"Investigation of individual differences in the metabolic elimination of drugs by the polymorphic enzymes CYP2C9, 2C19 and 2D6 based on metabolite profiling by LC-MS/MS"

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Abbreviations

Amino acids • C, Cys • I, Ile • K, Lys • L, Leu • P, Pro • R, Arg • S, Ser • T, Thr • V, Val • W, Trp	 • cysteine • isoleucine • lysine • leucine • proline • arginine • serine • threonine • valine • tryptophan
BSA	bovine serum albumin
CAD	collision gas for collisional activated dissociation
CE	collision energy
CID	collision induced dissociation
CNL	constant neutral loss
cps	counts per second
CUR	curtain gas
CXP	collision cell exit potential
CYP	cytochrome P450
Del	deleted
DEX	dextromethorphan
DNA	desoxyribonucleic acid
DNA-nucleotides • A • C • G • T	 • adenine • cytosine • guanine • thymine
dNTP	desoxyribonucleoside triphosphate
DOR	dextrorphan
DOR-Glu	dextrorphan glucuronide
DP	declustering potential
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EM	extensive metabolizer
EP	entrance potential
EtBr	ethidium bromide

FLB	flurbiprofen
FLB-Glu	flurbiprofen glucuronide
IM	intermediate metabolizer
Ins	inserted
IS	ion spray voltage
LOD	limit of detection
LOQ	limit of quantification
MR	metabolic ratio
MRM	multiple reaction monitoring
MS	mass spectrometer
MS/MS	tandem mass spectrometer
NADPH	nicotinamide adenine dinucleotide phosphate
NSAID	non-steroidal anti-inflammatory drug
OHF	4-hydroxy flurbiprofen
OHF-Glu	4'-hydroxy flurbiprofen glucuronide
OHOME	5-hydroxy omeprazole
OME	omeprazole
pK_a	logarithmic measure of the acid dissociation
PM	poor metabolizer
PPI	proton pump inhibitor
RNA	ribonucleic acid
rpm	rounds per minute
S/N	signal to noise
SNP	single nucleotide polymorphism
SSRI	selective serotonine re-uptake inhibitor
Taq polymerase	polymerase of bacterium *thermos aquaticus*
TCA	tricyclic antidepressant
UGT	uridine 5'-diphospho-glucuronosyltransferase
UM	ultrarapid metabolizer

Summary

In the 50's of the last century, the role of genetics for interindividual differences in drug response was discovered, and the field of pharmacogenetics was delineated and conceptualized.[1] The influence of genetic predisposition on the efficacy, susceptibility, and safety of drugs seems to be most important for drug metabolizing enzymes. Only 20 % of drugs in adverse drug reaction (ADR) studies are not metabolized by polymorphic enzymes.[2]

The most important human enzymes for oxidative drug metabolism are the cytochrome P450 monooxygenases of families 1 to 3 (CYP1–CYP3). Three of the isoforms belonging to these families (CYP2D6, 2C9 and 2C19) were found to possess a high rate of genetic polymorphisms with huge influence on drug response. Approximately 5–10 % of all Caucasians possess no active CYP2D6 enzyme,[3] about 40 % have reduced CYP2C9 enzyme activity,[4] and up to 6 % are deficient for CYP2C19.[5] These three enzymes take part in the oxidative metabolism of several drugs that can cause severe ADRs, like tricyclic antidepressants and antipsychotic drugs (CYP2D6), oral anticoagulants, and sulfonylurea antidiabetic drugs (CYP2C9), as well as anti-epileptics (CYP2C19). Therefore, the assessment of the individual enzyme activity of a patient before drug treatment can minimize the occurrence of ADRs and treatment failures. Two different approaches to determine enzyme activity are available. The first approach is based on the actual enzyme activity towards a carefully evaluated probe drug (phenotyping). The second approach examines the underlying genotype to predict the actual enzyme activity (genotyping).

This study should contribute to the important field of pharmacogenetics by: firstly, establishing an easy and safe phenotyping method that combines the activity determination of all three previously mentioned CYPs (CYP2D6, CYP2C9, and CYP2C19) into one phenotyping cocktail and secondly, improving the knowledge about the predictive power of the genotype for the measured phenotype.

It was indeed possible to develop a save, easy-to-use, fast and simultaneous phenotyping procedure for the important genetic polymorphic enzymes CYP2D6 and CYP2C9. To accomplish that, interaction studies with the chosen probe drugs dextromethorphan (DEX, CYP2D6), flurbiprofen (FLB, CYP2C9) and omeprazole (OME, CYP2C19) were conducted. It could be proven that DEX and FLB can be administered in combination, whereas OME alters the phenotyping results of CYP2C9. This is a new finding as in 2004 a phenotyping cocktail was published that used FLB and OME in combination. However, to our knowledge, no interaction tests were carried in that study.[6] The new phenotyping procedure is not only verified by prior probe drug interaction studies, it also has other advantages over

phenotyping cocktails found in literature. Firstly, save probe drugs are used in very small doses. This is possible due to the new sensitive LC-MS/MS methods that were evaluated. Secondly, the new phenotyping procedure is very fast and non-invasive. Urine has to be collected only for 2 h and the results also suggest that the time consuming glucuronide cleavage of the CYP2D6 dependent metabolite dextrorphan, usually carried out before CYP2D6 phenotyping, may be unnecessary.

Most importantly, however, new insights into the phenotype prediction from genotype for CYP2C9 and CYP2D6 could be gained within this study. Nearly 300 phenotyped Caucasian subjects were also genotyped for the most important known variant alleles for CYP2D6, CYP2C9 and CYP2C19 using several established and newly developed genoptyping methods. Therefore, a direct correlation between phenotype and genotype could be conducted for CYP2D6 and CYP2C9. Employing linear modeling, it was possible to assign activity coefficients to each of the detected CYP2D6 and CYP2C9 alleles, thereby estimating their contribution to the resulting enzyme activity. This might facilitate the prediction of the CYP2D6 and CYP2C9 metabolic status of a subject knowing only its respective genotypes. Especially the new CYP2D6 genotype phenotype correlation model might allow for more precise phenotype prediction for the included variant alleles than was possible until now. Additionally, it may also give new insides into the relative expression of the two alleles comprising a genotype.

Taken together, this study substantially contributes to the important research field of pharmacogenetics by (i) developing a save and easy-to-use phenotyping combination for CYP2D6 and CYP2C9, and (ii) by establishing activity coefficients for each of the detected CYP2D6 and CYP2C9 alleles, thereby allowing for a more precise prediction of the phenotype from genotype.

Zusammenfassung

In den 50er Jahren des letzten Jahrhunderts entdeckten Kliniker die Bedeutung der individuellen genetischen Ausstattung eines Patienten für dessen Reaktion auf eine Arzneimitteltherapie. Diese Beobachtungen eröffneten das neue Forschungsfeld der Pharmakogenetik.[1] Der Einfluss der genetischen Prädisposition auf die Effektivität und die Verträglichkeit eines Medikaments scheint vor allem für die Enzyme, die am Medikamentenstoffwechsel beteiligt sind, besonders groß zu sein. Nur 20 % aller Medikamente in Studien zu unerwünschten Arzneimittelwirkungen werden nicht von genetisch polymorphen Enzymen metabolisiert.[2]

Die wichtigsten humanen Enzyme des oxidativen Medikamentenstoffwechsels sind die Cytochrom P450 Monooxygenasen der Familien 1 bis 3 (CYP1–CYP3). Für drei Isoformen dieser Familien (CYP2D6, 2C9 und 2C19) wurden viele genetische Polymorphismen entdeckt, die einen entscheidenden Einfluss auf die Verträglichkeit und Effektivität vieler medikamentöser Therapien ausüben. Ungefähr 5–10 % aller Kaukasier besitzen kein aktives CYP2D6 Enzym,[3] etwa 40 % zeigen eine reduzierte Aktivität des CYP2C9 Enzyms,[4] und bis zu 6 % sind defizient für CYP2C19.[5] Diese eben genannten Enzyme sind am oxidativen Metabolismus einiger Medikamente beteiligt, die schwere Nebenwirkungen hervorrufen können. Dies sind unter anderem trizyklische Antidepressiva und Neuroleptika (CYP2D6), orale Antikoagulantien und Sulfonylharnstoffe (CYP2C9) und Antiepileptika (CYP2C19). Daher kann die Bestimmung der Enzymaktivität eines Patienten vor der Behandlung mit einem solchen Medikament das Auftreten von unerwünschten Nebenwirkungen und Therapiefehlschlägen minimieren. Zur Bestimmung der Enzymaktivität stehen zwei verschiedene Möglichkeiten zur Verfügung. Der erste Ansatz basiert auf der tatsächlichen Enzymaktivität bezüglich einer sorgfältig ausgewählten Testsubstanz (Phänotypisierung). Bei der zweiten Herangehensweise wird versucht die Enzymaktivität vom Genotyp ausgehend vorherzusagen (Genotypisierung).

Mit der vorliegende Studie sollte zu dem wichtigen Forschungsfeld der Pharmakogenetik beigetragen werden, indem zum einen eine einfache und sichere kombinierte Phänotypisierung der drei zuvor erwähnten CYPs (CYP2D6, CYP2C9 und CYP2C19) entwickelt, und zum anderen die Vorhersagekraft des Genotyps für den gemessenen Phänotyp näher untersucht werden sollte.

Es ist uns gelungen eine sichere, einfache, schnelle und kombinierte Phänotypisierung der beiden wichtigen Monooxygenasen CYP2D6 und CYP2C9 zu etablieren. Zunächst wurden dazu Wechselwirkungsstudien mit den ausgewählten Testsubstanzen Dextromethorphan (DEX, CYP2D6), Flurbiprofen (FLB, CYP2C9) und Omeprazole (OME, CYP2C19)

durchgeführt. Es konnte gezeigt werden, dass DEX und FLB als Kombination verabreicht werden können. Die Gabe von OME gemeinsam mit FLB verändert jedoch das Ergebnis der CYP2C9 Phänotypisierung. Dies ist eine neue Erkenntnis, denn noch 2004 wurde ein Phänotypisierungscocktail veröffentlicht, der die Kombination von FLB und OME enthielt. Bei der genannten Studie wurden jedoch, unseres Wissens nach, keine Wechselwirkungsstudien zu den einzelnen Testsubstanz-Kombinationen durchgeführt.[6]

Die von uns entwickelte Phänotypisierungsmethode wurde durch Wechselwirkungsstudien verifiziert. Sie ist jedoch auch in anderen Bereichen den bisher veröffentlichten Phänotypisierungscocktails überlegen. Zum einen wurden nur sehr kleine Dosen sicherer Testsubstanzen verwendet. Dies wurde durch Entwicklung neuer, sensitiver LC-MS/MS Methoden ermöglicht. Zum anderen ist diese neue Prozedur schnell und nicht-invasiv durchführbar. Nach Verabreichung der Testsubstanz muss der Urin nur für zwei Stunden gesammelt werden. Zudem weisen unsere Ergebnisse darauf hin, dass die normalerweise durchgeführte, aufwendige Glucuronidspaltung des CYP2D6 abhängigen DEX-Metaboliten, Dextrorphan, vermutlich vernachlässigt werden kann.

Die wichtigsten Ergebnisse dieser Studie sind jedoch die Einblicke, die in die Vorhersagekraft der CYP2D6 und CYP2C9 Genotypen für die entsprechenden Phänotypen gewonnen werden konnten. Fast 300 phänotypisierte Kaukasier wurden auch in Hinsicht auf die wichtigsten varianten Allele von CYP2D6, CYP2C9 und CYP2C19 mithilfe bekannter und neu etablierter Methoden genotypisiert. Aufgrund der parallelen Phäno- und Genotypisierung konnten Geno- und Phänotyp direkt korreliert werden. Mit linearen Modellen war es möglich, allen detektierten varianten CYP2D6- und CYP2C9-Allelen Aktivitätskoeffizienten zuzuweisen. Diese können nun verwendet werden, um den Beitrag der einzelnen Allele zur resultierenden Enzymaktivität zu bestimmen, wodurch sich die Vorhersage dieser Aktivität ausgehend vom Genotyp verbessern lassen sollte. Besonders für CYP2D6 ermöglicht das neue Korrelationsmodel präzisere Vorhersagen des Phänotyps als bisher veröffentlichte Modelle. Zudem könnte dieses Model neue Einblicke in die relative Expression zweier varianter CYP2D6 allele eines Genotyps erlauben.

Zusammengefasst leistet diese Studie durch die Entwicklung eines sicheren und einfachen Phänotypisierungsprozesses für CYP2D6 und CYP2C9 und durch die Bestimmung von Aktivitätskoeffizienten für alle einbezogenen CYP2D6 und CYP2C9 Allele und der damit verbundenen präziseren Vorhersage des Phänotyps ausgehend vom Genotyp einen wesentlichen Beitrag zum Forschungsfeld der Pharmakogenetik.

1 Introduction and Background

1.1 Aspects of Pharmacogenetics

Vast progress concerning the understanding of interindividual differences in drug response has been made in the last few decades. Until the middle of the last century, the differences in treatment efficacy or drug toxicity were only assigned to the obvious reasons like age, sex, nutritional status, renal and liver function of the patients, drug interaction, concomitant illnesses, course and severity of the treated disease. In the 50's of the last century, however, clinicians documented for the first time the huge influence of genetics on the response of an individual to a specific drug treatment. These findings concerned three widely used drugs, the anti malarial drug primaquine, the muscle relaxant succinylcholine and the anti tuberculosis drug isoniazid. The earliest report described the toxicity of primaquine for a small percentage of Caucasians and African-Americans. Approximately 1 % of the Caucasians and 5-10 % of the African-Americans contracted acute hemolysis after primaquine treatment.[7, 8] Subsequent studies revealed a genetic background to these observations. The absence of the enzyme glucose-6-dehydrogenase in the erythrocytes of affected individuals accounted for primaquine toxicity.[9, 10]

Also in the 1950's, a rare inherited deficiency of plasma cholinesterase was found to explain the prolonged muscle relaxation after treatment with the muscle relaxant succinylcholine during surgeries.[11, 12] And the peripheral neuropathy of some patients after administration of the antituberculosis drug isoniazid could be assigned to inherited differences in the acetylation of the drug.[13, 14]

The relations between genetics and adverse drug reactions, especially for primaquine and succinylcholine were discussed in an important review by Motulsky in 1957.[15] This article delineated and conceptualized the field of pharmacogenetics[1], a term first used in 1959 by Vogel[16] and soon adopted by others working in this field.[17]

In the course of this research field, different kinds of polymorphisms (mutations with a frequency of >1 % in the population) at molecular level are studied. As the most frequent polymorphisms, the single nucleotide polymorphisms (SNPs) play a crucial role in pharmacogenetics. SNPs describe the occurrence of at least two different alleles for one gene, differing only at one specific DNA position. It also includes deletions and insertions of a single nucleotide. A SNP therefore is defined as a point-mutation with a frequency of more than 1 % in a specific population. Occurrences of SNPs differ between different races like e.g. Caucasians, Asians and Colored.

As previously mentioned, pharmacogenetics is the study of genetic factors influencing drug

response. SNPs, which are the most common human genetic polymorphisms, play a crucial role for pharmacogenetics. To date, many SNPs with influence on pharmacokinetics e.g polymorphisms of transporters like MDR1 (the efflux pump p-glycoprotein),[18-20] and pharmacodynamics like polymorphisms of drug targets like the dopamine receptor D2[21, 22] are known. However, all the early examples for pharmacogenetic polymorphisms concern genetic variations of the pharmacokinetic pathway, more specifically the drug metabolizing enzymes. The reason for this is their huge impact on drug treatment response, because of their importance for the detoxification of drugs. First and foremost, the polymorphisms of the most important human enzymes of oxidative drug metabolism, the Cytochrome P450 monooxygenases - first described in the 1970's for the CYP2D6,[23, 24] and the CYP2C9 isoform[25] and in the 1980's for CYP2C19[26] - still seem to have the most extensive clinical consequences.

The ultimate aim of pharmacogenetics is to allow for "individualized medication". This means that, based on the genetic make-up of the patient (e.g. genotype for CYP-isoform metabolizing the administered drug), the phenotype (e.g. metabolizing activity for administered drug) is predicted, thereby facilitating e.g. dose adjustments. To achieve this aim, studies comparing phenotype and genotype have to be conducted. In the following chapters, human cytochromes P450, their importance for human drug metabolism, as well as their different geno- and phenotypes are discussed.

1.2 Human Cytochromes P450 and their role in Pharmacokinetics

Cytochrome P450s (CYPs) constitute a huge superfamily of heme-containing monooxygenases named after the specific absorption band of their reduced complex with carbon monoxide at 450 nm.[27] It is one of the oldest (approximately 1.5 billion years) and most widely spread enzyme family throughout nearly every life form on earth. CYPs can be found in archea, bacteria and in eucaryota. In mammals, they are present in every tissue except skeletal muscle and mature erythrocytes, usually incorporated in membranes of the endoplasmatic reticulum, some are found in the inner membranes of mitochondria.[28, 29]

1.2.1 Human Cytochrome P450

The sequencing of (nearly) the complete human genome in 2003 in context of the "Human Genome Project" and subsequent interpretation of the data, revealed 57 different human CYP genes and 58 pseudogenes (nonfunctional genes, no longer encoding proteins or no

longer expressed in the cell; http://drnelson.uthsc.edu/cytochromeP450.html). All CYPs consist of a heme-moiety as a cofactor and a protein. The iron ion of the heme moiety is complexed by a porphyrine ring. Protein and heme moiety are joined via a cysteine-residue of the protein acting as fifth ligand for the iron atom (Figure 1).[29]

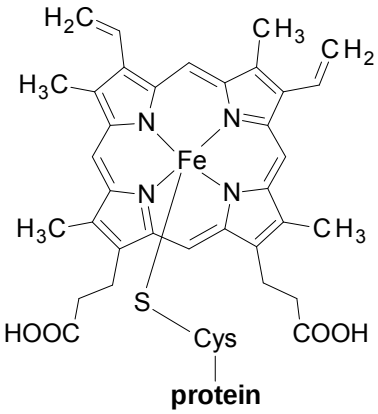


Figure 1 Heme moiety and linked protein, implied by the line.

The sequence of the protein is highly variable. Because of the numerous known variants of CYP proteins, a specific nomenclature has been invented and nearly all Cytochromes P450 are named according to the following scheme: beginning with the root “CYP” followed by an Arabic number denoting the CYP family (approximately 40 % amino sequence similarity), a capital letter naming the subfamily (approximately 50 % amino sequence similarity), ending with another Arabic number for a certain gene encoding a specific isoenzyme (Figure 2).[30]

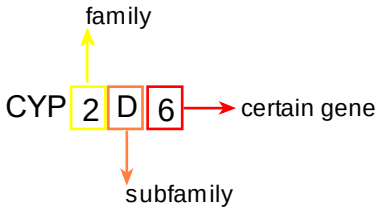


Figure 2 Nomenclature of CYPs.

According to this nomenclature there are 51 families (CYP1-CYP51) of human CYPs. These can be divided into three major groups. The first group consists of the evolutionary conserved families CYP5 – CYP51 with high substrate specificity and affinity. They take part in the metabolism of endogenous compounds. The second group, the CYP4 family, metabolizes fatty acids, related substrates, and a few xenobiotics. And the third group, families CYP1 – CYP3 exhibits less substrate specificity, shows a high rate of genetic polymorphisms and plays a major role in the oxidative metabolism of xenobiotics.[31]

CYPs are monooxygenases. This means that they add a single atom of molecular oxygen to their substrates, thereby catalyzing a broad variety of reactions like hydroxylations,

epoxidation, dealkylation, or sulfoxidation.[29] The details of the catalytic cycle of the CYPs, like electronic states of the complexes and conformational changes of the protein are still under research. The mechanism of substrate hydroxylation, however, is well understood. As depicted in Figure 3 it consists of eight main steps. Without a substrate, the ferric iron (Fe^{III}) is complexed by the four nitrogen atoms of the porphyrine molecule, the sulfur atom of the cysteine residue and the oxygen atom of a water molecule (**I**). After entering the protein pocket, a substrate molecule R-H (R= organic residue) replaces that water molecule (**II**). Then the ferric iron of **II** is reduced to ferrous iron (Fe^{2+}, **III**), which then binds molecular oxygen (**IV**). After another reduction to the negatively charged iron-peroxo intermediate **V** and protonation to complex **VI**, the O – O bond is cleaved, forming the iron-oxo complex **VII** and water is released. The oxygen atom of **VII** then reacts with the substrate R-H (**VIII**) and in the last step the hydroxylated substrate ROH is displaced by a water molecule, yielding the first complex **I**.[29, 32]

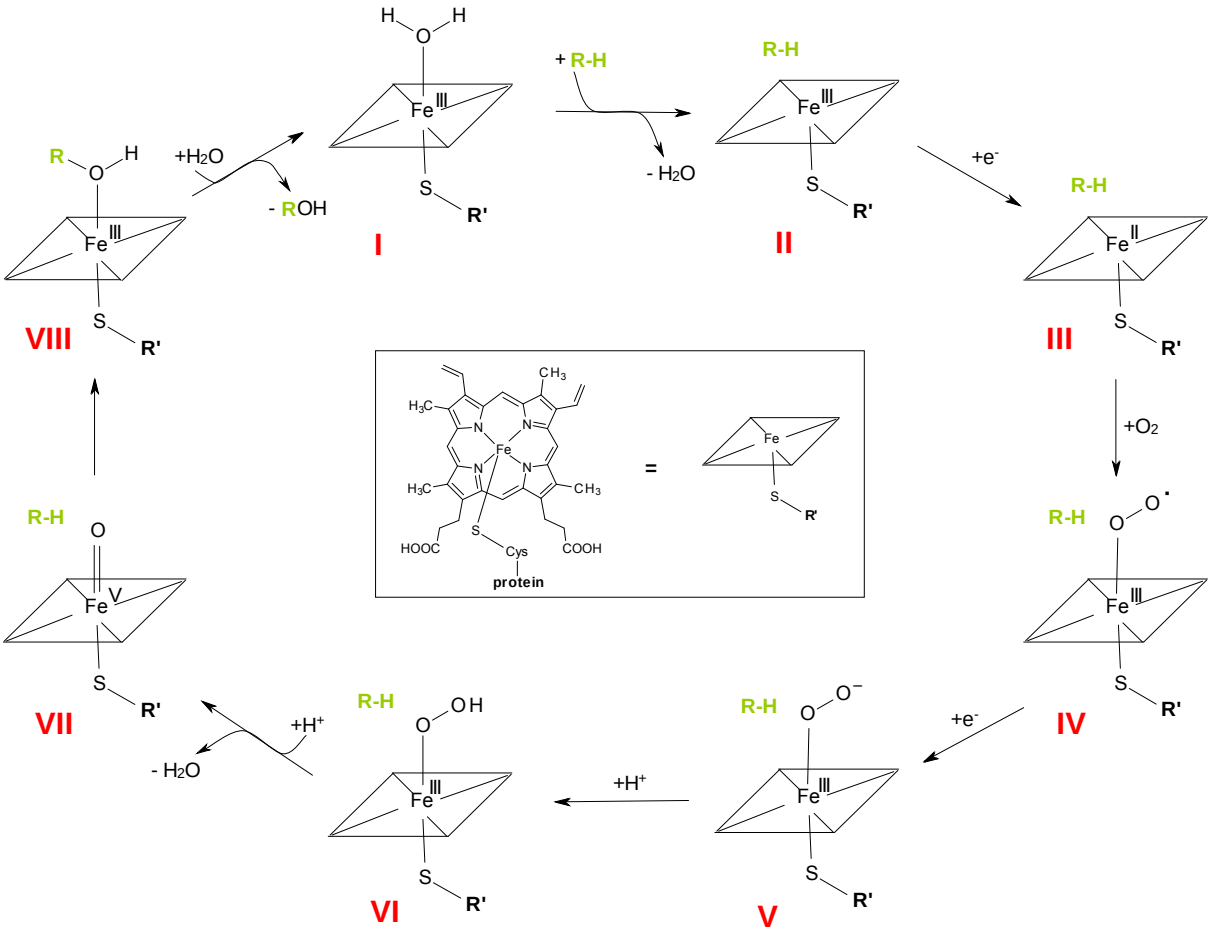


Figure 3 Catalytic cycle of cytochrome P450 as adapted from [24].

The electrons needed for this catalytic cycle are provided by a reducing partner. In humans endoplasmatic reticulum this is the flavoprotein NADPH-cytochrome P450 oxidoreductase.

1.2.2 The role of human CYPs in pharmacokinetics

As mentioned before, families CYP1, CYP2 and CYP3 play a major role in the oxidative metabolism and thereby the pharmacokinetics of drugs. In simple terms, pharmacokinetics describes how the body affects a specific drug after administration. All processes altering the concentration of the drug in the different body compartments over time, like absorption, distribution, metabolism and excretion are included in the concept of pharmacokinetics. Absorption is the movement of the drug into the bloodstream. Distribution concerns the spreading of the drug into different body compartments, like different organs or binding of the drug to specific cell structures. Metabolism is the term for the chemical alteration of the drugs e.g. via oxidation (CYPs) or conjugation to molecules like glutathione. Excretion via urine or feces is the last of the four processes summarized with the acronym ADME. Every drug has specific pharmacokinetic constants, depending on the application form and its physicochemical properties. The most important pharmacokinetic parameters are the bioavailability, the volume of distribution and the clearance of a drug.

The bioavailability is the amount of unchanged drug reaching system circulation. By definition, the bioavailability of an intravenously administrated drug is 100 %. However, if a drug is administered via another route e.g. orally or intramuscularly, the bioavailability decreases. This is due to two different factors. Firstly, the drug must be absorbed into the tissue and eventually into the blood. The absorption is not only influenced by the physicochemical properties of the drug, like solubility, hydrophobicity and pK_a but also by individual properties of the patient, like age, nutritional state, gastric emptying rate, disease state and activity of drug transporters. Secondly, orally administered drugs can posses an extensive first-pass effect. First-pass effect, also known as first-pass metabolism describes the metabolizing of the drug by CYPs and other enzymes in enterocytes (gut wall) and hepatocytes (liver) before the drug can reach system circulation. This effect is depending on the enzyme activity and therefore also on the genetic disposition of the patient concerning the CYPs.

The volume of distribution is the theoretical volume of fluid into which the total amount of administered drug would have to be diluted to produce the concentration in plasma. It is a virtual value not associated with the actual volume of the body, but it is a measure of the distribution of the drug between different body tissues. When a drug is e.g. highly lipophilic, most of the drug will accumulate in fatty tissues and only a small percentage will be present in blood plasma. This would then lead to a high volume of distribution. Therefore the volume of distribution is, like the bioavailability depending on either the physicochemical properties of the drug and also on factors like weight, sex, age and health status of the patient.

The clearance of a drug is the volume of plasma from which the drug is completely removed per unit time. It is proportional to the quantity of drug taken. The clearance includes not only the drug excreted unchanged via the urine (renal clearance) or feces, but also the amount of drug chemically changed by metabolism (intrinsic clearance). The metabolism is usually divided into Phase I and II, Phase I describing the chemical alteration of the drug by e.g. oxidation or reduction and Phase II constituting all conjugations of the drugs or products of Phase I metabolism with e.g. glucuronides or glutathion. The excretion of unchanged drug via bile (feces) and the drug metabolism is summarized as hepatic clearance, as biliary excretion and the majority of metabolism takes place in the liver. This parameter is again depending on drug and body properties and on the genetic disposition of the patient.

Knowing the volume of distribution and clearance, the elimination half life can be calculated. The elimination half life of a drug is the time it takes to halve the plasma concentration of the drug. After four half lifes, elimination of the drug is 94 % complete.

All these parameters do intertwine. Altering the degree or rate of one of these processes can have huge impact on all the other parameters. As explained in the last paragraphs, bioavailability and clearance are dependent on drug metabolism. This means that a patient possessing a decreased activity of one CYP isoform, because of his genetic disposition, will have a higher bioavailability and smaller clearance for drugs metabolized via this specific CYP. This is very important, as many drugs have to be taken daily or regularly, so that an increase of bioavailability, a decrease of clearance and thereby an increase of the half life can lead to accumulation of the drug in the body, ultimately leading to adverse drug reactions (ADRs) or even toxicity of the drug. Whereas a genetic alteration causing an increase of activity of a CYP enzyme can lead to impaired efficacy of the drug therapy, because therapeutic blood levels of the drug cannot be reached. With prodrugs, drugs which have to be metabolized to their active form, the reverse holds true. A carrier of alleles with decreased activity of the CYP enzyme activating the drug cannot reach the therapeutic blood levels, whereas a patient with increased CYP activity can be subject to severe ADRs, due to the accumulation of the active metabolite in the body.

About 56 % of all drugs in ADR studies are metabolized by polymorphic Phase I enzymes, 86 % of these by CYPs. Only 20 % of drugs in ADR studies are not metabolized by polymorphic enzymes.[2] For Germany the annual costs of drug-related hospitalizations were estimated to be 400 Mio Euros.[33] Many ADRs occur because of drug-drug interactions or prescription errors, but approximately 10-20 % of all drug therapies may be improved by a gene based dose adjustment.[2] Mainly for drugs with small therapeutic indices (ratio between the dose of the drug causing toxicity and the dose causing therapeutic effect), which are

metabolized primarily by one polymorphic CYP, the knowledge of the patient's enzyme activity could be relevant.

1.3 Important polymorphic human CYPs

Only CYPs of family CYP1, CYP2 and CYP3 take part in the metabolism of drugs. Approximately 78 % of all drugs metabolized in the liver are subject to a CYP of one these three families.[34] More than 90 % of human drug oxidations are catalysed by the following isoforms: CYP1A2 (4 %), CYP2A6, (2 %), CYP2C9 (10 %), CYP2C19 (2 %), CYP2E1 (2 %), CYP2D6 (30 %) and CYP3A4 (50 %).[2, 3] For CYP3A4 no genetic polymorphism with clinical effects has been discovered until now. Nevertheless, its activity shows a huge interindividual variability due to its sensitivity towards induction or inhibition by food ingredients or concomitantly administered drugs.

The CYP isoforms 2D6, 2C9, and 2C19, however, show a high rate of genetic polymorphisms, some of which have huge influence on drug susceptibility. The polymorphic alleles normally don't possess only one SNP or one deletion, but a certain combination of them. Each specific combination is a haplotype. Normally one of the SNPs or variations of the DNA is essential for the resulting phenotype. When an allele shows one of these essential polymorphisms, the allele is assigned with a star and a specific number after the name of the CYP e.g. CYP2D6*3 for the deletion of the nucleotide adenine at position 2549 (numbers according to gene m33388). However, there can be different combinations of SNPs all containing the phenotype distinguishing variation. For CYP2D6*3 there are two haplotypes, namely CYP2D6*3A and *3B. As they are associated with the same enzyme activity, because only the 2549delA polymorphism determines this, these details will be neglected in the following explanations. The wildtype allele, defined as the allele without polymorphisms and encoding an enzyme with extensive metabolism, is always named *1.

1.3.1 CYP2D6

In the early 1970's two groups Mahgoub et al. in England and Eichelbaum et al. in Germany independently discovered different phenotypes for the response of patients to the antiarrhythmic drug sparteine and the antihypertensive drug debrisoquine, respectively.[23, 24] A few years later the enzyme metabolizing these drugs was found to be CYP2D6.[35] Even though it has a low relative content of approximately 3.9 % in the liver,[36] it participates in the metabolism of 20-25 % of all important clinically used drugs.[37, 38]

CYP2D6 is the most extensively studied human CYP isoform. As depicted in Figure 4 the gene is located on chromosome 22q13.1, near the two CYP2D pseudogenes 7P and 8P. It

consists of nine exons and eight introns and is 4.38 kb long (B, Figure 4). Its open reading frame consists of 1491 base paires and encodes a protein with 497 amino acids.[3] The gene exhibits a high rate of genetic polymorphisms. The sequence of exon 2 is exemplarily shown in Figure 4 D, the red letters indicating positions of known SNPs.

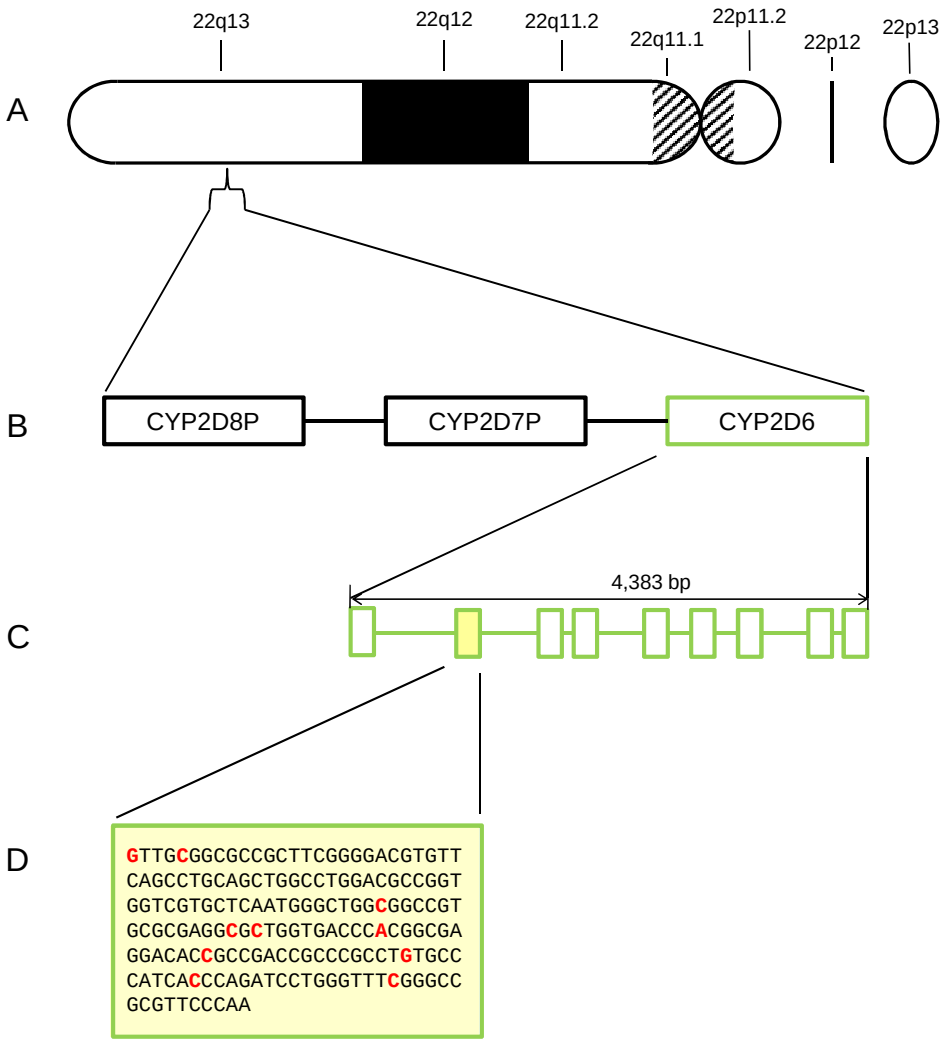


Figure 4 A: Ideogram of chromosome 22; B: Location of the CYP2D6 gene in relation to the pseudogenes; C: Exon and introns of CYP2D6; D: Sequence of exon 2, positions of known SNPs highlighted in red.

In 1997 only 15 polymorphic alleles plus the wildtype allele (CYP2D6*1) were known[39], in 2005 more than 46[38] and today there are over 78 alleles described for CYP2D6 (http://www.cypalleles.ki.se/cyp2d6.htm; access date: 7.11.2010). Many of these alleles encode enzymes with altered activity. Phenotyping studies with sparteine, debrisoquine and dextromethorphan - drugs known to be metabolized via CYP2D6 - showed four distinguished groups of metabolizers: (1) persons with no CYP2D6 activity named poor metabolizers (PMs), (2) persons with impaired enzyme activity named intermediate metabolizers (IMs), (3)

persons with normal enzyme activity named extensive metabolizers (EMs) and in newer studies also (4) persons with increased CYP2D6 activity named ultrarapid metabolizers. According to these phenotypes, the alleles can be roughly divided into four groups: alleles associated with no (null alleles), impaired (IM alleles), extensive (EM alleles) or ultrarapid (UM alleles) CYP2D6 metabolism.[3] Although this classification is not totally satisfying, it will be used in the following chapters, because of its simplicity and for lack of a much better system.

1.3.1.1 Null alleles and associated phenotypes

There are two different reasons why an allele can be associated with no enzyme activity. Either the enzyme encoded has no activity or no full enzyme is built. Only a few known alleles for CYP2D6 are non-functional but full length coded, like CYP2D6*12, *14 and *18. They have a very small allele frequency in Caucasians.[3] All other alleles associated with no enzyme activity don't encode a full enzyme because of their genetic alterations. A summary of the most important Caucasian null alleles, their specific gene variation and their allele frequency in Caucasians can be found in Table 1.

Allele	Major SNPs/alterations responsible for the phenotype, Gene M33388	Effect on protein level	Enzyme activity	Allele frequency in Caucasians[40]	First References
CYP2D6*3	2549delA	259Frameshift	None	0.3 %	[41]
CYP2D6*4	1846G>A	Splicing defect	None	17.2 %	[41-43]
CYP2D6*5	CYP2D6 deleted	CYP2D6 deleted	None	3.2 %	[44, 45]
CYP2D6*6	1707delT	118Frameshift	None	0.6 %	[46-49]

Table 1 Summary of important null alleles, their SNPs and allelic frequencies in Caucasians, the arrow (>) indicates a substitution of one nucleotide by another.

For CYP2D6*3 and CYP2D6*6 the deletion of adenine at position 2549 and the deletion of thymine at position 1707 causes a frameshift, creating a stop codon (TGA) at position 260 and 153, respectively.

The substitution of guanine by adenine at position 1846 (CYP2D6*4) leads to a shift of the consensus acceptor splice site of the third intron by one base. The resulting RNA has an altered reading frame and a premature stop codon.[37]

Even a deletion of nearly the whole CYP2D6 gene has been discovered (CYP2D6*5). The mechanism of this deletion is considered to be an unequal crossover between the two sister

chromatids.[5]

Homozygote carriers of these alleles have a phenotype with no CYP2D6 enzyme activity they are called poor metabolizers (PMs). Approximately 5-10 % of all Caucasians are PMs.[3] CYP2D6 *3, *4, *5 and *6 account for more than 80 % of these.[50] The PM phenotype is therefore relatively well predictable based on the genotype.

In many publications a heterozygous carrier of a null allele and a functional allele is defined as an intermediate metabolizer (IM). [17, 51-53] This definition is not appropriate. As previously mentioned, the classification of the alleles and the genotypes is derived from the resulting phenotype, this means from the actual enzyme activity a patient shows towards a certain drug. Mathematical modeling of early phenotyping studies[54, 55] revealed that approximately 10-15 % of Caucasians are IMs.[56] The Hardy–Weinberg equilibrium, however, predicts a heterozygote frequency of 35-40 % on base of the PM allele frequency.[37] While some of the heterozygous carriers of a null allele and a functional allele belong indeed to the IMs, many heterozygous carriers have a phenotype belonging to the extensive metabolizer (EM) category.[57] Also, not every IM is a carrier of a null allele. There are specific alleles associated with decreased enzyme activity, explaining the phenotype of many IMs.

1.3.1.2 IM alleles and associated phenotypes

As previously mentioned, there are specific alleles causing the IM phenotype in homozygous carriers or heterozygous carriers of an IM allele and a null allele. The most important IM alleles are shown in Table 2.

Allele	Major SNPs/alterations responsible for the phenotype, Gene M33388	Effect on protein level	Enzyme activity[40]	Allele frequency in Caucasians[40]	First References
CYP2D6*9	2615-2617delAAG	K281del	Decreased	2.5 %	[58]
CYP2D6*10	100C>T	P34S	Decreased	2.9 %	[59]
CYP2D6*17	1023C>T, 2850C>T	T107I, R296C	Decreased	-	[60]
CYP2D6*41	2988G>A	Splicing defect	Decreased	7 %	[61-63]

Table 2 Summary of important IM alleles, their polymorphisms and allelic frequencies in Caucasians

CYP2D6*9 lacks codon 281 (position 2615 to 2617 in the gene), therefore the encoded enzyme lacks the amino acid lysine at that position. It was established that this enzyme has decreased activity.[36, 64]

The substitution of cytosine by thymine at position 100 is characteristic for CYP2D6*10. It leads to a substitution of proline by serine at position 34 of the CYP2D6 enzyme. Among microsomal P450s there is a highly conserved proline rich region. Proline at position 34 is part of that region that probably acts as a hinge between the lipophilic membrane anchor and the active site (heme-moiety) of the enzyme. The change of an amino acid in this region may well decrease the activity of the enzyme.[37]

CYP2D6*17 was never found in Caucasians. However, because of its very high allelic frequency of up to 34 % in African or African-Americans,[60] it was included in Table 2. The substitution of threonine by isoleucine at enzyme positon 107 (T107I) seem to account for the decreased activity.

CYP2D6*9 and *10 are only responsible for approximately 20 % of all Caucasian IMs.[39] In 2000, Raimundo et al. discovered a new haplotype, which encodes for an enzyme with impaired activity.[61] Subsequent studies revealed that the important SNP for this new haplotype named CYP2D6*41 is 2988G>A.[62] This SNP seems to affect the splicing of the RNA, thereby decreasing the activity of the resulting enzyme.[65] The genotype consisting of one null allele and CYP2D6*41 accounts for more than 50 % of Caucasian IMs. Therefore approximately 70 % of Caucasian IMs can now be predicted, based on their genotype.[37]

1.3.1.3 EM alleles and associated phenotypes

There are two genes that encode for enzymes with normal activity (Table 3). Per definition, one is the wildtype allele CYP2D6*1, possessing no SNPs. The other one is CYP2D6*2. It is discussed that CYP2D6*2 encodes for an enzyme with impaired activity, but its higher expression levels in cells leads to an EM phenotype.[62] Approximately 70–80 % of all Caucasians are EMs.[3]

Allele	Major SNPs/alterations responsible for the phenotype, Gene M33388	Effect on protein level	Enzyme activity	Allele frequency in Caucasians[40]	First References
CYP2D6*1	None	None	Normal	34.4 %	[66]
CYP2D6*2	2850C>T, 4180G>C	R296C, S486T	Normal	28.7 %	[61, 67, 68]

Table 3 Summary of important EM alleles, their SNPs and allelic frequencies in Caucasians

1.3.1.4 UM alleles and associated phenotypes

The unequal crossover of sister chromatids, leading to the deletion of CYP2D6 (CYP2D6*5), can also cause duplication or multiplication of the gene. Other mechanisms are also discussed.[5] Duplications and multiplications (x2, x3, x4, x5, x13)[69] are known for the following previously discussed genes: *1, *2, *4, *6, *10, *17, *41.[34] The occurrence of two or more copies of an EM-gene (*1 and *2) causes the ultrarapid metabolizing phenotype (UM). This is due to a higher expression level because of the increased gene copy number and thereby a higher concentration of CYP2D6 enzyme in the cells. The allele frequencies of CYP2D6*1xN and CYP2D6*2xN are 0.6 % and 1.3 %, respectively. As the fraction of Caucasians with UM phenotype is 5-10 %, only 20-38 % of all UM phenotypes can be predicted based on allele duplication or multiplication.[37]

1.3.1.5 Important CYP2D6 substrates

CYP2D6 metabolizes a variety of chemical substances. Common characteristics of the substrates are: (1) at least one basic nitrogen atom (at physiological pH), (2) a distance of 5, 7 or for some substrates 10 Å between the basic nitrogen atom and the oxidation site and (3) an aromatic ring near the oxidation site.[70]
Many amino acid residues of the CYP2D6 enzyme have been implicated to play a role in substrate recognition and binding. The crystal structure of CYP2D6 published 2006 by Rowland et al. (Protein data bank code 2F9Q)[71] helps to explain the probable roles of four of these amino acids. Aspartic acid at position 301 (Asp-301) and glutamic acid at position 216 (Glu-216) can bind the substrates due to electrostatic interactions between the positively charged, protonated nitrogen atom of the substrates and the negatively charged carboxyl group of either aspartic or glutamic acid. Phenylalanine at positions 120 and 483 can form π-π interactions with the aromatic ring which is present in nearly every CYP2D6 substrate.[28, 71]

The common features for CYP2D6 substrates—aromatic ring and basic nitrogen atom—can be found in many drugs acting on the central nervous and the cardiovascular systems.[72] Therefore, CYP2D6 takes part in the metabolism of many CNS- and cardiovascular drugs. It catalyses hydroxylations or dealkylations of e.g. tricyclic antidepressants, selective serotonine reuptake inhibitors (SSRIs), antipsychotics, opiods, antihypertensives, beta adrenergic blocking agents and vasodilitators (Table 4).[37] An assembly of all known CYP2D6 substrates can be found online on the Cytochrome P450 Drug Interaction Table.[73]

Therapeutic class	Drug	Pathway catalysed by CYP2D6[37]
Tricyclic antidepressants	Amitriptyline	Benzylic hydroxylation
	Desipramine	Aromatic hydroxylation
	Nortriptyline	Benzylic hydroxylation
Selective serotonin reuptake inhibitors (SSRIs)	Fluoxetine	N-demethylation
	Paroxetine	Demethylenation
	Sertraline	N-demethylation[74]
Other non-tricyclic antidepressants	Venlafaxine	O-Demethylation
	Maprotiline	N-Demethylation[75]
Antipsychotics	Chlorpromazine	Aromatic hydroxylation[76]
	Perphenazine	N-dealkylation
	Risperidone	Aliphatic Hydroxylation
Opioids	Codeine	O-demethylation
	Dihydrocodeine	O-demethylation
	Tramadol	O-demethylation[77]
Antiemetics	Dolasetron	Aromatic hydroxylation
	Ondansetron	Aromatic hydroxylation
Antihypertensives	Debrisoquine	Benzylic hydroxylation
	Guanoxan	Aromatic hydroxylation
	Indoramins	Aromatic hydroxylation
Beta adrenergic blocking agents	Bufuralol	Benzylic hydroxylation
	Bupranolol	Aromatic hydroxylation
	Metoprolol	Aliphatic hydroxylation
	Propranolol	Aromatic hydroxylation
Vasodilitators	Cinnarizine	Aromatic hydroxylation
	Flunarizine	Aromatic hydroxylation

Table 4 Important drugs metabolized by CYP2D6 and the catalyzed pathways.

Many of these CYP2D6 substrates can cause severe ADRs and have narrow therapeutic indices. It is discussed that PMs administered these drugs are prone to dose-dependent ADRs or even drug toxicity due to high blood levels of the drug or drug accumulation. The tricyclic antidepressant nortriptyline, for instance, was shown to cause marked CNS toxicity (e.g. dizziness and sedation) in CYP2D6 PMs or patients receiving co-medication with CYP2D6 inhibitors.[78]

To avoid these ADRs, dose adjustments for CYP2D6 PMs, IMs or UMs can be advisable (Table 5).

Drug	Dose recommendation for genotype relative to recommended dose[79]
Amitriptyline	PMs: 50 %
Nortriptyline	PMs: 50 % IMs: 100 % EMs: 120 % UMs: up to 230 %
Desipramine	PMs: 30 % UMs: higher than average dose, therapeutic drug monitoring recommended
Clomipramine	PMs: 60 %
Fluoxetine	PMs: 70 % at the beginning
Paroxetine	PMs: 20 % at the beginning
Venlafaxine	PMs: 20 %
Maprotiline	PMs: 40 %

Table 5 Dose adjustments for some important antidepressants according to CYP2D6 phenotypes

To conduct these dose adjustments, the metabolic activity of the patient has to be tested. The two different ways to determine the CYP enzyme activity (pheno- and genotyping), their respective advantages and disadvantages will be discussed in chapter 1.4.

1.3.2 CYP2C9

A family study with the sulfonylurea oral hypoglycemic drug tolbutamide conducted by Scott et al. in the late 1970s revealed a trimodal distribution for tolbutamide clearance. This distribution together with pedigree analysis indicated an autosomal transmission of slow or rapid tolbutamide metabolism. Therefore it was concluded that the metabolizing enzyme is genetically polymorphic.[25] Subsequent studies showed that not CYP2D6 but another CYP, later named CYP2C9, was the polymorphic monooxygenase responsible for the metabolism of tolbutamide.[80, 81] The CYP2C9 gene is located within a cluster of CYP2C subfamily genes in the order CYP2C18—2C19—2C9—2C8, mapped on chromosome 10q24.[4] CYP2C9 consists of 50,734 bp, has 9 Exons and its open reading frame is 1,473 bp long.[82] It encodes for an enzyme with 490 amino acids (NCBI Reference Sequence: NP_000762.2). CYP2C9 is with approximately 20 % relative liver content the second most frequent CYP in the liver after CYP3A4. The encoded CYP2C9 enzyme takes part in the metabolism of ~15

% of all clinically used drugs (>100 drugs).[83] To date, more than 34 different alleles for CYP2C9 have been discovered (http://www.cypalleles.ki.se/cyp2c9.htm; access date: 22.11.2010), but only three alleles —*1, *2 and*3— seem to be of importance for Caucasians due to their high allele frequencies. They will be discussed in the following chapter.

1.3.2.1 Alleles and associated phenotypes

In contrast to CYP2D6, no null allele for CYP2C9 has been identified in Caucasians. However, a null allele (CYP2C9*6) was discovered in an African-American patient, showing severe ADRs while treated with the antiepileptic drug phenytoin, a drug known to be metabolized mainly by CYP2C9.[84] To our knowledge, this allele was never included in Caucasian genotyping studies and was therefore included in this work. CYP2C9*6 is characterized by a deletion of adenine at gene position 10601 (positions refer to following gene: GenBank: AL359672.19). This deletion causes a frameshift at protein position 273 thereby inserting an early stop codon leading to a truncated protein with no CYP2C9 activity (Table 6).[85]

Beside the wildtype allele with normal enzyme activity and a high allelic frequency of nearly 80 %, CYP2C9*2 and *3 are the most important known alleles in Caucasians with allele frequencies of approximately 13 % and 7 %, respectively. The nucleoside change from cytosine to thymine at gene position 3608 is the characteristic SNP for CYP2C9*2. It causes a substitution of arginine by cysteine at protein position 144 (Table 6). In vitro studies showed that this substitution has only minor effect on substrate affinity, but the maximum rate of metabolism (V_{max}) is reduced to approximately 50 % of the wildtype enzyme V_{max}.[86] Other publications state a reduction of enzyme activity of only ~20-30 %.[87] The reduction is probably caused by insufficient interaction of the altered enzyme with NADPH-cytochrome oxidoreductase (OR). The positive charge of arginine at position 144 seems to be critical for a sufficient CYP2C9/OR pairing and the substitution of Arg144 by the neutral cysteine therefore decreases the interaction efficacy, leading to reduced enzyme activity.[88] For hetero- and homozygous carriers of the CYP2C9*2 allele this reduction of enzyme activity leads to lower intrinsic clearances of the drugs metabolized by CYP2C9.[86]

The amino acid substitution at protein position 359 caused by the SNP characteristic for CYP2C9*3 (42614A>C; Table 6) is conservative, i.e. isoleucine (wdildtype protein) and leucine (*3-protein) belong to the same amino acid group as they both contain hydrophobic side chains. It is commonly assumed that conservative amino acid changes influence protein functions only marginally. Interestingly, the enzymatic activity of the *3-encoded protein is nevertheless significantly reduced for most of the CYP2C9 substrates.[86] Up to 70 % of the enzyme activity is lost compared with the wildtype enzyme.[87] The crystal structure of

CYP2C9, published 2003 (Protein data bank code 1OG2)[89] showed that position 359 is in proximity to the active site and part of the so-called recognition site 5 of the enzyme. A substitution of isoleucine by leucine at this position may therefore alter access of the substrate to the active centre or may influence substrate binding.[4]

Allele	Major SNPs/alterations responsible for the phenotype, AL359672	Effect	Enzyme activity	Allele frequency in Caucasians[90]	First References
CYP2C9*1	None	None	Normal	79.7 %	[91]
CYP2C9*2	3608C>T	R144C	Decreased	13.1 %	[92]
CYP2C9*3	42614A>C	I359L	Decreased	6.7 %	[93]
CYP2C9*6	10601delA	273Frameshift	None	0 %[94]	[84]

Table 6 Summary of important alleles for CYP2C9, their SNPs and allelic frequencies in Caucasians

The existence of three different alleles for CYP2C9 (*1, *2 and *3) leads to six possible genotypes, namely *1/*1, *1/*2, *1/*3, *2/*2, *2/*3 and *3/*3 with frequencies of approximately 65.3 %, 20 %, 12 %, 0.9 %, 1.4 % and 0.4 %, respectively.[4] To date, only these genotypes are known to be common for Caucasians. None of them leads to a phenotype with no enzyme activity or increased enzyme activity compared with the homozygous wildtype genotype (*1/*1). Therefore, a division of phenotypes into the four groups used for CYP2D6 (PM, IM, EM and UM) is not appropriate. However, the genotypes can be ranked according to the resulting CYP2C9 enzyme activity; for most substrates the metabolic activity decreases in the following order: *1/*1, *1/*2, *2/*2, *1/*3, *2/*3 and *3/*3.[4]

1.3.2.2 Important CYP2C9 substrates

CYP2C9 metabolizes predominantly relatively small lipophilic and weakly acidic molecules. It is still not clear which amino acid residues are crucial for this substrate specificity, even though many suggestions have been made. To date, three different crystal structures of CYP2C9 were published: one ligand-free enzyme (Protein data bank code 1OG2), and two in complex with either warfarin (Protein data bank code 1OG5) or flurbiprofen (Protein data bank code 1R9O).[89, 95] The X-ray structures in complex with the substrate molecules both suggest Val113, Phe114 and Leu366 to take part in the formation of a hydrophobic cleft.[89, 95] The important role of Val113 and Phe114 for substrate binding is also supported by early site-directed mutagenesis studies.[96, 97] However, the assumption that CYP2C9 metabolizes acidic substrates due to basic residues in the active pocket of the enzyme could not be confirmed by the first crystal structure with bound warfarin. Although site-directed

mutagenesis studies and computer-based models suggested that the basic residue of Arg108 binds to acidic substrates, the crystal structure 1OG5 showed that this residue points away from the cavity.[89, 98] In stark contrast, the X-ray structure 1R9O (CYP2C9 with bound flurbiprofen) revealed that the positively charged Arg108 residue points into the cavity site and is therefore able to interact with negatively charged substrates.[95, 98] In addition to that, the flurbiprofen molecule in the CYP2C9-flurbiprofen complex 1R9O is in the proper position for hydroxylation, approximately 4.5 Å away from the heme Fe atom, whereas warfarin in complex 1OG5 is far away (10Å) from the heme Fe atom. Therefore, it was concluded that the non-productive binding of warfarin is an alternative binding mode to the productive binding mode and that CYP2C9 can probably bind more than one molecule.[98] As the crystal structure of the CYP2C9-flurbiprofen complex supports the findings of the previously mentioned mutagenesis and computer-based studies and seems to show a productive binding mode, it is suggested that it is better suited to explain the substrate specificity of CYP2C9 than the warfarin complex.[98]

Obviously the structural determinants for CYP2C9 substrate specificity are still not fully understood. Even though we are not able to satisfyingly explain the preference of CYP2C9 towards small lipophilic and weakly acidic molecules — let alone the difference of substrate affinity between 2C9 and 2C19, which shows only one non-conservative amino acid substitution near the active site compared with 2C9, but does not prefer acidic substrates — many in vitro and in vivo studies prove the existence of that preference.[98]

Many drugs like non steroidal anti inflammatory drugs (NSAIDs), several oral anticoagulants, angiotensin II blockers and sulfonylurea hypoglycemic drugs share the common features for CYP2C9 substrates. A few examples of drugs and the respective pathway metabolized by CYP2C9 can be found in Table 7.

Therapeutic class	**Drug**	**Pathway catalysed by CYP2C9[5]**
NSAIDs	Ibuprofen	Aliphatic hydroxylation[99]
	Lornoxicam	Aromatic hydroxylation[100]
	Meloxicam	Aliphatic hydroxylation[101]
	Naproxen	O-Demethylation[4]
	Piroxicam	Aromatic hydroxylation[4]
	Flurbiprofen	Aromatic hydroxylation[4]
Oral Anticoagulants	S-Warfarin	Aromatic hydroxylation[102]
	Acenocoumarol	Aromatic hydroxylation[102]
	Phenprocoumon	Aromatic hydroxylation[102]
Angiotensin II Blockers	Losartan	Oxidation of hydroxyl function

Therapeutic class	Drug	Pathway catalysed by CYP2C9[5]
		to aldehyde/acid
	Irbesartan	Aliphatic hydroxylation
Sulfonylurea hypoglycemic drug	Glyburide	Aromatic hydroxylation
	Glimepiride	Aliphatic hydroxylation[98]
	Tolbutamide	Aliphatic hydroxylation
Other drugs	Amitriptyline	N-demethylation
	Fluoxetine	N-demethylation
	Fluvastatin	N-dealkylation, aromatic hydroxylation
	Phenytoin	Aromatic hydroxylation
	Torsemide	Aliphatic hydroxylation

Table 7 Important drugs metabolized by CYP2C9 and the catalyzed pathways.

As previously described for CYP2D6, carriers of CYP genes coding less active enzymes are prone to ADRs of drugs metabolized mainly by the affected CYP. This is especially important for drugs with narrow therapeutic indices. However, not all drugs metabolized mainly by CYP2C9 and possessing narrow therapeutic indices are influenced by CYP2C9 genetics. There are basically two possible reasons for that. Firstly, no drug is entirely metabolized by one CYP which means that alternative drug detoxification routes exist. Provided that there is not enough CYP2C9 to metabolize the drug, the effect of that deficiency depends on the amount of drug that may be metabolized by other CYPs or different enzymes. If other pathways e.g. drug oxidation via CYP2C8 can compensate the partly inactive CYP2C9 enzyme, the pharmacogentics of CYP2C9 may only have minor effects on the clearance of the drug and therefore on the caused ADRs. Secondly, it has been suggested that the degree of activity loss for several polymorphic CYP2C9 variants like *3 is substrate-dependant, i.e. the substitution of an amino acid in the heme protein of the CYP2C9 enzyme does not affect all substrates in the same way.[98, 103] Depending probably on the physicochemical features of the drug, amino acid substitution at a specific position may or may not change the enzyme affinity to the substrate. Therefore it is possible that even though a drug is primarily metabolized by CYP2C9, no effect of pharmacogenetic alterations on drug susceptibility can be observed.

Together with the absence of known null alleles in Caucasians, the alternative metabolic pathways especially via the highly similar CYP2C8 and the substrate-depending enzyme activities of the CYP2C9*3 enzyme seem to limit the predictive capability of CYP2C9 geno-

and phenotyping for drug adjustments. However, in vitro and in vivo studies suggest that the detoxification, effectiveness and ADR rates of important drugs like e.g. some NSAIDs or oral anticoagulants are influenced by the genetic polymorphism of CYP2C9.

Several studies investigated the correlation between NSAIDs-induced acute gastric bleeding and the pharmacogenetics of CYP2C9. It could be demonstrated that the frequency of heterozygous and homozygous carriers of the polymorphic alleles CYP2C9*2 and *3 was significantly higher in patients with gastric bleeding compared to a control group.[104]

Treatment with the oral anticoagulant acenocoumarol lead to statistically significantly higher incidence of major bleeding in heterozygous or homozygous carriers of variant CYP2C9 alleles than in carriers of the wildtype genotype *1/*1. Many authors recommend dose adjustments for anticoagulants and other affected drugs according to the CYP2C9 genotype of the patient.[4, 102]

1.3.3 CYP2C19

In 1979, a study on the stereoselective metabolism of S-mephenytoin was conducted at the Vanderbilt University in Nashville, Tennessee. One subject had to withdraw from the study due to extreme sedation after five days of S-mephenytoin administration. Urinary metabolic profiling indicated a deficiency of S-mephenytoin-4'-hydroxylase.[105] Subsequent family studies revealed that not only this subject, but also two of his brothers, but not his parents and the third brother were slow metabolizers for S-mephenytoin. This finding strongly suggested a genetic background for the *S*-mephenytoin-4'-hydroxylase deficiency. [26] It could be proven that not the CYP2D6 polymorphism was responsible for the mephenytoin poor metabolizer phenotype, but that a new pharmacogenetic polymorphism had been discovered.[106] In 1993, the enzyme responsible for the hydroxylation of mephenytoin was found to be CYP2C19.[107]

Like the CYP2C9 gene, the CYP2C19 gene is located within the cluster of CYP2C subfamily genes in on chromosome 10q24. It is 90,209 bp long and consists of nine exons. Its open reading frame of 1,473 bp encodes a protein with 490 amino acids.[108]

CYP2C19 is primarily found in the liver, but a considerable amount is also found in the gut wall. It constitutes 2–5 % of the microsomal hepatic P450 content and takes part in the metabolism of approximately 9 % of all oxidatively biotransformed drugs.[109]

To date nearly 30 allelic variants of CYP2C19*1 have been discovered (http://www.cypalleles.ki.se/cyp2c19.htm; access date: 29.11.2010), but there only seem to be two important alleles for Caucasians beside the wildtype allele, namely CYP2C19*2 and *17.

1.3.3.1 Alleles and associated phenotypes

CYP2C19 is the only enzyme of the CYP2C family which comprises null alleles in an allele frequency high enough to constitute a poor metabolizer phenotype like that for CYP2D6.[34] The allele primarily responsible for the poor metabolizer phenotype in Caucasians is CYP2C19*2 (Table 8).

Allele	Major SNPs/alterations responsible for the phenotype	Effect	Enzyme activity	Allele frequency in Caucasians[90]	First References
CYP2C19*1	None	None	Normal	69.1 %	[91]
CYP2C19*2	19154G>A	Splicing defect	None	12.8 %	[110]
CYP2C19*3	17948G>A	W212X	None	0.1 %	[111]
CYP2C19*17	-806C>T	I331V	Increased	18 %[112]	[113]

Table 8 Summary of important alleles for CYP2C19, their SNPs and allelic frequencies in Caucasians

The major SNP for CYP2C19*2 is the substitution of guanine by adenine at gene position 19154 (positions refer to following gene: NCBI Reference Sequence: NG_008384.1). It leads to an aberrant splice site, alters the reading frame of the mRNA from amino acid 215 and inserts a premature stop codon at position 235, thereby encoding a truncated protein with no enzyme activity.[105] As stated in Table 8 approximately 13 % of all Caucasians are carriers of this allele. Several studies assigned up to 93 % of all Caucasian PMs to be homozygous carriers of CYP2C19*2. [111, 114]

Another CYP2C19 null allele is *3, the single base substitution 17948G>A results in a premature stop codon in exon 4.[5] Even though it has a small allele frequency of 0.1-0.3 %[90] in Caucasians, it is very important for e.g. Asians (allele frequency ~6.8 %)[90] and was therefore included in this study.

Three genotypes lead to an EM phenotype, namely *1/*1, *1/*2 and *1/3. The other three genotypes: *2/*2, *2/*3 and *3/*3 are assigned to the PM phenotype.

Approximately 1–6 %[5] of all Caucasians are poor metabolizers (PMs) and until 2006 the remaining persons were assigned to the extensive metabolizer phenotype (EM). However, in 2006 a new allele (CYP2C19*17) with a high allelic frequency of 18 % in Caucasians and associated with an ultra rapid phenotype (UM) was discovered. In vivo experiments in mice suggest that the major CYP2C19*17 SNP (-806C>T) leads to an increased transcriptional activity of the allele. [113] Therefore, homozygous carriers of CYP2C19*17 possess an UM phenotype, whereas the phenotype of heterozygous carriers of *17 and *1 has an enzyme activity intermediate between EMs and UMs. Subsequent studies confirmed the high allelic

frequency of this polymorphic allele in Caucasians, therefore it was suggested that the former assignment of approximately 90 % of the Caucasians to the EM phenotype has to be reassessed.[115]

1.3.3.2 Important CYP2C19 substrates

CYP2C19 and CYP2C9 differ only in 43 amino acids out of 490 and there is only one non-conservative amino acid substitution near the active site. Nevertheless CYP2C19 shows no specific affinity for CYP2C9 substrates, i.e. small lipophilic and weakly acidic molecules.[98] In contrast, most CYP2C19 substrates are neutral or weakly basic with two or three hydrogen bond acceptors/donors about 4.5 Å apart and 5–8 Å away from the site of metabolism.[116] So far the crystal structure of CYP2C19 has not been solved. Therefore, several mutagenesis studies and in silico techniques, e.g. based on available crystal structures of other CYPs, have been conducted to determine the relevant residues for substrate binding and specificity. A 3D structure modeling of CYP2C19 using the CYP2C9 crystal structure 1R9O as template suggested the following residues to be of importance for CYP2C19 substrate binding: Ile331, Arg333, Asn334 and Gln454. The active cavity of CYP2C19 seems to be mainly hydrophobic.[117]

Many drugs like e.g. several proton pump inhibitors (PPIs), anti-epileptics, tricyclic antidepressants and SSRIs meet the characteristics of CYP2C19 substrates and are therefore metabolized mainly or in part by this enzyme (Table 9). A more detailed listing can be found online on the Cytochrome P450 Drug Interaction Table.[73]

Therapeutic class	**Drug**	**Pathway catalysed by CYP2C19[5]**
PPIs	Lansoprazole	Aromatic hydroxylation
	Omeprazole	Aliphatic hydroxylation
	Pantoprazole	O-Demethylation
	Rabeprazole	O-Demethylation
Anti-epileptics	Clobazam	N-Demethylation
	Diazepam	N-Demethylation
	S-Mephenytoin	Aromatic hydroxylation
	Phenobarbital	Aromatic hydroxylation
Tricyclic antidepressants	Amitriptyline	N-Demethylation
	Clomipramine	N-Demethylation
	Imipramine	N-Demethylation
Other drugs	Carisoprodol	N-Dealkylation[118]

Therapeutic class	Drug	Pathway catalysed by CYP2C19[5]
	Citalopram	N-Demethylation
	Hexobarbital	Allylic oxidation[119]
	Nelfinavir	Aliphatic hydroxylation
	Progesterone	Aliphatic hydroxylation[120]

Table 9 Important drugs metabolized by CYP2C19 and the catalyzed pathways.

Several studies confirmed that PMs of CYP2C19 only need a fraction of the recommended doses for some CYP2C19 metabolized drugs to reach therapeutic blood levels. An important example for this is the treatment with the PPI omeprazole in a dual regimen to eradicate *Helicobacter pylori*. It was found to be sufficient in 100 % of PMs, but only in 41 % of the EMs.[121]

Another drug which is metabolized by CYP2C19 is the anti-epileptic drug clobazam. It was suggested that PMs might be prone to ADRs after treatment with this drug.[105] A case study with a patient presenting unusual ADRs after clobazam administration further supported this hypothesis.[122]

Findings like these suggest that the assessment of CYP activity can be advantageous also for CYP2C19. The two major ways to determine enzyme activity will be discussed in the following chapter.

1.4 Methods for assessment of CYP activity

There are two possibilities to determine the activity of CYP monooxygenases in a living human based on either on the enzymatic phenotype (phenotyping) or the underlying genotype (genotyping).

1.4.1 Phenotyping

For enzyme phenotyping, a so called probe drug is administered. After a defined period of time either blood or urine of the tested person is collected. The concentrations of probe drug and enzyme-specific metabolite are determined in the fluids. Mostly the **m**etabolic **r**atio (**MR**= concentration of probe drug / concentration of metabolite) is used to describe the person`s metabolic activity. According to this MR definition, high MRs belong to a low, low MRs to a high enzyme activity.

1.4.1.1 Probe drugs

As described in the last chapters, the genetic polymorphisms of CYP monooxygenases were discovered due to phenotypic differences in drug response. In the first years after this discovery the drugs for which the phenotypic differences were described (sparteine and debrisoquine for CYP2D6, tolbutamide for CYP2C9 and *S*-mephenytoin for CYP2C19) were used as probe drugs. The formation of at least one of their metabolites depends nearly exclusively on one CYP and the formation rate is depending on the CYP-genotype. Therefore these drugs fulfill the most important characteristic of a probe drug: the concentration of one of their metabolites in urine, plasma or saliva provides a measure for the CYP enzyme activity. There are, however, various other characteristics a drug should fulfill to be classified as a good probe drug. Preferably, the drug has to be (i) as safe as possible, preferable an over-the-counter drug (OTC drug), (ii) still in clinical use so that it is easily available, (iii) easy to detect and stable under used conditions to simplify analytics and (iv) renally excreted to allow for non-invasive phenotyping in urine.

Due to the multitude of requirements it is not easy to establish new probe drugs. A choice of probe drugs based only on *in-vitro* experiments with isolated human CYP enzyme is not advisable, as some probe drugs used in *in-vitro* CYP activity tests do not reflect the enzyme genotype sufficiently. 4-Hydroxydiclofenac a metabolite of the NSAID diclofenac, for instance, was shown to be built nearly exclusively via CYP2C9 oxidation in *in-vitro* tests.[4] Several clinical studies, however, indicate that diclofenac pharmacokinetics are independent of the CYP2C9 genotype.[86] In addition to that, it was shown that it is not possible to distinguish between CYP2C9*1/*1 and CYP2C9*2/*2 based on the urinary MR of diclofenac *in-vivo*.[123]

Therefore, the best probe drug candidates are those which provide maximum interindividual differences that either match the differences detected with the traditional probe drugs sparteine, tolbutamide and S-mephenytoin or reflect the detected genotypes. In the following paragraphs, traditional and well established “newer” probe drugs for CYP2D6, 2C9 and 2C19 will be briefly described.

CYP2D6

The first probe drugs used for CYP2D6 were sparteine and debrisoquine, their CYP2D6-catalysed metabolisms are depicted in Figure 5.

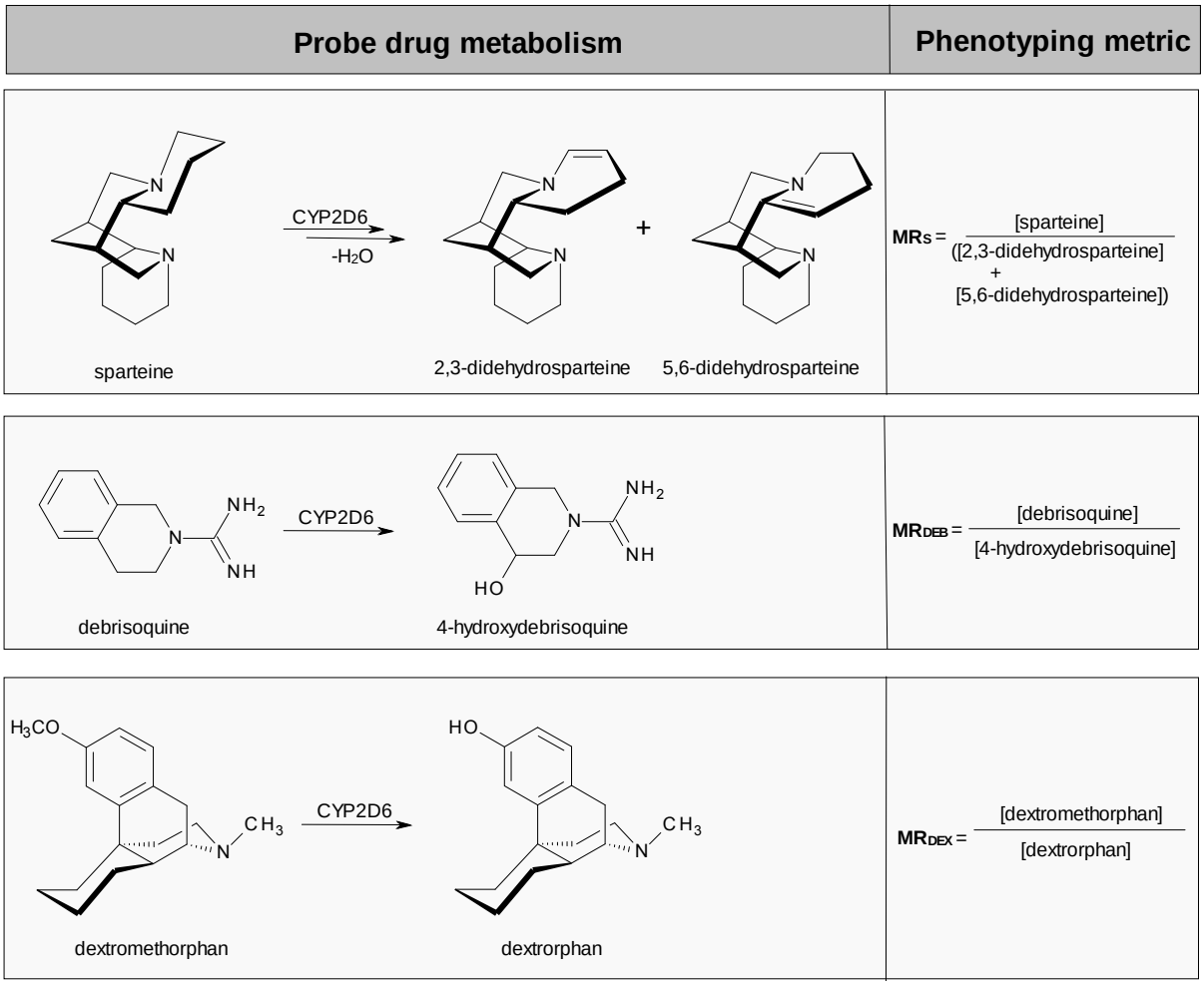


Figure 5 Probe drugs for CYP2D6 and their relevant metabolites.

The antiarrhythmic drug sparteine is oxidized by CYP2D6 and after dehydration the two enamines 2,3- and 4,5-didehydrosparteine are built. The MR_S (with S for sparteine) defined as the ratio of sparteine concentration to the sum of metabolite concentrations in urine at a certain time point after sparteine ingestion can be used as a CYP2D6 phenotyping metric. In most studies 100 mg sparteine sulphate pentahydrate (equivalent to 55.6 mg free base) were used and the drug and metabolite concentrations were measured in the 12 h urine.[124-126] With this dosage and the used time span the $\log_{10}MR_S$ histogram shows a trimodal distribution with antimodes at 1.2 and 20 (Figure 6). Persons with a $MR_S > 20$ are defined as poor metabolizers (PMs), persons with a MR < 1.2 are assigned to be extensive metabolizers (EMs), and a MR between 1.2 and 20 indicate an intermediate metabolizer (IM). In addition to that, an arbitrarily chosen upper limit of $MR_S = 0.2$ was imposed to define the group of ultrarapid metabolizers (UMs).[127]

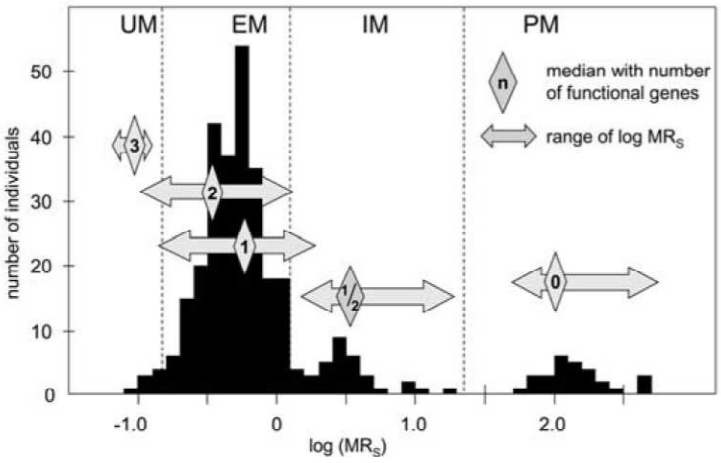


Figure 6 Phenotype definition and distribution in a random European population (N=316). With kind permission from Springer Science+Business Media. [37]

The CYP2D6-dependent metabolite of the antihypertensive drug debrisoquine, 4-hydroxydebrisoquine is formed by an aliphatic hydroxylation (Figure 5). The phenotyping metric is again the metabolic ratio in urine (MR_{DEB} = [debrisoquine]/[4-hydroxydebrisoquine]). A multitude of different dosages and time spans are documented for phenotyping.[124] The results of debrisoquine phenotyping are reported to be consistent with those achieved by sparteine phenotyping.[37]

Sparteine and debrisoquine have narrow therapeutic indices, can cause severe ADRs, and in addition to that are no longer clinically used in many countries including Germany. Therefore, the new probe drug dextromethorphan, an antitussive OTC drug, was validated. Dextromethorphan is demethylated to dextrorphan via CYP2D6 oxidation of the methyl group and elimination of formaldehyde (Figure 5). The urinary MR = [DEX]/[DOR] after different dextromethorphan dosages and time spans has been used in many studies as CYP2D6 phenotyping metric.[124] The distribution of the obtained MR is usually bimodal, which means that phenotyping with DEX in urine is supposed to be limited to distinguish between poor and extensive metabolizers. Nevertheless, until now dextromethorphan is thought to be the best alternative probe drug to the no longer available probe drugs sparteine and debrisoquine.

CYP2C9

The first probe drug used for CYP2C9 phenotyping was the sulfonylurea hypoglycemic drug tolbutamide. Tolbutamide is first oxidized to 4-hydroxytolbutamide via CYP2C9 and further oxidized by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) to yield 4-carboxytolbutamide (Figure 7).[124] The metric used for CYP2C9 phenotyping is again the MR in urine (MR_{TOL}). In contrast to the MRs of e.g. sparteine and dextromethorphan, tolbutamid MR was defined as the sum of metabolite concentrations (4-hydroxy- and 4-

carboxytolbutamide) divided by the concentration of starting compound (tolbutamide). This means that high MRs indicate high and low MRs low enzyme activity. It was supposed in 2003 that the urinary MR_{TOL} after 12 h is a better phenotyping metric than the MRs for losartan or flurbiprofen.[128] However, tolbutamide as a probe drug holds two problems. Firstly, tolbutamide administration is associated with the risk of hypoglycaemia and secondly only a very small amount of unchanged tolbutamide is excreted in urine which limits reliable measurement.[128, 129] Therefore, flurbiprofen a NSAID with a high therapeutic index and higher amounts of unchanged drug excreted in urine, is a good alternative CYP2C9 probe drug. Flurbiprofen is hydroxylated via CYP2C9 to 4-hydroxyflurbiprofen (Figure 7). In 2006, Zgheib et al. showed that the urinary MR after 8 h, originally defined as the concentration of 4.hydroxyflurbiprofen divided by the concentration of unchanged drug, is a suitable phenotyping index for CYP2C9 activity.[129]

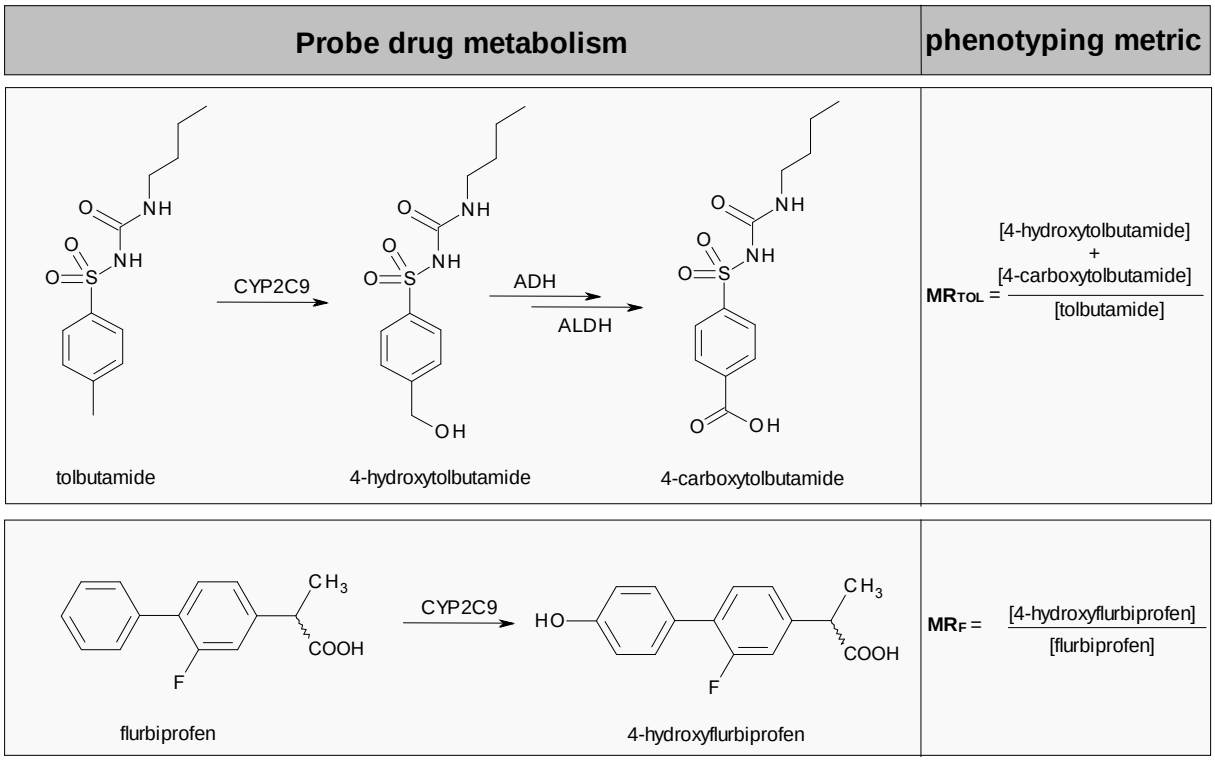


Figure 7 Probe drugs for CYP2C9 and their relevant metabolites. ADH: alcohol dehydrogenase; ALDH: aldehyde dehydrogenase.

CYP2C19

The antiepileptic drug *S*-mephenytoin and the proton-pump-inhibitor (PPI) omeprazole are validated probe drugs for CYP2C19. Their relevant metabolism is depicted in Figure 8.

After administration of racemic mephenytoin, *S*-mephenytoin undergoes fast CYP2C19 catalyzed aromatic hydroxylation to 4-hydroxymephenytoin (Figure 8).[106] *R*-mephenytoin does not appear to be a CYP2C19 substrate but is instead slowly demethylated to yield *R*-

phenylethylhydantoin.[26] It was suggested that the ratio between unchanged *S*-mephenytoin and *R*-mephenytoin (S/R ratio) in 8 h urine after ingestion of 100 mg racemic mephenytoin could be used as CYP2C19 phenotyping metric. The S/R ratio was found to be small in extensive metabolizers and close to one in slow metabolizers.[124] Even though it seems to be possible to distinguish between extensive and slow CYP2C19 metabolizers with *S*-mephenytoin phenotyping, the procedure has some major disadvantages: (i) mephenytoin is a drug with a narrow therapeutic index and the administration can cause severe ADRs, (ii) complex chiral chromatography is required to separate *S*- and *R*-mephenytoin, (iii) the levels of *S*-mephenytoin are often below the limit of quantification and (iv) the S/R ratio of urine samples is not stable with storage at -20 °C.[124]

As previously mentioned, there is another CYP2C19 probe drug, the PPI omeprazole, which has the advantage that its therapeutic index is much wider than that of mephenytoin. Omeprazole undergoes CYP2C19 catalyzed aliphatic hydroxylation to 5-hydroxyomeprazole (Figure 8). Its metabolic ratio ([omeprazole]/[5-hydroxyomeprazole]) in plasma 2 h after ingestion of omeprazole was shown to correlate well with the S/R ratio of mephenytoin.[124] Non-invasive phenotyping with omeprazole e.g. in urine, however, is not validated sufficiently. Regarding the alternative probe drug mephenytoin, omeprazole should nevertheless be preferred due to safety considerations.

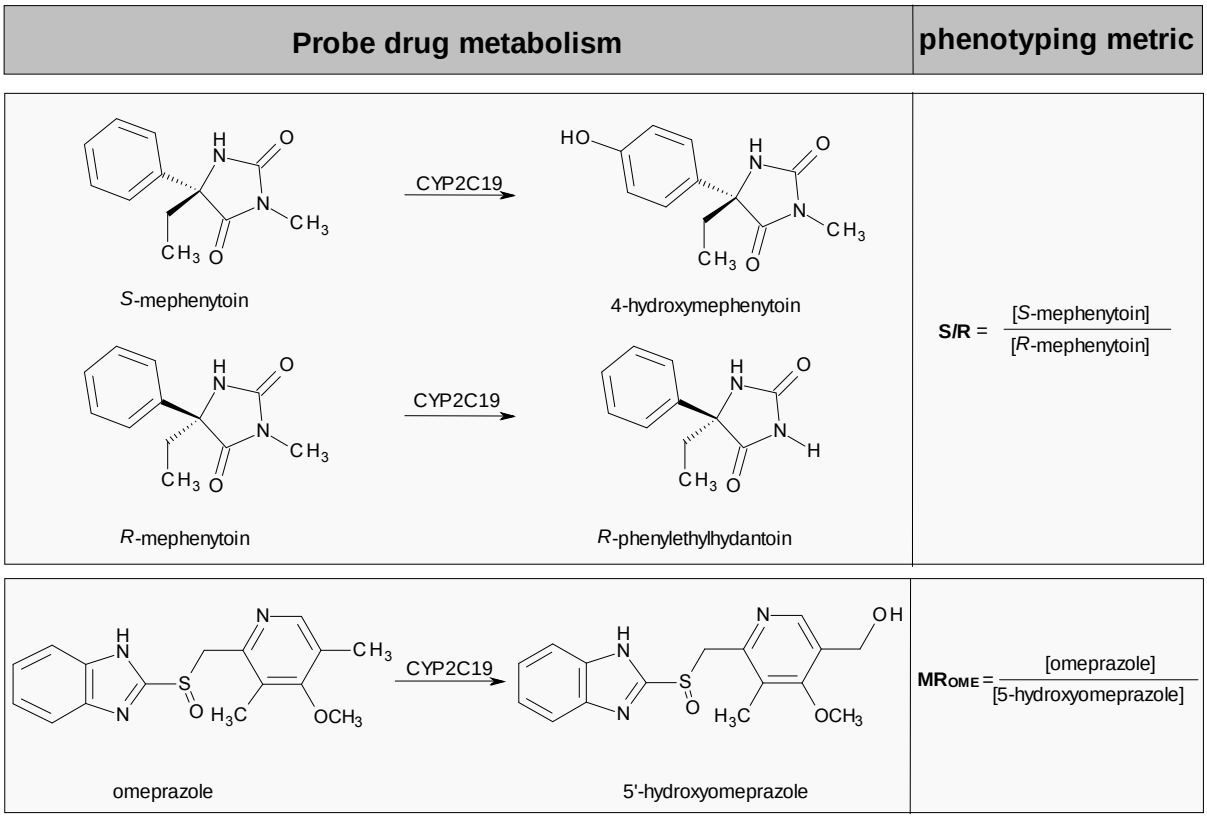


Figure 8 Probe drugs for CYP2DC19 and their relevant metabolites.

1.4.1.2 Analytical methods

To allow for phenotyping, the concentrations of the administered probe drug and its enzyme-specific metabolite have to be determined. In this context sensitive HPLC (high performance liquid chromatography) methods play a crucial role. Many methods based on HPLC coupled with UV or fluorescence detection have been published. HPLC coupled with tandem-mass spectrometry (LC-MS/MS), however, is the method of choice due to its high sensitivity and therefore the possibility to use very small probe drug dosages. The analytical methods used in this study are described in more detail in the method section chapter 6.1.

1.4.1.3 Cocktail approach

In the last decades, there have been attempts to simplify phenotyping procedures by combining up to five probe drugs into a so called probe drug cocktail. A selection of used phenotyping cocktails including CYP2D6, 2C9 and 2C19 are summarized in Table 10. All of these cocktails have at least one of the following disadvantages:

1. some probe drugs have severe ADRs (e.g. mephenytoin or warfarin),
2. high probe drug doses must be administered,
3. the interactions between probe drugs were not tested,
4. the phenotyping process is invasive (measurement in plasma),
5. several measurements at different time points are necessary for phenotyping.

The GW-cocktail which was invented in 1999 used chlorzoxazone together with midazolam.[130] These two probe drugs were later shown to interact so that they should not be used together in one cocktail.[131] A cocktail described in 2004 (cocktail 2004 switzerland Table 10) used the combination of flurbiprofen, omeprazole and dextromethorphan to phenotype CYP2C9, CYP2C19 and CYP2D6, respectively. However the interaction between these probe drugs was not tested before simultaneous administration.[6] Probe drugs with severe ADRs were used in therapeutic doses in the last two cocktails shown in Table 10: 100 mg mephenytoin and 10 mg warfarin in the Pittsburgh and New Cooperstown cocktail, respectively.[132] In addition to these limitations, all of the listed cocktails in Table 10 are invasive i.e. they require at least one blood sample for phenotyping and the time span between administration and collection of blood or urine is very long, rendering the procedure very time consuming.

Probe drug	Tested enzyme	Dose	Subjects	Specimen
GW-Cocktail UK[130, 131]			16	
Caffeine	CYP1A2	100 mg		plasma
Mephenytoin	CYP2C19	100 mg		urine
Diclofenac	CYP2C9	25 mg		urine
Debrisoquine	CYP2D6	10 mg		urine
Chlorzoxazone	CYP2E1	250 mg		plasma
Midazolam	CYP3A4	5 mg		plasma
Cocktail 2004 switzerland[6]			10	
Caffeine	CYP1A2	100 mg		plasma
Flurbiprofen	CYP2C9	50 mg		plasma
Omeprazole	CYP2C19	40 mg		plasma
Dextromethorphan	CYP2D6	25 mg		plasma
Midazolam	CYP3A4	7.5 mg		plasma
"New" Pittsburgh Cocktail[133]			24	
Caffeine	CYP1A2	100 mg		plasma
Mephenytoin	CYP2C19	100 mg		urine
Debrisoquine	CYP2D6	10 mg		urine
Chlorzoxazone	CYP2E1	250 mg		plasma
Dapsone	NAT2	100 mg		plasma
Flurbiprofen	CYP2C9	50 mg		urine
New Cooperstown 5+1[134]			15	
Caffeine	CYP1A2, NAT2, XO	200 mg		urine
Omeprazole	CYP2C19	40 mg		plasma
Dextromethorphan	CYP2D6	30 mg		urine
Warfarin	CYP2C9	10 mg		plasma
Vitamin K		10 mg		

Table 10 Selection of CYP phenotyping cocktails

1.4.2 Genotyping

For genotyping, genomic DNA of the patient has to be isolated from blood cells or tissue. The gene encoding for the respective enzyme is then analyzed by molecular biological means, scanning for polymorphisms known to influence enzyme activity.

Nearly all genotyping methods are based on the polymerase chain reaction (PCR). Kary Mullis developed this method in the early 1980s and was awarded the Nobel Price in Chemistry for this work in 1993.[135, 136] PCR is an easy enzymatic in-vitro method to specifically amplify DNA (chapter 7.1.2). The synthesized amplicons can then be further examined via RFLP (restriction fragment length polymorphism, chapter 7.1.4) or sequencing (chapter 7.1.5). Newer genotyping techniques like SNP multiplex methods (chapter 7.1.6) or DNA microarrays (e.g. Amplichip® from Roche) that allow for detection of multiple

polymorphic alleles simultaneously are also based on PCR and therefore depending on selective gene amplification. This is the biggest challenge for CYP genotyping as the different isoforms and CYP pseudogenes have highly similar DNA sequences.

2 Aim of this work

Cytochrome P450 enzymes of the families CYP1–3 are the most important monooxygenases of the Phase I metabolism of drugs. Many of the genes encoding for these enzymes are genetically polymorphic, leading to enzymes with altered activity. Therefore, the interindividual differences in drug metabolism have a genetic background in addition to environmental factors like age, sex, nutritional status, renal and liver function of the patients, drug interaction, concomitant illnesses, course, and severity of the treated disease. For drugs with small therapeutic indices, especially when given regularly, these genetic predispositions can account for up to 20–40 % of the interindividual differences in drug response. It is estimated that 10-20 % of all drug therapies may be improved by a gene based dose adjustment.[2]

Especially three genetically polymorphic CYP genes, CYP2D6, CYP2C9, and CYP2C19 are important in this context. The encoded enzymes take part in the oxidative metabolism of many administered drugs and as the enzymes encoded by some polymorphic alleles have different activities, the genetic polymorphisms were found to influence the clinical outcome of many drug therapies.

There are two possibilities to determine the activity of CYP monooxygenases in a living human based on either on the enzymatic phenotype (phenotyping) or the underlying genotype (genotyping). For enzyme phenotyping, a so called probe drug is administered. After a defined period of time either blood or urine of the tested person is collected. The concentrations of probe drug and enzyme-specific metabolite are determined in the fluids. For genotyping, genomic DNA of the patient has to be isolated from blood cells or tissue. The gene encoding for the respective enzyme is then analyzed by molecular biological means, scanning for polymorphisms known to influence enzyme activity.

The first aim of this study was to establish an easy phenotyping method that combines the activity determination of all three previously mentioned CYPs (CYP2D6, CYP2C9, and CYP2C19) into one phenotyping cocktail. This cocktail should not only use very small doses of safe probe drugs, preferably over-the-counter drugs, but should also allow for phenotyping by using only one urine sample. For this purpose, new analytical methods to quantify probe drugs and their metabolites using LC-MS/MS should be developed. To ensure that the probe drugs do not interact and mutually influence their respective phenotype, interaction studies should be conducted.

In addition to the development and evaluation of a new non-invasive phenotyping cocktail and appropriate LC-MS/MS analytics, the predictive power of the genotype for the measured phenotype should also be tested. Therefore, the Caucasian subjects phenotyped using the

newly developed cocktail should also be genotyped for the most important alleles of CYP2D6, CYP2C9 and CYP2C19.

3 Materials

3.1 Instrumentation

Equipment	Provider
Autosampler Series 1100	Applied Biosystems, Darmstadt, Germany
Balances, P1000 N and AG245	Mettler-Toledo GmbH, Giessen, Germany
Blood collection system, S-Monovette and multi adapter	Sarstedt, Nümbrecht, Germany
Centrifuges, Eppendorf 5403, 5412, 5414, 5810	Eppendorf, Hamburg, Germany
DC Power Supply, PS 3000	Hoefer, Inc., Holliston, USA
Disposable cup and screw cap, 100 mL	Sarstedt, Nümbrecht, Germany
Glassware	Schott, Mainz, Germany
Heating block, 2099-DA	Gebr. Liebisch Bielefeld, Germany
Horizontal gel systems: S, M, XL	Peqlab Biotechnology GmbH, Erlangen, Germany
HPLC detector, 1100 VWD	Agilent Technologies, Böblingen, Germany
HPLC pump, HP 1050	Hewlett Packard, Minnesota, USA
Imaging system, FluorChemQ	Cell Biosciences, Santa Clara, CA, USA
Magnetic stirrer, Ikamag® RCT	IKA Labortechnik, Staufen, Germany
MassARRAY iPLEX System	SEQUENOM, Inc., San Diego, USA
Mass spectrometer, QTRAP 2000	Applied Biosystems, Darmstadt, Germany
Mini-centrifuge, Microspin FV-2400	Biosan, Riga, Latvia
Microwave oven	De'Longhi, Seligenstadt, Germany
Nanodrop 2000C	Thermo Fisher Scientific, Hamburg, Germany
PCR cycler, Mastercycler gradient	Eppendorf, Hamburg, Germany
PCR tubes, 8-tube strips and caps, 0.2mL, Polypropylene	Thermo Fisher Scientific, Hamburg, Germany
PH-meter pH530	WTW Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany
Polypropylene reaction tubes (0.5 mL, 1.5 mL, 2.0 mL)	Sarstedt, Nümbrecht, Germany
Polypropylene tubes (15 mL, 50 mL)	Sarstedt, Nümbrecht, Germany
Polystyrene cuvettes (10 x 4 x 45 mm)	Sarstedt, Nümbrecht, Germany

Equipment	Provider
Quaternary solvent pump	Applied Biosystems, Darmstadt, Germany
Shaker, L-40	GLW, Würzburg, Germany
SpeedVac, VR-1	Heraeus, Hanau, Germany
Top-loading balance	Sartorius, Göttingen, Germany
TurboIonSpray® (ESI) source	Applied Biosystems, Darmstadt, Germany
Ultrasonic bath, Sonorex,	Bandelin, Berlin, Germany
Urine collecting flask, 2L	Sarstedt, Nümbrecht, Germany
UV-meter, Ultrospec 2000	Pharmacia Biotech, Cambridge, UK
Vortex Duo Press_To_Mix 52	Labinco, Giessen, Germany
Vortex, VF2	IKA Labortechnik, Staufen, Germany
Water Purification Systems, E-Pure Barnstead	Thermo Fisher Scientific, Hamburg, Germany
Winged infusion set, Venofix	B.Braun, Meisungen, Germany
3130 Genetic Analyzer	Applied Biosystems, Darmstadt, Germany

Table 11 Instrumentation

3.2 Software

Software	Provider
Graph pad prism	GraphPad Software, Inc., La Jolla, CA, USA
R Project for Statistical Computing	Free software environment, initially written at the Statistic Department of the University of Auckland, New Zealand
Symyx Draw 4.0	Symyx solutions, Inc., San Ramon, CA, USA
Basic Local Alignment Search Tool (BLAST)	public domain software, developed at the National Institutes of Health (NIH), Bethesda, MD, USA
Primer express	Applied Biosystems, Darmstadt, Germany
Analyst software 1.4.2.	Applied Biosystems, Darmstadt, Germany
NEBcutter V2.0	New England Biolabs, Frankfurt a. Main, Germany
MassArray Assay Design	Sequenom, San Diego, CA, USA
MassArray Typer 3.4	Sequenom, San Diego, CA, USA
Sequencing Analysis Software v5.3	Applied Biosystems, Darmstadt, Germany

Table 12 Software

3.3 Chemicals

Chemicals	Provider
4-Hydroxyflurbiprofen	Gift from Dept. of Experimental and Clinical Pharmacology, College of Pharmacy, University of Minnesota, Minneapolis, USA
5-Hydroxyomeprazole	TRC, Toronto, Canada
Acetic acid, 99.7 %, p.a.	Roth, Karlsruhe, Germany
Acetonitril, HPLC-grade	Roth, Karlsruhe, Germany
Agarose Broad Range	Roth, Karlsruhe, Germany
Agarose LE	Biozym Scientific GmbH, Oldendorf, Germany
Agarose Standard	Roth, Karlsruhe, Germany
Agarose Universal	Rapidozym GmbH, Berlin, Germany
Ammonium acetate, min. 98 %	Sigma-Aldrich, Steinheim, Germany
Dextromethorphan hydrobromide monohydrate	Fagron, Barsbüttel, Germany
Dextromethorphan-d3	TRC, Toronto, Canada
Dextrorphan-d3	TRC, Toronto, Canada
EDTA-dihydrat, 98 %	Sigma-Aldrich, Steinheim, Germany
Flurbiprofen	Sigma-Aldrich, Steinheim, Germany
Ethidium bromide solution 10 mg/mL in H_2O	Sigma-Aldrich, Steinheim, Germany
Flurbiprofen-d3	TRC, Toronto, Canada
Formic acid, ~98 %	Fluka, Sigma-Aldrich, Steinheim, Germany
Glucose-6-phosphate	
Hydrochloric acid, 25 %, ultra pure	Roth, Karlsruhe, Germany
Magnesium chloride	
Methanol, HPLC-grade	Roth, Karlsruhe, Germany
Omeprazole	Sigma-Aldrich, Steinheim, Germany
Omeprazole-d3	TRC, Toronto, Canada
Silver nitrate, ultra pure	AppliChem, Darmstadt, Germany
Sodium hydroxide, pellets, 97 %	Sigma-Aldrich, Steinheim, Germany
Tris, molecular biology grade	AppliChem, Darmstadt, Germany
Disodium hydrogen phosphate dihydrate	Merck KGaA, Darmstadt, Germany

Table 13 Chemicals.

3.4 Probe drugs

Probe drugs	Provider
Dextromethorphan hydrobromide monohydrate	Fagron, Barsbüttel, Germany
Dobendan® Direkt (8.75 mg Flurbiprofen)	Boots Healthcare, Hamburg, Germany Gift from Klosterfrau Healthcare Group, Köln, Germany
Gastracid® (20 mg Omeprazole)	AWD.pharma, Radebeul, Germany Gift from AWD.pharma

Table 14 Probe drugs.

3.5 Enzymes

Enzymes	Provider
AvaII	New England Biolabs, Frankfurt a. Main, Germany
KpnI	New England Biolabs, Frankfurt a. Main, Germany
NsiI	New England Biolabs, Frankfurt a. Main, Germany
BstNI	New England Biolabs, Frankfurt a. Main, Germany
HphI	New England Biolabs, Frankfurt a. Main, Germany
MboII	New England Biolabs, Frankfurt a. Main, Germany
BspMI	New England Biolabs, Frankfurt a. Main, Germany
TfiI	New England Biolabs, Frankfurt a. Main, Germany
LongAmpTaq polymerase	New England Biolabs, Frankfurt a. Main, Germany
Human CYP2C9*1 Supersomes™	BD Biosciences, Woburn, USA
ß-Glucuronidase Type HP-2	Sigma-Aldrich, Steinheim, Germany

Table 15 Used enzymes.

3.6 Buffers and Solutions

Ready-to-use buffers were purchased from New England Biolabs, Frankfurt a. Main, Germany.

Purchased buffers	**Composition**
NEB-Buffer 1	10 mM Bis Tris Propane-HCl 10 mM $MgCl_2$ 1mM DTT pH 7.0 at 25 °C Supplied as a 10X concentrated stock
NEB-Buffer 2	50 mM NaCl 10 mM Tris-HCl 10 mM $MgCl_2$ 1 mM DTT pH 7.9 at 25 °C Supplied as a 10X concentrated stock
NEB-Buffer 3	100 mM NaCl 50 mM Tris-HCl 10 mM $MgCl_2$ 1 mM DTT pH 7.9 at 25 °C Supplied as a 10X concentrated stock
NEB-Buffer 4	50 mM potassium acetate 20 mM Tris acetate 10 mM magnesium acetate 1 mM DTT pH 7.9 at 25 °C Supplied as a 10X concentrated stock
LongAmp Taq Buffer	60 mM Tris-SO_4 20 mM ammonium sulfate 2 mM $MgSO_4$ 3 % Glycerol 0.06 % NP-40 0.05 % Tween-20 pH 9.0 at 25 °C

Purchased buffers	Composition
	Supplied as a 5x concentrated stock
Gel Loading Dye, Blue	2.5 % Ficoll 400 11 mM EDTA 3.3 mM Tris-HCl 0.017 % SDS 0.015 % Bromophenol Blue pH 8.0 at 25 °C Supplied as a 6x concentrated stock

Table 16 Ready-to-use buffers and solutions.

Buffer preparation

DNA extraction:

a) Fresh blood

Lysis buffer

155 mL 1 M NH_4Cl

10 mL 1 M $KHCO_3$

200 µL 0.5M Na_2EDTA

Ad 1 L deionized water

autoclave

b) Frozen blood

Lysis buffer, pH 7.4

1 mL Nonidet

9 g NaCl

Ad 1 L deionized water

(autoclaved)

b) Fresh and frozen blood

SE (saline-EDTA) buffer, pH 8.0

4.38 g NaCl

50 mL EDTA-solution (0.5 M, pH 8.0)

Ad 1 L deionized water (autoclaved)

10 x TE (Tris-EDTA) buffer, pH 8

12.2 g Tris

2.9 g EDTA

Ad 1 L deionized water

(with HCl to pH 8)

Autoclave

1 x TE

100 mL 10 x TE buffer

900 mL deionized water

(autoclaved)

Gel electrophoreses:

10 x TAE (Tris-acetate-EDTA) buffer, pH 8

48.4 g Tris

11.4 mL acetic acid

20 mL EDTA (0.5 M)

Ad 1L deionized water

1 x TAE:

100 mL 10 x TAE buffer

900 mL deionized water

Agarose gel:

1 g (1 %), 2 g (2 %) or 3 g (3 %) agarose

100 mL 1 x TAE buffer (recipe see above)

Boil until agarose is completely dissolved

5 µL ethidium bromide solution (10 mg/mL in H_2O)

Glucuronide cleavage:

0.2 M Sodium acetate solution, pH 4.7

5.44 g $NaC_2H_3O_2 \cdot 3H_2O$

200 mL water (millipore)

Sequencing:

Precipitant:

10 mL Ethanol

400 µL 3 M Sodium acetate solution (pH 4.8)

6 mL deionized water

3.7 Kits

3.7.1 DNA extraction kit

NucleoSpin® Tissue Kit for isolation of DNA from buffy coats was purchased from Macherey-Nagel GmbH & Co. KG**,** Düren, Germany. It contains:

Macherey-Nagel name	**Purpose**	**Composition according to safety sheets**
buffer T1	lysis buffer	
buffer B1	mix to yield buffer B3: binding buffer	contains guanidinium chloride
buffer B2		
buffer BW	wash buffer	< 56 % guanidinium chloride dissolved in 25 %

Macherey-Nagel name	Purpose	Composition according to safety sheets
		isopropanol
buffer B5	wash buffer concentrate	
buffer BE	elution buffer	5 mM Tris/HCl solution at pH 8.5
proteinase K	proteinase	
buffer PB	proteinase buffer	

Table 17 Macherey-Nagel kit contents.

3.7.2 Sequencing Kit

For sequencing, the BigDye® Terminator v1.1 Cycle Sequencing Kit was purchased from Applied Biosystems, Darmstadt, Germany.

	Composition
Ready Reaction Mix	dNTPs, BigDye terminators, polymerase, sequencing Buffer (5X)

Table 18 BigDye® Terminator v1.1 Cycle Sequencing Kit contents.

3.8 Oligonucleotides

3.8.1 Primers used for PCR

All primers were purchased from invitrogen GmbH, Darmstadt, Germany.

Primer name	Primer sequence
cyp-17f	5'–TCCCCCACTGACCCAACTCT–3'
cyp-207f	5'–CCCTCAGCCTCGTCACCTCAC–3'
CYP2C9*2 SKf	5'–TACAAATACAATGAAAATATCATG–3'
CYP2C9*2 SKr	5'–CTAACAACCAGGACTCATAATG–3'
CYP2C9*3 SK f1	5'–AATAATAATATGCACGAGGTCCAGAGATGC–3'
CYP2C9*3 SK f2	5'–AATAATAATATGCACGAGGTCCAGAGGTAC–3'
CYP2C9*3 SK r	5'–GATACTATGAATTTGGGGACTTC–3'
CYP2D6 P81	5'–CGTCTAGTGGGGAGACAAAC–3'
CYP2D6 P92	5'–CTCAGCCTCAACGTACCCCT–3'
CYP2D6*10 Xu r UR	5'–CACCATCCATGTTTGCTTCTGGT–3'
CYP2D6*4 H1newf	5'–TCCCAGCTGGAATCCGGTGTCG–3'
CYP2D6*4 H2newr	5'–GGAGCTCGCCCTGCAGAGACTCCT–3'
CYP2D6*4 H7	5'–CGAAAGGGGCGTCC–3'
CYP2D6*4 HBmut	5'–TCTCCCACCCCCAA–3'
CYP2D6*6 H11	5'–TCCTCGGTCACCCA–3'
CYP2D6*6 HTmut	5'–GTCGCTGGAGCAGG–3'
CYP2D6*5-Dlow	5'–CAGGCATGAGCTAAGGCACCCAGAC–3'

Primer name	Primer sequence
CYP2D6*5-DPKlow	5'–GCCGACTGAGCCCTGGGAGGTAGGTA–3'
CYP2D6*5-DPKup	5'–GTTATCCCAGAAGGCTTTGCAGGCTTCA–3'
CYP2D6*5-Dup	5'–CACACCGGGCACCTGTACTCCTCA–3'
CYP2D6*10 P11	5'–TCAACACAGCAGGTTCA–3'
CYP2D6*10 P12	5'–CTGTGGTTTCACCCACC–3'
CYP2D6*10 Xu f	5'–CCATTTGGTAGTGAGGCAGGTAT–3'
CYP2D6*10 Xu r	5'–CACCACCCATGTTTGCTGGTGGT–3'
cyp-32r	5'–CACGTGCAGGGCACCTAGAT–3'
P5	5'–AGGCCTTCCTGGCAGAGATGAAG–3'
P62	5'–CCCCTGCACTGTTTCCCAGA–3'
Xu 1B1 forward	5'–GCAAATAAAGGGGAATATTTCTC–3'
Xu 1B1 reverse	5'–AGAGATGTAATTAAATGTATAC–3'

Table 19 primers used for PCR.

3.8.2 Primers used for MassArray iplex® method

(In collaboration with Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg)

SNP	Primary PCR primers	Extend primer
CYP2C19*2 (19154G>A)	F: 5'–***X***–CTTTCCATAAAAGCAAGGTT–3' R: 5'–***X***–GCAATAATTTTCCCACTATC–3'	5'–tcAGTAATTTGTTATGGGTTCC–3'
CYP2C19*3 (17948G>A)	F: 5'–***X***–GACTGTAAGTGGTTTCTCAG–3' R: 5'–***X***–AACATCAGGATTGTAAGCAC–3'	5'–CTTGGCCTTACCTGGAT–3'
CYP2C19*17 (-806C>T)	F: 5'–***X***–CAAATTTGTGTCTTCTGTTC–3' R: 5'–***X***–GAGCTGAGGTCTTCTGATGC–3'	5'–TTTGTGTCTTCTGTTCTCAAAG–3'
CYP2C9*3 (42614A>C)	F: 5'–***X***– CTACACAGATGCTGTGGTGC–3' R: 5'–***X***– TGTCACAGGTCACTGCATGG–3'	5'–TGCACGAGGTCCAGAGAT–3'
CYP2C9*6 (818delA)	F: 5'–***X***–CACAAATTCACAAGCAGTCAC–3' R: 5'–***X***–TATTGATTGCTTCCTGATG–3'	5'–TAAGCTTTTGTTTACATTTTACCT–3'
CYP2D6*2_1 (2850 C>T)	F: 5'–***X***–CCCTGAGAGCAGCTTCAATG–3' R: 5'–***X***–TGGTCACCATCCCGGCAGA–3'	5'–tCTTCAATGATGAGAACCTG–3'
CYP2D6*2_3 (1661G>C)	F: 5'–***X***–TTGCCCAGGCCCAAGTTGC–3' R: 5'–***X***–TAATGCCTTCATGGCCACGC–3'	5'–CAAGTTGCGCAAGGTGGA–3'

SNP	Primary PCR primers	Extend primer
CYP2D6*2_4 (3183G>A)	F: 5'–***X***–TGGTCACCCATCTCTGGTC–3' R: 5'–***X***–TGGGTGCTGACCCATTGTG–3'	5'–ctcgGGTCGCCGCACCTGCCCTATCA–3'
CYP2D6*3 (2549delA)	F: 5'–***X***–TCCAAAAGGCTTTCCTGACC–3' R: 5'–***X***–AAGGCCTCAGTCAGGTCTC–3'	5'–AGCTGCTAACTGAGCAC–3'
CYP2D6*6 (1707delT)	F: 5'–***X***–AACTTGGGCCTGGGCAAGAA–3' R: 5'–***X***–TTTGTGCCCTTCTGCCCATC–3'	5'–ggAGAAGTCGCTGGAGCAG–3'
CYP2D6*9 (2615-2617 delAAG)	F: 5'–***X***–ATGAGCTGCTAACTGAGCAC–3' R: 5'–***X***–AACCCACCACCCTTGCCCC–3'	5'–ACCTGACTGAGGCCTTCCT–3'
CYP2D6*17 (1023C>T)	F: 5'–***X***–GCCGACCGCCCGCCTGTG–3' R: 5'–***X***–GCTGCTTGCCTTGGGAACG–3'	5'–ccctACCGCCCGCCTGTGCCCATCA–3'
CYP2D6*41 (2988G>A)	F: 5'–***X***–GTGTCCCAGCAAAGTTCAT–3' R: 5'–***X***–TGAGCCCATCTGGGAAACA–3'	5'–CCCCCGCCTGTACCCTT–3'

Table 20 Primers used for MassArray iplex *X* = 10-mer tag: ACGTTGGATG

4 Methods for Phenotyping

4.1 Probe drugs

The probe drugs used in this study were carefully chosen according to the considerations explained in chapter 1.4.1.1. For CYP2D6 phenotyping, 10 mg dextromethorphan dissolved in 100 mL water was used. CYP2C9 was phenotyped with 8.75 mg flurbiprofen as lozenge (Dobendan Direkt®) and 20 mg omeprazole as enteric coated tablet (Gastracid®) was the probe drug for CYP2C19 in this study.

4.2 Subjects

In laboratory courses (WS07–SS09) in pharmacology and toxicology altogether 350 students of the Würzburg University registered for phenotyping in course of a study that had been approved by the Regional Ethical Committee of the Würzburg University (study number 37/07). Of those, 48 subjects were excluded from statistics because they were not of Caucasian origin. The resulting cohort consisted of 213 females with a mean ± SD age of 23 ± 2 years (range, 20–34) and 88 males aged 24 ± 2 years (range, 21–33). All tested subjects were of good health and gave informed written consent.

4.3 Phenotyping studies

4.3.1 General phenotyping procedure for flurbiprofen and dextromethorphan

All phenotyping experiments were carried out in the following manner: After voiding the bladder, the participants ingested the probe drugs (for dosage see 4.1). After 2 h, urine samples were collected, divided into aliquots, and stored at −20 ºC.
Individuals were classified on the basis of a metabolic ratio (MR) calculated by dividing the concentration of the parent drug DEX or FLB by the concentration of the CYP–dependent metabolite dextrorphan (DOR) or 4-OH flurbiprofen (OHF) in urine:

- MR_{DEX} = [DEX]/[DOR] (metric for CYP2D6 activity)
- MR_{FLB} = [FLB]/[OHF] (metric for CYP2C9 activity).

4.3.2 Preliminary studies for phenotyping with omeprazole

The proton pump inhibitor omeprazole (OME) should be used as probe drug for CYP2C19 phenotyping. OME is a so-called prodrug that rearranges under acidic conditions to an active sulfenamide analog. Due to this reaction, it was necessary to evaluate the stabilities of OME

and its CYP2C19-dependent metabolite 5-hydroxy omeprazole (OHOME) in urine with physiological pH values.
In addition to that, blood sampling for the genotyping of 60 subjects was conducted 2–3 hours after ingestion of 20 mg OME. This approach allowed the preliminary analysis of OME and OHOME concentrations in plasma.

4.3.3 Probe drug interaction studies

As previously mentioned, a probe drug cocktail containing dextrometorphan (DEX), flurbiprofen (FLB) and omeprazole (OME) should be designed to phenotype CYP2D6, CYP2C9 and CYP2C19, respectively. To exclude mutual influence on metabolism of DEX, FLB, and OME and therefore on the respective phenotypes, probe drug interaction studies were conducted. For this purpose, at least 30 subjects were administered one probe drug (probe drug 1) on the first day of the study. The associated phenotype was calculated based on the metabolic ratio. After a washout period of at least one week, the same subjects took probe drug 1 in combination with another probe drug (probe drug 2). The phenotype was calculated again and through statistical testing the influence of probe drug 2 on the phenotype of probe drug 1 was examined. The conducted studies are summarized in Table 21.

Interaction probe drug 2 → probe drug 1	**Participants**	**Day 1**	**Day 2**
FLB → DEX	44	DEX	DEX + FLB
DEX → FLB	30	FLB	FLB + DEX
OME → FLB	32	FLB	FLB + OME

Table 21. Interaction studies

4.3.4 Combined phenotyping with DEX and FLB

The interaction studies showed that the administered doses of DEX and FLB had no influence on the calculated MR_{FLB} and MR_{DEX}, respectively. Therefore phenotyping of CYP2D6 (DEX) and CYP2C9 (FLB) was combined. Altogether 353 participants were administered DEX directly after FLB ingestion. Urine was collected 2 h after administration.

4.4 LC-MS/MS analytics

4.4.1 Standard and sample preparation

4.4.1.1 Dextromethorphan

Initial stock solutions of dextromethorphan, dextrorphan, dextromethorphan-d3 and dextrorphan-d3 were prepared by dissolving 1 mg in 1 mL water.

4.4.1.1.1 Without glucuronide cleavage

Standard solutions, urine standard samples and sample preparation were done according to a method developed and published by our laboratory [137], however different internal standards were used.

Standard solutions and urine standard samples

For the method without glucuronide cleavage, six calibration standards were prepared by spiking 250 µL blank urine with 5µL of a standard solution (**A** to **F**, Table 22) containing DEX and DOR in H_2O to give concentrations from 2.5 nM to 1 µM in urine

[DEX] and [DOR]	**A**	**B**	**C**	**D**	**E**	**F**
in standard solution	125 nM	250 nM	1.25µM	5 µM	25 µM	50 µM
in calibration standard	2.5 nM	5 nM	25 nM	100 nM	500 nM	1 µM

Table 22 DEX and DOR standard solutions and calibration standards for method without glucuronide cleavage

Sample preparation

500µL urine were combined with 5µL internal standard (12 µM DEX-d3, 12 µM DOR-d3 in water). The sample was centrifuged at 10,000 × *g* for 10min and 10µL of the supernatant were injected into the LC-MS/MS.

4.4.1.1.2 With glucuronide cleavage

Standard solutions, urine standard samples and sample preparation were done according to a method developed in our laboratory.[138]

Standard solutions and urine standard samples

For the method with glucuronide cleavage, seven calibration standards were prepared by spiking a mixture of 6 µL blank urine, 6 µL sodium acetate solution (pH 4.7, see chapter 3.6), and 13 µL methanol with 8µL of a standard solution (**A** to **G**, Table 23) containing DEX and

DOR in H_2O to give concentrations from 1 nM to 5 µM in urine. Of these mixtures 10 µL were injected into the LC-MS/MS.

[DEX] and [DOR]	A	B	C	D	E	F	G
in standard solution	50 nM	250 nM	1.25µM	5 µM	25 µM	50 µM	250 µM
in calibration standard	1 nM	5 nM	25 nM	100 nM	500 nM	1 µM	5µM

Table 23 DEX and DOR standard solutions and calibration standards for method with glucuronide cleavage

Sample preparation

ß-Glucuronidase (100 000 u/mL) was diluted with sodium acetate solution (pH 4.7, see chapter 3.6) 1:50 (v/v) to yield a ß-glucuronidase solution with an activity of approximately 2000 U/mL. The solution was mixed gently by inverting the tube so that the enzyme was not destroyed.

50 µL urine collected 2 h after dextromethorphan ingestion were combined with 50 µL of the freshly prepared ß-glucuronidase solution (2000 u/mL) and mixed gently by pipetting several times. Then the mixture was incubated for 18h at 37 °C under gentle agitation. The reaction was stopped by adding 100 µL methanol (0 °C). The denatured proteins were separated by centrifugation (4 °C, 10 min, 20000xg). 26 µL supernatant was transferred into a glas vial and mixed with 370 µL H_2O and 4 µL internal standard (12 µM DEX-d3, 12 µM DOR-d3 in water). Of this mixture, 10 µL were injected into the LC-MS/MS.

4.4.1.2 Flurbiprofen

Synthesis and purification of 4-hydroxyflurbiprofen-d3

A 500 µL reaction mixture containing 100 pmol human CYP2C9*1, 0.65 µmol NADP+, 1.65 µmol glucose-6-phosphate, 0,2 U glucose-6-phophate dehydrogenase, 1.65 µmol magnesium chloride and 0.1 µmol flurbiprofen-d3 in 100mM Tris-HCl (pH 7.5) was incubated at 37 °C for 60 min. After incubation, the reaction was stopped by addition of 500 µL acetonitril and centrifuged (14 000 x g) for 5 min.

The supernatant was purified using HPLC-UV equipped with a hypersil ODS 5 µ (250 x 4.6 mm; Agilent, Böblingen, Germany):

The mobile phase consisted of (A) water, 0.1 % trifluoroacetic acid (TFA) (B) acetonitrile, 0.1 % TFA. The flow rate was 1 mL/min and the injection volume 50 µL. A linear gradient starting with 80 % A to 30 % A within 15 min was used. Within 1 min, the ratio was set back to starting conditions and the system was equilibrated for 10 min. The fraction containing OHF–d3 (retention time: 10 min) was collected and dried under vacuum in a SpeedVac.

The residue was dissolved in approximately 615 µL acetonitrile and a photometer was used to measure 4-hydroxyflurbiprofen-d3 concentration.

Quantification of 4-hydroxyflurbiprofen-d3

To quantify a substance using the Lambert-Beer law, the molar absorptivity α of that substance has to be established.

Lambert-Beer law: $E = l \cdot c \cdot \alpha$

E = extinction, c = substance concentration, *l* = path length and α = molare absorptivity.

The molare absorptivity α of 4-hydroxyflurbiprofen-d_3 is not known. Therefore the absorptivity of 4-hydroxyflurbiprofen was determined and used for calculation of OHF-d_3 concentration under the presumption that α is identical for the substances.
An absorption of 0.808 for a OHF solution (10 µg/mL in H_2O = 38 µM) at 260 nm was measured using a photometer with a path length *l* of 0.125 cm. The molare absorptivity α_{260}(OHF) is therefore 184.6 x 10^3 L mol^{-1} cm^{-1}.
With this value the amount of OHF-d_3 synthesized by CYP2C9 catalysed oxidation of FLB-d_3 could be calculated. The process yielded 34.3 nmol OHF-d_3 ([OHF-d_3] = 14.7 µg/mL CH_3CN).

Standard solutions and urine standard samples

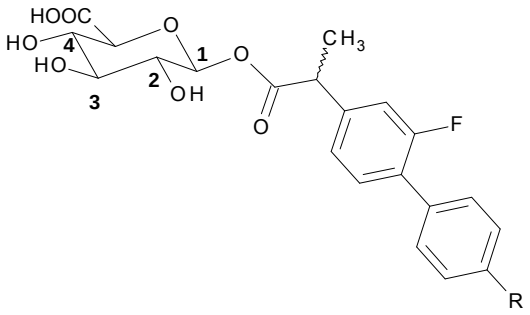
Flurbiprofen (FLB), 4-hydroxyflurbiprofen (OHF) and flurbiprofen-d_3 (FLB-d_3) were dissolved in acetonitrile (1 mg/mL) and stored at -20 °C.
Urine standard samples were prepared by spiking 200 µL blank urine with 5 µL of a standard solution. In that manner, 9 calibration standards were produced, containing FLB and OHF from 50 pmol/mL to 29 nmol/mL urine (Table 24).

[FLB] and [OHF]	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**
standard solution	2 µM	4 µM	8 µM	40 µM	80 µM	0.2 mM	0.4 mM	0.8 mM	1.2 mM
calibration standard	50 nM	0.1 µM	0.2 µM	1 µM	2 µM	5 µM	10 µM	20µM	30 µM

Table 24 FLB and OHF standard solutions and calibration standards.

Sample preparation

FLB- and OHF-acylglucuronides can rearrange to structural isomers via acyl migration of the drug from position 1 to positions 2, 3 and 4 of the glucuronic acid moiety (Figure 9).[139]



FLB-glucuronide : **R** = H

OHF-glucuronide: **R** = OH

Figure 9 FLB- and OHF acylglucuronides with positions of the glucuronic acid moiety.

Glucuronide cleavage with ß-glucuronidase is only possible for 1ß-glucuronides and therefore treatment of FLB and OHF-acylglucuronides with ß-glucuronidase does not lead to a complete cleavage of glucuronides.

To allow for complete glucuronide cleavage, a new cleaving method was developed based on the basic saponification of esters:

After centrifuging the urine samples for 10 min at 22 000 x g, 1 mL of the supernatant was mixed with 60 µL 3N NaOH, incubated at 90 °C for 10 min, cooled on ice and neutralized with 30µL 6N HCl. Afterwards, 5 µL IS (containing 100 pmol FLB–d3 and 25 pmol OHF–d3) were added to 75 µL of the treated sample and the mixture was analyzed.

4.4.1.3 Omeprazole

Initial stock solutions of omeprazole (OME), 5-hydroxyomeprazole (OHOME), and omeprazole-d_3 (OME-d_3) were prepared by dissolving 1 mg in 1 mL methanol/0.1 M Na_2CO_3 4/1 (v/v) and stored at -20 °C.

4.4.1.3.1 Stability test in urine

The pH of urine strongly depends on environmental factors like e.g. diet. To estimate the average pH value of urine, the urinary pH values of 31 different persons were measured at up to eight different time points. PH values from 4.8 to 8.6 were found, with a mean ± SD of 6.3 ± 0.7. As it is well known that omeprazole (OME) is not stable under acidic conditions, it was necessary to test how fast this analyte would decompose under “physiological” conditions. To evaluate the stabilities of OME and its metabolite 5-hydroxyomeprazole (OHOME) in urine at different pH-values, standard urine samples were prepared according to the following method.

Standard urine samples

A spike solution was prepared by combining 10 µL OME-, OHOME- and OME-d_3 solution (each 10 µg/mL in methanol/0.1 M Na_2CO_3 4/1) with 70 µL methanol.

The pH value of blank urine (pH 5) was adjusted to pH 6, 7, and 8 by adding sodium hydrogen carbonate. To 290 µL of these urines 10 µL of the spike solution were added. Of these mixtures, 10 µL were injected and measured directly (t=0) and after 75, 150, 225, 300, 375, 450 and 525 min.

4.4.1.3.2 In plasma

In a preliminary study, the blood of 60 subjects was sampled 2-3 hours after omeprazole ingestion.

Standard solutions and plasma standard samples

Seven calibration standards were prepared by spiking 250 µL blank plasma with 10 µL of a standard solution (**A** to **G**) containing OME and OHOME in methanol/0.1 M Na_2CO_3 4/1 to give concentrations from 0.75 µg/mL (OME: 2.2 µM, OHOME: 2.0 µM) to 5 ng/mL (OME: 14.5 nM, OHOME: 13 nM) in plasma (Table 25). These standard samples then underwent the same procedure as the samples (see following paragraph).

[OME] and [OHOME]	**A**	**B**	**C**	**D**	**E**	**F**	**G**
standard solution	3 µg/mL	2 µg/mL	0.8 µg/mL	0.3 µg/mL	0.1 µg/mL	40 ng/mL	20 ng/mL
calibration standard	0.75 µg/mL	0.5 µg/mL	0.2 µg/mL	50 ng/mL	25 ng/mL	10 ng/mL	5 ng/mL

Table 25 OME and OHOME standard solutions and calibration standards.

Sample extraction

Whole blood collected approximately 2-3 hours after omeprazole ingestion was centrifuged at 4000 rpm (= 2916 x g for Eppendorf centrifuge 5810) for 25 min. After centrifugation, the upper clear level was transferred to a tube and stored at -20 °C until further examination.

Plasma sample and standard sample preparation

250 µL plasma (samples and standard samples) were mixed with 10 µL internal standard (5 ng/mL OME-d_3 in methanol/0.1 M Na_2CO_3 4/1) and 100 µL 1M Na_2HPO_4 solution and extracted twice for 20 min with 1.5 mL dichloromethane/acetonitrile (9/1, v/v) as organic phase. The organic phases of the first and second extraction were combined and evaporated

to dryness in a Speed Vac. The residue was dissolved in 50 µL methanol/0.1 M Na_2CO_3 (1:4 v/v) and 10 µL were injected.

4.4.2 Analytical conditions

For all analytical methods, the Turbo Ionspray source was used with the following parameters: IS 4000V, TEM 400 °C; N_2 as curtain gas (40), gas 1 (45), gas 2 (65) and collision gas (CAD=4).
All compound specific parameters were obtained by infusion of the standards using the quantitative optimization function of Analyst software 1.4.2.

4.4.2.1 Dextromethorphan and dextrorphan in urine

4.4.2.1.1 Without glucuronide cleavage

For LC, a HyperClone C8 3µ 100x2 with a corresponding guard cartridge was used (Phenomenex Ltd., Aschaffenburg, Germany). Chromatography was performed isocratically with water/acetonitrile 70/30 (v/v), containing 0.1 % formic acid at a flow rate of 240 µL/min. 10 µL of the sample were injected.
Analytes were recorded by multiple reaction monitoring (MRM) in the positive ion mode. The compound specific parameters for DEX, DOR, DEX-d3, and DOR-d3 (transitions, declustering potentials and collision energies) are given in Table 26. For retention times see Figure 23, chapter 6.2.1.1.

Compound	Transition	DP (V)	CE (V)
DEX	272 → 128	41	73
DOR	258 → 157	91	53
DEX-d_3	275 → 128	51	75
DOR-d_3	261 → 157	61	51

Table 26 MS/MS-transitions, declustering potential (DP) and collision energy (CE)

4.4.2.1.2 With glucuronide cleavage

For LC, a HyperClone C8 3µ 100x2 with a corresponding guard cartridge was used (Phenomenex Ltd., Aschaffenburg, Germany). The mobile phase consisted of (A) acetonitrile with 0.1 % formic acid and (B) water with 0.1 % formic acid. The flow rate was 240 µL/min and the injection volume 10 µL. The following conditions were used: 98 % B isocratic for 1.5 min, followed by a linear gradient to 50 % B within 30 s then isocratic 50 % B for 5 min. Within 1 min, the ratio was set back to starting conditions and the system was equilibrated for 7 min. With this method only DOR was quantified. The DOR and DOR-d3 parameters were

identical to the ones used for the method without glucuronide cleavage (Table 26) For retention times see Figure 24, chapter 6.2.1.2.

4.4.2.2 Flurbiprofen and 4-hydroxyflurbiprofen in urine

For LC, a reprosil-pur phenyl 3µ column, 100mm×2mm with a corresponding guard cartridge was used (Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany). The mobile phase consisted of (A) 10 mM NH_4OAc buffer pH 5 and (B) acetonitrile/methanol (1/1). The flow rate was 200 µL/min and the injection volume 10 µL. For the analysis of FLB and OHF, the following conditions were used: 80 % A isocratic for 1 min, followed by a linear gradient to 10 % A within 1 min then isocratic 10 % A for 5 min. Within 1 min the ratio was set back to starting conditions and the system was equilibrated for 8 min.
Analytes were recorded by multiple reaction monitoring (MRM) in the negative ion mode. The compound specific parameters for FLB, OHF, FLB-d3 and OHF-d3 are given in Table 27. For retention times see Figure 26, chapter 6.2.2.

Compound	Transition	DP (V)	CE (V)
FLB	243 → 199	-26	-10
OHF	259 → 215	-16	-12
FLB-d_3	246 → 202	-21	-12
OHF-d_3	262 → 218	-21	-12
FLBGlu	419 → 113	-20	-20
OHFGlu	435 → 113	-20	-20

Table 27 MS/MS-transitions, declustering potential (DP) and collision energy (CE)

4.4.2.3 Omeprazole and 5-hydroxyomeprazole

4.4.2.3.1 Stability test in urine

For LC, a HyperClone C8 3µ column, 100mm×2mm with a corresponding guard cartridge was used (Phenomenex Ltd., Aschaffenburg, Germany). The mobile phase consisted of (A) acetonitrile with 0.1 % formic acid and (B) water with 0.1 % formic acid. The flow rate was 250 µL/min and the injection volume 10 µL. For the analysis of omeprazole (OME) and 5-hydroxyomeprazole (OHOME) the following conditions were used: starting at 5 % A, a linear gradient to 100 % A within 15 min then isocratic 100 % A for 2 min. Within 1 min the ratio was set back to starting conditions and the system was equilibrated for 7 min.
Analytes were recorded by multiple reaction monitoring (MRM) in the positive ion mode. The compound specific parameters for OME, OHOME, and OME-d3 are given in Table 28. For retention times see Figure 27, chapter 6.2.3.1.

Compound	Transition	DP (V)	CE (V)
OME	346 → 198	41	19
OHOME	362 → 214	21	17
OME-d_3	349 → 136	26	43

Table 28 MS/MS-transitions, declusterung potential (DP) and collision energy (CE)

4.4.2.3.2 In plasma

For LC, a HyperClone C8 3µ 100x2 with a corresponding guard cartridge was used (Phenomenex Ltd., Aschaffenburg, Germany). Chromatography was performed isocratically with water/acetonitrile 70/30 (v/v), containing 0.1 % formic acid at a flow rate of 240 µL/min. 10 µL of the sample were injected.

The detection parameters were identical with the ones used for the stability test in urine (Table 28). For retention times see Figure 28, chapter 6.2.3.2.

5 Methods for genotyping

5.1 Subjects

In laboratory courses (WS07–SS09) in pharmacology and toxicology altogether 291 Caucasian students of the Würzburg University that registered for phenotyping also registered for genotyping in course of a study that had been approved by the Regional Ethical Committee of the Würzburg University (study number 37/07). All tested subjects were of good health and gave informed written consent.

CYP2D6

234 subjects were successfully genotyped and therefore included in CYP2D6 statistics. The resulting cohort consisted of 168 females with a mean ± SD age of 23 ± 2 years (range, 20–31) and 66 males aged 24 ± 2 years (range, 21–33).

CYP2C9

283 subjects were included in CYP2C9 statistics. The resulting cohort consisted of 201 females with a mean ± SD age of 23 ± 2 years (range, 20–34) and 82 males aged 24 ± 2 years (range, 21–33).

CYP2C19

235 subjects were included in CYP2C19 statistics. The resulting cohort consisted of 159 females with a mean ± SD age of 23 ± 2 years (range, 20–34) and 75 males aged 24 ± 2 years (range, 21–33).

5.2 Polymorphic alleles included in the study

The SNPs and small deletions included in the study and the methods used for detection are summarized in Table 29. Not all SNPs and small deletions could be successfully determined using the fast and easy iplex gold® method, therefore methods like RFLP-PCR had to be applied as well.

Method of detection	SNPs and small deletions	important associated alleles	Chapter
tetra primer PCR	1707delT[1]	CYP2D6*6	5.4.3
RFLP-PCR	1846G>A[1]	CYP2D6*4	5.4.4.2
	2615-2617delAAG[1]	CYP2D6*9	5.4.4.3
	100C>T[1]	CYP2D6*10, CYP2D6*4	5.4.4.4
	3608C>T[2]	CYP2C9*2	5.4.4.5

Method of detection	SNPs and small deletions	important associated alleles	Chapter
	42614A>C[2]	CYP2C9*3	5.4.4.6
iplex gold®	2549delA[1]	CYP2D6*3	5.6
	1707delT[1]	CYP2D6*6	5.6
	1023C>T[1]	CYP2D6*17	5.6
	2988G>A[1]	CYP2D6*41	5.6
	2850C>T[1]	CYP2D6*2, CYP2D6*17	5.6
	1661G>C[1]	CYP2D6*2, CYP2D6*4, CYP2D6*6, CYP2D6*10, CYP2D6*17, CYP2D6*41	5.6
	10601delA[2]	CYP2C9*6	5.6
	19154G>A[3]	CYP2C19*2	5.6
	17948G>A[3]	CYP2C19*3	5.6
	-806C>T[3]	CYP2C19*17	5.6

Table 29 SNPs and small deletions included in the study with their associated alleles, the method of detection and the respective method chapter. Numbers according to [1]: gene M33388; [2]: gene AL359672; 3: gene NG_008384.1.

In addition to these SNPs and small deletions, CYP2D6 duplication and multiplication (see chapter 5.4.2.1) and the deletion of nearly the whole CYP2D6 gene (CYP2D6*5, see chapter 5.4.2.2) were included in the study.

5.3 DNA extraction

5.3.1 Fresh and frozen blood

The DNA isolation from fresh blood was mainly done at the Dept. of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg in cooperation with Prof. Andreas Reif.

For buffer recipes see chapter 3.6

Lysis of erythrocytes

10 mL blood were mixed with 30 mL RBC lysis buffer or lysis buffer for fresh or frozen blood, respectively. After cooling the mixture (0 °C, 30 min) it was centrifuged (4 °C, 15 min,

3000x*g*) and the supernatant was carefully discarded. The remaining pellet was washed with 10 mL RBC lysis buffer (or lysis buffer), centrifuged again, and after discarding of the supernatant the cap was left inverted on a clean sheet of absorbant paper.

Lysis of leucocytes and nuclei

After drying, the pellet was redissolved in 5 mL SE-buffer and 250 µL pronase E (20 mg/mL) and 250 µL 10 % SDS were added. The mixture was vortexed and incubated over night at 37 °C.

Precipitation of genomic DNA

1.5 mL saturated NaCl solution (6M) was added to the lymphocyte suspension and the mixture was vortexed for 15 s. After centrifuging (20 min, 3000x*g*) the supernatant was transferred into a clean tube. 7.5 mL isopropanol were added to the supernatant and the tube was inverted a few times until the DNA was visible.
The DNA was removed using a sterile pipette tip, transferred to a new tube, covered with foil and left to dry. Over night, the DNA was redissolved in TE buffer (500 µL) at 4 °C.

5.3.2 Buffy coat

Preparation of buffy coat

Whole blood was centrifuged at 4000 rpm (= 2916 x g for eppendorf centrifuge 5810) for 25 min. After centrifugation, the three layers (i) concentrated erythrocytes (bottom layer), (ii) leukocyte-enriched fraction (buffy coat, intermediate layer), and (iii) plasma (upper clear layer) were easily distinguishable. With an autoclaved pipette tip, the buffy coat was carefully removed, and stored at -20 °C until DNA isolation.

Preparation of buffers according to NucleoSpin® manual

- Buffer B1 and B2 were mixed to yield binding buffer B3.
- Wash buffer B5 was prepared by diluting B5 concentration with 28 mL ethanol.

Lyophilized proteinase K was dissolved in 260 µL proteinase buffer (buffer PB)

Isolation of DNA using the NucleoSpin® Tissue DNA kit

The frozen buffy coat was thawed quickly in a 37 °C water bath. Then, DNA was isolated following the instructions of the NucleoSpin® handbook:

Prelysis:

180 µL lysis buffer T1 and 25 µL Proteinase K were added to the buffy coat and the mixture

was vortexed and incubated at 56 °C over night.

Lysis:

200 µL binding buffer (buffer B3) containing the chaotropic agent guanidium chloride were added to the preincubated buffy coat, the mixture was vigorously vortexed for 20 s and incubated for 15 min at 70 °C.

Adjust DNA binding conditions:

210 µL Ethanol were added and the sample was vortexed.

Bind DNA:

A NucleoSpin® column was placed in a collection tube, loaded with the sample, and centrifuged 1 min at 11000x*g*. The flow-through and collection tube were discarded.

Wash silica membrane:

The column was placed in a new collection tube, 500 µL wash buffer (buffer BW) were added, and the tube was centrifuged 1 min at 11000 x *g*. The flow-through and collection tube were discarded. The column was again placed in a new collection tube, 600 µL wash buffer (buffer B5) were added and the tube was centrifuged 1 min at 11000 x *g*. The flow-through was discarded and collection tube was reused.

Dry silica membrane:

The column was placed back into the tube and centrifuged 1 min at 11000 x *g*, to remove residual ethanol.

Elute highly pure DNA:

50 µL preheated elution buffer (Buffer BE, 70 °C) was added to the column and incubated for 3 min. Then the tube was centrifuged 1 min at 11000 x *g*. The procedure was repeated once.

5.4 Polymerase chain reactions (PCR)

5.4.1 PCR optimization

All PCR based methods were optimized for the correct annealing temperature, amount of polymerase, DNA and DMSO by using positive and negative control samples obtained from the Institute for Clinical Pharmacology and Toxicology, Charitè Berlin. The perfect conditions were those resulting in only the specific amplicon.

5.4.2 Long range PCR

Amplification of DNA sequences with a length of several kb is called long range PCR (LR-PCR). This method was used to detect duplication or multiplication (CYP2D6xN) and deletion (CYP2D6*5) of the CYP2D6 gene.

5.4.2.1 CYP2D6 duplication and multiplication

For the detection of CYP2D6 duplication and multiplication, previously published and validated primers (cyp-17f and cyp-32r, see Table 19) were used.[140] Figure 10 depicts the theory behind this long range PCR detection method. Without gene duplication or multiplication (**A.**) only one product with a length of 5.2 kb can be amplified as the reverse primer (empty diamond) only has one annealing position near the CYP-REP-7 region. Because of the insertion of one or more additional CYP2C6 genes regions, another annealing position for the reverse primer is added to the sequence, leading to one secondary amplicon with a length of 3.6 kb (**B.**).[140] The 5.2 kb amplicon should be detected in both cases and can therefore be used to verify PCR efficacy.

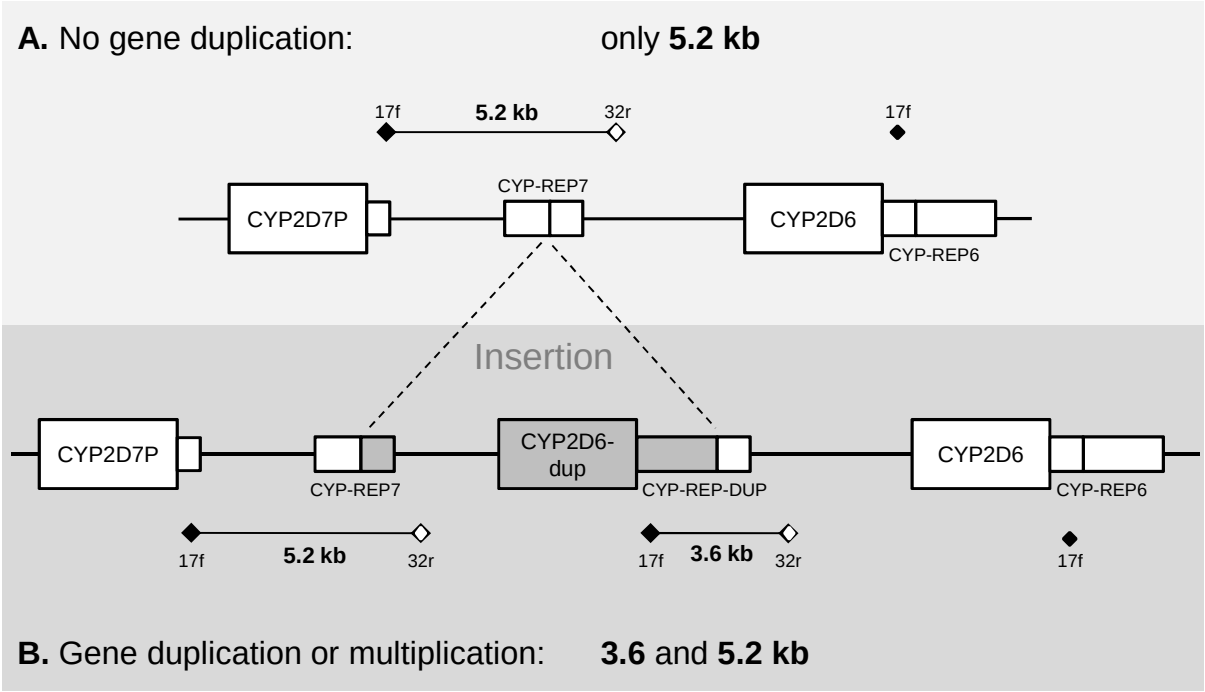


Figure 10 Schematic of the CYP2D6 gene locus without (A.) and with gene duplication or multiplication (B.). Filled diamonds indicate the annealing places for forward primer 17f, empty diamonds those for the reverse primer 32r.

The 25 µL reaction mixture for each sample contained LongAmp buffer (1x concentrated), LongAmp Taq polymerase (1.9 units), 0.5 µM forward primer (cyp-17f), 0.5 µM reverse primer (cyp-32r), 0.2 µM of each dNTP, ca. 40 ng DNA, and water. The reaction conditions are shown in Table 30. The resulting amplicons were separated by 0.8 % agarose gel electrophoreses for 2.5 h (Figure 38, chapter 7.2.1.1).

step	temperature	duration	
1	94 ℃	3 min	
2	94 ℃	30 s	35 cycles
3	62 ℃	30 s	
4	65 ℃	5.5 min	
5	65 ℃	10 min	
6	4 ℃	10 min	

Table 30 PCR reaction conditions

*5.4.2.2 DelCYP2D6 (CYP2D6*5)*

The deletion of the CYP2D6 gene was detected using two combined (multiplexed) long range PCRs with four validated primers.[141] The 3.2 kb DNA product (primers CYP2D6*5-Dlow and CYP2D6*5-Dup, Table 19) indicates a gene deletion (CYP2D6*5), whereas the 5.1 kb product (primers CYP2D6*5-DKlow and CYP2D6*5-DKup, Table 19) not only indicates that there is no CYP2D6 deletion but also works as a control for the PCR amplification.
The 25 µL reaction mixture for each sample contained LongAmp buffer (1x concentrated), LongAmp Taq polymerase (1.9 units), 0.5 µM forward primer 1 (CYP2D6*5-Dup), 0.5 µM reverse primer 1 (CYP2D6*5-Dlow), 0.5 µM forward primer 2 (CYP2D6*5-DKup), 0.5 µM reverse primer 1 (CYP2D6*5-DKlow), 0.2 µM of each dNTP, ca. 40 ng DNA, and water.

The reaction conditions were similar to those used for duplication and multiplication of CYP2D6 (Table 30). Again the resulting amplicons were separated by 0.8 % agarose gel electrophoreses for 2.5 h (Figure 38, chapter 7.2.1.1).

5.4.3 Special nested PCR: 1707delT (CYP2D6*6)

Nested PCRs use two sets of primers in two successive PCR runs, the first set to amplify a longer sequence of the DNA and the second set to amplify a target within the first DNA product. In this manner, product contamination due to unspecific primer binding sites can be minimized. In the tetra primer assay for CYP2D6*6 detection, published by Hersberger et al. a special kind of nested PCR was used.[141] The first set of primers (1newf and 2newr) lead to non-allele specific amplification of a part of the CYP2D6 gene (750 bp) containing gene position 1707. As previously mentioned, CYP2D6*6 carries a deletion of thymine at this gene position. The 750 bp product can be used as a control for PCR amplification and serves as a template for the second PCR reaction, which is an allele specific amplification (ASA). The second two primers H11 and HTmut are allele specific for the wildtype and CYP2D6*6 allele,

respectively (Figure 11). Due to their 3'-ends, primer H11 can only bind to the wildtype allele whereas primer HTmut only anneals to the *6 allele.

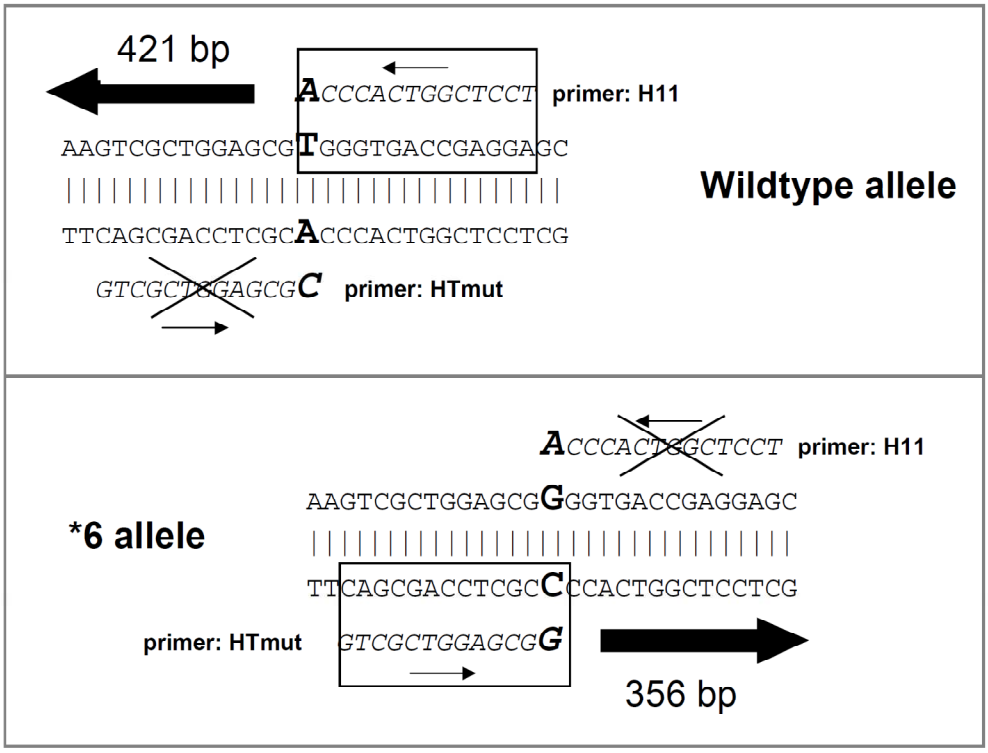


Figure 11 Allele specific primers H11 and HTmut for wildtype and CYP2D6*6 allele, respectively.

The special feature of the nested PCR used in this case is the recycling of the first primer set. As shown in Figure 12, the primers H11 and HTmut define in the second reaction cycle the allele specific amplicons together with 1newf and 2newr, respectively.

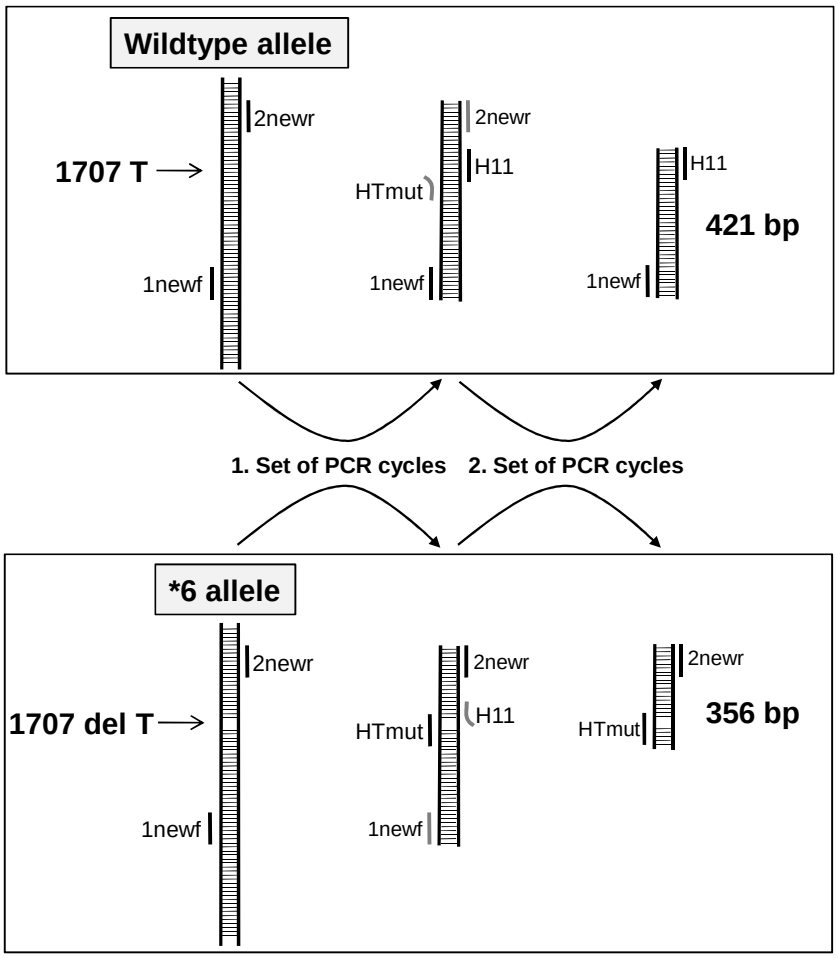


Figure 12 Schematic of the nested PCR used for CYP2D6*6 detection. Primers 1newf and 2newr are used for amplification of the template sequence. ASA is achieved by using the allele specific primers H11 and HTmut which define the allele specific amplicons in combination with 1newf (wildtype, 421 bp) and 2newr (CYP2D6*6, 356 bp), respectively.

The 25 µL reaction mixture for each sample contained 2.5 % DMSO, LongAmp buffer (1x concentrated), LongAmp Taq polymerase (1.9 units), 0.5 µM forward primer (cyp-1 of each of the primers of the first cycle (1newf and 2newr), 0.5 µM of each of the allele specific primers (H11 and HTmut), 0.2 µM of each dNTP, ca. 40 ng DNA, and water.

step	temperature	duration	
1	94 ℃	3 min	
2	94 ℃	30 s	15 cycles
3	65 ℃	30 s	
4	65 ℃	1 min	
2	94 ℃	30 s	29 cycles
3	54 ℃	30 s	
4	65 ℃	1 min	

step	temperature	duration
5	65 ℃	7 min
6	4 ℃	10 min

Table 31 PCR reaction conditions for nested PCR.

The reaction conditions are shown in Table 31. The resulting amplicons were separated by 2.5 % agarose gel electrophoreses for 3 h (Figure 39, chapter 7.2.1.2).

5.4.4 RFLP-PCR

5.4.4.1 General procedure

Six different RFLP analyses with previous PCR amplification were used in this study to identify three common CYP2D6 SNPs and deletions, two SNPs for CYP2C9, and one SNP for the Solute Carrier Organic Anion transporter (SLCO1B1 or OATP1B1). The recipe for the PCR reaction mixture of all six RFLP analyses is presented in Table 32, the reaction conditions in Table 33.

Reagent	Stock concentration	Theoretical volume for one sample*
LongAmp buffer	5x	5 µL
dNTPs	10 µM	0.5 µL
Primers	25 µM	0.5 µL each
LongAmp Taq	2.5 units/µL	0.5 µL
H_2O		ad 24.6 µL
DNA	ca. 100 ng/µL	ca. 0.4 µL

Table 32 PCR reaction mixture for RFLP analyses. *The reaction mixture was prepared for at least 10 samples, so that no volumes smaller than 1 µL had to be used.

Briefly, ca. 40 ng DNA, LongAmp Buffer (1x concentration), 0.2 µM of each dNTP, 0.5 µM of each primer (used primers are given in the respective chapters), 1.25 units LongAmp Taq, and water were combined to yield a 25 µL reaction mixture. This mixture was then heated to 94 °C to separate the DNA strands, cooled to the primer specific annealing temperature to allow primer binding to the DNA (annealing temperature is given in the respective chapter) and then brought to 65 °C to elongate the primers. The three steps, denaturation (94 °C), hybridization (annealing temperature, T_{anneal}) and synthesis (65 °C) are repeated 34 times followed by a longer synthesis step (7 min) and a cooling step of the reaction mixture at 4 °C.

step	temperature	duration	
1	94 °C	3 min	
2	94 °C	30 s	35 cycles
3	T_{anneal}	30 s	
4	65 °C	1 min	
5	65 °C	7 min	

Table 33 Reaction conditions for RFLP analyses.

The PCR product mixture was then digested using restriction endonucleases. The used enzymes, their specific recognition sites, and the digestion conditions are given in the following chapters.

*5.4.4.2 CYP2D6*4, 1846G>A*

For the detection of the guanine substitution with adenine at position 1846 characteristic for CYP2D6*4, primers previously used for CYP2D6*6 detection (1newf and 2newr) were used. The reaction mixture was prepared according to Table 32 and the reaction conditions were those given in Table 33 with an annealing temperature of 65 °C. The resulting 752 bp product was then digested with the restriction enzyme BstNI. The recognition site of BstNI and the part of CYP2D6 sequence containing position 1846 are shown in Figure 13, the cutting positions are indicated by filled triangles. The substitution of guanine with adenine anticipates the binding of BstNI so that only the wildtype allele (1846 G) is cut by the enzyme at this position.

Figure 13 BstNI recognition site and part of CYP2D6 sequence containing position 1846. 1846 A = CYP2D6*4. W = A or T.

The 752 bp PCR product is cut by BstNI at five more positions, altogether leading to six cuts for the wildtype allele and five for the CYP2D6*4 allele. Agarose gel electrophoreses after the digestion with BstNI shows therefore a band at 161 bp characteristic for the wildype and one

band at 366 bp characteristic for the CYP2D6*4 allele (Figure 14). Samples showing both bands can be identified as heterozygous carriers of CYP2D6*4.

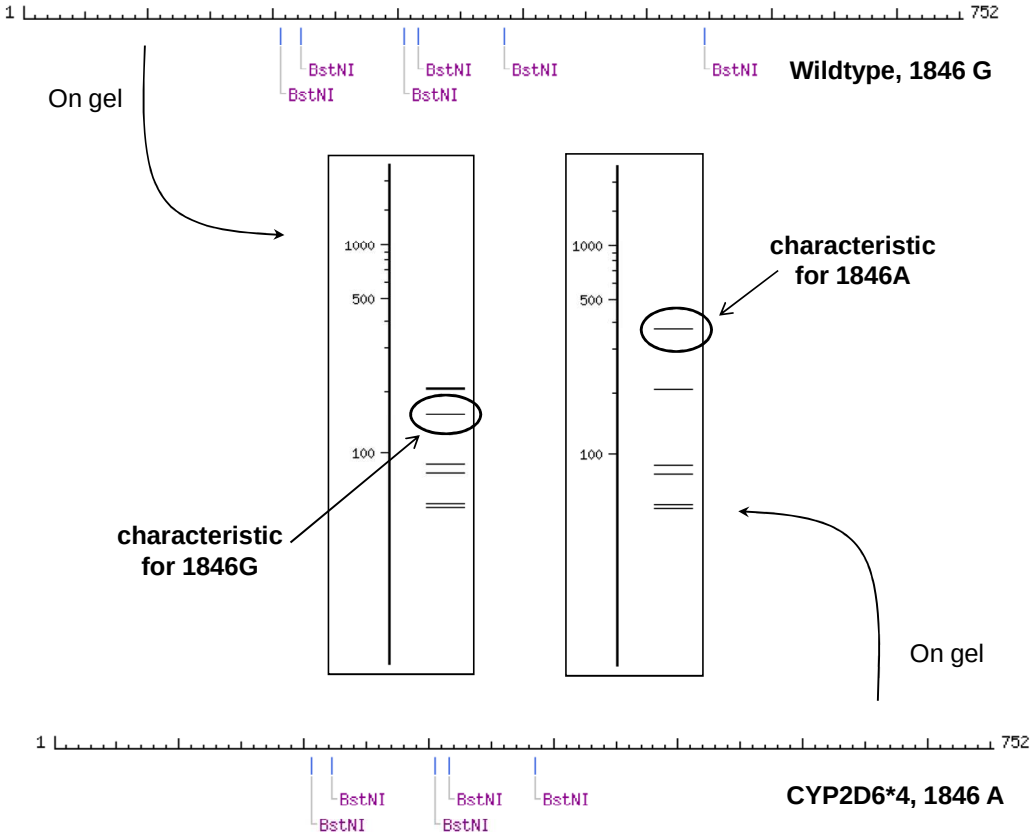


Figure 14 Schematic of theoretical gel electrophoreses output of either the wildtype or the CYP2D6*4 allele.

For digestion of the PCR product, 10 µL PCR product mixture, 12 µL water, 1x concentrated buffer (NEB buffer 2, see Table 16), 1x concentrated bovine serum albumin (BSA) and 1.5 units BstNI enzyme were mixed and incubated at 60 °C for 3 h. The products were separated by 3 % agarose gel electrophoreses for 3 h (Figure 40, chapter 7.2.1.3).

*5.4.4.3 CYP2D6*9, 2615-2617delAAG*

The deletion of the three nucleotides AAG at position 2615 to 2617 characteristic for CYP2D6*9 was also detected via RFLP using the validated primers P5 and P62 (for sequences, see Table 19).[39] The reaction mixture was prepared according to Table 32 and the reaction conditions were those given in Table 33 with an annealing temperature of 60 °C. The PCR product, a 387 bp amplicon, was subsequently digested with MboII. The recognition site and the part of the amplicon containing positions 2615 to 2617 for the wildtype and the polymorphic allele CYP2D6*9 are given in Figure 15. Only the wildtype

amplicon carries one MboII recognition site, whereas the CYP2D6*9 amplicon cannot be cut by the enzyme.

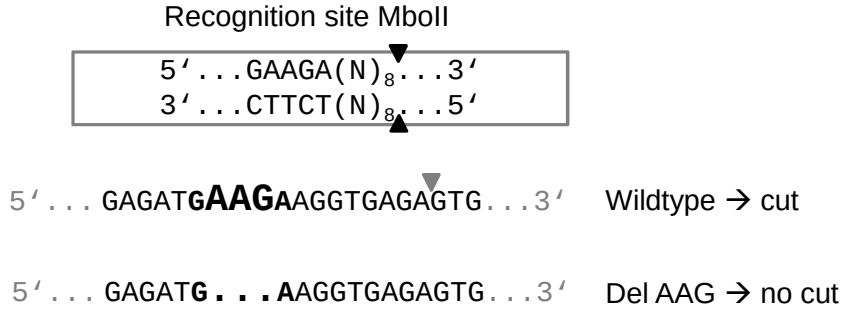


Figure 15 MboII recognition site and part of CYP2D6 sequence containing positions 2615 to 2617. Del AAG = CYP2D6*9. N = A, T, C or G.

Subsequent length determination of the digestion products by agarose gel electrophoreses will therefore show a band at 355 bp for the wildtype allele and an uncut PCR amplicon (387 bp) for CYP2D6*9 carriers. Heterozygote carriers will have both bands.
For digestion of the PCR product, 10 µL PCR product mixture, 12 µL water, 1x concentrated buffer (NEB buffer 3, see Table 16) and 1 unit MboII enzyme were mixed and incubated at 37 °C for 3 h. The products were separated by 3 % agarose gel electrophoreses for 3 h (Figure 40, chapter 7.2.1.3).

*5.4.4.4 100 C>T (amongst others CYP2C6*10)*

The substitution of cytosine with thymine at position 100 of the CYP2D6 gene was also detected with RFLP. The primers used for the amplification were P11 and P12 (for sequences, see Table 19), which were published by Sachse et. al.[39] The reaction mixture was prepared according to Table 32 and the reaction conditions were those given in Table 33 with an annealing temperature of 60 °C. The PCR product with a length of 433 bp was subsequently digested with the restriction endonuclease HphI, its recognition site is shown in Figure 16. Only the amplicon of the allele carrying the 100 C>T SNP is cut by HphI between position 91 and 92 of the CYP2D6 gene which is between position 258 and 259 of the PCR product.

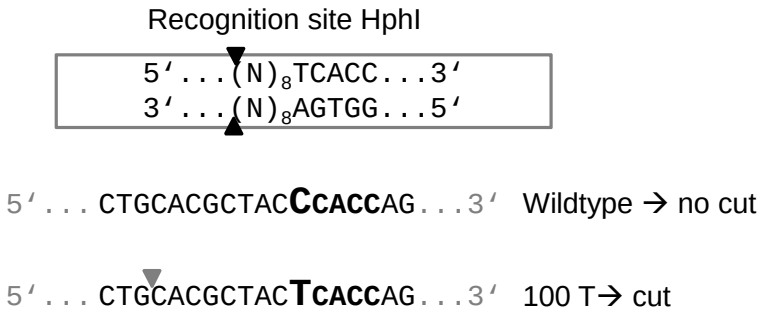


Figure 16 HphI recognition site and part of CYP2D6 sequence containing positions 100. N = A, T, C or G.

As HphI also cuts at position 362 of the product irrespective of the amplified allele, the digestion leads to a fragment with a length of 362 bp for the wildtype allele and to a 262 bp product and a 100 bp for the allele carrying the 100 C>T substitution. These fragments can be separated by using gel electrophoreses. For digestion of the PCR product, 10 µL PCR product mixture, 12 µL water, 1x concentrated buffer (NEB buffer 4, see Table 16) and 2 units HphI enzyme (5 units/µL) were mixed and incubated at 37 °C for 3 h. The products were separated by 3 % agarose gel electrophoreses for 3 h (Figure 40, chapter 7.2.1.3).

*5.4.4.5 CYP2C9*2 (3608C>T)*

CYP2C9*2, the allele carrying a substitution of cytosine by thymine at position 3608 of the CYP2C9 gene, was detected using primers CYP2C9*2 SKf and CYP2C9*2 SKr (for sequences see Table 19), published by Sullivan-Klose et. al.[93] The reaction mixture was prepared according to Table 32 and the reaction conditions were those given in Table 33 with an annealing temperature of 55 °C. The PCR product with a length of 690 bp was subsequently digested with the restriction endonuclease AvaII, its recognition site is shown in Figure 17. Only the amplicon of the allele carrying the 3608 C>T SNP is cut by AvaII between position 3607 and 3608 of the CYP2C9 gene which is between position 521 and 522 of the PCR product.

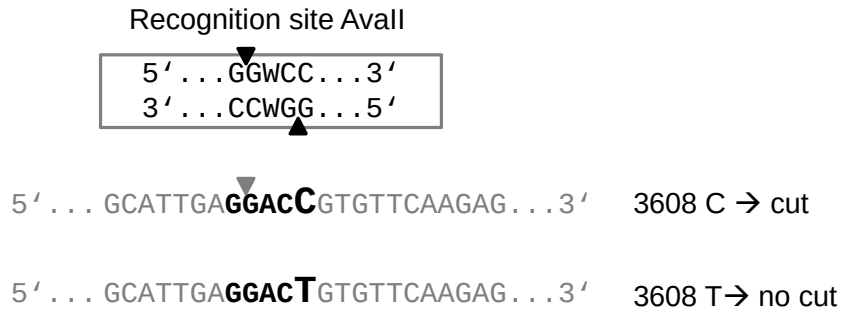


Figure 17 AvaII recognition site and part of CYP2D6 sequence containing position 3608. 3608 T = CYP2C9*2. W = A or T.

Therefore, after separation by gel electrophoreses, the wildtype allele and CYP2C9*2 can be determined as the wildtype allele shows the uncut 690 bp PCR product, whereas CYP2C9*2 is characterized by a band at 521 bp.
For digestion of the PCR product, 10 µL PCR product mixture, 12 µL water, 1x concentrated buffer (NEB buffer 4, see Table 16) and 2 units AvaII enzyme (10 units/µL) were mixed and incubated at 37 °C for 3 h. The products were separated by 3 % agarose gel electrophoreses for 3 h (Figure 41, chapter 7.3).

*5.4.4.6 CYP2C9*3 (42614A>C)*

For the detection of the polymorphic allele CYP2C9*3, a special kind of RFLP analysis was used. In some cases, it is necessary to insert a new recognition site with the used primers, because otherwise no distinction between wildtype allele and allele carrying the SNP can be achieved with RFLP. This may be due to a missing recognition site at the position of the nucleotide substitution or because the nucleotide substitution has no influence on the efficiency of the restriction enzyme. Therefore, for CYP2C9*3 detection two different forward primers CYP2C9*3 SK f1 and CYP2C9*3 SK f2 and one reverse primer CYP2C9*3 SK r were used, according to a method by Sullivan-Klose et al.[93] SK f1 and f2 lead together with the reverse primer to two different amplicons for the wildtype allele (Figure 18) and two different amplicons for the CYP2C9*3 allele (Figure 19). In two separate restriction reactions the PCR products are then digested with either NsiI or KpnI. The amplicon of the wildtype allele defined by primer SKf1 carries the recognition site for NsiI and can therefore be cut by this enzyme, whereas the amplicon defined by primer SKf2 has no complete recognition site for KpnI and cannot be cut (Figure 18).

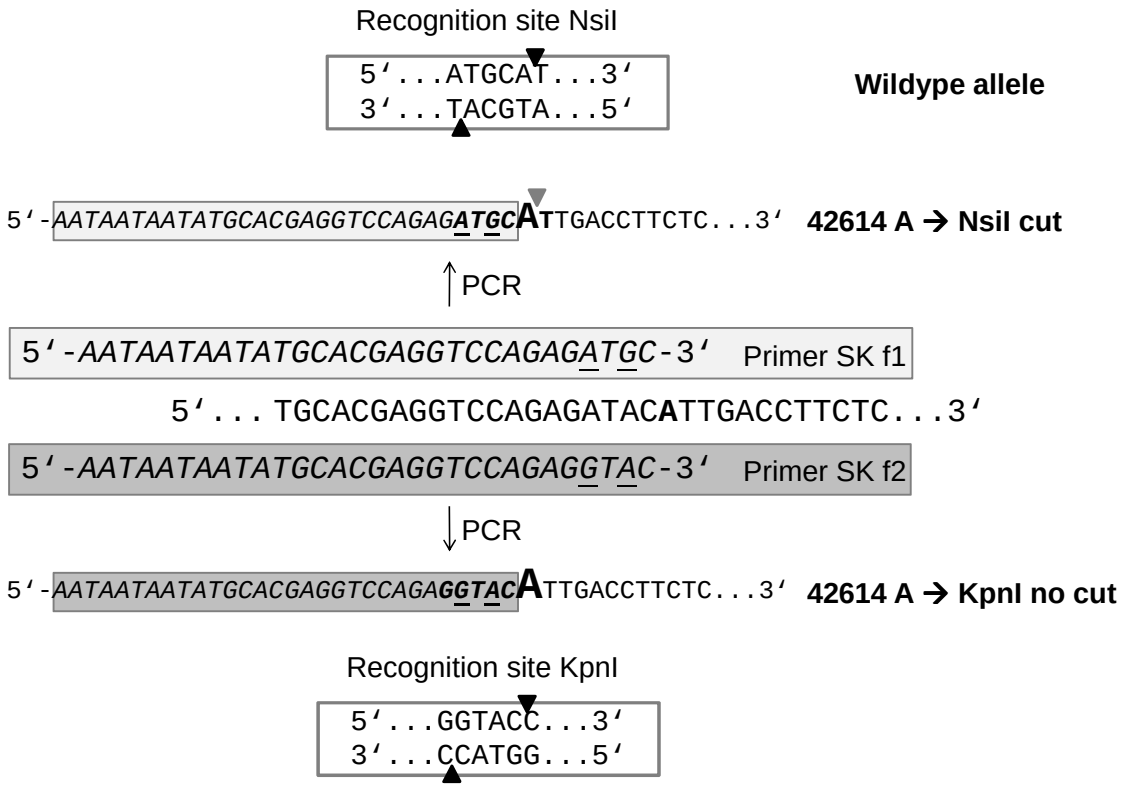


Figure 18 Schematic of the two amplicons for the wildtype allele defined by primers SK f1 and SK r or SK f2 and SK r. Also shown are the recognition sites for NsiI and KpnI.

For the respective amplicon of the polymorphic CYP2C9*3 allele, however, the KpnI recognition site is complete, whereas NsiI cannot cut the amplicon defined by primer SKf1 (Figure 19).

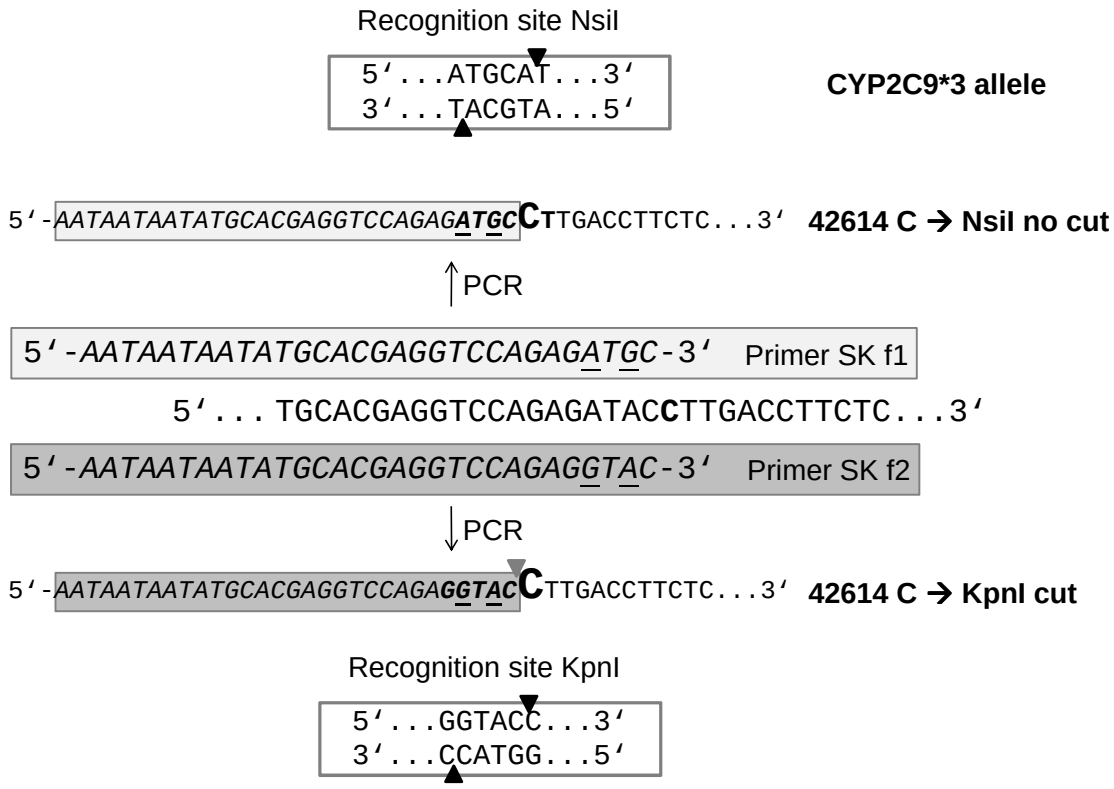


Figure 19 Schematic of the two amplicons for the CYP2C9*3 allele defined by primers SK f1 and SK r or SK f2 and SK r. Also shown are the recognition sites for NsiI and KpnI.

The PCR products are cut directly at the SNP position which is in this case directly behind the used forward primers. To facilitate the distinction of cut and uncut product with gel electrophorese, the two forward primers are extended by 10 non binding bp (AATAATAATA) at their 5'-end. In contrast to the published length of 141 bp[93] a BLAST analysis with the used primers showed that the amplicon defined by the primers is 166 bp long, the potential cut taking place at position 30 of the amplicon. For homozygous carriers of either the wildtype allele (only KpnI cuts) or the CYP2C9*3 allele (only NsiI cuts) and heterozygous carriers of both alleles (KpnI and NsiI cut) specific band pattern ensue as depicted in Figure 20.

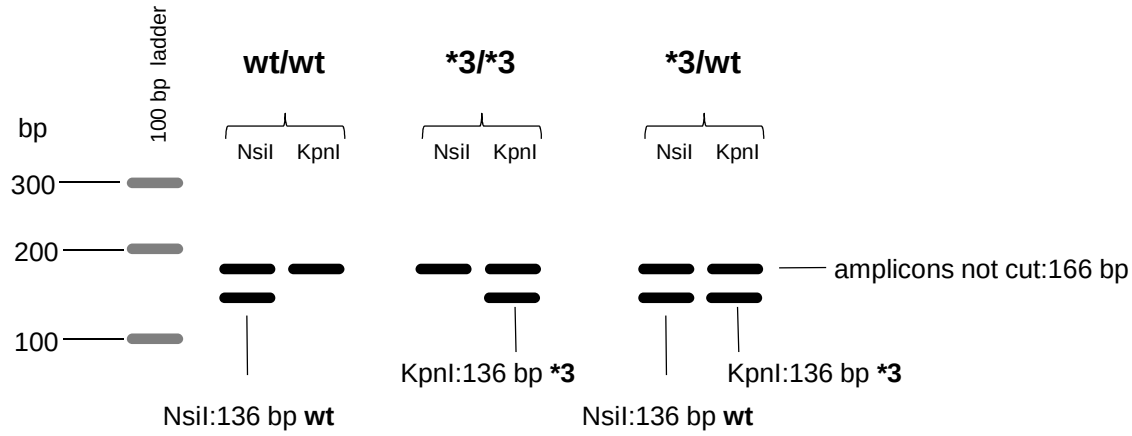


Figure 20 Schematic of the gel electorphoreses output of three different samples. From left to right: homozygote wt, homozygote CYP2C9*3, heterozygote *3/wt.

The PCR reaction mixture was prepared according to Table 32 and the reaction conditions were those given in Table 33 with an annealing temperature of 59 °C. Then two separate digestion reactions were carried out.

For the digestion with Nsi I 10 µL PCR product mixture, 12 µL water, 1x concentrated buffer (NEB buffer 3, see Table 16) and 1 unit NsiI enzyme (10 units/µL) were mixed and incubated at 37 °C for 3 h.

For the digestion with KpnI, 10 µL PCR product mixture, 12 µL water, 1x concentrated buffer (NEB buffer 1, see Table 16), 1x concentrated BSA (100 x) and 1 unit NsiI enzyme (10 units/µL) were mixed and incubated at 37 °C for 3 h. The products were separated by 3 % agarose gel electrophoreses for 3 h (Figure 41, chapter 7.3).

5.5 DNA sequencing

Verification of long PCR CYP2D6*5 detection

The CYP2D6 gene deletion CYP2D6*5 was detected using a long PCR method (chapter 5.4.2.2). For 7 samples, however, the iplex gold® results (multiple SNP detection, see chapter 5.6) for the SNP at gene position 2850 hinted towards heterozygocity of the CYP2D6 gene. This is impossible for carriers of the *5 allele, because they only possess one CYP2D6 gene and must therefore always show “homozygocity” for all other CYP2D6 polymorphisms.

To clarify whether the long PCR detection of CYP2D6*5 or the multiplexed SNP detection at position 2850 (C>T) produced reliable resultst, sequencing of the relevant DNA part was conducted. Amplicons of 18 samples which were found to carry the CYP2D6*5 allele

(including some not used for the phenotype-genotype correlation) containing position 2850 were sequenced.

The sequencing reactions were carried out according to the following protocol.

The DNA part containing position 2850 was amplified using normal PCR with a reduced amount of primers and polymerase. In this manner, purification of the PCR product before sequencing can mostly be avoided. The recipe and reaction conditions for the PCR with primers P5 and P62 (for sequence see Table 19) can be found in Table 34.

Reagent	Stock concentration	Theoretical volume for one sample*
LongAmp buffer	5x	5 µL
dNTPs	10 µM	0.4 µL
Primers	10 µM	0.3 µL each
LongAmp Taq	2.5 units/µL	0.4 µL
H_2O		ad 24.6 µL
DNA	ca. 100 ng/µL	0.4 µL

step	temperature	duration	
1	94 ℃	3 min	
2	94 ℃	30 s	x 35
3	60 ℃	30 s	
4	65 ℃	1 min	
5	65 ℃	7 min	

Table 34 Reaction mixture and conditions for sequencing. *The reaction mixture was prepared for at least 10 samples, so that no volumes smaller than 1 µL had to be used.

Then, for each sample two reaction mixtures containing 1 µL PCR product, 2 µL ready reaction mix (BigDye termination kit) and 6 µL water were prepared, one of which was mixed with the 1 µL of forward (P*5, 10 mM) one with 1 µL of the reverse primer (P62, 10 mM) of the first PCR reaction. These reaction mixtures were then again thermocycled, thereby conducting a chain termination reaction (reaction conditions see Table 35).

step	temperature	duration	
1	95 ℃	2 min	
2	96 ℃	15 s	x 24
3	45 ℃	15 s	
4	60 ℃	4 min	

Table 35 Reaction conditions for chain termination reaction.

The products of the chain termination reaction were precipitated by adding 340 µL precipitant (for recipe see chapter 3.6). The mixture was vortexed, centrifuged for 15 min at 10,000 × *g*, and the supernatant was carefully drawn of and discarded. The pellet was washed with 200

µL ethanol (70 %), centrifuged for 5 min at 10,000 × *g*, and the supernatant was again carefully drawn of and discarded. After left to dry for a few minutes, the pellet was resuspended in 20 µL Hi-Di formamide (highly deionized formamide) loading buffer. For DNA denaturation, the sample was heated to 94 °C for 3 min, and afterwards placed into the genetic analyzer for capillary electrophoreses and detection of the chain termination products.

5.6 Multiplex SNP detection (Iplex gold®)

The multiplex SNP detection was done in cooperation with Prof. Andreas Reif at the Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg.

Assay design

To design the amplification and extension primers suitable for a multiplexed single base extension reaction with the iplex gold® technology, a txt file (text file) containing the sequence 300 bp before and after each SNP or small deletion was written and computed online. The following four online tools supplied with the SNP analyzer were used subsequently:

Software program	Input	Function according to Sequenom[xx]
1. ProxSNP	written txt file	Compares input sequences against genome build 36 (gene database) and looks for registered SNPs that are proximal to the SNP of interest
2. PreXTEND	ProxSNP output	Aligns the input sequences against genome build 36 (gene database) to determine the best locations for PCR primers that would result in a unique amplification product containing the target for the extension primer
3. Assay Design	PreXTEND output	Designs multiplexed genotyping assays
4. PleXTEND	Assay Design output	Validates all primers in the entire multiplex by blasting them against each other to check for crosshybridization

Table 36 Software program functions.

In this manner, the primer sequences shown in Table 20 were achieved. It was not possible to design an assay including all SNPs, therefore two assays were used (Table 37).

Assay number	SNPs
1.	**CYP2D6*41** (2988G>A) **CYP2C19*3** (17948G>A) **CYP2D6*2_3** (1661G>C) **CYP2D6*9** (2615-2617delAAG) **CYP2D6*6** (1707delT) **CYP2C19*2** (19154G>A) **CYP2C9*6** (818delA) **CYP2D6*17** (1023C>T) **CYP2D6*2_4** (3183G>A)
2.	**CYP2D6*3** (2549delA) **CYP2C9*3** (42614A>C) **CYP2D6*2_1** (2850 C>T) **CYP2C19*17** (-806C>T)

Table 37 Iplex gold® assays and covered SNPs.

The following steps for the single base extension reaction were carried out according to the iplex gold application guide in the laboratory of Prof. Andreas Reif et al. at the Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg.

PCR

Briefly, for each of the two assays (Table 37) the previously isolated DNA samples were first amplified in a 384-well plate using the primers enlisted in Table 20 as a Primer mix. The reaction mixture and thermocycling conditions can be found in Table 38.

Reagent	Stock concentration	Theoretical volume for one sample*
PCR buffer (20 mM $MgCl_2$)	10x	0.5 µL
$MgCl_2$	25 mM	0.4 µL
dNTPs	25 mM	0.1 µL
Primer Mix	500 nM	0.3 µL
polymerase	5 units/µL	0.4 µL
H_2O		ad 4 µL
+ DNA	5-10 ng/µL	1 µL

step	temperature	duration	
1	94 ℃	4 min	
2	94 ℃	20 s	x 45
3	56 ℃	30 s	
4	72 ℃	1 min	
5	72 ℃	3 min	

Table 38 PCR reaction mixture and thermocycling conditions. *The reaction mixture was prepared for at least 10 samples, so that no volumes smaller than 1 µL had to be used.

SAP treatment

The PCR products were then incubated for 40 min at 37 °C with 2 µL of shrimp alkaline phosphatase (SAP) solution containing 0.5 units SAP enzyme and 1x concentrated SAP buffer in water (HPLC grade). To deactivate the enzyme, the reaction mixture was subsequently heated to 85 °C for five minutes.

IPLEX gold reaction

After the SAP treatment, the single base extension reaction using the extension primers also depicted in Table 20 was performed. To equilibrate signal-to-noise ratios when conducting multiplexing experiments, it is recommended to adjust primer concentration. Therefore, for each of the two assays the extension primers were divided into four groups depending on their masses, from lowest mass (primer mix 1) to highest mass (primer mix 4). The concentrations of the primers were than adapted as shown in Table 39.
To each of the SAP treated samples, 2 µL of the iPLEX Gold master mix was added (for recipe see Table 39) and the reaction mixture was thermocycled according to the conditions listed in Table 40.

Reagent	Stock concentration	Theoretical volume for one sample*
iPLEX Buffer Plus	10x	0.2 µL
iPLEX termination mix	20x	0.2 µL
Primer mix 1	7 µM	0.25 µL
Primer mix 2	9.3 µM	0.25 µL
Primer mix 3	11.7 µM	0.25 µL
Primer mix 4	14 µM	0.25 µL
iPLEX enzyme		0.04 µL
H_2O		ad 2 µL

Table 39 Reaction mixture for SBE.*The reaction mixture is always prepared as a mastermix for at least 96 samples.

step	temperature	duration	
1	94 ℃	30 s	
2	94 ℃	5 s	x 40
3	52 ℃	5 s	x 5
4	80 ℃	5 s	
5	72 ℃	3 min	

Table 40 Reaction conditions for SBE.

This program uses two cycling loops, one of five cycles integrated into one of 40 cycles, thereby resulting in a 200-short-step program. The sample is denatured at 94 °C (step 2), the

extension primers anneal at 52 °C (step 3) and the single base extension (SBE) takes place at 80 °C (step 4). Step 3 and 4 are repeated four more times and then the sample is again denatured for 5 s at 94 °C before again entering the primer annealing and extension loop. After the total of 200 steps, the sample is again brought to 72 °C for final extension.

MALDI-TOF analysis

To allow for mass detection using matrix-assisted laser desorption/ionization coupled with a time of flight detector (MALDI-TOF) with the MassARRAY analyzer, the SBE products have to be preconditioned. Buffer ingredients and detergent inhibit the crystallization process of the MALDI sample vital to the MALDI process.[142] Therefore, each sample was diluted with 16 µL ultra pure water and treated with approximately 6 mg of an ion-exchange resin loaded with ammonium ions (Clean resin). This conditions the phosphate backbone of nucleic acid fragments for chip-array based MALDI-TOF MS analysis. Then, the samples were loaded onto two SpectroCHIPs (one for each assay) in 384 well format pre-spotted with a MALDI matrix (specially formulated silicon dioxide). The MS spectrum of the SpectroCHIP was then acquired using the MassARRAY Analyzer and computed to give the SNP data using the MassArray Typer 3.4 software.

6 Results of Phenotyping studies

6.1 Principles of LC-MS/MS analytics

An LC-MS/MS system equipped with an electron spray ionisation source (see 3.1, Table 11) was used for the sensitive quantification of probe drugs and their metabolites in urine or plasma. Electron spray ionization (ESI) is an extensively used method for the analysis of drugs, carbohydrates, oligopeptides and also for protein sequencing. The schematic of the ESI process is depicted in Figure 21. The HPLC mobile phase containing the analytes separated by reverse phase chromatography is pumped through a needle (sprayer probe) to which a high voltage is applied. Due to the electric field gradient between sprayer probe and curtain plate, a so called Tayler cone with an excess of either positive charge (positive ion mode, Figure 21) or negative charge (negative ion mode), depending on the applied voltage, is built. Charged droplets consisting of solvent and ionized substances form at the tip of the Tayler cone and move down a potential and pressure gradient towards the curtain plate. In the course of that, the solvent evaporates supported by the hot nebulizer gas (N_2). Finally, fully desolvated ions pass through the orifice into increasingly higher-vacuum regions of the mass spectrometer. There are a few different postulated mechanisms for the formation of the fully desolvated analyte ions, one of which is the Coulomb fission mechanism. According to this mechanism, Coulomb explosions occur to form smaller and smaller droplets because the evaporation of solvent results in an increase of charge density until the Coulomb repulsion reaches the surface tension of the solvent (Rayleigh limit). Eventually the small “droplets” only contain one fully desolvated analyte ion.[143]

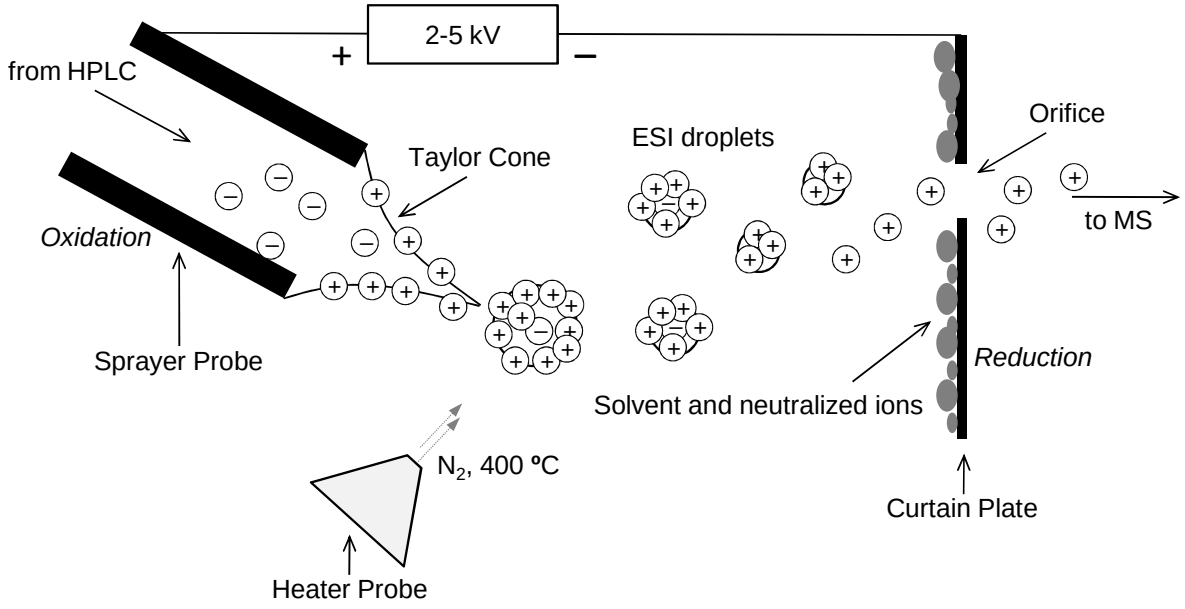


Figure 21 Schematic of the ESI process in positive ion mode (Turbolonspray) as adapted from Cech et al., 2001.[143]

These substance ions can then be analyzed by mass spectrometry. The mode in which the triple quadrupole mass spectrometer was used in this study is the multiple reaction mode (MRM, Figure 22). In MRM, the first of the three quadrupoles (Q1) is used as a mass filter, only allowing the ions with the right mass to charge ratio (m/z) to pass into the second quadrupole (Q2). In Q2, the ions are collided with the collision gas (N_2) leading to substance specific fragmentation. The fragments pass on into the third quadrupole which, like Q1, functions as a mass filter only detecting specific fragments so that substrate specific transitions are measured rather than m/z ratios.

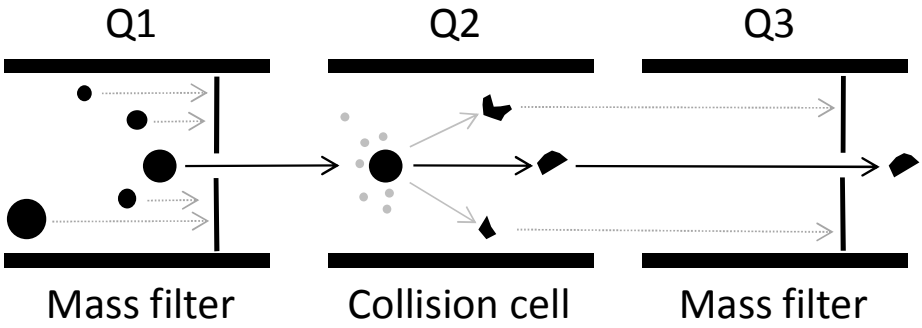


Figure 22 Multiple reaction monitoring

This maximizes the specificity and sensitivity by minimizing the background and therefore MRM is well suited to detect and quantify analytes in complex mixtures and matrices.

6.2 Analytical procedures

6.2.1 Dextromethorphan for CYP2D6

For CYP2D6 phenotyping, dextromethorphan (DEX) was used as probe drug. The concentrations of DEX and its CYP2D6-dependent metabolite dextrorphan (DOR) were measured in urine 2 h after DEX ingestion without prior glucuronide cleavage and with prior glucuronide cleavage.

6.2.1.1 Without glucuronide cleavage

The analytical method used for quantification of DEX and its metabolite DOR was developed in our working group and is based on a previously published method.[137] The original method was already optimized for fast sample preparation, chromatographic separation and reproducible quantification in urine. However, due to the isocratic elution used in this study, the analysis time for one sample is shortened to only 10 minutes. In addition to that, no subsequent re-equilibration of the column is required. An exemplary chromatogram is depicted in Figure 23, the retention times being 2.1 minutes and 5.1 minutes for DOR and DEX, respectively.

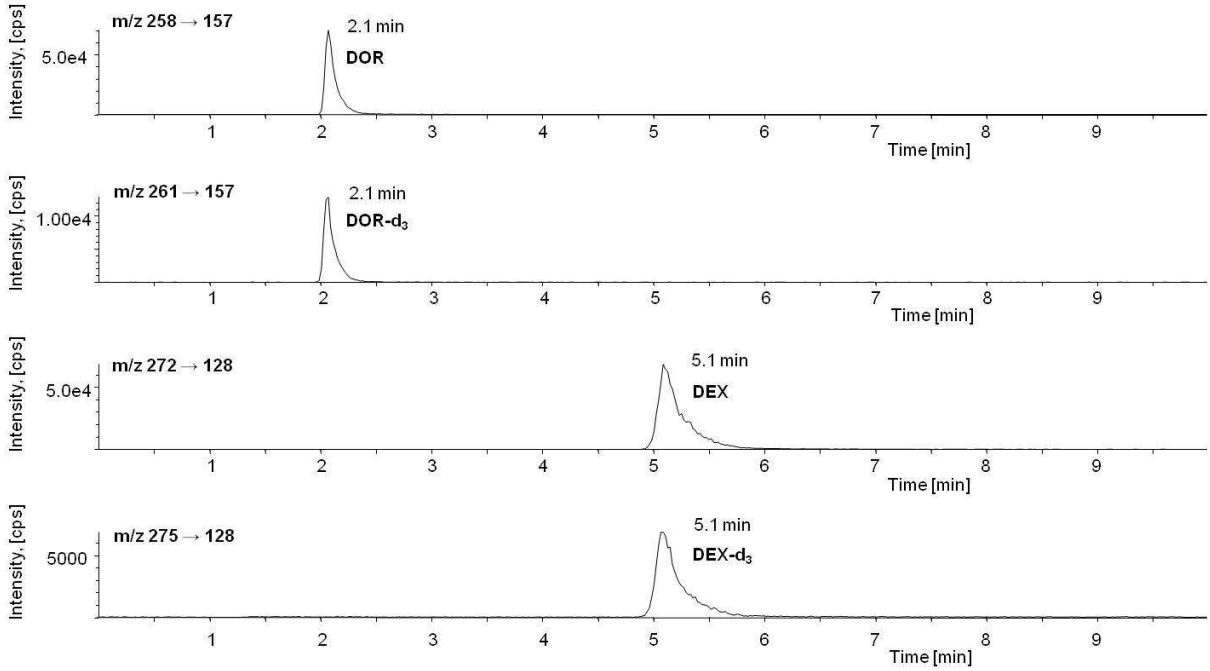


Figure 23 Chromatogram of DEX and DOR in urine.

Although urine was injected without prior extraction of the analytes, no interference with matrix components was observed. Good intra- and interday accuracy and precision could be achieved as shown in Table 41 (for each concentration n=3).

	Expected concentration (nM)	DEX				DOR			
		Mean	S.D.	%CV	Accy	Mean	S.D.	%CV	Accy
intraday	5	5.29	0.31	5.78	110	5.17	0.67	12.9	103
	100	95.1	6.04	6.35	95.1	98	1.66	1.71	98
	1000	1010	55.7	5.51	101	1017	20.8	2.05	102
interday	5	4.72	0.35	7.45	94.3	5.71	0.31	5.35	114
	100	88.3	5.95	6.74	88.3	101	10.8	10.7	101
	1000	1021	42.4	4.16	102	1009	141	14.0	101

Table 41 Analytical performance for LC–MS/MS analysis of DEX and DOR in urine without glucuronide cleavage. Mean and standard deviation (S.D.; n=3), intra- and interday precision (coefficient of variation, %CV) and accuracy (Accy).

The limit of detection (LOD) and the limit of quantification (LOQ) were determined as the concentrations with a signal-to-noise ratio (s/n) of approximately 5 (LOD) and 10 (LOQ). Because of matrix effects and resulting differences in the background we defined our LODs as the concentrations with a signal-to-noise ratio of approximately 5 (LOD) in contrast to the FDA guidelines, where a ratio of 3 is recommended. LOD and LOQ for both analytes were 2.5 nM and 5 nM, respectively. Best fit for accuracy (83–112 % for DEX and 87–116 % for DOR) for the standard curves was achieved with a linear regression and 1/*x* weighting.

6.2.1.2 With glucuronide cleavage

To investigate the influence of DOR glucuronidation on the calculated MR_{DEX}, DOR glucuronides were cleaved using ß-glucuronidase and DOR was measured according to a method developed and published by our working group.[138] The retention time of DOR was 7.6 min as shown in the chromatogram (Figure 24).

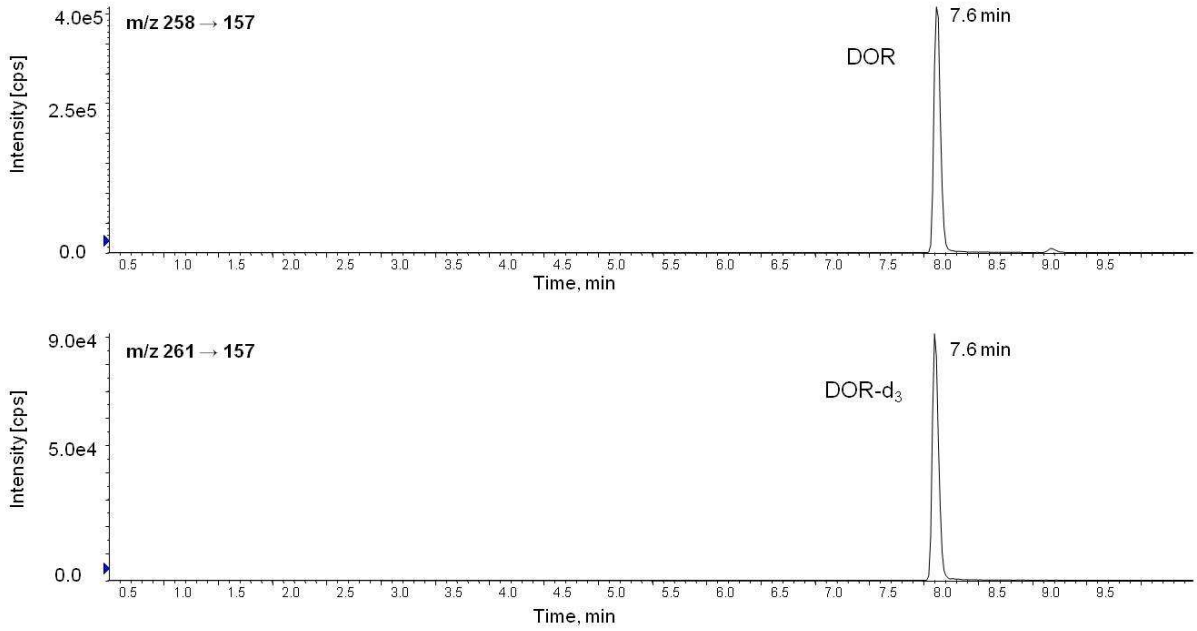


Figure 24 Chromatogram of DOR in urine after glucuronide cleavage.

This method also showed high intra- and interday accuracy and precision (Table 42, for each concentration n=3).

	Expected concentration (nM)	DOR			
		Mean	S.D.	%CV	Accy
intraday	5	5.19	0.14	2.65	104
	100	103	1.00	0.97	103
	5000	4890	150	3.07	97.8
interday	5	5.14	0.13	2.57	103
	100	103	1.53	1.49	103
	5000	4830	173	3.59	96.6

Table 42 Analytical performance for LC–MS/MS analysis of DEX and DOR in urine with glucuronide cleavage. Mean and standard deviation (S.D.; n=3), intra- and interday precision (coefficient of variation, %CV) and accuracy (Accy).

The LOD (s/n = 5) and LOQ (s/n = 10) for DOR were 2.5 nM and 5 nM, respectively. Like previously mentioned, we defined our LODs as the concentrations with a signal-to-noise ratio of approximately 5 (LOD) in contrast to the FDA guidelines, where a ratio of 3 is recommended because of matrix effects and resulting differences in the background. Best fit for accuracy (95–108 %) for the standard curve was achieved with a linear regression and 1/*x* weighting.

6.2.2 Flurbiprofen for CYP2C9

Flurbiprofen (FLB) was used as probe drug for CYP2C9 phenotyping. For the quantification of FLB and its CYP2C9-dependent metabolite 4-hydroxy flurbiprofen (OHF) a new analytical method was developed. With this method, the analytes were quantified in urine 2 h after FLB ingestion with prior glucuronide cleavage. FLB and OHF form acylglucuronides that can undergo acyl migration resulting in glucuronides that cannot be cleaved by ß-glucuronidase. Therefore, a new fast cleavage method based on the basic saponification of esters was established. The new method was verified in that neither FLB glucuronide (FLBGlu) nor OHF glucuronide (OHFGlu) were detectable by LC-MS/MS after the treatment (Figure 25).

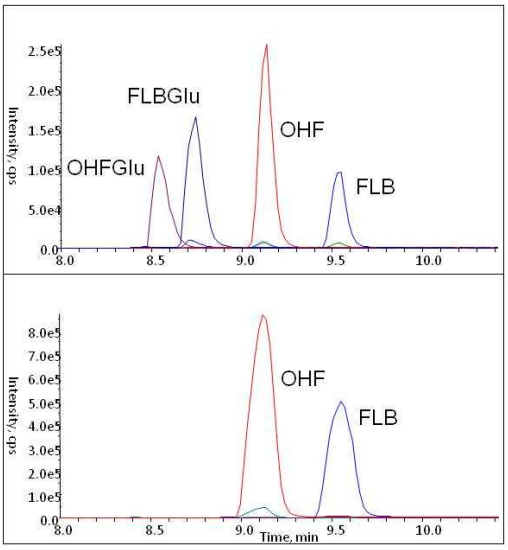


Figure 25 Chromatograms of the same sample before and after glucuronide cleavage

Instead of extracting the analytes after glucuronide cleavage, the treated urine was injected directly. In contrast to the method used for DEX and DOR, this leads to differences in analyte retention on the column depending on the sample matrix. To allow for definite peak assignment it was therefore essential to use deuterated FLB and OHF (FLB-d3 and OHF-d3) as internal standards, because their interactions with the stationary phase are influenced by the matrix similarly to those of the analytes. As OHF-d3 was not commercially available, a new method to synthesize this standard using FLB-d_3 as starting compound and human CYP2C9 enzyme as oxygenase was developed.

Figure 26 shows the resulting chromatogram with retention times for an exemplary sample.

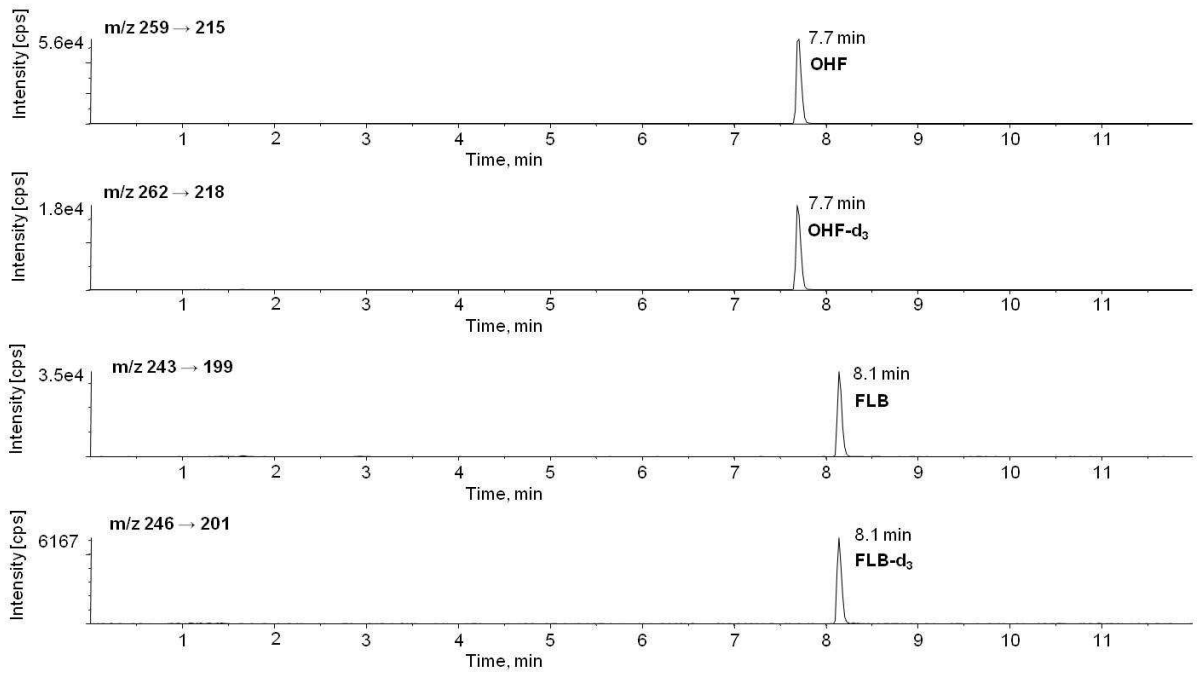


Figure 26 Chromatogram of FLB and OHF in urine.

Three sets of calibration standards were prepared in urine and treated with sodium hydroxide separately (n=27). The resulting calibration curves for FLB and OHF were linear from 50 nM to 29 µM with correlation coefficients of 0.992 and 0.994 and slopes of 0.0046 and 0.0025, respectively. Linear regression and 1/x weighing gave the best fit for accuracy with 86–120 % for FLB and 85–120 % for OHF. LODs and LOQs (s/n = 10) can be found in Table 43. Again the limits of detection were defined as the concentrations with a signal-to-noise ratio (s/n) of approximately 5 (LOD) in contrast to the FDA guidelines, were a ratio of 3 is recommended. However, due to matrix effects and resulting differences in the background, we chose a higher ratio for our LOD.

Compound	LOD	LOQ
FLB	20 nM	50 nM
OHF	20 nM	40 nM

Table 43 Limits of detection (LOD) and Limits of quantification (LOQ) for FLB and OHF.

The method also showed high intra- and interday precision and accuracy (Table 44, for each concentration n=3).

	Expected concentration (nM)	FLB				OHF			
		Mean	S.D.	%CV	Accy	Mean	S.D.	%CV	Accy
intraday	50.5	52.10	2.76	5.30	103.2	50.37	0.87	1.73	99.7
	1010	1014	50.0	4.93	100.4	1073	5.77	0.54	106.3
	10100	10567	550.8	5.21	104.6	10090	115	1.14	99.9
interday	50.5	49.83	1.48	2.98	98.7	51.53	4.70	9.12	102.0
	1010	1009	81.54	1.97	99.9	1073	37.86	3.53	106.3
	10100	10733	378.6	3.53	106.3	10127	161.66	1.60	100.3

Table 44 Analytical performance for LC–MS/MS analysis of FLB and OHF in urine with glucuronide cleavage. Mean and standard deviation (S.D.; n=3), intra- and interday precision (coefficient of variation, %CV) and accuracy (Accy).

6.2.3 Omeprazole for CYP2C19

6.2.3.1 In urine

It was planned to use the metabolic ratio calculated from the concentrations of omeprazole (OME) and its CYP2C19 dependent metabolite 5-hydroxyomeprazole (OHOME), preferably in urine, as the CYP2C19 phenotypic metric. For this purpose, stability test for OME and OHOME in urine of different pH values (pH 5, 6, 7 and 8) were conducted.

The analytical method should only be used to test whether and how fast the two analytes and the internal standard decompose in urine and the method was therefore not optimized for precision and accuracy. It was not used for analyte quantification, only relative analyte amounts on the basis of peak areas were measured over time.

An exemplary chromatogram with the retention times for OME and OHOME is depicted in Figure 27.

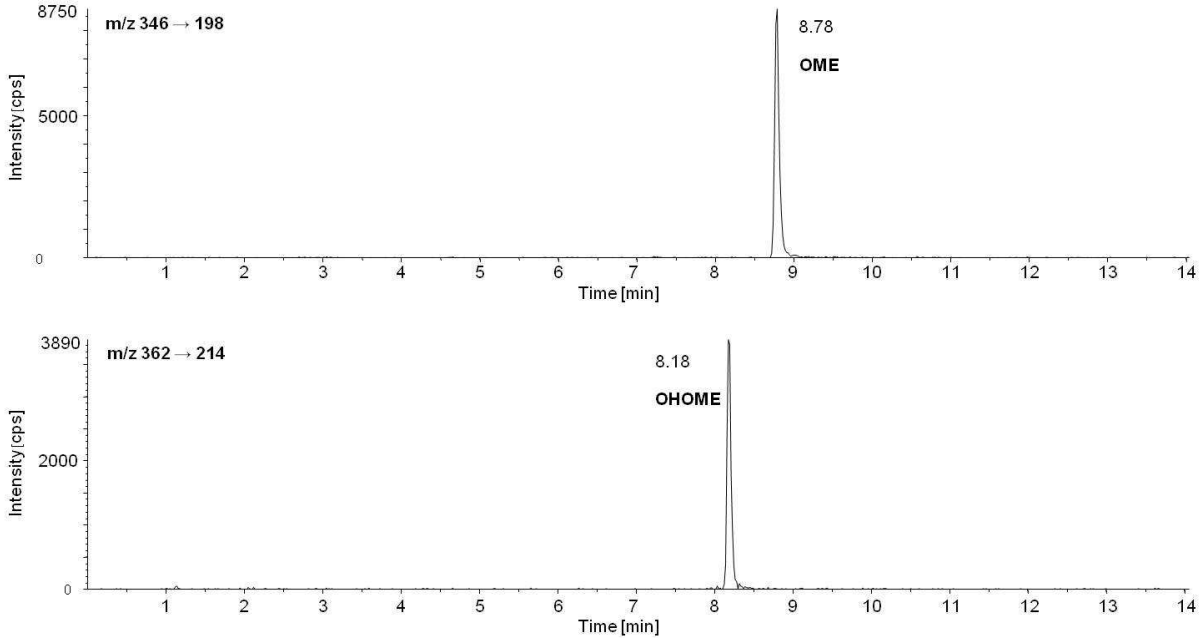


Figure 27 Chromatogram of OME and OHOME in urine.

6.2.3.2 In plasma

It was observed that OME and its metabolite (OHOME) were not stable enough in urine to provide a reliable CYP2C19 phenotyping metric (see chapter 6.5.1). Therefore, a method to measure OME and OHOME in plasma had to be developed.

To minimize plasma matrix effects and to enrich the analyte concentrations, a liquid-liquid extraction under basic conditions was carried out. In addition to the extraction method, a method using an isocratic mobile phase with a very short analysis time of six minutes per sample was designed (exemplary chromatogram see Figure 28). Three calibration curves (seven standards each) were prepared in plasma and extracted separately. The high accuracy (87–106 % for OME, 88–114 % for OHOME) and high correlation coefficients of 0.999 (for OME and OHOME) for the resulting calibration curve proved the reliability of the extraction procedure. The LOQs (s/n = 10) for OME and OHOME were 250 pg/mL each.

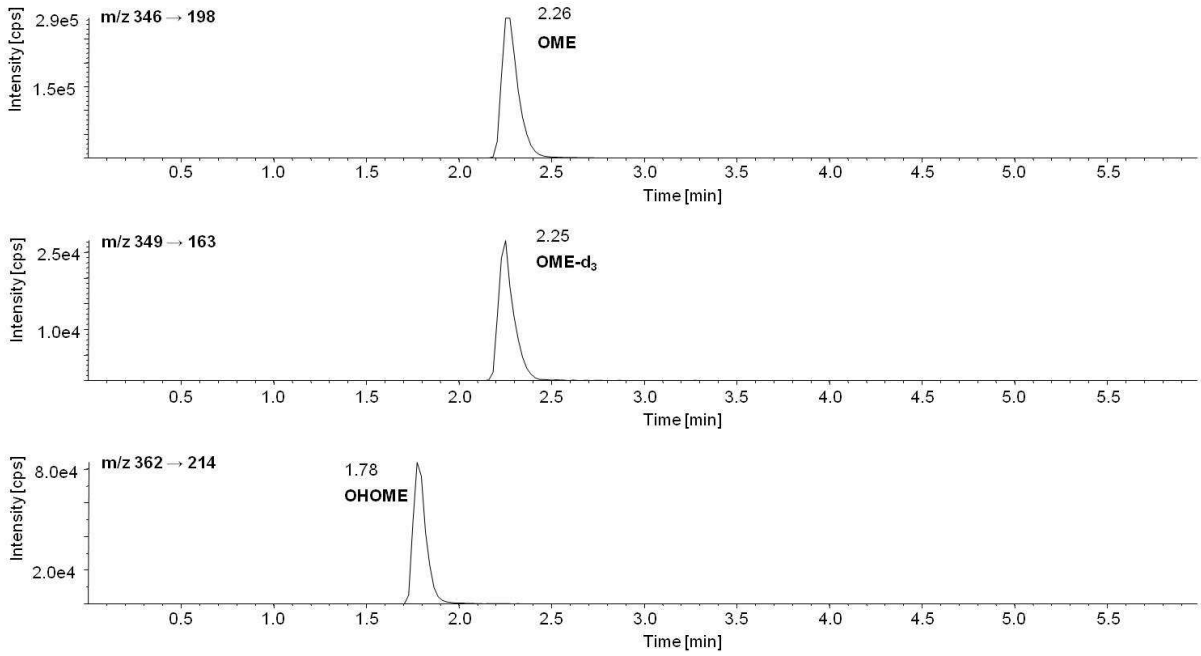


Figure 28 Chromatogram of OME and OHOME in plasma after liquid-liquid extraction.

Due to the results of the interaction studies (see chapter 6.3) and the preliminary results of OME and OHOME quantification in plasma of 60 subjects 2–3 h after OME ingestion (see chapter 6.5.2), it was concluded that there was no need to validate the method any further, as OME had to be excluded from the phenotyping cocktail.

6.3 Interaction studies

To examine the mutual influences of the probe drugs on their respective phenotypic metrics, interaction studies were conducted. The MR_{FLB} ([FLB]/[OHF]) of 32 subjects was calculated after intake of FLB alone and after simultaneous administration of OME. As can be seen in Figure 29, for the majority of subjects (28 out of 32) the calculated MR_{FLB} values were smaller after ingestion of the FLB and OME combination. A paired wilcoxon signed rank test confirmed that OME administration had a significant influence on the metabolic ratio of FLB (p value = 0.0004). Therefore OME had to be excluded from the phenotyping cocktail.

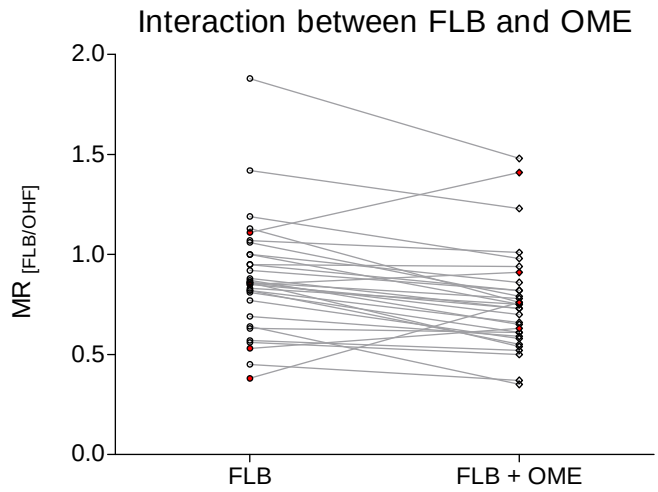


Figure 29 "Before and after" graph of MR_{FLB} after ingestion of FLB alone or in combination with OME.

The same tests were conducted for FLB and DEX. 44 subjects took DEX alone and after a wash out period of at least one week they took DEX and FLB in combination. There was no significant influence of FLB on MR_{DEX}, the p-value of the paired wilcoxon signed rank test being bigger than 0.05. Moreover, there was no significant influence of DEX on MR_{FLB} measured for 30 subjects (p-value = 0.3). As these results suggest that there is no mutual influence of FLB and DEX on their respective phenotypic metrics, these two probe drugs were combined to a phenotyping cocktail.

6.4 Combined phenotyping with dextromethorphan and flurbiprofen

The interaction studies showed that the administered doses of DEX and FLB had no influence on the calculated MR_{FLB} and MR_{DEX}, respectively. Therefore phenotyping of CYP2D6 (DEX) and CYP2C9 (FLB) could be combined. The values for DEX, DOR, FLB and OHF concentrations and metabolic ratios presented in the next paragraphs are those which were included in the phenotype genotype correlations. As CYP2D6 genotyping, due to its complexity, failed in more subjects than CYP2C9 genotyping, the number of subjects for DEX phenotyping (234 subjects) is smaller than the number for FLB phenotyping (283 subjects). However, all data shown was obtained 2 h after simultaneous ingestion of FLB and DEX.

6.4.1 Results of dextromethorphan phenotyping

The concentration of DEX and DOR measured in urine of 234 subjects varied up to 2784- and 3746-fold, respectively. For DEX values from 2.2 to 6180 pmol/mL urine were measured, for DOR concentrations from 2.4 to 8840 pmol/mL urine were found. The metabolic ratio

(MR_{DEX}) calculated as the concentration of DEX divided by the concentration of DOR ranged from 0.0036 to 208 (> 58 000-fold variation). The histogram for $log_{10}MR_{DEX}$ values is depicted in Figure 30. It suggests a bimodal distribution, the nonlinear fitting being a sum of two Gaussian functions. This distribution is in line with a discrimination between CYP2D6 poor metabolizers (PM, $log_{10}MR_{DEX}>0.5$) and extensive metabolizers (EM, $log_{10}MR_{DEX}<0.5$). 211 persons could be assigned the EM, 23 the PM phenotype. The percentage of PMs in the study cohort would therefore be 9.8 %.

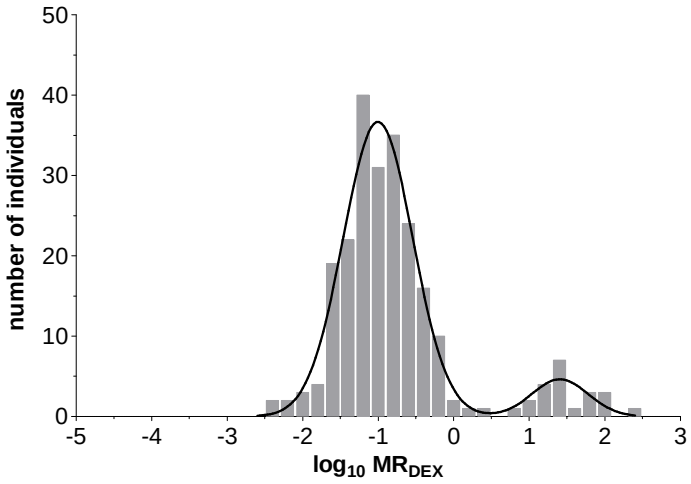


Figure 30 Histogram of $log_{10}(MR_{DEX})$ without glucuronide cleavage.

With the used method, only “free” DOR, the CYP2D6-dependant DEX metabolite, is detected. DOR, however, is extensively glucuronidated in the human body by uridine 5'-diphospho-glucuronosyltransferases (UGTs). As the activity of these enzymes can differ interindividually, it may be of advantage for the validity of the DEX phenotyping metric to cleave the DOR glucuronides before DOR quantification. Therefore DOR was also quantified after glucuronide cleavage. The measured DOR concentrations were much higher than those measured before cleavage, and varied up to 1784 fold, from 69 pmol/mL to 123.75 nmol/mL urine. The MR_{DEX} calculated with this DOR concentration ranged from $7 \cdot 10^{-5}$ to 30.5 (430 000-fold variation). The resulting second histogram for the $log_{10}MR_{DEX}$ values is depicted in Figure 31. Again a bimodal distribution for $log_{10}MR_{DEX}$ can be assumed. This leads to a clear separation of the groups of EM and PM, comprising 214 and 20 individuals, respectively. The percentage of subjects with PM phenotype determined after DOR glucuronide cleavage is 8.5 %.

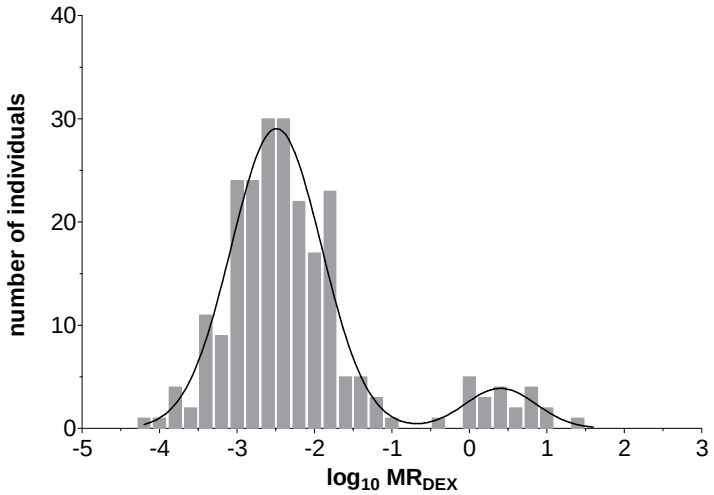


Figure 31 Histogram of $log_{10}(MR_{DEX})$ with glucuronide cleavage.

6.4.2 Results of flurbiprofen phenotyping

283 subjects were phenotyped for CYP2C9 2 h after ingestion of FLB and DEX. The CYP2C9 phenotyping metric used for this purpose was MR_{FLB}, calculated as the ratio of FLB to OHF concentrations in urine. FLB concentrations varied 79-fold, from 816 nM to 64.7 µM, OHF concentrations 209-fold from 388 nM to 81.1 µM. A 24-fold difference was observed between the lowest (0.43) and the highest calculated MR_{FLB} (10.4). Figure 32 shows the frequency distribution of $log_{10}MR_{FLB}$. In contrast to the distribution of $log_{10}MR_{DEX}$, no bimodal distribution can be observed. One subject, however, showed a metabolic ratio of 10.4 ($log_{10}MR_{FLB}$ = 1.02). This indicates that this person is a slow metabolizer for CYP2C9, probably a carrier of at least one variant allele (*2 and /or *3).

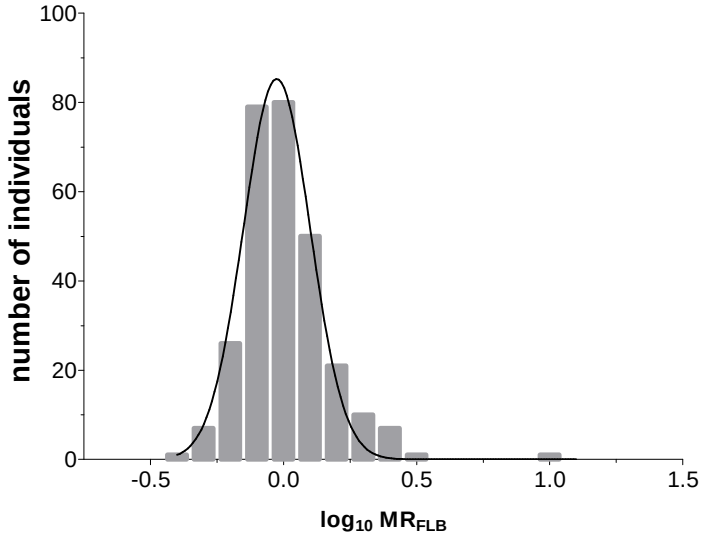


Figure 32 Histogram of $log_{10}(MR_{FLB})$ with glucuronide cleavage.

6.5 Results of omeprazole studies

6.5.1 Stability test in urine

As previously mentioned, the pH of urine depends on environmental factors like e.g. diet or amount of beverage taken in. The measured urinary pH values of 31 different persons at up to eight different time points ranged from acidic (pH 4.8) to basic (pH 8.6). Therefore the stabilities of OME and OHOME were determined in urine of pH = 5, 6, 7 and 8. The half-lifes of OME and OHOME were calculated from decomposing rate constants *k* determined from the slope of the ln-linear concentration time curve using the relationship $t_{1/2} = \ln 2/k$.

As can be seen in Figure 33, OME and its metabolite decompose in urine with a pH value of 5. No OME could be detected after 225 minutes. OHOME was no longer detectable at the last time point at 525 minutes. The half-lifes for OME and OHOME at pH 5 were 35 and 103 min, respectively. At pH 6, the analytes are also instable. OME and OHOME are reduced after 525 min by approximately 32 % and 15 %, respectively.

The metabolic ratio of OME (MR_{OME}) calculated as the quotient of measured OME concentration and OHOME concentration in urine 2 h after OME ingestion should originally be used as the CYP2C19 phenotyping metric. However, the findings of the stability test suggest that urinary MR_{OME} cannot be used as a reliable phenotyping metric due to interindividual differences in urinary pH value and the different half-lifes of OME and OHOME in urine.

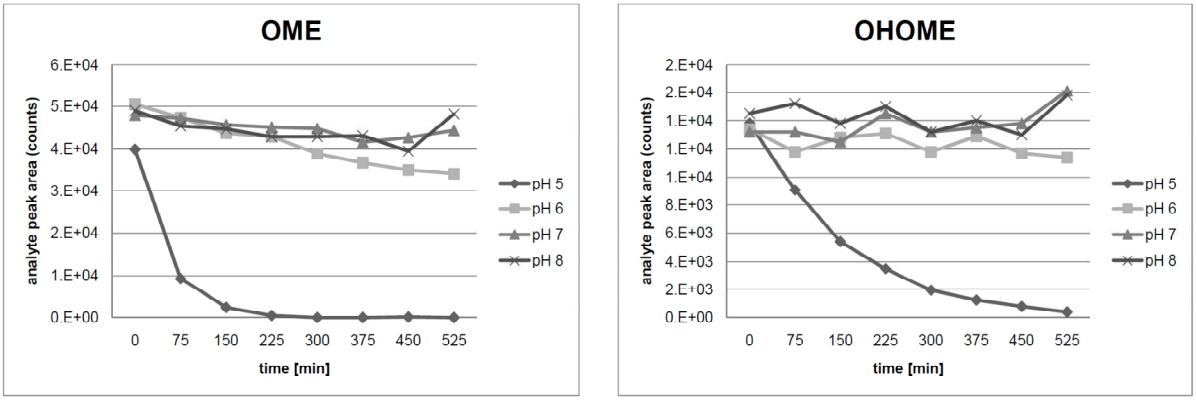


Figure 33 Diagrams depicting the stability of OME and OHOME in urine of different pH-values.

6.5.2 Omeprazole in plasma

The time span between probe drug ingestion and sampling of either urine or blood is essential for the validity of a calculated phenotyping metric. It was logistically impossible to ensure blood sampling from each participant after exactly the same time span following OME ingestion. This means that no stable phenotyping metric could be established using our study

design. In a preliminary study, however, OME and OHOME were quantified in plasma of 60 subjects 2–3 h after OME ingestion. The concentrations varied widely, they spread from under the limit of detection (LOQ for OME and OHOME = 250 pg/mL) to approximately 500 ng/mL (OME) and 400 ng/mL (OHOME). This finding further supported the thesis that phenotyping with OME could not be successfully accomplished using our study design.

7 Results of genotyping studies

7.1 Principles of used methods

7.1.1 Principles of DNA extraction

There are different methods that can be used to isolate DNA from blood. Two different methods, a salting out (fresh and frozen blood) and spin column-based (buffy coat: leukocyte-enriched blood fraction) DNA purification were used in this study.

The salting out DNA purification procedure is a simple and fast deproteinization method based on salting out of the cellular proteins by dehydration with a saturated sodium chloride solution.[144] Briefly, blood is mixed with RBC lysis buffer (**r**ed **b**lood **c**ells, erythrocytes) and centrifuged to precipitate the leucocytes. The supernatant containing the erythrocytes is discarded, the leucocyte membranes are lysed with the detergent sodium dodecylsulfate (SDS), and the proteins are digested with protease. To precipitate and remove the digested proteins, saturated sodium chloride solution is added to the mixture. The DNA stays in solution and can be easily precipitated by adding isopropanol.

The NucleoSpin® Tissue kit, a spin column-based DNA purification kit was used for DNA extraction from buffy coat. The principle of membrane lysis and protein digestion with proteinase is the same as in the previously described method. However, the method of DNA isolation differs. In presence of high concentrations of chaotropic salts like guanidinium chloride, DNA binds to silica materials, like the membrane of the spin column. The chaotropic salts destroy the relatively ordered structure of the DNA hydrate shell, thereby creating hydrophopic conditions in which the DNA binds to silica materials. Guanidinium cations may also saturate the negatively charged deprotonated hydroxyl groups of the silica membrane and could act as a “linker” between the negative phosphate groups in the DNA and the stationary phase. As proteins, metabolites, and other contaminants do not bind to the membrane, they are washed away in several washing steps. Using buffers with low salt concentrations, the DNA binding to the silica membrane is reversed. The DNA solution obtained in this fashion needs no further purification and can be used for various techniques.

7.1.2 Principles of PCR

As previously mentioned in chapter 1.4.2, PCR is an easy, fast, and sensitive enzymatic in-vitro method to amplify DNA. With the choice of specific short oligonucleotides (primers) which are complementary to opposite strands of the DNA, the target for DNA amplification is defined.

The reaction consists of three steps which are repeated up to 50 times: denaturation, hybridization and synthesis. The PCR reaction mixture containing DNA template, primers, dNTPs (deoxyribonucleotide triphosphates), and DNA polymerase is first heated (94 °C) to separate the two DNA strands (denaturation). During the second step (hybridization), the primers anneal to their complementary DNA sequences. The temperature at which this step is carried out is strongly depending on the used primers. The third step (synthesis) is the actual amplification step. The reaction mixture is brought to a temperature at which the DNA-polymerase is most active. The polymerase elongates both primers beginning at their 3'-end by condensing dNTPs. In this manner, specifically the sequence between the primers accumulates. It is theoretically doubled with each repetition of the three step reaction cycle, leading to an exponential amplification. In practice however, the product concentration reaches a plateau due to loss of polymerase activity over time, reannealing of the product strands and decrease of primer concentration.

7.1.3 Principles of length separation of DNA fragments

The sugar-phosphate backbone of DNA is negatively charged and therefore DNA travels towards the positive electrode in an electric field. If a sieving matrix like e.g. an agarose gel is placed into the electric field this movement towards the positive electrode leads to the separation of the DNA fragments due to their length.
For longer DNA fragments (100 to several thousand bp) agarose gels prepared by melting agarose in electrophoreses buffer (for recipe see chapter 3.6) are used as sieving matrices. The pore size of the agarose gel and therefore the length resolution is depending on the amount of agarose heated in buffer. The DNA bands in the resulting gels can be dyed by using a fluorescing intercalating agent like ethidium bromide and detected using a device equipped with UV light (for ethidium bromide exitation) and a camera to record the emission as photographs. Ethidium bromide can be added to the agarose gel either before electrophoreses or used diluted in buffer as a dying bath for the gel after the run.
Separation of DNA fragments that differ in length by only one bp, as needed for DNA sequencing, can be achieved using denatured acrylamide gels. Capillary electrophoreses with denaturing liquid polymers is also a suitable method and is often used in automated sequencing instruments, like the ABI genetic analyzer used in this study (see chapter 5.5).

7.1.4 Principles of RFLP-PCR

Restriction fragment length polymorphism (RFLP) is an often used method to detect SNPs or small deletions. A PCR product of known length containing the potential SNP is subjected to

digestion with a restriction enzyme (restriction endonuclease) that cleaves DNA at a specific recognition site. The endonuclease is chosen to cut either the wildtype or the polymorphic allele but not both. Subsequent length determination by agarose gel electrophoreses allows to distinguish between homozygous carriers of the wildtype allele, the polymorphic allele and heterozygous carriers of both alleles due to the pattern of the digestion products.

7.1.5 Principles of DNA sequencing

In 1977 two publications changed the field of genetics massively. Sanger et al.[145] and Maxam and Gilbert et al.[146] independently published two DNA sequencing methods, for which they were rewarded the Nobel Price in Chemistry in 1980.[147] What was once a cumbersome procedure only conducted in few laboratories became a much easier broadly available method. These “easy” sequencing methods gave also rise to the “Human Genome Project” (HGP), which had the goal of sequencing the whole human genome, which was more or less achieved in 2003.
Maxam and Gilbert’s method depends on chemical cleavage of the DNA fragment to be sequenced,[146] whereas Sangers method is based on a so called chain-termination process.[145] Meanwhile, many other methods like pyrosequencing or ligation sequencing have been developed. Nevertheless sequencing based on the chain termination method is still widely used. As the method applied in this study is also derived from Sanger’s sequencing, the chain termination sequencing will be described in detail in the following.

To synthesize DNA, polymerase condenses the 5’-phosphate group of one dNTP with the 3’-hydroxylgroup of another dNTP. Dideoxyribonucleotide triphosphates (ddNTPs) can also be incorporated into the newly synthesize DNA strand, but as ddNTPs lack the hydroxyl group in 3’-position (dashed circle in Figure 34) a condensation with another nucleotide to elongate the DNA chain is not possible. Therefore, the chain is terminated at the position of the ddNTP.

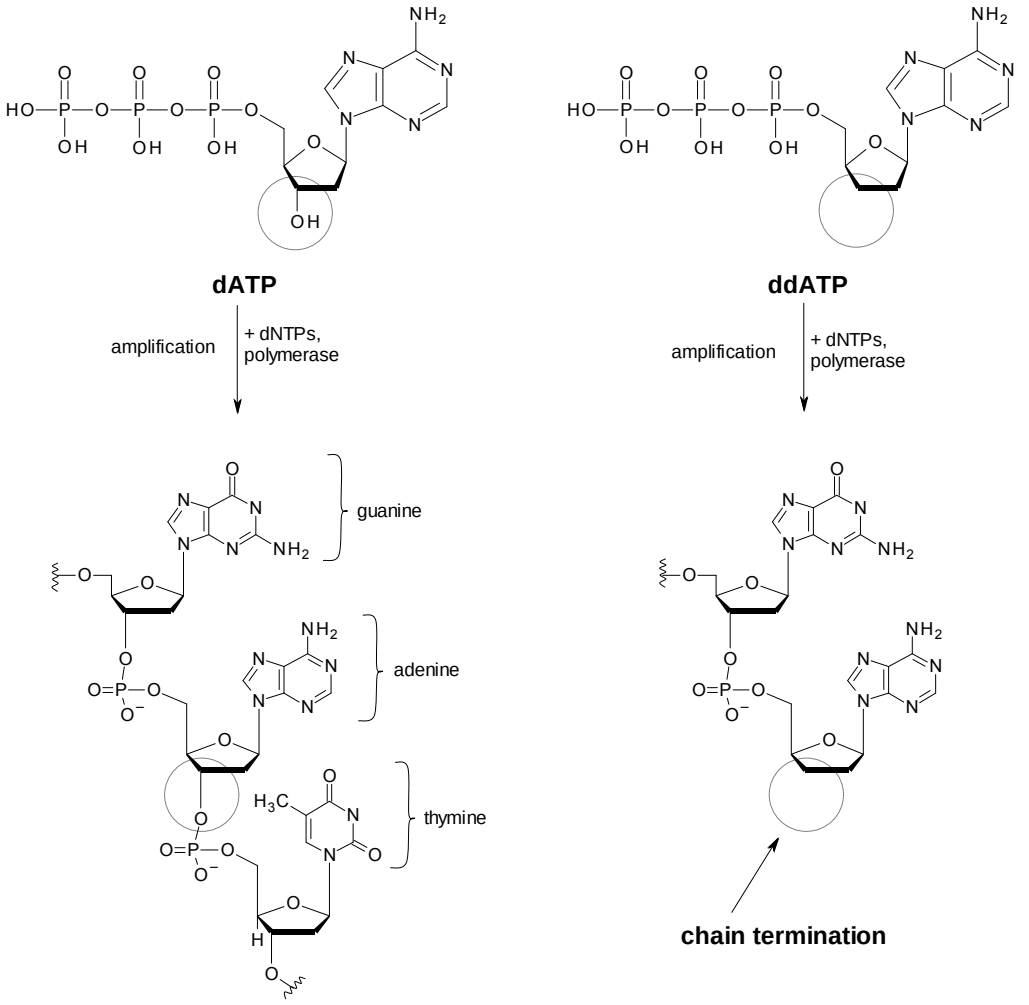


Figure 34 Principle of the chain termination reaction.

When the PCR of a DNA template is conducted with a mixture of dNTPs (dATP, dCTP, dGTP, dTTP) and a small amount of the four ddNTPs, the reaction product will contain not only the whole amplicon – synthesized with dNTPs – but also small amounts of all possible chain terminated products. The determination of the length and the 5'-end (position of ddNTP) of all chain terminated products allows the determination of the DNA template sequence.

The identification of the nucleotide at the 5'-end of the chain terminated products can be easily accomplished by labeling the four ddNTPs (also called terminators) with different fluorophores, thereby labeling the chain termination products depending on their 5'-end (Figure 35, A and B).

The separation of the oligonucleotides that differ in length by only one bp is achieved by either electrophoreses on denatured acrylamide gels or capillary electrophoreses using denaturing liquid polymers (Figure 35, C). Online fluorescence detection combined with

previous size separation of the labeled oligonucleotides can then be computed to give the desired DNA sequence (Figure 35, C and D).

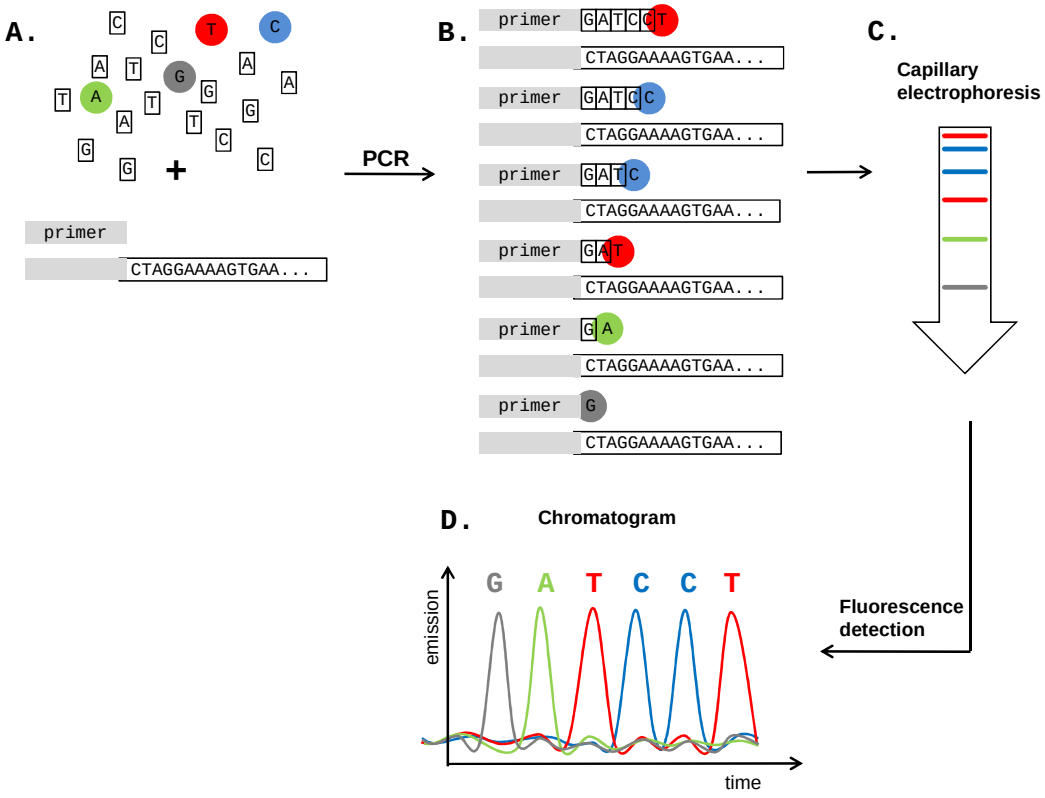


Figure 35 Schematic of DNA sequencing using fluorescence labeled ddNTPs.

In this study, a genetic analyzer (see Table 11) equipped with a capillary electrophoreses device and an online fluorescence detector was used. The device is coupled to a computer with an adequate software program (see Table 12) which is employed to transfer the measured fluorescence chromatogram into a DNA sequence.

The oligonucleotide length separation with capillary electrophoreses (for details see chapter 7.1.3) is achieved using a specific patented liquid **p**erformance **o**ptimized **p**olymer (POP-7®) as sieving material.

For the chain termination reaction the BigDye® Terminator v1.1 Cycle Sequencing Kit (see Table 18) was used. The ready reaction mixture contains dNTPs, polymerase and charge transfer dye-labeled terminators (labeled ddNTPs), which were synthesized and named "BigDyes" in 1997.[148] The thymine terminator is depicted in Figure 36. The charge-transfer dyes utilize fluorescein derivatives as donor and rhodamine derivatives as acceptor dyes. The two moieties are linked with 4-aminomethylbenzoic acid and bound to the ddNTPs using a propargyl ethoxyamino linker.[148, 149]

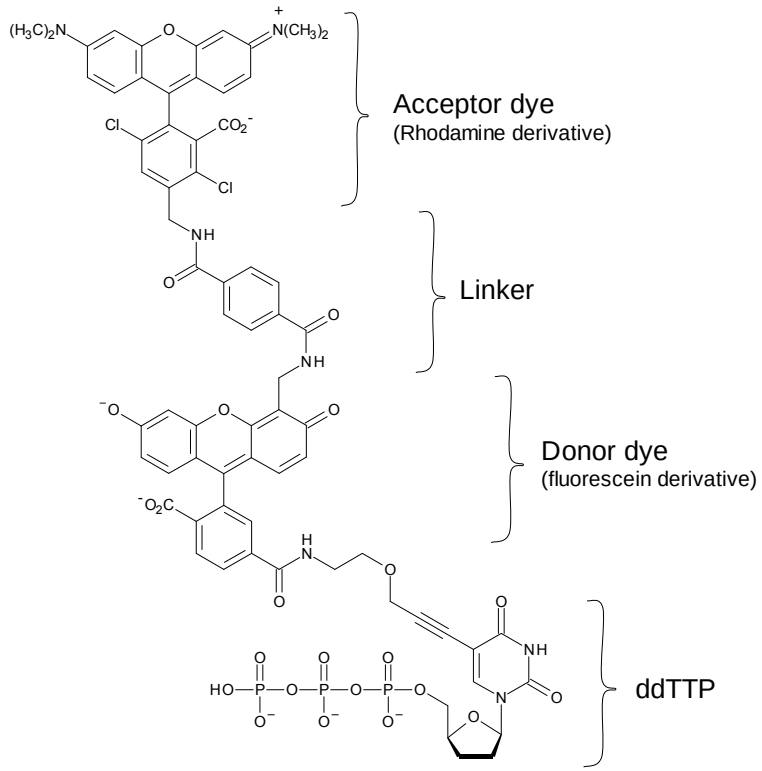


Figure 36 Structure of the ddT-BigDye terminator.

7.1.6 Principles of multiplex SNP detection (Iplex gold®)

The principle of the SNP detection with the iplex gold® technology is very similar to the sequencing technique described in the previous chapter. As depicted in Figure 37 a part of the genomic DNA containing a potential SNP position is first amplified in a PCR using the amplification primers f and r. The reaction mixture containing the amplicon and unincorporated dNTPs is then treated with shrimp alkaline phosphatase (SAP), which cleaves a phosphate group from the free dNTPs converting them to dNDPs (deoxyribunucleoside diphosphates) that cannot be used by the polymerase for DNA chain elongation. After this step, the extension primer, the ddNTPs and the polymerase are added and the mixture is thermocycled again. As the only building blocks available for the polymerase are ddNTPs, the primer can only be elongated by one bp. The extension primer is designed to anneal exactly one bp beside the potential SNP so that the primer is extended into the SNP site. The ddNTPs have different masses and therefore the masses of the produced oligonucleotides differ depending on the nucleotide at the SNP site. Mass determination using mass spectrometry leads to the identification of either wildtype amplicon or amplicon carrying the SNP.

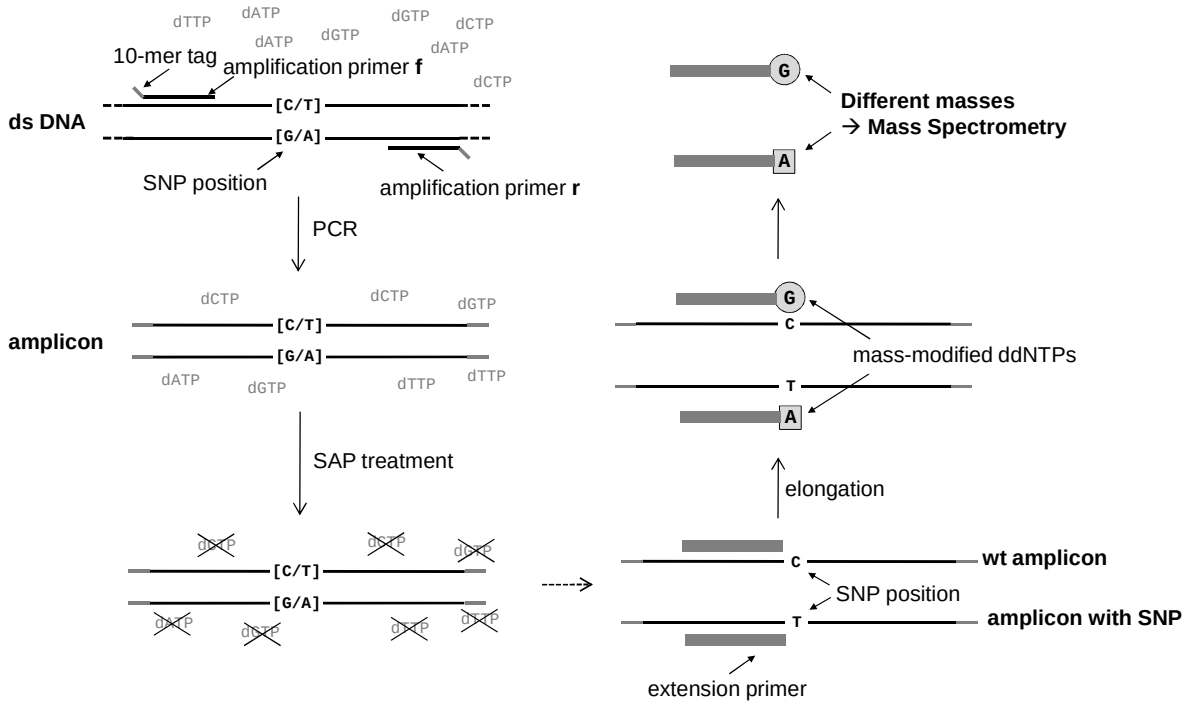


Figure 37 Schematic of the SNP detection using single base extension technologies.

The iplex gold® assay used in this study provides modified ddNTPs that differ in mass by at least 16 Da.[150] It allows the detection of up to 30 SNPs simultaneously (multiplex SNP detection). However, special requirements have to be fulfilled to provide for efficient SNP identification.

Firstly, all amplification primers must differ in mass from all extension primers and products, to avoid confusion in the mass spectrum. Therefore, it is recommended to add a 10-mer tag (sequence: 5'-ACGTTGGATG-3') to the 5'-end of each amplification primer but not to the extension primer. The addition of the tag also approximates the annealing temperatures of the amplification primers, thereby maximizing PCR efficiency.

Secondly, extension primers should be between 15 and 30 nucleotides long with a mass of approximately 4500 to 9000 Da (optimal mass for used mass spectrometer), their melting temperature should be approximately 60 °C or higher.

Thirdly, for best results all extension primers and products for the detection of different SNPs should differ in mass by at least 30 Da.

To design primers that fulfill these requirements, a software program should be used that calculates the best possible primers for given sequences and also checks for their sequence specificity using a genome wide data base.

7.2 CYP2D6

For CYP2D6, more than 78 polymorphic alleles and even more different haplotypes are known. Therefore only the most important alleles for Caucasians could be included in this study, namely CYP2D6*1, *2, *3, *4, *5, *6, *9, *10, *17, *41 and the gene duplication and multiplication. In addition to that, genotyping was also conducted for CYP2D6*17. It has a very high allelic frequency of up to 34 % in African or African-Americans and not only Caucasians took part in the study, even though the Non-Caucasians were excluded from the statistics.

To determine the 12 different polymorphic alleles, 10 SNPs and small deletions as well as the deletion of nearly the whole gene (*5) and the gene duplication and multiplication had to be detected. Three SNPs were covered with RFLP-PCR, one SNP using a tetra primer assay and the multiplex SBE method and six SNPs were determined using only multiplex SBE (see Table 29, chapter 5.2). In addition to that two long range PCR methods were used to detect CYP2D6*5 and CYP2D6 x N.

7.2.1 Results of PCR based methods

7.2.1.1 Long PCR

Figure 38 shows exemplary ethidium bromide stained gels of the PCR products of the method used for detection of CYP2D6 duplication and multiplication (**A**) and the one used for detection of CYP2D6 gene deletion (CYP2D6*5, **B**).

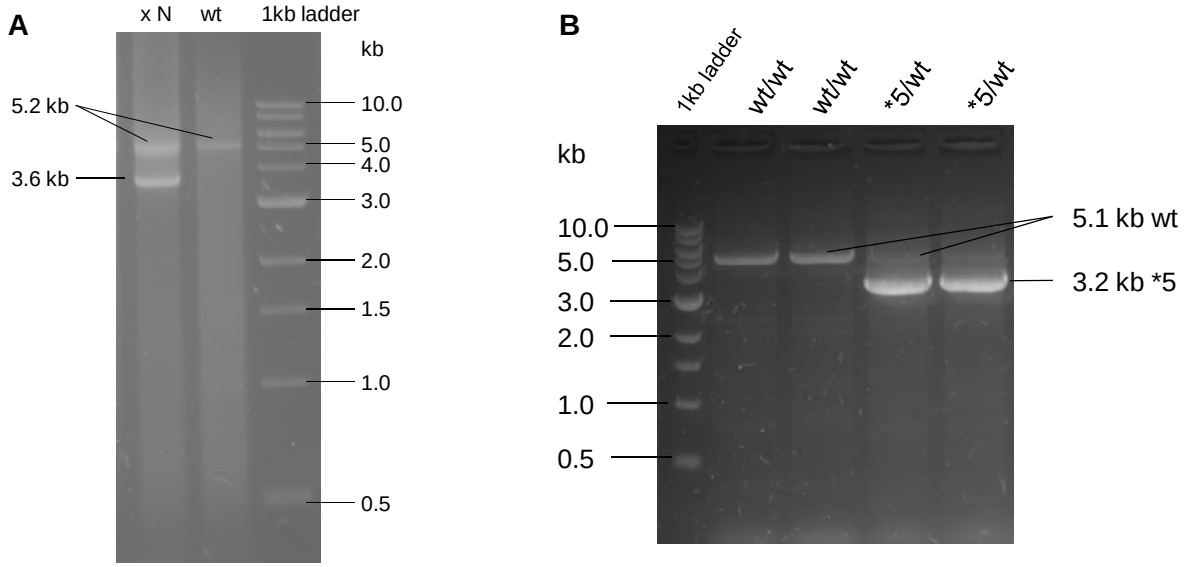


Figure 38 Ethidium bromide stained 0.8 % agarose gels. A 3.6 kb product indicates CYP2D6 duplication or multiplication. B 3.2 kb product indicates CYP2D6*5.

4 subjects of the 234 Caucasians included in the CYP2D6 genotype phenotype correlation showed a 3.6 kb band, and were therefore considered to carry a gene duplication or multiplication. The characteristic band for CYP2D6*5 (3.2 kb) was found in 13 persons.

*7.2.1.2 Special nested PCR: 1707delT (CYP2D6*6)*

For the detection of CYP2D6*6 a special nested PCR was used. After gel electrophoreses characteristic DNA bands at 356 bp and 421 bp can be seen for the *6 and the wildtype allele, respectively (Figure 39). CYP2D6*6 was found in five subjects of the 234 Caucasians.

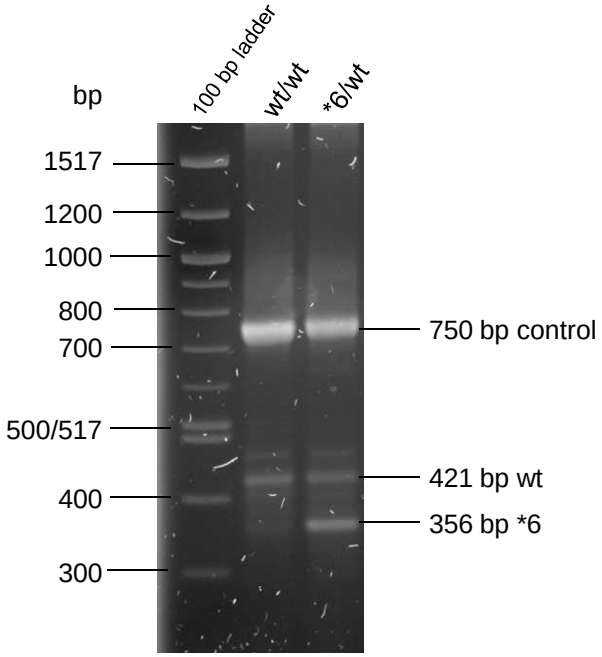


Figure 39 Ethidium bromide stained 2 % agarose gel. 356 bp product indicates CYP2D6*6.

7.2.1.3 RFLP-PCR

Three RFLP-PCRs were conducted to detect 1846G>A (CYP2D6*4, Figure 40 **A**), 2615–2617delAAG (CYP2D6*9, Figure 40 **B**) and 100C>T (found in e.g. CYP2D6*4 and CYP2D6*10, Figure 40 **C**).

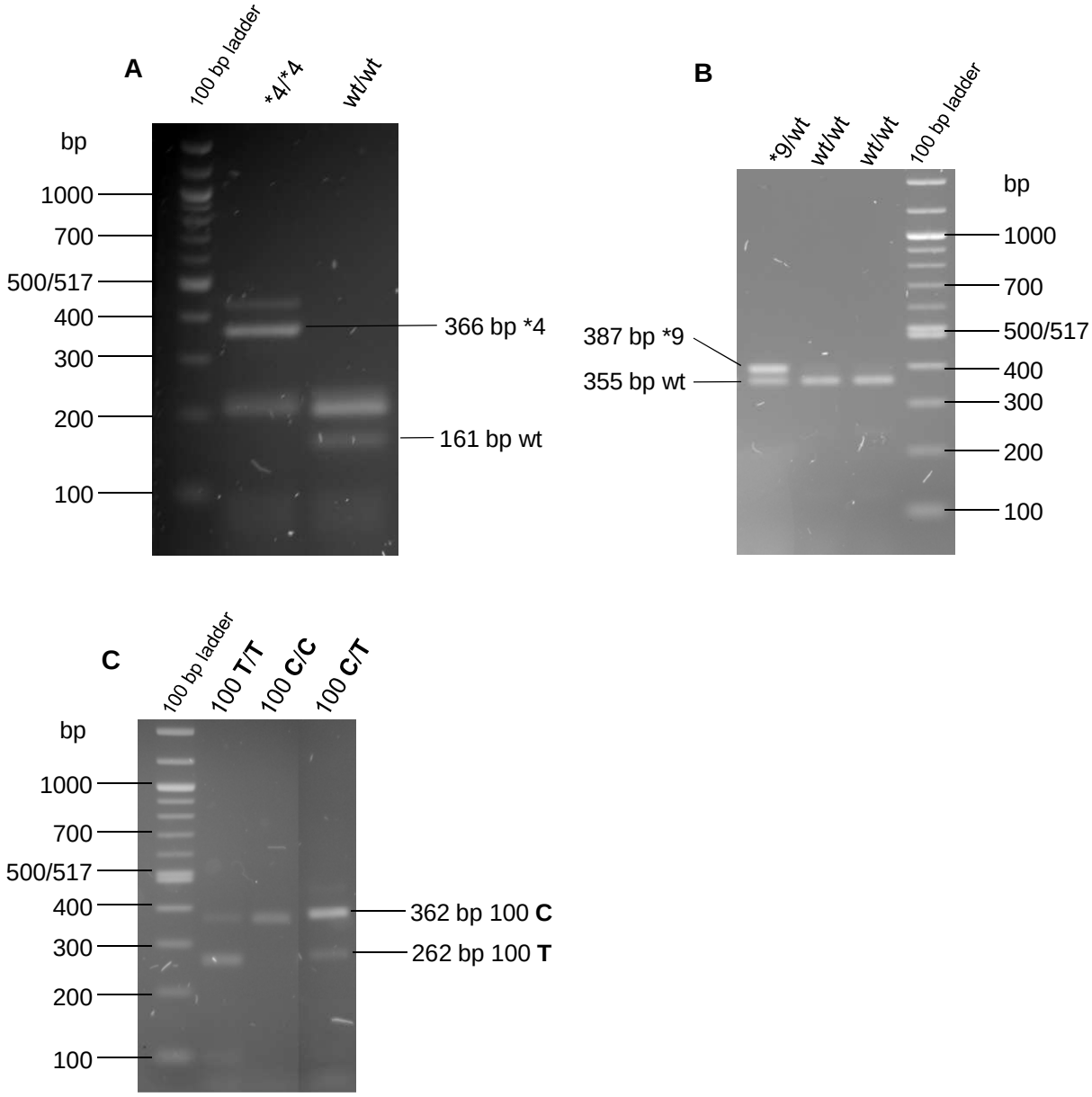


Figure 40 Ethidium bromide stained 2 % agarose gels. A 366 bp product indicates CYP2D6*4. B 387 bp product indicates CYP2D6*9. C 362 bp product indicates 100C, 262 bp product indicates 100T.

15 subjects were found to be homozygous for CYP2D6*4, 89 subjects showed the characteristic digestion product at 366 bp for one allele. The CYP2D6*9 allele was detected in 10 subjects, and the nucleotide substitution of cytosine by thymine at position 100 could be identified in 118 subjects. Of these, 43 were homozygous carriers of this SNP. The faint DNA band at 362 bp in the first lane of gel **C** is the uncut pseudogene CYP2D7P, which is amplified together with CYP2D6 in this method. However, by comparison with validated control DNA (homozygous and heterozygous for 100T) it was possible to distinguish between heterozygous and homozygous carriers.

7.2.2 Results of multiplex SNP detection

Four SNPs and two small deletions for CYP2D6 were examined using the multiplex SNP detection system iplex® gold. These were 1023C>T for CYP2D6*17, 1707delT for CYP2D6*6, 2549delA for CYP2D6*3, 2850C>T for CYP2D6*2 and *17, 2988G>A for CYP2D6*41, and 4180G>C for CYP2D6*2, *4, *6, *10, *17 and *41. The number of subjects carrying these variations can be found in Table 45. Not every SNP and deletion could be detected for every DNA sample. For the SNP characteristic for CYP2D6*17, for instance, four samples were not processed correctly. However, only samples which could be doubtlessly assigned to other alleles were included in the statistics and can be found in Table 45.

The designed multiplex assays also contained the SNP 3183G>A, which did not give results for any of the processed DNA samples.

SNPs and small deletions	important associated alleles	heterozygous carriers	homozygous carriers of SNP	homozygous carriers of wt
1023C>T	CYP2D6*17	0	0	230
1707delT	CYP2D6*6	5	0	222
2549delA	CYP2D6*3	5	0	199
2850C>T	CYP2D6*2, CYP2D6*17	90	1	114
2988G>A	CYP2D6*41	22	1	129
1661G>C	CYP2D6*2, CYP2D6*4, CYP2D6*6, CYP2D6*10, CYP2D6*17, CYP2D6*41	113	69	48

Table 45 Results for CYP2D6 SNPs and small deletions with the multiplex SNP detection method

For three subjects of those included in the statistics of this work, heterogenecity of the SNP at position 2850 was found, even though they were detected to carry the gene deletion (CYP2D6*5) on one allele. As previously mentioned these two results are not consistent and therefore sequencing of the relevant DNA part was conducted.

7.2.3 Results of sequencing

Some samples (three of those included in the statistics of this work) were found to be both carriers of the CYP2D6*5 allele and heterozygous for the SNP at position 2850. This is

impossible as a person only possessing one CYP2D6 gene can only show homozygocity for all other CYP2D6 alleles. To clarify whether the long range PCR method for CYP2D6*5 detection or the detection for 2850C>T with the iplex® gold method delivered reliable results, sequencing was employed. Amplicons of 18 samples (including samples not used for statistics in this work) containing position 2850 were sequenced. All samples were found to be homozygous for the SNP at position 2850. Therefore it was concluded that the detection of this SNP with the multiplex detection method failed, whereas the CYP2D6*5 detection method was further supported.

7.2.4 Resulting allele and genotype frequencies

As previously mentioned, many variant CYP2D6 alleles do not possess a characteristic SNP but show a distinct combination of SNPs. To detect those haplotypes, a thorough data acquisition had to be conducted. Table 46 shows a characteristic part of CYP2D6 genotyping data (for whole data set see Annex, Table 72). In the following paragraph, the used approach to determine the CYP2D6 genotype will be described with the help of the samples shown in Table 46.

Allele	?	*3	*4	*5	*6	?	*41*	
SNP or del	1661G>C	2549delA	1846G>A	gene del	1707delT	100C>T	2988G>A	genotype
1	GG	AA	GG	XX	TT	CC	GG	*1/*1
2	GC	AA	GG	XX	TT	CC	GG	*1/*2
3	GG	A.DEL	GG	XX	TT	CC	GG	*1/*3
4	GC	AA	GA	XX	TT	CT	GG	*1/*4
5	GG	AA	GG	X DEL	TT	CC	GG	*1/*5
6	GC	AA	GG	XX	TT	CC	GA	*1/*41
7	CC	AA	GG	XX	TT	CC	GG	*2/*2
8	CC	AA	GA	XX	TT	CT	GG	*2/*4
9	CC	AA	AA	XX	TT	TT	GG	*4/*4
10	CC	AA	GA	XX	TT	TT	GA	*4/*69

Table 46 Genotype acquisition table. *The SNP at position 2988 was thought to be characteristic for CYP2D6*41. In 2008 however, another haplotype was found (CYP2D6*69) carrying this SNP.

Samples possessing SNPs or deletions which only occur in one variant allele are easily genotyped. This is the case for samples 3 and 5 that only carried the SNP and the gene deletion characteristic for *3 and *5, respectively. As they do not show any other nucleotide changes, the second allele was found to be the wildtype allele *1. Sample 3 was therefore genotyped as CYP2D6 *1/*3 and sample 5 was assigned to the *1/*5 genotype.

For sample 1, no nucleotide changes were detected, therefore it was shown to be homozygous for the wildtype allele (*1/*1).

The genotyping of DNA number 2 is more complicated. It possesses one allele carrying 1661C instead of G. According to the CYP2D6 allele nomenclature homepage (http://www.cypalleles.ki.se/cyp2d6.htm; access date: 7.11.2010) which is a homepage regularly updated including all described CYP2D6 alleles, this SNP (1661G>C) is not characteristic for one polymorphic allele, but is present in many. However, of these only CYP2D6*2, CYP2D6*4, CYP2D6*10 and CYP2D6*41 are important for Caucasians. The characteristic nucleotide substitutions for *4 and *41 were not detected in sample number 2, and *10 can be excluded due to the missing nucleotide substitution at position 100. Therefore all named alleles except CYP2D6*2 can be excluded for sample 2. As no other nucleotide changes were detected, the genotype of DNA number 2 is CYP2D6*1/*2. Samples 4 and 6 were found to carry 1661C instead of G like DNA number 2. In addition to that, 4 and 6 possess the nucleotide changes characteristic for *4 and *41, respectively. Therefore, these two samples do not carry the CYP2D6*2 allele, but rather *4 (sample 4) and *41 (sample 6). Sample 6 could therefore be assigned to the *1/*41 genotype.

Sample 4, however, carries another nucleotide exchange, namely 100T. It is known that this exchange is also found in *4 alleles. As no other SNPs or deletions were detected for this sample, sample 4 was assigned *1/*4. In the same manner, sample 9 was assigned to the *4/*4 genotype.

Like for sample 6 (*1/*41) one allele of sample 10 was found to carry adenine at position 2988. Until 2008 this nucleotide exchange was thought to be characteristic for CYP2D6*41. In 2008, however, another haplotype for CYP2D6 was discovered (CYP2D6*69).[151] In contrast to CYP2D6*41, this polymorphic allele also shows the nucleotide exchange 100T. In sample 11 this newly described allele is found heterozygotely together with *4.

In this manner, all 234 DNA samples were genotyped. This leads to the allele and genotype frequencies shown in Table 47 and Table 48.

allele	frequency, %
CYP2D6*1	38.9
CYP2D6*4	25.4
CYP2D6*2	14.7
CYP2D6*10	7.9
CYP2D6*41	4.1
CYP2D6*5	2.8
CYP2D6*9	2.1
CYP2D6*3	1.1
CYP2D6*6	1.1

allele	frequency, %
CYP2D6*69	1.1
CYP2D6*1 x N	0.6
CYP2D6*2 x N	0.2
CYP2D6*17	0.0

Table 47 CYP2D6 allele frequencies.

genotype	frequency, %	genotype	frequency, %
CYP2D6*1/*4	20.5	CYP2D6*2/*5	0.9
CYP2D6*1/*2	17.9	CYP2D6*2/*10	0.9
CYP2D6*1/*1	12.8	CYP2D6*4/*9	0.9
CYP2D6*4/*10	9.4	CYP2D6*10/*10	0.9
CYP2D6*4/*4	6.4	CYP2D6*10/*41	0.9
CYP2D6*1/*41	4.7	CYP2D6*1/*3	0.4
CYP2D6*2/*4	3.0	CYP2D6*2/*6	0.4
CYP2D6*1/*5	2.6	CYP2D6*2/*9	0.4
CYP2D6*1/*10	2.1	CYP2D6*2xN/*9	0.4
CYP2D6*1/*9	1.7	CYP2D6*3/*4	0.4
CYP2D6*2/*2	1.7	CYP2D6*3/*5	0.4
CYP2D6*2/*41	1.7	CYP2D6*4/*6	0.4
CYP2D6*4/*5	1.7	CYP2D6*6/*9	0.4
CYP2D6*4/*69	1.7	CYP2D6*9/*10	0.4
CYP2D6*1/*1xN	1.3	CYP2D6*10/*69	0.4
CYP2D6*1/*6	0.9	CYP2D6*41/*41	0.4
CYP2D6*2/*3	0.9		

Table 48 CYP2D6 genotype frequencies.

7.3 CYP2C9

In contrast to the vast variety of polymorphic alleles for CYP2D6, CYP2C9 only seems to have three important alleles for Caucasians. The wildtype allele CYP2C9*1, and CYP2C9*2 and *3, both possessing a characteristic nucleotide exchange. Therefore it is seemingly simple to genotype for CYP2C9.

CYP2C9*2 (3608C>T) and *3 (42614A>C) were detected using RFLP-PCR methods. Figure 41 shows exemplary ethidium bromide stained agarose gels after digestion with AvaII (**A**: CYP2C9*2) and NsiI and KpnI (**B**: CYP2C9*3).

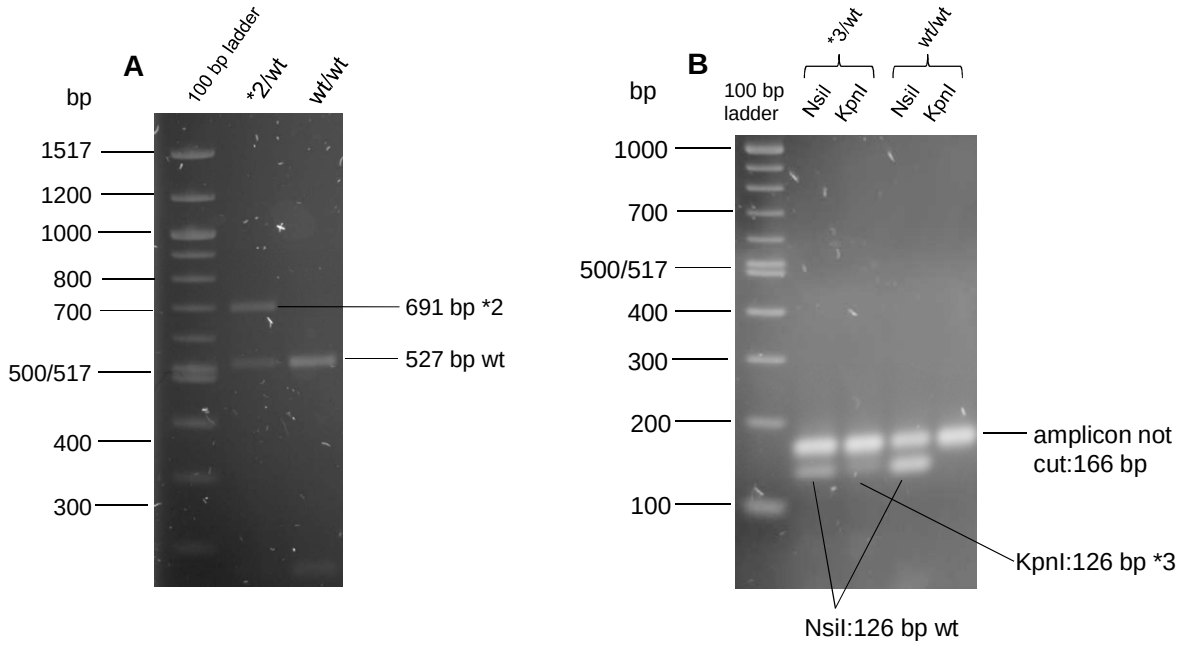


Figure 41 Ethidium bromide stained 2 % agarose gels. A 527 bp product after AvaII digestion indicates wildtype, 691 bp product indicates CYP2C9*2. B two samples are shown, both digested separately with NsiI (first and third lane) and KpnI (second and fourth lane). Digestion with NsiI leads to a product (126 bp) only for the wildtype allele (both samples). Digestion with KpnI leads to a product (126 bp) only for the *3 allele (left sample, lane 2).

91 subjects of the 283 Caucasians included in the CYP2C9 genotype phenotype correlation showed a 691 bp band and one at 527 bp after AvaII digestion and were therefore considered to carry one CYP2C9*2 allele. Five subjects were assigned to the *2/*2 genotype as no digestion product with a length of 527 bp could be detected.

For CYP2C9*3, 44 subjects were found to be heterozygotes due to the presence of digestion products (166 bp) after NsiI and KpnI treatment. One subject was identified to be a homozygous carrier of CYP2C9*3 since no digestion product was found after the reaction with KpnI.

As previously mentioned, a null allele (CYP2C9*6) was discovered in an African-American patient, which was, to our knowledge, never included in Caucasian genotyping studies. Therefore, this allele with the characteristic deletion of adenine at gene position 10601 was included in this study and detected using the multiplex SNP detection method. No subject was positive for this deletion.

All alleles negative for the nucleotide substitutions at position 3608 (*2) and 42614 (*3) were presumed to be CYP2C9*1. For resulting allele and genotype frequencies see Table 49 and Table 50, respectively.

allele	frequency, %
CYP2C9*1	80
CYP2C9*2	11.8
CYP2C9*3	8.1
CYP2C9*6	0

Table 49 CYP2C9 allele frequencies.

genotype	frequency, %
CYP2C9*1/*1	64.0
CYP2C9*1/*2	18.4
CYP2C9*1/*3	13.8
CYP2C9*2/*2	1.8
CYP2C9*2/*3	1.8
CYP2C9*3/*3	0.4

Table 50 CYP2C9 gene frequencies.

7.4 CYP2C19

For CYP2C19, four alleles with high frequencies in Caucasians are known. Beside the wildtype allele CYP2C19*1 there are CYP2C19*2,*3, and *17. These three variant alleles possess characteristic nucleotide changes. In this study 19154G>A (CYP2C19*2), 17948G>A (CYP2C19*3), and -806C>T (CYP2C19*17) were identified using the multiplex SNP detection method iplex® gold. The resulting allele and gene frequencies are listed in Table 51 and Table 52, respectively.

allele	frequency
CYP2C19*1	77.9
CYP2C19*2	13.1
CYP2C19*17	8.8
CYP2C19*3	0.2

Table 51 CYP2C19 allele frequencies.

genotype	frequency
CYP2C19*1/*1	63.1
CYP2C19*1/*2	21.5
CYP2C19*1/*17	7.7
CYP2C19*17/17	4.3
CYP2C19*2/*17	1.3
CYP2C19*1/*3	0.4

Table 52 CYP2C19 gene frequencies.

8 Correlation between phenotype and genotype

One aim of the study was to test the predictive power of the determined CYP2D6 and CYP2C9 genotypes for the measured dextromethorphan (DEX) and flurbiprofen (FLB) phenotypes, respectively. The idea was to assign activity coefficients to each of the detected CYP2D6 and CYP2C9 alleles, thereby estimating their contribution to the resulting enzyme activity and to facilitate the prediction of the CYP2D6 and CYP2C9 metabolic status of a subject knowing only its respective genotypes.

To this end, linear models to correlate phenotypes and genotypes were constructed by Dr. Roman Lutz, using the software environment for statistical computing R. The function used for this purpose was the linear modeling function. This function (see below) is used to calculate a value Y as a sum of independent variables e.g. X_1, and X_2 by assigning different coefficients e.g. b_1 and b_2.

$$\boldsymbol{Linear\ modeling\ function}: \quad Y = b_1 \cdot X_1 + b_2 \cdot X_2 + \cdots + b_n \cdot X_n$$

For the phenotype genotype correlation, Y are the measured phenotyping values, X have values of either 0, 1, or 2, depending on how often the alleles are present in the respective genotype and the assigned coefficients (b) can be used as metrics for the contribution of each allele to the resulting phenotype and therefore the enzyme activity. For example, if X_1 is the variable assigned to CYP2D6*1 and we regard a homozygote carrier of *1 with a $\log_{10}MR_{DEX}$ of -1.60, the equation for this subject would be:

$$\boldsymbol{example}: \quad -1.60 = b_1 \cdot 2 + b_2 \cdot 0 + \cdots + b_n \cdot 0$$

The solution for this equation is:

$$\boldsymbol{example}: \quad -0.8 = b_1$$

As many persons have the same genotype but different values for their phenotyping metric, every coefficient is over-determined. The linear model therefore calculates a “mean” coefficient and its standard deviation for each allele using regression analysis. These “simple” linear models are consistent with the presumption of alleles being co-dominantly expressed.

It is also possible to calculate linear models which consider possible interactions between the alleles. This is achieved by adding a so called “interaction term” to the linear modeling function (see below).

$$\boldsymbol{Linear\ modeling\ function\ with\ interaction\ term}:$$

$$Y = b_1 \cdot X_1 + b_2 \cdot X_2 + \cdots + b_n \cdot X_n + a_1 \cdot X_1 \cdot X_2 + \cdots + a_m \cdot X_{(n-1)} \cdot X_n$$

With these models, each allele is again assigned a specific coefficient (e.g. b_1 and b_2). However, additional coefficients (e.g. a_1) are allocated to every genotype consisting of two

interacting alleles (e.g. X_1 and X_2). Therefore, the predicted value for the phenotyping metric is calculated as the sum of the two allele coefficients plus the genotype coefficient. These models comprise that the alleles may influence each other, e.g that the alleles are recessively or dominantly expressed in certain combinations.

8.1 DEX phenotype and CYP2D6 genotype

For CYP2D6, measured DEX phenotyping metrics (with and without prior dextrorphan glucuronide cleavage) and the determined CYP2D6 genotypes of 230 persons were correlated using linear models. 4 Persons carrying CYP2D6 gene multiplications were excluded, because the number of gene-repeats was not determined. Therefore, an inclusion of these values would have corrupted the models.

To ensure the validity of a linear model, the used data has to be “normally-like” distributed. As can be seen in the exemplary boxplots for phenotyping data before dextrorphan glucuronide cleavage (Figure 42), $\log_{10}MR_{DEX}$ (**B**) shows a better distribution of values than MR_{DEX} (**A**). Therefore the models were calculated using the logarithmic phenotyping data ($\log_{10}MR_{DEX}$).

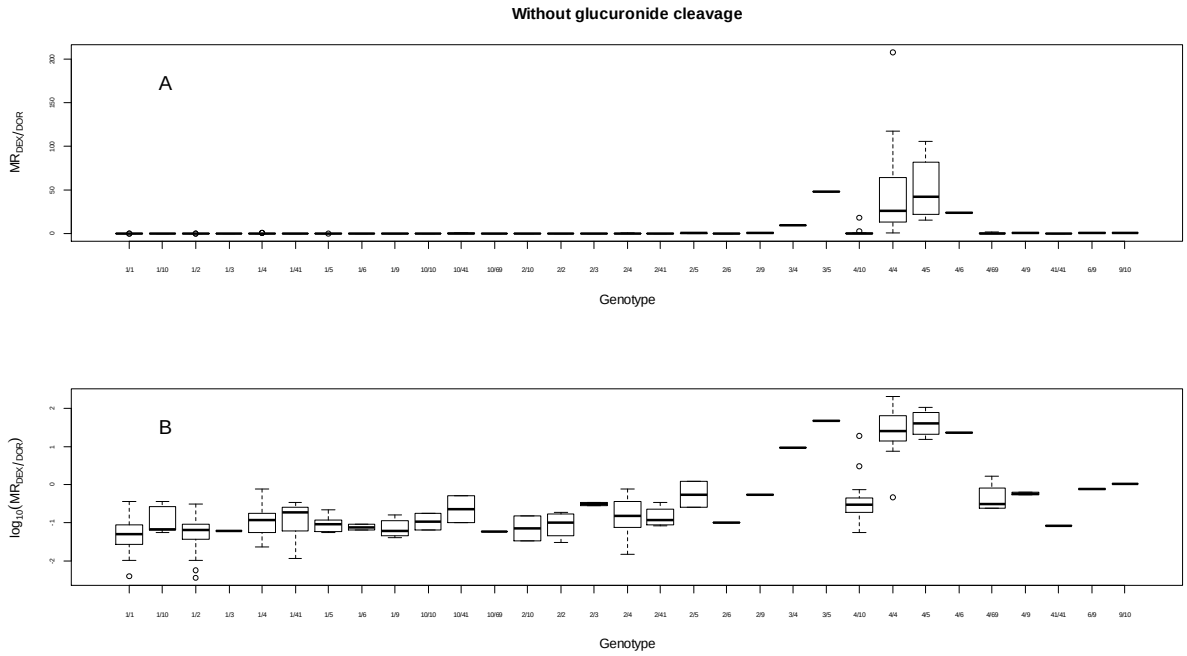


Figure 42 Tukey Boxplots of phenotyping data without prior dextrorphan glucuronide cleavage.

8.1.1 Linear models without interaction term

Linear models with independent variables were calculated using the phenotyping data with and without prior dextrorphan glucuronide cleavage. The results are shown in the next two chapters.

8.1.1.1 Without glucuronide cleavage

Coefficients for 10 alleles, CYP2D6*1,*2,*3,*4,*5,*6,*9,*10,*41, and *69 were calculated using the logarithmic phenotyping data ($log_{10}MR_{DEX}$) without glucuronide cleavage. The determined values (mean ± S.D.) are listed in Table 53.

Allele	Calculated coefficient (mean ± S.D.)
CYP2D6*1	-0.81 ± 0.04
CYP2D6*2	-0.57 ± 0.07
CYP2D6*3	0.33 ± 0.26
CYP2D6*4	0.28 ± 0.05
CYP2D6*5	0.46 ± 0.16
CYP2D6*6	0.03 ± 0.26
CYP2D6*9	-0.18 ± 0.20
CYP2D6*10	-0.57 ± 0.10
CYP2D6*41	-0.25 ± 0.13
CYP2D6*69	-0.64 ± 0.26

Table 53 Calculated coefficients for all included CYP2D6 alleles (data without glucuronide cleavage, linear model with independent variables).

With these coefficients it is possible to predict $log_{10}MR_{DEX}$ by simply adding the coefficients of the alleles composing the genotype. All predicted phenotyping metrics, together with the number of subjects possessing this genotype and the actual measured $log_{10}MR_{DEX}$ (mean ± S.D.) are shown in Table 54. 11 genotypes were only carried by one, 7 genotypes only by two subjects. Therefore, the predicted values for these show the largest deviation from the measured values. For all other genotypes, the deviation of means of predicted and measured ranges between 3 and 66 %.

Genotype	Predicted $log_{10}MR_{DEX}$ (mean ± S.D.)	Subjects	Measured $log_{10}MR_{DEX}$ (mean ± S.D.)	Deviation of means [%]
CYP2D6*1/*1	-1.63 ± 0.06	30	-1.30 ± 0.40	25
CYP2D6*1/*2	-1.39 ± 0.08	42	-1.29 ± 0.41	8
CYP2D6*1/*3	-0.48 ± 0.27	1	-1.21[1)]	60
CYP2D6*1/*4	-0.53 ± 0.07	48	-0.97 ± 0.36	45
CYP2D6*1/*5	-0.35 ± 0.17	6	-1.02 ± 0.23	66
CYP2D6*1/*6	-0.79 ± 0.27	2	-1.12[2)]	30
CYP2D6*1/*9	-0.99 ± 0.20	4	-1.15 ± 0.26	14
CYP2D6*1/*10	-1.38 ± 0.10	5	-0.93 ± 0.39	48
CYP2D6*1/*41	-1.06 ± 0.14	11	-0.97 ± 0.49	9

Genotype	Predicted $log_{10}MR_{DEX}$ (mean ± S.D.)	Subjects	Measured $log_{10}MR_{DEX}$ (mean ± S.D.)	Deviation of means [%]
CYP2D6*2/*2	-1.14 ± 0.10	4	-1.06 ± 0.37	8
CYP2D6*2/*3	-0.24 ± 0.27	2	-0.50[2)]	52
CYP2D6*2/*4	-0.29 ± 0.09	7	-0.84 ± 0.60	66
CYP2D6*2/*5	-0.11 ± 0.18	2	-0.26[2)]	58
CYP2D6*2/*6	-0.54 ± 0.27	1	-0.99[1)]	45
CYP2D6*2/*9	-0.75± 0.21	1	-0.27[1)]	179
CYP2D6*2/*10	-1.14 ± 0.12	2	-1.16[2)]	2
CYP2D6*2/*41	-0.82 ± 0.15	4	-0.85 ± 0.28	4
CYP2D6*3/*4	0.61 ± 0.27	1	0.98[1)]	37
CYP2D6*3/*5	0.80 ± 0.31	1	1.68[1)]	53
CYP2D6*4/*4	0.57 ± 0.07	15	1.39 ± 0.62	59
CYP2D6*4/*5	0.75 ± 0.17	4	1.61 ± 0.36	54
CYP2D6*4/*6	0.31 ± 0.27	1	1.37[1)]	77
CYP2D6*4/*9	0.10 ± 0.20	2	-0.24[2)]	143
CYP2D6*4/*10	-0.28 ± 0.11	22	-0.46 ± 0.52	39
CYP2D6*4/*69	-0.36 ± 0.27	4	-0.35 ± 0.40	3
CYP2D6*6/*9	-0.15 ± 0.33	1	-0.11[1)]	38
CYP2D6*9/*10	-0.74 ± 0.22	1	0.02[1)]	3825
CYP2D6*10/*10	-1.13 ± 0.13	2	-0.97[2)]	17
CYP2D6*10/*41	-0.81 ± 0.16	2	-0.65[2)]	25
CYP2D6*10/*69	-1.21 ± 0.28	1	-1.24[1)]	3
CYP2D6*41/*41	-0.49 ± 0.18	1	-1.08[1)]	54

Table 54 Comparison between calculated and measured values for $log_{10}MR_{DEX}$ (without glucuronide cleavage, model with independent variables). [1)] Only one subject possessed the respective genotype, therefore no mean and standard deviation can be given. [2)] Only two subjects possessed the respective genotype, therefore no standard deviation can be given.

More important than the prediction of $log_{10}MR_{DEX}$, however, is the possibility to evaluate the metabolic activity of the enzymes encoded by the 10 different variant alleles. A relative metabolic activity based on the assumption of an activity of 100 % for CYP2D6*1 can easily be calculated using the coefficients produced by the linear model. The antilog of these coefficients is proportional to the metabolic ratio of DEX (MR_{DEX}). As MR_{DEX} is defined as the concentration of DEX divided by the concentration of DOR, MR_{DEX} is inversely proportional to the metabolic activity of CYP2D6 towards DEX. By inverting the antilog of the coefficients and defining the value for CYP2D6*1 as 100 %, relative metabolic activities ranging from 5 % (CYP2D6*5) to 67 % (CYP2D6*69) can be calculated for the variant alleles (Table 55).

Allele	Relative metabolic activity [%]
CYP2D6*1	100
CYP2D6*2	57
CYP2D6*3	7
CYP2D6*4	8
CYP2D6*5	5
CYP2D6*6	14
CYP2D6*9	23
CYP2D6*10	56
CYP2D6*41	27
CYP2D6*69	67

Table 55 Relative metabolic activity (assuming an activity of 100 % for CYP2D6*1, data without glucuronide cleavage, model with independent variables).

8.1.1.2 With glucuronide cleavage

For the phenotyping data measured after DOR glucuronide cleavage, a similar linear model was calculated. The resulting coefficients are given in Table 56

Allele	Calculated coefficient (mean ± S.D.)
CYP2D6*1	-1.63 ± 0.05
CYP2D6*2	-1.28 ± 0.08
CYP2D6*3	-0.13 ± 0.31
CYP2D6*4	-0.32 ± 0.06
CYP2D6*5	-0.08 ± 0.19
CYP2D6*6	-0.71 ± 0.31
CYP2D6*9	-0.92 ± 0.23
CYP2D6*10	-1.45 ± 0.11
CYP2D6*41	-0.91 ± 0.15
CYP2D6*69	-1.16 ± 0.31

Table 56 Calculated coefficients for all included CYP2D6 alleles (data with glucuronide cleavage, linear model with independent variables).

The predicted values for $\log_{10}MR_{DEX}$ were again calculated (Table 57). As for the predicted values of the previously discussed model, the largest discrepancies between predicted and measured phenotyping metric were found for the underrepresented genotypes.

Genotype	Predicted $log_{10}MR_{DEX}$ (mean ± S.D.)	Subjects	$log_{10}MR_{DEX}$ (mean ± S.D.)	Deviation of means [%]
CYP2D6*1/*1	-3.25 ± 0.07	30	-2.93 ± 0.52	11
CYP2D6*1/*2	-2.90 ± 0.10	42	-2.76 ± 0.51	5
CYP2D6*1/*3	-1.75 ± 0.31	1	-2.47[1)]	29
CYP2D6*1/*4	-1.95 ± 0.08	48	-2.43 ± 0.48	20
CYP2D6*1/*5	-1.71 ± 0.20	6	-2.39 ± 0.45	28
CYP2D6*1/*6	-2.34 ± 0.31	2	-2.59[2)]	10
CYP2D6*1/*9	-2.55 ± 0.24	4	-2.64 ± 0.29	4
CYP2D6*1/*10	-3.07 ± 0.12	5	-2.57 ± 0.29	20
CYP2D6*1/*41	-2.54 ± 0.16	11	-2.44 ± 0.54	4
CYP2D6*2/*2	-2.55 ± 0.12	4	-2.61 ± 0.21	2
CYP2D6*2/*3	-1.40 ± 0.32	2	-1.87[2)]	25
CYP2D6*2/*4	-1.60 ± 0.10	7	-2.19 ± 0.55	27
CYP2D6*2/*5	-1.36 ± 0.21	2	-1.75[2)]	22
CYP2D6*2/*6	-1.99 ± 0.32	1	-2.49[1)]	20
CYP2D6*2/*9	-2.20 ± 0.25	1	-1.47[1)]	49
CYP2D6*2/*10	-2.72 ± 0.14	2	-2.63[2)]	3
CYP2D6*2/*41	-2.18 ± 0.17	4	-2.17 ± 0.66	1
CYP2D6*3/*4	-0.45 ± 0.32	1	0.02[1)]	2350
CYP2D6*3/*5	-0.21 ± 0.37	1	0.97[1)]	122
CYP2D6*4/*4	-0.65 ± 0.09	15	0.28 ± 0.78	332
CYP2D6*4/*5	-0.41 ± 0.20	4	0.51 ± 0.21	180
CYP2D6*4/*6	-1.03 ± 0.32	1	-0.10[1)]	935
CYP2D6*4/*9	-1.24 ± 0.24	2	-1.75[2)]	29
CYP2D6*4/*10	-1.77 ± 0.13	22	-1.99 ± 0.69	11
CYP2D6*4/*69	-1.48 ± 0.32	4	-1.54 ± 0.28	4
CYP2D6*6/*9	-1.63 ± 0.39	1	-1.55[1)]	5
CYP2D6*9/*10	-2.37 ± 0.26	1	-1.82[1)]	30
CYP2D6*10/*10	-2.89 ± 0.16	2	-2.68[2)]	8
CYP2D6*10/*41	-2.35 ± 0.19	2	-2.16[2)]	9
CYP2D6*10/*69	-2.61 ± 0.33	1	-2.38[1)]	9
CYP2D6*41/*41	-1.82 ± 0.22	1	-2.56[1)]	29

Table 57 Comparison between calculated and measured values for $log_{10}MR_{DEX}$ (with glucuronide cleavage, model with independent variables). [1)] Only one subject possessed the respective genotype, therefore no mean and standard deviation can be given. [2)] Only two subjects possessed the respective genotype, therefore no standard deviation can be given.

Using the calculation described in the previous chapter, relative metabolic activities of the variant alleles were again calculated using the “new” coefficients (Table 58).

Allele	Relative metabolic activity [%]
CYP2D6*1	100
CYP2D6*2	45
CYP2D6*3	3
CYP2D6*4	5
CYP2D6*5	3
CYP2D6*6	12
CYP2D6*9	20
CYP2D6*10	66
CYP2D6*41	19
CYP2D6*69	34

Table 58 Relative metabolic activity (assuming an activity of 100 % for CYP2D6*1, data with glucuronide cleavage, model with independent variables)

8.1.2 Linear models with interaction term

The models that were described in chapter 8.1.1 assume independent variables. This is consistent with the presumption of alleles being co-dominantly expressed. However, to test the hypothesis of co-dominant expression, models with interaction term were also calculated and compared with the primal “simple” models. In a prior test model, all possible genotypes were included into the model, i.e. for all allelic combinations an interaction term and therefore a coefficient was calculated. Then, the allele combinations for which this coefficient was significantly unequal zero (i.e. the Null Hypotheses could be rejected) were included in a new model with six interaction terms. The allele combinations for which a genotype coefficient was calculated were: CYP2D6*1/*4, CYP2D6*1/*5, CYP2D6*2/*4, CYP2D6*2/*5, CYP2D6*4/*9, and CYP2D6*4/*10. In “biological terms” this means that these models comprise that the alleles may influence each other, e.g that the alleles are recessively or dominantly expressed in certain combinations.

The models were again used to match the logarithmic phenotyping metric $\log_{10}MR_{DEX}$ without and with prior glucuronide cleavage. The results can be found in the following two chapters.

8.1.2.1 Without glucuronide cleavage

All allele and genotype coefficients calculated with the phenotyping data maintained without glucuronide cleavage are listed in Table 59.

Allele	Calculated coefficient
CYP2D6*1	-0.67 ± 0.03
CYP2D6*2	-0.59 ± 0.06
CYP2D6*3	0.11 ± 0.20
CYP2D6*4	0.70 ± 0.05
CYP2D6*5	1.04 ± 0.20
CYP2D6*6	-0.11 ± 0.20
CYP2D6*9	-0.17 ± 0.17
CYP2D6*10	-0.37 ± 0.10
CYP2D6*41	-0.34 ± 0.10
CYP2D6*69	-1.02 ± 0.20
CYP2D6*1/*4	-0.67 ± 0.09
CYP2D6*1/*5	-0.59 ± 0.27
CYP2D6*2/*4	0.11 ± 0.18
CYP2D6*2/*5	0.70 ± 0.37
CYP2D6*4/*9	1.04 ± 0.35
CYP2D6*4/*10	-0.11 ± 0.15

Table 59 Calculated coefficients for all included CYP2D6 alleles and genotypes (data without glucuronide cleavage, linear model with interaction terms for CYP2D6*1/*4, CYP2D6*1/*5, CYP2D6*2/*4, CYP2D6*2/*5, CYP2D6*4/*9, and CYP2D6*4/*10).

Summing up these coefficients as described in the first paragraph of this chapter the phenotyping metrics can be predicted (Table 60). The introduction of the six interaction terms leads to a significant better description of the relevant genotypes. The implication of this finding will be further discussed in chapter 9.

Genotype	Predicted $log_{10}MR_{DEX}$ (mean ± S.D.)	Subjects	$log_{10}MR_{DEX}$ (mean ± S.D.)	Deviation of means [%]
CYP2D6*1/*1	-1.34 ± 0.05	30	-1.30 ± 0.40	3
CYP2D6*1/*2	-1.26 ± 0.07	42	-1.29 ± 0.41	2
CYP2D6*1/*3	0.23 ± 0.20	1	-1.21[1)]	119
CYP2D6*1/*4	-0.97 ± 0.11	48	-0.97 ± 0.36	0
CYP2D6*1/*5	-1.02 ± 0.34	6	-1.02 ± 0.23	0
CYP2D6*1/*6	-0.78 ± 0.20	2	-1.12[2)]	30
CYP2D6*1/*9	-0.84 ± 0.17	4	-1.15 ± 0.26	27
CYP2D6*1/*10	-1.04 ± 0.11	5	-0.93 ± 0.39	11
CYP2D6*1/*41	-1.01 ± 0.10	11	-0.97 ± 0.49	4
CYP2D6*2/*2	-1.18 ± 0.08	4	-1.06 ± 0.37	12

Genotype	Predicted $log_{10}MR_{DEX}$ (mean ± S.D.)	Subjects	$log_{10}MR_{DEX}$ (mean ± S.D.)	Deviation of means [%]
CYP2D6*2/*3	-0.48 ± 0.21	2	-0.50[2)]	4
CYP2D6*2/*4	-0.84 ± 0.20	7	-0.84 ± 0.60	0
CYP2D6*2/*5	-0.26 ± 0.43	2	-0.26[2)]	0
CYP2D6*2/*6	-0.70 ± 0.20	1	-0.99[1)]	29
CYP2D6*2/*9	-0.76 ± 0.18	1	-0.27[1)]	182
CYP2D6*2/*10	-0.96 ± 0.12	2	-1.16[2)]	17
CYP2D6*2/*41	-0.93 ± 0.11	4	-0.85 ± 0.28	9
CYP2D6*3/*4	0.81 ± 0.21	1	0.98[1)]	17
CYP2D6*3/*5	1.15 ± 0.28	1	1.68[1)]	31
CYP2D6*4/*4	1.40 ± 0.08	15	1.39 ± 0.62	1
CYP2D6*4/*5	1.74 ± 0.21	4	1.61 ± 0.36	8
CYP2D6*4/*6	0.59 ± 0.20	1	1.37[1)]	57
CYP2D6*4/*9	-0.24 ± 0.39	2	-0.24[2)]	1
CYP2D6*4/*10	-0.46 ± 0.19	22	-0.46 ± 0.52	0
CYP2D6*4/*69	-0.32 ± 0.21	4	-0.35 ± 0.40	10
CYP2D6*6/*9	-0.28 ± 0.26	1	-0.11[1)]	156
CYP2D6*9/*10	-0.54 ± 0.19	1	0.02[1)]	2779
CYP2D6*10/*10	-0.73 ± 0.14	2	-0.97[2)]	25
CYP2D6*10/*41	-0.70 ± 0.14	2	-0.65[2)]	8
CYP2D6*10/*69	-1.38 ± 0.22	1	-1.24[1)]	12
CYP2D6*41/*41	-0.68 ± 0.14	1	-1.08[1)]	37

Table 60 Comparison between calculated and measured values for $log_{10}MR_{DEX}$ (without glucuronide cleavage, model with interaction terms for CYP2D6*1/*4, CYP2D6*1/*5, CYP2D6*2/*4, CYP2D6*2/*5, CYP2D6*4/*9, and CYP2D6*4/*10). [1)] Only one subject possessed the respective genotype, therefore no mean and standard deviation can be given. [2)] Only two subjects possessed the respective genotype, therefore no standard deviation can be given.

Using only the calculated allele coefficients, relative metabolic activities were again calculated (Table 61).

Allele	Relative metabolic activity [%]
CYP2D6*1	100
CYP2D6*2	83
CYP2D6*3	16
CYP2D6*4	4
CYP2D6*5	2
CYP2D6*6	28

Allele	Relative metabolic activity [%]
CYP2D6*9	32
CYP2D6*10	50
CYP2D6*41	46
CYP2D6*69	222

Table 61 Relative metabolic activity (assuming an activity of 100 % for CYP2D6*1, data without glucuronide cleavage, model with interaction terms for CYP2D6*1/*4, CYP2D6*1/*5, CYP2D6*2/*4, CYP2D6*2/*5, CYP2D6*4/*9, and CYP2D6*4/*10)

8.1.2.2 With glucuronide cleavage

A similar model with interaction terms for CYP2D6*1/*4, CYP2D6*1/*5, CYP2D6*2/*4, CYP2D6*2/*5, CYP2D6*4/*9, and CYP2D6*4/*10 was calculated using the logarithmic phenotyping metric obtained after DOR glucuronide cleavage. The resulting allele and genotype coefficients, the predicted phenotyping metrics, and the calculated relative enzyme activity can be found in Table 62, Table 63, and Table 64, respectively.

Allele	Calculated coefficient
CYP2D6*1	-1.48 ± 0.04
CYP2D6*2	-1.29 ± 0.07
CYP2D6*3	-0.37 ± 0.25
CYP2D6*4	0.14 ± 0.07
CYP2D6*5	0.57 ± 0.25
CYP2D6*6	-0.87 ± 0.24
CYP2D6*9	-0.87 ± 0.21
CYP2D6*10	-1.21 ± 0.13
CYP2D6*41	-1.01 ± 0.12
CYP2D6*69	-1.58 ± 0.25
CYP2D6*1/*4	-1.09 ± 0.11
CYP2D6*1/*5	-1.48 ± 0.34
CYP2D6*2/*4	-1.04 ± 0.23
CYP2D6*2/*5	-1.03 ± 0.46
CYP2D6*4/*9	-1.01 ± 0.44
CYP2D6*4/*10	-0.92 ± 0.18

Table 62 Calculated coefficients for all included CYP2D6 alleles and genotypes (data with glucuronide cleavage, linear model with interaction terms for CYP2D6*1/*4, CYP2D6*1/*5, CYP2D6*2/*4, CYP2D6*2/*5, CYP2D6*4/*9, and CYP2D6*4/*10).

Genotype	Predicted $log_{10}MR_{DEX}$ (mean ± S.D.)	Subjects	$log_{10}MR_{DEX}$ (mean ± S.D.)	Deviation of means [%]
CYP2D6*1/*1	-2.95 ± 0.06	30	-2.93 ± 0.52	1
CYP2D6*1/*2	-2.76 ± 0.08	42	-2.76 ± 0.51	0
CYP2D6*1/*3	0.29 ± 0.25	1	-2.47[1)]	112
CYP2D6*1/*4	-2.43 ± 0.14	48	-2.43 ± 0.48	0
CYP2D6*1/*5	-2.39 ± 0.42	6	-2.39 ± 0.45	0
CYP2D6*1/*6	-2.35 ± 0.25	2	-2.59[2)]	9
CYP2D6*1/*9	-2.35 ± 0.21	4	-2.64 ± 0.29	11
CYP2D6*1/*10	-2.68 ± 0.13	5	-2.57 ± 0.29	4
CYP2D6*1/*41	-2.48 ± 0.13	11	-2.44 ± 0.54	2
CYP2D6*2/*2	-2.58 ± 0.10	4	-2.61 ± 0.21	1
CYP2D6*2/*3	-1.66 ± 0.26	2	-1.87[2)]	11
CYP2D6*2/*4	-2.19 ± 0.25	7	-2.19 ± 0.55	0
CYP2D6*2/*5	-1.75 ± 0.53	2	-1.75[2)]	0
CYP2D6*2/*6	-2.16 ± 0.25	1	-2.49[1)]	13
CYP2D6*2/*9	-2.16 ± 0.22	1	-1.47[1)]	47
CYP2D6*2/*10	-2.50 ± 0.14	2	-2.63[2)]	5
CYP2D6*2/*41	-2.30 ± 0.14	4	-2.17 ± 0.66	6
CYP2D6*3/*4	-0.24 ± 0.26	1	0.02[1)]	1278
CYP2D6*3/*5	0.19 ± 0.35	1	0.97[1)]	80
CYP2D6*4/*4	0.27 ± 0.10	15	0.28 ± 0.78	2
CYP2D6*4/*5	0.70 ± 0.26	4	0.51 ± 0.21	38
CYP2D6*4/*6	-0.73 ± 0.25	1	-0.10[1)]	633
CYP2D6*4/*9	-1.75 ± 0.49	2	-1.75[2)]	0
CYP2D6*4/*10	-1.99 ± 0.23	22	-1.99 ± 0.69	0
CYP2D6*4/*69	-1.44 ± 0.26	4	-1.54 ± 0.28	7
CYP2D6*6/*9	-1.74 ± 0.32	1	-1.55[1)]	12
CYP2D6*9/*10	-2.08 ± 0.24	1	-1.82[1)]	14
CYP2D6*10/*10	-2.41 ± 0.18	2	-2.68[2)]	10
CYP2D6*10/*41	-2.21 ± 0.17	2	-2.16[2)]	3
CYP2D6*10/*69	-2.78 ± 0.28	1	-2.38[1)]	17
CYP2D6*41/*41	-2.02 ± 0.17	1	-2.56[1)]	21

Table 63 Comparison between calculated and measured values for $log_{10}MR_{DEX}$ (with glucuronide cleavage, model with interaction terms for CYP2D6*1/*4, CYP2D6*1/*5, CYP2D6*2/*4, CYP2D6*2/*5, CYP2D6*4/*9, and CYP2D6*4/*10). [1)] Only one subject possessed the respective genotype, therefore no mean and standard deviation can be given. [2)] Only two subjects possessed the respective genotype, therefore no standard deviation can be given.

Allele	Relative metabolic activity [%]
CYP2D6*1	100
CYP2D6*2	65
CYP2D6*3	8
CYP2D6*4	2
CYP2D6*5	1
CYP2D6*6	25
CYP2D6*9	25
CYP2D6*10	54
CYP2D6*41	34
CYP2D6*69	126

Table 64 Relative metabolic activity (assuming an activity of 100 % for CYP2D6*1, data with glucuronide cleavage, model with interaction terms for CYP2D6*1/*4, CYP2D6*1/*5, CYP2D6*2/*4, CYP2D6*2/*5, CYP2D6*4/*9, and CYP2D6*4/*10)

8.1.3 Comparison of models

Altogether four different linear models to correlate either the phenotypic metric $\log_{10}MR_{(DEX/DOR)}$ without or with prior dextrorphan glucuronide cleavage with the determined CYP2D6 genotype were calculated.

The first two models (Model 1A and B) are simple linear models in which the alleles are treated as independent variables (A: without glucuronide cleavage, chapter 8.1.1.1; B: with glucuronide cleavage, chapter 8.1.1.2).

The second two models (Model 2A and B) include six interaction terms, thereby introducing the possibility of dominant and recessive expression of variant genes of the following genotypes: CYP2D6*1/*4, CYP2D6*1/*5, CYP2D6*2/*4, CYP2D6*2/*5, CYP2D6*4/*9, and CYP2D6*4/*10.

In Table 65, R^2 (coefficients of determination), and the ranges of deviation between predicted and measured $\log_{10}MR_{DEX}$ are listed for the different models. Model 2A, the linear model using phenotyping data without prior glucuronide cleavage and including interaction terms for the above mentioned genotypes, is best suited for phenotype prediction.

Model	R^2	range of deviation of means*
1A	0.57	3 – 66 %
1B	0.57	1 – 332 %
2A	0.77	0 – 27 %
2B	0.74	0 – 38 %

Table 65 Comparison of the four calculated linear models for CYP2D6 genotype phenotype correlation. *The given range if deviation of means excludes values for genotypes with a number of subjects ≤ 2.

For illustration this result is also graphically presented (Figure 43). The figure depicts the phenotyping data as Tukey boxplots (whiskers with a maximum of 1.5 x interquartile range), and the predicted values using model 1A (orangered squares), and model 2A (green triangles). Except for six genotypes (CYP2D6*1/*9, CYP2D6*10/*10, CYP2D6*10/*69, CYP2D6*2/*2, CYP2D6*4/*69, and CYP2D6*6/*9) all values for log10MRDEX are predicted more precisely using Model 2A, i.e. the linear model with interaction terms. This finding will be further discussed in chapter 9.

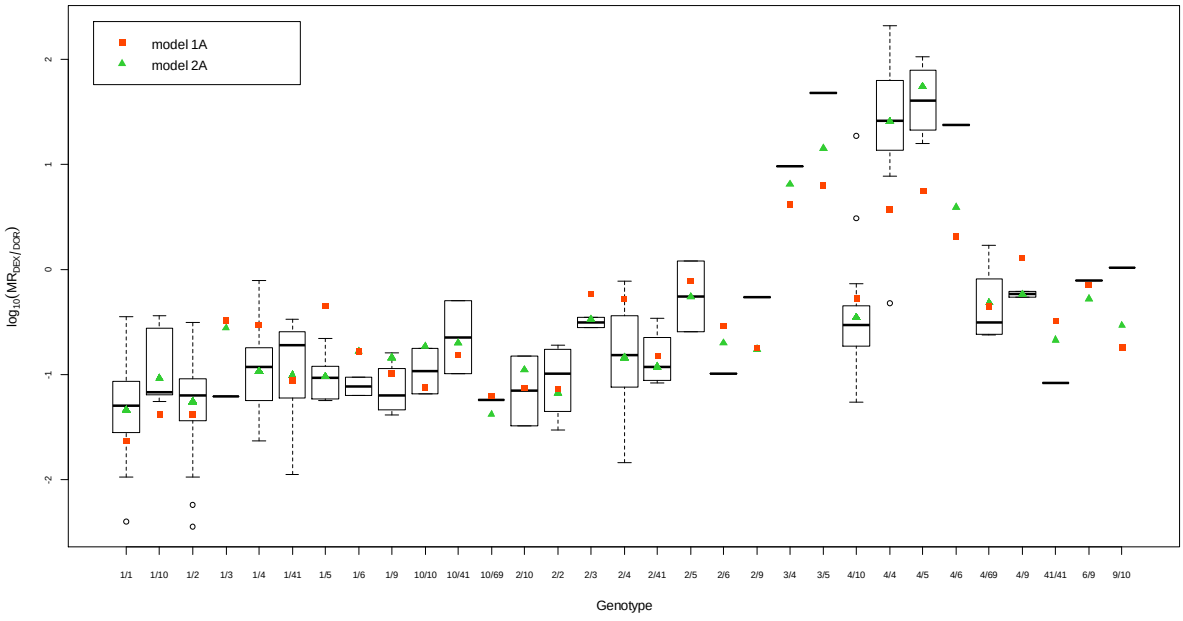


Figure 43 Tukey boxplots of the phenotyping data $log_{10}MR_{DEX}$ without glucuronide cleavage and the predicted values for $log_{10}MR_{DEX}$ using models 1A and 2A.

8.2 FLB phenotype and CYP2C9 genotype

A correlation between the measured FLB phenotype and the determined CYP2C9 genotype of 283 persons was also conducted. To estimate the contribution of each of the three variant CYP2C9 alleles (*1, *2 and *3) to the metabolic activity of a subject, the phenotyping data

($log_{10}MR_{FLB}$) was transformed to 1/MR. $1/MR_{FLB}$ shows the best distribution of values, because the value for the individual with the *3/*3 genotype is situated “correctly” relative to the values of the heterozygous *3 carriers (Figure 44).

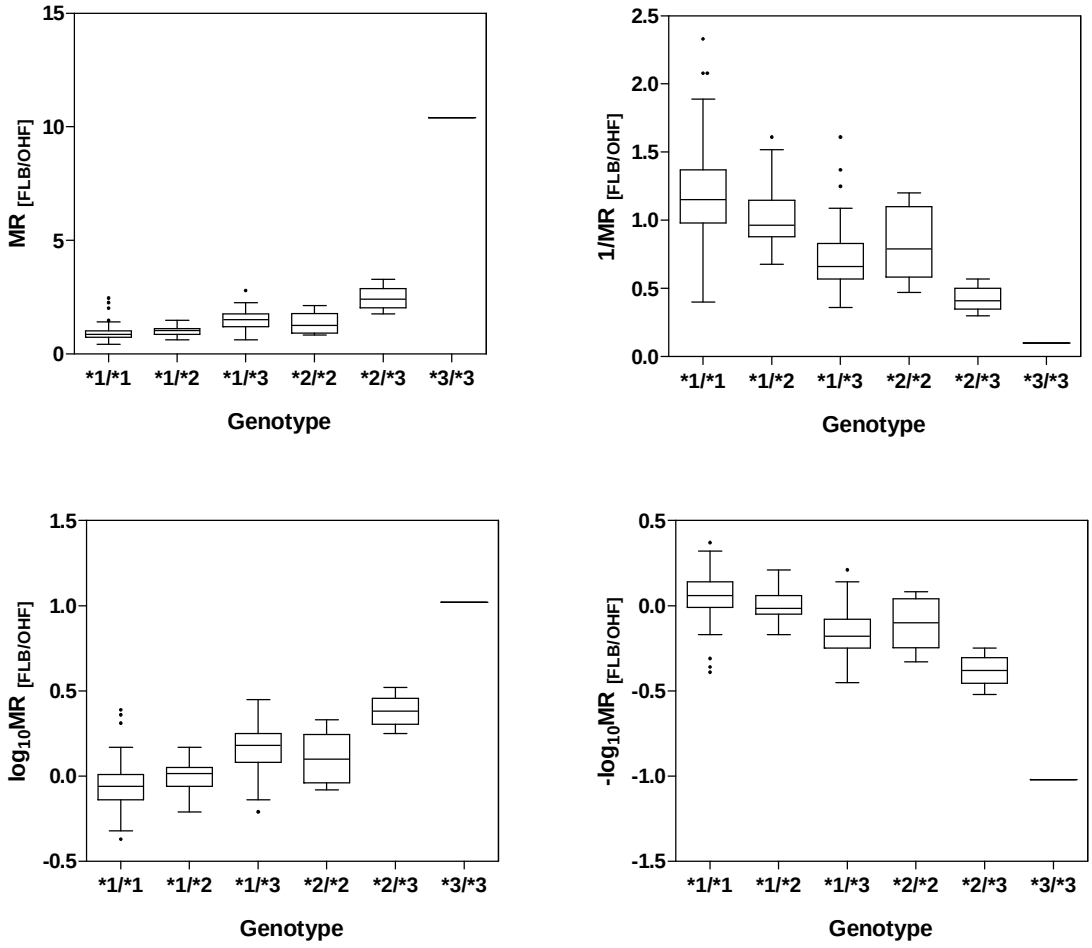


Figure 44 Tukey boxplots of the FLB phenotyping data.

Using this „new“ metric ($1/MR_{FLB}$), which is proportional to the metabolic activity, a linear model (independent variables) to assess the activity coefficients of the different alleles was calculated. With the highly significant model, a mean value ± S.D. of 0.596 ± 0.010 could be determined as the contribution of CYP2C9*1 to 1/MR. CYP2C9*2 and *3 contribute 0.405 ± 0.033 and 0.113 ± 0.042 to the 1/MR value, respectively. In comparison with the wildtype allele CYP2C9*1 (100 %), CYP2C9*2 therefore accounts for a metabolic activity of 68 % whereas CYP2C9*3 can be assigned to a metabolic activity of 19 %.

With these new activity coefficients, the values for $1/MR_{FLB}$ can be predicted:

genotype	predicted 1/MR	number of subjects	$1/MR_{FLB}$ (mean ± S.D.)	Deviation of means [%]
CYP2C9*1/*1	1.19 ± 0.01	181	1.19 ± 0.31	0
CYP2C9*1/*2	1.00 ± 0.03	52	1.00 ± 0.20	0
CYP2C9*1/*3	0.71 ± 0.04	39	0.73 ± 0.26	3
CYP2C9*2/*2	0.81 ± 0.05	5	0.83 ± 0.28	2
CYP2C9*2/*3	0.51 ± 0.05	5	0.42 ± 0.10	23
CYP2C9*3/*3	0.23 ± 0.06	1	10.4*	98

Table 66 Comparison between calculated and measured values for $1/MR_{FLB}$.*Only one subject possessed the CYP2C9*3/*3 genotype, therefore no mean and standard deviation can be given.

The biggest discrepancy between calculated and measured value is found for the subject with the *3/*3 genotype (0.23 versus 0.096). This is due to the fact that only one individual showed this genotype and was included in the linear model.

As previously mentioned, the used linear model working with the sum of the three variant alleles was highly significant. An enhancement of the model adding the homozygocity as an additional factor (i.e. using a model with an interaction term) did not significantly improve the model. Nor was a model using the six possible genotypes instead of the three variant alleles better than the original model. These findings are consistent with a co-dominant expression of the three variant alleles.

9 Discussion

In the 50's of the last century, clinicians discovered the role of genetics for the interindividual differences in drug response to the anti Malarial drug primaquine and muscle relaxant succinylcholine. A review by Motulsky published in 1957[15] delineated and conceptualized the field of pharmacogenetics.[1] This term describing the influence of the genetic disposition of an individual on its response to drug treatment was first used in 1959 by Vogel[16] and was soon adopted by others working in this field.[17] The first examples for pharmacogenetics concerned drug metabolizing enzymes. To date, other examples for genetic polymorphisms with effect on the appearance of adverse drug reactions (ADRs) or resistance of an individual towards drug treatment have been described. However, the described polymorphisms of drug metabolizing enzymes seem to have the biggest influence on drug treatment tolerance and efficacy. Only 20 % of drugs in ADR studies are not metabolized by polymorphic enzymes.[2]

The most important human enzymes for oxidative drug metabolism are the Cytochrome P450 monooxygenases of family 1 to 3 (CYP1–CYP3). Enzymes of these families take part in the oxidative metabolism of 78 % of all drugs metabolized in the liver.[34] Of these, more than 90 % are oxidized by the following CYP isoforms: CYP1A2 (4 %), CYP2A6, (2 %), CYP2C9 (10 %), CYP2C19 (2 %), CYP2E1 (2 %), CYP2D6 (30 %), and CYP3A4 (50 %).[2, 3]

Three of these isoforms (CYP2D6, 2C9 and 2C19) were found to possess a high rate of genetic polymorphisms with huge influence on drug susceptibility. This is on the one hand due to the occurrence and relative high frequency of alleles which encode for enzymes without enzyme activity (for CYP2D6 and CYP2C19) and on the other hand depending on the therapeutic indices of the metabolized drugs. CYP2D6 e.g. takes part in the oxidative metabolism of drugs with severe ADRs like tricyclic antidepressants, antipsychotic drugs, and beta adrenergic blocking agents. CYP2C9 oxidizes non Steroidal anti inflammatory drugs (NSAIDs), several oral anticoagulants, angiotensin II blockers, and sulfonylurea hypoglycemic drugs. Several proton pump inhibitors (PPIs), anti-epileptics, tricyclic antidepressants, and SSRIs are partly metabolized by CYP2C19.

To minimize the occurrence of ADRs and treatment failures with drugs that are metabolized by these polymorphic CYPs, it is recommended to assess the enzyme activity of a patient before drug treatment. For this purpose, two different approaches are available. The first approach is based on the actual enzyme activity towards a carefully evaluated probe drug (phenotyping). The second approach examines the underlying genotype to predict the actual enzyme activity. Both approaches have their advantages and disadvantages.

For phenotyping, a probe drug has to be found for which the formation of at least one of its

metabolites depends nearly exclusively on the CYP isoform to be examined. In addition to that, the metabolite formation rate has to depend on the CYP-genotype. Provided this, the concentration of its CYP isoform-dependant metabolite in urine, plasma or saliva provides a index for the CYP enzyme activity (phenotyping metric). The best available probe drugs are additionally (i) as safe as possible, preferable an over-the-counter drug (OTC drug), (ii) still in clinical use so that they are easily available, (iii) easy to detect and stable under used conditions, to simplify analytics and (iv) renally excreted to allow for non-invasive phenotyping in urine. Most established probe drugs do not fulfill all mentioned characteristics.[124] In addition to that one can argue about the transferability of results for enzyme activity obtained with one specific probe drug to all the other drugs metabolized by this enzyme.

Genotyping, however, is very complicated due to the multitude of known polymorphic alleles especially for CYP2D6 (http://www.cypalleles.ki.se/cyp2d6.htm; access date: 7.11.2010). Only known alleles can be detected in routinely conducted genotyping and as more alleles are discovered every month, it is obvious that not all alleles have yet been described. In addition to “knowing the alleles” a kind of metric for the activity of the encoded enzyme has to be established.[57] On top of this, the occurrence of an allele does not necessarily lead to its transcription and translation to the encoded enzyme. In the last few years more and more influence on gene expression and translation is acknowledged to epigenetic mechanisms. As Feinberg et al. wrote “the modern definition of epigenetics is modifications of the DNA or associated proteins, other than DNA sequence variation, that carry information content during cell division”.[152] To our knowledge, in every CYP genotyping study published, DNA was isolated from blood and not from liver. Therefore, it is not possible to examine epigenetic mechanisms influencing the CYP gene expression and translation in the most important organ for drug metabolism. Nevertheless, those mechanisms have to be kept in mind when discussing the predictive power of the genotype for the phenotype of an individual.[34]

Even if both available methods for CYP activity assessment have their pitfalls, the determination of enzyme activity cannot be neglected. As previously mentioned, 80 % of all drugs in ADR studies are metabolized by genetically polymorphic enzymes. It is believed that 10–20 % of all drug therapies could profit from predictive genotyping.[153]

The aim of this study was to contribute to this important research field by (i) developing a save and easy-to-use phenotyping cocktail, and (ii) investigating the predictive power of the genotype for the phenotype determined with the new cocktail.

Many phenotyping cocktails have been described in literature. However, they have at least one of the following disadvantages. Firstly, some of the used probe drugs can cause severe ADRs (e.g. mephenytoin or warfarin).[124, 130] Secondly, the probe drugs are administered in

high (therapeutic) doses, because of insensitive analytical methods used to quantify the probe drug and its metabolite in the body fluids.[133] Thirdly, for most phenotyping procedures, the phenotyping metric is determined in plasma. Mostly, several blood samples have to be taken.[154] Therefore the phenotyping procedure is invasive and cumbersome. Fourthly, most researchers did not test whether the probe drugs mutually influence their respective phenotyping metric.[6]

For this study, the best available probe drugs for CYP2D6, 2C9 and 2C19, meeting as many of the mentioned characteristics for a good probe drug as possible, were chosen (see chapter 1.4.1.1). The antitussive agent dextromethorphan (DEX), the NSAID flurbiprofen (FLB) and the PPI omeprazole (OME) should be used as probe drugs for CYP2D6, CYP2C9 and CYP1C19, respectively.

Before these drugs could be combined to a phenotyping cocktail, interaction studies had to be conducted to exclude mutual influences of the probe drugs on their respective phenotypic metric. The studies showed an influence of OME on the MR_{FLB} calculated as the ratio of FLB concentration to the concentration of the CYP2C9 dependent metabolite 4'-hydroxy flurbiprofen (OHF) in urine sampled 2 h after FLB administration. The calculated MR_{FLB} after administration of an OME-FLB combination were significantly lower than after administration of FLB alone. The amount of unchanged FLB did not significantly change, but a significantly higher amount of OHF could be found in urine after OME ingestion (data not shown). To our knowledge the influence of OME on FLB metabolism was never described. Figure 45 shows the assumed metabolism of flurbiprofen in humans. As FLB was given as a racemate and analyzed using achiral analytical methods, the stereoselectivity of FLB metabolism was neglected in Figure 45. The higher amount of OHF found in urine after simultaneous OME and FLB ingestion cannot result from CYP2C9 enzyme induction. As the mechanism of induction is thought to involve an induction of enzyme expression which leads to a higher amount of enzyme in the liver, the enzyme induction is supposed to take a few hours to days before an effect can be seen.[155] However, it is known that OME is not only substrate but also an inhibitor for CYP2C19. One explanation for the enhanced excretion of OHF might be the inhibition of another metabolic route of FLB including an oxidation via CYP2C19. When this alternative metabolism is hindered, this may well lead to an increased amount of FLB excreted as the CYP2C9 dependent metabolite OHF.

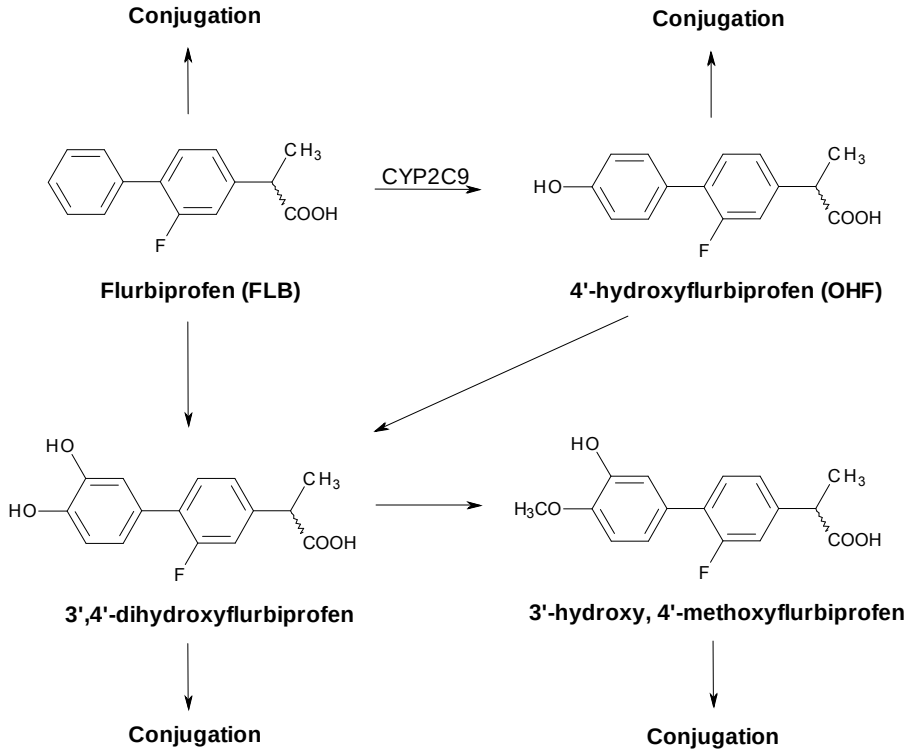


Figure 45 Simplified metabolism of flurbiprofen in humans.[156]

The discovered interaction between FLB and OME supports the importance of preliminary interaction tests. FLB and OME cannot be administered simultaneously without influencing the validity of the phenotyping metric for CYP2C9. In 2004, however, they were used together in a phenotyping cocktail in much higher doses (50 mg FLB and 40 mg OME) without prior interaction tests.[6]

Administration of DEX directly followed by FLB ingestion did not significantly alter the two phenotyping metrics, so that these two probe drugs could be used as a CYP2D6-CYP2C9 phenotyping combination.

Phenotyping with OME would have to be conducted separately. Only few data exist concerning the use of urinary phenotyping metrics of OME. Streetman et al. suggested that this is probably due to “unfavourable pharmacokinetics” of OME. Approximately 80 % of the dose is excreted as unchanged drug and only a very small amount of the CYP2C19-dependant metabolite 5'-hydroxyomeprazole (OHOME) can be found in urine.[124] Another obstacle, when searching for an OME phenotyping metric in urine, is the instability of this substance under acidic conditions. OME is a proton pump inhibitor and is administered as a prodrug which rearranges to its active sulfenamide form after protonation. In order to use a urinary phenotyping metric, it has to be proven that OME and its metabolite are stable enough in urine of physiological pH. Therefore, a preliminary measurement of human urinary pH values was conducted. The detected pH values ranged from acidic (4.8) to basic (8.6)

with a mean ± S.D. of 6.3 ± 0.7. Stability tests for OME and OHOME in urine of pH = 5, 6, 7 and 8 could show that OME decomposes fast ($t_{1/2}$ = 35 min) at pH 5. OHOME also decomposes at this pH, but rather slower with a half-life of 103 min. At pH 6, the analytes are also instable. These findings suggest that urinary MR_{OME} may not be used as a reliable phenotyping metric due to interindividual differences in urinary pH value and the different half-lifes of OME and OHOME in urine. Therefore further studies using a urinary phenotyping metric for CYP2C19 were neglected.

CYP2C19 phenotyping using an OME phenotyping metric in plasma, however, was further investigated. From the subjects that registered for CYP genotyping a blood sample had to be taken in any case. Therefore this sampling was done 2–3 h after OME ingestion for 60 subjects. Due to logistical issues it was impossible to ensure blood sampling from each participant after exactly the same time span following OME ingestion. The measured concentrations for OME and OHOME varied widely, they spread from under the limit of detection (LOQ for OME and OHOME = 250 pg/mL) to approximately 500 ng/mL (OME) and 400 ng/mL (OHOME). This is in line with a finding described by Balian et al. who found that 18 out of 95 subjects had a OME concentration not detectable 2 h after ingestion of 20 mg OME.[157] It was suggested that these subjects had concentration peaks within one hour after the OME dose.[124]

Because of these findings, CYP2C19 phenotyping had to be excluded from this study. CYP2C19 genotyping however was conducted. The allele frequency of the null alleles CYP2C19*2 and *3 are very similar to those published for other Caucasian cohorts (Table 67). In our study, an allele frequency of only 8.8 % was calculated for CYP2C19*17. This differs from the published value of 18 %. The discrepancy might nevertheless be explained by differences in the study cohorts.

Allele	Allele frequency in this study [%]	Allele frequency in literature[90] [%]
CYP2C19*1	77.9	69.1
CYP2C19*2	13.1	12.8
CYP2C19*17	8.8	18[112]
CYP2C19*3	0.2	0.1

Table 67 Comparison of CYP2C19 allele frequencies found in this study and literature values.

As previously described, FLB and DEX could be used as a CYP2D6-CYP2C9 phenotyping combination. In addition to the exclusion of probe drug interactions, the new phenotyping cocktail should also use very small probe drug doses. To our knowledge, phenotyping with FLB was never done with a dose smaller than 50 mg.[6, 128] Our cocktail, however, only contains 8.75 mg FLB, as Dobendan Direct® lozenges are used for phenotyping. This was

possible, due to a highly sensitive LC-MS/MS method, developed for this purpose. The best internal standards available for MS analytics are the deuterium labeled analytes. OHF-d3, however, was not commercially available. Therefore it was synthesized using FLB-d_3 as starting compound and human CYP2C9 enzyme as oxygenase. In addition to that, a new cleavage method based on the saponification of esters was developed. As indicated in Figure 45, FLB and OHF are conjugated in the human body. They primarily form acylglucuronides which can rearrange to glucuronide derivatives that cannot be cleaved by ß-glucuronidase. To our knowledge the resulting analytical method is the most sensitive ever published for FLB and OHF in urine.

The measured FLB and OHF concentrations were used to calculate the urinary MR_{FLB}. Displayed on a logarithmic scale the distribution resembles a normal distribution, except for one value. One subject showed a 3-fold higher MR_{FLB} than the subject with the highest MR still under the fitted Gaussian curve. It indicates that this person is a slow metabolizer for CYP2C9, probably a carrier of at least one variant allele (*2 and /or *3).

In addition to CYP2C9 phenotyping, genotyping was conducted using RFLP-PCR and a multiplex SNP detection method. It could be shown that the null allele (CYP2C9*6) found in an African-American patient [84] was not present in any of the 283 Caucasian participants of the study. The allele frequencies CYP2C9*2 and *3, both associated with decreased enzyme activity, where consistent with those found in literature (Table 68).

Allele	**Allele frequency in this study [%]**	**Allele frequency in literature[90] [%]**
CYP2C9*1	80	79.7
CYP2C9*2	11.8	13.1
CYP2C9*3	8.1	6.7

Table 68 Comparison of CYP2C9 allele frequencies found in this study and literature values.

The successful accomplishment of both pheno- and genotyping allowed a direct correlation of these to techniques. The subject with the conspicuously high MR_{FLB} could indeed be identified as a homozygous carrier of CYP2C9*3. This finding supports the quality of FLB as a CYP2C9 probe drug. In addition to comparing single values, a linear model to assess the enzyme activities for the different alleles was calculated. The highly significant model, which is in line with a co-dominant expression of the alleles, resulted in the following values:

- CYP2C9*1 (wildtype allele) = 100 % enzyme activity,
- CYP2C9*2 = 68 % enzyme activity,
- CYP2C9*3 = 19 % enzyme activity towards FLB.

These values refer to the contribution of metabolic activity towards the hydroxylation of FLB. Even if those values may not be transferred uncritically to other drugs, the percentages nevertheless give a valuable hint towards the activity of the encoded enzymes. In literature different values for the enzyme activity of the CYP2C9*2 and *3 enzymes are described. Mostly, these values were determined *in-vitro*. For CYP2C9*2 it was found that the rate of metabolism (V_{max}) is reduced to approximately 50 % of the wildtype enzyme V_{max}.[86] Other publications state a reduction of enzyme activity of only ~20-30 %.[87] Our finding, a reduction of enzyme activity of 32 %, is in line with the latter publication. For CYP2C9*3, a loss of up to 70 % of the enzyme activity is published.[87] Our results suggest an even higher activity loss. However, the number of subjects with the CYP2C9*3 genotype in our study was very low so that it might be possible to underestimate the enzyme activity of the encoded enzyme due to statistical issues.

For CYP2D6, phenotyping and genotyping was successful carried out for 234 subjects. As already described, it was possible to combine CYP2D6 and CYP2C9 phenotyping. Therefore, CYP2D6 phenotyping was conducted after the administration of the FLB–DEX combination. Figure 46 shows the supposed metabolism of DEX in humans. After absorption, DEX is demethylated either at the O-methyl or the N-methyl group by CYP2D6 or CYP3A4 to yield dextrorphan or 3-methoxymorphinan, respectively. Dextrorphan (DOR) can be glucuronidated by UDP-glucuronosyltransferase (UTP) to yield dextrorphan glucuronide or demethylated via CYP3A4 to yield 3-hydroxymorphinan. 3-Hydroxymorphinan is also the product of the O-demethylation of 3-methoxymorphinan, a reaction catalyzed by CYP2D6. Like DOR, 3-hydroxymorphinan can be glucuronidated by UDP-glucuronosyltransferase. All five DEX metabolites are found in human urine.

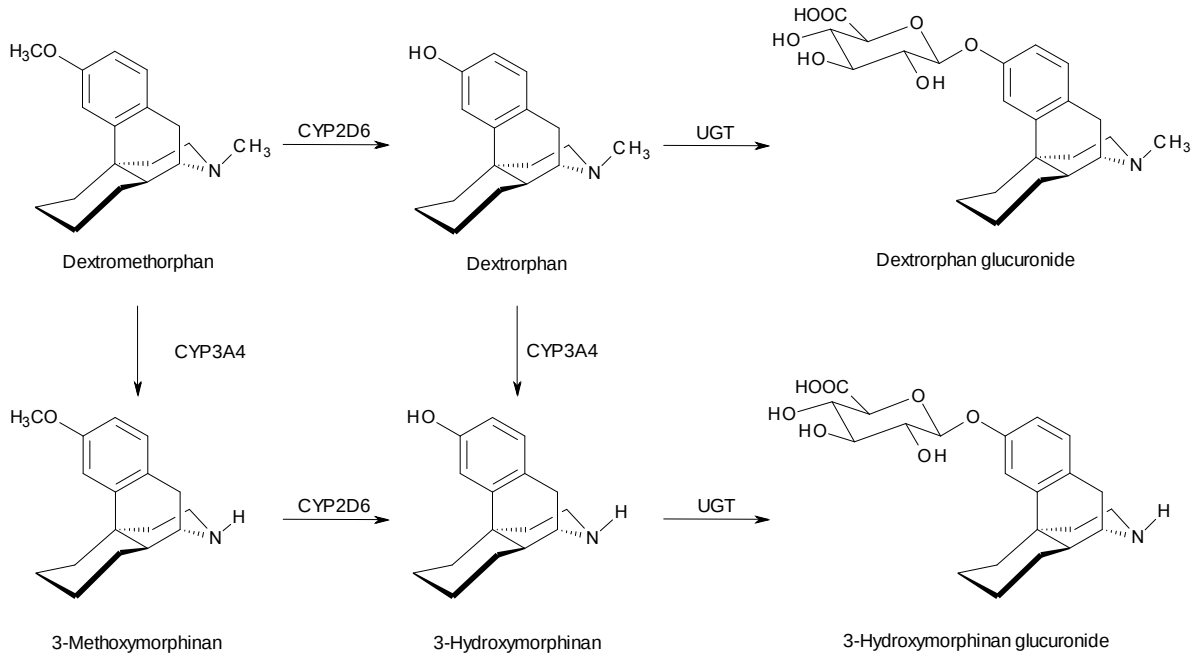


Figure 46 Simplified metabolism of dextromethorphan in humans.[137]

To phenotype for CYP2D6, the metabolite of choice is dextrorphan (DOR). In most studies the used phenotyping metric is $\log_{10}MR_{DEX}$, the logarithmic value of the metabolic ratio which is most often defined as the concentration of dextromethorphan (DEX) divided by the concentration of DOR. These concentrations are measured in urine collected over a period of 8 – 12 h.[158] To detect the whole amount of built DOR, DOR glucuronide is usually cleaved by treating the urine with ß-glucuronidase prior to DOR detection. The majority of DEX phenotyping procedures are therefore time-consuming and expensive as they require urine collection over up to 12 h, and on average an 18 – 24 hour treatment with ß-glucuronidase.[51] In addition to that, relatively high doses of 15 – 50 mg DEX have to be administered.

In our study we measured the concentration of DEX and DOR in urine 2 h after DEX administration before and after glucuronide cleavage. We used this data to generate two phenotyping datasets of $\log_{10}MR_{DEX}$ with and without glucuronide cleavage to examine the necessity of glucuronide cleavage. Additionally, only a small dose of 10 mg DEX was needed to phenotype for CYP2D6. To our knowledge, this is the smallest dose of DEX ever used for phenotyping, except for those studies conducted by our own laboratory.[137, 138] This could be achieved by using a very sensitive analytical method. The results of our own method, however, are as conclusive as those achieved with the more cumbersome methods. The logarithmic values of MR_{DEX} with and without glucuronide cleavage are bimodaly distributed, which is in accordance with the occurrence of extensive and poor metabolizers (EMs and

PMs). This bimodal distribution is usually found in CYP2D6 phenotyping study using DEX as probe drug.[124]

CYP2D6 genotyping was also conducted. Using long range PCR, nested PCR, RFLP-PCR, multiplex SNP detection, and sequencing, altogether six SNPs, four small deletions, the deletion of the whole gene, and gene duplication, and multiplication were detected. 234 subjects were successfully genotyped and the resulting allele frequencies together with those found in literature are listed in Table 69. The minor departures of the found frequencies from those published earlier may be explained by the different study cohorts and lie within the usual limits.[40] CYP2D6*69, however, is especially interesting, as it was only discovered in August 2008 and therefore its actual frequency of approximately 1.1 % in Caucasians has not been described until now.[151]

Allele	Allele frequency in this study [%]	Allele frequency in literature[40] [%]
CYP2D6*1	38.9	34.4
CYP2D6*2	14.7	28.7
CYP2D6*3	1.1	0.3
CYP2D6*4	25.4	17.2
CYP2D6*5	2.8	3.2
CYP2D6*6	1.1	0.6
CYP2D6*9	2.1	2.5
CYP2D6*10	7.9	2.9
CYP2D6*17	0.0	0.0
CYP2D6*41	4.1	7.0
CYP2D6*69	1.1	not determined[151]
CYP2D6*1 x N	0.6	0.6
CYP2D6*2 x N	0.2	1.3

Table 69 Comparison of CYP2D6 allele frequencies found in this study and literature values.

The effective phenotyping and genotyping of the subjects included in this study allowed a direct correlation of these two techniques. For 230 persons linear models were calculated, to test the predictive power of the determined CYP2D6 genotype for the measured dextromethorphan (DEX) phenotype. Two different kinds of linear models were used, one assuming independent variables (consistent with the presumption of alleles being co-dominantly expressed) and one allowing interaction terms for certain allelic combinations (consistent with the presumption of interacting alleles, e.g. dominant or recessive expression). With linear models it is possible to assigned coefficients to the variant alleles that can be used as metrics for the contribution of each allele to the resulting phenotype and

therefore the enzyme activity. In addition to these allele coefficients, models with interaction terms output genotype coefficients that describe allele-allele interaction. The two different model types were calculated with both datasets of $log_{10}MR_{DEX}$ (without and with prior DOR glucuronide cleavage).

Surprisingly, the best correlation between genotype and phenotype could be achieved by using $log_{10}MR_{DEX}$ without glucuronide cleavage and a model with interaction terms for CYP2D6*1/*4, CYP2D6*1/*5, CYP2D6*2/*4, CYP2D6*2/*5, CYP2D6*4/*9, and CYP2D6*4/*10 (model 2A, see chapter 8.1.3). This finding suggests two conclusions. Firstly, CYP2D6 phenotyping seems to be also possible without prior DOR glucuronide cleavage, leading to a much simpler phenotyping process. Secondly, the alleles for which the interaction terms were included might interact, e.g. one allele of each allele combination might be dominantly expressed. Table 70 shows these allele combinations, their predicted $log_{10}MR_{DEX}$ without and with interaction term, and the measured phenotyping metric. The predicted phenotyping metric were calculated using the coefficients output by model 2A (chapter 8.1.2.1, Table 59).

Genotype	Predicted $log_{10}MR_{DEX}$ without interaction term (mean ± S.D.)	Predicted $log_{10}MR_{DEX}$ with interaction term (mean ± S.D.)	Measured $log_{10}MR_{DEX}$ (mean ± S.D.)
CYP2D6*1/*4	0.03 ± 0.06	-0.97 ± 0.11	-0.97 ± 0.36
CYP2D6*1/*5	0.37 ± 0.20	-1.02 ± 0.34	-1.02 ± 0.23
CYP2D6*2/*4	0.11 ± 0.08	-0.84 ± 0.20	-0.84 ± 0.60
CYP2D6*2/*5	0.45 ± 0.21	-0.26 ± 0.43	-0.26 [1)]
CYP2D6*4/*9	0.53 ± 0.18	-0.24 ± 0.39	-0.24 [1)]
CYP2D6*4/*10	0.34 ± 0.11	-0.46 ± 0.19	-0.46 ± 0.52

Table 70 Comparison between calculated and measured values for $log_{10}MR_{DEX}$ (genotypes with interaction terms). [1)] Only two subjects possessed the respective genotype, therefore no standard deviation can be given.

The computed $log_{10}MR_{DEX}$ including the interaction terms predicts the measured value much better than $log_{10}MR_{DEX}$ without interaction term. It is also noticeable that the inclusion of the genotype coefficients (calculated with the interaction term) leads to smaller values for $log_{10}MR_{DEX}$ in all cases. As $log_{10}MR_{DEX}$ is inversely proportional to the metabolic activity of CYP2D6, this might suggest that the alleles coding for CYP2D6 enzymes with higher activity are dominantly expressed in the discussed cases. Based on the results that will be discussed in the next paragraph CYP2D6*1, *2, *9 and *10 might therefore be dominant over CYP2D6*4 and *5.

The question which alleles encode for enzymes with higher metabolic activity can be

addressed by calculating relative metabolic activities. This calculation is done based on the assumption of an activity of 100 % for CYP2D6*1 and by using the allele coefficients produced by the linear model (for details see chapter 8.1.1.1). Table 71 lists the predicted relative enzyme activities for the 10 different variant alleles together with the information found on http://www.cypalleles.ki.se/cyp2d6.htm (access date: 7.11.2010).

	Allele	Protein level	Enzyme activity	Predicted relative enzyme activity*
PM alleles	CYP2D6*3	259Frameshift	None	16 %
	CYP2D6*4	Splicing defect	None	4 %
	CYP2D6*5	CYP2D6 deleted	None	2 %
	CYP2D6*6	118Frameshift	None	28 %
IM alleles	CYP2D6*9	K281del	Decreased	32 %
	CYP2D6*10	P34S	Decreased	50 %
	CYP2D6*41	Splicing defect	Decreased	46 %
EM alleles	CYP2D6*1	None	Normal	100 %
	CYP2D6*2	R296C, S486T	Normal	83 %
	CYP2D6*69[151]	Splicing defect	Decreased	222 %

Table 71 Predicted relative enzyme activities and information for the 10 different variant alleles (http://www.cypalleles.ki.se/cyp2d6.htm; access date: 7.11.2010). *Calculated using model 2A.

For the alleles with the highest allele frequencies in this study, CYP2D6*1 (38.9 %), CYP2D6*4 (25.4 %), CYP2D6*2 (14.7 %), CYP2D6*10 (7.9 %), and CYP2D6*41 (4.1 %) the predicted relative enzyme activities are consistent with those expected from their alterations on protein level. Except for CYP2D6*69, all other values also show the right tendencies. CYP2D6*69 was only found in 5 subjects, four times in combination with CYP2D6*4. Interestingly, this novel variant allele was discovered in an individual possessing the CYP2D6*4/*69 genotype.[151] Probably, CYP2D6*69 is not well described using model 2A ($log_{10}MR_{DEX}$ without glucuronidation, model with interaction terms). Both linear models without interaction terms using $log_{10}MR_{DEX}$ without and with glucuronidation predict a relative metabolic activity of <70 %, more precisely 67 % and 34 %, respectively. ‘

Nevertheless, this new CYP2D6 genotype phenotype correlation model might allow for more precise phenotype prediction for the other included variant alleles than was possible until now. To our knowledge, the most accurate way for phenotype estimation starting from a subject’s genotype was the so-called Activity Score (AS). It assigns a value of 0 to the null, 0.5 to the intermediate (IM), 1 to the extensive (EM), and 2 to the ultrarapid (UM) alleles.[159] With our model, however, it is not only possible to distinguish between single variant alleles instead of using predefined groups (null, IM, EM and UM alleles), it may also give new

insides into the relative expression of the two alleles comprising a genotype. Using the established linear model for a bigger cohort might specify the described findings.

Taken together, this study contributes substantially to the important research field of pharmacogenetics. A save, easy-to-use, and fast phenotyping procedure for the important genetic polymorphic enzymes CYP2D6 and CYP2C9 was developed. The results for CYP2C19 phenotyping with OME question the application of this drug as a probe drug for CYP2C19 and support the necessity of thorough probe drug and probe drug interaction studies. Most importantly, however, new insights into the phenotype prediction from genotype for CYP2C9 and CYP2D6 could be gained.

10 References

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11 Annex

Allele	?	*3	*4	*5	*6	*9	?	*17	*41		gene
SNP or del	1661 G>C	2549 delA	1846 G>A	gene del	1707 delT	2615-2617 delAAG	100 C>T	1023 C>T	2988 G>A	genotype	duplication or multiplication
1	GC		GA	X	T	AAG	CT	C	G	*1/*4	x
2	C		G	X	T	AAG	CC	C	AA	*41/*41	x
3	C	A	GA	X	T	AAG	TT	C	G	*4/*10	x
4	C		AA	X	T	AAG	TT	C	G	*4/*4	x
5	GC		GA	X	T	AAG	CT	C	G	*1/*4	x
6	GC		G	X	T	AAG	CC	C	G	*1/*2	x
7	C		GA	X	T	AAG	TT	C	G	*4/*10	x
8	GC	A	G	X	T	AAG	CC	C	GA	*1/*41	x
9	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
10	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
11	C	A	GA	X	T	AAG	CT	C	G	*2/*4	x
12	C	A	G	X	T	AAG	CC	C	G	*2/*2	x
13	C	A	GA	X	T	AAG	TT	C	G	*4/*10	x
14	G	A	G	X	T	AAG	CC	C	G	*1/*1	xN
15	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
16	C	A	G	X	T	AAG	CC	C	GA	*2/*41	x
17	GC	A	GA	X	T	AAG/ DEL	CT	C	G	*4/*9	x
18	C	A	G	X	T	AAG	CC	C	GA	*2/*41	x
19	GC		G	X	T	AAG	CC	C	G	*1/*2	x
20	C	A	GA	X	T	AAG	TT	C	G	*4/*10	x
21	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
22	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
23	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
24	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
25	GC	A	G	X	T	AAG	CC	C	GA	*1/*41	x
26	G	A	G	DEL	T	AAG	CC	C	G	*1/*5	x
27	GC		GA	X	T	AAG	CT	C	G	*1/*4	x
28	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
29	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
30	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
31	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
32	G	A	G	X	DEL/T	AAG	CC	C	G	*1/*6	x
33	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
34	G		G	X	DEL/T	AAG	CC	C	G	*1/*6	x
35	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x

36	C	A	GA	X	T	AAG	TT	C	GA	*4/*69	x
37	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
38	G	A	G	DEL	T	AAG	CC	C	G	*1/*5	x
39	G	A	G	X	T	AAG/DEL	CC	C	G	*1/*9	x
40	C	A	GA	X	T	AAG	TT	C	G	*4/*10	x
41	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
42	C	A	AA	X	T	AAG	TT	C	G	*4/*4	N.A
43	C	A	AA	DEL	T	AAG	TT	C	G	*4/*5	x
44	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
45	C	A	AA	X	T	AAG	TT	C	G	*4/*4	x
46	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
47	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
48	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
49	C	A	AA	X	T	AAG	TT	C	G	*4/*4	x
50	C	A	AA	X	T	AAG	TT	C	G	*4/*4	x
51	C	A	G	X	T	AAG	TT	C	G	*10/*10	x
52	C	A	G	DEL	T	AAG	CC	C	G	*2/*5	x
53	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
54	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
55	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
56	C	A	GA	X	T	AAG	CT	C	G	*2/*4	x
57	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
58	GC	A.DEL	G	X	T	AAG	CC	C	G	*2/*3	x
59	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
60	GC	A	G	X	T	AAG	CC	C	G	*1/*2	xN
61	C	A	G	DEL	T	AAG	CC	C	G	*2/*5	x
62	C	A	GA	X	T	AAG	TT	C	G	*4/*10	x
63	C	A	AA	X	T	AAG	TT	C	G	*4/*4	x
64	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
65	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
66	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
67	C	A	G	X	T	AAG	CC	C	G	*2/*2	x
68	GC		G	X	T	AAG	CC	C	GA	*1/*41	x
69	C	A	GA	X	T	AAG	TT	C	GA	*4/*69	x
70	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
71	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
72	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
73	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
74	GC		G	X	T	AAG/DEL	CT	C	G	*9/*10	x

75	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
76	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
77	G	A	G	X	T	AAG/ DEL	CC	C	G	*1/*9	x
78	GC	A	G	X	DEL/T	AAG	CC	C	G	*2/*6	x
79	GC		GA	X	T	AAG	CT	C	G	*1/*4	x
80	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
81	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
82	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
83	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
84	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
85	C	A	G	X	T	AAG	TT	C	G	*10/*10	x
86	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
87	C	A	GA	X	T	AAG	TT	C	G	*4/*10	x
88	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
89	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
90	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
91	C	A	AA	DEL	T	AAG	TT	C	G	*4/*5	x
92	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
93	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
94	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
95	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
96	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
97	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
98	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
99	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
100	C	A	GA	X	T	AAG	TT	C	G	*4/*10	x
101	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
102	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
103	C	A	GA	X	T	AAG	TT	C	G	*4/*10	x
104	C	A	GA	X	T	AAG	TT	C	GA	*4/*69	x
105	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
106	C	A	G	X	T	AAG	CT	C	GA	*10/*41	x
107	G	A	G	DEL	T	AAG	CC	C	G	*1/*5	x
108	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
109	C	A	G	X	T	AAG	CC	C	GA	*2/*41	x
110	C	A	G	X	T	AAG	TT	C	GA	*10/*69	x
111	C	A	GA	X	T	AAG	TT	C	G	*4/*10	x
112	C	A	AA	X	T	AAG	TT	C	G	*4/*4	x
113	G	A	G	DEL	T	AAG	CC	C	G	*1/*5	x
114	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
115	G	A	G	X	T	AAG	CC	C	G	*1/*1	xN

116	C	A	GA	X	T	AAG	CT	C	G	*2/*4	x
117	C	A	GA	X	T	AAG	TT	C	G	*4/*10	x
118	G	A/ DEL	G	X	T	AAG	CC	C	G	*1/*3	x
119	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
120	GC	A	G	X	T	AAG	CT	C	G	*1/*10	x
121	GC	A/ DEL	G	X	T	AAG	CC	C	G	*2/*3	x
122	C	A	G	X	T	AAG	CT	C	GA	*10/*41	x
123	G	A	G	DEL	T	AAG	CC	C	G	*1/*5	x
124	GC	A	G	X	T	AAG	CC	C	GA	*1/*41	x
125	C		GA	X	T	AAG	TT	C	G	*4/*10	x
126	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
127	C	A	AA	X	T	AAG	CT	C	G	*4/*4	x
128	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
129	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
130	GC	A	G	X		AAG	CC	C	GA	*1/*41	x
131	GC	A	G	X	T	AAG	CC	C	GA	*1/*41	x
132	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
133	C	A	GA	X	T	AAG	TT	C	G	*4/*10	x
134	GC		G	X	T	AAG	CC	C	G	*1/*2	x
135	C	A	GA	X	T	AAG	TT	C	GA	*4/*69	x
136	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
137	C	A	GA	X	T	AAG	TT	C	G	*4/*10	x
138	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
139	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
140	C	A	G	X	T	AAG	CC	C	GA	*2/*41	x
141	C	A	GA	X	T	AAG	CT	C	G	*2/*4	x
142	C	A	GA	X	T	AAG	TT	C	G	*4/*10	x
143	GC	A	G	X	T	AAG	CT	C	G	*1/*10	x
144	G	A/ DEL		DEL	T	AAG	CC	C	G	*3/*5	x
145	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
146	GC		GA	X	T	AAG	CT	C	G	*1/*4	x
147		A	AA	X	T	AAG	CT		G	*4/*4	x
148	C	A	G	X	T	AAG	CC	C	G	*2/*2	x
149	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
150	G	A	G	X	T	AAG/D EL	CC	C	G	*1/*9	x
151	G		G	X	T	AAG	CC	C	G	*1/*1	x
152	GC		GA	X	T	AAG	CT	C	G	*1/*4	x
153	C	A	AA	X	T	AAG	TT	C	G	*4/*4	x

154	GC		G	X	T	AAG	CC	C	GA	*1/*41	x
155	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
156	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
157	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
158	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
159	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
160	GC		GA	X	T	AAG	CT	C	G	*1/*4	x
161	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
162	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
163	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
164	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
165	C	A	GA	X	T	AAG	TT	C	G	*4/*10	x
166	GC	A	GA	X	DEL/T	AAG	CT	C	G	*4/*6	x
167	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
168	GC		GA	X	T	AAG	CT	C	G	*1/*4	x
169	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
170	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
171	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
172	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
173	C	A	G	X	T	AAG	CT	C	G	*2/*10	x
174	GC	A	G	X	T	AAG	CC	C	GA	*1/*41	x
175	C		AA	X	T	AAG	TT	C	G	*4/*4	x
176	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
177	C	A	GA	X	T	AAG	TT	C	G	*4/*10	x
178	C	A	AA	X	T	AAG	TT	C	G	*4/*4	N.A.
179	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
180	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
181	C	A	AA	X	T	AAG	TT	C	G	*4/*4	x
182	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
183	C	A	GA	X	T	AAG	TT	C	G	*4/*10	x
184	C	A	GA	X	T	AAG	CT	C	G	*2/*4	x
185	C	A	G	X	T	AAG	CT	C	G	*2/*10	x
186	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
187	C	A	GA	X	T	AAG	TT	C	G	*4/*10	x
188	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
189	C	A	GA	X	T	AAG	TT	C	G	*4/*10	x
190	G		GA	X	T	AAG	CT	C	G	*1/*4	x
191	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
192	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
193	C	A	GA	X	T	AAG	CT	C	G	*2/*4	x
194	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
195	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x

196	GC		G	X	T	AAG	CT	C	G	*1/*10	x
197	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
198	GC	A	G	X	T	AAG	CC	C	GA	*1/*41	x
199	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
200	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
201	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
202	GC		G	X	T	AAG	CT	C	G	*1/*10	x
203	C		AA	DEL	T	AAG	TT	C	G	*4/*5	x
204	GC		G	X	T	AAG	CC	C	GA	*1/*41	x
205	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
206	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
207	C	A	AA	DEL	T	AAG	TT	C	G	*4/*5	x
208	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
209	GC	A	G	X	T	AAG	CC	C	GA	*1/*41	x
210	C		GA	X	T	AAG	TT	C	G	*4/*10	x
211	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
212	C	A	GA	X	T	AAG	CT	C	G	*2/*4	x
213	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
214	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
215	GC	A	GA	X	T	AAG/ DEL	CT	C	G	*4/*9	x
216	C	A	AA	X	T	AAG	TT	C	G	*4/*4	x
217	GC		GA	X	T	AAG	CT	C	G	*1/*4	x
218		A	G	DEL	T	AAG	CC			*1/*5	x
219	G	A	G	X	T	AAG	CC	C	G	*1/*1	x
220	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
221	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
222	GC	A	GA	X	T	AAG	CT	C	G	*1/*4	x
223	C	A	G	X	T	AAG	CC	C	G	*2/*2	x
224	C	A	GA	X	T	AAG	TT	C	G	*4/*10	x
225	GC	A	G	X	T	AAG	CT	C	G	*1/*10	x
226	G	A	G	X	DEL/T	AAG/ DEL	CC	C	G	*6/*9	x
227	GC	A	G	X	T	AAG/ DEL	CC	C	G	*2/*9	x
228	C		AA	X	T	AAG	TT	C	G	*4/*4	x
229	GC	A	G	X	T	AAG	CC	C	G	*1/*2	x
230	G	A	G	X	T	AAG	CC	C	G	*1/*1	xN
231		A	GA	X	T	AAG	CT			*1/*4	x
232		A	GA	X	T	AAG	CT			*1/*4	x
233	G		G	X	T	AAG/ DEL	CC	C	G	*1/*9	x

234	GC	A/ DEL	GA	X	T	AAG	CT	C	G	*3/*4	x

Table 72 Genotyping data for CYP2D6. ? indicate that these SNPs are present in more than one haplotype and can therefore not be assigned to only one variant alleles.

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