	0.62	19.25	15.78	53.86	20.02	0.004	0.04
6	2.17	0.67	15.95	5.93	17.39	6.79	0.12	0.09
7	1.46	0.85	36.93	3.74	41.39	8.59	0.04	0.04
8	0.88	0.19	10.91	3.55	12.43	9.74	0.11	0.02
9	1.05	0.89	12.41	9.34	13.40	10.51	0.13	0.09
10	3.00	1.32	29.36	12.08	31.40	13.47	0.12	0.10
Mean	2.23	1.14	26.86	11.82	32.86	16.81	0.09	0.06
SD	1.70	0.85	12.07	6.86	14.42	11.03	0.06	0.04
p-value	0.007		0.001		0.004		0.029	

Table 6 Effect of phenytoin on efavirenz pharmacokinetic parameters in each of ten rabbits (continue)

	Efavirenz							
Rabbit	$t_{1/2,z}$ (h)		T_{max} (h)		$Cl_{/f}$ (L/h/kg)		$V_{z/f}$ (L/kg)	
No	efavirenz	efavirenz + phenytoin	efavirenz	efavirenz + phenytoin	efavirenz	efavirenz + phenytoin	efavirenz	efavirenz + phenytoin
1	3.71	5.08	1.00	4.00	1.43	2.73	7.65	19.99
2	31.17	91.42	8.00	2.00	1.89	1.61	84.94	211.92
3	16.53	30.60	8.00	1.00	1.78	3.98	42.37	175.91
4	5.20	7.70	8.00	2.00	2.10	5.73	15.77	63.65
5	171.36	19.60	8.00	0.50	1.30	3.50	321.29	98.85
6	5.88	7.45	0.50	1.00	4.03	10.31	34.13	110.72
7	18.18	17.68	8.00	1.00	1.69	8.15	44.36	207.88
8	6.60	35.80	8.00	1.00	5.63	7.18	53.64	370.98
9	5.44	7.38	8.00	4.00	5.21	6.66	40.89	70.89
10	5.90	6.91	2.00	0.50	2.23	5.20	18.96	51.83
Mean	27.00	22.96	5.95	1.70	2.73	5.50	66.40	138.26
SD	51.46	26.34	3.32	1.32	1.61	2.66	92.19	105.26
p-value	0.823		0.005		0.003		0.132	

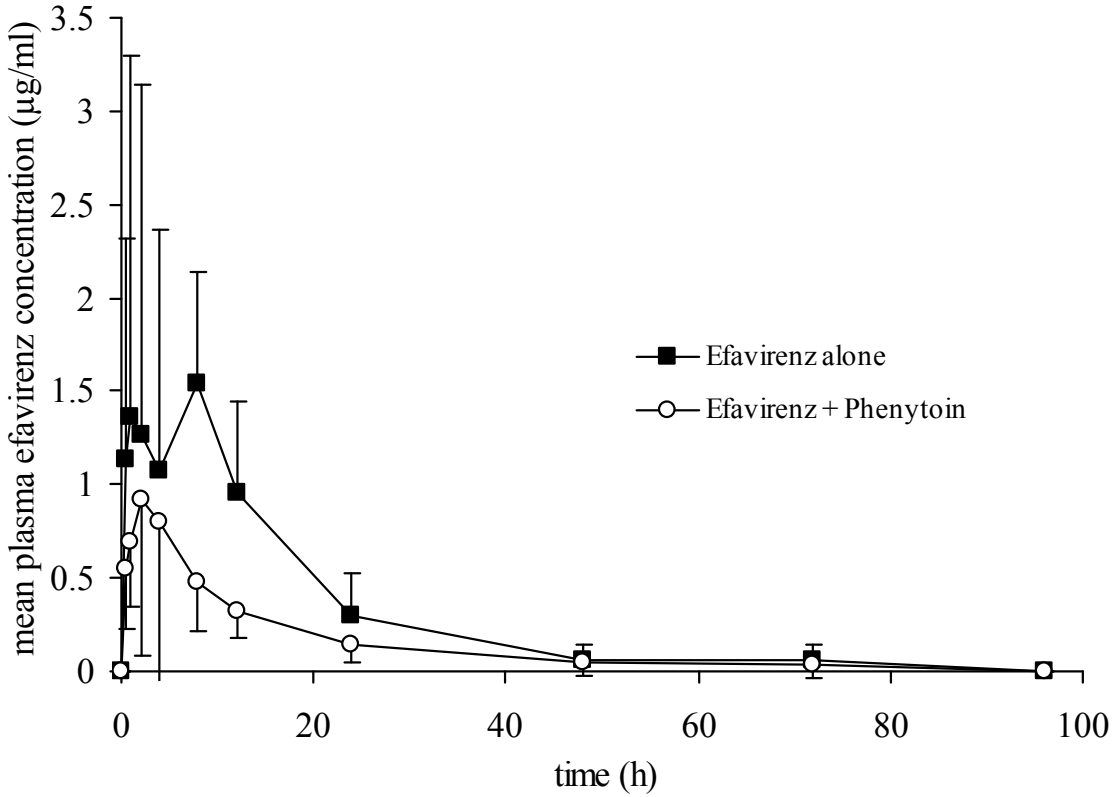


Figure 12 : The mean plasma concentration-time curves of efavirenz in 10 rabbits after a single oral dose of 70 mg/kg efavirenz alone (*filled squares*) and after pretreatment with 30 mg/kg phenytoin once daily for 7 days (*open circles*).

CHAPTER 5

DISCUSSION AND CONCLUSION

Drug–drug interactions are the important consideration when initiating anticonvulsant therapy in HIV infected patient. Current management guidelines for HIV infection advocate the use of potent three-drug antiretroviral regimens. These regimens most commonly include two nucleoside reverse transcriptase inhibitors and either a protease inhibitor or a non- nucleoside reverse transcriptase inhibitors (DHHS, 2009).

Efavirenz is one of the non-nucleoside reverse transcriptase inhibitors which has been shown to have good inhibitory activity againts HIV-1. It is primarily metabolized by CYP3A4 and CYP2B6 to inactive hydroxylated metabolites (Adkin *et al*., 1998). Drugs that inhibit or induce either of these enzymes are expected to alter plasma efavirenz concentration. Ketoconazole has been shown to increased the AUC of efavirenz by 44.5% as a result of CYP3A4 inhibition (Saadeddin *et al*., 2009). Concurrent administration of rifampin, a potent inducer of CYP3A4 and CYP2B6 was found to reduce the AUC of efavirenz by 26 % (Sustiva, 2004). Efavirenz is not a substrate of P-gp (Stormer *et al*., 2002) and therefore the absorption of efavirenz is unlikely to be affected by inducers or inhibitors of P-gp.

Phenytoin is an effective and a major first line antiepileptic drug which widely used for the treatment of partial and generalized epilepsy in Thailand (Chinvarun, 2009). Phenytoin is useful for controlling seizures without the sedative effects. It is extensively metabolised in the liver by CYP 2C9 and CYP 2C19 (Bajpai *et al*, 1996) to several inactive hydroxylated metabolites. It is an inducer of CYP3A4 and CYP2B6 (Patsalos *et al*., 2002). Phenytoin has been reported to decreased the AUC of lopinavir/ritonavir by approximately 30 % (Lim *et al*., 2004) and decreased the AUC of itraconazole by more than 90% (Ducharme *et al*., 1995) as a result of CYP3A4 induction. It has been reported that phenytoin altered the pharmacokinetics of cyclophosphamide by increasing the clearance of (*R*)- and (*S*)-cyclophosphamide by inducing CYB2B (William *et al*.,1999). In addition, the *N*-dechloroethylated

metabolites of ifosfamide was increased in a pediatric patient receiving phenytoin combination with ifosfamide as a result of CYP2B6 induction (Duchrame *et al*., 1997). There has been a case report of the lower-than-expected concentration (12-h concentration, < 1 μg/ml.) after efavirenz was given to a patient receiving phenytoin (Robertson *et al*., 2005). Coadministration of antiviral drugs and antiepileptic drugs is very often in HIV-infected patients, therefore clinically important interaction can occur and may lead to failure of treatment with efavirenz. To date, there has been no study aimed at determining the pharmacokinetic drug interaction of efavirenz and phenytoin. Therefore, in this study, we carried out a preclinical investigation of a possible pharmacokinetic interaction between efavirenz and phenytoin in rabbits.

New Zealand White rabbit has become the one most commonly used in pharmacology and medical research. Since rabbits can be easily restrained in stocks and are generally docile and cheap to maintain. In addition, the general physiology of rabbits is similar to that of humans, like mice and rats. They have been used for a wide range of toxicity testing and drug interaction studies (Elmas *et al*, 2008; Nworu *et al*, 2008; Matin-Suarez *et al*, 2003; Fernandez *et al*, 1996; Krishnaiah *et al*, 1993).

The rabbit was used to study the CYP3A inductive potential because the rabbit CYP3A6 is similar to CYP3A4 in several key characteristics. Like CYP3A4, CYP3A6 is predominant in the liver and intestine (McKinnon *et al*., 1993); these CYP3As share similar substrate selectivity, and both of these enzymes are inducible in the liver by rifampin by activation of the pregnane X receptor (PXR) (Savas *et al*., 2000; Daujat *et al*., 1991). CYP2B is well conserved between rodents and rabbits, but is poorly expressed in human liver. In humans, CYP2B6 has 76% amino acid sequence identity with rat CYP2Bl (Yamano *et al*., 1989). Rabbit CYP2B4 appears to have a catalytic specificity similar to that of rat CYP2B1 (Guengerich *et al*., 1997).

For the above reasons, this study was designed to investigate the effect of phenytoin on the pharmacokinetics of a single oral dose of efavirenz in rabbits. In our study, phenytoin was given to the rabbits at the dose of 30 mg/kg once daily orally for 7 days. In studies using phenytoin as CYP induction, generally at least 7 days of pretreatment are used to ensure maximum induction (Nation *et al*., 1990). While efavirenz was given orally 70 mg/kg (Balani *et al*., 1998). The results of the

present study showed that the plasma concentration–time profile of efavirenz was fitted to noncompartment model because of the variation of the pharmacokinetic profile of each rabbit in this study.

When a single oral dose of efavirenz was administered after pretreatment with phenytoin for 7 days, the mean of C_{max}, AUC_{0-96}, $AUC_{0-\infty}$ and t_{max} were significantly decreased by phenytoin by 48.88%, 55.99%, 48.84% and 71.43%, respectively and the mean of Cl/f was significantly increased by 101.47% when compared to efavirenz alone. The increase in the clearance of efavirenz led to corresponding markedly decreases in C_{max} and AUC of efavirenz, suggesting that the metabolism of efavirenz was increased.

The mean Cl/f of single oral dose of efavirenz after pretreatment with phenytoin 7 days was significantly increased, which was thought to be due to increase in hepatic clearance. Indeed, the total clearance is defined by renal clearance and non renal clearance, but there has been the report that haemodialysis does not affect pharmacokinetics of efavirenz (Izzedine *et al*., 2000). Because efavirenz is primarily metabolized by liver, and the elimination of unchanged drug and metabolites takes place primarily through the biliary tract. Only 1% of the dose is recovered in the urine as parent compound, it has been suggested that kidney function is not the principal determinant of the elimination of efavirenz (Adkins *et al*., 1998). By theory, if a drug is metabolized exclusively by liver, the total clearance ($Cl_{/f}$) of the drug is equal to the hepatic clearance (Cl_H). Hepatic clearance is defined as the volume of blood perfusing the liver that is cleared of drug per unit of time. Calculation of hepatic clearance based on total hepatic blood flow (Q_H), fraction unbound of drug in the blood (f_u) and the free intrinsic clearance (Cl_{int}): $Cl_H = Q_H \times f_u \times Cl_{int} / Q_H + f_u \times Cl_{int}$. In addition, the plasma drug concentration data can also be obtained from the equation: hepatic clearance (Cl_H) = (rate of elimination by liver/ plasma drug concentration). The Cl_{int} was increased in phenytoin combination since a major part of this parameter is metabolism. It is important to note that the pharmacokinetic consequences of enzyme induction should always be a decrease in plasma concentrations, because cytochrome P450 induction will cause an increase in the hepatic metabolism led to corresponding markedly decreases in $t_{1/2}$ and increases in λ_z. But in the present study, the $t_{1/2}$ was not significantly decrease and λ_z was decreased, thus phenytoin was not induced hepatic

clearance of efavirenz. Then phenytoin may induce the intestinal clearance of efavirenz throught CYP3A and CYP2B induction because both enzymes were also found in intestine.

The mean t_{max} of single oral dose of efavirenz after pretreatment with phenytoin 7 days was significantly decreased, which may indicate that the absorption process of efavirenz was markedly faster when it was administered with phenytoin. The intestinal first pass metabolism of efavirenz induced by phenytoin may led to corresponding markedly decreases in T_{max} of efavirenz induce. Althought phenytoin is an inducer of P-gp with may affect the efflux of P-gp substrate causing decrease in T_{max} but efavirenz is not a substrate of P-gp, it is a substrate of BCRP(ABCG2) (Peroni *et al*., 2011), thus decrease in T_{max} is not caused by intestinal P-gp induction.

Several clinical studies have reported a reduction in the plasma levels of other CYP3A4 and CYP2B6 substrates such as lopinavia/ritonavir, quatiapine, itraconazole, cyclophosphamide when they are coadministered with phenytoin (Lim *et al*., 2004; Wong *et al*., 2001; Ducharme *et al*., 1995; William *et al*.,1999). Chhun et al, (2009) has been reported a reduction in the plasma levels of gefitinib, CYP3A4 substrate when coadministered with phenytoin throught induction of presystemic CYP3A-mediated intestinal first-pass. The mechanism may be via phenytoin activated PXR, resulting in transcriptional activation of the CYP3A4 gene (Luo *et al*., 2002). Phenytoin induction of CYP2B6 gene expression in human primary hepatocytes is mediated predominantly through activation of human CAR (Wang *et al*., 2004).

In conclusion, in a long term phenytoin treatment, it has been shown that phenytoin increases the clearance of efavirenz. These effects may be probably due to the intestinal induction of CYP3A and CYP2B leading to a reduction in the plasma concentration of efavirenz. Further studies are needed to find out the clinical relevance of this efavirenz-phenytoin interaction in humans.

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APPENDIX

APPENDIX A

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MOE 0521.11/516 Ref.21/2012

May 30 , 2012

This is to certify that the research project entitled "Effect of Phenytoin on the Pharmacokinetic of Efavirenz in Rabbits" which was conducted by Assoc. Prof. Dr.Werawat Mahatthanatrakul, Faculty of Sciences, Prince of Songkla University, has been approved by The Animal Ethic Committee, Prince of Songkla University.

Kitja Sawangjaroen.

Kitja Sawangjaroen, Ph.D.
Chairman,
The Animal Ethic Committee, Prince of Songkla University

APPENDIX B

Profile of efavirenz concentration at each time concentration of blood drawn from each rabbit

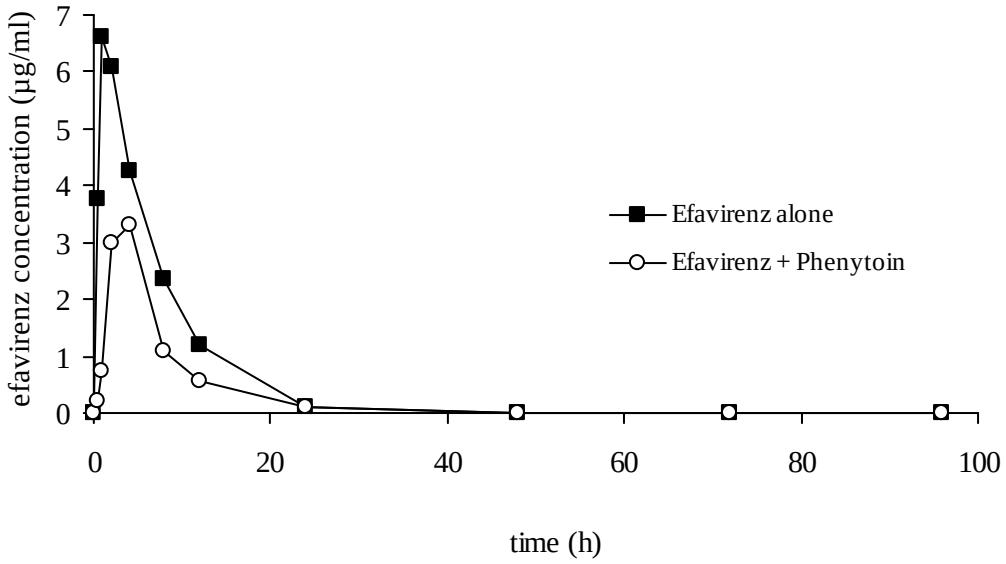

Figure 13 : Plasma efavirenz concentration-time curve of rabbit No. 1

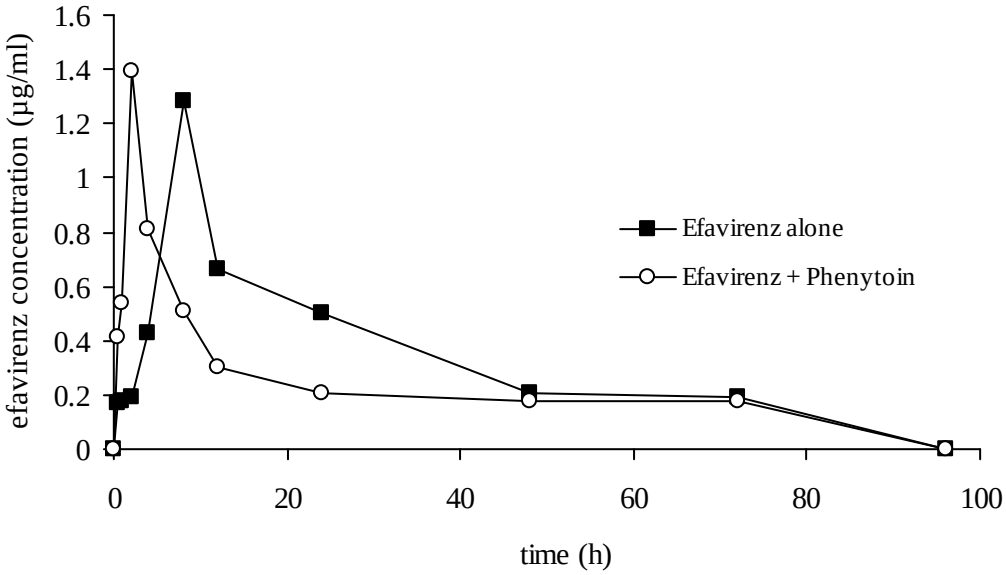

Figure 14 : Plasma efavirenz concentration-time curve of rabbit No. 2

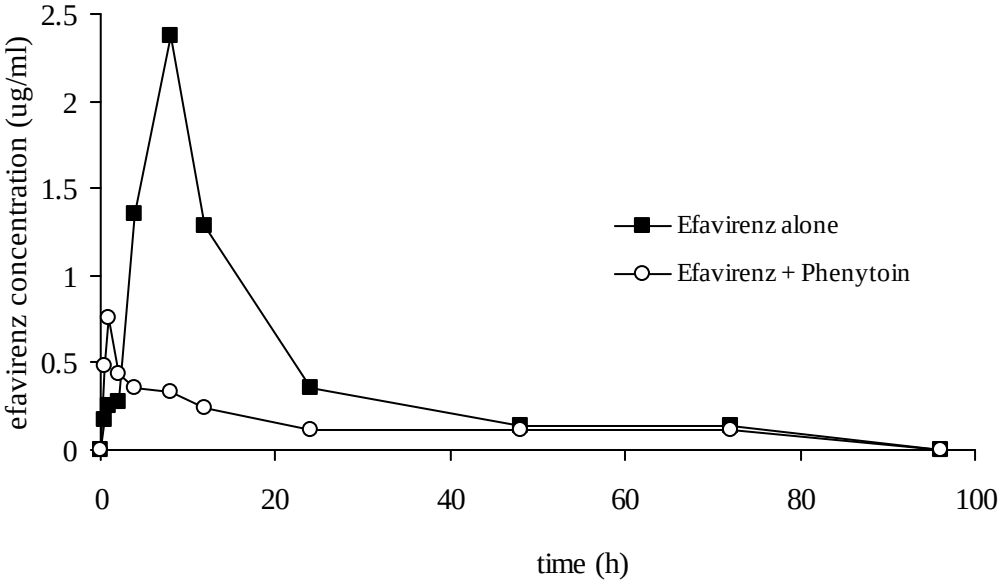

Figure 15 : Plasma efavirenz concentration-time curve of rabbit No. 3

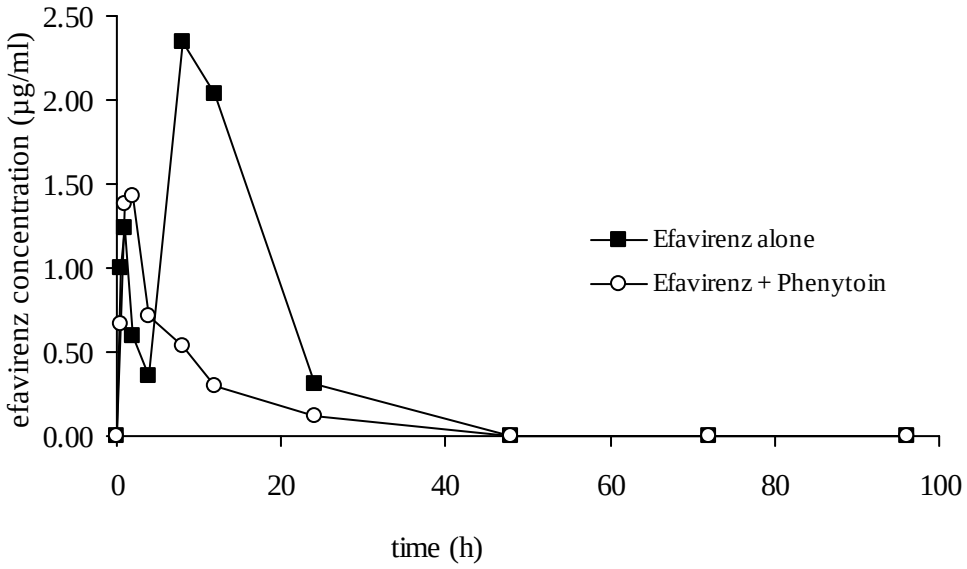

Figure 16 : Plasma efavirenz concentration-time curve of rabbit No. 4

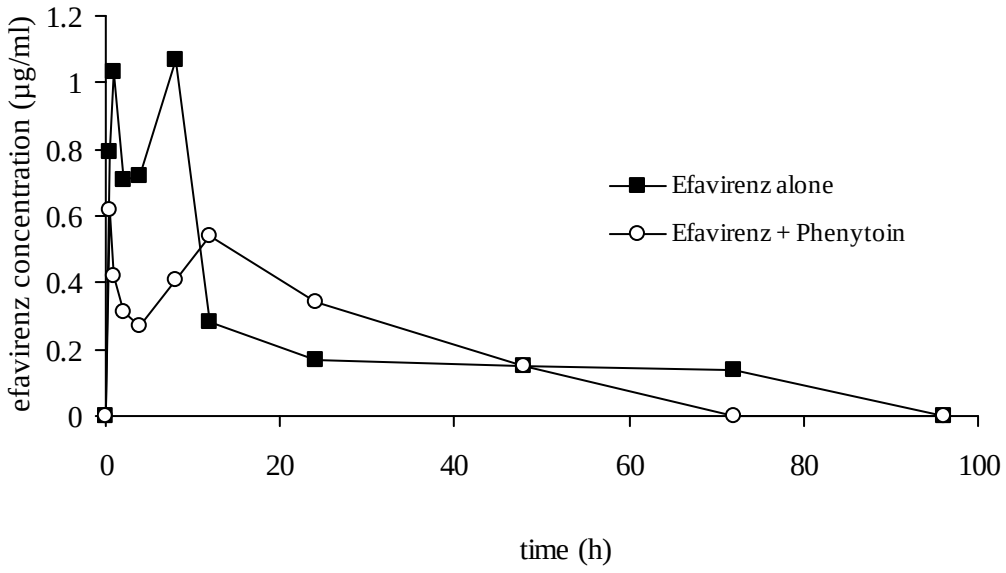

Figure 17 : Plasma efavirenz concentration-time curve of rabbit No. 5

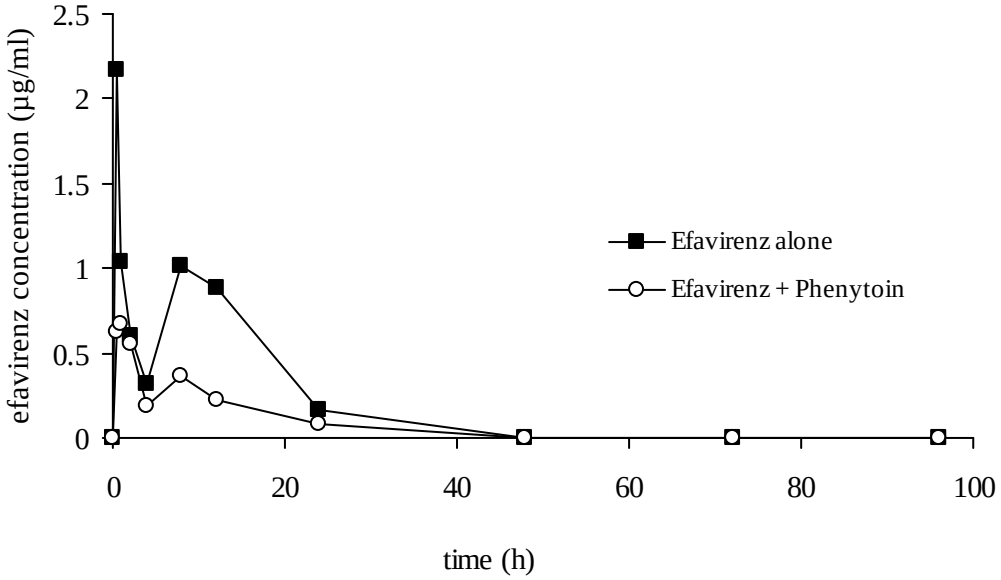

Figure 18 : Plasma efavirenz concentration-time curve of rabbit No. 6

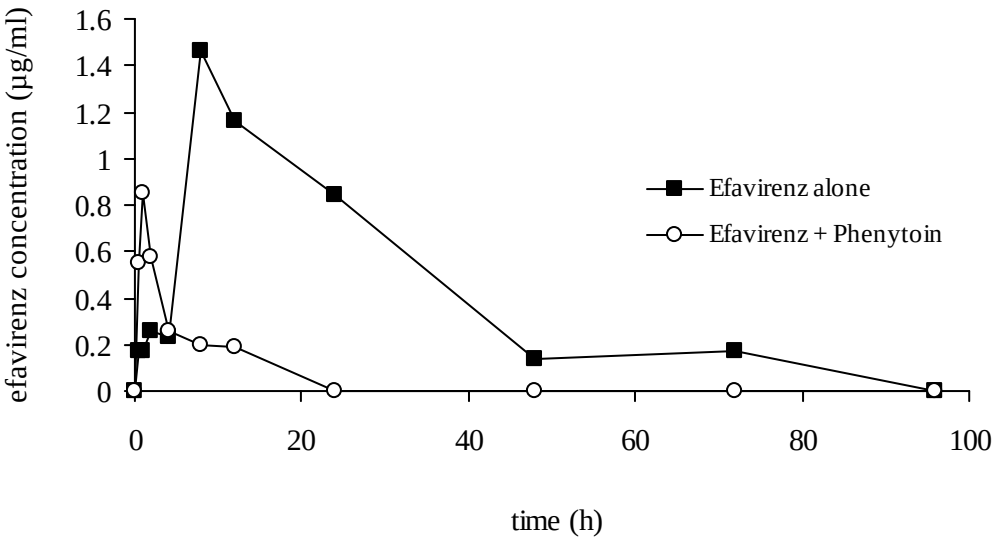

Figure 19 : Plasma efavirenz concentration-time curve of rabbit No. 7

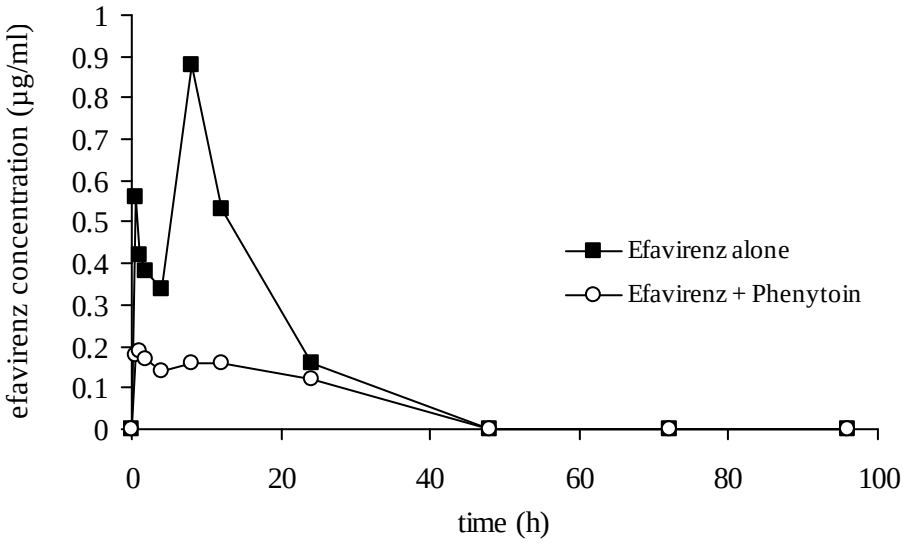

Figure 20 : Plasma efavirenz concentration-time curve of rabbit No. 8

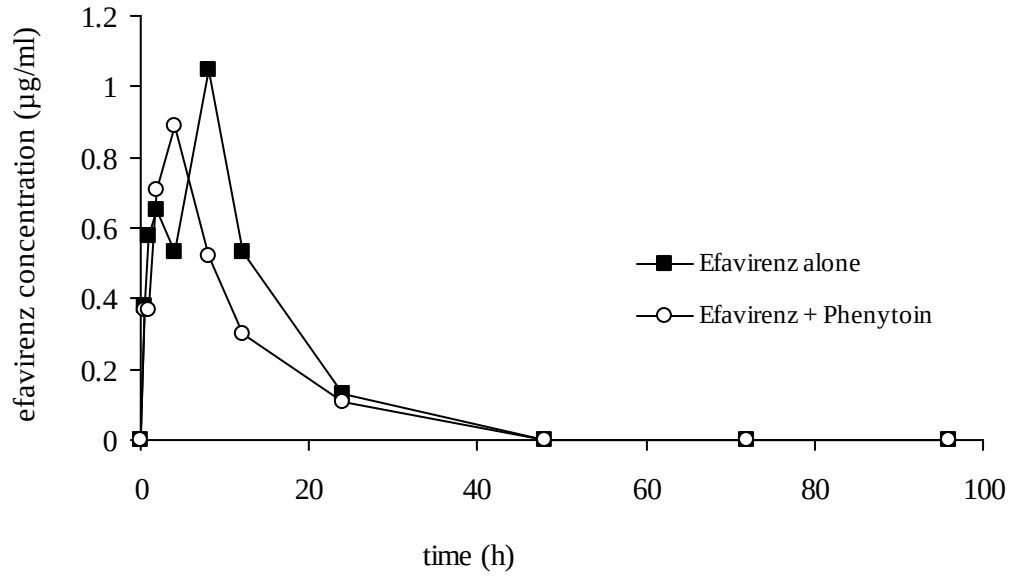

Figure 21 : Plasma efavirenz concentration-time curve of rabbit No. 9

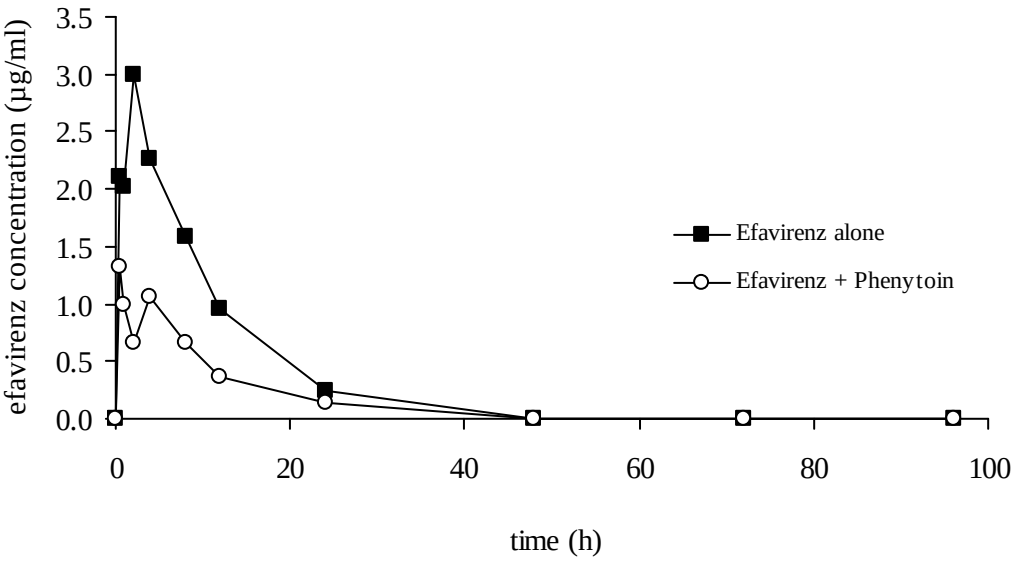

Figure 22 : Plasma efavirenz concentration-time curve of rabbit No. 10