1 PART I GENERAL INTRODUCTION

1.1 Introduction
The biological response of the human body to an exogenous compound e.g. a drug is dependent on a complex network of factors, as is illustrated in figure 1. Most drugs are effective because they bind to a particular target protein like enzymes, ion channels or receptors. In general, the intensity of drug effect (pharmacodynamics) is determined by the concentration of the drug in the direct environment (biophase) of the target and the affinity of the drug for the target. The drug concentration in blood should be considered as a surrogate marker but a relationship should exist because all organs and tissues are supplied with blood. Pharmacokinetics deals with drug plasma concentration-time relationships in the body.

Figure 1: Schematic illustration of the complex interrelationships of factors that influence drug response

1.2 Pharmacokinetic Aspects

1.2.1 Pharmacokinetic processes
These basic processes of absorption, distribution and elimination are responsible for the transport and distribution of drug molecules and therefore influence their overall behaviour in the body.\(^2\) Absorption is defined as the passage of a drug from its site of administration into the systemic circulation. For the oral administration route (see figure 2), drugs are absorbed from the gastrointestinal tract, for other routes other organs are involved e.g. the skin
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(percutaneous absorption) or the lungs (inhalation). The extent of absorption determines the fraction of a dose that gains access to the circulation (the bioavailability). In addition, for some drugs, the bioavailability may be influenced by metabolism in the gut and the liver (see figure 2) before reaching the systemic circulation.\textsuperscript{3,4} After reaching the systemic circulation the drug is distributed into the various body compartments. The four main compartments are plasma water (~5% of body weight), interstitial fluid (~16%), intracellular water (~35%) and fat (~20%).\textsuperscript{5} Elimination comprises all processes, which result in removal of the drug from the body. The major elimination processes are excretion and biotransformation. Excretion is the elimination of the compound in the unchanged form or as metabolites and the main routes are: the urine (renal excretion) and the faeces (hepato-biliary system). The polarity of a drug is often the limiting factor in excreting the drug from the body. Enzymatic biotransformation (metabolism) reduces the lipid solubility of a drug and thereby increases the possibilities for excretion of the drug. Biotransformation reactions are generally divided in phase I and phase II reactions. Phase I reactions consist of oxidation, reduction and hydrolysis and usually form more reactive products. Phase II reactions consist of conjugation e.g. glucuronidation, sulfation, acetylation to form highly polar and readily excretable conjugates. Phase I reactions are mainly catalysed by a complex enzyme system known as the mixed function oxygenase system which is based on cytochrome P450.\textsuperscript{5} Besides the cytochrome P450 enzymes other non-P450 enzymes like flavine mono-oxygenase, monoamine oxidase, alcohol dehydrogenase, xanthine oxidase etc, may catalyse oxidative pathways as well.\textsuperscript{5}

![Figure 2: The barriers that an orally administered drug encounters before reaching the circulation to exert its biological activity.](image-url)
1.2.2 Pharmacokinetics of Elimination

The elimination of a drug can be described by the clearance: the proportionality factor relating rate of drug elimination to the plasma (drug) concentration. It can also be described from a physiologic approach, namely the loss of a drug across an organ of elimination, which is summarized in figure 3.

**Figure 3:** Schematic representation of clearance as extraction by an organ of elimination.

The rate of entry of a drug into an organ of elimination is the product of blood flow, \( Q \), and concentration in blood entering the arterial side, \( C_A \) (\( Q \cdot C_A \)). Similarly, the rate at which drug leaves at the venous side is \( Q \cdot C_v \), where \( C_v \) is the concentration in the returning venous blood. The difference between these rates is the rate of drug extraction (elimination) by the organ (\( Q \cdot (C_A - C_v) \)).

The extraction ratio is defined as:

\[
E = \frac{(C_A - C_v)}{C_A} \quad (1)
\]

The clearance is defined as:

\[
CL = \frac{Q(C_A - C_v)}{C_A} \quad (2)
\]

(1) and (2) combined gives the following important relationship

\[
CL = Q \cdot E \quad (3)
\]

The clearance may be regarded as the volume of blood from which all the drug is removed per unit time. Although drug metabolism can take place in many organs, the liver frequently has the greatest metabolic capacity. Hepatic clearance includes biliary excretory clearance and hepatic metabolic clearance.
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\[ CL_H = Q_H \cdot E_H \] (4)

where \( CL_H \) = hepatic blood clearance, \( Q_H \) = hepatic blood flow (usually 1.5 L.min\(^{-1}\)) and \( E_H \) = hepatic extraction ratio.

As shown in Equation (2) and (3) the clearance will be influenced by changes in blood flow. When the extraction ratio of a drug is low e.g. < 0.3 the arterial and venous blood concentration are similar and therefore changes in blood flow will hardly affect the clearance. However, when the hepatic extraction ratio is high e.g. > 0.7, changes in blood flow produce substantial changes in clearance. In the latter case the clearance is perfusion rate-limited and changes in enzyme activity cause little or no change in clearance. Conversely, if enzyme activity is the rate-limiting step, the clearance is dependent and directly proportional to enzyme activity. Intrinsic clearance is defined as the maximum capacity of an organ to eliminate drug in the absence of limitations due to flow and/or plasma protein binding.

The enzymatic transformation of a drug can be symbolized by the following reaction:

\[ E + S \rightleftharpoons ES \rightarrow E + p \]

Where \( E \) = Enzyme, \( S \) = substrate (= drug) and \( p \) = product (= metabolite)

According to Michaelis and Menten the relationship between the rate of transformation (V) and substrate concentration is as follows (figure 4):

\[ V = \frac{V_{max} \cdot [S]}{K_M + [S]} \] (5)

![Michaelis-Menten Kinetics](image)

**Figure 4**: Michaelis-Menten Kinetics of an enzyme as function of substrate concentration. \( V_{max} \) = maximal velocity and \( K_M \) = Michaelis constant.
If the free drug concentration in plasma ($C_U$) is equal to drug concentration at the sites of the enzymes and the therapeutic free drug concentration is far lower than the $K_M$ the following equation is valid:

$$V = \frac{V_{\text{max}}}{K_M} \cdot C_U \quad (6)$$

Since metabolic clearance is defined as the rate of metabolism relative to the plasma concentration ($V = CL \cdot C_U$) the clearance is:

$$CL = \frac{V_{\text{max}}}{K_M} \quad (7)$$

This means that if $C_U \ll K_M$ the clearance is a constant which is called the intrinsic metabolic clearance.

The loss as drug passes, for the first time, through the gastrointestinal membranes and the liver during absorption is known as the first-pass effect. High clearance drugs will show a large first-pass effect leading to a low bioavailability. A decreased enzyme activity due to genetic polymorphism or enzyme inhibition can have large effects on the bioavailability for these drugs. A clear example is the dramatic effect of inhibition of CYP3A4 and the bioavailability of terfenadine (see section 1.4.4).

1.3 Genetic polymorphism and the diversity of human genes Genetics

The human genome contains three billions of base pairs of nucleotides in the haploid genome of which about only 3% are genes. A gene can be defined as the basic unit of heredity that contains the information for making one RNA and, in most cases, one polypeptide. The number of genes in humans is estimated at 40,000 to 100,000. Extensive genetic variation occurs in most populations at several different levels, including variants affecting colour, chromosomal structure, and protein characteristics.

Polymorphism is defined as the existence of two or more genetically determined forms (alleles) in a population in substantial frequency. In practice, a polymorphic gene is one at which the frequency of the most common allele is less than 0.99. In humans polymorphism is rather common: it has been estimated that in each human individual 20% of the proteins and hence the genes exist in a form that is different from the majority of the population. Similarly, in a sample of 71 human genes it was observed that 28% was polymorphic and that the average heterozygosity was 0.067. Heterozygosity is defined as the proportion in a population of diploid genotypes in which the two alleles for a given gene are different.

In a population the allelic frequencies may follow the Hardy-Weinberg Law that states: under certain conditions of stability both allelic frequencies and genotypic
ratios remain constant from generation to generation in sexually reproducing populations. Condition for this genetic equilibrium are (i) the population must be large enough, (ii) mutations must not occur, (iii) there must be no immigration or emigration and (iii) reproduction must be totally random. The Hardy-Weinberg equilibrium is as follows:

\[ p^2 + 2pq + q^2 = 1 \]  

where \( p \) = frequency of one allele and \( q \) = frequency of the other allele

Polymorphism in drug metabolising enzymes is caused by mutations in genes that code for the specific biotransformation enzyme. Generally, they follow the autosomal recessive trait that means that the mutation is not sex linked (autosomal) and that one mutated allele does not express the phenotype when combined with a normal, not mutated (dominant) allele.

Genomes can be mutated in several ways: a nucleotide can be changed by substitution (e.g. a C changes into a T), insertion or deletion of a base. If changes refer to one or a few bases these mutations are called point mutations. Larger changes can exist also, e.g. deletion of the entire gene or duplication of the entire gene.

Some point mutations are silent mutations, that is, they have no consequences at the protein level. Yet, other point mutations will affect amino acid sequence and thereby will affect the biological function of the protein. There are several mechanisms at which a point mutation can affect amino acid sequence and or expression. Mutations can be missense mutations, in which a base change alters the sense of a codon (a three base sequence that is specific for an amino

**Figure 5:** Model for control of protein synthesis by the genes.
Part I

acid) from one amino acid to another. For example, a missense mutation might change the proline codon (CCG) to the arginine codon (CGG). A mutation may introduce a preliminary stop codon: the cysteine codon (TGC) for example can be mutated to a stop codon (TGA). Insertion or deletion may lead to a frameshift, which means that the base sequence will be read incorrect from the insertion or deletion because the reading frame has shifted. Another frequently occurring consequence of a mutation is incorrect splicing (splicing defect). A gene consists of introns (non-coding sequences) and exons (coding sequences). The messenger RNA (mRNA; Figure 5) is read from the DNA and the primary transcription product contains both introns and exons. The primary product is further matured by removal of its introns in a process called splicing. Splicing occurs via splicing-sites: GU (first two bases of an intron) and AG (almost always last two bases). Mutations in these sequences or mutations at other sites may lead to abnormal splicing.

1.3.1 Development of pharmacogenetics / pharmacogenomics
The factors that influence drug response are a mixture of individual physiological / pathophysiological (e.g. age, liver/kidney function, etc) and inherited genetic markers (figure 6). Within the pharmacology the genetic markers are addressed by the pharmacogenetics.

Figure 6: Genetic factors determining drug response and toxicity

In 1957 it was suggested by Motulsky that certain adverse drug reactions could be related to genetically determined variation of drug-metabolizing enzymes in
the liver.\textsuperscript{15} This was the starting point of the pharmacogenetics. In 1960 it was discovered that the metabolism of isoniazid was under genetic control and “slow” and “rapid” metabolizers were identified.\textsuperscript{16} Today, this large interindividual difference is associated with polymorphism in the $\text{NAT2}$ gene for which several allelic variants exists that code for non-functional enzymes.\textsuperscript{17} Until now, many clinically, important polymorphisms have been discovered, e.g. butyrylcholinesterase, methyltransferase, UDP-glucuronosyltransferase, sulfotransferase, alcohol dehydrogenase, CYP2D6, CYP2C19, CYP2C9. Pharmacogenetics describe inherited differences in expression and catalytic activities of drug metabolizing enzymes and genetic mechanisms of differences in expression and regulation of transporters and receptors for drugs.\textsuperscript{18} These genetically determined differences often lead to differences in response to therapeutically used drugs. Pharmacogenetics combines the techniques of pharmacology, biochemistry, genetics, analytical chemistry and molecular biology, to understand the genetic basis of unusual reactions or responses to drugs. It may affect pharmacodynamics (clinical pharmacogenetics) and pharmacokinetics (metabolic pharmacogenetics). In addition, although outside the scope of pharmacogenetics, the pathophysiology may be genetically determined as well (see figure 6).\textsuperscript{14} Recently, pharmacogenetics has been merged with the newest techniques of genomics to pharmacogenomics.\textsuperscript{19} This field focuses on comparison of DNA sequences of responders and non-responders and it tries to identify genes and alleles, which differs between these groups. Thus, aberrant genes can be found without any knowledge of biochemistry of the response. For this approach, sophisticated high throughput techniques like DNA array microchip or cleavable mass spectrometer tags are needed.\textsuperscript{20} A nice example of this approach is a study to predict clozapine response in schizophrenic patients. In total 19 genes were tested and 6 of them were identified as possibly related to clozapine response resulting in 77\% success in the prediction of clozapine response.\textsuperscript{21}

1.4 Oxidative drug metabolism

1.4.1 Cytochrome P450 enzymes
Cytochrome P450 is a superfamily of heme-containing mono-oxygenases. The P450 system is located in the smooth endoplasmic reticulum of the cell and is particularly abundant in the liver. On homogenizing these tissues, the endoplasmic reticulum is disrupted to form small vesicles called microsomes. For this reason, metabolising enzymes of the endoplasmic reticulum are called microsomal enzymes. At the active site of cytochrome P450 is an iron atom which, when in the oxidized form ($\text{Fe}^{3+}$), binds the substrate (Figure 7: step 1). Reduction of the enzyme-substrate complex occurs, with an electron being transferred from NADPH via NADPH cytochrome P450 reductase (step 2).
Figure 7: The cytochrome P450 mono-oxygenase system\(^2\)
P450\(^{+++}\) = Cytochrome P450 in oxidized state (Fe\(^{+++}\)). P450\(^{++}\) = Cytochrome P450 with iron in reduced state. S = substrate. E = electron.

This reduced (Fe\(^{2+}\)) enzyme-substrate complex then binds molecular oxygen (step 3) and is then reduced further by a second electron (step 4 or step 4'). The enzyme-substrate-oxygen complex splits into water, oxidized substrate and the oxidized form of the enzyme. Carbon monoxide, which binds with the reduced form of the cytochrome, inhibits oxidation and gives a complex with an absorption peak at 450 nm, the origin of the name of the enzyme.

The P450 superfamily is composed of families and subfamilies of enzymes that are defined on the basis of their amino acid sequence similarities.\(^2\) Members of a family are at least 40% identical and members of a subfamily have at least 55% sequence similarity. P450's are named with the root "CYP" followed by a number (the family) and a letter (subfamily) and another number denoting the individual P450 form. At least 74 CYP gene families, of which 14 are ubiquitous in all mammals, have been described.\(^2\) Enzymes from families 1, 2 and 3 are involved in metabolizing xenobiotics like drugs; other families e.g. 7, 17, 19, 21 and 27 are involved in steroid and cholesterol metabolism.\(^2\)
### Table 1: Human xenobiotics-metabolizing cytochromes P450\textsuperscript{22}

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Tissue</th>
<th>Polymorphism#</th>
<th>Model substrate(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1</td>
<td>Many</td>
<td>No</td>
<td>Benzopyrene</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>Liver</td>
<td>No</td>
<td>Caffeine, Phenacetin</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Liver</td>
<td>Yes</td>
<td>Coumarin, Diethylnitrosamine</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Liver, intestine</td>
<td>Yes</td>
<td>Tolbutamide, S-warfarin</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>Liver</td>
<td>Yes</td>
<td>Mephenytoin, omeprazole</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Liver, intestine, kidney</td>
<td>Yes</td>
<td>Dextromethorphan, sparteine, debrisoquine</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>Liver, intestine, leukocytes</td>
<td>No</td>
<td>Ethanol</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Gastrointestinal tract, liver</td>
<td>No</td>
<td>Testosterone, nifedipine, erythromycin</td>
</tr>
</tbody>
</table>

\#: Polymorphically expressed due to variation in the genes coding for these enzymes\textsuperscript{24,25,26}.

#### 1.4.2 CYP2D6

Between 1975 and 1977 two independent studies led to the discovery of the genetic deficiency of debrisoquine and sparteine metabolism, which could later on be related to genetic polymorphism in the CYP2D6 gene. In a study in volunteers on debrisoquine, a sympatholytic antihypertensive drug, one individual showed a much more pronounced hypotensive response than the others. This was being found due to impaired 4-hydroxylation of debrisoquine.\textsuperscript{27}

In 1975, during a study on the kinetics of a slow release preparation of sparteine, an oxytocic and antiarrhythmic alkaloid, two subjects developed more severe side effects than the others, and their plasma levels were 3 to 4 times higher, although they received the same dose as the others. This difference was caused by a defective N-oxidation of sparteine to sparteine-N\textsubscript{1}-oxide.\textsuperscript{28} Family studies on this impaired metabolism showed a monogenic autosomal Mendelian recessive inheritance.\textsuperscript{29,30}
The concept of genetic polymorphism is illustrated in figure 8. It gives an idea how a mutant gene results in mutant mRNAs, yielding a [non-detectable and] non-functional protein. Individuals with a non-functional CYP2D6 variant have impaired metabolism of CYP2D6 substrates and are called poor metabolisers. The CYP2D6 gene is located on chromosome 22 and is part of the CYP2D gene cluster with two pseudogenes (CYP2D7P and CYP2D8P) and one functional gene (CYP2D6). It contains 9 exons within a total of 4378 base pairs. From several large studies in Caucasians (1456 Europeans) it can be estimated that for CYP2D6 67% of all alleles encode for enzymes with normal activity, 4% encode for enzymes with decreased activity, 27% lead to non functional enzymes and 2% encode for increased activity.
Table 2: The most common allelic variants of the CYP2D6 gene

<table>
<thead>
<tr>
<th>Allele</th>
<th>Trivial name</th>
<th>Nucleotide Changes</th>
<th>Effect</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2D6*1</td>
<td>Wild-type</td>
<td>None</td>
<td>-</td>
<td>Normal</td>
</tr>
<tr>
<td>CYP2D6*2</td>
<td>CYP2D6L</td>
<td>G1749C; C2938T; G4268C</td>
<td>R296C; S486T</td>
<td>Decreased</td>
</tr>
<tr>
<td>CYP2D6*2xN</td>
<td></td>
<td></td>
<td>N active genes</td>
<td>Increased</td>
</tr>
<tr>
<td>CYP2D6*3</td>
<td>CYP2D6A</td>
<td>A2637 deletion</td>
<td>Frameshift</td>
<td>None</td>
</tr>
<tr>
<td>CYP2D6*4</td>
<td>CYP2D6B</td>
<td>C1749T; C1085G; G1749C</td>
<td></td>
<td>Splicing defect</td>
</tr>
<tr>
<td>CYP2D6*5</td>
<td>CYP2D6D</td>
<td>CYP2D6 deleted</td>
<td>CYP2D6 deleted</td>
<td>None</td>
</tr>
<tr>
<td>CYP2D6*6</td>
<td>CYP2D6T</td>
<td>T1795 deleted</td>
<td>Frameshift</td>
<td>None</td>
</tr>
<tr>
<td>CYP2D6*7</td>
<td>CYP2D6E</td>
<td>A1072G; G2938C</td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>CYP2D6*8</td>
<td>CYP2D6G</td>
<td>G1749C; G1846T; C2938T; G4268C</td>
<td>CYP2D6 deleted</td>
<td>Stop codon</td>
</tr>
<tr>
<td>CYP2D6*9</td>
<td>CYP2D6C</td>
<td>A3023C</td>
<td></td>
<td>None</td>
</tr>
<tr>
<td>CYP2D6*10</td>
<td>CYP2D6J</td>
<td>C1749C; G4268C</td>
<td></td>
<td>None</td>
</tr>
</tbody>
</table>

The most common non-coding alleles are CYP2D6*4 (about 71% of null alleles), CYP2D6*5 (about 16% of null alleles), CYP2D6*3 (about 6% of null alleles) and CYP2D6*6 (about 4% of null alleles) all the other non-coding alleles are rare and have a prevalence of 1% or lower of the non-coding alleles. Allelic variants that encode for enzymes with decreased activity are rare in Caucasians and the most common alleles are CYP2D6*9 and CYP2D6*10, each accounting for about 2% of all alleles. Ultra-rapid metabolism (CYP2D6*2xN) is caused by multiple functional CYP2D6 genes, causing an increased amount of CYP2D6 to be expressed. It was proposed that the latter gene duplication occurs as a reciprocal of gene deletion (CYP2D6*5), which is thought to be caused by homologous, unequal recombination. Therefore, the prevalence of gene duplication and gene deletion are likely to be equal. A homozygous combination of non-coding alleles leads to the poor metabolizer (PM) phenotype, whereas heterozygous wild type or combinations of alleles with diminished enzyme activity lead to reduced CYP2D6 activity. In addition, gene duplication, or sometimes multiplication lead to the ultra-rapid (UR) phenotype. Individuals with normal metabolic enzyme activities are often called extensive metabolizer (EM). In-vivo enzyme activity can be assessed by measurement of the metabolic ratio of an enzyme specific probe; for CYP2D6 dextromethorphan, sparteine, debrisoquine and metoprolol have been described as probe drugs.

The prevalence of CYP2D6 PM phenotype differs per race and is reported to be 5 to 10% in white populations and 1 to 2% in Orientals. This difference between races can be explained by the very low prevalence of the CYP2D6*4 mutation in Orientals. Another major interethic difference is a shift in the metabolic ratio distribution to higher values (lower enzyme activity) in the Chinese population due to the high prevalence of *10 alleles with lower enzyme activity.
In table 3 an overview of typical CYP2D6 substrates is given. It can be seen that especially cardiovascular agents and psychoactive drugs are metabolized via CYP2D6. Therefore, the clinical impact of impaired metabolism is thought to be the greatest in these classes of drugs.

**Table 3: An overview of typical CYP2D6 substrates**

<table>
<thead>
<tr>
<th>Class</th>
<th>Drug Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Narcotics</td>
<td>Hydrocodone, Oxycodone, Tramadol</td>
</tr>
<tr>
<td>Cardiovascular agents: antiarrhythmics</td>
<td>Encainide</td>
</tr>
<tr>
<td>Cardiovascular agents: β blockers</td>
<td>Flecaïnide, Mexilitine, Propafenone</td>
</tr>
<tr>
<td>Cold and allergy agents</td>
<td>Metoprolol, Propranolol, Timolol</td>
</tr>
<tr>
<td>Gastrointestinal agents</td>
<td>Ondansetron, Dextromethorphan, Methamphetamine</td>
</tr>
<tr>
<td>Antidepressants, tricyclic</td>
<td>Amitriptyline, Clomipramine, Desipramine, Imipramine, Nortriptyline, Trimipramine</td>
</tr>
<tr>
<td>Antidepressants, others</td>
<td>Fluoxetine, Citalopram, Fluvoxamine, Maprotiline, Mianserine, Moclubemide, Paroxetine, Trazodone, Venlafaxine</td>
</tr>
<tr>
<td>Neuroleptics</td>
<td>Chlorpromazine, Perphenazine, Risperidone, Thioridazine, Sertindole, Flufenazine, Zuclopentixol</td>
</tr>
</tbody>
</table>
1.4.3 CYP2C19

The anticonvulsant mephenytoin is the drug that led to the discovery of the polymorphic character of CYP2C19.\textsuperscript{42} The S-enantiomer (but not the R-enantiomer) of mephenytoin (MEP) is metabolised to 4'-OH-mephenytoin. It was shown that this route could be blocked due to impaired metabolism. It was suggested that individuals, with impaired 4'-hydroxylation were most likely to have a homozygous genotype for an autosomal recessive mutant allele of a gene coding for (S)-mephenytoin 4'-hydroxylase.\textsuperscript{42} In 1994, Goldstein and co-workers could prove that (S)-mephenytoin 4'-hydroxylase is coded by the CYP2C19 gene.\textsuperscript{43} At least seven genes or pseudogenes of the CYP2C subfamily appear to be localized on chromosome 10.\textsuperscript{24} The complete CYP2C19 gene has not been published yet, but it is closely related to other genes of the 2C subfamily, e.g. CYP2C9, with 95% similarity.

Table 4: The most common allelic variants of the CYP2C19 gene\textsuperscript{44,45,46}

<table>
<thead>
<tr>
<th>Allele</th>
<th>Trivial name</th>
<th>Nucleotide Changes</th>
<th>Effect</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C19*1A</td>
<td>CYP2C19 wt1</td>
<td>None</td>
<td>-</td>
<td>Normal</td>
</tr>
<tr>
<td>CYP2C19*1B</td>
<td>CYP2C19 wt2</td>
<td>C_{99}T; A_{991}G</td>
<td>Ile331Val</td>
<td>Normal</td>
</tr>
<tr>
<td>CYP2C19*2</td>
<td>CYP2C19 m1</td>
<td>C_{99}T; G_{681}A; C_{990}T; A_{991}G</td>
<td>Splicing defect</td>
<td>None</td>
</tr>
<tr>
<td>CYP2C19*3</td>
<td>CYP2C19 m2</td>
<td>G_{636}A; A_{991}G; A_{125}C</td>
<td>Stop codon</td>
<td>None</td>
</tr>
<tr>
<td>CYP2C19*4</td>
<td>CYP2C19 m3</td>
<td>A_{1}G; A_{991}G; C_{99}T; C_{129}T</td>
<td>GTG initiation codon</td>
<td>None</td>
</tr>
<tr>
<td>CYP2C19*5A</td>
<td>CYP2C19 m4</td>
<td>A_{991}G; C_{129}T</td>
<td>Arg_{433}Trp</td>
<td>None</td>
</tr>
<tr>
<td>CYP2C19*5B</td>
<td>CYP2C19 m5</td>
<td>C_{99}T; A_{129}T; A_{991}G</td>
<td>Arg_{433}Trp</td>
<td>None</td>
</tr>
</tbody>
</table>

Two null-alleles in the CYP2C19 gene, *2 and *3, have been described to account for approximately 87% of all PMs in Caucasians and 100% of all PMs in Orientals.\textsuperscript{44,45} In addition, three non-coding alleles (CYP2C19*4, CYP2C19*5 and CYP2C19*6) have been described but the frequencies of these alleles are expected to be below 1% in Caucasians.\textsuperscript{46} Deficiency of CYP2C19 occurs with a prevalence of PMs of 2-5% among white Europeans, Black Africans 4-5%, Black Americans 6% and 12% to 23% among Orientals.\textsuperscript{40,47} In-vivo enzyme activity can be assessed by measurement of the metabolic ratio of an enzyme specific probe for CYP2C19 and besides mephenytoin, omeprazole and proguanil have been described as probe drugs.\textsuperscript{48,49} Well known substrates of CYP2C19 are given in table 5, among them frequently used drugs like the sedative drug diazepam, and the proton pump inhibitor omeprazole. Furthermore, the
activation of proguanil to cycloguanil, which has shown to be an effective prophylaxis for malaria, is catalysed by CYP2C19. Deficiency for CYP2C19 is therefore thought to decrease the efficacy of this drug, although clinical studies showed no clear evidence for this hypothesis until now.\textsuperscript{50,51,52}

**Table 5: An overview of typical CYP2C19 substrates\textsuperscript{41,53}**

<table>
<thead>
<tr>
<th>Class</th>
<th>Drugname</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anticoagulants</td>
<td>R-warfarin</td>
</tr>
<tr>
<td>Anticonvulsant</td>
<td>Mephenytoin</td>
</tr>
<tr>
<td></td>
<td>Phensuximide</td>
</tr>
<tr>
<td>Anti-infectives</td>
<td>Proguanil</td>
</tr>
<tr>
<td>Antidepressants, tricyclic</td>
<td>Amitriptyline</td>
</tr>
<tr>
<td></td>
<td>Clomipramine</td>
</tr>
<tr>
<td></td>
<td>Imipramine</td>
</tr>
<tr>
<td>Antidepressants, others</td>
<td>Citalopram</td>
</tr>
<tr>
<td></td>
<td>Moclobemide</td>
</tr>
<tr>
<td>Gastrointestinal agents</td>
<td>Lansoprazole</td>
</tr>
<tr>
<td></td>
<td>Omeprazole</td>
</tr>
<tr>
<td>Sedative/ hypnotics</td>
<td>Diazepam</td>
</tr>
<tr>
<td></td>
<td>Hexobarbital</td>
</tr>
<tr>
<td>Immunosuppressants</td>
<td>Cyclophosphamide</td>
</tr>
</tbody>
</table>

1.4.4 CYP3A4

Cytochrome P450 3A4 (CYP3A4) is an iso-enzyme involved in Phase I oxidative metabolism of many endogenous and exogenous substances. From a quantitative point of view it is the most important hepatic CYP-enzyme, accounting for approximately 25% of all liver cytochrome P450s.\textsuperscript{54} Since CYP3A4 is also present in the small intestine, it has a significant effect on the first-pass metabolism of CYP3A4 substrates. The gene coding for CYP3A4 is situated on chromosome 7 and at this moment no genetic polymorphisms have been described that are associated with changed enzyme activity.\textsuperscript{55} Recently, a polymorphism has been detected in the 5'-flanking promoter region (G\textsubscript{399}A) but several studies could not find a genotype/phenotype relationship and therefore this polymorphism is thought to have only modest clinical relevance.\textsuperscript{56,57,58}

Nevertheless, the variability of CYP3A4 activity has appeared to be quite severe. The intrinsic clearance for CYP3A4 metabolised substances can vary dramatically amongst individuals, with inter-individual differences of factors of 10 or higher.\textsuperscript{59} The iso-enzyme is quite sensitive to inhibition and induction, which is perhaps the largest cause for the high variability. Inhibition of CYP3A4 in the intestine by, amongst others, grapefruit juice may lead to a substantial increase of bioavailability. Such interactions are clinically relevant for CYP3A4 substrates with a low bioavailability, such as terfenadine and saquinavir.\textsuperscript{60,61}

One of the most striking examples is the interaction between CYP3A4 inhibitors and the non-sedating antihistamine, terfenadine.\textsuperscript{60,62} Terfenadine undergoes
extensive pre-systemic elimination by CYP3A4 to terfenadine carboxylate. Terfenadine is a potent blocker of myocyte delayed rectifier potassium current, whereas the metabolite is inactive. This blockade may lead to prolongation of the QTc interval and development of a serious ventricular tachyarrhythmia, torsade de pointes, and may finally lead to death. Inhibition of CYP3A4 activity due to concomitant medication or intake of grapefruit juice lead to increased plasma levels of terfenadine with serious side effects as described above. Approximately 125 deaths linked to terfenadine have been reported, showing the relevance of this interaction.60

In table 6 it can be seen that the number of drugs metabolised chiefly by CYP3A4 is large and, including a wide variety of compounds in many different therapeutic classes.

**Table 6**: An overview of typical CYP3A4 substrates

<table>
<thead>
<tr>
<th>Class</th>
<th>Drugname</th>
</tr>
</thead>
<tbody>
<tr>
<td>Narcotics</td>
<td>Alfentanil</td>
</tr>
<tr>
<td></td>
<td>Fentanyl</td>
</tr>
<tr>
<td></td>
<td>Methadone</td>
</tr>
<tr>
<td>Anticonvulsants</td>
<td>Carbamazepine</td>
</tr>
<tr>
<td></td>
<td>Ethosuximide</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Azithromycin</td>
</tr>
<tr>
<td></td>
<td>Clarithromycin</td>
</tr>
<tr>
<td></td>
<td>Erythromycin</td>
</tr>
<tr>
<td>Antifungals</td>
<td>Fluconazole</td>
</tr>
<tr>
<td></td>
<td>Ketoconazole</td>
</tr>
<tr>
<td></td>
<td>Miconazole</td>
</tr>
<tr>
<td>Antiparasitics</td>
<td>Quinine</td>
</tr>
<tr>
<td>Antivirals and HIV drugs</td>
<td>Indinavir</td>
</tr>
<tr>
<td></td>
<td>Ritonavir</td>
</tr>
<tr>
<td></td>
<td>Saquinavir</td>
</tr>
<tr>
<td>Cancer Chemotherapy</td>
<td>Etoposide</td>
</tr>
<tr>
<td></td>
<td>Ifosfamide</td>
</tr>
<tr>
<td></td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>Cardiovascular agents: antiarrhythmics</td>
<td>Amiodarone</td>
</tr>
<tr>
<td></td>
<td>Disopyramide</td>
</tr>
<tr>
<td></td>
<td>Lidocaine (lignocaine)</td>
</tr>
<tr>
<td></td>
<td>Quinidine</td>
</tr>
</tbody>
</table>

Table 6 - continued -

<table>
<thead>
<tr>
<th>Class</th>
<th>Drugname</th>
</tr>
</thead>
</table>
Part I

Cardiovascular agents: calcium channel blockers
- Amplodipine
- Diltiazem
- Felodipine
- Nicardipine
- Nifedipine
- Nimodipine
- Nitrendipine
- Verapamil

Cardiovascular agents: hypolipidaemics
- Fluvastatin
- Pravastatin

Antihistamines
- Loratadine
- Terfenadine

Gastrointestinal agents
- Cisapride

Immunosuppressants
- Cyclosporin
- Tacrolimus

Antidepressants
- Nefazodone
- Sertraline

Sedatives / hypnotics
- Alprazolam
- Midazolam
- Triazolam
- Zolpidem

Steroids
- Dexamethasone
- Finasteride
- Methylprednisolone
- Prednisone
- Testosterone

1.5 Phenotyping

1.5.1 Assessment of enzyme activity using probe drugs
The individual status of the activity of drug metabolizing enzymes can be assessed using enzyme specific probe drugs. To this end, the drug is administered to an individual and the excretion rate e.g. metabolic ratio, is measured after several hours. In selecting a probe drug the following basic elements should be considered: kinetics/metabolite formation should be determined predominantly by metabolism and not by liver blood flow or protein binding, the probe drug should not inhibit other enzymes of interest, the probe drug should be able to detect environmental or host influences and it should be safe. Furthermore, some practical issues play a role: are the clinical procedures not too inconvenient for patients or volunteers, is the analytical methodology not too demanding and is the probe easily available (preferably a clinically used drug). Simultaneous assessment of in-vivo activities of more than one enzyme may be performed by a multi-enzyme probe approach or by the cocktail
approach. The multi-enzyme approach also called ‘the metabolic fingerprinting approach’ seems to be a practical approach: only one drug has to be administered yielding information on several drug-metabolising enzymes. In the eighties and early nineties antipyrine was considered a promising multi-enzyme probe but nowadays its use as test compound has decreased because it failed to predict liver disease correctly and furthermore inhibition or induction of antipyrine metabolism cannot directly be coupled to a specific P450 enzyme.

At least six P450 enzymes are involved in the metabolism of antipyrine and none of the routes to the major metabolites are single enzyme routes. Therefore, it was concluded that antipyrine is not well suited as a probe for distinct human cytochrome P450 enzymes. Other examples of multi-enzyme probes are caffeine (NAT2, XO and CYP1A2), warfarine (CYP3A4, CYP1A2 and CYP2C19), dextromethorphan (CYP2D6 and CYP3A4) and omeprazole (CYP2C19 and CYP3A4).

The cocktail method is a multi-drug approach in which enzyme specific probes are co-administered. Several combinations have been used, validated and applied successfully in both clinical and epidemiological studies. Examples of well-established cocktails for simultaneous assessment of several polymorphic and non-polymorphic enzymes are given in table 7.

Table 7: An overview of multi-drug cocktails to assess P450 activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>Enzymes to be assessed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextromethorphan, mephenytoin</td>
<td>CYP2D6 and CYP2C19</td>
<td>70</td>
</tr>
<tr>
<td>Sparteine, mephenytoin</td>
<td>CYP2D6 and CYP2C19</td>
<td>71</td>
</tr>
<tr>
<td>Debrisoquine, mephenytoin</td>
<td>CYP2D6 and CYP2C19</td>
<td>72</td>
</tr>
<tr>
<td>Debrisoquine, mephenytoin and dapsone</td>
<td>CYP3A4</td>
<td>73</td>
</tr>
<tr>
<td>Dextromethorphan, proguanil</td>
<td>CYP2D6, CYP2C19</td>
<td>49</td>
</tr>
<tr>
<td>Dextromethorphan, proguanil and caffeine</td>
<td>CYP2D6, CYP2C19 and</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>NAT2</td>
<td></td>
</tr>
<tr>
<td>Dextromethorphan, caffeine</td>
<td>CYP2D6, NAT2, XO,</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>CYP1A2</td>
<td></td>
</tr>
<tr>
<td>‘Pittsburgh Cocktail’ caffeine, chlorzoxazone, dapsone, debrisoquine, mephenytoin</td>
<td>NAT2, CYP1A2, CYP2E1, CYP3A4, CYP2D6 and CYP2C19</td>
<td>76</td>
</tr>
</tbody>
</table>

Some combinations, however, may lead to misinterpretations, for example the combined administration of omeprazole and caffeine showed significant induction of CYP1A2 by omeprazole. Furthermore, care should be taken when CYP2D6 has to be assessed in populations other than Caucasians. Dissociation has been observed in the control of metoprolol, sparteine and debrisoquine oxidation in Nigerians and of metoprolol and debrisoquine oxidation in Zambians. Similar results were observed in a study to compare sparteine, debrisoquine and dextromethorphan as CYP2D6 probes in Ghanaians, Chinese
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and Caucasian. Again, dissociation was found in the oxidation of the probes in Ghanaians whereas such dissociation did not exist in Caucasians and Chinese. It was suggested that this dissociation might be explained by allele(s), which alter substrate specificity of CYP2D6. These studies highlight the difficulties in the interpretation of data from pharmacogenetic studies in ethnic groups.

1.5.2 Dextromethorphan

Dextromethorphan is a highly effective and widely used non-opioid antitussive drug. Dextromethorphan has been marketed since the early 1950s and is widely available as an over-the counter drug. Dextromethorphan has a wide margin of safety: adverse drug reactions are infrequent and usually not severe. The metabolism of dextromethorphan to its main metabolite dextrorphan was shown to co-segregate with the debrisoquine O-demethylation. It has been shown that it can be used as a safe phenotyping probe and nowadays it is widely used as CYP2D6 probe. Extensive dextromethorphan metabolizers (EMs) excrete more than 30% of the administered dose in urine as the O-demethylated metabolite dextrorphan within eight hours, whereas in poor metabolizers (PM) this amount is less than 5%. When the metabolic ratio is calculated, i.e. the ratio of urinary dextromethorphan/dextrorphan excretion, a bimodal distribution is found with a gap between the metabolic ratios of 0.2 and 0.6 and an antimode of 0.3, separating the EMs from the PMs. Subjects with a metabolic ratio > 0.3 are classified as PM.

Studies have been published on the applicability of dextromethorphan as CYP3A4 probe, in the demethylation of dextromethorphan to 3-methoxymorphinan (3MM), where the authors studied inhibition detection with dextromethorphan with regard to correlation with verapamil and tamoxifen metabolism. However, dextromethorphan as a probe for CYP3A4 has limitations, according to Kashuba et al. They state that there is a large intra-individual variability of the DEX/3MM urinary ratio and that using this ratio does not enable investigators to discriminate moderate CYP3A4 inhibition.
Impaired CYP2D6 metabolism does not have a major impact on drug safety of short-term treatment with standard doses of dextromethorphan, although pharmacokinetics may have a 20-fold difference for EMs as compared with PMs. On the other hand, the therapeutic efficacy was higher in PMs as well in patients with decreased CYP2D6 activity due to inhibition with quinidine.

1.5.3 Mephenytoin

Mephenytoin (3-methyl-5-phenyl-5-ethylhydantoin) was developed in the early 1940s and introduced into medical practice in 1945 as an anti-epileptic drug. Mephenytoin has been shown to be effective in the control of seizures of focal origin and for major motor seizures but it is of little use in petit mal attacks. Due to its toxicity the use is limited mostly to patients who are refractory to other anticonvulsants. Today, mephenytoin is marketed in only a limited number of countries.

Mephenytoin is extensively metabolized with less than 5% of an orally administered dose being excreted unchanged in the urine. In most individuals, the metabolism of mephenytoin is highly stereoselective with the (S)-enantiomer being rapidly oxidized to 4'-hydroxymephenytoin whereas (R)-mephenytoin is more slowly demethylated to (R)-nirvanol. For the disposition of (S)- and (R)-mephenytoin this resulted in a 100- to 200-fold difference in the mean oral clearance and a 30- to 40-fold difference in elimination half-life (2 vs. 76 hr). In subjects with deficient 4'-hydroxylation, so-called poor metabolizers (PMs), plasma levels of (S)-mephenytoin were increased significantly as compared to extensive metabolizers and the metabolism was no longer stereoselective. In 1993 it was discovered that CYP2C19 was strongly correlated with the (S)-mephenytoin hydroxylation and in 1994 the molecular basis for the
impaired metabolism was found. The current knowledge of the biotransformation of mephénytoïn is summarized in Figure 10. Mephénytoïn is widely used as a probe drug for the assessment of CYP2C19 activity. Other, more recently implemented probes for CYP2C19 are the proton pump inhibitor omeprazole and the anti-malarial drug proguanil. Although especially omeprazole is being increasingly used, the majority of the studies on CYP2C19 activity are still performed using mephénytoïn. Extensive and poor metabolizers can be characterized using racemic mephénytoïn by measurement of the S/R-ratio of the parent drug in urine or the 4'-hydroxylation index, i.e. the amount of (S)-mephénytoïn administered (50% of racemic dose) divided by the amount of 4'-hydroxymephénytoïn eliminated over 8 hours. Since the S-enantiomer is rapidly hydroxylated in EMs, the S/R-ratio will be low in 8-hours urine (usually < 0.05) and an antimode of 0.8 can be used to separate EMs from PMs. Some disadvantages of mephénytoïn as probe drug have been reported: the difficulty to obtain mephénytoïn medication and analytical reference materials due to reduced clinical use, stability problems of urine samples of extensive metabolizers due to an acid-labile component and the possibility of a sedative side effect as reported in a study in South-eastern Oriental subjects. The stability problem in urine concern an acid labile cysteine conjugate that is formed in EMs but not in PMs. Since this conjugate is instable it is easily hydrolysed back to (S)-mephénytoïn, thus influencing the S/R-ratio and increasing the risk in an incorrect phenotype classification. This risk on misclassification can be eliminated by re-analysis of PMs after acid treatment of the urine. Since in PMs this acid labile cysteine conjugate is not formed acidification will not influence the S/R-ratio in true PMs whereas in falsely classified PMs, the S/R-ratio will increase after acidification.
1.5.4 Metoprolol

Metoprolol is a class II antiarrhythmic drug that is used since 1975. It is a cardioselective $\beta_1$-adrenoceptor antagonist for the treatment of hypertension and angina pectoris. It has relatively little effect on $\beta_2$-receptors, which is likely to reduce the unwanted bronchospasm in patients with asthma or other form of obstructive airway disease. However, $\beta_1$-selectivity is a relative property that is lost at higher plasma concentrations of metoprolol. It was shown that the maximum tolerated concentration in asthmatics is much lower than the therapeutically effective concentration. Metoprolol belongs to the lipophilic beta-blockers, which are eliminated from the body almost entirely by metabolism. It was shown that the metabolism of metoprolol to $\alpha$-hydroxymetoprolol was under the same control as the debrisoquine
hydroxylation, which nowadays is known as the polymorphic CYP2D6 metabolism.\textsuperscript{105} CYP2D6 PMs had six fold higher area under the plasma curve and a threefold prolongation of the elimination half-life of metoprolol than EMs. Since the $\alpha$-hydroxylation is only a minor pathway of metoprolol metabolism (about 10% of an oral dose) the major pathway, $\Omega$-demethylation (65% of the dose) must also be polymorphic.\textsuperscript{104}

**Figure 11**: Plasma concentration versus time curves for (S)-metoprolol [(S)-M] and (R)-metoprolol [(R)-M] after administration of a single 200 mg oral dose of racemate to one EM and to one PM of debrisoquine.\textsuperscript{104}

This hypothesis is supported by several pieces of evidence although in-vitro data showed that $\Omega$-demethylation of metoprolol is catalysed by CYP2D6 for about 60% and that another enzyme is involved as well.\textsuperscript{104} Metoprolol is administered as a racemic mixture, with (S)-metoprolol responsible for the $\beta$-blocking activity.\textsuperscript{106} As can be seen in figure 11 the plasma levels of (S)-metoprolol are higher in EMs compared to the (R)-metoprolol levels indicating a more rapid clearance for the (R)-metoprolol. This stereo-selectivity seems to be absent in the metabolism of metoprolol in PMs.

In addition, to mephenytoin this is another example of the stereo-selectivity of cytochrome P450 enzymes. Metoprolol has been validated as probe for CYP2D6 activity. To this end metoprolol (usually 100 mg) is administered as a single oral dose and the metoprolol over $\alpha$-hydroxymetoprolol ratio can be assessed in an 8-hours post dose urine collection interval or in a one-point (3-hours post dose) plasma sample.\textsuperscript{107} Phenotyping on CYP2D6 using metoprolol has been applied in Caucasians, Orientals and Africans.\textsuperscript{78,79,108,109} In Caucasians and Orientals good correlations between metoprolol and debrisoquine were observed. However, in Africans dissociation in the control of debrisoquine and metoprolol oxidation has been observed.
1.5.5 Omeprazole
Omeprazole, a substituted benzimidazole, has been shown to be effective in the suppression of gastric acid secretion.\textsuperscript{110} It acts by inhibiting H\textsuperscript{+}, K\textsuperscript{+}-ATPase in the parietal cell. The suppression of gastric acid secretion is correlated with the plasma omeprazole concentration-time curve (AUC) and is not directly related to the plasma concentration of the drug at a particular timepoint. Omeprazole is generally rapidly eliminated from plasma, the plasma elimination half-life ($t\textsubscript{1/2}$) is usually 0.5-1h. In some subjects the $t\textsubscript{1/2}$ is three times longer and the plasma omeprazole concentration-time curve was ten-fold higher than the average values. It was found that this slow elimination co-segregated with the (S)-mephenytoin 4'-hydroxylation.\textsuperscript{111} Eighty percent of a given dose of omeprazole is excreted as 5-hydroxyomeprazole and its corresponding carboxylic acid.\textsuperscript{112} The metabolism of omeprazole to 5-hydroxyomeprazole is clearly catalysed by CYP2C19. (see figure 12).\textsuperscript{112,113,114} Both in vitro and in vivo studies showed that omeprazole sulphoxidation is catalysed by CYP3A4 and, moreover, that both metabolites undergo further oxidation to a secondary metabolite 5-hydroxyomeprazole sulphone, mediated by CYP3A4 and CYP2C19, respectively.\textsuperscript{112,115,116} The hydroxylation of omeprazole has shown to be enantioselective: (+)-omeprazole is metabolized via CYP2C19 whereas (-) omeprazole is only partly metabolized by CYP2C19 and mainly metabolized by another enzyme, CYP3A4, to the sulphone metabolite.\textsuperscript{118} Omeprazole has shown to be an effective probe for CYP2C19 phenotyping and has been applied in both Caucasians and Orientals.\textsuperscript{97,119,120,121} Given the safety of omeprazole, its common use, ease of handling and its possibility to use it as dual probe on both CYP2C19 and CYP3A4, it seems a promising probe, possibly replacing mephenytoin as favourite CYP2C19 probe. On the other hand CYP2C19 phenotyping using omeprazole seems to be restricted to individuals without liver disease, or using co-medication.\textsuperscript{122,123} It has been shown in healthy volunteers that the pharmacodynamic effects as measured by intragastric pH levels and plasma gastrin levels, are more pronounced in PMs as compared to EMs.\textsuperscript{124} It was suggested that CYP2C19 genotyping might be a useful tool for an optimal long-term treatment with omeprazole.
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Figure 12: Oxidative metabolism of omeprazole in humans

1.6 Genotyping

1.6.1 RFLP and Southern Blotting

Detection of mutations in genomic DNA is not an easy task if one realizes that sometimes one single point mutation has to be determined in the midst of three billions of base pairs. The classical method is restriction fragment length polymorphism (RFLP), followed by Southern blotting.

Figure 13: RFLP analysis and Southern blotting for the detection of mutated alleles of CYP2D6 locus on human chromosome 22.
An example of RFLP analysis on the CYP2D6 locus is depicted in figure 13. To this end, blood (30-50 mL) was used to isolate DNA from peripheral leukocytes. DNA (5 µg) was digested with XbaI, a restriction endonuclease that specifically cuts DNA between thymine and cytosine in the nucleotide sequence TCTAGA. DNA restriction fragments are isolated by agarose gel electrophoresis and transferred to a membrane (Southern blot). Subsequently, the membrane was hybridised with a CYP2D6 specific $^{32}$P-labeled probe (single stranded DNA fragment) and the membrane is exposed to an X-ray film. The 29-kb fragment is specific for CYP2D6 extensive metabolisers, whereas in poor metabolisers a 44-kb or an 11.5-kb fragment is found. This approach has some disadvantages: large volumes of blood are needed, time-consuming procedures are involved, and it can only detect mutations that affect restriction sites (about 5% of all mutations).

### 1.6.2 The Polymerase Chain Reaction

The polymerase chain reaction (PCR) has been developed by Mullis and others in the mid-1980s. PCR has revolutionized the analysis of genetic diseases and polymorphisms and is applied in many fields of research and diagnostics. Today, it is the basis for almost all methods for the detection of single nucleotide polymorphisms (SNIPs). In addition, many PCR based tests have been developed for monitoring cancer therapy, characterization of bacterial and viral infections (e.g. HIV, HCV), prenatal diagnosis and forensic applications. PCR has also been shown to be a highly effective tool in gene cloning and manipulation and can be used in studies on gene expression (reverse transcriptase (RT)-PCR). The most important reagent for PCR is a heat-stable DNA polymerase obtained from *Thermus Aquaticus*, a bacteria that lives in hot springs. Its polymerase, Taq Polymerase, has a temperature optimum of 72 °C and is even stable at 94 °C. This property of Taq polymerase makes it possible that DNA that is normally double stranded can be replicated to single stranded DNA at 72°C after denaturation at 94 °C. The principle of the test and the different temperature phases per cycle are shown in figure 14. Initially DNA is heated to 94°C to separate the DNA strands and become template for the primers (short DNA sequences that are complementary for a specific part of the template DNA).
Figure 14: Schematic presentation of the Polymerase Chain Reaction and the PCR cycle

Then the temperature is lowered to 50 - 65 °C for annealing of the primers (recombination of the single stranded DNA to double stranded DNA). Finally, the temperature is raised to 72 °C (66 - 75 °C) so that Taq polymerase can synthesize new DNA strands. The cycle is repeated about 25-35 times and since each template / target will form two new targets, after n cycles $2^n$ new targets will be formed. Theoretically after 30 cycles, $268.10^6$ new copies will be formed. In practice however, this number will be lower due to the efficiency of the PCR reaction. PCR reactions are performed in a thermocycler, which is basically a sophisticated and highly accurate heating and cooling equipment.

The specificity of the PCR assay is highly determined by the choice of the primer sequence and primer length. To ensure successful amplification, a minimum primer length between 15 and 20 nucleotides is required. Longer primers enable
higher annealing temperatures and result in a higher specificity. As source for DNA all nucleus containing cells can be used: blood (leukocytes), urine, semen, biopsy material, hair roots etc.\(^{\text{129}}\) Before PCR, DNA has to be isolated and purified. Whole blood, a frequently used source in clinical diagnostics will yield about 0.5 - 1 µg of dsDNA per 30 µL sample, which is usually sufficient for 5 - 10 PCR reactions. A PCR reaction can be optimized in several ways improving PCR efficiency or specificity. Some parameters that can be optimized are: purity of input DNA, PCR buffer composition especially MgCl\(_2\) concentration, choice of polymerase e.g. proofreading activity for high fidelity applications, thermocycler conditions e.g. hot start PCR, anneal temperature.\(^{\text{129}}\) After PCR an additional technique has to be used to assess the allelic constitution of the amplified fragments. To this end, the gold standard is DNA sequencing of the amplified fragments and it is advised that each alternative approach should be confirmed by sequencing.\(^{\text{130}}\)

If the mutation to be detected alters a restriction site, RFLP can be used after PCR and the analysis of the CYP2C19*2 mutation (figure 15) is an example of such an assay.

**Figure 15:** Analysis of the CYP2C19*2 mutation by PCR and restriction fragment length polymorphism (RFLP)\(^{\text{44}}\)

The wild type sequence of CYP2C19 contains a Sma I restriction site (Sma I cuts between C and G at CCCGGG), whereas the mutant allele (CYP2C19*2) lacks this restriction site. Using PCR, a fragment of 169 basepairs is amplified which is subjected to restriction with Sma I and the resulting fragments are analysed to determine the basepair size using agarose gel electrophoreses. In case of a homozygous wild type both alleles will be restricted and therefore two bands will be observed. Contrary, if a subject is homozygous mutant the restriction site is not present in both alleles and therefore no restriction can occur and thus the unrestricted product of 169 basepairs will be observed. Analogous, the heterozygous mutant subject contains both alleles and therefore the restriction products and the unrestricted fragment are observed. This mutation
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detection method is generally reliable and robust but can only be applied if a restriction site is involved, which is not always the case (see 1.6.1). An alternative mutation detection method is the allele-specific PCR (AS-PCR) of which the principle is illustrated by the method for the 5 most common non-coding alleles for CYP2D6. The assay starts with a long range PCR amplifying the CYP2D6 gene but not CYP2D7 and CYP2D8. This amplicon is then amplified again in two parallel reactions. In the first reaction the reverse primers for the several mutations are complementary to the wild-type sequence; in the second reaction, the reverse primers are complementary to the mutant sequences. In both reactions the forward primer is the same. Both reactions are applied separately to agarose electrophoreses to determine the product sizes. A band observed in the wild reaction only, indicates homozygous wild type for this mutation, a band observed in the mutant reaction indicates homozygous mutant and a band in both reactions means heterozygous mutant.

Figure 16: Strategy of rapid detection of CYP2D6 null alleles by long distance and allele-specific-polymerase chain reaction

This type of PCR should be well controlled by both mutation positive and negative controls since the risk on false positivity and false negativity is relatively high. Especially in a multiplex setting, the primer concentration of both wild type and mutations should be well balanced. In addition, the anneal temperature may influence the specificity of this PCR variant as well and should be thoroughly examined before implementing as a routine test.

1.7 Phenotyping versus Genotyping
In general there are three ways to get information on metabolizing enzyme activities: (i) study the genes that code for the enzyme, (ii) study the level of enzyme expression in a certain tissue or (iii) assess actual enzyme activity using an enzyme specific probe. Some relations are obvious e.g. individuals, who lack
wild type alleles, cannot produce the enzyme and therefore lack enzyme expression, resulting in absence of the enzyme specific metabolites. Other relations however are more complex e.g. an individual genotyped as EM does not necessarily express enzymes or possess enzyme activity. Today, genotyping is rapidly replacing phenotyping as the tool to describe populations, mainly through recent developments in molecular biology. Genotyping is a more simple procedure compared to phenotyping: no probe drugs are necessary, a blood sample or even a cheek mucosa swab sample is convenient and the analytical equipment is relatively simple and cheap.

Furthermore, genotyping bypasses the confounding factors involved in phenotyping and directly identify the possibility to express the iso-enzyme of interest.

There are several reasons that advocate phenotyping instead of, or in addition to genotyping. If a drug metabolising enzyme lacks clear genetic polymorphism e.g. CYP1A2, CYP2E1 and CYP3A4, genotyping is no option to assess enzyme activity. Furthermore, the enzyme activity range within a genotypic group might have a large variation. This is nicely illustrated for CYP2D6 by study in Germans, in which extensive genotyping in a large population only poorly predicted actual enzyme activity. In population studies, phenotyping in addition to genotyping might be helpful in detecting interethnic differences. For example, the relatively high frequency of the CYP2D6*10 mutation in Orientals, coding for the enzyme with decreased activity, was detected by a higher mean metabolic ratio (decreased enzyme activity) in a Chinese population as compared to a Swedish population (see figure 17).

Finally, in all situations where quantitative information on the enzyme activity is needed, e.g. in drug interaction studies to detect enzyme inhibition or induction, genotyping does not yield information and phenotyping should therefore be applied. The concurrency of genotype and phenotype should always be carefully investigated, to show the validity of the genotyping assay in predicting the phenotype. On the other hand, application of phenotyping methods to predict dosage requirements or to understand mechanism leading to unexpected blood levels may have clinical and ethical problems. Phenotyping often requires that the patient has to be drug free, implying that its normal pharmacotherapy has to be interrupted.
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**Figure 17**: Distribution of the urinary debrisoquine/4-hydroxy-debrisoquine metabolic ratio (MR) in healthy Chinese (n = 695) and Swedish (n = 1011) individuals. The arrows indicate a metabolic ratio of 12.6, the antimode between EMs and PMs.\(^{135}\)

### 1.8 Clinical consequences of genetic polymorphism

#### 1.8.1 Metabolic pharmacogenetics and clinical drug development

Concerning metabolic pharmacogenetics the main question during drug development is which iso-enzymes are involved in drug metabolism and what are their contributions to the wanted and unwanted effects of a drug.\(^{138}\) Studies have to be performed to serve to\(^{139}\):
- Determine the overall metabolism of a drug
- Identify the (polymorphic) enzymes involved and establish their relative contribution
- Determine the contribution of the various pathways to the overall elimination of a drug
- Identify different levels of expression of enzymes and predict interindividual variability
This information can be obtained in several ways and at several stages during drug development and is vital for the estimation of the clinical impact of a polymorphic enzyme to drug metabolism. These data are especially important if it concerns a drug with a narrow therapeutic index or if a drug is metabolised from a prodrug. Until now no guidelines exist that solely concern pharmacogenetics, but pharmacogenetics is seen as one of the many factors that contribute to drug response.\textsuperscript{143} The American Food and Drug Administration (FDA) states that identifying metabolic differences based on genetic polymorphisms, could help guide the design of dose-ranging studies for such populations and can aid interpreting results.\textsuperscript{141,142} The FDA advises phenotype or genotype determinations to identify genetically determined metabolic polymorphisms, especially mediated by CYP2D6 and CYP2C19, of patients or healthy subjects in clinical studies. The European Agency for Evaluation of Medical Products (EMEA) states that subjects participating in metabolic in vivo interaction studies should be appropriately genotyped and / or phenotyped if any of the active enzymes involved in the metabolism are polymorphically distributed in the population.\textsuperscript{143} In some cases, clinically relevant interactions may occur in a subset of the total population, for instance in poor metabolizers, when an (alternative) route of metabolism is inhibited. The International Conference on Harmonisation of Technical Requirement for Registration of Pharmaceuticals for Human Use (ICH) identifies clearance by an enzyme showing genetic polymorphism as an important factor that make ethnic differences more likely.\textsuperscript{144} Again knowledge of metabolism and assessing the role of genetic polymorphism helps identifying risk factors on ethnic differences and may be useful in designing bridging studies.

In clinical studies information on drug metabolising status of volunteers / patients is often needed for inclusion or to explain pharmacokinetic or pharmacodynamic observations. Rather than selecting subjects at random, genotyping on for example genes that code for drug metabolising enzymes, could be helpful in selecting subjects that are most likely to respond or might be helpful in the elucidation of side effects.\textsuperscript{145} It has been suggested that even "dead-drugs" due to efficacy or toxicity problems might be rescued by genotyping potential users before use and adjust doses to enhance efficacy and / or to avoid toxicity.\textsuperscript{140,145} Early screening during drug discovery (before development of a candidate drug) on the involvement in polymorphic enzymes in the metabolism is frequently performed nowadays.\textsuperscript{138} These data however are not primarily to prevent molecules from attaining development status, but are part of a set of routine tests. The decision to advance a particular candidate is made on information of a variety of disciplines typically pharmacology, toxicology and metabolism. It should be noted that deleting a candidate drug only on the basis of its polymorphic metabolism might result in the loss of a drug with potential clinical value. This is exemplified by many successful psychoactive drugs and omeprazole, which would never have been developed if the above deletion criterion had been applied.\textsuperscript{14}
1.8.2 The role of pharmacogenetics in clinical practice

In clinical practice information about drug metabolising enzyme capacities might be helpful to optimise pharmacotherapy and may reduce risks of unwanted drug reactions in e.g. psychiatry or cardiology. Morbidity and mortality induced by adverse drug reactions are important both from a health care point of view as from a pharmaco-economic point of view. Adverse drug reactions in hospitalised patients are countable, dangerous, and evaluable events and are associated with a significantly prolonged length of stay in the hospital, increased economic burden, and an almost 2-fold increased risk of death. In The Netherlands, each year about 1,700 victims of drug associated poisonings are treated at emergency departments, and some 11,000 hospital admissions take place due to unwanted effects of drugs/biological compounds in therapeutic dosages. In 1996, the National Poisons Control Centre of the National Institute of Public Health and the Environment received 15,647 requests for information on suspected drug overdosing. A total of 6,388 (41%) concerned drugs that act on the central nervous system. Especially in this class of drugs, metabolic polymorphism is widely present. CYP2D6, and to a lesser extent CYP2C19, plays an important role in the metabolism of psychoactive drugs. According to an extensive review by Bertz and Granneman, it is estimated that CYP2D6 is involved in the metabolism of about 50% of all psychoactive drugs, whereas CYP2C19 is involved in about 15% of these drugs.

The clinical consequences of impaired metabolism have been studied extensively during recent years. The following effects have been described: (i) increased side effects (e.g. in the case of amitriptyline and nortriptyline); (ii) drug interactions (e.g. fluoxetine); (iii) shift to more toxic metabolites (e.g. phenacetin) or (iv) therapeutic failure due to lack of the formation of active metabolite (e.g. codeine). For psychotropic medication it is well documented that 20 to 30% of patients do not respond to therapy. Therapeutic drug monitoring (TDM) may decrease the proportion of non-responders to 10-20%, indicating a relationship between blood levels and clinical effect.

The metabolism of tricyclic antidepressants (TCAs) such as desipramine, amitriptyline, imipramine, clomipramine and nortriptyline is clearly associated with CYP2D6 activity. Impaired metabolism may result in high plasma concentrations, long half lives and increased excretion of the parent compound, accompanied by decreased plasma levels of metabolites. For TCAs clear relations between blood level and side effects have been shown. In a prospective study in which the clinical effect of CYP2D6 polymorphism was investigated in depressed psychiatric outpatients receiving desipramine, it was concluded that identifying polymorphism may help in preventing adverse experiences (AEs) but that it does not yield information on the efficacy of treatment.

Novel antidepressants (serotonin selective re-uptake inhibitors) like paroxetine, fluoxetine, citalopram and fluvoxamine are also metabolised by CYP2D6. They have a wider therapeutic index compared to the TCAs, making them safer. It has been suggested that the plasma concentration is not always related to the therapeutic effectiveness. This may be due to the fact that the role of...
metabolites in the therapeutic effect is not clear. In some cases active metabolites exist (fluoxetine and sertraline) while in other cases the metabolites are inactive (fluvoxamine and paroxetine). Some of the novel antidepressants proved to be potent inhibitors of CYP2D6 mediated metabolism. This may result in clinically relevant interactions, e.g. fluoxetine or fluvoxamine with TCAs. Interactions have also been shown for fluoxetine with diazepam.\textsuperscript{154} Also, for neuroleptics no clear relationship between concentration and side effects have been demonstrated and therefore the clinical relevance of polymorphism has been less well studied. For various neuroleptics, e.g. perphenazine, thioridazine, chlorpromazine, inhibition of CYP2D6 has been shown. Perphenazine metabolism has been shown to be strongly dependent on CYP2D6, which influences first-pass metabolism and thereby the oral bioavailability of this drug.\textsuperscript{155} CYP2D6 is less prominently involved in the metabolism of zuclopenthixol: impaired metabolism results in decreased oral clearance but does not change oral bioavailability.\textsuperscript{154} CYP2D6 is also involved in the oxidation of reduced haloperidol to haloperidol and there are indications that PMs are prone to experience serious side effects.\textsuperscript{156} Population studies have shown that the metabolism of thioridazine, remoxipride and risperidone also co-segregate with CYP2D6.\textsuperscript{156} A prospective study to assess the relation between AEs (extrapyramidal) and CYP2D6 polymorphism showed that the prevalence of PMs in the AE group was significantly higher (45\%) than in the non-AE group (14\%).\textsuperscript{157} It was concluded that impaired metabolism by CYP2D6 was a contributing factor in extrapyramidal side effects.

CYP2C19 is involved in the metabolism of some antidepressant and anxiolytic drugs. The metabolism of amitriptyline, citalopram, clomipramine, diazepam, imipramine and moclobemide is catalysed by CYP2C19.\textsuperscript{156}

1.9 Objectives of the studies in this thesis

Ten years ago a European consensus conference (COST 1990) advocated routine phenotyping to improve the efficacy of drug therapy and to reduce the need for TDM.\textsuperscript{139} However, phenotyping is still not common practice, perhaps because its cost-effectiveness is not yet generally accepted. More recently, retrospective studies have been published indicating genotyping as a clinically relevant and cost effective tool.\textsuperscript{146,158,159} These studies have tried to establish the efficacy of pharmacotherapy by also including drug related adverse events since cost-effectiveness is strongly related to the clinical cost related to severe adverse events.

At Pharma Bio-Research, phenotyping on drug metabolism has been applied since 1990 to serve as a tool for recruitment of volunteers in clinical pharmacology studies. However, phenotyping has some disadvantages e.g. administration of a drug, expensive analytical equipment and environmental factors which may influence the outcome of the test. In 1995, it was decided to develop genotyping techniques on the most common polymorphic drug metabolizing enzymes to overcome these disadvantages. However, at that time
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the value of genotyping with respect to the predictability of the phenotype and the clinical value of the knowledge of an individual metabolizing status was not clear yet. In addition, genotyping requires an analytical approach that is different from the analytical chemistry needed for phenotyping (chromatography techniques). The knowledge of the molecular biology, the technique needed for genotyping, was not present at Pharma Bio-Research at that time.

For these reasons it was decided to start a scientifically driven project to develop, implement and evaluate genotyping and phenotyping techniques to assess polymorphic drug metabolizing enzymes.

Therefore:
- Phenotyping data on CYP2D6 and CYP2C19 have been investigated and a new phenotyping approach that allows combined phenotyping and genotyping has been developed. In addition, the current analytical methodology for CYP2D6 phenotyping has been expanded to be used for simultaneous phenotyping on CYP2D6 and CYP3A4.
- Genotyping tests have been developed for CYP2D6 and CYP2C19 and the analytical methodology has been validated. Correlation between phenotype and genotype has been evaluated to assess the predictability of the developed genotyping tests.
- Both phenotyping and genotyping tests have been applied in clinical studies in both Phase I drug research and in a clinical setting (psychiatry).

Answers were sought on the following questions:
- What is the value of genotyping on polymorphic drug metabolising enzymes compared to the classical phenotyping strategies?
- What is the situation concerning CYP2D6 and CYP2C19 polymorphism in the Dutch population?
- Is knowledge of the individual metaboliser status of CYP2D6 or CYP2C19 valuable in clinical pharmacological research and practice of pharmacotherapy?

1.10 References


37. Løvlie R, Daly AK, Molven A, Idle JR, Steen VM. Ultrarapid metabolizers of debrisoquine: characterization and PCR-based detection of alleles with duplication of the CYP2D6 gene. Febs Letters 392:1


53. Flockhart DA Web site of the Georgetown University Medical Center (Washington DC, USA) on http://www.dml.georgetown.edu/depts/pharmacology/davetab.html


General Introduction

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89. Wilkinson GR, Guengerich FP, Branch RA. Genetic polymorphism of S-mephenytoin hydroxylation. Pharmac Ther 1989; 43: 53-76


111. Andersson T, Regårdh CL, Dahl-Puustinen ML, Bertilsson L. Slow omeprazole metabolizers are also poor S-mephenytoin hydroxylators. Ther Drug Monit 1990; 12: 415-416


114. Karam WG, Goldstein JA, Lasker JM, Ghanayem BI. Human CYP2C19 is a major omeprazole 5-hydroxylase, as demonstrated with recombinant cytochrome P450 enzymes. Drug Metab Dispos 1996; 24: 1081-1087


125. Skoda RD, Gonzalez FJ, Demierre A, Meyer UA. Two mutant alleles of the human cytochrome P-450db1 gene (P450C2D1) associated with genetically deficient metabolism of debrisoquine and other drugs. Proc Natl Acad Sci 1988; 85: 5240-5243


137. Dahl ML, Sjöqvist F. Pharmacogenetic methods as a complement to therapeutic monitoring of antidepressants and neuroleptics. Ther Drug Monit 2000; 22: 114-117


148. Avorn J. Putting adverse drug events into perspective. JAMA, 1997; 277: 341-342


