ความหลากหลายของยีน *CYP2D6* และผลการทำงานของเอนไซม์ CYP2D6 ในคนไทย

นาง พเยียรัตน์ นาคมหาชลาสินธุ์

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GENETIC POLYMORPHISMS AND CYP2D6 ACTIVITY IN THAI SUBJECTS

Mrs. Payiarat Nakmahachalasint

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Thesis Title	Genetic Polymorphisms and CYP2D6 Activity in Thai Subjects
By	Mrs. Payiarat Nakmahachalasint
Field of Study	Pharmacology
Thesis Advisor	Associate Professor Pornpen Pramyothin, Ph.D.
Thesis Co-advisor	Chanin Limwongse, M.D.

Accepted by the Faculty of Pharmaceutical Sciences, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

Boonyong Tantisira ..... Dean of the Faculty of Pharmaceutical Sciences
(Associate Professor Boonyong Tantisira, Ph.D.)

THESIS COMMITTEE

Siriporn Foongwithaya ..... Chairman
(Associate Professor Siriporn Foongwithaya, M.Sc.)

Pornpen Pramyothin ..... Thesis Advisor
(Associate Professor Pornpen Pramyothin, Ph.D.)

..... Thesis Co-advisor
(Chanin Limwongse, M.D.)

C. Itthipanichpong ..... Member
(Associate Professor Chandhanee Itthipanichpong, M.Sc.)

Boonsri Ongpipattanakul ..... Member
(Assistant Professor Boonsri Ongpipattanakul, Ph.D.)

พเยียรัตน์ นาคมหาชลาสินธุ์: ความหลากหลายของยีน *CYP2D6* และผลการทำงานของเอนไซม์ CYP2D6 ในคนไทย (GENETIC POLYMORPHISMS AND CYP2D6 ACTIVITY IN THAI SUBJECTS) อ. ที่ปรึกษาวิทยานิพนธ์: รศ.ดร. พรเพ็ญ เปรมโยธิน อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: นพ. ชนินทร์ ลิ่มวงศ์, 62 หน้า ISBN: 974-17-5592-9

CYP2D6 มีบทบาทสำคัญในการเปลี่ยนแปลงยาที่ใช้มากในทางคลีนิกและเป็นที่ทราบดีว่าเอนไซม์นี้มีความหลากหลายทางพันธุกรรม ทำให้ผลการรักษาด้วยยามีความแตกต่างกันมากในแต่ละบุคคล ได้ทำศึกษาลักษณะของ *CYP2D6* อัลลีลทั้งหมดแปดอัลลีลและตรวจพบหกอัลลีลในคนไทย ผล genotype โดยทั่วไปแล้วคล้ายคลึงกับผลที่พบในคนเอเชียนแต่แตกต่างกับผลของคนคอเคเซียน ความถี่ของยีนที่พบมากที่สุดในคนไทยคือ *CYP2D6**10 (69.49%) โดยที่พบ *10A ได้น้อยกว่า *10B การที่พบอัลลีล *CYP2D6**10 มากมีผลให้ความถี่ในการพบ *CYP2D6**2 (1.69%) น้อยลง *CYP2D6**4 และ *5 พบได้มากกว่าคนเอเชียกลุ่มอื่นเล็กน้อย พบ homozygous ที่ *CYP2D6**5 ซึ่งการผันแปรของทั้งสองอัลลีลดังกล่าวพบได้น้อยมากในคนเอเชีย แต่เราก็พบลักษณะดังกล่าวในคนไทยถึง 2 คนจาก 7 คนที่มีการผันแปรที่ยีน ทุกคนที่ทำการศึกษาพบการเปลี่ยนแปลงที่ตำแหน่ง $C_{2938}\rightarrow T$ ร่วมกับ *CYP2D6**1, *4 and *10 เสมอ ซึ่งอาจจะเป็นลักษณะพิเศษเฉพาะของ *CYP2D6* อัลลีลในคนไทยที่มีการเปลี่ยนแปลงที่ $C_{2938}\rightarrow T$ เพิ่มเข้ามา แตกต่างจากอัลลีลที่มีมาแล้ว การทำงานของ CYP2D6 เอนไซม์สามารถวัดได้จากปฏิกิริยา dextromethorphan O-demethylation โดยใช้ HPLC ตรวจวัดเมตาบอไลท์ที่เกิดขึ้น เมื่อวิเคราะห์เปรียบเทียบแต่ละ genotype กับ phenotype ที่ได้แล้ว พบว่ามีความสัมพันธ์กัน การทำงานของเอนไซม์ในกลุ่ม EM/EM เมื่อเปรียบเทียบแล้วสูงกว่ากลุ่ม IM/PM และ PM/PM ($p<0.05$) กลุ่ม extensive metabolizer (EM) มีค่า V_{max} สูงกว่า และ ค่า K_m ต่ำกว่ากลุ่ม intermediate metabolizer (IM) มีสองคนที่เป็นกลุ่ม PM/PM พบว่ามี enzyme activity ต่ำมากอย่างชัดเจน การศึกษานี้ได้แสดงให้เห็นการศึกษาความสัมพันธ์ระหว่าง genotype-phenotype ของ CYP2D6 เป็นครั้งแรกในประเทศไทย.

ภาควิชา	เภสัชวิทยา	ลายมือชื่อนิสิต.....
สาขาวิชา	เภสัชวิทยา	ลายมือชื่ออาจารย์ที่ปรึกษา.....
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PAYIARAT NAKMAHACHALASINT: GENETIC POLYMORPHISMS AND CYP2D6 ACTIVITY IN THAI SUBJECTS. THESIS ADVISOR: ASSOC. PROF. PORNPEN PRAMYOTHIN, Ph.D., THESIS CO-ADVISOR: CHANIN LIMWONGSE, M.D., 62 pp, ISBN 974-17-5592-9

CYP2D6 plays a major role in the metabolism of many clinically used drugs and is known to be genetically polymorphic resulting in a high degree of interindividual variation in drug therapy. Eight of *CYP2D6* alleles have been characterized and six of them were found in Thai subjects. The genotype results are generally in agreement with those found in other Asians but different from those of Caucasians. *CYP2D6**10 has the highest frequency (69.49%) with *10A being less frequent than *10B. The high prevalence of *CYP2D6**10 results in a lower frequency of *CYP2D6**2 (1.69%). *CYP2D6**4 and *5 are found slightly more frequent than those found in other Asian population. Homozygous of *CYP2D6**5 which is rare in Asians was unexpectedly detected in 2 individuals out of 7 mutants. All subjects constantly exhibits $C_{2938}\rightarrow T$ together with *CYP2D6* *1, *4, and *10 alleles which possibly are the unique characteristic of *CYP2D6* alleles in Thais that have an additional $C_{2938}\rightarrow T$ change different from the proposed alleles. CYP2D6 activity was measured from the dextromethorphan O-demethylation reaction using HPLC for the detection of metabolite. Each genotype was compared with corresponding phenotype and the correlation was shown. EM/EM group has significantly ($p<0.05$) higher enzyme activity when compared to IM/PM and PM/PM group. Extensive metabolizer (EM) presents higher V_{max} and lower apparent K_m than those of intermediate metabolizers (IM). Two individuals with PM/PM were found with a remarkably low enzyme activity. This study represents the first genotype-phenotype correlation study of CYP2D6 in Thailand.

Department	Pharmacology	Student's signature P. Nakmahachalasint
Field of study	Pharmacology	Advisor's signature Pornpen Pramyothin
Academic year	2003	Co-advisor's signature

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ABBREVIATIONS

Arg	=	Arginine
ASA	=	Allele Specific Amplification
cDNA	=	Complimentary Deoxyribonucleic Acid
CYPs	=	CytochromeP450s
Cys	=	Cysteine
dNTPs	=	Deoxyribonucleoside Triphosphates
EDTA	=	Ethylenediaminetetraacetic Acid
EM	=	Extensive Metabolizer
FAD	=	Flavin Adenine Dinucleotide
FMN	=	Flavin Mononucleotide
HiQC	=	High Quality Control
HPLC	=	High Performance Liquid Chromatography
IM	=	Intermediate Metabolizer
LoQC	=	Low Quality Control
MeQC	=	Medium Quality Control
MR	=	Metabolic Ratio
NADP	=	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	=	Nicotinamide Adenine Dinucleotide Phosphate
OD	=	Optical Density
PBS	=	Phosphate Buffer Saline
PCR	=	Polymerase Chain Reaction
PM	=	Poor Metabolizer
RBC	=	Red Blood Cell
RFLP	=	Restriction Fragment Length Polymorphism
SNPs	=	Single Nucleotide Polymorphisms
UM	=	Ultrarapid Metabolizer
WBC	=	White Blood Cell

Chapter 1

INTRODUCTION

1.1 Rationale and Background

Genetic polymorphisms have been considered to be one of the critical factors causing interindividual variation in drug therapy. Some people show response to the prescribed drug without any serious side effects, whereas others experience adverse reactions. When a drug is administered, it will be normally absorbed into human body and undergoes biotransformation (metabolism) to be a more hydrophilic product, readily excreted from the body. Metabolism involves in detoxification and elimination of most drugs or activation of the prodrug to the biologically active therapeutic metabolite or toxin. When the pharmacological activity of a drug is linked to the catalytic activity of a specific enzyme, factors that influence the activity of that enzyme will affect the clinical response of that agent. The cytochromeP450 (CYP) enzyme system plays an important role in the metabolism and elimination of numerous widely used drugs. The capacity of this system varies from one person to another according to the expression of CYP enzymes due to genetic and environmental factors and some diseases. These factors produce inter-individual variation in the rate and metabolic pathways of xenobiotics. For this reason, therapeutic response and side-effects vary widely between patients treated with the same dose of drug.

The CYP superfamily is divided into families and subfamilies on the basis of their nucleotide and amino acid sequence homology. A number of CYP enzymes are known to be genetically polymorphic. Examples of genetic polymorphism influencing interindividual variation are the polymorphic expression of CYP2A6, CYP2C9, CYP2C19 and CYP2D6. Polymorphism in CYP genes carrying certain nucleotide substitutions, deletion, insertions or gene

conversions are known, which may result in CYP enzymes with abnormal activity. The simplest form of these variations is the substitution of one single nucleotide for another, termed SNP (single nucleotide polymorphism). SNPs possibly change amino acid sequences which may reduce or increase enzyme activity. On the other hand, SNPs may not change encoded amino acids and that results in normal enzyme activity. Based on the extent of drug metabolism, genetic polymorphism has been linked to three classes of phenotypes: EM, PM and UM. Extensive metabolizer (EM) is characterized as normal population while poor metabolizer (PM) is associated with the accumulation of specific drug substrates and is prone to drug toxicity. It is typically an autosomal recessive trait requiring mutation and/or deletion of both alleles for phenotypic expression. Ultraextensive metabolizer (UM) results in increased drug metabolism which leads to subtherapeutic responses. The extent of drug metabolism which stands between EM and PM may be classified into another extra class, called intermediate metabolizer (IM).

CYP2D6 enzyme is one of the subfamilies of cytochromeP450 that has a major role in metabolism accounted for 25 % [1] of all commonly prescribed drugs such as antidepressant, antiarrythmics, neuroleptic drugs, opiates, and antihypertensive drugs. Many compounds currently in clinical development are known to be CYP2D6 substrates. This enzyme has a wide range of activity within human populations, with interindividual metabolic rates ranging from 0 to 10,000 folds [2]. CYP2D6 gene is highly polymorphic and more than 70 alleles have been so far identified (http://www.imm.ki.se/CYPalleles/). The *CYP2D6**3, *CYP2D6**4 and *CYP2D6**5 alleles are found mostly in Caucasian as poor metabolizer [1, 3]. In Asian population, those alleles found in Caucasian are rare but *CYP2D6**10 is common in Asians with reduction of function allele [4, 5]. Both Africans and African Americans have reduced function alleles representing 35% of allele variation, mainly *CYP2D6**17 [1]. There are significant interethnic differences in the prevalence of the PM phenotype of CYP2D6. For example, in North Americans and European Caucasians have consistently shown that 5-10%

of the population is PMs [3], whist in Oriental only 0-2 % were found to be PMs [6]. African populations have yielded inconsistent results with prevalence of PMs ranging from 0-19% [7]. The clinical significance of these enzyme polymorphisms has been widely studied especially on the filed of psychopharmacology [8]. The knowledge of CYP2D6 genotype status may be of clinical value in predicting adverse or inadequate response to certain therapeutic agents and in predicting the increased risk of environmental or occupational exposure-linked diseases.

In Asian, allele frequency characterizations in a wide-range of population are still needed to be investigated. Allele frequencies of CYP2D6 in Thai population have not yet been characterized. Only one study conducted by Wanwimoluk et al. [9] determined the prevalence of CYP2D6 poor metabolizers in Thai population. Phenotypic approach has been done by examining urine metabolic ratio of debrisoquine and 1.2 % of Thai were found to be poor metabolizers. Although a prevalence of Thai PMs has been reported, none of genotypic data is revealed in Thai. Thus, this study aims to determine allele type and frequency of *CYP2D6* gene in Thai together with liver enzyme activity of each subject for the analysis of genotype-phenotype correlation. The knowledge of CYP2D6 genotype would assist in the prediction of toxic effects or in the appropriate selection of an alternative therapeutic drug or dosing.

1.2 Hypothesis

CYP2D6 allele frequencies in Thai subjects are different from those of other ethnic groups and phenotypic consequences of each subject are correlated with their genotypes.

1.3 Objectives

1.3.1 To determine allelic variation and frequency of *CYP2D6* gene in Thai subjects.

1.3.2 To determine CYP2D6 enzyme activity in liver of each subjects and to analyze the genotype-phenotype correlation.

1.4 Expected Outcomes

1.4.1 *CYP2D6* allele frequency and polymorphisms in Thai subjects will be characterized; the ratio of poor metabolizer will be revealed and compared to other ethnic groups.

1.4.2 The data from the study can be used as preliminary data for a further study of *CYP2D6* gene in the future.

Chapter 2

LITERATURE REVIEWS

2.1 Introduction to CytochromeP450

The mechanisms that serve to protect animals (and plants) against chemical insults are metabolism and elimination pathways. There are various enzymes involved in this protection as we can classify into phase I (oxidative) and phase II (conjugative) enzyme systems (Fig. 2.1). These two enzyme systems catalyze the reactions often complement each other in function. The major route of phase I drug metabolism is the oxidation by cytochromeP450 (CYP) mixed-function monooxygenases located within the endoplasmic reticulum. CYPs are intracellular hemeproteins that activate the oxidative, peroxidative and reductive metabolism of a wide variety of endogenous and exogenous compounds. CYPs mediate biotransformation to polar metabolites which can be excreted by liver and the kidney. The name P450 is derived from the property of pigments in a microsomal fraction with an absorbance band maximum at 450 nm. This optical absorbance characteristic proves to be a fortuitous property useful in spectrophotometrically identifying and quantifying P450s since no other mammalian hemeproteins (except nitric oxide synthase) significantly absorb light at this wavelength. The human hepatic CYP system consists of over 30 related isoenzymes with different, and sometimes overlapping, substrate specificities [10]. The extent of isoenzymes proliferates differently in liver and each isoenzyme has its catalytic reaction on a limited number of xenobiotics and endogenous compounds. The capacity of this system varies widely from one person to another, leading to variable drug excretion rates and final drug concentrations. The intersubject variability in metabolic rate is largely determined by genetic factors since a number of CYP enzymes are genetically polymorphic. The prevalence of polymorphism in population is different among ethnic groups.

2.2 Nomenclature

The CYP enzymes have been classified in a systematic way on the basis of their amino acid sequences. A standard nomenclature system classified enzymes into gene families and subfamilies. Families are indicated by the abbreviation for cytochromeP450 (CYP), followed by an Arabic number. Members of a family are at least 40% identical. Within a family, enzymes with a greater than 55% sequence homology are included in the same subfamily. Subfamilies are indicated by a letter following the family number. Individual genes, coding for one specific isoenzyme, have a second Arabic number after the letter. The nomenclature system and a main substrate specificity of each isoenzyme are depicted in Table 2.1 and Table 2.2, respectively. At least 74 CYP gene families, of which 14 are ubiquitous in all mammals, have been described so far [11]. Enzymes belonging to families CYP1, CYP2 and CYP3 catalyze the oxidative biotransformation of exogenous compounds, including many drugs, (pro)carcinogens, (pro)mutagens and alcohols. The other CYP families are involved in the metabolism of endogenous substances, such as fatty acids, prostaglandins, steroid and thyroid hormones.

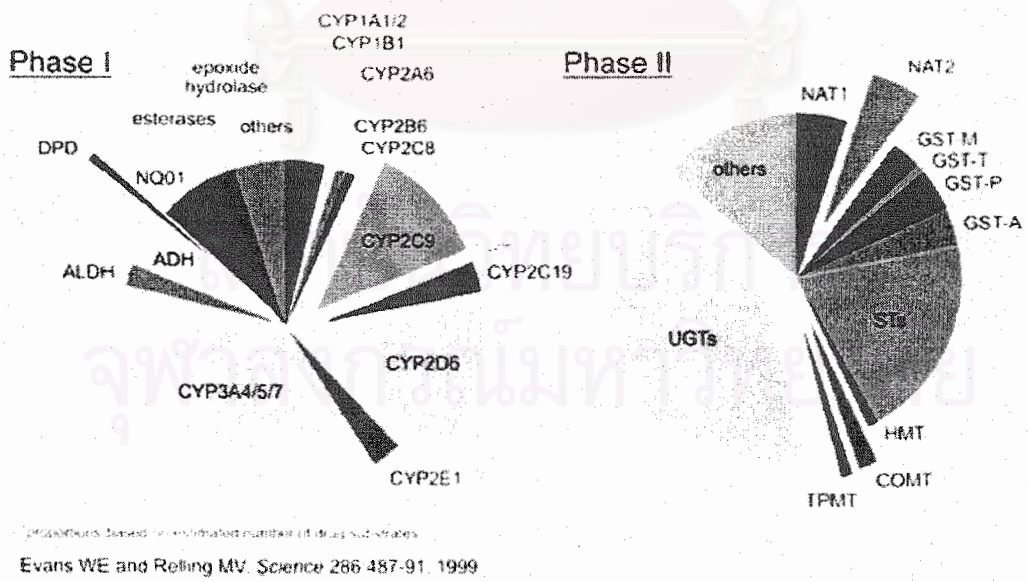


Figure 2.1 The proportion of metabolizing enzymes in Phase I and Phase II reactions.

Table 2.1 CytochromeP450 (CYP) nomenclature system

Family	subfamily	individual
CYP1		
CYP2 →	CYP2C →	CYP2C9
	CYP2D	CYP2C19

Table 2.2 Characteristics of the major human cytochromeP450s [12]

CYP450	Approx. % of liver	Polymorphic CYP	Significant for:		Representative substrates
			First pass metabolism of drug	Metabolism of carcinogen	
CYP1A1	-	Yes	No	Yes	Carcinogen polymorphic aromatichydrocarbon, e.g. benz[α]pyrene
CYP1A2	13	Yes	Yes	Yes	Arylamines, nitrosamines, aflatoxinB1, caffeine, paracetamol, theophylline, imipramine, fluvoxamine
CYP2A6	4	Yes	No	Yes	Coumarin, nicotine
CYP2C9	18	Yes	Yes	No	Tolbutamide, ibuprofen, mefenamic acid, tetrahydrocannabinol, losartan, diclofenac
CYP2C19		Yes	Yes	No	S-mephenytoin, amitriptyline, diazepam, omeprazole, proguanil
CYP2D6	2	Yes	Yes	No	Debrisoquine, metoprolol, sparteine, propranolol, encainide, codeine, dextromethorphan, clozepine, imipramine
CYP2E1	7	Yes	No	Yes	Ethanol, nitrosamines, paracetamol, chlorzoxazone
CYP3A4	29	No	Yes	Yes	Erythromycin, ethinyl estradiol, nifedipine, triazolam, cyclosporine, amitriptyline, imipramine, aflatoxine B1

2.3 Structure and Catalytic Activity

CytochromeP450 is a membrane-bound hemeprotein, each containing about 500 amino acids with iron-protoporphoryrin IX as the prosthetic group. The number of chemicals that can serve as substrates metabolized by P450 is enormous and is certainly greater than 1000. P450 enzyme catalyzes reactions for the oxidative conversion of a chemical following the equation illustrated in Fig. 2.2. Two electrons originating from NADPH are transferred to the hemeprotein by a flavoprotein (or a flavoprotein/iron sulfur protein) in the presence of an organic chemical and molecular oxygen. The organic chemical is oxidized and the atom of molecular oxygen is incorporated into the chemical product. Most biotransformation of xenobiotics is done by enzymes from families CYP1, CYP2 and CYP3. Other families are mainly involved in the metabolism of endogenous compounds, such as fatty acids, bile acids, and hormones [13].

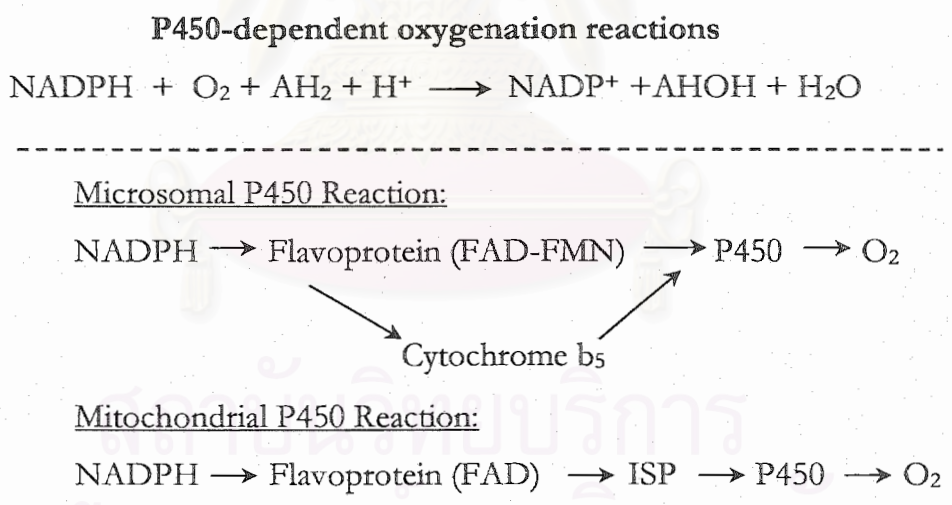


Figure 2.2 The equation for P450-dependent mixed-function oxidase (oxygenase) reaction and the two types of electron transport carrier systems functional with different P450s depending on their sub-cellular localization [12].

The CYP2 family has been under intensive studied using rat, mouse and rabbit as model systems. The CYP2 family includes seven subfamilies in mammals. In human, the most important CYP2s from the point of view of drug

metabolism are CYP2A6, CYP2C9, CYP2C19, CYP2D6 and CYP2E1. The role of CYP2B6 in drug metabolism has not yet been clarified thoroughly. Although CYP2B6 represents only about 1% of total P450 content in human liver, there is some evidence suggesting its significant participation in the metabolism of certain drugs. About 70% of CYP enzymes in the human liver belong to the families which participate in drug metabolism as determined immunochemically. Of these, CYP3A4 represents about 30% and CYP2C about 20% of total CYP enzymes. These enzymes are the major P450 forms in human liver microsomes [14]. Of the CYP2 enzymes, CYP2F1 has not been found to be expressed in adult livers [15], and it seems to be expressed only in lungs.

2.4 Pharmacogenetics of CytochromeP450

Metabolism of most drugs influences their pharmacological and toxicological effects. Drugs particularly affected are those with a narrow therapeutic window and which are subject to considerable first pass metabolism. Much of the interindividual and interethnic differences in effects of drugs are now attributable to genetic differences in their metabolisms. Mutations in a gene coding for a drug metabolizing enzyme can give rise to enzyme variants with higher, lower, or no activity (functional mutations) or may have no effect on enzyme activity. If the mutant allele occurs with a frequency of at least 1% in the normal population and causes a different drug response or phenotype, this phenomenon is termed a pharmacogenetic polymorphism [16] .

Polymorphisms of drug metabolism divide a population into at least two phenotypes, extensive and poor metabolizers giving rise to a bimodal frequency distribution. Polymorphisms have been detected in many drug metabolizing enzymes, including the cytochromeP450 (CYP), at both genotypic and phenotypic levels. While some allelic variants, such as the *CYP2D6**5 (gene deletion), are common to all populations studied i.e., African, Caucasian and Oriental, others seem to be the characteristic for a particular population e.g., *CYP2D6**4 in

Caucasian, *CYP2D6**10 in Asians [17] and CYP2D6*17 in Africans [1]. Type and prevalence of allelic variants present in an individual or in a population will influence the pharmacological and toxicological effects of drugs, toxins and carcinogens leading to interindividual and interethnic differences in effects of drugs and other xenobiotics. This becomes important for individual drug therapy, for clinical trials appropriated for a particular ethnic group and also the search for possible relationships between genotypes and their susceptibility to cancer and other diseases. The cytochromeP450 for which phenotype and/or genotypic polymorphisms have been described include CYP 1A1, 1A2, 2A6, 2C9, 2C19, 2D6 and 2E1. CYP3A4 is subject to wide inter-individual variation but no data exist to support a possible genetic polymorphism. The two most studied and best characterized CYP polymorphisms are those for debrisoquine 4-hydroxylase (CYP2D6) and S-mephenytoin hydroxylase (CYP2C19).

2.5 CYP2D6 (debrisoquine hydroxylase) Polymorphism

Genetic polymorphism is defined as the inheritance of a trait controlled by a single genetic locus with two alleles, in which the least common allele has a frequency of about 1% or greater. One of the most extensively studied genetic polymorphism known to influence drug metabolism and response is the debrosquine type (CYP2D6) oxidation polymorphism. The discovery of CYP2D6 polymorphism created new interest in the role of pharmacogenetics in clinical pharmacology.

2.5.1 Evolution of CYP2D6 polymorphism

Between 1975 and 1977 two groups independently discovered the genetic deficiency of debrisoquine and sparteine metabolism [18]. The discovery of genetic polymorphism in the metabolism of two prototype drugs was not the result of a planned strategy but rather an incidental observation. A dramatic event in a pharmacokinetic study prompted the initial search for a specific metabolic

defect. The investigator, Dr. Smith, who was participating in a study on debrisoquine, a sympatholytic antihypertensive drug, had a much more pronounced hypotensive response than his colleagues, collapsing from a sub therapeutic dose. This was found to be due to the impairment of debrisoquine 4-hydroxylation [18].

Similarly in 1975, during the course of kinetic studies by Eichelbaun et al. with a slow release preparation of sparteine, two subjects developed side effects such as diplopia, blurred vision, dizziness and headache. When analyzing the plasma levels of sparteine in those subjects the reason for the development of side effects became evident. Compared to all other subjects studied, their plasma levels were 3 to 4 times higher, although the same dose had been given to every subject [19].

Family and population studies uncovered a genetic polymorphism and later work established that the two independently discovered defects in drug oxidation co-segregated in Caucasians (PM for sparteine exhibit impaired debrisoquine metabolism and vice versa) and the term sparteine/debrisoquine polymorphism was coined [20]. However, there are apparent exemptions to this rule. For instance, in a study in Ghana, the ability of Ghanaians to oxidaize sparteine was independent from their capacity for debrisoquine oxidation. Numbers of drugs metabolized by CYP2D6 enzyme are shown in Table 2.3

2.5.2 Nomenclature

Guidelines on nomenclature for individual cytochromeP450 isoform have been internationally agreed upon and are regularly updated. Genes encoding the P450 enzyme are designated as CYP. Because of the diversity of the cytochrome family, a nomenclature system based on sequence identity has been developed to assist in unifying scientific efforts in this area and to provide a basis

for nomenclature of newly regcognized members of this gene superfamily. For example, CYP2D6 is isoform 6 of subfamily D included in The 2CYP family [21].

Table 2.3 Some drugs whose metabolism is catalyesed by CYP2D6

β-Adrenoceptor blocker	*Antidepressant*	*Neuroleptic*
Metoprolol	Amitriptylline	Haloperidol
Propranolol	Clomipramine	Perphenazine
Timodol	Desipramine	Risperidone
	Fluoxetine	Thioridazine
Antiarrythmic drugs	Fluvoxamine	Zuclopenthixol
Encainide	Imipramine	
Flecainide	Mianserin	*Miscellaneous*
Perhexilene	Nortriptyline	Codeine
Propafenone	Paroxetine	Debrisoquine
Sparteine	Venlafaxine	Phenformin
	Dextromethorphan	Tramadol
	Tolterodine	

In the past, *CYP2D6* alleles have been named arbitrarily using a single letter after the gene name [22] but with increasing numbers of alleles being detected, this system is now inadequate. The general recommendation is that the gene and allele and the entire gene allele symbol is italicized e.g. *CYP2D6**1A [23]. Since a number of *CYP2D6* alleles share common key mutations but differ with respect to other base changes, these should be given the same Arabic number (denoting their allele group) and distinguished by capitalized Latin letters (denoting the allele sub groups). For example, both *CYP2D6**4A and *CYP2D6**4B have the same mutation but differ by a single silent base substitution [23].

Extra copies of an allele (duplicated or amplified) may exist in tandem; for example, the *CYP2D6*L2 allele contains two copies of *CYP2D6*L. Here the entire arrangement of alleles should be referred to as *CYP2D6**2×2. When duplication is not with the same subgroup, they are separated with a coma e.g., *CYP2D6**10B, 10C. A non-italicized form of the allele is used to name the protein

with asterisk omitted and replaced by a single spacing e.g., *CYP2D6* 1 /. Both alleles italicized and separated by slash to name the genotype designation (*CYP2D6**1/ *CYP2D6**4A). Description of the alleles as well as the nomenclature and relevant references are continuously updated at the new web page (http://www. imm.ki.se /CYPalleles/).

2.5.3 Molecular genetics

Gonzalez et al. [24] showed that poor metabolizers have negligible amounts of the cytochromeP450 enzyme product of the CYP2D locus (called P450db1 by them). They cloned cDNA for the gene. By directly cloning and sequencing cDNAs from several poor-metabolizer livers, they identified 3 variant mRNAs that are products of mutant genes producing incorrectly spliced pre-mRNA, thus providing a molecular explanation for one of man's most commonly defective genes (frequency of mutant alleles, 35-43%). Skoda et al. [25] demonstrated restriction fragment length polymorphisms associated with the P450db1 locus and observed more frequently in individuals with poor-metabolizer phenotype. A different, 29-kb XbaI fragment was presented in all individuals with extensive-metabolizer phenotype. The authors proposed that these polymorphisms identified 2 independent mutant alleles of the P450db1 gene. Eight additional RFLPs associated with this gene were also reported

Kimura et al. [26] have reported the *CYP2D6* gene structure and its sequences in 1989. They found that *CYP2D6* (4,378 basepaires) contains nine exons resided in the *CYP2D6*-8 clusters on chromosome 22 associated with two other genes, designated *CYP2D7* and *CYP2D8P*. *CYP2D8P* contains several gene-disrupting insertions, deletions, and termination codons within its exons, indicating that this is a pseudogene. *CYP2D7,* which is just downstream of *CYP2D8P* is apparently normal, except for the presence, in the first exon, of an insertion that disrupts the reading frame. The structure of *CYP2D6* gene with its

pseudogene and the localization of all nine exons of CYP2D6 are demonstrated in Fig. 2.3 and 2.4, respectively.

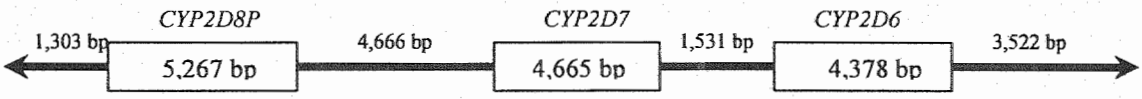


Figure 2.3 The structure of *CYP2D6* gene and its pseudogene on chromosome 22.

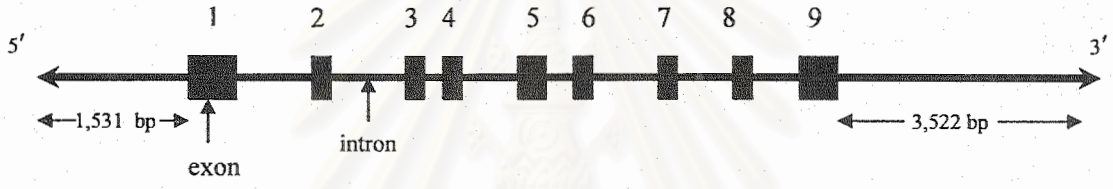


Figure 2.4. The localization of nine exons of *CYP2D6* gene.

Defective alleles can be the result of gene deletion, gene conversions with related pseudogenes and single base mutations causing frameshift, missense, nonsense or splice-site mutation [27]. The most common polymorphism found in this gene is a single nucleotide polymorphism (SNP) such as base pair substitution, deletion and conversion that are localized in nine exons as depicted in Fig. 2.5. The homozygous presence of such allele leads to a total absence of active enzyme and an impaired ability to metabolize probe drugs specific for the drug-metabolizing enzyme. These subjects are classified as poor metabolizer (PM) [13, 27, 28]. More than 50 different alleles have so far been identified. Their expanding number has led to agreements on a common nomenclature, which has recently been updated (www.http//imm.ki.se/CYPalleles/). The allelic variants are divided into subgroups, sharing the same characteristic mutation(s). The comparison of percent frequency in Caucasians with Asians is shown in Table 2.4.

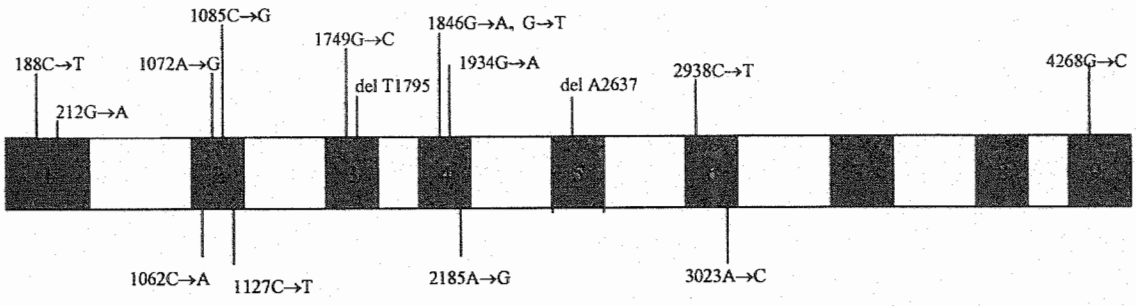


Figure 2.5. The example of single nucleotide polymorphisms (SNPs) in *CYP2D6* gene.

Table 2.4 *CYP2D6* allele subgroup, characteristic mutation(s), enzyme activity and frequency among Caucasians and Asians [4, 6].

Designation	Characteristic Mutations	Enzyme Activity	% frequency (Caucasians)	% frequency (Asians)
*CYP2D6**1	wild type	normal		
*CYP2D6**2	G_{1749}→C, C_{2938}→T, G_{4268}→C sub	normal	30	7.98
*CYP2D6**3	A_{2637} del	deficient	2	0*
*CYP2D6**4	G_{1934}→A sub, C_{188}→T, G_{1749}→C and G_{4268}→C sub	deficient	22	0.2*
*CYP2D6**5	whole gene deletion	deficient	2	4.62, 7.2*
*CYP2D6**6	T_{1795} del	deficient	2	0
*CYP2D6**7	A_{3023}→C sub	deficient	0.1	N/A
*CYP2D6**8	G_{1846}→T sub	deficient	0.1	0
*CYP2D6**9	A_{2701}, A_{2703} or G_{2702}, A_{2704} del	decrease	1.5	N/A
*CYP2D6**10	C_{188}→T, C_{1127}→T, G_{1749}→C and G_{4268}→C sub	decrease	1.5	**10A*=10.51 **10B*=54.2, 51.3*
*CYP2D6**11	G_{971}→C sub	deficient	0.1	N/A
*CYP2D6**12	G_{212}→A sub	deficient	0.1	N/A
*CYP2D6**13	hybrid: *2D7*exon 1, *2D6* exon 2-9	deficient	0.1	N/A
*CYP2D6**14	G_{1846}→A sub	deficient	0.1	0, 2.0*

Note: sub = basepair substitution, del = basepair deletion, N/A = data not available, * = Mainland Chinese [6] and without * = Hong Kong Chinese [4].

In addition to defective CYP genes, there are also alleles that cause diminished or altered drug metabolism. This results in enzyme products that exhibit impaired folding capacity and therefore the expression of the functional

enzyme is severely diminished [28]. Among extensive metabolizers, heterozygotes (one functional gene) have higher medium metabolic efficacy than those who are homozygous for the wild-type allele (two functional genes), but with pronounced overlap [5, 29]. Another type of metabolism is known as ultra rapid metabolism (UM) and is caused by occurrence of duplicated, multidupicated or amplified *CYP2D6* genes. At present, alleles with two, three, four, five and 13 gene copies in tandem have been reported and the number of individuals carrying multiple *CYP2D6* genes copies is highest in Ethiopia and Saudi Arabia, where up to one third of the population displays this phenotype. In a Swedish family, a father, a daughter and a son were shown to have 12 copies of a functional *CYP2D6*L gene with one normal gene and showed extremely high CYP2D6 activity [30].

Although clear criteria have not been formed to structurally assess whether a compound should be metabolized by this enzyme, it is observed that most of CYP2D6 substrates and inhibitors have a basic nitrogen and are oxidized at a site within 0.5-0.7 nm of this basic nitrogen. It may also have a flat lipophilic region and functional groups which have capacity for electrostatic interactions or the ability to from hydrogen bonds [31, 32]. The enzyme also shows stereoselectivity. In extensive metabolizers, inactive R-metoprolol is metabolized faster than the active S-enantiomer whereas this metabolism is not sterioselective in poor metabolizers [33] .

2.5.4 Population genetics

Bradford [1] has reviewed the median frequency for CYP2D6 alleles classified as functional, non-functional and reduced functional in Caucasians, Asians, Africans and African Americans which is illustrated in Fig. 2.6. In Caucasian populations, 71% of *CYP2D6* alleles code for normal enzymatic activity, while 26% are non-functional, mainly *CYP2D6**4 and *CYP2D6**5. Future refinements of variants with certain SNPs could improve predictability of drug response. Many Asians and those of Asian descent metabolize CYP2D6-medaited

drugs more slowly than Caucasians, due predominantly to high frequencies of variants of *CYP2D6**10, a reduced function allele. Further allele frequency characterizations in wide-range of Asian populations, and those of Asian descent are needed.

Almost 50% of allele frequencies in African and African Americans are either reduced function or code for no CYP2D6 enzymatic activity. Discovery in these populations have lagged behind other populations. Black populations may also be higher carriers of SNPs which are associated with reduced CYP2D6 enzymatic activity. More clinical studies are needed in these populations to fully characterize factors leading to lower metabolic rates compared with Caucasians. New allele discoveries, frequency determination, gene sequence, SNP information combined with high throughput genotyping technology are leading to the increased predictability of drug response on an individual, rather than population-based, empirical approach. In addition to the improved pharmacotherapy, these discoveries could facilitate and validate new drug targets.

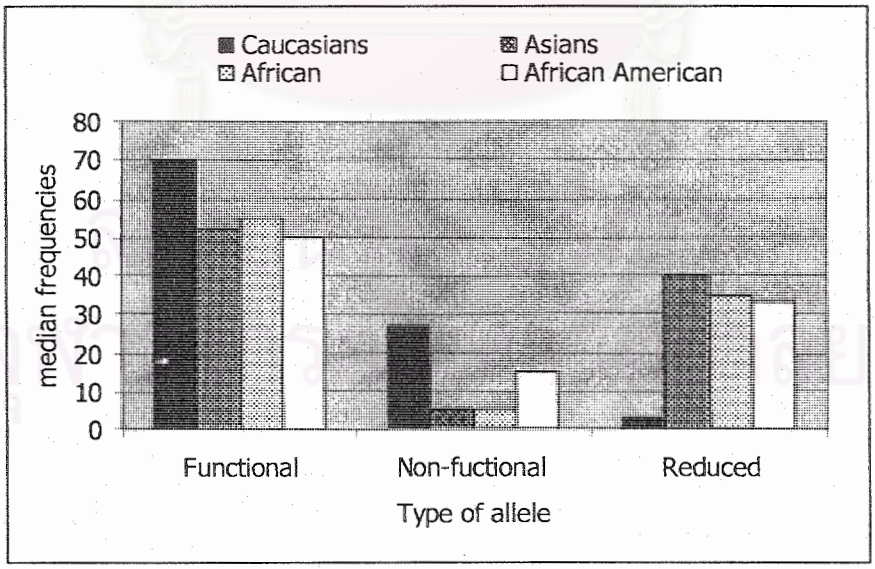


Figure 2.6 Median frequency for CYP2D6 alleles classified as functional, non-functional and reduced functioning in Caucasians, Asians and African Americans [1].

CYP2D6 activity ranges from complete deficiency to ultrafast metabolism, depending on at least 16 different known alleles. Sachse et al. [3] d etermined their frequencies in 589 unrelated German volunteers and correlated enzyme activity measured by phenotyping with dextromethorphan or debrisoquine as substrates. For genotyping, they developed nested PCR-RFLP tests from a PCR amplification of the entire *CYP2D6* gene. The frequency of the *CYP2D6**1 allele coding for extensive metabolizer (EM) phenotype was 0.364. The alleles coding for slightly (*CYP2D6**2) or moderately (*CYP2D6**9 and *CYP2D6**10) reduced activity (Intermediate Metabolizer phenotype, IM) showed frequencies of 0.324, 0.018, and 0.015, respectively. By use of novel PCR tests for discrimination, CYP2D6 gene duplication alleles were found with frequencies of 0.005 (*CYP2D6**1x2), 0.013 (*CYP2D6**2x2), and 0.001 (*CYP2D6**4x2). Frequencies of alleles with complete deficiency (Poor Metabolizer phenotype, PM) were 0.207 (*CYP2D6**4), 0.020 (*CYP2D6**3 and *CYP2D6**5), 0.009 (*CYP2D6**6), and 0.001 (*CYP2D6**7, *CYP2D6**15, and *CYP2D6**16). The defective *CYP2D6* alleles *8, *11, *12, *13, and *14 were not found. All 41 PMs (7.0%) in this sample were explained by 5 mutations detected by 4 PCR-RFLPs, which may suffice, together with the gene duplication test, for clinical prediction of CYP2D6 capacity. Sachse et al. [3] found 3 novel variants of known *CYP2D6* alleles. Analysis of variants showed significant differences in enzymatic activity measured by the dextromethorphan metabolic ratio (MR) between carries of EM/PM (mean = 0.006) and IM/PM (mean = 0.014) alleles and between carriers of 1 (mean MR = 0.009) and 2 (mean MR = 0.003) functional alleles. They commented that their results provided a solid basis for prediction of CYP2D6 capacity, as required in drug research and routine drug treatment

In contrast with poor metabolizers, individuals with a capacity to rapidly metabolize CYP2D6 drug substrate also exist. Bertilsson et al. [34] reported cases where higher than normal doses of drugs were required to attain therapeutic concentrations. Bertilsson et al. [35] and Johansson et al. [30] showed that the genetic basis of this phenomenon is gene duplication or amplification of

functionally active *CYP2D6* genes, resulting in higher levels of enzyme being expressed. In addition, subjects with multiduplicated *CYP2D6* genes present in 3, 4, or 5 copies on one allele have been seen [36, 37]. The occurrence of gene duplication has been found to vary between populations, as individuals carrying extra genes are seen in Ethiopia at a frequency of 29% [37], whereas a frequency of 1 to 2% was observed in Swedish, German, Chinese, and black Zimbabwean populations. McLellan et al. [38] found duplication of the *CYP2D6* gene in 21 of 101 Saudi Arabians studied. In contrast, only 2 individuals were heterozygous for a deletion of the whole gene. The allele frequency of *CYP2D6**4, the most common defective allele among Caucasians, was only 3.5% in the Saudi Arabian population. These findings were in agreement with earlier Saudi Arabian phenotyping studies reported a low frequency (1 to 2%) of poor metabolizers for CYP2D6-probe drugs.

2.5.5 Assessment of individual CYP2D6 activity

The activity of CYP2D6 enzyme can be assessed by means of pheynotyping or genotyping

Phenotyping

Phenotyping requires intake of a probe drug; the metabolism of which is known to be solely dependent on CYP2D6 enzyme. The excretion of parent compound and/or metabolite in urine allow to calculate the metabolic ratio, which is a measure of individual CYP2D6 activity [20, 21]. In typical phenotyping experiment, individuals were administered an oral dose of the probe drug usually at a subtherapeutic level, and urine was collected over a period of 8-12 hours. Total yield of parent compound and metabolites were determined and the parent/metabolite compound ratio, termed metabolic ratio (MR) was plotted as frequency distribution histogram (Fig. 2.7). A polymorphism is indicated by bimodal between the two populations. Antimode which separates the extensive

metabolizers from poor metabolizers serves as a baseline to distinguish these two groups [39]. A probit plot or normal test variable (NTV) plot can also be used to express the bimodal distribution. Different probe drug are used for CYP2D6 phenotyping. Earlier phenotyping studies have been performed with debrisoquine and sparteine and with dextromethorphan [40], metoprolol [41] and codeine [42] were also used for phenotyping CYP2D6 activity. The antimodes of this bimodal distribution in Caucasians are about 20, 0.3 and 12.6 for sparteine [21], dextromethorphan [21, 40] and debrisoquine [21, 40]/metopolol [40], respectively. The metabolic ratio is a function of factors such as renal drug clearance as well as enzyme activity. Environmental factors may modify these variables, which may give rise to differences in the antimode of MR between ethnic groups.

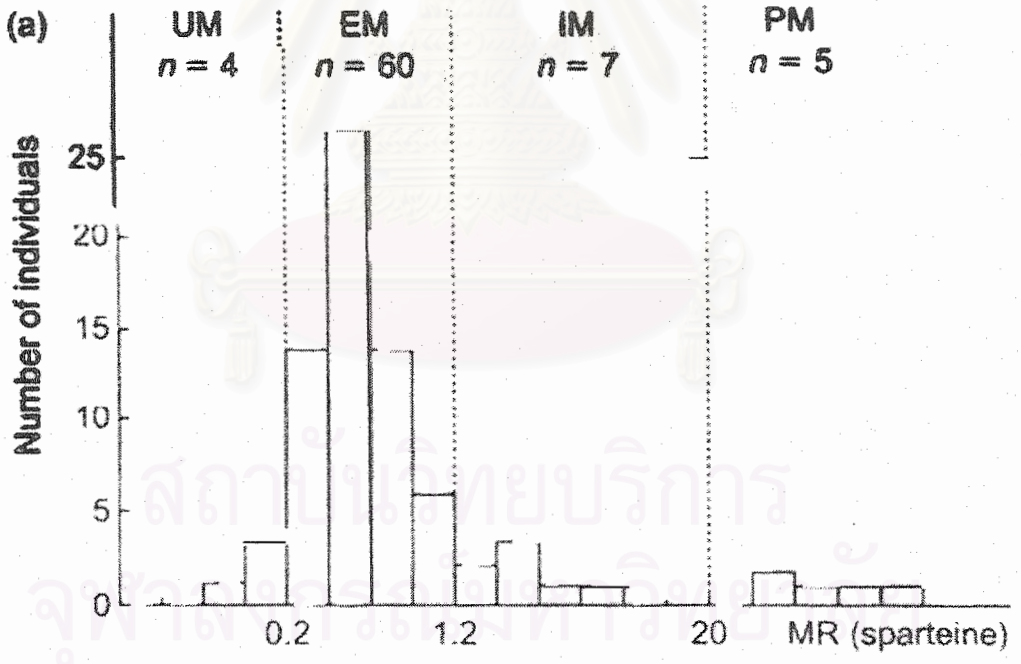


Figure 2.7 Histogram of the metabolic ratio (MR) for sparteine oxidation [43].

Dextromethorphan represents the only probe drug readily available as OTC drug in most of the countries. It is also considered safe for children and pregnant women. However metabolism of this drug proceeds simultaneously via other enzymes such as CYP3A4 (see metabolic pathway of dextromethorphan in Fig. 2.8) and results should therefore be interpreted with some caution. Blood and salivary analysis also have been used for phenotyping studies.

In vitro study of CYP2D6 using native human liver microsomes is another method for phenotyping approach. Start with initiating enzyme reaction by adding liver microsome to the prewarmed mixture of buffer, substrate and NADPH regenerating system. Incubation time is usually 20-60 minutes. The metabolite will be detected with a sensitive fluorometric or radiometric method. The enzyme activity can be derived from the calculation of the amount of metabolite per milligram protein used per hours of incubation time. Enzyme activity is found to be significantly lower with poor metabolizer compared to extensive metabolizers.

However, phenotyping is the only approach to evaluate enzyme function. If post-translational variation contributes to the individual CYP2D6 activity then phenotyping will be the only way to identify such phenomena. Phenotyping is useful in revealing drug-drug interactions or defect in overall process of drug metabolism [21] .

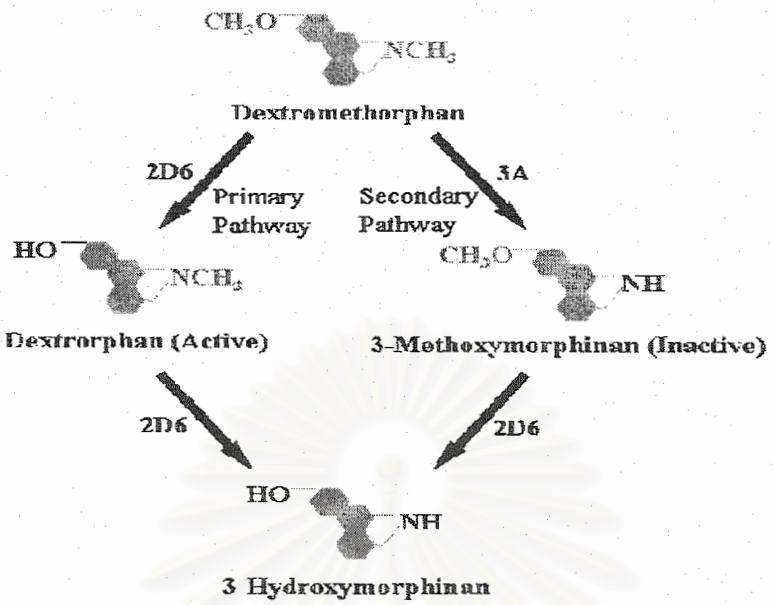


Figure 2.8 Metabolic pathway of dextromethorphan
(from http://www.uchsc.edu/sm/psych/ppfr/cyp_metabolism.htm)

Genotyping

Genotyping involves identification of defined genetic mutation that give rise to the specific drug metabolic phenotype. These mutations include genetic alterations that lead to over expression (gene amplification), absence of an active protein product (null allele), or production of a mutant protein with diminished catalytic capacity (inactivating allele) [21] .

DNA isolated from peripheral lymphocytes can be used for genotyping . Two commonly used methods in genotyping are PCR-RFLP method and allele-specific PCR [21]. In the former technique, specific region of the gene of interest is amplified by PCR followed by digestion of the amplified DNA product with restriction endonucleases. The size of the digestion products is easily evaluated by agarose gel electrophoresis with ethidium bromide staining and UV transullumination [3, 21]. In allele specific PCR amplification, oligonucleotides specific for hybridizing with the common or variant alleles are used for parallel

amplification reactions. Analysis for the presence or absence of the appropriate amplified product is accomplished by agarose gel electrophoresis.

These genotyping methods require small amount of blood or tissue, are not affected by underlying disease or drugs taken by the patient and provide results within 48-72 hours, allowing for rapid intervention. The number of known defective alleles is growing and a total of more than 30 different defective *CYP2D6* and 55 *CYP2D6* variations have been identified [44]. However, it appears that depending on the ethnic group, genotyping for only 5-6 most common defective alleles will predict the CYP2D6 phenotype with about 95-99% certainty [44]. For example the most common *CYP2D6* variant alleles in Caucasian, Chinese/Japanese and Black African/Afro-American population are *CYP2D6**4, *10 and *17 respectively.

2.5.6 Clinical significance

Although CYP2D6 is only a relatively minor form in a human liver (2% of total cytochromeP450 isoform), it metabolizes up to one quarter of all prescribed drugs, Drugs metabolized by CYP2D6 are targeted to the central nervous system. Brosen and Grams suggest [45] that clinical significance of polymorphism can be evaluated by asking the following questions; Does the kinetics of an active principle of a drug depend significantly on a specific enzyme? Does the resulting pharmacokinetic variability have any clinical importance? Can the variation in response be assessed by direct clinical or paraclinical measurement? On the basis of these criteria, it provided the significance exists for those drugs for which plasma concentration measurement are considered useful and for which the elimination of the drug and/or its active metabolite is mainly determined by CYP2D6 enzyme.

The PM trait is characterized clinically by an impressive deficiency in forming the relevant metabolite(s) of affected substrate, which can result in either

drug toxicity or inefficacy. The reverse is in case of UM. The polymorphism of CYP2D6 is clinically more significant for tricyclic antidepressants, certain neuroleptics, antiarrhythmics, antihypertensives, β-blocker and morphine derivatives [31]. For tricyclic antidepressants, both the PM and UM phenotypes of CYP2D6 is at risk of adverse reactions. PM individuals given standard doses of these drugs will develop toxic plasma concentrations, potentially leading to unpleasant side effects including dry mouth, hypotension, sedation and tremor or in some cases life threatening cardiotoxicity [21]. Administration of CYP2D6 substrates to UM individual may result in therapeutic failure because plasma concentrations of active drug at standard doses will be far too low [46]. The clinical presentation of UM and PM patients are at time similar, leading to confusion in understanding the basis of adverse drug reaction. Because of lack of dose individualization, patients requiring treatment with antidepressant or antipsychotic substrates of CYP2D6 may begin the normal treatment regimen. Because of the long half-life of these drugs, toxic drug concentrations may take 5-7 weeks to develop. Therefore, it is suggested that the patients should be phenotyped before starting the treatment with drugs which are metabolized mainly by CYP2D6 enzyme [21]. A recent US study showed that in patients prescribed with psychiatric drugs that are CYP2D6 substrate, adverse reaction were observed in every patient with inherited mutations inactivating the *CYP2D6* gene.

Chapter 3

RESEARCH METHODOLOGY

3.1 Chemicals and Instruments

Most Chemicals used in this study were of molecular biological grade. Solvents used in metabolite analysis were HPLC grade. All are commercially available as listed: The chemicals used in DNA preparation; NaCl, KCl, Na_2HPO_4, KH_2PO_4, NH_4Cl, $KHCO_3$, Phenol, 8-hydroxyquinoline, Cholofrom, Isoamyl alcohol, Absolute ethanol, were purchased from Merck®, USA. $Na_2EDTA{\cdot}H_2O$, EDTA wes from Biobasic®, USA. Trizma® base and Sodium Decadocyl Sulfate (SDS) were from Sigma chemical Co. Lymphoprep™ was from Pacific Science, USA, ProteinaseK® from Amercham, USA, Seakem® LE Agarose from FMC Bioproduct. The chemicals used in microsomal preparation are NADPH regenerating system from BD Gentest™. Glycerol, Dextromethophan, Dextrophan, Bovine Serum Albumin (BSA), Folin& Ciocalten's phenol reagent, Na_2CO_3, sodium citrate, $CuSO_4$, and NaOH were purchased from Sigma Chemical CO. For PCR; Quigen® Taq polymerase and buffer, Immulase® hot start Taq polymerase and buffer, Elongase® mix enzyme and buffer, and dNTPs were from Perkin Elmer®. Generuler™ 100 bp DNA ladder and 1 kb DNA extension ladder from Gibco BRL were used as DNA markers and ethidium bromide was from Pacific Science, USA. PCR primers were ordered from Bio Service Unit (BSU), Thailand. Restriction enzymes (B*sm*AI, *Hha* I, *Bst*EII, *Bsa*AI, *Bst*NI, *Hph*I, *Msp*I) were from Biolabs Inc., USA. HPLC solvents; acetonitrile, methanol, triethanolamine, perchloric acid were purchased from LabScan Co., Thailand.

The instruments used in this study were; refrigerated water bath (Jalabo F-1250), fume hood (ASTEC sensair), centrifuge (Hermle ZK380), microcentrifuge

(Biofuge Pico kendro) , spectrophotometer (Genesys 10 UV and Shimadzu UV-160A), horizontal electrophoresis set (GelMate 2000m TOYOBO), vortex mixer (vortex-gene 2 ™), homogenizer, ultracentrifuge (BECKMAN L-80), PCR system (Touchgene™ Gradient and GeneAmp PCR system 2400, Perkin Elmer™ and PTC-100™, MJ Researcher), gel documentation system (Sysgene, GeneGenuis and Gene Tool Match), HPLC autoinjection system (Shimadzu, Japan).

3.2 Subjects and Specimens

Subjects in this study were recruited from patients who underwent hepatectomy according to the diagnosis of liver tumor in Siriraj Hospital. Patients who were continually medicated with enzyme inducers (e.g., cabamazepine, phenobarbital) or enzyme inhibitors (e.g., quinidine, fluoxetine) until the day of operation were excluded except for whom discontinued within one week before that. Sixty of EDTA blood tubes and normal liver tissue were evaluated in this study. 10 ml of blood were drawn from venous vessel and kept in screwed-cap tube containing 200 µl of 20% EDTA for the DNA preparation. About 2-3 grams of normal liver tissue were incised from resected liver pieces and immediately transferred to the laboratory room using the portable cooler. The tissue were constantly frozen under liquid nitrogen and stored at -80 °C for the microsomal preparation. Tissue resections were done using the standard procedures by sophisticated surgeons. All subjects were given consent and research protocol approved by Ethical Review Board of Siriraj Hospital.

3.3 DNA Preparation

EDTA blood were centrifuged at 3,000 rpm for 5 minutes for precipitating lymphocyte. Discarded the supernatant (plasma) using plasture pipette then washed the whole cell twice with phosphate-buffered saline (PBS) and discarded PBS. 3-5 volumes of lysis buffer were added and incubated at room temperature for 10 minutes then centrifuged and discarded RBC lysate. Washed WBC with PBS twice and stored WBC for DNA preparation at -20 °C.

3.3.1 DNA isolation from WBC

3.3.1.1 Added 4 ml of TE20-5 (20 mM Tris-HCl pH 7.5 and 5 mM EDTA) and resuspended WBC pellet by vigorous shaking until all clumps disappeared then added 200 µl of 10% SDS and 200 µl of Proteinase K. Incubated mixture at 37 °C overnight.

3.3.1.2 Added ½ volumes of phenol and ½ volumes of chloroform-isoamyl alcohol (24:1) to the mixture, and mixed gently but thoroughly. Centrifuged at 2,500 rpm for 10 minutes.

3.3.1.3 Inserted the plasture pipette into the bottom layer and suctioned out organic phase. Repeated step 3.3.1.2 and 3.3.1.3 twice.

3.3.1.4 One volume of chloroform-isoamyl alcohol (24:1) was added then centrifuged, suctioned out the organic phase and repeated this step twice.

3.3.1.5 Added 1/10 volume of 4 M NaCl and 2 volumes of chilled absolute alcohol for precipitating DNA out of aqueous phase. Collected DNA by centrifuged at 3,000 rpm for 10 minutes.

3.3.1.6 Decanted solution and washed DNA once with 5 ml of 70% ethanol then centrifuged at 3,000 rpm, 10 minutes, decanted, and left the tubes open at room temperature.

3.3.1.7 Dissolved DNA in 0.5-1.0 ml of distilled water.

3.3.1.8 Measured OD at 260 and 280 nm.

3.3.1.9 Calculated OD 260/280 ratio to observe purity and estimated concentration of DNA following this formula.

DNA concentration in µg/ml or ng/µl = OD260 × 50 × dilution factor

3.4 Analysis of CYP2D6 Polymorphism

CYP2D6 gene is highly polymorphic. The novel alleles and known alleles have been widely reported – more than 70 alleles have been characterized so far. The distribution of genotypic frequency is interethnic difference. It is impractical to detect all discovered alleles in Thai population since some alleles may not be found. Thus, the alleles that were found more frequent in Caucasian and Asian population were listed and selected for the test in Thai subjects, presented in Table 3.1.

CYP2D6 polymorphisms were analyzed by the amplification of the *CYP2D6* gene, 4,681 basepair long, containing all 9 exons using long PCR method. The subsequent nested PCRs were performed for the specific amplification of sequences that contain SNPs and gene deletions. PCR-RFLP and PCR-ASA techniques were used to detect polymorphisms which are summarized in Table 3.1, designated name and sequence of primers used in the experiment are shown in Table 3.2.

Table 3.1 Primers, restriction enzymes and diagnostic fragments pattern for polymorphism detections.

mutant alleles	detected mutations	primers	restriction enzymes	Diagnostic fragments pattern		reference
				wild type	mutant	
*CYP2D6**2	$G_{1749} \rightarrow C$	D3, D4	BsmAI	311/56	209/156/102	[3]
	$C_{2933} \rightarrow T$	G1, G2	Hha I	386	260/126	[3],[4]
	$G_{4268} \rightarrow C$	H1, H2	BstE II	866	246/640	[5]
*CYP2D6**3	A_{2637} deletion	F1, F2	BsaAI	201	180/20	[3]
*CYP2D6**4D	$G_{1934} \rightarrow A$ and all form *10B	E1, D3	BstNI	190/163	353	[3]
*CYP2D6**5	gene deletion	I1,I2,I3,I4		5.1 kb	3.2 kb	[47]
*CYP2D6**10A	$C_{188} \rightarrow T$	D1,D2	HphI	362/71	262/100/71	[3]
	$G_{1749} \rightarrow C$	D3, D4	BsmAI	311/156	209/156/102	[3]
	$G_{4268} \rightarrow C$	H1, H2	BstE II	866	246/640	[5]

*CYP2D6**10B	C_{1127}→ T and all from *10A	B1, B2 C1, C2				[4]
*CYP2D6**14	G_{1846}→ T/A	E1, D3	MspI	278/75	353	[3]
*CYP2D6**1	wild type					

Table 3.2 Names and sequence of primers used in this study [3, 4, 5, 47].

Name	Sequence	Position
A1	5′-GGC CTA CCC TGG GTA AGG GCC TGG AGC AGG A-3′	-180 – -150
A2	5′-CTC AGC CTC AAC GTA CCC CTG TCT CAA ATG CG-3′	+123 – +92
B1	5′-CCA TTT GGT AGT GAG GCA GGT AT-3′	68 – 90
B2	5′-CCC CAC TCG CTG GCC TGT TTC A-3′	1261 – 1237
C1	5′-TCA CCC AGA TCC TGG GTT TC-3′	1105 – 1127
C2	5′-TCA CCC AGA TCC TGG GTT TT-3′	1105 – 1127
D1	5′-TCA ACA CAG CAG GTT CA-3′	-82 – -66
D2	5′-CTG TGG TTT CAC CCA CC-3′	351 – 335
D3	5′-GAG ACT CCT CGG TCT CTC G-3′	2122 –2104
D4	5′-TAA TGC CTT CAT GGC CAC GCG-3′	1651 – 1671
E1	5′-CCT GGG CAA GAA GTC GCT GGA CCAG-3′	1770 – 1794
F1	5′-GCT GGG GCC TGA GAC TT-3′	2457 – 2473
F2	5′-GGC TGG GTC CCA GGT CAT AC-3′	2657 – 2638
G1	5′-AGG CCT TCC TGG CAG AGA TGA AG-3′	2680 – 2702
G2	5′-CCC CTG CAC TGT TTC CCA GA-3′	3066 – 3047
H1	5′-GAG ACA AAC CAG GAC CTG CCA-3′	3632 – 3652
H2	5′-GCC TCA ACG TAC CCC TGT CTC-3′	+118 – +98
I1	5′-GTT ATC CCA GAA GGC TTT GCA GGC TTC A-3′	-259 – -232
I2	5′-GCC GAC TGA GCC CTG GGA GGT AGG TA-3′	4844 – 4819
I3	5′-CAG GCA TGA GCT AAG GCA CCC AGA C-3′	7846 – 7822
I4	5′-CAC ACC GGG CAC CTG TAC TCC TCA-3′	43 – 66

The conditions of PCR for each reaction were applied from the PCR method of Merce Gracia-Barcelo et al.[4] as following

3.4.1 Whole gene amplification

The whole gene was amplified using Elongase® enzyme for long PCR, A1 and A2 were used as primers of this reaction.

Mixture of 50 µl whole gene PCR reaction

PCR buffer A	2.5	µl
PCR buffer B	7.5	µl
2 mM dNTPs	5.0	µl
10 pmol/µl primer A1	1.0	µl
10 pmol/µl primer A2	1.0	µl
1 u/µl Elongase (Taq polymerase mix)	1.0	µl
Sterile distilled water (SDW)	27.0	µl
20 ng/µl genomic DNA	5.0	µl
Total	50.0	µl

Thermal cycle condition.

94.0°C	2 min	
94.0°C	30 sec	30 cycles
60.0°C	30 sec	
68.0°C	5 min	
68.0°C	7 min	
4.0°C	.....	

PCR products were checked by 1% agarose gel electrophoresis for 1 hour, then stained with ethiduim bromide, destained in water and checked the product under the UV light. If the PCR was successful, the products were diluted with distilled water to 1:10^4 dilutions and stored at 4 °C for nested PCR amplification.

3.4.2 The subsequent nested PCR amplification

The subsequence nested PCR were performed to amplify the sequence of following polymorphisms; $C_{188}\rightarrow$ T, $G_{1749}\rightarrow$ C, $G_{1846}\rightarrow$ T/A, $G_{1934}\rightarrow$ A, A_{2637} deletion, $C_{2938}\rightarrow$ T, $C_{1127}\rightarrow$ T and $G_{4268}\rightarrow$ C. The PCR conditions of these polymorphisms were divided into 3 groups according to their similarities of thermal cycle and reaction mixtures.

3.4.2.1 Group I; $C_{188}\rightarrow$ T, $G_{1749}\rightarrow$ C, $G_{1846}\rightarrow$ T/A, $G_{1934}\rightarrow$ A, A_{2637} deletion and $C_{2938}\rightarrow$ T, were amplified by the following condition.

Mixture of 25 µl PCR reaction

10x PCR buffer	2.5	µl
2 mM dNTPs	2.5	µl
*10 pmol/µl forward primer	1.0	µl
*10 pmol/µl reverse primer	1.0	µl
5U/µl Taq (Qiagen)	0.125	µl
Sterile distilled water	16.875	µl
Primary PCR product	1.0	µl
Total	25.0	µl

* Each reaction used different primers depended on which reaction was performed.

Thermal cycle condition.**

94.0°C	5 min	
94.0°C	30 sec	30 cycles
60.0°C	30 sec	
72.0°C	30 sec	
72.0°C	7 min	
4.0°C	.....	

**for $G_{1934}\rightarrow$ A and $C_{188}\rightarrow$ T used annealing temp. = 62 °C.

3.4.2.2 Group II; $C_{1127}\rightarrow$ T, PCR-ASA was used to detect this polymorphism. First, nested PCR of $C_{1127}\rightarrow$ T was amplified by the following condition then the PCR-ASA was further carried out.

Mixture of 25 µl PCR reaction

10x PCR buffer	2.5	µl
25mM $MgCl_2$ (total = 2.5)	1.0	µl
6x Q solution	5.0	µl
2mM dNTPs	2.5	µl
10pmol/µl B_1	1.0	µl
10pmol/µl B_2	1.0	µl
5U/µl Taq (Qiagen)	0.125	µl
Primary PCR product	1.0	µl
Sterile distilled water	10.825	µl
Total	25.0	µl

Thermal cycle condition.

94.0°C	5 min	
94.0°C	30 sec	} 30
62.0°C	30 sec	
72.0°C	1.30 min	
72.0°C	7 min	
4.0°C	.....	

3.4.2.3 Group III , $G_{4268}\rightarrow$ C, was amplified by the following condition

Mixture of 25 µl PCR reaction

10 x PCR buffer	2.5	µl
2 mM dNTP	2.5	µl
10 pmol/µl H1	1.0	µl
10 pmol/µl H2	1.0	µl
5U/µl Taq (Imulase)	0.125	µl
Sterile distilled water	16.875	µl
Primary PCR product	1.0	µl
Total	25.0	µl

Thermal cycle condition.

94.0°C	5 min	
94.0°C	30 sec	} 30 cycles
65.0°C	30 sec	
72.0°C	45 sec	
72.0°C	7 min	
4.0°C	.....	

The nested PCR products from group I and group III ware analyzed by 2% agarose gel electrophoresis. If the PCR was successful, the products were further digested with the respective restriction endonuclease using 10 µl of nested PCR product. The enzymes and restriction fragment lengths for all tests are given in Table 3.1. The conditions of incubation for each restriction enzyme are shown in Table 3.3. After 16 hours of incubation, the restriction products were detected by 2% agarose gel electrophoresis for 45 minutes.

Table 3.3 the conditions of restriction enzyme incubation

Position	Enzyme	BSA	Temp	Buffer	Cutting site	Inactivate
188	HphI	-	37 °C	4	GGTGA(N_8):	65°C, 20 min
1749	BsmAI	-	55 °C	2/3	GTCTC (N_1):	80°C, 20 min
1846	MspI	-	37 °C	2	5'…..C'CGG…3'	65°C, 20 min
1934	BstNI	+	60 °C	2/3	CC:(A/T)GG	-
2637	BsaAI	-	37 °C	3	5'...PyAC'GTP	80°C, 20 min
2938	HhaI	+	37 °C	2/3/4	GCG:C	65°C, 20 min
4268	BstEII	-	60 °C	3	G:GTNACC	-

3.4.3 Detection of whole gene deletion (*CYP2D6**5)

For detection of *CYP2D6**5, a 50-µl multiplex long PCR reaction was performed by the following condition [47].

Mixture of 50 µl multiplex long PCR reaction

PCR buffer A	2.5	µl
PCR buffer B	7.5	µl
2 mM dNTPs	5.0	µl
10 pmol/µl primer I_1	1.0	µl
10 pmol/µl primer I_2	1.0	µl
10 pmol/µl primer I_3	1.0	µl
10 pmol/µl primer I_4	1.0	µl
1 u/µl Elongase (Taq polymerase mixture)	1.0	µl
sterile distilled water (SDW)	25.0	µl
20 ng/µl genomic DNA	5.0	µl
total	50.0	µl

Thermal cycle condition.

94.0°C	2 min	
94.0°C	30 sec	30cycles
60.0°C	30 sec	
68.0°C	5 min	
68.0°C	7 min	
4.0°C	.....	

The PCR products were then analyzed directly by 1% agarose gel electrophoresis for 1 hour. In this multiplex long PCR, the 5.1 kb product was interpreted as wild type and the 3.2 kb indicated the deletion of *CYP2D6*.

3.4.4 Detection of C_{1127}→ T (*CYP2D6**10B)

The mutation of C_{1127}→ T was checked by amplifying 1 µl of nested PCR product from group II with allele-specific primer C1 and C2 and the reverse primer B2. Using The PCR condition was as following [4].

Mixture of $C_{1127} \rightarrow T$ 25 µl nested PCR reaction

10 x PCR buffer	2.5	µl
2 mM dNTP	2.5	µl
50 mM $MgCl_2$	0.5	µl
10pmol/µl C1 or C2	1.0	µl
10pmol/µl B2	1.0	µl
10pmol/µl D1	1.0	µl
10pmol/µl D2	1.0	µl
5U/µl Taq (Imulase)	0.125	µl
Sterile distilled water	14.375	µl
Nested PCR product	1.0	µl
Total	25.0	µl

Thermal cycle condition		
94.0°C	10 min	
94.0°C	30 sec	30 cycles
62.0°C	30 sec	
72.0°C	30 sec	
72.0°C	7 min	
4.0°C	.....	

The PCR products were then analyzed directly by 2% agarose gel electrophoresis for 30 minutes. In this allele specific amplification, allele specific wild type primer C1 and mutant primer C2 were used as indicator for the band of wild type and mutant, respectively. D1 and D2 primer were used for the internal standard along with ASA primers.

3.5 Human Microsomal Preparation

Liver microsome preparation was prepared followed the method described previously by Christ Von Bahr et al. [48] but some procedures were applied differently for the suitable method and material availability.

1. Firstly, frozen liver tissue were weighed then thawed and washed in 10 mM Tris-HCl pH 7.5 containing 10 mM EDTA and 100 mM NaCl chilled solution.
2. Tissues were minced thoroughly by a scissor and 15 ml of chilled buffer in step 1 were added then homogenized using Teflon-glass homogenizer for approximately 15 strokes. The homogenizer glass was submersed in a small bucket of ice during all homogenization.

3. The upper lipid layer and cytosolic supernatant were removed. The pellets were harvested and resuspended in 10 mM Tris-HCl pH 7.4 containing 1.0 mM EDTA and 20% glycerol v/v. Some of microsomal suspension were kept under -80 °C for further determination of enzyme activity and other part were used for protein content measurement.

3.6 Determination of Protein Content

Protein measurement was followed the method described previously by Oliver H. Lowry [49]. First, labeled tubes in duplicate for 7 concentration-standard tubes and sample tubes (see table). The reagents were added in micro liter to each standard and sample with specific amount of solution represented in table.

Standard tube	1	2	3	4	5	6	7	sample
1 mg/ml BSA	0	50	100	150	200	250	300	490
0.5 M NaOH	500	450	400	350	300	250	200	10 (microsome)

1. Each tube was mixed thoroughly after adding 6.5 ml of working and left in room temperature for 10 minutes.

2. 200 µl of Folin & Ciocalteu's phenol reagent was added to each tube, the tubes were vortexed thoroughly for a minimum of 30 sec.

3. After the tubes were allowed to stand at room temperature for a minimum of 30 minutes, the absorbances of the solution were measured by spectrophotometer against standard tubes number 1 at 500 nm.

Calculation of protein content

The average absorbance of each standard was plotted against its amount of protein. The best fit regression line was drawn through the points. The amount of protein in each unknown sample was obtained by comparing its absorbance against the standard curve. The protein concentration (mg/ml or μg/μl) in each unknown sample was obtained by dividing its amount of protein (from the first step) with the volume of microsomal used (i.e., 10 μl) in the reaction.

3.7 CYP2D6 Enzyme Activity Assay

Reagents used in the enzyme activity reaction.

solution I	1 mM dextromethorphan in 0.1 M potassium phosphate, pH 7.4
solution II	20 mg/ml glucose-6-phosphate, 20 mg/ml NADP, 13.3 mg/ml $MgCl_2$-H2O
solution III	40U/ml glucose-6-phosphate dehydrogenase in 5 mM Na citrate (tribasic)
solution IV	0.1 M potassium phosphate pH 7.4
solution V	70 % perchoric acid

Assay condition (250 μl final volume)

25 μl of solution I (final concentration 100 μM, a saturating concentration)
12.5 μl of solution II
2.5 μl of solution III
XX μl of microsome
210 – XX μl of solution IV

The assay condition was followed the method suggested by the kit.

1. Solution I, II, III and IV were mixed together and were preincubated at 37 °C for 5 minute before the addition of microsome.

2. Started the reaction by adding microsome and the incubation was carried out at 37 °C for 20 minutes then terminated reaction by adding 15 μl of solution V.

3. The mixtures were centrifuged by 10,000 g for 5 minutes and the aliquot of supernatant were used for the determination of dextrorphan (Dextromethorphan metabolite) by HPLC method.

3.8 Assay of Dextrorphan

The supernatant from enzyme activity assay was determined for the concentration of dextrorphan which is a metabolite of dextromethorphan O-demethylation by CYP2D6. Reverse-phase HPLC was used to detect metabolite. Aliquots of supernatant 50 µl were injected directly, by means of automated system (Shmadzhu, Japan), to Alltech® C18 column (4.6mm×250mm) with guard column. The mobile phase which consisted of 30% acetonitrile, 1 mM perchloric acid and 200 µl/L triethanolamine were delivered at a flow rate of 1.5 ml/minute. The elution of metabolites was monitored with a fluorescence detector at an excitation/emission wavelength pair of 270/312 nm. The peaks were analyzed by an LC-10 system of auto injection HPLC (Shimadzhu, Japan).

HPLC method validation were carried out, six concentrations in the range of 0.1-2.0 µM were used to construct a calibration curve. A calibration curve was generated by plotting the ratio of the peak area of the analyte against theoretical concentrations. Three quality control samples were prepared. The concentration was equal to 0.15, 0.85 and 1.8 µM for the low quality control (LoQC), the medium quality control (MeQC) and the high quality control (HiQC), respectively. The intra- and inter-day accuracy and precision of the method were performed using five and ten replicates of each quality control, respectively.

3.9 Data analysis

The frequency of a determined *CYP2D6*X allele in a sample of N individuals was estimated by $(2n_{x/x} + n_{x/-})/2N$ where $n_{x/x}$ is a number of individuals homozygous for X, and $n_{x/-}$ is a number of individuals heterozygous for the X allele [4]. The allele frequency was represented in percent of frequency.

CYP2D6 activity was expressed in nanomoles of dextrorphan per mg protein of microsome per hour (nmol/mg protein/hr). Enzyme activities were plotted in frequency histogram and normal distribution plot for the estimation of cut off points. A one-way ANOVA was used to determine the coefficient of variation based on enzyme activity values for each genotype.

Chapter 4

RESULTS

4.1 Characterization of *CYP2D6* Genetic Polymorphism in Thais

A total of 8 types of CYP2D6 genetic polymorphism were analyzed, they were; *CYP2D6**1, *CYP2D6**2, *CYP2D6**3, *CYP2D6**4, *CYP2D6**5, *CYP2D6**10A, *CYP2D6**10B, and *CYP2D6**14. Sixty subjects were recruited for this study, 37 were men and 23 were women and their ages ranged between 40 to 78 years.

PCR-RFLP and PCR-ASA techniques were used to detect these polymorphisms and the result of RFLP and ASA band patterns were presented in Fig. 4.1 – 4.6. Allelic variants detected from the subjects were classified into genotype groups followed the report of Sachse et al. [3]. Allele frequencies were calculated and the outcome was slightly different from those found in Asians (see Table 4.1). $C_{2938}\rightarrow T$ was found at the highest frequency in Thai (83.83%). It was surprisingly found that all subjects had a nucleotide change in this position. The less commonly found variants were at position $G_{1749}\rightarrow C$ followed by $G_{4268}\rightarrow C$ with a frequency of 74.17% and 72.25%, respectively. $C_{188}\rightarrow T$ and $C_{1127}\rightarrow T$, the characteristic of *CYP2D6**10A and *CYP2D6**10B were detected at 70.83% and 45.83%, respectively which were concordant with those found in other Asians. *CYP2D6**3 (2637del) and *CYP2D6**14 ($G_{1846}\rightarrow A/T$) were not detected in these subjects. Whole gene deletion or *CYP2D6**5 was found in 7.50 % and 2 subjects surprisingly showed homozygous mutant (Fig 4.5) causing the remarkably low enzyme activity. $G_{1934}\rightarrow A$ which was represented of *CYP2D6**4 was detected in only 2.50% of Thais whereas this had a higher frequency in Caucasians (~20%). One subject had an unclassifiable genotype group. This genotype was shown to be homozygous mutant at $G_{1749}\rightarrow C$ and $C_{2938}\rightarrow T$ and heterozygous at $C_{1127}\rightarrow T$

and this caused a low enzyme activity. These variants would be classified as *CYP2D6**2 but lacking G_{4268}→C and had an additional change at C_{1127}→T. Therefore, further investigation is needed to verify a novel allele. Frequencies of point mutations and gene deletion are shown in Table 4.1. The summery of genotype pattern and allele frequency are presented in Table 4.2 and Table 4.3, respectively.

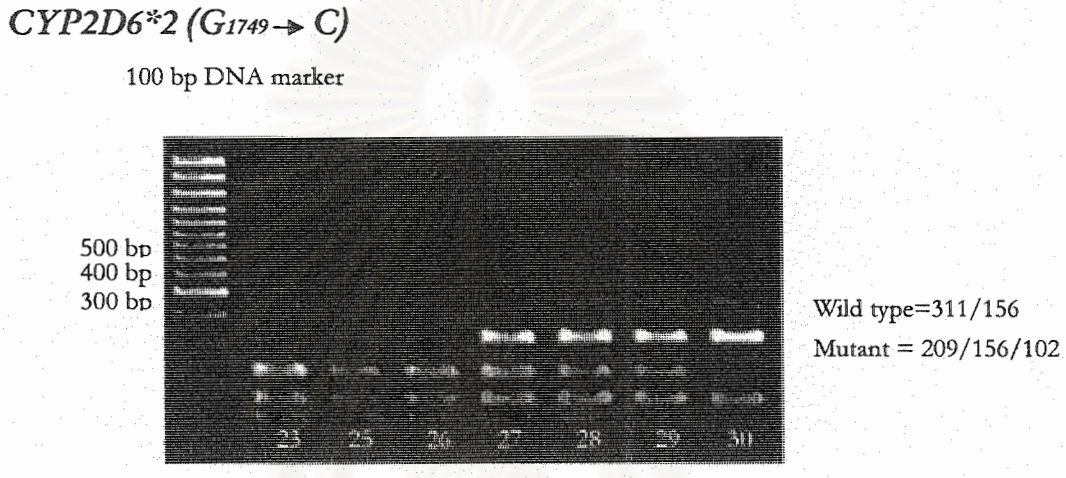


Figure 4.1 G_{1749}→C restriction band labeled 23, 25, and 26 are mutant with 209/156/102 fragments when cut with *Bsm*AI. Number 30 is wild type with non 209 and 102 fragments and number 27-29 are heterozygous band.

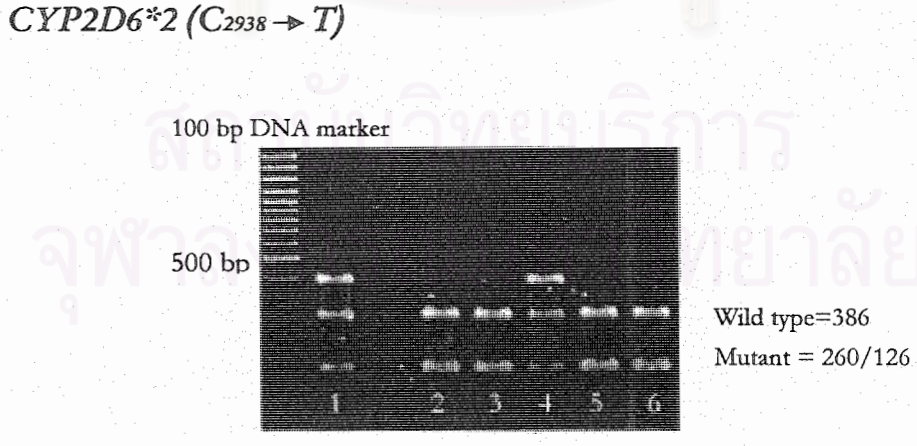


Figure 4.2 C_{2938}→T restriction band number 2, 3, 5 and 6 are mutant fragments when cut with *Hba*I. Number 1 and 4 are heterozygous bands.

*CYP2D6*2 ($G_{4268} \rightarrow C$)*

Figure 4.3 $G_{4268} \rightarrow C$ restriction band number 4, 5 and 8 are mutant fragments when cut with *Bst*EII. Number 1 and 7 are heterozygous band and number 6 is uncut representing wild type.

*CYP2D6 *4 ($G_{1934} \rightarrow A$) and CYP2D6*3 (A_{2637} deletion)*

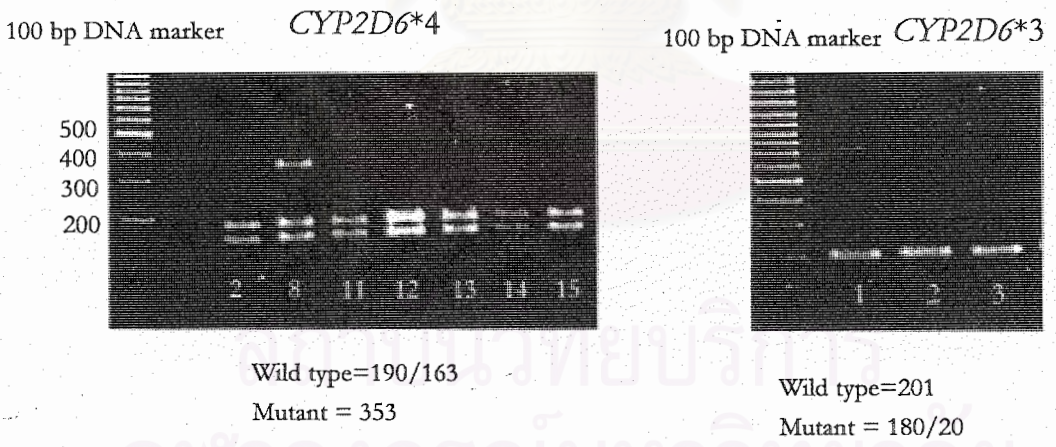
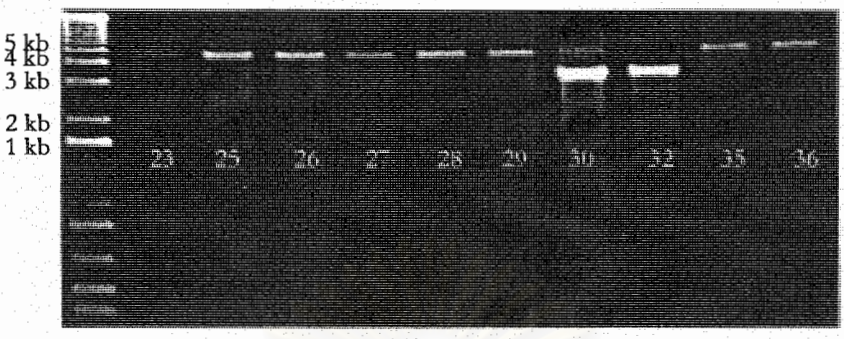


Figure 4.4 *CYP2D6*4* analysis (left panel) representing only one heterozygous (8) and wild type restriction band when cut with *Bst*NI. On the other hand, *CYP2D6*3* analysis (right panel) representing only wild type band as reported in most of Asian population.

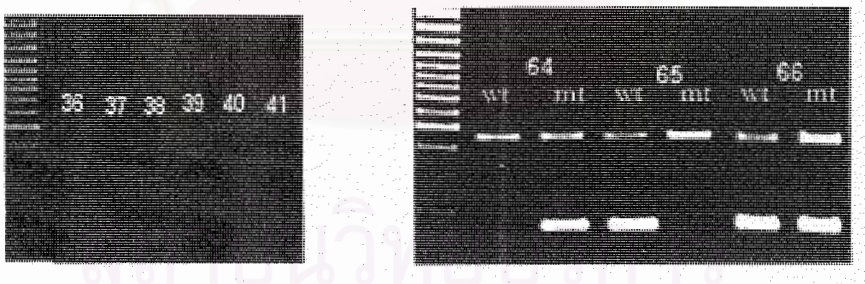
*CYP2D6*5 (Whole gene deletion)*



1 kb DNA marker

Figure 4.5 Band labeled 23-29 and 35, 36 are wild type. Number 32 is homozygous of *CYP2D6**5 indicated for *CYP2D6* whole gene deletion from multiplex long PCR technique and number 30 is heterozygous band of *CYP2D6**5.

*CYP2D6*10A (C188 → T)* and *CYP2D6*10B (C1127 → T)*



Wild type = 362/71

Mutant = 262/100/71

ASA pattern of C1127T

Figure 4.6 *CYP2D6**10A analysis (left panel) shows homozygous mutant fragment (36-38), heterozygous (39-40) and wild type (41) when cut with *Hph*I. An analysis of *CYP2D6**10B (right panel) using ASA technique, the upper bands is an internal standard, the lower band representing mutant band (64), heterozygous band (66) and wild type band (65).

Table 4.1 Frequency of point mutations and gene deletion in Thai subjects

mutation	number of homozygous	number of heterozygous	number of wild type	total alleles	allele frequencies	percent allele frequencies
C188T	33	19	6	120	0.7083	70.83
G1749C	35	19	4	120	0.7417	74.17
G1846T/A	0	0	58	120	0.0000	0.00
G1934A	0	3	55	120	0.0250	2.50
A2637del	0	0	58	120	0.000	0.00
C2938T	39	19	0	120	0.8083	80.83
G4268C	34	19	5	120	0.7225	72.25
Gene del	2	5	53	120	0.075	7.50
C1127T	3	49	6	120	0.4583	45.83

Table 4.2 Distribution of CYP2D6 genotype pattern

nucleotide position change	genotype pattern 188	1127	1749	1846	1934	2637	2938	4268	gene	distribution	
	C→T	C→T	G→C	G→T/A	G→A	A del	C→T	G→C	del	n	%
CYP2D6 genotype											
*1/*1[a]	○	○	○	○	○	○	▲	○	○	2	3.33
*1/*1[b]	○	○	○	○	○	○	●	○	○	1	1.67
*1/*2[a]	○	○	▲	○	○	○	●	▲	○	1	1.67
non classifiable	○	▲	●	○	○	○	●	○	○	1	1.67
*1/*4D[b]	▲	▲	▲	○	▲	○	●	▲	○	1	1.67
*1/*5[b]	○	○	○	○	○	○	●	○	▲	1	1.67
*1/*10B[b]	▲	▲	▲	○	○	○	●	▲	○	12	20.00
*1/*10B[b,c]	▲	●	▲	○	○	○	●	▲	○	1	1.67
*2/*10B[a]	▲	●	●	○	○	○	●	●	○	1	1.67
*4D/*10A[b]	●	▲	●	○	▲	○	●	●	○	2	3.33
*5/*5									●	2	3.33
*5/*10B[b]	●	●	●	○	○	○	●	●	▲	4	6.67
*10A/*10B[b]	●	▲	●	○	○	○	●	●	○	15	25.00
*10A/*10B[a]	●	▲	●	○	○	○	▲	●	○	12	20.00
*10B/*10B[b]	●	●	●	○	○	○	●	●	○	3	5.00
*10B/*10B[a]	●	●	●	○	○	○	▲	●	○	1	1.67
	total									60	100

● = homozygous mutant, ▲ = heterozygous mutant, ○ = wild type, a= heterozygous for C2938→T and b= homozygous mutant for C2938→T, c=heterozygous for C1127→T.

Table 4.3 *CYP2D6* allele and genotype frequency in Thais.

Genotype	n	% Frequency	Allele	Number of variants	% Frequency
*1/*1	3	5.00	*1	22	18.64
*1/*2	1	1.67	*2	2	1.69
*1/*4D	1	1.67	*3	0	0.00
*1/*5	1	1.67	*4D	3	2.54
*1/*10B	13	21.67	*5	9	7.63
*2/*10B	1	1.67	*10A	29	24.58
*4D/*10A	2	3.39	*10B	53	44.92
*10A/*10B	27	45.00	*14	0	0
*5/*10B	4	6.67			
*10B/*10B	4	6.67			
*5/*5	2	3.39			
unclassified	1	1.67			

Table 4.4 The comparison of *CYP2D6* allele frequency in different ethnic groups

Nationality	n	*1	*2	*3	*4	*5	*10	*14
Thai	**60**	**18.64**	**1.69**	**0**	**2.54#**	**7.63**	**69.5**	**0**
Hong Kong Chinese [4]	119	22.69	7.98	N/A	N/A	4.62	64.71	N/A
Chinese Taiwanese [5]	124	N/A	N/A	N/A	0.8#	N/A	70	1.2
Mainland Chinese [50]	21	21	26	N/A	N/A	2	48	N/A
Chinese Living in Sweden [51]	113	29.6	13.4	N/A	N/A	5.7	50.7	N/A
Chinese *	179	23	20	1	0	5.7	50	N/A
Japanese 1*	162	N/A	12.9	N/A	N/A	6.2	38.6	N/A
Japanese 2*	206	43	12.3	N/A	N/A	4.5	38.1	N/A
Japanese 3*	98	42.3	9.2	N/A	0.5	6.1	N/A	N/A
Malaysian*	107	36	N/A	N/A	2.8	5.1	49.5	N/A
Ghanaian*	193	43.7	10.9	0	7	0.6	3.1	N/A
Tanzanian*	106	27.8	20.3	0	0.9	6.1	3.8	N/A
German [3]	589	36.4	32.4	2.04	20.7	1.95	1.53	N/A
American [52]	195	35.6	28.5	1	19.5	4.1	2	N/A

N/A = not determined, * = data from [1] and # = *CYP2D6**4D

When compared *CYP2D6* allele frequency of Thai subjects with other ethnic groups, it was found that *CYP2D6**2 in Thai was less frequent (1.69%) than that of other groups. *CYP2D6**10 (69.49%) was the highest frequent allele found in Thais and was in agreement with those found in most Asians especially Chinese. *CYP2D6**4D was detected in 2.54% of subjects whereas it was not found from some studies of Chinese and Japanese [53] . In Caucasian group, *CYP2D6**4 allele was reported at a much higher frequency (~20%) [3]. *CYP2D6**5 or whole gene deletion was detected in Thais slightly higher (7.63%) than those of other Asians, and it was surprising that 2 of 7 *CYP2D6**5, a nonfunctioning mutant, was found in homozygous state which was rare in other reports. Two genotypes were not detected in Thai subjects; *CYP2D6**3 and *CYP2D6**14 which also had low prevalences in other groups. In general, *CYP2D6* allele frequencies of Thais are concordant with Asian population except that the change at position $C_{2938} \rightarrow T$ was constantly found together with allele *CYP2D6**1, *4D and *10 in all Thais. In Chinese, only 2 individuals have $C_{2938} \rightarrow T$ changed with *CYP2D6**10 while in Caucasians, only 32.4% of people have this position changed. The comparison of *CYP2D6* allele frequency was described in Table 4.4.

4.2 CYP2D6 Enzyme Activity

CYP2D6 enzyme activity of all subjects was determined from dextromethorphan O-demethylation reaction. The formation of dextrorphan metabolite was indicative of CYP2D6 enzyme activity. Dextrorphan was a metabolite formed by the oxidation of specific CYP2D6 substrate, dextromethorphan. HPLC technique was used to detect this metabolite and the chromatogram showed dextrorphan peak differently in EM, IM and PM group. The highest peak of dextrorphan is demonstrative of EM, the next peak was IM and PM was the lowest peak as depicted in Fig. 4.7- 4.9. Table 4.5 showed the inter-day and intra-day assay of HPLC validation which were all in acceptable ranges.

In this study, CYP2D6 phenotype were classified into 6 groups according to their previously reported genotype-phenotype correlation [3]; EM/EM (*1/*1, *1/*2), EM/IM (*1/*10B, *2/*10B), EM/PM (*1/*4D, *1/*5), IM/IM (*10A/*10B, *10B/*10B), IM/PM (*4D/*10B, *5/*10B) and PM/PM (*5/*5). Extensive metabolizer (EM) was characterized by a high enzyme activity. In this study, only 4 subjects showed extensive metabolizers, classified by genotype. This group displayed large variations of enzyme activity as showed in a normal distribution plot (Fig. 4.11). Due to large variations in enzyme activity of EM and IM group, enzyme activity of these groups could not possibly be used as a distinguishing value between these two groups. Nevertheless, two representatives from PM/PM group showed extremely low enzyme activity (0.299 and 0.641 nmol/mg protein/hr) which could definitely be defined as PM genotype. Three outliers from box plot (Fig.4.10) and one unclassified genotype were excluded from one way ANOVA test. A normal distribution plot (Fig. 4.11) separated 3 groups in the ambiguous cut off point of enzyme activity for phenotype groups. EM/EM group or normal group showed highest value of enzyme activity significantly different ($p<0.05$) from IM/PM and PM/PM grou p. Descriptive statistic of CYP2D6 enzyme activity in each genotype group were presented in Table 4.6.

Table 4.5 Intra-and inter-day validation of HPLC detection

Nominal concentration (μM)	LoQC	MeQC	HiQC
	0.150	0.850	1.800
Intra-day validation (n)	**5**	**5**	**5**
Measured mean concentration (μM)	0.164	0.810	1.793
SD (μM)	0.007	0.109	0.071
Precision (%)	4.09	13.42	3.97
Accuracy (%)	109.18	95.29	99.64
Inter-day validation (n)	**10**	**10**	**10**
Measured mean concentration (μM)	0.154	0.842	1.789
SD (μM)	0.011	0.088	0.067
Precision (%)	6.85	10.47	3.74
Accuracy (%)	102.85	99.05	99.40

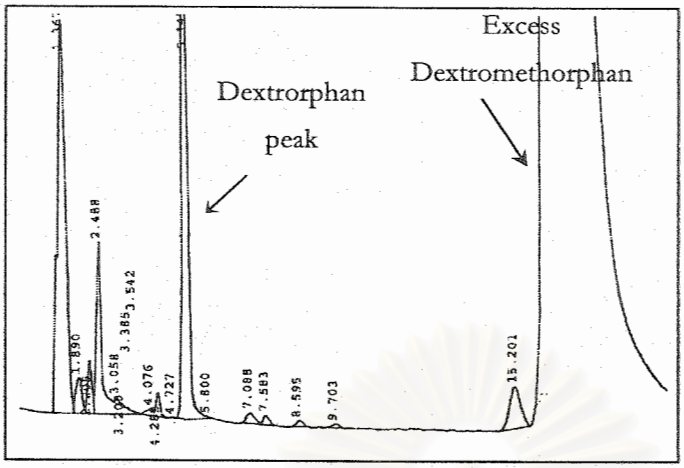


Figure 4.7
Chromatogram of an extensive metabolizer showed highest peak height.

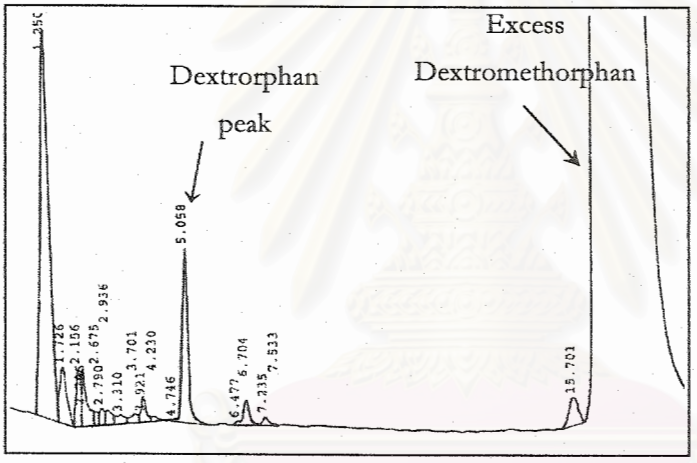


Figure 4.8 Chromatogram of an intermediate metabolizer showed a medium peak height.

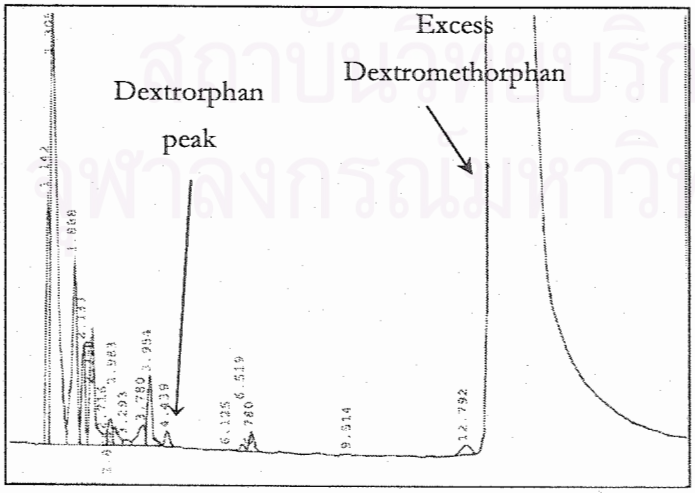


Figure 4.9
Chromatogram of a poor metabolizer showed almost no peak height.

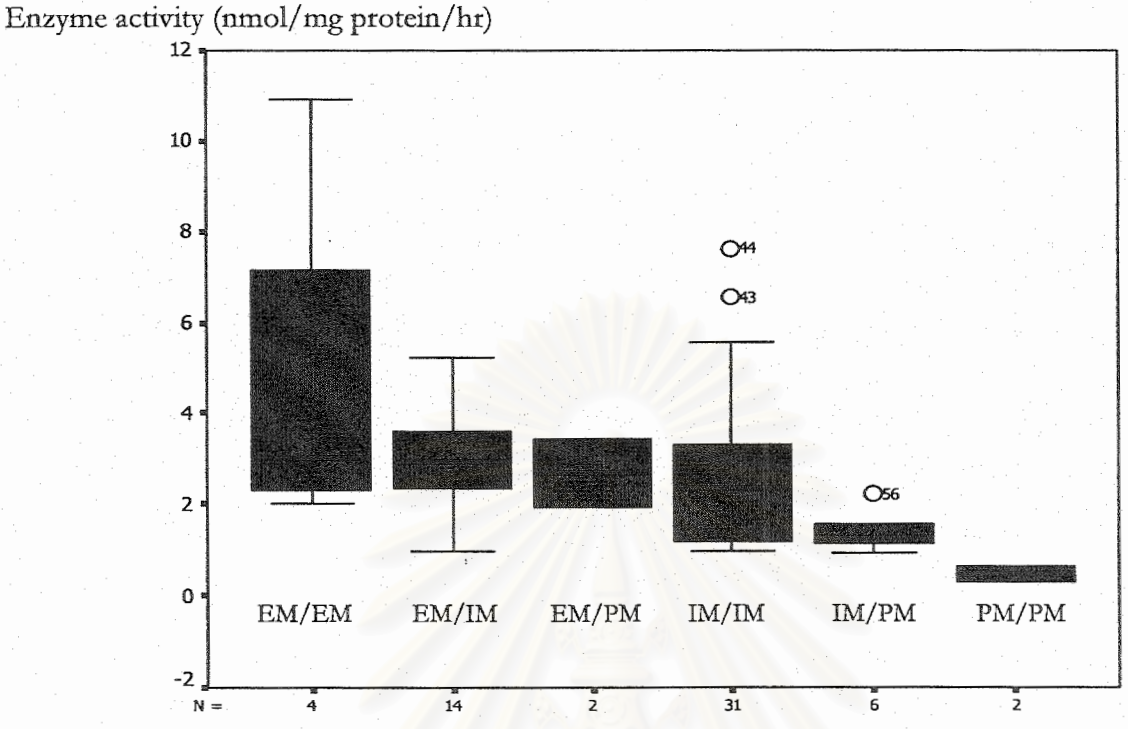


Figure 4.10 Box plot of enzyme activity of each genotype.

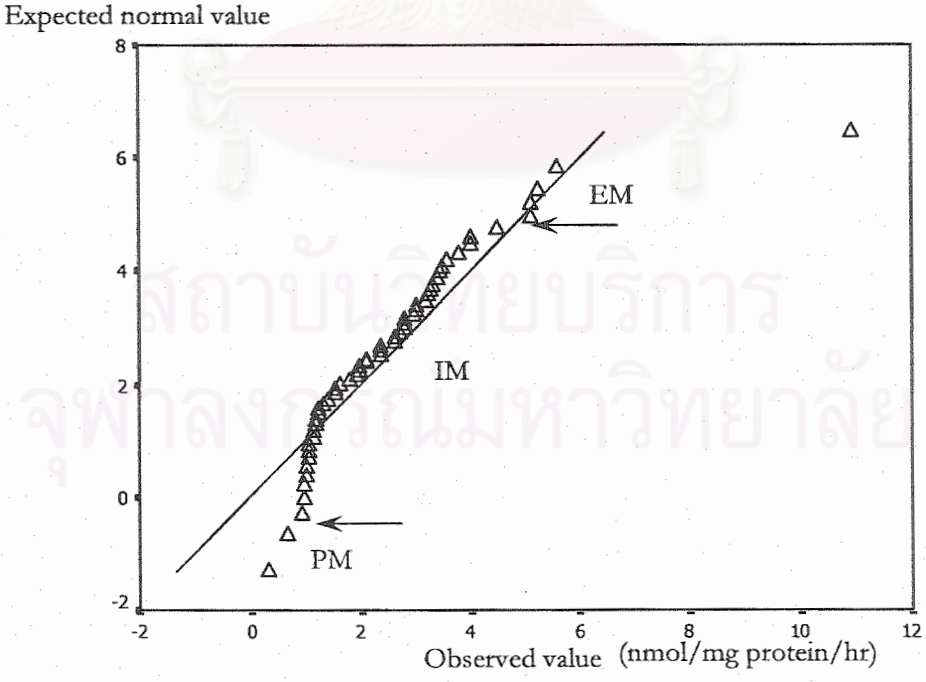


Figure 4.11 Normal P-P plot of enzyme activity

Table 4.6 Descriptive statistic of enzyme activity for each genotype group.

Genotype	N	Mean	Stdev	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower	Upper		
EM/EM *	4	4.737	4.157	2.078	-1.877	11.372	1.999	10.911
EM/IM	14	3.016	1.229	0.329	2.306	3.726	0.955	5.227
EM/PM	2	2.694	1.067	0.755	-6.893	12.28	1.939	3.448
IM/IM	29	2.400	1.322	0.245	1.898	2.903	0.978	5.579
IM/PM	5	1.227	0.222	0.099	0.951	1.003	0.935	1.538
PM/PM	2	0.470	0.241	0.171	-1.703	2.643	0.299	0.641
Total	56	2.568	1.731	0.233	2.100	3.036	0.299	10.911

* = different from group IM/PM and PM/PM at $p < .05$.

Enzyme activity of all subjects was plotted in histogram graph (Fig.4.12) which was indicative of 3 phenotypes; poor metabolizers in left, the middle was intermediate and in right was extensive metabolizers. Most of Thai were intermediate metabolizers which enzyme activities were mainly dispersed in the range of 0.9-7.0 nmol/mg protein/hr. Scatter plot (Fig. 4.13) represented the distribution of enzyme activity in each genotype and also displayed large variations within group. Group *5/*5 showed the lowest enzyme activity which represented poor metabolizer and its value was definitely separated from other groups. Intermediate metabolizer group exhibited values not different from extensive metabolizer group due to large variations of enzyme activity within the group. However, in general, CYP2D6 phenotype was concordant with its genotype.

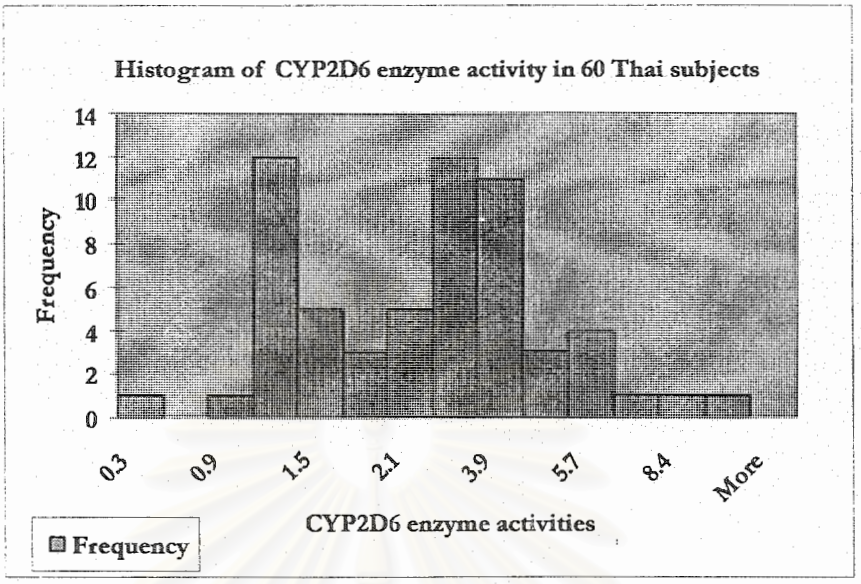


Figure 4.12 Histogram of CYP2D6 enzyme activity in 60 Thai subjects.

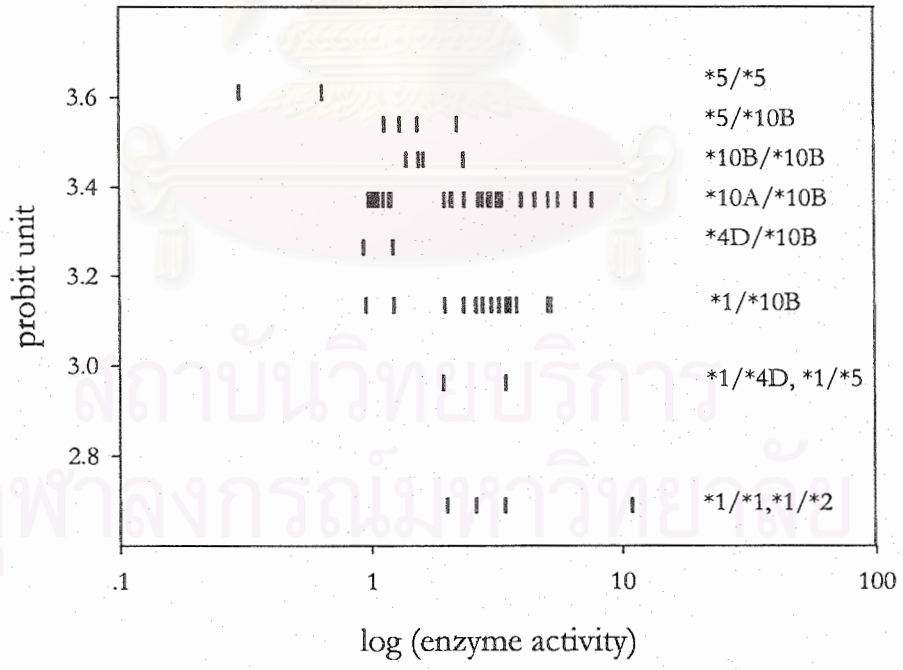


Figure 4.13 Scatter plot of CYP2D6 phenotype versus genotype.

4.3 CYP2D6 enzyme kinetics

Enzyme activities were classified into 3 phenotypes; EM, IM and PM according to the genotype, at least one sample from each phenotype were selected for enzyme kinetic test. The formation of dextrorphan by microsomes from extensive, intermediate and poor metabolizers was shown in Fig. 4.14. One microsome from extensive metabolizers showed high oxidation rate (V_{max} =9.66) and had a lower Michaelis-Menten constant (K_m=2.35) than those of intermediate metabolizer group (K_m =14.66). There were 6 representatives for intermediate metabolizer group showed lower V_{max} (3.24) than those of extensive metabolizer. Poor metabolizer exhibited the lowest oxidation rate as previously reported [54] but its K_m of this study was not higher than IM group due to the limitation in sample size and accuracy of measuring extremely low enzyme activity.

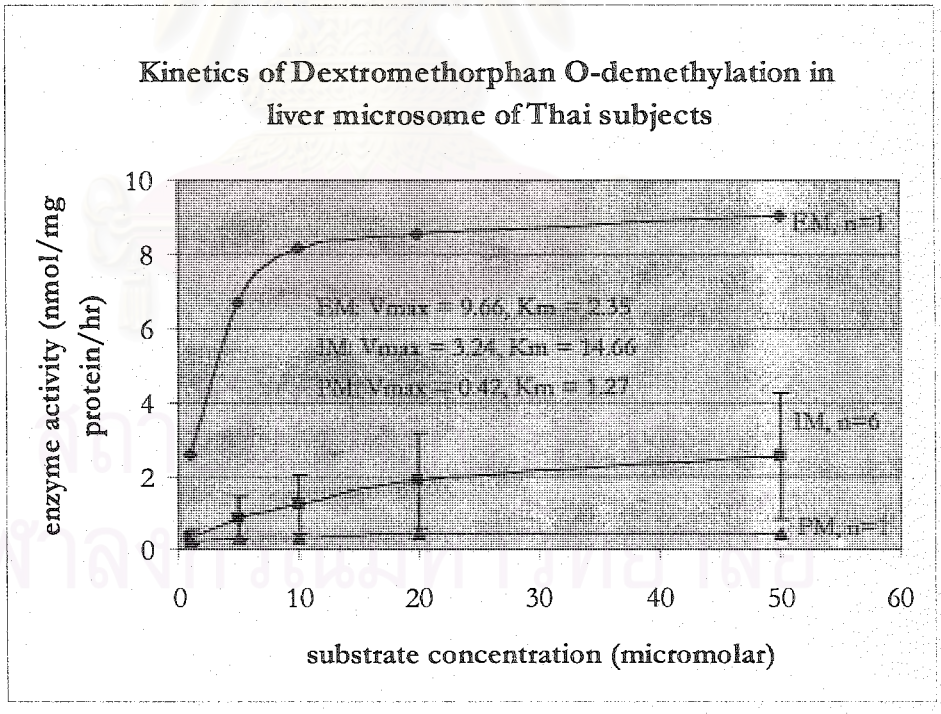


Figure 4.14 Enzyme kinetic of CYP2D6 in 5 substrate concentrations.

Chapter 5

DISCUSSION AND CONCLUSION

The aim of this study was to characterize *CYP2D6* allele and its frequency together with liver enzyme activity in Thai subjects. There were 8 *CYP2D6* alleles investigated and 6 known alleles were detected in different frequencies. *CYP2D6**10 is the highest allele (69.49%) found in Thai subjects as expected and the results are in agreement with most studies conducted in Asians [3, 4, 5] especially Chinese population. For Japanese, this allele was found at a lower frequency, accounted for about 38% (Table 4.4) and only *CYP2D6**10B was detected [53]. It was striking that all Thai subjects had a nucleotide change at position C_{2938}→T which was the characteristic of allele *CYP2D6**2, *4 and *17 only. In this study, C_{2938}→T also presented with *CYP2D6**1, *4D and *10 allele which caused an Arg296→Cys amino acid substitution. The change can be considered of high functional importance as exemplified by its significant role in the *CYP2D6**17 allele [4]. This is the first time that C_{2938}→T has been found with *CYP2D6**10 in population study. This co-existence may signify a unique allele specific to Thais. Further investigation is necessary to unravel this phenomenon.

The frequency of allele *CYP2D6**2 (1.69%) in Thai was somewhat lower than those found in other studies. This could be the consequence of high frequency of *CYP2D6**10 allele which often resulting in a lower frequency of the *CYP2D6**2 variation [54]. *CYP2D6**4 was found at a high frequency in Caucasians but it exhibited less than 1% in Chinese and Japanese [1]. In Thai, *CYP2D6**4D was also detected. This is a subgroup of *CYP2D6**4 allele that have C_{1127}→T change as an additional variant. It was found at a slightly higher frequency in Thais (2.54%) compared with some studies conducted in Chinese and Japanese which found none. The observed low frequency of *CYP2D6**4 in Asians has been related to the low incidence of PM (1-2%) compared to

Caucasians (5-10%). This allele can be found in Caucasians as high as 20%. *CYP2D6**5 was found at a slightly higher frequency (7.63%) than those of other Asians ranging constantly between 4-6%. Two individuals of this study possessed homozygous mutant of gene deletion (*5/*5) which was rarely found from other previous reports of Asians. An unclassifiable genotype also found in one subject which could be a novel allele – further study should be conducted to confirm this finding.

Liver microsome of all subjects was determined for CYP2D6 enzyme activity by detection of dextrorphan metabolite from dextromethorphan O-demethylation reaction. Phenotype can be classified into 3 groups; EM, IM and PM according to their genotypes. Due to the large variations in enzyme activity of EM and IM group, phenotypic cut off points from normal distribution plot were ambiguous. However, enzyme activity of PM/PM group can be used to indicate PM genotype since it was remarkably low. When genotypes were plotted versus phenotype (Fig. 4-13), the value of *5/*5 group was lowest and dispersed separately from other groups but the rest was not different and showed large variations in enzyme activity within group. Only enzyme activity of EM/EM was significantly ($p<0.05$) higher than IM/PM and PM/PM group when excluded 3 outliers from box plot (Fig. 4-10) and other 1 unclassifiable genotype. The number of sample in some phenotype group was too few to be a good representative of that phenotype.

Samples from EM, IM and PM were selected for the enzyme kinetic test. EM presented the highest rate of oxidation (V_{max}) and low apparent K_m constant which resulted from a mixed contribution of two isozymes, showing high and low affinity. On the other hand, IM group showed higher Km that could be the absence of the high affinity component resulted in a shift to a higher apparent Km [55]. PM group exhibited the lowest V_{max} but the K_m was not high as IM group. This could be the limitation of detection when the enzyme activity was extremely low. Enzyme activity of subjects from EM/EM group (n=4) exhibited

large variations within group ranging from 1.999-10.911 nmol/mg protein/hr. This variation could be the effect of other unknown variants which need further investigation.

In conclusion, CYP2D6 genotype results are generally in agreement with the previous reports of Asians, except for these following finding. The exceptional finding of $C_{2938}\rightarrow T$ in all Thai subjects, consequently, causes *CYP2D6**1, *4D and *10 to be different from the proposed allele since they exhibit an additional $C_{2938}\rightarrow T$ change. Two individuals presented homozygous mutant of whole gene deletion which was infrequently found in Asians. One unclassified genotype which possibly belongs to *CYP2D6**2 but lacking $G_{4268}\rightarrow C$ and possessing $C_{1127}\rightarrow T$ could be a novel allele. Further investigation is needed for this allele. *CYP2D6**4D was found in Thai with a higher frequency (2.54%) than other reports from Chinese and Japanese [4, 53] in which it was not detected.

Genotype-phenotype of CYP2D6 was relatively correlated. Extensive metabolizers exhibited higher enzyme activity and large variations within group whereas poor metabolizers showed extremely low activity. Intermediate metabolizers had a wide range of enzyme activity. It would have a low activity when IM combined with PM genotype.

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APPENDICES

Reagent Preparation

Reagent for microsomal preparation

Solution I : 10 mM Tris-HCl pH 7.5 + 10 mM EDTA + 100 mM NaCl
Solution II : 10 mM Tris-HCl pH 7.4 + 1.0 mM EDTA + 20% glycerol v/v.

10 mM Tris-HCl : dissolve 1.211 g of Trizma in 1,000 ml ultrapure water. Adjust the pH with concentrated HCl. Adjust 500 ml of solution for pH 7.5 and other 500 ml for pH 7.4.

10 and 1 mM EDTA: dissolve 1.4612 g of EDTA in 10 mM Tris-HCl pH 7.5, 500 ml and dissolve 0.146 g of EDTA in 10 mM Tris-HCl pH 7.4, 500 ml.

100 mM NaCl: □ dissolve 0.2 g of NaCl in 10 mM Tris-HCl pH 7.5 + 10 mM EDTA

- add 20 % glycerol v/v in solution II before use

Reagent use in CYP2D6 assay

1 mM dextromethorphan in 0.1 M potassium phosphate buffer

0.1 M potassium phosphate buffer dissolve 4.35 g of dibasic K_2HPO_4 in 250 ml ultrapure water and dissolve 3.4 g of monobasic KH2PO4 in 250 ml ultrapure water. Place 125 ml of KH_2PO_4 onto a magnetic stirrer and insert a pH electrode. Add 125 ml K_2HPO_4 slowly to adjust the pH to 7.4 and adjust the final volume to 250ml with water if necessary.

1 mM dextromethorphan dissolve 0.0373 g of dextromethorphan in the final volume of 10 ml potassium phosphate buffer (above solution). Make a 10X dilution by pipette 100 µl of dextromethorphan solution then adjust the final

volume to 1 ml with the 0.1 M potassium phosphate buffer. The final concentration of dextromethorphan is 1 mM.

Reagent used in protein assay (30 tubes)

1. Bovine serum albumin (BSA), 1 mg/ml in 0.5 NaOH.
2. Folin & Ciocalteu's phenol reagent.
3. working solution composed of 100 ml of 2% w/v Na_2CO_3 + 20 ml of 0.5 M NaOH + 2 ml of 2% w/v sodium citrate + 2 ml of 1% w/v cupric sulfate.

2 % w/v Na_2CO_3 : dissolve 4 g of Na2CO3 in 200 ml of water.

0.5 M NaOH: dissolve 2 g of NaOH in 100 ml of water.

2% w/v sodium citrate: dissolve 0.2 g of sodium citrate in 10 ml of water.

1% w/v cupric sulfate: dissolve 0.05 g of cupric sulfate in 2 ml of water.

Reagent used in CYP2D6 enzyme assay

solution I	1 mM dextromethorphan in 0.1 M potassium phosphate, pH 7.4
solution II	20 mg/ml glucose-6-phosphate, 20 mg/ml NADP, 13.3 mg/ml MgCl2-H2O.
solution III	40U/ml glucose-6-phosphate dehydrogenase in 5 mM Na citrate (tribasic).
solution IV	0.1 M potassium phosphate pH 7.4.
solution V	70 % perchoric acid.

VITA

Payiarat Nakmahachalasint was born in Nakorn Panom on June 8, 1977. She spent her childhood in Mukdahan. In 1993, she went to Holland and had lived for 1 year as an exchanged student. She graduated from Silpakorn University with the bachelor of Pharmacy, majored in Pharmaceutical Technology in the year of 2000. After the year of graduate, she worked as a pharmacist in Watson Co. for almost a year and then went to California, USA and had lived there for 6 months. Admitted to Faculty of Pharmaceutical Sciences, Department of Pharmacology, Chulalongkorn University in the year 2002.