Development, Validation and Application of the Basel Phenotyping Cocktail

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1. Summary

Personalized therapy is a matter of current discussion in the medical community. It consists of adapting the choice of a drug and its posology to the specific profile of a subject (genotype and phenotype) or to its disease (i.e. specific cancer biology). Activity of drug metabolizing enzymes depends on the genotype and extrinsic factors which together determine the phenotype of a subject. Therefore a precise characterization of a patient phenotype will guide the physicians efforts to personalize therapy and thus improve efficacy and reduce side effects. This approach requires specific and easy to use diagnostic tools that are not yet available in clinical routine. With our work, we explored possible ways to promote phenotyping of drug metabolizing enzymes as a valuable tool for personalized medicine.

In this dissertation we describe the development of a new phenotyping cocktail (Basel cocktail) containing caffeine, efavirenz, losartan, omeprazole, metoprolol, and midazolam as probe drugs for CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4, respectively. We validated the new phenotyping procedure in two clinical studies in healthy volunteers: the Basel cocktail studies I and II. With the data acquired in study I, the absence of mutual interactions between the new combinations of probe drugs selected for the Basel phenotyping cocktail was established. Furthermore we showed that evaluation of the metabolic ratios in plasma at single time-points can replace conventional full AUC measurements. Additionally, the use of dried blood spots (DBS) and saliva as minimally or non-invasive sampling collection procedures were explored.

For a comprehensive evaluation of the new phenotyping cocktail, the influence of genetic factors or co-administration of an enzyme inducer or inhibitor on the metabolic ratios of the Basel cocktail had to be assessed. The Basel cocktail study II was performed to characterize the range of the chosen phenotyping metrics under conditions of induction and inhibition. Additionally we measured the metabolic ratios in saliva and DBS.

Bioanalysis and genotype determination were key tools for this project. We developed and validated bioanalytical methods for simultaneous detection of probe drugs and their main metabolites in different human matrices. To maintain clinical applicability, short return times were required. Therefore highly sensitive bioanalytical methods with simple sample work-up and short run-times were developed. Study subjects providing consent were genotyped for the most relevant single-nucleotide polymorphisms and when possible correlations between genotype and phenotype were assessed.

In the Basel cocktail studies I and II, the lowest commercially available doses and formulations of the phenotyping drugs were used. To simplify probe drug administration, a prototype of a combi-pill containing all six cocktail probe drugs was developed and tested in a single subject in a clinical pilot study. Pharmacokinetic profiles of the new formulation were comparable with the ones obtained after intake of the individual probe drugs in the commercial formulations. This new cocktail combi-pill will be formally evaluated in a clinical study (Basel cocktail study III) in healthy volunteers.

The use of phenotyping information to personalize cancer therapy with tyrosine kinase inhibitors is under investigation in an ongoing clinical trial. Individual CYP3A4 and CYP1A2 phenotype will be assessed using probe drugs of the Basel cocktail and the correlation of the phenotyping metrics with sunitinib, pazopanib or erlotinib exposure will be analyzed.

Taken together the work performed during this dissertation was important to improve the clinical applicability of phenotyping, which is essential to move this important tool for personalized medicine closer to the clinical routine.

2. Abbreviations

AUC	Area under the curve
CYP	Cytochrome P450
C_{max}	Peak plasma concentration
CTC	Common Toxicity Criteria
DBS	Dried Blood Spot
DMSO	Dimethyl sulfoxide
EU	European Union
FDA	Food and Drug Administration
HILIC	Hydrophilic Interaction Liquid Chromatography
HPLC	High Performance Liquid Chromatography
i.v.	Intravenous
KTI	Kommission für Technologie und Innovation
LC-MS/MS	Liquid Chromatography-Triple quadrupole Mass Spectrometry
MR	Metabolic Ratio
SPE	Solid Phase Extraction
t_{max}	Time corresponding to peak plasma concentration
$t_{½}$	Half-time
TDM	Therapeutic Drug Monitoring
UHPLC	Ultra-high Pressure Liquid Chromatography
UPLC®	Ultra Performance Liquid Chromatography (Trademark of Waters Corporation)
VAS	Visual Analog Scale

3. Introduction

3.1. Phenotyping of Drug Metabolizing Enzymes using a "Cocktail" Approach

Cytochrome P450 (CYP) enzymes, a superfamily of haemoproteins, are the terminal oxidases of the mono-oxygenase system. They are involved in the oxidative, reductive and peroxidative metabolism of numerous endogenous compounds and xenobiotics [1].

The six major human CYP enzymes involved in the metabolism of drugs are (in order of quantitative contribution) CYP3A4, CYP2D6, CYP2C9, CYP2C19, CYP2E1, and CYP1A2 [2]. CYP2D6, CYP2C9, CYP2C19, and CYP1A2 are polymorphically expressed, leading to high interindividual variation in CYP enzyme activities [3].

In subjects with an increased enzyme activity due to induction or over-expression of the CYP enzyme, such as CYP2D6 ultrarapid metabolizers (UMs), a normal therapeutic doses can result in therapeutic failure due to decreased systemic exposure [4]. At the other end of the spectrum, reduced activity of CYP enzymes due to inhibition of CYP activity or in genetically poor metabolizers (PMs), can result in increased systemic exposure of their respective substrates, leading to a pharmacodynamic effect of increased intensity and/or duration.

To optimize therapeutic effect, drug therapy has to be individualized according to a patient's genotype and/or phenotype [5]. Extensive efforts have been directed to develop genotyping methods for detecting specific DNA mutations that determine individual drug metabolizing capacity. However, genotyping is not able to monitor changes of phenotype caused by non-genetic factors such as e.g. drug-drug interactions. Combination drug therapy is common in clinical practice and increases the risk for drug–drug interactions (DDIs). Many DDIs occur due to inhibition or induction of the cytochrome P450 system. In vitro hepatic microsomal studies can provide preliminary information about the CYP isoforms that are likely to be affected clinically. However, the potential to cause relevant DDIs in vivo needs to be investigated in clinical studies. In addition to interference with a patient's phenotype, DDIs can cause significant safety problems resulting in delayed drug registration or early withdrawal from the market [6].

Therefore, there is a need for an easy and reliable phenotyping tool in clinical drug development for early identification of clinically relevant DDIs in vivo and in clinical practice to guide dose individualization in patient treatment.

Phenotyping itself involves the administration of an appropriate substrate (i.e. probe drug) that is selectively metabolized to a known metabolite by a given CYP isoform. Metabolic ratios, i.e. the ratio of the concentration of the substrate to that of a specific metabolite in urine, plasma or saliva produced by the CYP isoform of interest are already extensively used as an index of CYP activity [7].

Although many CYP isoforms exhibit overlapping substrate specificity [7], for some substrates a single CYP isoform is exclusively or to a major extent involved in a particular pathway of their metabolism [8]. For instance, metoprolol α-hydroxylation is mediated by CYP2D6 [7,9] and omeprazole 5-hydroxylation by CYP2C19 [10].

Phenotyping provides a measure of the activity of a given drug-metabolizing enzyme at a given time-point, and takes into account not only genetic but also non-genetic and environmental (e.g. DDI) factors (Figure 1).

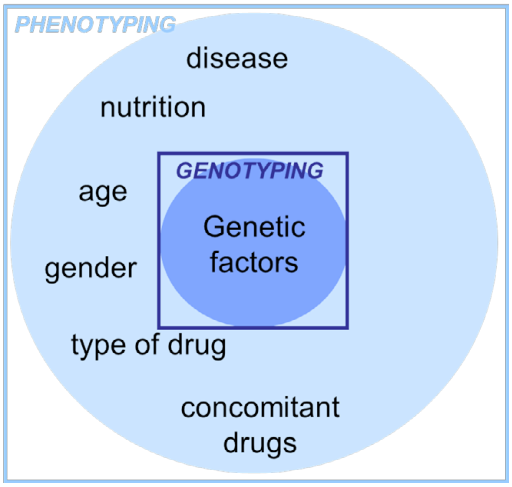

Figure 1. Phenotyping and Genotyping

When a compound with inhibitory potential is co-administered, the metabolism of the probe drug is impaired. When two or more isoforms are involved, several DDI studies may have to be performed to understand the full clinical interaction potential. In this case, a 'cocktail' approach provides a more efficient procedure than the traditional single probe, single study approach.

The 'cocktail' approach involves the simultaneous administration of two or more probe drugs to a single subject to assess the activity of several distinct CYP isoforms [5]. By sequential administration of a well-defined cocktail of probe drugs, the *in vivo* effect of a test drug (e.g. a new compound in clinical development) on clinically relevant CYP isoforms can be established within one clinical study.

Before a combination of probe drugs can be used as a phenotyping cocktail, it has to be established that no pharmacokinetic interactions occur between the probe drugs of the cocktail. Furthermore, pharmacodynamic interactions would be of particular concern. Several probe drugs (e.g. losartan, metoprolol) can affect blood pressure when applied at therapeutic doses and could influence hepatic blood flow and consequently the metabolic clearance of co-administered probe drugs [11].

Although several phenotyping cocktails using different combinations of probe drugs have been published so far [8, 11-13], none of the proposed cocktails have gained clinical acceptance due to several draw-backs such as limited availability of the probe drugs, complicated sampling procedures or pharmacodynamic effects due to high doses of the probe drugs [5].

Several improvements such as simplified phenotyping metrics [14], low- or micro-dose approach [15] or use of non-invasively accessible biological matrices, as for example oral fluid or dried blood spot (DBS), have been shown for midazolam or flurbiprofen [16, 17] but not for most of the other probe drugs. In a review paper, Fuhr et al. [18] pointed out the need for optimizing phenotyping procedures. Replacement of individual probe drugs, better validated metrics, doses reduction and simplification of the sampling procedures are mentioned as possible improvement to promote clinical use of the cocktail approach.

Therefore, the main goal of this project was to develop and validate a new phenotyping cocktail using easily available probe drugs at the lowest clinically approved doses. To simplify sampling procedures, an appropriate phenotyping metric and optimal time-point for sparse sampling had to be defined. Furthermore we wanted to evaluate whether minimally or non-invasive sampling methods such as collection of dried blood spots or saliva samples could be used for phenotyping. In the following sections, selection and validation criteria of the new probe drug combination are described. The project involved the development and validation of two bioanalytical methods, two clinical studies, the Basel cocktail basic interaction study and the Basel cocktail induction/inhibition study, and a pilot study with a combi-pill containing in a single formulation the phenotyping drugs that constitute the cocktail. Finally an application of the phenotyping procedure for optimizing doses of tyrosine kinase inhibitors is presented.

3.2.Phenotyping cocktail combinations already published

Several different phenotyping cocktails have already been described in literature (Table 1 adapted from Ghassbian [19] and Fuhr [18]). The name of the cocktail is often associated with the town where it was developed, hence the cocktail we developed is called the “Basel” cocktail.

Table 1. Summary of published phenotyping cocktails

Cocktail	CYP isoforms	Substrates	Doses (mg)	Availability of probe drug	Phenotyping Metric	Matrix
Changsha[13]	1A2	Caffeine	100	●	MR P 6h	P, U
	2C19	Mephenytoin	100	○	Ae 4'OHM 0-8h	
	2D6	Metoprolol	100	●	MR U 8h	
	2E1	Chlorzoxazone	200	●	MR P 4h	
	3A4	Midazolam	7.5	●	MR P 1h	
Cologne[20-23]	1A2	Caffeine	150	●	MR P 6h	P, U
	2B6	Mephenytoin	50	●	Ae Nirvanol 0-96h	
	2C9	Tolbutamide	125	○	[Tolbutamide] P 24h	
	2C19	Mephenytoin	50	○	Ae 4'OHM 0-12h	
	2D6	Dextrometorphan	30	●	MR U 8h	
	3A4	Midazolam	2 (1 i.v.)	●	Fi P - CL P 0-12h	
	NAT2	Caffeine		●	MR U 6h	
	P-gp	Digoxin	0.5	●	Cmax P 0-12h	
Cooperstown 5+1[24]	1A2	Caffeine	2 mg/kg	●	MR U 0-12h	P, U
	2C9	S-warfarine(+Vit K)	10	●	AUC 0-96h	
	2C19	Omeprazole	40	●	MR P 2h	
	2D6	Dextrometorphan	30	●	MR U 0-12h	
	3A4	Midazolam	0.025 mg/kg	●	CL P 0-6(-12)h	
	NAT2	Caffeine	2 mg/kg	●	MR U 0-12h	
	XO	Caffeine	2 mg/kg	●	MR U 0-12h	
Darmstadt[25]	1A2	Caffeine	100	●	AUC P 0-24h	P, U
	2C9	Diclofenac	50	●	AUC P 0-24h	
	2C19	Mephenytoin	100	○	Ae 4'OHMn 0-8h	
	2D6	Metoprolol	100	●	AUC P 0-72h	
	3A4	Midazolam	7.5	●	AUC P 0-24h	
Indianapolis[26, 27]	1A2	Caffeine	100	●	MR P 6h	P, U
	2C9	Tolbutamide	500	○	CL/F P 24h	
	2D6	Dextrometorphan	30	●	MR U 24h	
	3A4	Midazolam	5 (50 µg/kg i.v.)	●	CL/F O 0-12h (CL P 0-12h)	

Cocktail	CYP isoforms	Substrates	Doses (mg)	Availability of probe drug	Phenotyping Metric	Matrix
Inje[28]	1A2	Caffeine	93	●	MR P 4h	P, U
	2C9	Losartan	30	●	MR U 8h	
	2C19	Omeprazole	20	●	MR P 4h	
	2D6	Dextrometorphan	30	●	Log MR U 8h	
	3A4	Midazolam	2	●	[Mid] P 4h	
Inje modified by Ghassabian[19]	1A2	Caffeine	100	●	MR P 4h	P
	2C9	Losartan	25	●	AUC R 0-6h	
	2C19	Omeprazole	20	●	MR P 4h or 6h	
	2D6	Dextrometorphan	30	●	AUC R 0-6h	
	3A4	Midazolam	2	●	[Mid] P 4h and AUC R 0-6h	
Jena[29]	1A2	Caffeine	200	●	AUC P 0-12h	P, U
	2D6	Debrisoquine	10	○	MR U 0-6h	
	NAT2	Sulphamethazine	500	○	MR U, P 0-6h, 6h	
Karolinska[30]	1A2	Caffeine	100	●	MR P 4h	P, U
	2C9	Losartan	25	●	MR U 0-8h	
	2C19	Omeprazole	20	●	MR P 3h	
	2D6	Debrisoquine	10	○	MR U 0-8h (*)	
	3A4	Quinidine	250	○	MR P 16h	
Leiden[31]	2C19	Mephenytoin	100	○	Ae 4'OHMn 0-8(-48)h	P, U
	2D6	Spartein	25-90	○	MR U 0-8h	
	3A4	Nifedipine	5-20	●	CL/F 0-8h	
Loughborough[11]	1A2	Caffeine	50	●	MR P 6.5h	P, U
	2C9	Tolbutamide	250	○	MR U 6-12h	
	2D6	Debrisoquine	5	○	MR U 0-6h	
	2E1	Chlorzoxazone	250	●	MR P 2.5h	
	3A4	Midazolam	25 μg/kg i.v.	●	CL P 0-12h	
Pittsbourgh[32]	1A2	Caffeine	100	●	MR P 8h	P, U
	2C9	Flurbiprofen	50	●	MR U 0-8h	
	2C19	Mephenytoin	100	○	Ae 4'OHM 0-8h	
	2D6	Debrisoquine	10	○	MR U 0-8h	
	2E1	Chlorzoxazone	250	●	MR P 4h	
	3A4	Dapsone	100	○	MR U 0-8h	
	NAT2	Dapsone	100	○	MR U 0-8h	
Quebec[12]	1A2	Caffeine	100	●	MR U 0-8h	U
	2C9	Tolbutamide	250	○	MR U 0-8h	
	2D6	Metoprolol	25	●	MR U 0-8h	
	2E1	Chlorzoxazone	250	●	MR U 0-8h	
	3A4	Dapsone	100	○	MR U 0-8h	
	XO	Caffeine		●	MR U 0-8h	
	NAT2	Caffeine		●	MR U 0-8h	

Cocktail	CYP isoforms	Substrates	Doses (mg)	Availability of probe drug	Phenotyping Metric	Matrix
Yin[33]	1A2	Caffeine	100	●	MR P 2/3h	P, U
	2C9	Tolbutamide	500	○	MR U 6-12h	
	2C19	Omeprazole	40	●	MR P 2/3h	
	2D6	Debrisoquine	10	○	MR U 0-6h	
	3A4	Midazolam	3.75	●	MR P 2/3h	
Basel	1A2	Caffeine	100	●	MR P 8h	P
	2B6	Efavirenz	50	●	MR P 8h	
	2C9	Losartan	12.5	●	MR P 8h	
	2C19	Omeprazole	10	●	MR P 2h	
	2D6	Metoprolol	12.5	●	MR P 8h	
	3A4	Midazolam	2	●	MR P 2h	

(*)	Interference observed
Ae	Amount excreted
AUC R	Area under the curve ratio
CL	Clearance
F	bioavailability
Fi	intestinal bioavailability
MR	Metabolic ratio
P	Plasma
U	Urine
4'OHM	4'hydroxy-mephenytoin
●	Widely available (Switzerland or European Union)
○	Not available (Switzerland or European Union)

None of the proposed cocktails have so far gained sufficient acceptance to be used as a tool to phenotype patients in clinical practice.

Currently available cocktails have several limitations, such as limited availability of probe drugs, complicated sampling procedures, and/or insufficiently validated phenotyping metrics.

Several drugs such as mephenitoine, tolbutamide, debrisoquine, and sparteine are specific CYP substrates and are no longer available on the Swiss market or no longer used in clinics and therefore difficult to obtain. This makes their use for phenotyping more complicated.

Only one matrix, urine or plasma, is used in the Quebec cocktail and modified Inje cocktail respectively. All the other cocktails recommend metrics in plasma and urine. The use of only one matrix would facilitate sampling procedures.

In the modified Inje cocktail, plasma AUC of dextromethorphan is used as CYP2D6 metric. Validation data are reported for dextromethorphan in urine, but not in plasma.

Based on this analysis of strengths and weaknesses of published phenotyping procedures our goal was to select the most promising probe drugs and make a new phenotyping cocktail.

3.3.Specific CYP substrates used in the Basel Cocktail study I – selections criteria

The selection of caffeine, efavirenz, losartan, omeprazole, metoprolol, chlorzoxazone and midazolam for phenotyping of CYP 1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4, respectively, is the result of an extensive evaluation. The criteria that guided our choices are described in the following paragraphs.

3.3.1. Selection criteria of Caffeine as a specific substrate for CYP1A2

Caffeine is a natural product present in coffee, tea, soft drinks and chocolate. It is a bronchial smooth muscle relaxant, a CNS stimulant, a cardiac muscle stimulant, and a diuretic.

Despite several alternative substrates being available, caffeine is the most commonly used substrate for CYP1A2 and arylamine N-acetyltransferase 2 (NAT2) phenotyping. The first step in its metabolism is almost exclusively mediated by CYP1A2, while NAT2 is mainly responsible for the formation of 5-acetylamino-6-formylamino-3-methyluracil (AFMU), one of the final metabolites of caffeine (Figure 2)[34].

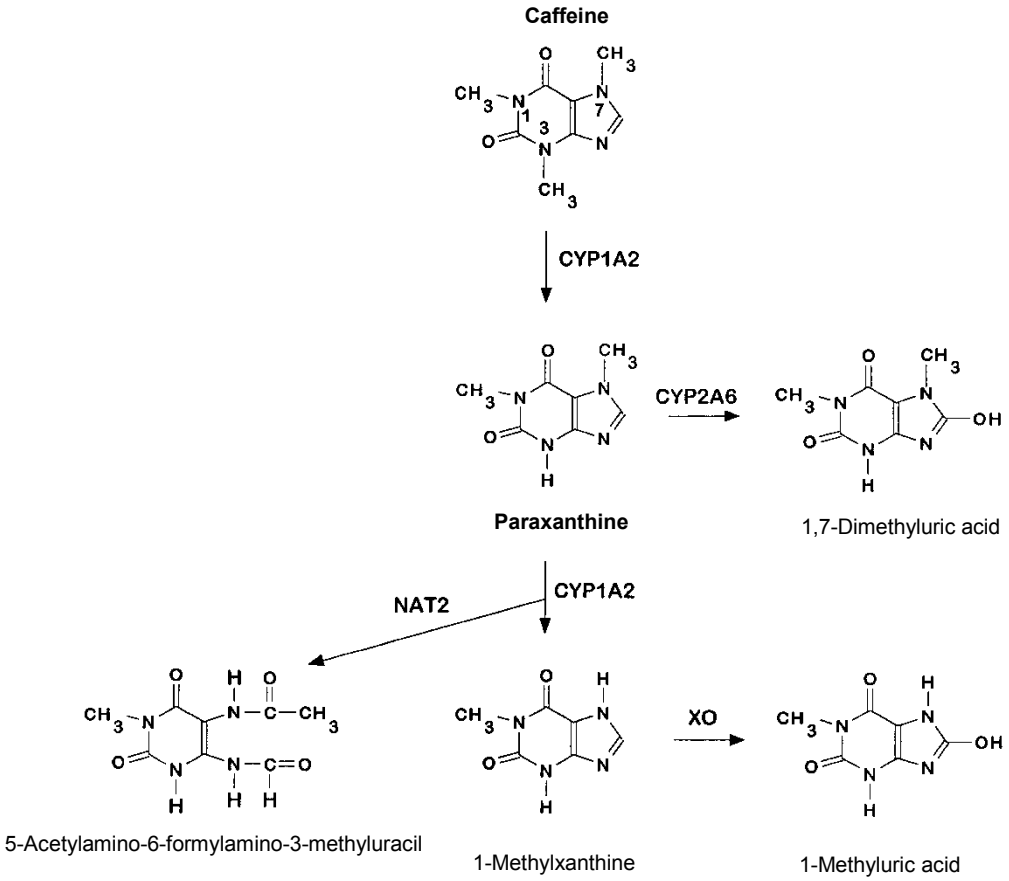

Figure 2. Main pathway of caffeine metabolism in human healthy subjects and enzymes involved [35].

The major primary metabolite is paraxanthine; other primary metabolites are theobromine and theophylline. The dimethylxanthines are pharmacologically active and may contribute to the effects of caffeine. Sequential metabolic steps include acetylation, 8-hydroxylation, and metabolism by xanthine oxidase.

Caffeine is well tolerated and easily available therefore several studies have been performed and extensive validation data are available.

Caffeine is completely absorbed from caffeinated beverages and reaches peak concentrations in blood in about 30-60 min. The rate of metabolism is variable, with a half-life ranging from 2 to 12 hours in healthy adults. On average, the half-life is 4-6 hours, with shorter half-life in smokers and longer half-life in people with chronic liver disease or in pregnant women. Plasma caffeine concentrations in the general population have not been well characterized. In a group of 600 medical outpatients, plasma caffeine averaged 2.1 mg/L (range 0.2-13.1 mg/L). In an experimental study of 17 health care personnel who were all moderate to heavy coffee drinkers, plasma caffeine averaged (24-h mean) 4.4 mg/L (range 1.2-9.7) [36].

Theophylline, tizanidine and melatonin are also specific substrates of CYP1A2. Even if they are valuable alternatives for phenotyping, their application is much less common. Validation data are lacking, theophylline has a narrow therapeutic range, and melatonin is not available in Switzerland.

Duloxetine, another specific substrate of CYP1A2, is also a moderate inhibitor of CYP2D6, therefore cannot be used in phenotyping cocktail approach.

3.3.2. Selection criteria of Efavirenz as a specific substrate for CYP2B6

Efavirenz (Stocrin®) is a non-nucleoside reverse transcriptase inhibitor (NNRTI) and is used as part of highly active antiretroviral therapy (HAART) for the treatment of HIV type 1.

Efavirenz has never been tested as part of a phenotyping cocktail. It is an established probe for determining CYP2B6 activity [37] and its use is recommended by the FDA for this purpose [38]. CYP2B6 is responsible for the formation of the main metabolite 8-hydroxy-efavirenz and subsequently 8,14-dihydroxy-efavirenz. Phase II reactions mediated by UGT produce the glucuronide derivative of the two metabolites (Figure 3).

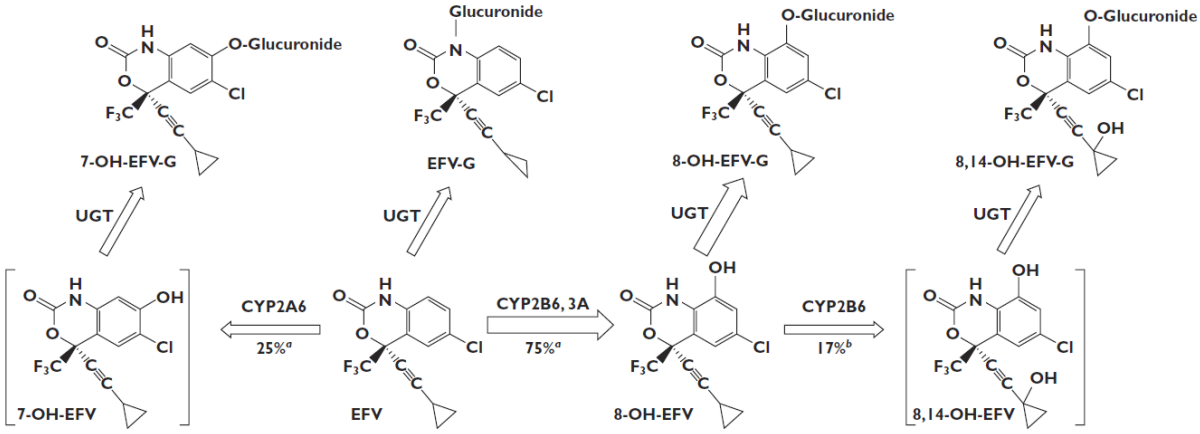

Figure 3. Metabolism of efavirenz as proposed by Jiang al [39]. EFV, Efavirenz; EFG(-G), EFV-glucuronide; 7-OH-EFV, 7-hydroxyefavirenz; 7-OH-EFV(-G), 7-hydroxyefavirenz-glucuronide; 8-OH-EFV, 8-hydroxyefavirenz; 8-OH-EFV(-G), 8-hydroxyefavirenz-glucuronide; 8, 14-OH-EFV, 8,14-hydroxyefavirenz; 8, 14-OH-EFV(-G), 8,14-hydroxyefavirenz-glucuronide

After repeated dosing it is a moderate inducer of CYP3A4 [40] and there are no published *in vivo* data available about CYP inhibition by efavirenz. The only retrievable data show *in vitro* inhibition of several CYP isoforms (CYP1A2, 2C9, 2C19, 2D6 and 3A4). When the study was planned, pharmacokinetic data after administration of 50 mg efavirenz were not available. We expected plasma concentrations would be reached in vivo after oral administration of 50 mg efavirenz of the drug to be much lower than the *in vitro* IC50 reported for 1A2, 2C9, 2C19, 2D6 and 3A4 [41]. On the basis of *in vitro* data (and assuming no relevant accumulation of efavirenz into hepatocytes), we expected plasma concentrations at least twenty times lower than the lowest IC50 (20 µM for 2C9, 2C19 and 3A4). Based on these considerations, we hypothesized that after low-dose efavirenz, relevant *in vivo* inhibition will not occur.

The elimination half-life of efavirenz is 52-76 h (after single dose) and 40-55 h (after multiple dose), making longer sampling intervals and washout periods necessary. The usual adult dose is 600 mg once a day. In our studies we used single doses of 50 mg efavirenz separated by sufficiently long wash-out periods to ensure that no accumulation occurs. As known from Marzolini *et al.* CNS symptoms occur more frequently at higher plasma concentrations [42]. So we expect that a dose of 50 mg will cause fewer and/or milder adverse effects.

The use of alternative substrates has been evaluated. Bupropion is also a specific substrate of CYP2B6. However, bupropion inhibits CYP2D6 and the only formulation available is a slow release tablet, therefore it was not suitable for the cocktail approach planned in this study. Propofol, ketamine or prasugrel were not selected due to lack of an oral formulation, inhibition of 2C9 and 3A4, or insufficient selectivity.

3.3.3. Selection criteria of Losartan as specific substrate for CYP2C9

Losartan is an angiotensin II receptor (type AT_1) antagonist. It is mainly indicated for the treatment of hypertension and to reduce the risk of stroke in patients with hypertension and left ventricular hypertrophy.

Oxidation of Losartan to its metabolite E-3174 is primarily mediated by CYP2C9. The elimination half-life of losartan is 1.5-2 h, and for E3147 4-9 h.

CYP3A4 appears to play a very minor role at the concentrations corresponding to therapeutic plasma levels. Losartan has already been used in several phenotyping cocktails [28, 30] and recent investigations have concluded that it may be a useful CYP2C9 phenotypic probe in humans. The ratio of losartan plasma AUC_{total} to the E-3174 plasma AUC_{total} was used by Yasar et al. [43] to characterize poor and enhanced CYP2C9 metabolizers. The ratio was about 30-fold higher in poor compared to enhanced metabolizers. The same trend was expected for the ratio between the plasma concentration of losartan and E-3174 four hours postdose.

Tolbutamide, warfarin, flurbiprofen, and losartan have already been used as probe drugs for CYP2C9 in cocktail combinations. Tolbutamide is an oral hypoglycemic drug no longer available in Switzerland and in several European Union (EU) countries. Tolbutamide plasma clearance and plasma concentrations 24 hours after administration were shown to correlate to CYP2C9 phenotype [20]. Warfarine is an anticoagulant metabolized by CYP2C9. The pharmacological effect of warfarine is a limitation to its phenotyping application. It was included in phenotyping cocktail in association with vitamin K. Flurbiprofen is also a specific substrate of CYP2C9. Zgheib *et al.* have evaluated flurbiprofen urinary ratios as phenotyping indices for CYP2C9 activity [32, 44]. Warfarine is no longer available in Switzerland. Flurbiprofen was not considered for the Basel cocktail because when the first study was planned, validation data were only available in urine. Recently, Daali et al. [17] described CYP2C9 phenotyping using 4'-hydroxy-flurbiprofen/flurbiprofen metabolic ratio in plasma and dried blood spot 2 hours after administration of 50 mg flurbiprofen. According to these data, flurbiprofen seems to be a valuable alternative to losartan.

3.3.4. Selection criteria of Omeprazole as specific substrate for CYP2C19

Omeprazole is indicated for treatment of peptic ulcer disease in adults.

CYP2C19 and CYP3A4 exhibit stereoselective metabolism of omeprazole. 5-hydroxylation of R-Omeprazole is exclusively mediated by CYP2C19, while S-isomer is metabolized by CYP3A4 to sulphone and by CYP2C19 to 5-O-desmethyl-omeprazole and only 27% to 5-hydroxy-omeprazole (Figure 4) [45, 46].

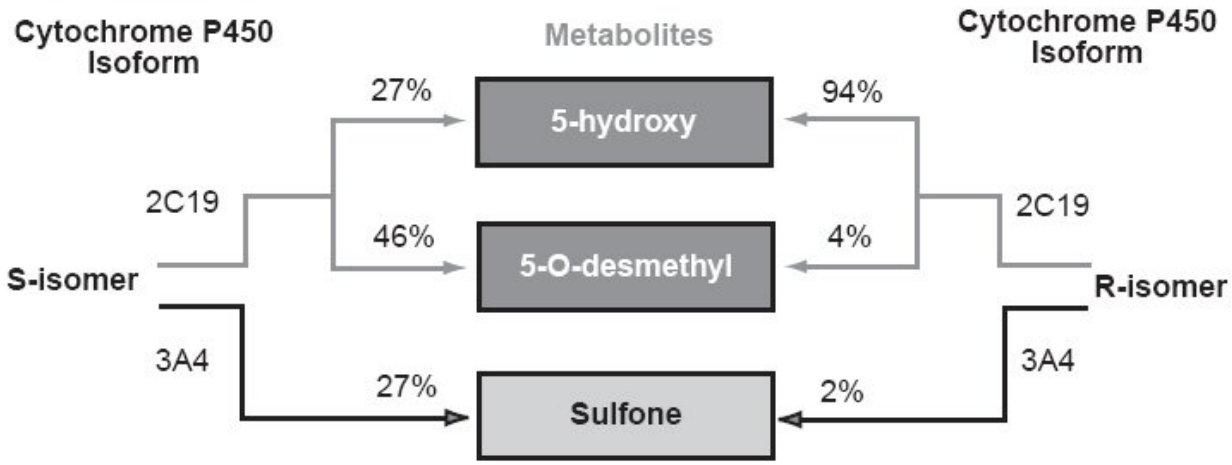

Figure 4. Stereoselective metabolism of omeprazole [47].

The plasma elimination half-life is very short (0.5-1 h), which requires sampling within a few hours after administration.

The reference compound for CYP2C19 phenotyping is mephenytoin. Since mephenytoin is no longer available, omeprazole is often used instead. According to Tamminga et al, all correlations between AUC 0-12 hours and individual metabolic ratios at 3 hours postdose were significant. The ratio between the plasma concentration of omeprazole and 5-hydroxyomeprazole 3 hours postdose was assumed to be a good measure for the activity of CYP2C19 [9]. Omeprazole has already been applied in several cocktails [28, 30, 48] and was also selected for our cocktail.

3.3.5. Selection criteria of Metoprolol as specific substrate for CYP2D6

Metoprolol is a selective β_1-adrenoreceptor antagonist. It is indicated for the treatment of hypertension, for the long-term treatment of angina pectoris and in the treatment of hemodynamically stable patients with definite or suspected acute myocardial infarction to reduce cardiovascular mortality. The only formulation available in Europe is the sustained release formulation.

Metoprolol is a specific and extensively validated substrate of CYP2D6. The measure of plasma AUC or the metabolic ratios between metoprolol and OH-metoprolol in urine have already been used as phenotyping index in several cocktails, while the use of metabolic ratio in plasma has never been evaluated [13, 25]. In the Basel cocktail, the plasma metabolic ratio has been evaluated for the first time.

Absorption of metoprolol in man is rapid and complete. Plasma concentrations following oral administration, however, approximate 50% of levels following intravenous administration, indicating about 50% first-pass metabolism. Plasma concentrations achieved are highly variable after oral administration. Only a small fraction of the drug (about 12%) is bound to human serum albumin. Metoprolol is a racemic mixture of *R*- and *S*-enantiomers. Less than 5% of an oral dose is recovered

unchanged in the urine, the rest is excreted by the kidneys as metabolites that appear to have no clinical significance.

There are marked ethnic differences in the prevalence of the CYP2D6 poor metabolizers (PM) phenotype. Approximately 7% of Caucasians and less than 1% Asians are poor metabolizers. Poor CYP2D6 metabolizers exhibit several-fold higher plasma concentrations of metoprolol than extensive metabolizers with normal CYP2D6 activity [9]. The elimination half-life of metoprolol is about 7.5 hours in poor metabolizers and 2.8 hours in extensive metabolizers. None of the metabolites of metoprolol contribute significantly to its beta-blocking effect. The estimation of the ratio between the plasma concentrations of metoprolol and OH-metoprolol in poor and extensive metabolizers four hours postdose shows a bimodal distribution.

Dextromethorphan, sparteine, debrisoquine and metoprolol have been described as probe drugs for CYP2D6 [9].

Sparteine and debrisoquine are not widely available, therefore their use is no longer possible.

The molar ratio of dextromethorphan over dextrorphan in urine collected 0-8 h postdose has been extensively validated. When the same ratio is measured in plasma the concentrations of parent compound are extremely low, especially in extensive metabolizers, making detection of dextromethorphan difficult. In this study we planned to analyze phenotyping indexes in plasma, blood, and oral fluid, but not in urine, therefore dextromethorphan was not selected for our cocktail.

3.3.6. Selection criteria of Chlorzoxazone as specific substrate for CYP2E1

Chlorzoxazone is a centrally-acting agent for painful musculoskeletal conditions with an elimination half-life of 1.1 h. The clearance of chlorzoxazone and the ratio 6-hydroxy-chlorzoxazone over chlorzoxazone in plasma 2 to 4 h after oral administration of a 250-500 mg dose are validated CYP2E1 markers. However, *in vivo* inhibition of CYP3A4 by chlorzoxazone has been documented, but the available data are not conclusive. Palmer *et al.* have reported an interaction between chlorzoxazone at the dose of 250 mg and midazolam [49], while according to Zhu *et al.* when chlorzoxazone was administered in combination with midazolam at the dose of 200 mg no significant interactions were observed [13].

In order to minimize inhibition of CYP3A4 a dose of 125 mg was applied.

Felbamate and theophylline were reviewed as alternative probe drugs for CYP2E1. 85% of felbamate is unchanged in plasma and it is metabolized only to a minor extent by CYP2E1 and CYP3A4 [50]. Theophylline is also metabolized by CYP3A4 and CYP1A2[50]. No other suitable probe drugs for CYP2E1 apart from chlorzoxazone could be identified.

3.3.7. Selection criteria of Midazolam as specific substrate for CYP3A4

Midazolam was introduced in the late 1970s as short-acting benzodiazepine. It has potent anxiolytic, anesthetic, hypnotic, anticonvulsant, skeletal muscle relaxant, and sedative properties. It is used in many countries as a premedication before surgery or for sedation before short minor procedures such as dental extraction. Midazolam is one of the most extensively validated and specific CYP3A4 substrates and its application has already been described in several cocktails [25, 28]. The broad experience of our group using midazolam for phenotyping guided our choice to this substrate [16].

The usual oral dose for insomnia treatment is 7.5-15 mg. The effect usually begins after 10-20 min, reaches the peak after 30-60 min and reduces after 1-2 h. The elimination half-life is 1.8-6.4 h. For our studies we used a formulation containing a lower dose of 2 mg of midazolam to minimize unwanted sedative effects. Feasibility of using low- or micro doses of midazolam for phenotyping has already been shown by another group [15].

The erythromycin breath test [51] has been used to characterize CYP3A4 phenotypes. A 3 µCi ^{14}C-N-methyl-erythromycin dose is administered i.v. CYP3A4 is responsible of the N-demethylation of the labeled methyl group. The reaction produces formaldehyde that is excreted as carbon dioxide in the breath. The $^{14}CO_2$ concentration detected in breath 20 minutes after dosing correlates with the subject phenotype. The i.v. administration of a labeled compound was probably an obstacle to the clinical acceptance of this procedure, not routinely applied nowadays.

Quinine is mentioned in FDA guidelines as specific CYP3A4 with a narrow therapeutic range. A cocktail approach including quinidine was not explored because of CYP2D6 quinidine inhibition [52].

Dapsone is also a well-accepted CYP3A4 specific substrate. Its use was not considered because according to Gass et al dapsone recovery in urine is not sensitive to inhibition [53].

3.4.How to study the possible interactions between the seven components of the Basel cocktail?

The fundamental requirement of cocktail combinations is that no mutual interactions between substrates occur. Potential interactions are estimated measuring AUC of the substance of interest alone and co-administered with other components of the cocktail.

To systematically verify the interactions between seven substrates would have required testing of 21 possible combinations, an approach not feasible for a clinical study. To simplify the procedure, we used information already available in literature (drug-drug interaction studies or substrates already validated in a cocktail mix). In order to visualize the interactions, green lines were used to link substrates where no interactions have previously been reported. Interactions that were already reported in literature were linked with red lines and where there was contradictory or missing data, links were grey.

In Figure 5 interactions of each probe drug with the other substrates of Basel cocktail are shown.

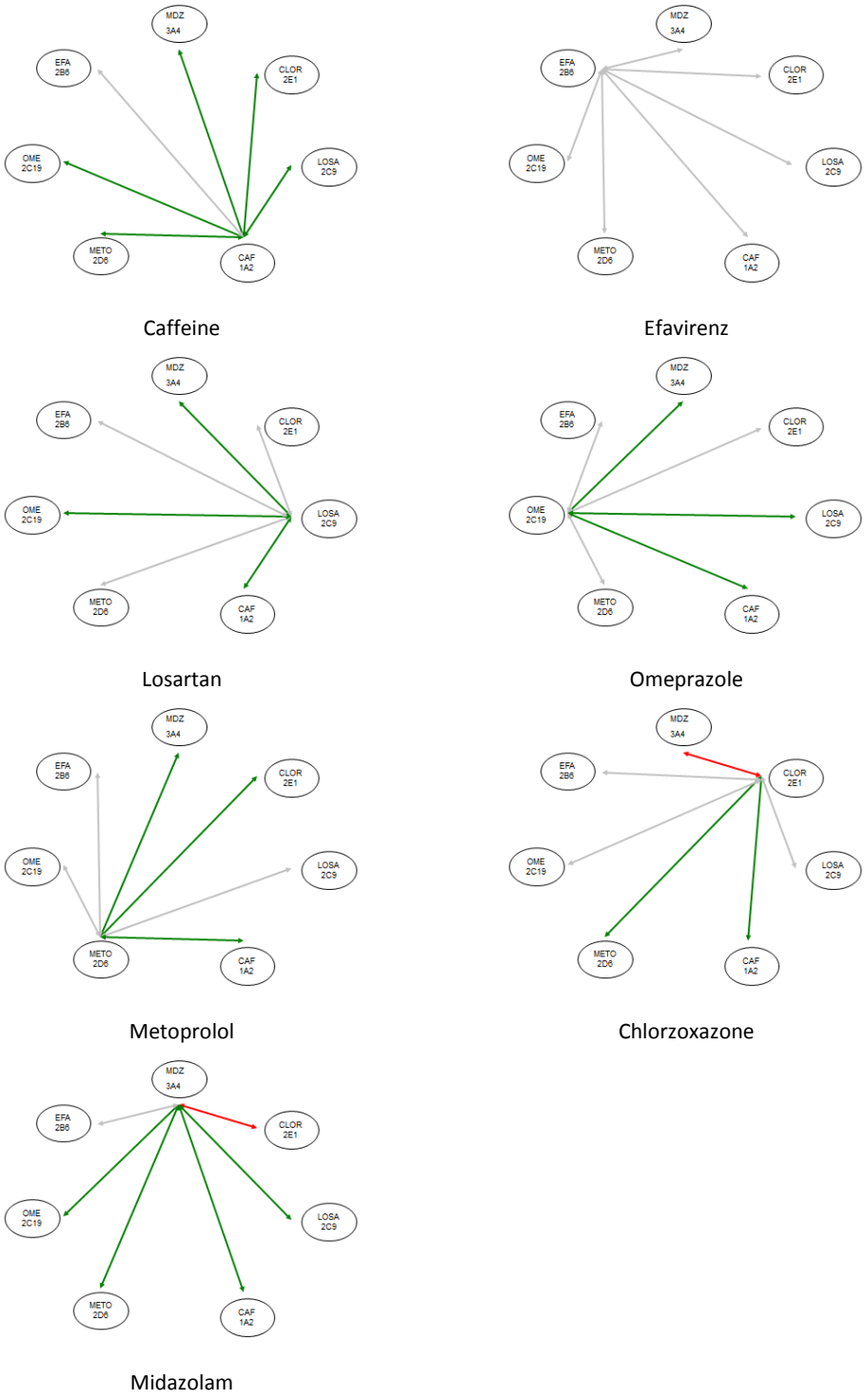

Figure 5. Visualization of interactions for each component of the Basel cocktail. CAF, caffeine; EFA, efavirenz; LOSA, losartan; OME, omeprazole; METO, metoprolol; MDZ, midazolam.

Figure 6 combines all the information reported in Figure 5 and summarizes all the possible interactions between the Basel cocktail substrates.

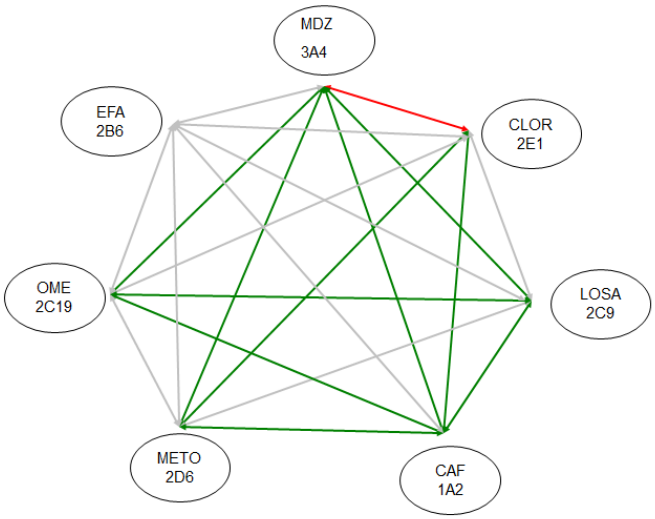

Figure 6. Visualization of all the possible interactions

To evaluate the interactions using a minimum number of study days, we grouped substrates for which no interactions have been documented into “safe” 3-drug combinations to acquire reference pharmacokinetic profiles for each substrate. For the 3-drug combination A (Figure 7) which included losartan, omeprazole and midazolam, only green arrows are visualized, therefore these three probe drugs could be combined without mutual interactions.

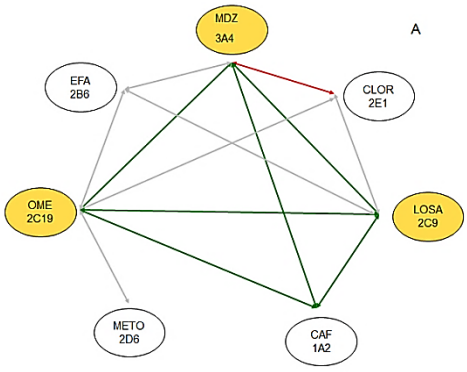

Figure 7. Treatment A: Losartan, omeprazole and midazolam

Treatment B (Figure 8) consisted of metoprolol, caffeine, and chlorzoxazone. Also for this combination no interactions have previously been documented.

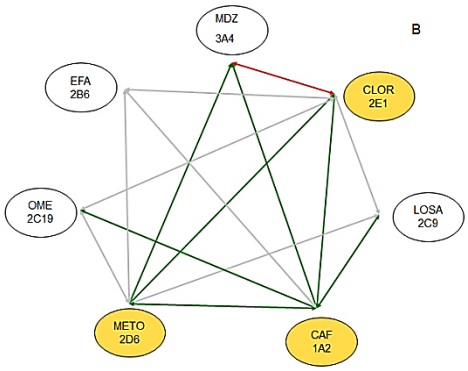

Figure 8. Treatment B: Metoprolol, caffeine, and chlorzoxazone

Treatment C (Figure 9) was used to explore interactions between losartan, metoprolol, chlorzoxazone, and midazolam and to test whether a reduction of the chlorzoxazone dose would allow minimizing or avoiding the possible interaction between midazolam and high dose chlorzoxazone. As mentioned above, contradictory information about the interaction between chlorzoxazone and its inhibitory effect on CYP3A4 are available. Chlorzoxazone has already been used in combination with midazolam in the Changsa cocktail. To evaluate this possible interaction, an ad-interim analysis of the data after treatment C was planned with the option of removing chlorzoxazone from the final cocktail.

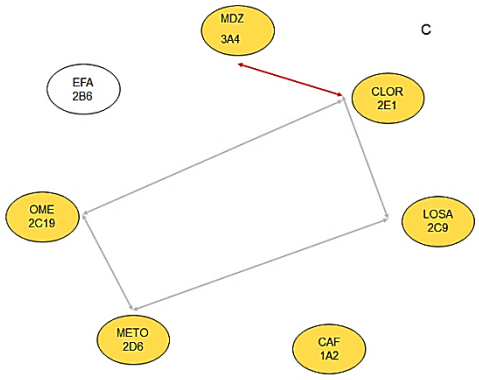

Figure 9. Treatment C: Midazolam, chlorzoxazone, losartan, caffeine, metoprolol and omeprazole

Since efavirenz has never been used as part of a phenotyping cocktail and no pharmacokinetic data for the 50 mg dose in healthy volunteers were available, efavirenz was given alone in treatment D (Figure 10).

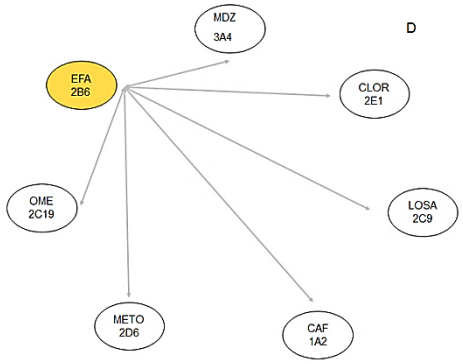

Figure 10. Treatment D: Efavirenz alone

According to the results of the ad-interim analysis, treatment E (Figure 11) or Ebis (Figure 12) was applied.

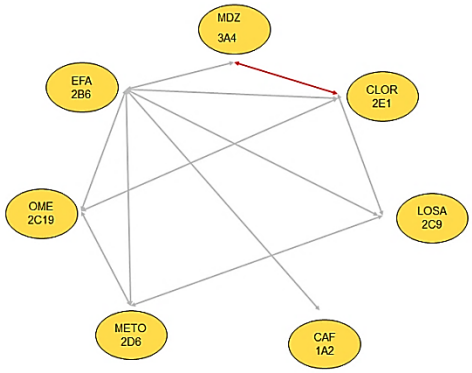

Figure 11. Basel cocktail, treatment E including chlorzoxazone, depending on the result of the inter-analysis after treatment C.

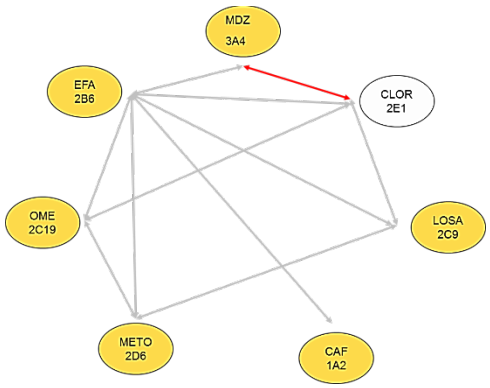

Figure 12. Basel cocktail, treatment Ebis.

3.5.Drugs used as inhibitors and inducers in Basel cocktail study II

The effects of inducers and inhibitors on the Basel cocktail substrates was evaluated in a clinical study using specific drugs selected according to the criteria described in the following paragraphs.

3.5.1. Selection criteria of Ciprofloxacin

Ciprofloxacin is a fluorochinolone with a broad antibacterial spectrum and is used in numerous infection diseases.

After administration of a single-dose of 250 mg, 500 mg, 750 mg and 1'000 mg, ciprofloxacin peak concentrations of 0.94-1.53 mg/L (2.84-4.62 µM), 2-2.9 mg/L (6.04-8.75 µM), 2.6-4.3 mg/L (7.85-12.98 µM) and 5.4 mg/L (16.3 µM), respectively, were reached after 1-2 h. The elimination half-life was 3-6 h [54] i.e. >90% of the steady state concentration is achieved after 10-20 h.

According the FDA-Guidance ciprofloxacin is a moderate inhibitor of CYP1A2 [38]. *In vitro* and *in vivo* data suggest that ciprofloxacin does not inhibit other cytochromes than CYP1A2 [55].

An interaction study with ciprofloxacin and caffeine in ten healthy volunteers showed a significant increase of the half-life of caffeine (from 5.2 ± 1.2 to 8.2 ± 2.5 h) and the caffeine AUC (from 16.3 ± 6.6 to 25.9 ± 7.8 mg·h/L) while a decrease of the total body clearance (from 106 ± 41.6 to 58.2 ± 28.8 mL/min per 1.73 m^2) was observed. In addition, the rate of conversion of caffeine to paraxanthine was significantly delayed [56].

In another study in 12 healthy volunteers a two fold increase of the area under the plasma concentration-time curve (77.8 vs. 31.8 mg·h/L) and terminal-phase half-life (9.7 vs. 4.5 h) of caffeine was observed in the presence of ciprofloxacin [57]. In both studies ciprofloxacin was administered in a dose of 750 mg twice daily.

Based on these data ciprofloxacin was administered as an inhibitor of CYP1A2 in the Basel cocktail II study at a dose of 750 mg, 24 h, 12 h and 1 h before administering the Basel cocktail.

3.5.2. Selection criteria of Fluconazole

Fluconazole is a triazole antifungal, which is used in various infections with *Candida species* and *Cryptococcus neoformans*.

Following oral administration of a single-dose of 400 mg of fluconazole in fasted healthy volunteers, the C_{max} was 6.72 mg/L (range 4.12-8.08 mg/L) after 1-2 h. Fluconazole has a high oral bioavailability (90 %) and a half-life of 30 h, therefore the steady state is reached after 5-10 days [54].

According to the FDA-Guidance fluconazole is a moderate inhibitor of CYP2C9 [38].

In addition to the inhibition of CYP2C9, fluconazole was also shown to be a strong inhibitor of CYP2C19 and CYP3A4, whereas *in vitro* data suggest only minor effects on CYP1A2, CYP2B6 and CYP2D6 activity [55].

In a study with 12 volunteers receiving fluconazole and midazolam, a single dose of fluconazole increased the area under the oral midazolam concentration-time curve ($AUC_{0-\infty}$) 3.5-fold ($p < 0.001$) and the peak concentration 2-fold ($P < 0.05$) compared to placebo. On the sixth day the $AUC_{0-\infty}$ of oral midazolam was 3.6 times higher with fluconazole ($P < 0.001$) than without the antimycotic. The psychomotor effects of midazolam were also profoundly increased ($P < 0.001$) [58].

Another study investigated the interaction of fluconazole and omeprazole in 18 healthy male volunteers. After inhibition with fluconazole, the area under the plasma concentration-time curve of omeprazole from time zero to time infinity ($AUC_{0-\infty}$) increased significantly (3090 vs 491 µg h/L), terminal half-life of omeprazole was prolonged (2.59 vs. 0.85 h), and peak plasma concentration of omeprazole (C_{max}) was higher (746 vs. 311 µg/L) [59].

Based on these data, fluconazole was used to inhibit CYP2C9, CYP2C19 and CYP3A4. The inhibitory effect on CYP1A2, CYP2B6, and CYP2D6 was expected to be minimal.

3.5.3. Selection criteria of Paroxetine

Paroxetine is a phenylpiperidine antidepressant agent which selectively inhibits serotonin reuptake. Paroxetine was developed as an alternative to tricyclic antidepressants that interfere with the reuptake of both serotonin and other neurotransmitters [54]. Paroxetine is approved for the treatment of depression, panic, and generalized anxiety disorders as well as social phobias [50].

After oral administration of 20 mg paroxetine, mean peak serum concentration was 10.7 µg/L (range 0.8-32.5 µg/L). With oral doses of 30 and 40 mg, mean peak concentrations were 17.6 µg/L and 26.6 µg/L after 3 and 8 h respectively [54].

According to FDA-Guidance paroxetine is a strong inhibitor of CYP2D6 [38].

An interaction study with metoprolol and paroxetine in eight healthy male volunteers showed a significant increase of the mean area under the plasma concentration-time curve extrapolated to infinity ($AUC_{0-\infty}$) of *R*- and *S*-metoprolol (169 vs. 1340 µg·h/L [$P < 0.001$] and 279 vs. 1418 µg·h/L [$P < 0.001$], respectively), with an approximately 2-fold increase in both maximum plasma concentration and terminal elimination half-life. The mean metoprolol metabolic ratio (metoprolol / 5-OH-metoprolol) was significantly increased from 0.17 to 5.69 ($P < 0.05$) [60].

In another study, 17 depressed patients with acute myocardial infarction received metoprolol as a routine part of their therapy (mean dose 75 ± 39 mg/day). Paroxetine 20 mg daily was then administered. Mean metoprolol areas under the concentration-time curve (AUC) increased 4-fold (1064 ± 1213 to 4476 ± 2821 nM·h/mg per kg, $P = 0.0001$), while metabolite AUCs decreased (1492 ± 872 to 348 ± 279 nM·h/mg per kg, $P < 0.0001$), with an increase of metabolic ratios (0.9 ± 1.3 to 26 ± 29; $P < 0.0001$). A reduction of metoprolol dose was required in two patients due to excessive bradycardia and severe orthostatic hypotension. No other adverse effects of the drugs were identified [61].

In contrast to CYP2D6, little is known about the effects of paroxetine on the activity of CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP3A4.

In an *in vivo* single-dose study, 24 young, healthy men received increasing doses (10, 20, 40 and 80 mg) of paroxetine, fluoxetine, fluvoxamine and citalopram. The subjects were phenotyped for CYP2D6, CYP2C19 and CYP1A2 by co-administration of spartein, mephenytoin and caffeine. Potent inhibition of CYP2D6 and moderate inhibition of CYP2C19 was observed after administration of paroxetine and fluoxetine. Fluvoxamine showed an inhibitory effect on CYP1A2 [62].

In vitro data suggest no effect of paroxetine on CYP3A4 [55] and CYP2C9 [63].

In summary, paroxetine is a strong inhibitor of CYP2D6 with no effects on CYP1A2, CYP2C9, CYP2C19 and CYP3A4. The initial therapeutic dose is 20 mg daily. The dose may be increased of 10 mg/day at intervals of at least 1 week. Paroxetine has an elimination half-life of 15-22 h, therefore 90% of the steady state concentration is reached after 50-72 h. Using loading doses in the therapy with paroxetine is unusual because higher doses in previously untreated patients lead to more adverse effects. For these

reasons paroxetine was administered in a dose of 20 mg daily up to 72 h before applying the cocktail drugs (i.e. 72 h, 48 h, 24 h, and 1 h).

3.5.4. Selection criteria of Rifampicin

Rifampicin is a semi-synthetic antibiotic derived from a fermentation product of *Nocardia Mediterranei*. The drug has a wide antibacterial spectrum and is approved for the treatment of several infectious diseases, especially tuberculosis.

Rifampicin is one of the stronger inducers of cytochromes and therefore recommended by the FDA for induction studies [38].

A mean C_{max} of 10.54 mg/L following 600 mg oral doses was found in 14 healthy adult volunteers. Time to peak concentration observed after oral administration was 1-4 h. A high oral bioavailability, 90 to 95 % and a half-life of 1.5-5 h [54] was reported.

Rifampicin is a known inducer of CYP3A4 and CYP2C9 *in vivo* [64, 65]. It has also been reported that rifampicin is an inducer of CYP1A2 (only weak effect), CYP2C8 and CYP2C19 [66-68].

Kanebratt *et al.* investigated the inducing effect of rifampicin on the cytochromes CYP1A2, CYP2C9, CYP2C19 and CYP3A4 using the Karolinska Cocktail. Caffeine (CYP1A2), losartan (CYP2C9), omeprazole (CYP2C19), quinine (CYP3A4) and 4β-hydroxycholesterol (as an endogenous CYP3A4 marker) were used as specific phenotyping substrates. After administration of Rifampicin (20, 100 or 500 mg daily) for 14 days to eight healthy subjects a four-fold induction of CYP3A4 was observed at the highest dose by both quinine/3'-hydroxyquinine and 4β-hydroxycholesterol measurements ($P < 0.001$). CYP3A4 induction was also observed at lower doses of rifampicin. CYP1A2, CYP2C9 and CYP2C19 were induced after daily treatment with 500 mg rifampicin (1.2-fold, $P < 0.05$; 1.4-fold, $P < 0.05$; and 4.2-fold, $P < 0.01$, respectively) [69].

In contrast to CYP1A2, CYP2C9, CYP2C19 and CYP3A4, cytochrome CYP2D6 does not seem to be inducible. To date no CYP2D6-inducer has been identified.

The effect of rifampicin on the metabolism of efavirenz was published by Yenny *et al.* [70]. Co-administration of a single dose of Efavirenz 600 mg after 1-week rifampicin treatment (450 mg/day) significantly reduced efavirenz bioavailability in healthy volunteers.

In the Sustiva® medication instruction sheet a rifampicin related reduction of efavirenz C_{max} and AUC by 20 % (11-28%) and by 26% (15-36%), respectively is reported.

In summary, rifampicin is a strong inducer of CYP3A4 and especially CYP2C19 and a weak inducer of CYP1A2 and CYP2C9. Data published on CYP2B6 induction are contradictory and no effect on CYP2D6 has been observed. FDA-Guidance recommends a rifampicin dose of 600 mg daily for multiple days. The time required to achieve a complete induction depends on the cytochrome [71]. On average 1 week seems to be enough for a sufficient induction. On the other hand, the baseline activity after

discontinuing rifampicin treatment would be attained in about 2 weeks. The uncertainty of the time period required for complete induction is reflected in the numerous dosing regimens used in other studies [15, 69, 70, 72-76]. On average most studies used a dose of 600 mg rifampicin daily for 7 days. FDA guidelines suggest evaluating the effect of CYP1A2-induction by comparative PK studies in smokers vs. non-smokers. This procedure would have required a different study design, not appropriate for other CYP450 isoforms monitored in our study. On the basis of these considerations rifampicin was administered in a dose of 600 mg for 7 days before applying the cocktail drugs.

3.6.Genotyping of Drug Metabolizing Enzymes

In the Basel cocktail I and II studies the most relevant single-nucleotide polymorphisms (SNP) associated with altered enzyme function were determined. Genetic polymorphism of CYP1A2, CYP2D6, CYP2C9 and CYP2C19 have been shown to impact the pharmacokinetics of drugs metabolized by these isoforms [77].

While genotyping only detects genetic variations, phenotyping provides a measure of the activity of a drug-metabolizing enzyme at a given point in time, and reflects all relevant factors that influence drug metabolism (e.g. genetic variations, drug-drug interactions, hormonal and environmental influences).

Genotyping of enzymes responsible for drug metabolism has become more important but is still reserved to a few specific applications. One example is thiopurine S-methyl-transferase (TPMT), a polymorphic enzyme responsible for S-methylation of azathioprine (AZA), 6-mercaptopurine (6-MP) and thioguanine (TG). Since there is a significant correlation between TPMT genotype and TPMT activity the Clinical Pharmacogenetics Implementation Consortium provided dosing recommendation for AZA, 6-MP and TG based on TPMT genotype [78]. Similarly, guidelines for CYP2D6 and CYP2C19 genotypes and dosing of tricyclic antidepressant [79], or for CYP2C19 genotyping and clopidogrel therapy [80] have been published.

From the technical perspective, automation of genotype testing has been implemented. The Roche AmpliChip® CYP450 test delivers information on CYP2D6 and CYP2C19 polymorphisms in the form of a genotype and predicted metabolizer status. Despite approval of this test by the FDA in 2005, CYP2D6 and CYP2C19 genotyping is not yet commonly used in clinical practice.

3.7.Minimally-invasive and non-invasive sample collection: dried blood spot technique and saliva sampling as tools for easier phenotyping

Introduction of new diagnostic procedures such as phenotyping into clinical practice may encounter resistance, especially if complicated and invasive sampling is required. The easier a procedure, the more likely it will be accepted and used in daily practice. For this reason we explored the possibility of minimizing the number of samples to be collected as well as invasiveness, using single time point saliva or dried blood spot sampling instead of collecting full 24 h pharmacokinetic plasma profiles.

3.7.1. Dried blood spot technique

DBS collection technique due its popularity to the neonatal screening application introduced by Guthrie in 1963 [81]. Few applications for quantitation of drugs in DBS were reported in the literature between 1980 and 2000. The main limitation to its application was the high sensitivity required to accurately quantify drugs or biomarkers in a single drop of a complex matrix such as blood. With the sensitivity

increase of LC-MS/MS instruments, these limitations have been overtaken and an increased interest of hospitals, academia and pharmaceutical industries has appeared (Figure 13).

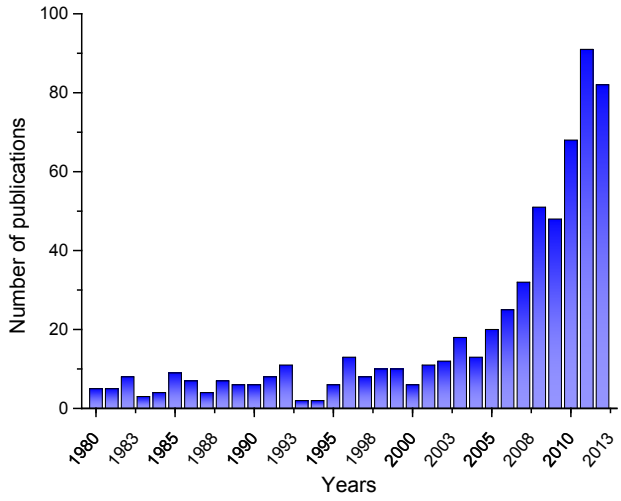

Figure 13. Number of publications per year about DBS in Pubmed (1980-2012) using the search term "dried blood spot" in the title.

DBS sampling involves collection of a few drops of blood either by heel prick (in newborns) or by finger prick on special blood collection cards. After drying for 2 hours the blood spots are ready for analysis or storage.

The main advantages of this approach are a less invasive and easier blood collection. If correctly instructed, the patient can perform finger prick and collect DBS without the support of medical staff. If the dugs of interest are stable at room temperature, DBS samples can be shipped easily by normal mail. DBS have been proposed also as solution to stability issues. The removal of water can stabilize the sample as described by Heinig et al [82]. The advantages mentioned would also support the choice of DBS when a clinical study is performed in regions with limited logistics support [83].

Since DBS are obtained by prick of the finger, the collected blood is capillary blood. For classic diagnostic purposes blood is collected from a forearm or cubital vein. Before replacing conventional sampling with DBS, it is necessary to demonstrate that the sampling site does not influence the measurement. This approach is the "clinical validation" and consists in collecting at the same time venous and capillary blood, compare the measurement, and establish to what extent venous and capillary blood concentration correlates.

Our interest for DBS in phenotyping is related to the low invasiveness of this technique. In chapters 6, 7, and particularly in chapter 8.2 the results of DBS applications are described.

From a technical perspective, analysis of DBS is a challenging task. Each DBS is manually processed. A manual puncher is used to cut out a disc with 3 or 6 mm diameter, transferred in a test tube, extracted

with a suitable solvent, and analyzed by LC-MS. Handling of a high number of DBS cards following the manual procedure is time consuming and could generate errors. A technical solution has been proposed by Camag that has developed and now commercializes the DBS 500, an online DBS extraction system. In the framework of a KTI project, we established collaboration with Camag to develop DBS analysis for phenotyping and TDM of tyrosine kinase inhibitors. The technical details are reported in chapter 8.2.

3.7.2. Oral fluid sampling

If DBS is an easy and low invasive sampling approach, collection of oral fluid is even easier and non-invasive.

Saliva has been used for monitoring of drugs of abuse, while TDM applications are less common [84].

Quantification of midazolam in oral fluid for phenotyping purpose has already been described by Link et al. The possibility to extend it to other phenotyping substrates has been explored in the two Basel cocktail studies (chapters 6 and 7)

4. Aims of the thesis

The overall objective of this thesis was to develop and characterize a new phenotyping procedure with the potential to be used not only in the classical phase I research setting but also for clinical applications.

To reach this goal, the work was divided into three projects:

(A) Bioanalytical method development (Chapter 5)
(B) Clinical trial to rule out mutual interactions between phenotyping probe drugs (Basel cocktail study I, Chapter 6)
(C) Characterization of phenotyping metrics under conditions of induction and inhibition (Basel cocktail study II, Chapter 7)

The objective of the first project was to develop and validate bioanalytical methods for simultaneous detection of probe drugs and their main metabolites in different human matrices. To limit side effects, it was planned to use the lowest commercially available doses of probe drugs. It was anticipated that under induction or inhibition very low concentrations of either parent or metabolite would occur compared to baseline conditions. To maintain clinical applicability, short return times are required. Therefore highly sensitive bioanalytical methods with simple sample work-up and short run-times had to be developed.

Clinical validation of the new phenotyping procedure involved two clinical studies in healthy volunteers: the Basel cocktail studies I and II. The objective of the second project (Basel cocktail study I) was to prove the absence of interactions between the new combination of probe drugs selected for the Basel phenotyping cocktail. Furthermore the possibility of using metabolic ratios in single time-point plasma measurements to replace the full AUC measurements was tested. Additionally, the use of DBS and saliva as minimally or non-invasive sampling collection procedures was explored.

The goal of the third project (Basel cocktail study II) was to characterize the range of the chosen phenotyping metrics under conditions of induction and inhibition. For a comprehensive evaluation of the new phenotyping cocktail, the influence of genetic factors or co-administration of an enzyme inducer or inhibitor on the metabolic ratios of the Basel cocktail had to be assessed. Additionally, the measurements of metabolic ratio in saliva and DBS were repeated to evaluate whether oral fluid or DBS sampling could also be used to phenotype individuals in situations where very low concentrations of either parent or metabolite are expected.

In the last part of the thesis an outlook on planned phenotyping projects is provided. In the Basel cocktail studies I and II, the commercially available formulations of the phenotyping drugs were used. To simplify probe drug administration, a prototype of a combi-pill containing all six cocktail probe drugs was developed, and tested in a single subject in a clinical pilot study. Pharmacokinetic profiles of the new formulation were comparable with the ones obtained after intake of the individual probe drugs in

the commercial formulations. This new cocktail combi-pill will be formally evaluated in a clinical study (Basel cocktail study III) in healthy volunteers.

The use of phenotying information to personalize cancer therapy with tyrosine kinase inhibitors is under investigation in an ongoing clinical trial. Individual CYP3A4 and CYP1A2 phenotype will be assessed using probe drugs of the Basel cocktail and the correlation of obtained phenotyping metrics with sunitinib, pazopanib or erlotinib exposure will be analyzed.

5. Development and validation of a bioanalytical method for simultaneous quantification of the BASEL phenotyping cocktail probe drugs and metabolites in plasma, saliva and dried blood spots

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5.1. Introduction

The first and fundamental step in developing a new phenotyping cocktail is to have the analytical tools that reliably quantify low concentrations of the phenotyping dugs and their metabolites. To limit side effects, the lowest possible doses of probe drugs should be applied. Furthermore, under induction or inhibition very low concentrations of either parent or metabolite compared to baseline conditions are to be expected. To maintain clinical applicability, short return times of the results are required. Therefore high sensitivity, a simple sample work-up effective for all the analytes, and short run-times were the prerequisites for developing the analytical method to be used for the samples of the Basel cocktail studies.

The analytical methods for the analysis of already published phenotyping cocktails are quite diverse. The probe drug combination we selected was new and therefore already published methods could not be used. The most recent methods are summarized below. All these authors tried to achieve our objectives in terms of sensitivity and speed, reducing sample pretreatment with online solid phase extraction (SPE), improving chromatographic separation with UHPLC, and increasing sensitivity with high end mass spectrometry detection.

Lin et al. [85] recently published a cocktail method with online SPE-HPLC separation and diode array detection. The limit of quantitation (LOQ) achieved (50 ng/mL for omeprazole, 200 ng/mL for metoprolol) clearly support the need for a more sensitive detection mode such as MS/MS if sub-therapeutic doses are administered. Ghassabian et al. [86] presented a method to quantify the Injie cocktail drugs using liquid-liquid extraction for caffeine and paraxanthine and SPE for all the other analytes, followed by a 6 min LC-MS/MS run. An 8 min UHPLC-MS/MS method was proposed by Stewart et al [87] for the quantification of the 6 probe drugs and metabolites of the Pittsburg cocktail. Petsalo et al [88] have developed a sensitive cocktail method to quantify 12 CYP-specific probe metabolites and their nine parent drugs in urine, using a sub 2 µm particle size column and a 5 min LC-MS/MS run. This method was developed for urine and detected only few of the drugs used in the Basel cocktail.

Within the Basel cocktail study I and II the application of less invasive sampling techniques was explored. Quantification of low concentrations of drugs in plasma, blood and especially in saliva and DBS, is a challenging process. Use of DBS for caffeine determination in preterm infants [89] or for quantification of midazolam for CYP3A4 phenotyping [90] has already been reported. Daali et al [17] have demonstrated the possibility of using flurbiprofen quantitation in DBS for phenotyping of CYP2C9.

Saliva or oral fluid is a matrix much less used. Phenotyping of CYP1A2 measuring caffeine and its metabolites in saliva has already been reported by Perera et al [91], while determination of midazolam in saliva to phenotype CYP3A4 has been explored by Link et al [16].

The optimization and partial validation of two analytical methods, the first developed in 2010 for the analysis of the first Basel cocktail (study I) and a second optimized method (higher sensitivity, shorter runtime) developed in 2011 for the analysis of the second Basel cocktail (study II) are described in the following chapters.

5.2.Material and Methods

5.2.1. Chemicals and reagents

Caffeine-d9, chlorzoxazone, 6-hydroxy chlorzoxazone, chlorzoxazone-d3, 8-hydroxy efavirenz, efavirenz-d4, losartan, losartan carboxylic acid, losartan-d4, hydroxy-metoprolol, metoprolol-d7, omeprazole, 5-hydroxy omeprazole, omeprazole-d3, were purchased from TRC (Toronto, Canada). 1'-hydroxy-midazolam and midazolam-d6 were from Lipomed (Arlesheim, Switzerland). Metoprolol and paraxanthine were obtained from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Midazolam (Hoffmann-La Roche, Basel, Switzerland) and efavirenz (Merck, NJ, USA) were kindly provided by the producer.

Formic acid, HPLC-grade methanol, and water were purchased from Merck (Darmstadt, Germany). Schleicher & Schuell #903® filter paper was purchased from Schleicher & Schuell GmbH (Dassel, Germany). Manual hole punchers with a diameter of 3mm, Harris punch and cutting mats were from Whatman (Sanford, USA).

5.2.2. Stock solutions, standard solutions and quality controls

Fresh stock solutions containing caffeine, paraxanthine, chlorzoxazone, 6-hydroxy-chlorzoxazone, efavirenz, 8-hydroxy-efavirenz, losartan, losartan carboxylic acid, metoprolol, hydroxy-metoprolol, midazolam, 1'-hydroxy-midazolam, omeprazole, and 5-hydroxy-omeprazol were prepared in dimethyl sulfoxide (DMSO) at a concentration of 2 mg/mL. Calibration spiking solutions containing the cocktail components at concentrations of 100, 50, 25, 10, 5, 2.5, 1, 0.5, 0.25, 0.1 and 0.05 µg/mL and quality controls (QC) at concentrations of 50, 5, and 0.5 µg/mL were prepared from the concentrated DMSO solutions. Calibration standards ranging from 0.5 to 1000 ng/mL and quality controls at 5, 50, and 500 ng/mL were prepared by enriching caffeine free blank human plasma, blood and saliva using the corresponding spiking solutions. 50 µL aliquots of plasma, blood and saliva were stored frozen at -20 °C until use.

DBS calibration and quality control samples were prepared by transferring 15 µL of enriched blood on filter paper.

Stock solutions containing metoprolol-d7, omeprazole-d3, losartan-d4, midazolam-d6, caffeine-d9, efavirenz-d4, and chlorzoxazone-d3 were prepared in DMSO at a concentration of 2 mg/mL. An internal standard (ISTD) solution containing the deuterated compounds mentioned above at a concentration of 0.1 µg/mL was prepared in methanol and used for the analysis of samples from study I. A 10 ng/mL solution of the same deuterium labeled compound was used for the analysis of samples from the study II. For the extraction of DBS the same concentration of deuterated compounds was spiked in methanol/water (1/1, v/v).

5.2.3. Preparation of plasma, blood and saliva samples

Sample work up was carried out with aliquots of 50 µL human plasma, blood or saliva. 50 µL aliquots were mixed with 150 µL ISTD solution. After vortex mixing for at least 30 s and centrifugation (3220 g, 30 min, 10°C), samples were analyzed by LC-MS/MS.

5.2.4. Treatment of plasma and saliva samples with glucuronidase

Plasma and saliva samples collected during the Basel cocktail study II were treated with glucuronidase. 50 µL of plasma or saliva were incubated with ≥ 500 units of β-glucuronidase Type HP 2 (Sigma G7017, Sigma-Aldrich, Buchs, Switzerland) at 37°C for 16h. Sample processing was as described above for plasma and saliva samples.

5.2.5. Preparation of dried blood spot samples

A disc with a diameter of 3 mm was cut out of the center of the dried blood spots into a clean tube using a manual hole punching device. 200 µL of ISTD solution were added to each tube, containing one blood spot. The vials were vortex-mixed for 10 min and centrifuged (3220 g, 30 min, 10 °C).

5.2.6. LC-MS/MS instrumentation and conditions used in study I - (BC-LCMS-01)

Chromatographic separation was performed on a Shimadzu HPLC system (Shimadzu AG, Reinach, Switzerland). Samples were eluted on an Atlantis T3 column (2.1X50 mm, 3 µm, Waters, Baden-Dättwil, Switzerland). The injection volume was 10 µL and total run time was 5.5 min. The following gradient was applied: from 0 to 0.5 min 100% solvent A (0.1% formic acid in water), from 0.5 to 4.3 min the proportion of solvent B (0.1% formic acid in methanol) linearly increased from 0 to 70%, at 4.31 the percentage of B was increased to 95% and maintained for 0.7 min. At 5.0 min the initial conditions were re-established. The flow rate was 0.35 mL/min, the column was placed in a column oven at 40°C and the samples were stored in the autosampler at 10°C.

The LC system was interfaced with a triple quadrupole mass spectrometer (API4000, AB/MDS Sciex, Concord, Canada) equipped with an ESI source. Positive ESI was applied for the first 4.25 min (ion spray voltage 5500 eV). Polarity was then switched to negative mode (ion spray voltage -4500 eV) for the detection of efavirenz and 8-hydroxy-efavirenz. The probe temperature was 350°C. The selected mass-to-charge (m/z) ratio transitions of the analytes and internal standards used in selective reaction monitoring mode are reported in Table 2. Efavirenz probes were reanalyzed using an optimized method

to improve the detection of efavirenz and 8-hydroxy-efavirenz. The same gradient was applied but only the negative mode transitions were monitored.

Table 2. Multiple reaction monitoring (MRM) transitions monitored in the analytical method used to quantify the samples of Basel cocktail study I

Analyte	Precursor (m/z)	Product (m/z)	Ionization mode
Metoprolol	268	116	ESI+
1-Hydroxy-metoprolol	284	116	ESI+
Metoprolol-d7	275	123	ESI+
Omeprazole	346	198	ESI+
3-Hydroxy-omeprazole	362	214	ESI+
Omeprazole-d3	349	198	ESI+
Losartan	423	405	ESI+
E-3174	437	235	ESI+
Losartan-d4	427	409	ESI+
Midazolam	326	291	ESI+
1'-Hydroxy-midazolam	342	324	ESI+
Midazolam-d6	332	297	ESI+
Caffeine	195	138	ESI+
Paraxanthine	181	124	ESI+
Caffeine-d9	204	144	ESI+
Efavirenz	314	244	ESI-
8- Hydroxy -efavirenz	330	258	ESI-
Efavirenz-d4	318	69	ESI-

5.2.7. LC-MS/MS instrumentation and conditions used in study II (BC-LCMS-02)

The analytical method applied for the analysis of the Basel cocktail study I, was adapted to additionally monitor the concentrations of the inhibitors ciprofloxacin, fluconazole, paroxetine, and the inducer rifampicin, as well as the metabolite 8,14-dihydroxy-efavirenz.

Chromatographic separation was performed using a Shimadzu HPLC system (Shimadzu AG, Reinach, Switzerland).

Samples were eluted on an Atlantis T3 column (2.1X50 mm, 3 µm, Waters, Baden-Dättwil, Switzerland). The injection volume was 10 µL and total run time was 2.5 min. The following gradient was applied: from 0 to 0.5 min 100% solvent A (0.1% formic acid in water), from 0.5 to 2 min the proportion of

solvent B (0.1% formic acid in methanol) linearly increased from 50 to 70%, at 2.01 the percentage of B was increased to 95% and maintained for 0.5 min. At 2.5 min the initial conditions were re-established. The flow rate was 0.8 mL/min, the column was placed in a column oven at 60°C and the samples were stored in the autosampler at 10°C.

The LC system was interfaced with a triple quadrupole mass spectrometer (API4000, AB/MDS Sciex, Concord, Canada) equipped with an ESI source. Positive ESI was applied for the first 1.8 min (ion spray voltage 5500 eV). Polarity was then switched to negative mode (ion spray voltage -4500 eV) for the detection of EFA, OH EFA and 8,14 di OHEFA. The probe temperature was 450°C.

The selected mass-to-charge (m/z) ratio transitions of the analytes and internal standards used in selective reaction monitoring mode were already reported in Table 2. The MRM transitions added to the present method are reported in Table 3.

Table 3. MRM transitions additionally monitored in the Basel cocktail II analytical method

Analyte	Precursor (m/z)	Product (m/z)	Ionization mode
Ciprofloxacin	332	288	ESI+
Fluconazole	307	238	ESI+
Paroxetine	330	192	ESI+
Rifampicin	823	791	ESI+
8,14-Dihydroxy-efavirenz	346	262	ESI-

Efavirenz probes were reanalyzed using an optimized method to monitor 8,14-dihydroxy-efavirenz. The same gradient was applied but only the negative mode transitions were monitored.

5.3.Results

Two bioanalytical methods were used for the analysis of the samples collected in two Basel cocktail studies. The second method was developed to monitor the concentrations of the compounds used for inhibition and induction as well as an additional efavirenz metabolite.

5.3.1. Performance of the analytical method used to quantify plasma, blood, saliva and DBS samples of study I

The first LC-MS/MS method, BC-LCMS-01, was developed and partially validated for the quantification of metoprolol, OH-metoprolol, caffeine, paraxanthine, omeprazole, 5-OH-omeprazole, midazolam, 1'-OH-midazolam, losartan, E-3174, efavirenz, and 8-OH-efavirenz.

A representative chromatogram is reported in Figure 14.

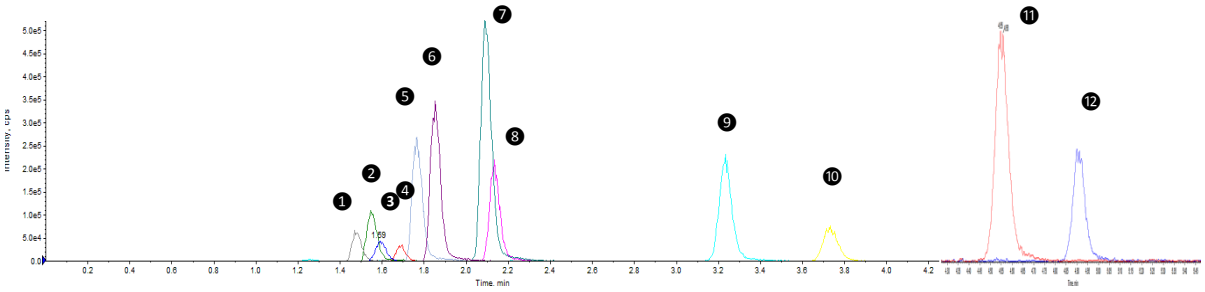

Figure 14. Representative chromatogram of a calibration sample containing 1000 ng/mL of each analyte in human plasma. OH-metoprolol (1), Metoprolol (2), Paraxanthine (3), Caffeine (4), Midazolam (5), 5-OH-omeprazole (6), Omeprazole (7), 1'-OH-midazolam (8), Losartan (9), E-3174 (10), 8-OH-efavirenz (11), Efavirenz (12).

The lower limits of quantification (LLOQ) achieved in plasma, saliva, blood and DBS are reported in Table 4.

Table 4. LLOQ in plasma, saliva, blood, and DBS (Basel cocktail study I).

Analyte	LLOQ plasma (ng/mL)	LLOQ saliva (ng/mL)	LLOQ blood (ng/mL)	LLOQ DBS (ng/mL)
Metoprolol	1	0.5	0.5	2.5
OH-metoprolol	0.5	2.5	1	25
Omeprazole	0.5	0.5	0.5	1
5-OH-omeprazole	0.5	0.5	0.5	5
Losartan	1	0.5	1	10
E3174	1	0.5	1	10
Midazolam	0.5	0.5	0.5	2.5
1'-OH-midazolam	0.5	0.5	0.5	5
Caffeine	10	5	5	25
Paraxanthine	2.5	2.5	2.5	25
Efavirenz	1	2.5	2.5	10
8-OH-efavirenz	1	2.5	2.5	25

The performance of the method was monitored using quality control (QC) samples at low, medium and high concentrations.

In plasma, inter-assay accuracy (determined as the percent bias) for QC samples ranged from -4.7 to 11.6 and inter-assay precision (determined as the relative standard deviation) was lower than 10.3 for all the analytes (Table 5).

Table 5. Inter-assay accuracy and precision for QC plasma samples (Basel cocktail study I).

nominal concentrations		QC1 = 5 ng/mL	QC2 = 50 ng/mL	QC3 = 500 ng/mL
Metoprolol	Overall Mean (n)	5.58 (18)	49.9 (21)	494 (21)
	Accuracy (Bias%)	11.6	-0.26	-1.25
	Precision (CV %)	2.09	4.00	3.69
OH-metoprolol	Overall Mean (n)	5.15 (18)	49.8 (21)	476 (21)
	Accuracy (Bias%)	2.94	-0.37	-4.68
	Precision (CV %)	7.53	7.57	6.17
Omeprazole	Overall Mean (n)	5.11 (21)	50.4 (21)	484 (21)
	Accuracy (Bias%)	2.17	0.881	-3.30
	Precision (CV %)	2.44	2.38	1.83
5-OH-omeprazole	Overall Mean (n)	4.97 (21)	49.7 (21)	499 (21)
	Accuracy (Bias%)	-0.686	-0.719	-0.133
	Precision (CV %)	4.48	3.38	2.36
Losartan	Overall Mean (n)	5.05 (21)	50.1 (21)	493 (21)
	Accuracy (Bias%)	0.910	0.324	-1.33
	Precision (CV %)	4.39	2.11	2.26
E-3174	Overall Mean (n)	4.97 (21)	50 (21)	494 (21)
	Accuracy (Bias%)	-0.624	-0.100	-1.30
	Precision (CV %)	5.60	3.52	2.94
Midazolam	Overall Mean (n)	4.9 (21)	49.3 (21)	494 (21)
	Accuracy (Bias%)	-2.02	-1.38	-1.20
	Precision (CV %)	3.70	3.92	3.08
1'-OH-midazolam	Overall Mean (n)	4.82 (21)	48.3 (21)	498 (21)
	Accuracy (Bias%)	-3.68	-3.44	-0.457
	Precision (CV %)	5.79	4.38	3.90
Caffeine	Overall Mean (n)	not measured	51.8 (21)	500 (21)
	Accuracy (Bias%)		3.68	-0.062
	Precision (CV %)		5.17	2.10
Paraxanthine	Overall Mean (n)	5.12 (19)	50.4 (21)	496 (21)
	Accuracy (Bias%)	2.41	0.819	-0.762
	Precision (CV %)	8.52	3.85	2.51
Efavirenz	Overall Mean (n)	4.94 (19)	51.3 (21)	501 (21)
	Accuracy (Bias%)	-1.17	2.74	0.167
	Precision (CV %)	10.30	5.08	2.67
8-OH-efavirenz	Overall Mean (n)	4.89 (21)	50.1 (21)	486 (21)
	Accuracy (Bias%)	-2.16	0.229	-2.78
	Precision (CV %)	7.46	4.00	3.40

In saliva, inter-assay accuracy (determined as the percent bias) for QC samples ranged from -6.81 to 10.4 and inter-assay precision (determined as the relative standard deviation) was lower than 7.52 for all the analytes (Table 6).

Table 6. Inter-assay accuracy and precision for QC saliva samples (Basel cocktail study I).

nominal concentrations		QC1 = 5 ng/mL	QC2 = 50 ng/mL	QC3 = 500 ng/mL
Metoprolol	Overall Mean (n)	4.82(21)	50(21)	511(21)
	Accuracy (Bias%)	-3.5	0.02	2.25
	Precision (CV %)	4.97	1.62	3.82
OH-metoprolol	Overall Mean (n)	4.69(16)	50(21)	552(17)
	Accuracy (Bias%)	-6.18	-0.25	10.4
	Precision (CV %)	7.52	5.65	5.03
Omeprazole	Overall Mean (n)	4.79(21)	50(21)	487(21)
	Accuracy (Bias%)	-4.11	0.11	-2.58
	Precision (CV %)	2.13	1.97	4.03
5-OH-omeprazole	Overall Mean (n)	4.78(21)	50.3(21)	534(20)
	Accuracy (Bias%)	-4.46	0.67	7.22
	Precision (CV %)	3.51	3.10	4.54
Losartan	Overall Mean (n)	4.81(21)	49.1(21)	509(21)
	Accuracy (Bias%)	-3.90	-1.83	1.85
	Precision (CV %)	6.02	2.75	4.88
E-3174	Overall Mean (n)	4.86(21)	50.6(20)	541(21)
	Accuracy (Bias%)	-2.90	1.20	8.11
	Precision (CV %)	4.21	4.31	4.84
Midazolam	Overall Mean (n)	4.76(21)	49.7(21)	513(21)
	Accuracy (Bias%)	-4.76	-0.73	2.69
	Precision (CV %)	4.38	2.13	4.92
1'-OH-midazolam	Overall Mean (n)	4.76(21)	49.5(21)	477(19)
	Accuracy (Bias%)	-4.92	-1.03	-4.60
	Precision (CV %)	5.56	4.29	7.52
Caffeine	Overall Mean (n)	not measured	49.1(21)	514(21)
	Accuracy (Bias%)		-1.86	2.70
	Precision (CV %)		3.87	4.71
Paraxanthine	Overall Mean (n)	not measured	49.6(21)	506(21)
	Accuracy (Bias%)		-0.88	1.17
	Precision (CV %)		3.12	4.53
Efavirenz	Overall Mean (n)	4.99(21)	50.1(21)	524(21)
	Accuracy (Bias%)	-0.08	0.11	4.80
	Precision (CV %)	6.57	7.42	4.95
8-OH-efavirenz	Overall Mean (n)	4.99(20)	50.1(21)	500(21)
	Accuracy (Bias%)	-0.09	0.19	0.00
	Precision (CV %)	6.66	5.91	4.46

In DBS, inter-assay accuracy (determined as the percent bias) for QC samples ranged from -8.5 to 1.0 and inter-assay precision (determined as the relative standard deviation) was lower than 10.6 for omeprazole, 5-OH-omeprazole, caffeine, and paraxanthine. For the other analytes LLOQ was too high to quantify study samples, therefore performance of the method was not reported (Table 7).

Table 7. Inter-assay accuracy and precision for QC DBS samples (Basel cocktail study I).

nominal concentrations		QC1 = 5 ng/mL	QC2 = 50 ng/mL	QC3 = 500 ng/mL
Omeprazole	Overall Mean (n)	4.58(20)	48.4(20)	476(20)
	Accuracy (Bias%)	-8.53	-3.19	-4.76
	Precision (CV %)	7.04	4.38	7.30
5-OH-omeprazole	Overall Mean (n)	5.02(16)	47.8(18)	470(20)
	Accuracy (Bias%)	0.38	-4.43	-5.96
	Precision (CV %)	9.22	8.54	5.14
Caffeine	Overall Mean (n)	not measured	50.5(18)	474(21)
	Accuracy (Bias%)		1.02	-5.32
	Precision (CV %)		10.6	7.60
Paraxanthine	Overall Mean (n)	not measured	48.8(19)	494(21)
	Accuracy (Bias%)		-2.40	-1.29
	Precision (CV %)		10.33	4.88

Clinical validation data for caffeine and paraxanthine 6 hours after administration of a caffeinated drink had already been acquired by Berger et al (Poster 2, page 145). Caffeine and paraxanthine concentration in DBS were highly correlated with venous concentrations. Efavirenz concentrations in DBS were measured using a prototype of an online extraction system and as well after manual preparation. With both methods comparable results were obtained (Poster 3, page 147). Further comparison between caffeine and efavirenz PK profiles in venous and capillary (DBS) was performed for the Basel cocktail study I and results are summarized in Poster 4 (page 149). Overall correlation between concentrations of caffeine and efavirenz in DBS and venous blood was good (R^2 0.881 and 0.908, respectively). During the absorption phase, higher concentrations were observed in DBS compared to venous blood samples, while in the elimination phase (after Tmax) the correlation for both caffeine and efavirenz improved (R^2 0.969 and 0.942, respectively).

5.3.2. Performance of the analytical method used to quantify plasma, blood, saliva and DBS samples of study II

Samples collected in the Basel cocktail study II were analyzed with a different method, BC-LCMS-02. Representative chromatograms are reported in Figure 15, Figure 16, and Figure 17. In addition to the probe drugs and metabolites already analyzed with the previous method, fluconazole, ciprofloxacin, paroxetine, rifampicin, and 8,14-diOH-efavirenz were included.

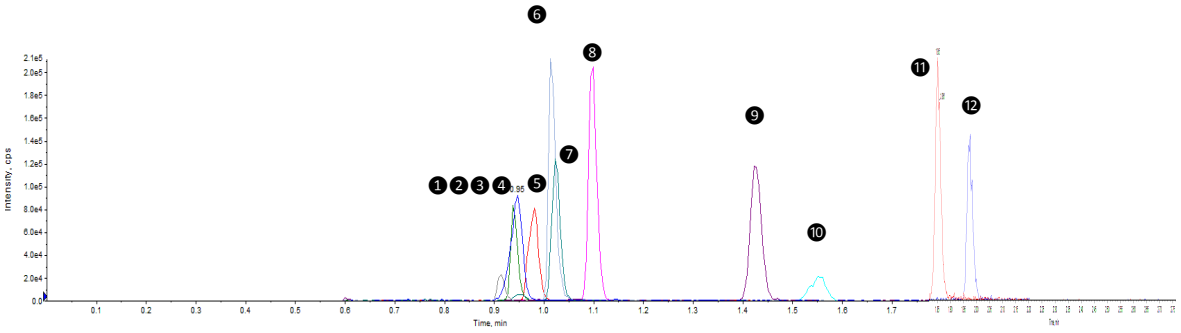

Figure 15. Representative chromatogram of a calibration sample containing 1000 ng/mL of each phenotyping analyte in human plasma. OH-metoprolol (1), Metoprolol (2), Paraxanthine (3), Caffeine (4), Midazolam (5), 5-OH-omeprazole (6), Omeprazole (7), 1'-OH-midazolam (8), Losartan (9), E-3174 (10), 8-OH-efavirenz (11), Efavirenz (12).

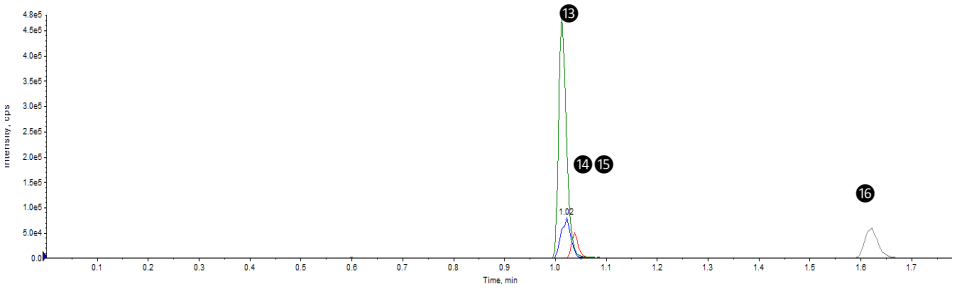

Figure 16. Representative chromatogram of a calibration sample containing 1000 ng/mL of inhibitors and inducer in human plasma. Fluconazole (13), Ciprofloxacin (14), Paroxetine (15), Rifampicin (16). Negative mode MRM window not reported.

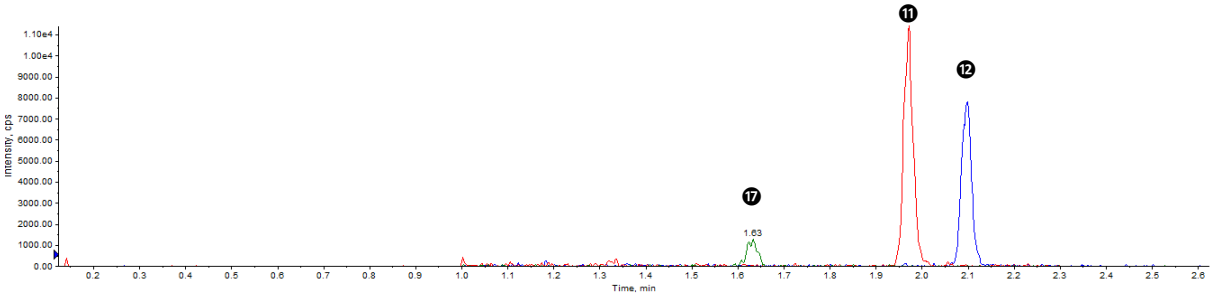

Figure 17. Representative chromatogram of a calibration sample containing 1000 ng/mL of 8,14-diOH-efavirenz (17), 8-OH-efavirenz (11), Efavirenz (12) in human plasma.

The lower limits of quantification (LLOQ) achieved in plasma, saliva, and blood are reported in Table 8.

Table 8. LLOQ in plasma, saliva and blood (Basel cocktail study II).

Analyte	LLOQ plasma (ng/mL)	LLOQ saliva (ng/mL)	LLOQ blood (ng/mL)	LLOQ DBS (ng/mL)
Metoprolol	1	0.5	0.5	1
OH-metoprolol	0.5	2.5	1	1
Omeprazole	0.5	0.5	0.5	1
5-OH-omeprazole	0.5	0.5	0.5	1
Losartan	1	0.5	1	25
E3174	1	0.5	1	25
Midazolam	0.5	0.5	0.5	1.0
1'-OH-midazolam	0.5	0.5	0.5	2.5
Caffeine	10	10	10	50
Paraxanthine	10	10	10	10
Efavirenz	1	0.5	1	5
8-OH-efavirenz	1	0.5	1	25
8,14-$(OH)_2$-efavirenz	0.50			

The performance of the method was monitored using quality control (QC) samples at low, medium and high concentrations.

In plasma, inter-assay accuracy (determined as the percent bias) for QC samples ranged from -11.6 to 10.2 and inter-assay precision (determined as the relative standard deviation) was lower than 10.4 for all the analytes (Table 9).

Table 9. Inter-assay accuracy and precision for QC plasma samples (Basel cocktail study II).

nominal concentrations		QC1 = 5 ng/mL	QC2 = 50 ng/mL	QC3 = 500 ng/mL
Metoprolol	Overall Mean (n)	5.51 (9)	50.9 (9)	454 (9)
	Accuracy (Bias%)	10.2	1.74	-9.11
	Precision (CV %)	3.55	3.19	3.70
OH-metoprolol	Overall Mean (n)	5.36(8)	49.9 (9)	488 (9)
	Accuracy (Bias%)	7.10	-0.76	-2.36
	Precision (CV %)	8.49	8.67	5.42
Omeprazole	Overall Mean (n)	5.06 (9)	50.6 (9)	457 (9)
	Accuracy (Bias%)	1.36	1.02	-8.62
	Precision (CV %)	4.84	3.15	2.48
5-OH-omeprazole	Overall Mean (n)	5.05 (9)	50.3 (9)	442 (9)
	Accuracy (Bias%)	0.860	0.670	-11.6
	Precision (CV %)	5.20	5.33	3.28
Losartan	Overall Mean (n)	5.24 (9)	51.4 (9)	not measured
	Accuracy (Bias%)	4.80	6.68	
	Precision (CV %)	8.10	6.68	
E-3174	Overall Mean (n)	5.28 (9)	52.4 (9)	460 (9)
	Accuracy (Bias%)	5.57	4.76	-8.04
	Precision (CV %)	8.24	5.70	4.58
Midazolam	Overall Mean (n)	5.41 (6)	49.9 (9)	not measured
	Accuracy (Bias%)	8.12	-0.18	
	Precision (CV %)	6.20	6.98	
1'-OH-midazolam	Overall Mean (n)	5.11 (8)	49.3 (8)	486 (9)
	Accuracy (Bias%)	2.16	-1.49	-2.90
	Precision (CV %)	9.11	4.29	2.64
Caffeine	Overall Mean (n)	not measured	50.8(9)	506(9)
	Accuracy (Bias%)		1.64	1.28
	Precision (CV %)		10.4	6.22
Paraxanthine	Overall Mean (n)	not measured	50.7 (9)	505 (9)
	Accuracy (Bias%)		1.37	1.01
	Precision (CV %)		8.27	5.06
Efavirenz	Overall Mean (n)	5.05 (9)	48.6 (9)	480 (9)
	Accuracy (Bias%)	0.88	-2.86	-3.99
	Precision (CV %)	5.59	2.66	1.89
8-OH-efavirenz	Overall Mean (n)	5.31 (9)	50.0 (9)	445 (9)
	Accuracy (Bias%)	6.10	0	-10.9
	Precision (CV %)	4.58	5.00	2.35

In saliva, inter-assay accuracy (determined as the percent bias) for QC samples ranged from -7.3 to 10.2 and inter-assay precision (determined as the relative standard deviation) was lower than 12.1 for all the analytes (Table 10).

Table 10. Inter-assay accuracy and precision for QC saliva samples (Basel cocktail study II).

nominal concentrations		QC1 = 5 ng/mL	QC2 = 50 ng/mL	QC3 = 500 ng/mL
Metoprolol	Overall Mean (n)	5.52(9)	51.6(9)	not measured
	Accuracy (Bias%)	10.2	3.30	
	Precision (CV %)	3.15	3.75	
OH-metoprolol	Overall Mean (n)	4.99(8)	51.2(9)	not measured
	Accuracy (Bias%)	-0.3	2.56	
	Precision (CV %)	11.4	7.32	
Omeprazole	Overall Mean (n)	5.37(9)	50.2(9)	not measured
	Accuracy (Bias%)	7.22	0.4	
	Precision (CV %)	4.81	3.34	
5-OH-omeprazole	Overall Mean (n)	5.38(9)	51.3(9)	not measured
	Accuracy (Bias%)	7.63	2.52	
	Precision (CV %)	5.14	4.96	
Losartan	Overall Mean (n)	5.27(8)	50.8(9)	not measured
	Accuracy (Bias%)	5.39	1.70	
	Precision (CV %)	4.82	3.45	
E-3174	Overall Mean (n)	5.47(9)	51.2(9)	not measured
	Accuracy (Bias%)	9.38	2.33	
	Precision (CV %)	4.60	2.76	
Midazolam	Overall Mean (n)	5.44(9)	52.6(9)	not measured
	Accuracy (Bias%)	8.78	5.13	
	Precision (CV %)	3.55	5.40	
1'-OH-midazolam	Overall Mean (n)	5.29(7)	51.1(8)	not measured
	Accuracy (Bias%)	5.86	2.29	
	Precision (CV %)	4.45	6.50	
Caffeine	Overall Mean (n)	not measured	53.7(9)	467(9)
	Accuracy (Bias%)		7.56	-6.58
	Precision (CV %)		3.27	6.25
Paraxanthine	Overall Mean (n)	not measured	52.9 (9)	463(9)
	Accuracy (Bias%)		5.67	-7.30
	Precision (CV %)		3.16	6.93
Efavirenz	Overall Mean (n)	5.35(9)	52.9(9)	478(9)
	Accuracy (Bias%)	6.89	5.91	-4.33
	Precision (CV %)	6.86	5.12	12.1
8-OH-efavirenz	Overall Mean (n)	5.49(8)	53.3(9)	not measured
	Accuracy (Bias%)	9.88	6.57	
	Precision (CV %)	2.37	4.70	

Rifampicin, fluconazole, ciprofloxacin and paroxetine were measured to verify treatment adherence and to estimate the extent of exposure. Accuracy and precision were not evaluated.

5.4.Discussion

The substances used in the Basel Cocktail have quite diverse physical and chemical properties. Our aim was to quantify the parent compound and the phase I metabolites expected to be more hydrophilic than the parent compound with a single method. The selection of the column chemistry was mainly based on LogP values of the analytes that had to be analyzed. A LogP value above 0 indicates a more hydrophobic molecule, usually better retained and separated by a reversed phase (RP) chromatography column. LogP values below 0 indicate a more hydrophilic analyte that will be better analyzed using a column with hydrophilic interaction liquid chromatography (HILIC) chemistry. Paraxanthine and caffeine have LogP values of -0.94 and -0.13 respectively, while efavirenz at the other end of the spectrum has a LogP of 3.03. To retain and separate a mixture characterized by such a broad polarity range, several RP column chemistries (C8, C18, and pentafluorophenyl) were tested. A C18 column with special affinity for hydrophilic compounds, the Atlantis T3 column, exhibited the best separation performance for the components of the Basel cocktail and the metabolites generated in vivo.

To improve sample clean up and to increase the amount of sample injected and consequently the sensitivity of the analytical method, we explored the application of online solid phase extraction (SPE). Several trapping columns were tested (Oasis HLB, Clipeus, Halo EXP). The most promising results were observed with the Halo EXP trap. Very hydrophilic compounds such as metoprolol and OH-metoprolol were retained by the trapping column, while efavirenz and OH-efavirenz were not retained.

For the analysis of DBS, online extraction with online dilution using a 20 µL mixing chamber to improve sample and mobile phase mixing was evaluated. Sensitivity slightly increased but only for a few of the analytes. Therefore online extraction was abandoned and the method was optimized and validated using the Atlantis T3 analytical column. To increase sample throughput, analysis time was considerably reduced from 5.5 min in BC-LCMS-01 to 2.5 min in BC-LCMS-02 by increasing mobile phase flow and column oven temperature (Table 11).

Table 11. Comparison between relevant parameters in analytical method BC-LCMS-01 and BC-LCMS-02.

Analytical method	Flow (mL/min)	T (°C)	Total run time (min)	N° of samples analyzed
BC-LCMS-01	0.35	40	5.5	circa 9000
BC-LCMS-02	0.8	60	2.5	circa 4300

Over the past few years, there has been increasing interest in using very high pressure conditions (namely UHPLC, for ultra-high pressure liquid chromatography) with columns packed with porous sub-2µm particles. This approach increases the resolving power and speeds up the analytical separation process, therefore the transfer of the HPLC method to UHPLC conditions was also evaluated. Although Waters, the supplier of the Atlantis T3 column, claims the HSS T3 to be an equivalent UHPLC column, our tests of UHPLC conditions were not successful for this application. The hydrophilic compounds paraxanthine and OH-metoprolol for example were not retained by the HSS T3 column under UHPLC conditions.

All the analytes with the exception of efavirenz and its metabolites were detected in positive ion mode. E-3174, detected in positive mode, was eluted shortly before OH-efavirenz and efavirenz, the only two analytes detected in negative mode. Fast polarity switching is one of the options of the API4000 that could have been used for these analyses. The disadvantage is that for each cycle a pause time is necessary between the positive and negative mode detection, resulting in a lower sensitivity. This kind of approach is necessary when the components detectable with different polarity co-elute. For this application, compounds detected in positive mode were separated by analytes detectable in negative mode. We therefore used within the same method a positive MRM window followed by a negative MRM window. This implied a fine tuning of the switching time. Just after the elution of the E3174 peak, polarity was switched to negative and efavirenz and its metabolites were acquired.

Due to the low absolute amount of analyte contained in a DBS, sensitivity of the DBS method was the limiting factor for using DBS instead of venous blood sampling. Therefore, clinical validation of DBS with the Basel cocktail study I samples could only be performed for caffeine and efavirenz. The high LLOQ, particularly for losartan and E-3174, did not allow quantification of these analytes in DBS and the comparison with venous blood concentration was not possible. Another limitation of using DBS is the extensive sample work-up requiring manual punching, transfer of the DBS to a test tube and offline extraction of the paper disc with a suitable solvent. A set of operations that for 96 samples took 2 to 3 hours. An automated online extraction system, the CAMAG DBS-MS 500 was tested for analysis of efavirenz (Poster 3, page 147

Poster 3) and a more extensive validation was performed for the analysis of TKIs in DBS (Poster 6, page 153). Use of such automated systems reduces manual workload and increases sample throughput. While chromatographic separation is performed, the DBS-MS 500 prepares for the extraction of the subsequent sample, minimizing the delay between sample analyses. On-line extraction takes 2 minutes per sample and can be performed in parallel with the chromatographic separation. However such systems are not yet available in clinical routine and were also not available long enough to analyze the samples from the two Basel cocktail studies.

5.5.Conclusions

For the development of the new phenotyping cocktail fast, sensitive and robust bioanalytical methods were required. Both developed methods proofed to be valuable tools, capable of analyzing thousands of study samples in diverse matrices from the two Basel cocktail studies and the combi-pill pilot study. Both methods fulfilled requirements of accuracy, precision and robustness. The second method was faster, allowing shorter return times if phenotyping is used in clinical applications. DBS as a less invasive sampling method is currently only feasible for selected analytes. Sensitivity needs considerable improvements and the observed difference between capillary and venous concentrations during absorption phase should be further investigated. A more efficient and automated extraction system such as the CAMAG DBS-MS 500 autosampler will allow a reduction of the manual workload and speed up sample processing times.

6. The Basel cocktail for simultaneous phenotyping of human cytochrome P450 isoforms in plasma, saliva and dried blood spots

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6.1. Introduction

The advantages of the cocktail approach have already been mentioned in the introduction section and are summarized in brief here. Phenotyping cocktails enable the simultaneous characterization of cytochrome P450 enzymes using a combination of specific substrates. Many phenotyping cocktails have already been published and are used to investigate drug interactions in early clinical drug development. The cocktail approach could also be applied to predict drug exposure of a patient before administering a medication personalizing the therapy and avoiding exposure to potential risk, particularly when the treatment consists of a drug with narrow therapeutic range.

None of the published cocktails have so far gained sufficient acceptance to be used as a tool to phenotype patients in clinical practice. Currently available cocktails have several limitations. Some of the probe drugs used in many of these cocktails, such as tolbutamide, mephenytoin, or debrisoquine are no longer available as licensed drugs in most countries [18]. Another limitation is complicated sampling procedures, requiring collection of multiple plasma samples at appropriate timepoints for specific CYP isoforms or for the determination of AUCs. Insufficiently validated phenotyping metrics (e.g. dapsone for CYP3A4) and/or complicated bioanalytical procedures (e.g. off-line sample purification, multiple methods for different analytes or biological matrices) are further limitations.

To improve clinical applicability of CYP phenotyping, our primary objective was to develop a new cocktail (Basel cocktail) based on probe drugs that are widely used in clinical practice and therefore easily available. To minimize probe drug exposure and adverse effects, the lowest commercially available doses were chosen. A secondary objective was to test whether alternative minimally or non-invasive sampling methods (dried blood spots or saliva samples) could be used to simplify the sampling process.

6.2.Material and Methods

6.2.1. Clinical study

All subjects signed informed consent prior to any study-mandated procedure. Screening consisted of medical history, physical examination, including heart rate, systolic and diastolic blood pressure, standard 12-lead electrocardiogram, routine blood tests and urine drug screen. Exclusion criteria included any clinically relevant abnormality identified at the physical examination or laboratory screening, positive results from urine drug test (amphetamines/methamphetamines, cocaine, opiates, phencyclidine, tetrahydrocannabinol, acetaminophen, barbiturates, benzodiazepines, methadone, propoxyphene and tricyclic antidepressants), smoking within the last 3 months prior to screening, history or clinical evidence of alcoholism or drug abuse within the 3-year period prior to screening, consumption of alcohol within 14 days before the first drug administration, consumption of more than 800 mg caffeine daily, previous treatment with any prescribed or over the counter (OTC) medications within 2 weeks prior to screening, treatment with another investigational drug within 30 days prior to screening.

A single-center, randomized, five-way crossover study was performed at the Phase I Research Center, University Hospital Basel, Basel, Switzerland (ClinicalTrials.gov Identifier: NCT01187862).

6.2.2. Experimental procedures

Each subject first received the treatments A, B, C and D in a randomized order: 4 subjects received the sequence ABCD, 4 subjects the sequence BCDA, 4 subjects the sequence CDAB, and 4 subjects the sequence DABC. The allocation to one of the groups was drawn by lot. Single doses of 12.5 mg losartan (Cosaar®) for CYP2C9, 10 mg omeprazole (Antramups®) for CYP2C19, 2 mg midazolam (Midazolam oral solution 2mg/ml) for CYP3A4, 100 mg caffeine (Coffeinum® N 0.2) for CYP1A2, 12.5 mg metoprolol (Belok ZOK®) for CYP2D6, 125 mg chlorzoxazone (Paraflex®) for CYP2E1and 50 mg efavirenz (Stocrin®) for CYP2B6 were administered in different combinations:

Treatment A:	Losartan-Omeprazole-Midazolam
Treatment B:	Caffeine-Metoprolol-Chlorzoxazone
Treatment C:	Losartan-Omeprazole-Midazolam-Caffeine-Metoprolol-Chlorzoxazone (A+B)
Treatment D:	Efavirenz
Treatment E:	Losartan-Omeprazole-Midazolam-Caffeine-Metoprolol-Efavirenz (Basel Cocktail)

After the first 10 subjects had completed treatments A and C, an ad interim analysis was performed to evaluate a possible interaction of chlorzoxazone on probe drugs of treatment A. Interactions between chlorzoxazone and midazolam were observed and therefore chlorzoxazone was excluded from the final cocktail (Treatment E).

On study days, the subjects remained fasted from at least 10 hours prior to and up to 4 hours after study drug intake. No food was allowed for at least 4 hours postdose. Water was allowed as desired except for one hour before and after drug administration. The drugs were administered with 240 ml of tap water. On the day of drug administration, subjects received standardized meals: lunch approximately 4 hours after drug administration (after blood sampling), and snack approximately 8 hours after drug administration. Drinking of alcoholic beverages or xanthine-containing food or beverages was not permitted during the time in the clinic. No concomitant medications were allowed, except for the treatment of adverse events (AE). Use of herbal medicines, OTC medications or food products known to be inducers or inhibitors of CYP450 (e.g. grapefruit juice) was forbidden during the entire study. Consumption of alcoholic beverages was not allowed for 2 weeks prior to first dosing until after the end of the study to avoid induction of CYP2E1. Caffeine-containing beverages were not allowed for 48h prior to dosing until after the last sampling time-point of the study period. The washout time between different study periods was 14 days.

6.2.3. Pharmacokinetic sampling

Blood samples for pharmacokinetic analysis were taken before and 0.25, 0.50, 0.75, 1, 2, 3, 4, 6, 8, 12, 24, 48, and 72 h after drug administration (72 h only treatment D and E). About 2.7 ml blood were collected by direct venous puncture (for 24, 48 and 72 h samples) or via an i.v. catheter placed in an antecubital vein in the arm in Monovette® tubes containing EDTA. The indwelling catheter was inserted in the arm at a maximum of 1 hour before the start of blood sampling. In order to keep the catheter patent, 0.9% sodium chloride i.v. drip at a very slow rate, i.e. one drop every 2 to 3 seconds was infused. To avoid any dilution artifacts the i.v. drip was stopped and 1-2 ml of blood was drawn through the catheter and wasted prior to collection of the blood sample. Immediately following collection of the required blood volume, the Monovettes® were slowly tilted backwards and forwards (no shaking) to bring the anti-coagulant into solution, and immediately cooled on ice. Within 30 minutes of collection, an aliquot of 1 ml of blood was transferred in a labeled tube. The Monovettes® containing the remaining blood were centrifuged at approximately 1’500 g for 10 minutes at 4°C. The plasma was transferred into one labeled polypropylene tube to avoid carry-over of erythrocytes. All samples were stored in an upright position at -80°C.

Dried blood spot (DBS) and oral fluid samples were collected before dosing and 0.5, 1, 2, 4, 8, 12 and 24 h (plus 48 and 72h for treatments containing efavirenz) after study drug administration. DBS samples were collected via capillary puncture of the fingertip using a finger pricker (Accu-Check Softclix Pro, Roche, Basel, Switzerland). A drop of blood (approximately 20 µL) was transferred on filter paper (Schleicher & Schuell GmbH ,Dassel, Germany). After drying for at least 2 h at room temperature the DBS samples were stored at -80°C until analysis. Oral fluid (5 mL) was collected in polypropylene tubes. To avoid contamination, subjects were asked to rinse their mouth before sampling. After centrifugation (1500 g for 10 min at 4°C) the supernatant was stored at -80°C until analysis.

6.2.4. Pharmacodynamic measurements and adverse events

Pharmacodynamic assessments (heart rate, blood pressure, sedation score) were performed at baseline (up to 30 min before drug intake) and 1, 2, 4, 6, and 12 h after drug administration. Subjects rated their level of sedation on a visual analog scale (VAS) ranging from 0, “not tired” to 10, “very tired”.

All AEs, regardless of severity or relationship to the study drug were recorded.

6.2.5. Study drugs

The following study drugs were purchased through the University Hospital Pharmacy, Basel, Switzerland:

- Caffeine (Coffeinum N 0.2g, Mylan Dura GmbH, Darmstadt, Germany)
- Efavirenz (Stocrin®, Merck Sharp & Dohme-Chibret AG, Opfikon, Switzerland)
- Losartan (Cosaar®, Merck Sharp & Dohme-Chibret AG, Opfikon, Switzerland)
- Metoprolol (Belok ZOK®, AstraZeneca AG, Zug, Switzerland)
- Omeprazole (Antramups®, AstraZeneca AG, Zug, Switzerland)
- Chlorzoxazone (Paraflex®, BioPhausia AB, Stockholm, Sweden)
- Midazolam (midazolam oral solution, 2 mg/ml, in-house formulation University Hospital Pharmacy, Basel, Switzerland)

6.2.6. Bioanalytical analysis

The bioanalytical method used for the analysis of the plasma, saliva, blood and DBS samples is described in chapter 5.

6.2.7. Data Analysis

Area under concentration time curve from time zero to 24 hours (AUC) after dosing and half-life were estimated using non-compartmental methods, maximal concentrations (C_{max}) and time to reach C_{max} (t_{max}) were directly taken from observed data. AUC was estimated using the linear trapezoidal method. Bioequivalence (BE) tests were performed with a linear mixed effects model using WinNonlin (Pharsight, Mountain View, CA). Results are represented as geometric mean and 90% confidence intervals. To determine the appropriate BE acceptance limits, within-subject coefficients of variation (CV%) for the AUCs were calculated using the root mean square approach. For not highly variable drugs with a within-subject CV% below 30%, the BE acceptance limits of 0.8 and 1.25 had to be met by the point estimate and both 90% confidence intervals. After the first four treatment sequences (treatments A to D) a preplanned interim analysis was performed to detect a possible interaction of chlorzoxazone with CYP3A4. A bioequivalence test was performed using the midazolam AUCs of treatment A (no interaction) and treatment C (midazolam in combination with chlorzoxazone). To conclude absence of a relevant interaction, point estimate and 90% confidence intervals were required to fall completely within the BE limits stated above. Correlations between single time point concentration ratios and AUC ratios were tested using linear regression analysis. Concentration ratios of different genotype groups were compared using the nonparametric Kruskal-Wallis test.

6.2.8. Genotype analysis

DNA was isolated from 400 µL EDTA-blood on an m2000sp instrument (Abbott Molecular, De Plaines, IL, USA) following the manufacturer's protocol. Final elution volume was 100 microliter. Specific single nucleotide polymorphisms in the genes encoding CYP1A2 (alleles *1A, *1F), CYP2B6 (allele *6), CYP2C9

(alleles *2, *3) and CYP2C19 (alleles *1, *2, *3) were detected using primer / probe real time PCR reagent (Lightmix, TIB MOLBIOL, Berlin, Germany) on a Lightcycler 1.5 instrument (Roche Diagnostics, Rotkreuz, Switzerland). For detection of CYP2C19 (*2, *3, *4, *6, *7, *8, *9, *10, *17), CYP2D6 (*2, *3, *4, *5, *6, *7, *8, *9, *10, *12, *14, *17, *28, *41, *XN) and CYP3A4 (alleles *1B, *2, *3, *12, *17) hybridization of amplified products was performed using the Infiniti chip technology (Autogenomics, Carlsbad, USA) according to the manufacturer's instructions.

6.3.Results

6.3.1. Pharmacodynamics and adverse events

Sixteen healthy male volunteers (mean age 26 years, range 18-45 years, mean BMI 23.4 kg/m2, range 20.6-26.3 kg/m^2) completed the study according to the protocol. All subjects were with no history of relevant disease and no history of drug or substance abuse. All study subjects were of Caucasian ethnicity, with normal findings on physical exam, screening laboratory (including drugs of abuse screen), and ECG. Simultaneous administration of all probe drugs was well tolerated. No clinically significant adverse effects and no relevant changes in heart rate, blood pressure or sedation score (Figure 18) were observed.

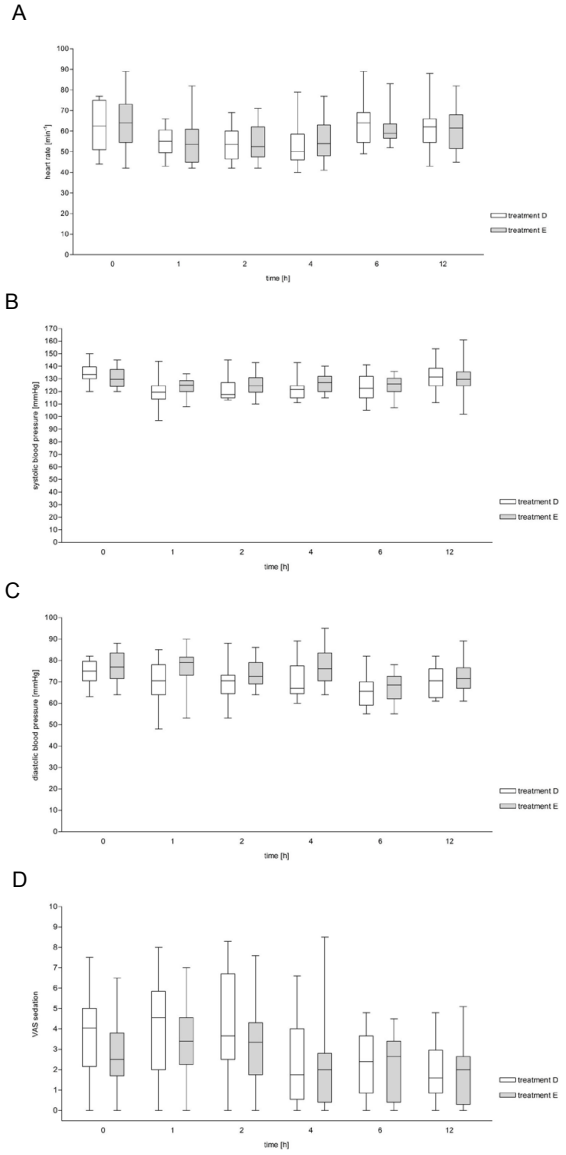


Figure 18. Changes of heart rate (Figure 18A), systolic and diastolic blood pressure (Figure 18 B and C) and sedation scores (Figure 18D) were compared after administration of efavirenz (treatment D, white boxes) and after administration of the final 6-drug cocktail (treatment E, grey boxes). Treatment D was chosen as "negative control", as there are no relevant effects of efavirenz on heart rate, blood pressure or alertness at the low dose of 50mg used in this study. The whiskers represent the range with minimum and maximum. The boxes represent the 25.-75. percentile and the median.

6.3.2. Pharmacokinetic interactions and bioequivalence testing

Bioequivalence tests based on full pharmacokinetic profiles of the probe drugs were performed to test for mutual interactions. In a pre-planned interim analysis, chlorzoxazone, the probe drug for CYP2E1, was found to cause a significant increase of midazolam AUC (geometric mean AUC ratio 1.77, 90%CI 1.57-1.99, Figure 19) and had to be excluded from the final cocktail.

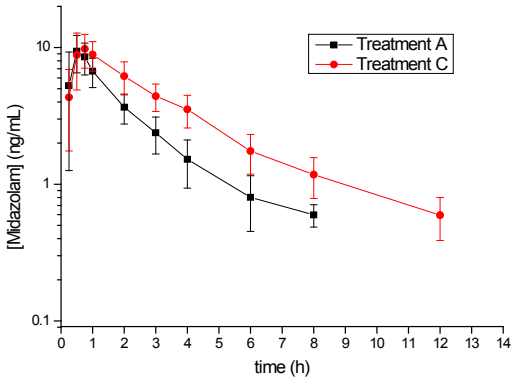


Figure 19. Comparison of plasma concentration time-profile of midazolam obtained after application in a safe 3-drug combination (together with two other probe drugs with documented lack of interaction, treatment A) and in a combination containing chlorzoxazone (treatment C)

For the remaining six probe drugs of the final cocktail there was no evidence for pharmacokinetic interactions (Figure 20). Pharmacokinetic profiles of the probe drugs in the final six drug combination were almost identical with profiles obtained when the probe drug of interest was administered in combination with two other, not-interacting probe drugs (“safe” 3-drug combination, see methods section for further details).

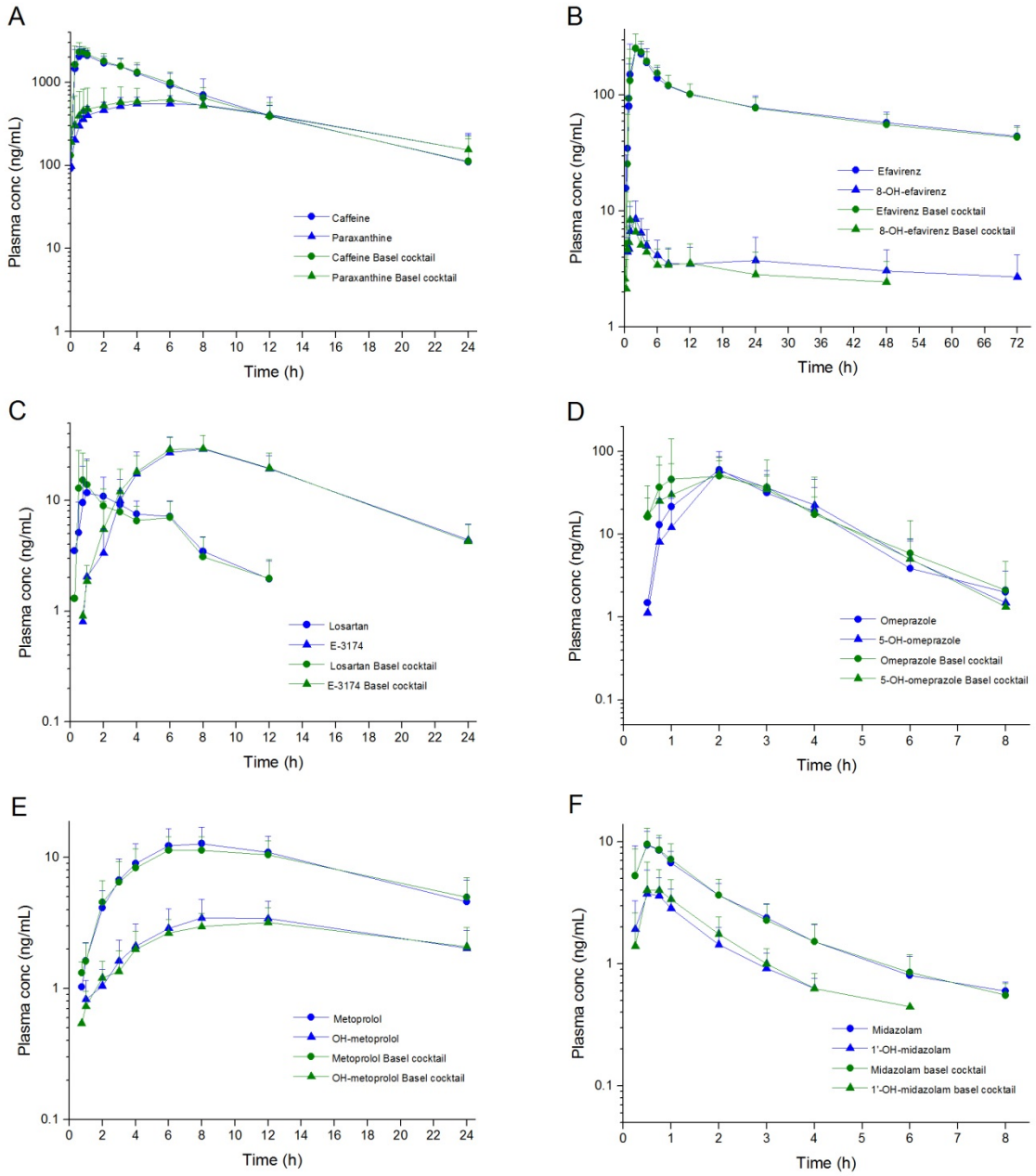


Figure 20. Comparison of plasma concentration time-profiles of cocktail probe drugs obtained after application in a safe 3-drug combination (together with two other probe drugs with documented lack of interaction) and in the final 6-drug cocktail. Concentration-time profiles are shown for parent drugs and corresponding metabolites for CYP1A2 (A), CYP2B6 (B), CYP2C9 (C), CYP2C19 (D), CYP2D6 (E) and CYP3A4 (F). For all concentration-time profiles no significant difference between application in the 3-drug combination and in the final 6-drug cocktail is observed.

All within-subject coefficients of variation (CV%) for the AUCs were below 30%, hence the bioequivalence (BE) acceptance limits were set to 0.8 and 1.25. The point estimates and the 90% confidence intervals for the AUC ratios (AUC of probe drug in final cocktail vs. AUC of probe drug in combination with two known, non-interfering probe drugs) were all within the chosen BE acceptance limits (Table 12 and Figure 21).

Table 12. Geometric mean and 90% confidence interval for AUC ratios of probe drugs in final 6-drug cocktail compared to 3-drug cocktail

Cytochrome	Probe drug	n	geometric mean AUC ratio	90% CI lower	90% CI upper	within subject CV%
CYP1A2	caffeine	16	1.04	0.94	1.15	15.5
CYP2B6	efavirenz	16	1.01	0.96	1.05	6.9
CYP2C9	losartan	16	0.99	0.90	1.10	13.7
CYP2C19	omeprazole	16	1.06	0.92	1.23	24.5
CYP2D6	metoprolol	16	0.97	0.89	1.05	12.6
CYP3A4	midazolam	16	0.98	0.89	1.07	14.4

AUC, area under the curve; CI, confidence interval; CV, coefficient of variation; 3-drug cocktail, probe drug of interest combined with two other, non-interacting probe drugs

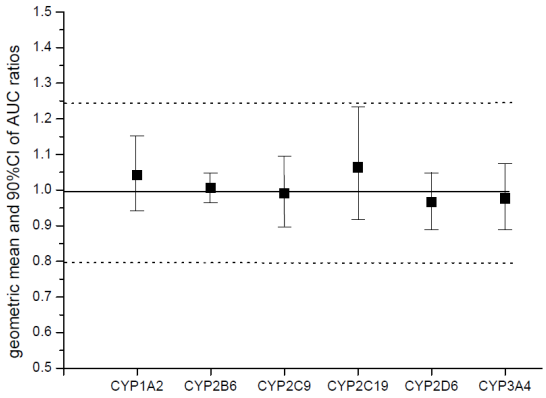


Figure 21. Point estimate and 90% confidence interval of geometric mean of AUC ratios of probe drug in final 6-drug cocktail compared to 3-drug cocktail (probe drug combined with two other, non-interfering probe drugs, e.g. for CYP3A4 midazolam combined with omeprazole and losartan). Dotted lines represent bioequivalence acceptance limits of 0.8 and 1.25.

6.3.3. Single sampling for phenotyping applications

In a next step, limited sampling procedures were evaluated. Parent drug concentrations measured at a single time-point during the first 12 hours after dosing were correlated with full pharmacokinetic profiles of the parent drug (AUC_{parent}).

For all final cocktail probe drugs, correlations of single point parent concentrations with the respective AUC_{parent} were higher for samples taken during the elimination phase compared to earlier time-points (Table 13). Since CYP activity is not only reflected in changes of the parent, but also of the metabolite concentration, we also tested the correlation of parent drug to metabolite concentration ratios (metabolic ratio) measured at a single time-point with the corresponding AUC ratios (i.e. AUC_{parent} to $AUC_{metabolite}$).

Table 13. Correlation of single point measurements with AUC in plasma

		coefficient of determination (R^2)					
	time (h)	**CYP1A2**	**CYP2B6**	**CYP2C9**	**CYP2C19**	**CYP2D6**	**CYP3A4**
parent concentration vs AUC	1						0.886
	2				0.290		0.910
	3				0.577		0.955
	4	0.863	0.269	0.431	0.590	0.527	0.929
	6	0.949	0.484	0.620		0.771	
	8	0.865	0.591	0.681		0.829	
	12	0.915	0.863			0.867	
Parent/metabolite concentration ratio vs $AUC_{parent}/AUC_{metabolite}$ ratio	1						0.944
	2				**0.892**		**0.959**
	3				0.903		0.950
	4	0.844	0.817	0.197	0.936	0.888	0.894
	6	0.927	0.897	0.409		0.904	
	8	**0.910**	**0.888**	**0.592**		**0.808**	
	12	0.967	0.954			0.987	

For all cocktail probe drugs, correlations of parent concentrations with AUC were higher for samples taken during the elimination phase compared to earlier time-points. For caffeine and midazolam, correlations between parent concentration and AUC were comparable with correlations between metabolic ratios (i.e. single point parent to metabolite concentration ratio and AUC_{parent} to $AUC_{metabolite}$ ratio). For efavirenz, omeprazole and metoprolol, on the other hand, a better correlation was found between metabolic ratios than with single point parent concentration and AUC_{parent}. Therefore metabolic ratios were used as simplified phenotyping metric for the final cocktail. Due to large pharmacokinetic differences between the probe drugs, two sampling time-points (2h and 8h after dosing) were necessary to obtain sufficiently high correlations between single point concentration ratios and AUC ratios. Correlations of individual single point concentration ratios with the corresponding AUC ratios for the two selected time-points (2h after dosing for omeprazole and midazolam and 8h after dosing for caffeine, efavirenz, losartan and metoprolol) are shown in Figure 22. The coefficient of determination R^2 was >0.8 for all correlations except for losartan with an R^2 of 0.59.

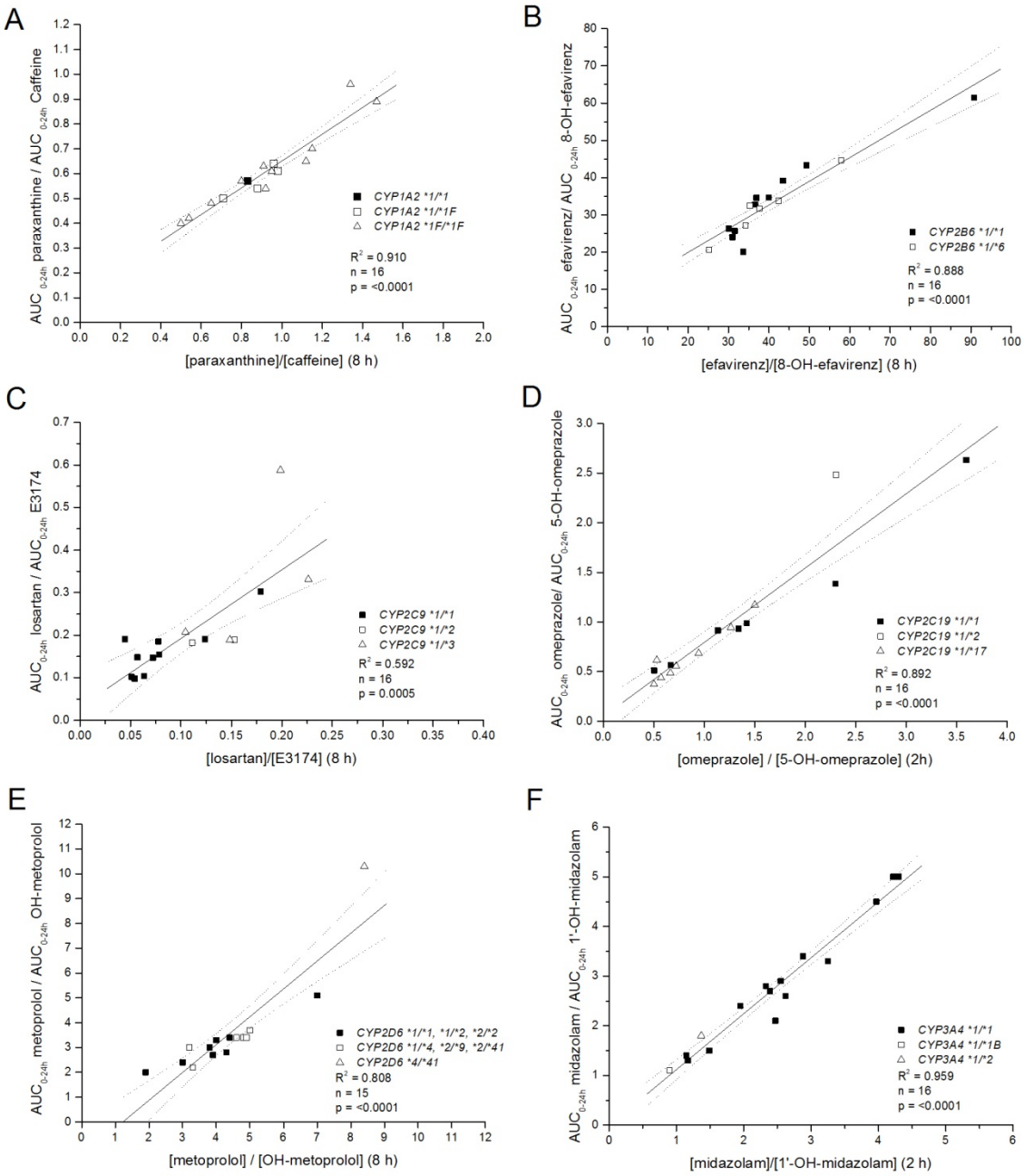


Figure 22. Correlations of individual single point concentration ratios with the corresponding AUC ratios 2h after dosing for omeprazole(D) and midazolam (F) and 8h after dosing for caffeine (A), efavirenz (B), losartan (C) and metoprolol (E).

6.3.4. Genotyping

All study participants were genotyped for altered function alleles. CYP2D6 genotyping identified one poor, one intermediate, 14 extensive and no ultrarapid metabolizers. The poor metabolizer was compound heterozygous for two loss-of-function alleles (CYP2D6*3/*4). In this subject no OH-metoprolol could be detected and no metabolic ratio could be calculated. The intermediate metabolizer had a combination of a loss-of-function and a diminished-function allele (CYP2D6*4/*41). This subject showed the highest metabolic ratio at 8h (Figure 22E). For CYP2B6, CYP2C9 and CYP2C19 no poor metabolizers were identified. Six subjects were heterozygous for diminished-function alleles of CYP2B6 (CYP2B6*6, n=6) and/or CYP2C9 (CYP2C9*2, n=2 and CYP2C9*3, n=4). The metabolic ratios of the four subjects heterozygous for the CYP2C9*3 allele were not significantly higher (p=0.074) compared to subjects with *1 or *2 alleles. One subject was heterozygous for the loss-of-function allele CYP2C19*2 and 8 subjects for the enhanced function allele CYP2C19*17. Genotyping of CYP1A2 identified 11 subjects homozygous and four subjects heterozygous for the *1F allele, which is associated with increased inducibility (**Fehler! Verweisquelle konnte nicht gefunden werden.**).
Metabolic ratios for heterozygous carriers of altered function alleles did not show significant differences compared to non-carriers (Figure 22, p>0.05 for all correlations between genotype and AUC ratios).

Table 14 (subject 1-8). Results of genotyping analysis (Basel cocktail study I). WT, wildtype; HT, heterozygous; HO, homozygous; neg, gene deletion or duplication not observed.

CYP	*Allele*	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7	Subject 8
1A2	*CYP1A2*1F*	***HO***	**HT**	***HO***	***HO***	***HO***	***HO***	***HO***	***HO***
2B6	*CYP2B6*6*	WT	**HT**	WT	WT	**HT**	WT	WT	WT
2C9	*CYP2C9*2*	WT	WT	WT	WT	WT	**HT**	WT	WT
	*CYP2C9*3*	WT	WT	WT	WT	WT	WT	**HT**	WT
2C19	*CYP2C19*2*	WT	**HT**	WT	WT	WT	WT	WT	WT
	*CYP2C19*3*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2C19*4*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2C19*6*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2C19*7*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2C19*8*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2C19*9*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2C19*10*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2C19*17*	**HT**	WT	WT	WT	WT	**HT**	WT	WT
2D6	*CYP2D6*2*	**HT**	**HT**	**HT**	**HT**	WT	WT	***HO***	**HT**
	*CYP2D6*3*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2D6*4*	WT	WT	**HT**	**HT**	**HT**	WT	WT	WT
	*CYP2D6*5*	neg	neg	neg	neg	neg	neg	neg	neg
	*CYP2D6*6*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2D6*7*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2D6*8*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2D6*9*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2D6*10*	WT	WT	**HT**	**HT**	**HT**	WT	WT	WT
	*CYP2D6*12*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2D6*14*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2D6*17*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2D6*29*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2D6*41*	WT	WT	WT	WT	WT	WT	WT	**HT**
	*CYP2D6*XN*	neg	neg	neg	neg	neg	neg	neg	neg
3A4	*CYP3A4*1B*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP3A4*2*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP3A4*3*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP3A4*12*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP3A4*17*	WT	WT	WT	WT	WT	WT	WT	WT

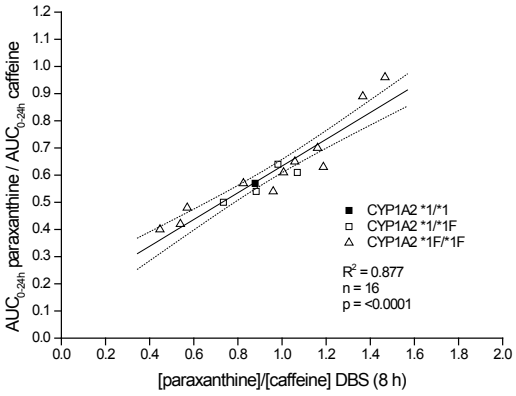
Table 14 (Subject 9-16). Results of genotyping analysis (Basel cocktail study I). WT, wildtype; HT, heterozygous; HO, homozygous; neg, gene deletion or duplication not observed.

CYP	*Allele*	Subject 9	Subject 10	Subject 11	Subject 12	Subject 13	Subject 14	Subject 15	Subject 16
1A2	*CYP1A2*1F*	***HO***	**HT**	***HO***	***HO***	***HO***	**HT**	**HT**	WT
2B6	*CYP2B6*6*	**HT**	WT	WT	**HT**	**HT**	**HT**	WT	WT
2C9	*CYP2C9*2*	WT	WT	WT	**HT**	WT	WT	WT	WT
	*CYP2C9*3*	**HT**	WT	WT	WT	WT	**HT**	**HT**	WT
2C19	*CYP2C19*2*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2C19*3*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2C19*4*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2C19*6*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2C19*7*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2C19*8*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2C19*9*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2C19*10*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2C19*17*	**HT**	WT	**HT**	**HT**	**HT**	WT	**HT**	**HT**
2D6	*CYP2D6*2*	**HT**	WT	**HT**	**HT**	**HT**	**HT**	WT	WT
	*CYP2D6*3*	WT	WT	WT	WT	WT	WT	WT	**HT**
	*CYP2D6*4*	**HT**	**HT**	WT	WT	WT	**HT**	**HT**	**HT**
	*CYP2D6*5*	neg	neg	neg	neg	neg	neg	neg	neg
	*CYP2D6*6*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2D6*7*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2D6*8*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2D6*9*	WT	WT	WT	WT	**HT**	WT	WT	WT
	*CYP2D6*10*	**HT**	**HT**	WT	WT	WT	**HT**	**HT**	**HT**
	*CYP2D6*12*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2D6*14*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2D6*17*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2D6*29*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2D6*41*	**HT**	WT	WT	WT	WT	WT	WT	WT
	*CYP2D6*XN*	neg	neg	neg	neg	neg	neg	neg	neg
3A4	*CYP3A4*1B*	WT	WT	WT	WT	WT	**HT**	WT	WT
	*CYP3A4*2*	WT	WT	WT	**HT**	WT	WT	WT	WT
	*CYP3A4*3*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP3A4*12*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP3A4*17*	WT	WT	WT	WT	WT	WT	WT	WT

6.3.5. DBS as a minimally invasive sampling procedure for phenotyping

In DBS only caffeine, paraxanthine, omeprazole, 5-OH-omeprazole, efavirenz and midazolam could be quantified. The concentrations of the metabolites of efavirenz and midazolam as well as losartan and metoprolol (and their respective metabolites) were below the limit of quantification in DBS at the time points 2 and 8 h. The correlation of single point concentration ratios in DBS for CYP1A2 at 8h and for CYP2C19 at 2h with the corresponding AUC ratios in plasma were good (R^2 >0.8 for both, Figure 23A and B) and comparable to correlations between single point concentration ratios and AUC ratios determined in plasma.

A



B

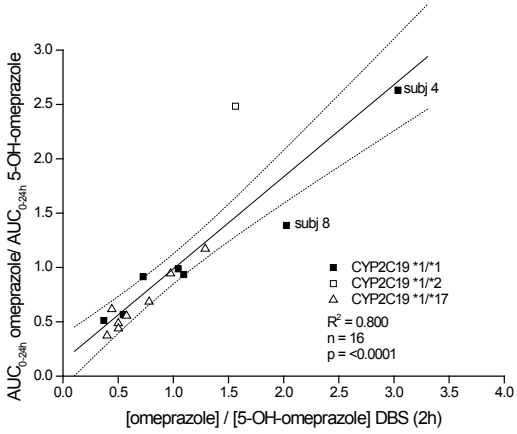


Figure 23. Correlations of individual single point concentration ratios in DBS with the corresponding AUC ratios 2h after dosing for omeprazole (B) and 8h after dosing for caffeine (A).

Efavirenz concentrations at 8h or 12h and midazolam concentrations at 1h or 2h in DBS showed only moderate correlation (R^2 of 0.44 to 0.57) with the corresponding AUCs in plasma (Table 15).

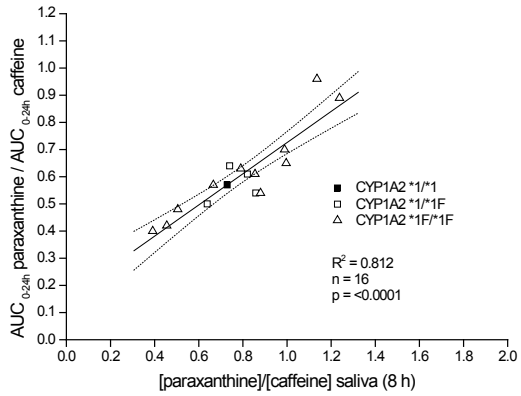
Table 15. Correlation of single point DBS measurements with AUC in plasma

		coefficient of determination (R^2)					
	time (h)	CYP1A2	CYP2B6	CYP2C9	CYP2C19	CYP2D6	CYP3A4
parent concentration in DBS vs AUC	1				0.011		0.442
	2				0.436		0.571
	3						
	4	0.937	0.091				
	6						
	8	0.926	0.443				
	12	0.936	0.522				
Parent/metabolite concentration ratio in DBS vs $AUC_{parent}/AUC_{metabolite}$ ratio	1				0.484		
	2				**0.800**		
	3						
	4	0.925					
	6						
	8	**0.877**					
	12	0.787					

6.3.6. Oral fluid as a non-invasive sampling procedure for phenotyping

In saliva the concentrations of all analytes except caffeine, paraxanthine, omeprazole, 5-hydroxy-omeprazole and metoprolol were too low to allow reliable quantification (LLOQ between 0.5 and 5ng/ml, Table 4). The correlation of metabolic ratios for CYP1A2 in saliva at 8h with the corresponding AUC ratios in plasma was good (Figure 24A), whereas the metabolic ratios for CYP2C19 in saliva at 2h only showed moderate correlation to the corresponding AUC ratios in plasma (Figure 24B).

A



B

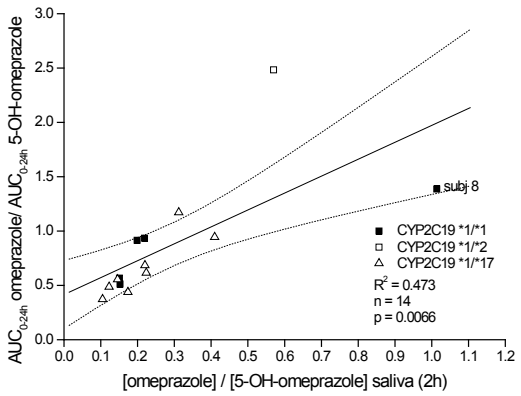


Figure 24. Correlations of individual single point concentration ratios in saliva with the corresponding AUC ratios 2h after dosing for omeprazole (B) and 8h after dosing for caffeine (A).

Table 16. Correlation of single point saliva measurements with AUC in plasma.

		coefficient of determination (R^2)					
	time (h)	**CYP1A2**	**CYP2B6**	**CYP2C9**	**CYP2C19**	**CYP2D6**	**CYP3A4**
parent concentration in saliva vs. AUC	1				0.959		
	2				0.63		
	3						
	4	0.873			0.201		
	6						
	8	0.883				0.306	
	12	0.894				0.279	
Parent/metabolite concentration ratio in saliva vs $AUC_{parent}/AUC_{metabolite}$ ratio	1				0.905		
	2				**0.473**		
	3						
	4	0.918			0.544		
	6						
	8	**0.812**				**0.192**	
	12	0.719				0.008	

Since OH-metoprolol could not be quantified in saliva only data for metoprolol could be examined. In contrast to plasma, metoprolol concentrations at 8h and metoprolol AUC0-24h in saliva in the intermediate and poor metabolizers were significantly higher ($p \leq 0.01$) compared to extensive metabolizers. Within the extensive metabolizer group the same was observed for subjects heterozygous for a diminished- or a loss-of-function allele (*4, *9, *41) compared to subjects with two normal function (*1 or *2) alleles ($p<0.05$ for AUC and 8h concentration, respectively, Figure 25C and Figure 25D). In plasma, however, only metoprolol AUC0-24h, but not 8h concentrations were higher for intermediate and poor metabolizers compared to extensive metabolizers with two normal function alleles (Figure 25 A and B). No significant difference between extensive metabolizers with two and those with only one normal function allele was found.

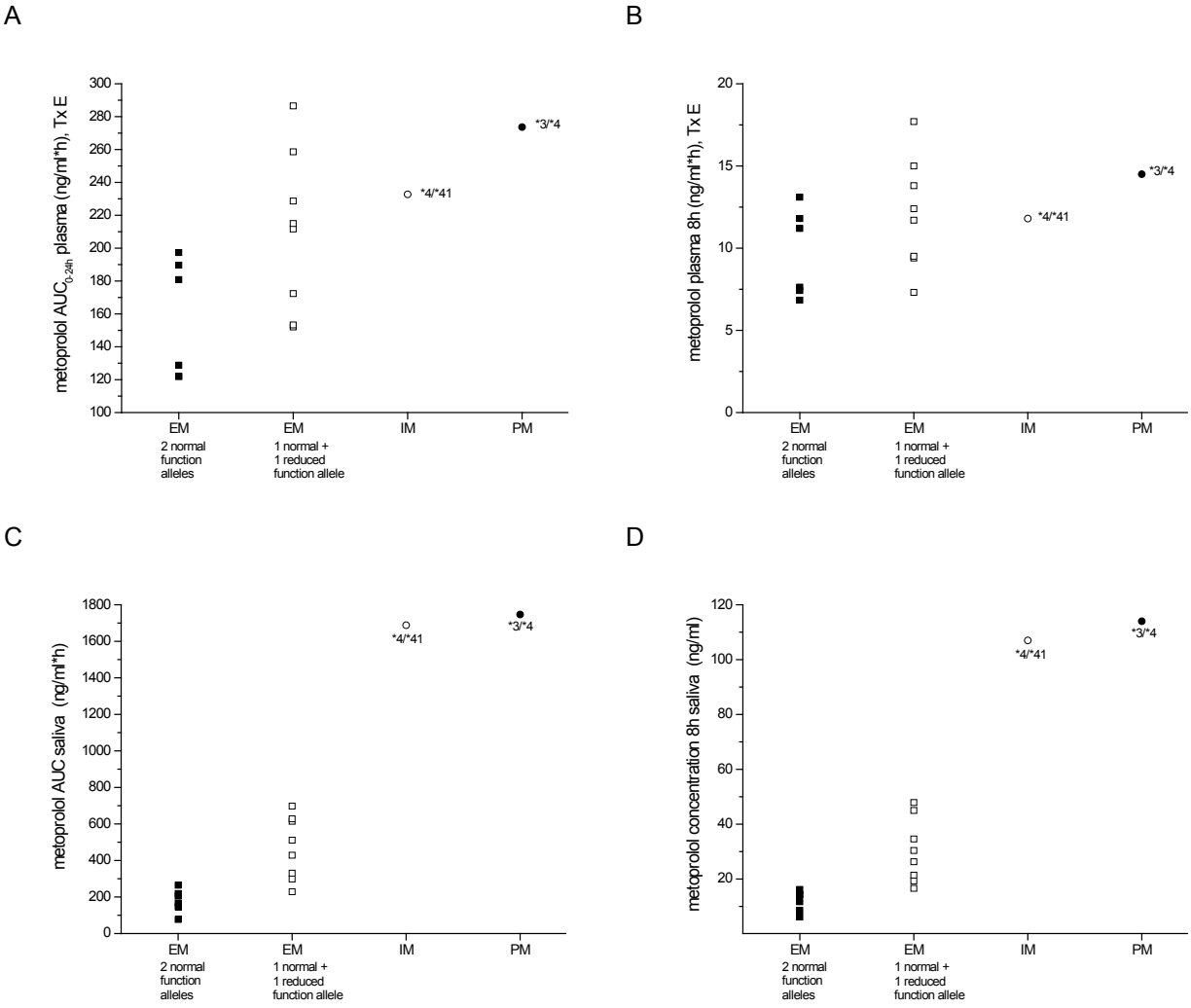


Figure 25. Metoprolol AUC in plasma and saliva versus CYP2D6 genotype (A and C), metoprolol concentration in plasma and saliva versus CYP2D6 genotype (B and D).

6.4.Discussion

In our study we investigated a new combination of probe drugs for simultaneous phenotyping of six major human cytochromes in plasma, saliva and dried blood spots. Limited availability and tolerability of specific probe drugs are major limitations of many published cocktails, preventing more widespread use of this phenotyping tool [18]. One of our main goals was to develop a phenotyping cocktail that causes minimal pharmacodynamic effects and thus could also be applied to patients. In contrast to many existing cocktails, all probe drugs of our cocktail are approved for clinical use and commercially available in many countries. They were administered at the lowest approved oral dose and the selected combination proved to be safe and did not cause any relevant effects on vital signs or vigilance in our study subjects.

After excluding chlorzoxazone from the initial drug combination, we did not find evidence for mutual pharmacokinetic interactions between the final six cocktail probe drugs. In contrast to many published phenotyping cocktails which used changes in phenotyping metrics as an indicator for pharmacokinetic interactions [8, 11-13, 24, 30], we performed bioequivalence tests based on full pharmacokinetic profiles of all our cocktail probe drugs. All intra-subject CV% were lower than 30%, hence the standard bioequivalence limits for low-variability drugs of 0.8-1.25 were applied and met by all six probe drugs [92].

Another draw-back of many available phenotyping methods are complicated sampling procedures requiring collection of more than one sample matrix at multiple time-points after dosing. We present one of the first phenotyping cocktails that is completely based on plasma as single sample matrix. Apart from the Quebec cocktail, which only uses urine samples [12], most other cocktails are based on at least two different matrices, usually plasma and urine. Use of a single matrix not only offers the advantage of easier sample processing, but also reduces the complexity of bioanalytical method validation and pre-analytical sample work-up.

To promote clinical application of phenotyping procedures, a single sampling time-point as early as possible after dosing would be ideal. With our cocktail a single plasma sample 4 hours after dosing could be proposed for five of the six CYP isoforms (CYP1A2, 2B6, 2C19, 2D6 and 3A4) with good correlations (R^2 >0.82) between concentration ratios and AUC ratios (Table 13). The only exception is CYP2C9, where the correlation between losartan metabolic ratios and AUC ratios at 4h was poor and a later time point was needed for better correlation. On the other hand, at time-points later than 4 hours, midazolam and omeprazole could not be reliably quantified due to their short plasma half-lifes. Therefore two sampling time points, the first 2 hours and the second 8 hours after dosing are proposed to obtain reasonable correlations for all six CYP isoforms of the cocktail.

Efavirenz is a specific substrate for CYP2B6 *in vitro* [37] and CYP2B6 poor metabolizer genotypes are associated with elevated efavirenz exposure in vivo [93, 94]. Efavirenz is listed as a sensitive CYP2B6 substrate in the FDA/CDER draft guidance on drug interaction studies but has so far not been used as part of a phenotyping cocktail [92]. Therapeutic doses of efavirenz are associated with side effects such as vivid and unpleasant dreams which typically occur after the first one or two doses. In our cocktail

efavirenz was used at a dose of 50mg, which is more than ten times lower than the recommended therapeutic dose. At this low dose it was well tolerated without causing any relevant side effects. Efavirenz can thus be safely used as part of a phenotyping cocktail without interfering with phenotyping metrics of other CYP isoforms.

Metoprolol is an established probe drug for CYP2D6, which is mainly responsible for α-hydroxylation of metoprolol [95]. Urinary metoprolol/α-hydroxymetoprolol concentration ratios show a bimodal distribution corresponding to extensive and poor metabolizer subgroups and they are highly correlated with urinary debrisoquine/4-hydroxydebrisoquine ratios [96]. In contrast to metoprolol concentration ratios in urine, which have previously been used as phenotyping metric [12, 13], metoprolol metabolic ratios in plasma have so far not been used in phenotyping cocktails.

Our data showed a high correlation between metoprolol metabolic ratio in plasma at 8h and plasma AUC ratios for extensive metabolizers.

The only intermediate metabolizer in the sample had a distinctly higher metabolic ratio of 8.3 compared to the extensive metabolizers, while for the poor metabolizer no ratio could be calculated due to undetectable hydroxymetoprolol. For the poor metabolizer subject no metabolic ratio could be calculated due to undetectable hydroxymetoprolol. In such cases an alternative metabolic ratio could be obtained by substituting the lower limit of quantification of the bioanalytical method for the non-detectable metabolite concentration. For our subject this would have resulted in a ratio of 29. Although not a “true” metabolic ratio, this alternative ratio clearly indicates poor metabolic activity and could be useful to detect poor CYP2D6 metabolizers. The proposed procedure should to be tested in a larger sample with a sufficient number of poor metabolizer subjects.

Our observations are in line with data published on metoprolol metabolic ratios in plasma. In a study with healthy Asian subjects, the 3h metoprolol/hydroxymetoprolol concentration ratios in plasma correlated with 0-8h urinary metabolic ratios and were distinctly higher for poor compared to extensive metabolizers [97]. In another study hydroxymetoprolol could not be detected in poor metabolizers, due to insufficient sensitivity of the analytical method (lower limit of quantification 5ng/ml) [9]. In a prospective clinical study with primary care patients treated with metoprolol, concentrations ratios taken at the end of the dose interval were higher for poor metabolizers compared to intermediate and extensive metabolizers, without an overlap between the ratios of intermediate and poor metabolizers [98].

Losartan has so far been used as a probe drug for CYP2C9 in two phenotyping cocktails. In both cocktails the 0-8h urinary losartan/E-3174 ratio was used [28, 30]. Although less well validated than tolbutamide, the urinary losartan/E-3174 ratios have been shown to be significantly higher in subjects carrying a CYP2C9*3 diminished-function allele compared to subjects with wild-type alleles [43, 99, 100] and plasma $AUC_{losartan}/AUC_{E-3174}$ ratios were highly correlated with the corresponding urinary ratios [43]. Furthermore, the plasma $AUC_{losartan}/AUC_{E-3174}$ ratio is also significantly altered by the CYP2C9 inhibitor fluconazole [101]. In our study we found a reasonable correlation between the plasma losartan/E-3174 metabolic ratios at 8h with the plasma $AUC_{losartan}/AUC_{E-3174}$ ratios. A possible explanation for the lower correlation with plasma AUC ratios compared to the other probe drugs of the cocktail could be the fact

that losartan undergoes enterohepatic circulation which increases variability of concentrations around 4-8 hours after dosing [102].

Availability of minimally or non-invasive sampling procedures such as DBS or saliva would further increase attractiveness of phenotyping outside of clinical research settings. However, the low systemic drug exposures reached with our low-dose cocktail pose bioanalytical challenges when using alternative matrices. In DBS reliable single point concentration ratios could only be obtained for the probe drugs of CYP1A2 and 2C19. Feasibility of using DBS for phenotyping of CYP3A4 has been shown by another group using a higher midazolam dose (7.5mg) together with extensive pre-analytical sample work-up and detection with a UPLC-LCMS system [90]. For CYP2C9, DBS has recently been evaluated using flurbiprofen as probe drug [17].

In saliva, a useful correlation between single point concentration ratios and AUC ratios was only found for CYP1A2, whereas the correlation for CYP2C19 was moderate. Using a higher midazolam dose of 7.5mg we have previously shown the usefulness of saliva for non-invasive phenotyping of CYP3A4 [16], again emphasizing the need for ultrasensitive bioanalytical methods before alternative matrices such as DBS or saliva can be used for the phenotyping of all major CYP isoforms. Although we were not able to quantify OH-metoprolol in saliva, inspection of the metoprolol data in saliva showed that in contrast to the corresponding plasma values, both metoprolol AUC and metoprolol concentrations at 8h were able to distinguish intermediate and poor metabolizers from extensive metabolizers. The reason for this finding is not clear, however, if confirmed in a larger sample, this would be an attractive alternative for non-invasive CYP2D6 phenotyping.

6.5.Conclusions

We have validated a new phenotyping cocktail that offers several improvements compared to established procedures. The required probe drugs are all approved for clinical use, commercially available in many countries and well tolerated at the low doses used. For the first time low-dose efavirenz has been included in a cocktail and correlation of metabolic ratios with AUC ratios in plasma has been evaluated for losartan and metoprolol. With the exception of losartan, all probe drugs are listed as sensitive in vivo CYP substrates by the FDA. Collection of a single plasma sample 4 hours after dosing for simultaneous assessment of five CYP isoforms, or two plasma samples 2 hours and 8 hours after dosing for the assessment of six major CYP isoforms are proposed and should be further evaluated. To assess validity of this approach, additional studies in subjects with altered CYP activity (either genetically or due to CYP induction or inhibition) are required. Use of DBS or saliva samples seems feasible for phenotyping of selected CYP isoforms at present. With more sensitive analytical methods in the future, minimally or non-invasive phenotyping procedures can be expected to become available for other major CYP isoforms.

7. Effects of induction and inhibition on the Basel Phenotyping Cocktail

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7.1. Introduction

In the "Basel" Phenotyping Cocktail Study we showed that caffeine, midazolam, losartan, omeprazole and efavirenz do not interfere with each other and can be safely administered together as phenotyping probe drugs. We also collected information about the variation of the metabolic ratios in a small population of healthy subjects that, with the only exception of an intermediate and a poor metabolizer for CYP2D6, did not show altered metabolism.

One of the classic applications of phenotyping cocktails is the identification of inhibiting or inducing effects of new investigational drugs on cytochromes during drug development. Changes of the metabolic ratios of the cocktail probe substrates after exposure to the investigational drug compared with the metabolic ratios under baseline conditions reliably indicates whether the investigational drug inhibits or induces a certain cytochrome [103].

After excluding mutual interactions between the chosen combination of probe drugs as a first validation step of our cocktail, we wanted to know how co-administration of an inducer or an inhibitor and genetic factors (poor or extensive metabolizers) would influence the metabolic ratios of the Basel cocktail.

With these objectives, we tested the Basel cocktail alone, in combination with the known inhibitors ciprofloxacin, fluconazole and paroxetine and with the known inducer rifampicin in a three way crossover design. These drugs were selected, with the only exception of losartan, on the basis of the FDA "Guidance for Industry for Drug interactions Studies" [38] and/or available clinical data indicating that the compound could be used as a specific probe drug.

A second aim of the "Basel" Phenotyping Cocktail Study was to evaluate whether minimally or non-invasive sampling techniques such as collection of saliva or dried blood spots could be used instead of the more invasive venous blood sampling. In the first Basel Phenotyping Cocktail Study the correlation between probe substrates and their metabolites measured in saliva and DBS with the corresponding metabolic ratios measured in plasma was evaluated. In the second study, these measurements were repeated to evaluate whether DBS or oral fluid sampling could also be used under conditions of

induction or inhibition where very low concentrations of either metabolite or parent compound will occur.

7.2.Material and Methods

7.2.1. Clinical study

A single-center, randomized, three-way crossover study was performed at the Phase I Research Center, University Hospital Basel, Basel, Switzerland (ClinicalTrials.gov Identifier: NCT01386593). Screening procedures and exclusion criteria were the same as used in the 1st Basel cocktail study (6.2.1).

Single doses of caffeine, efavirenz, losartan, omeprazole, metoprolol and midazolam were administered together as a cocktail with and without preliminary inhibition with fluconazole, ciprofloxacin and paroxetine or induction with rifampicin. Each treatment was investigated in the same group of 16 healthy male subjects. The study was divided in three treatment arms A, B, and C (Figure 26, Table 17).

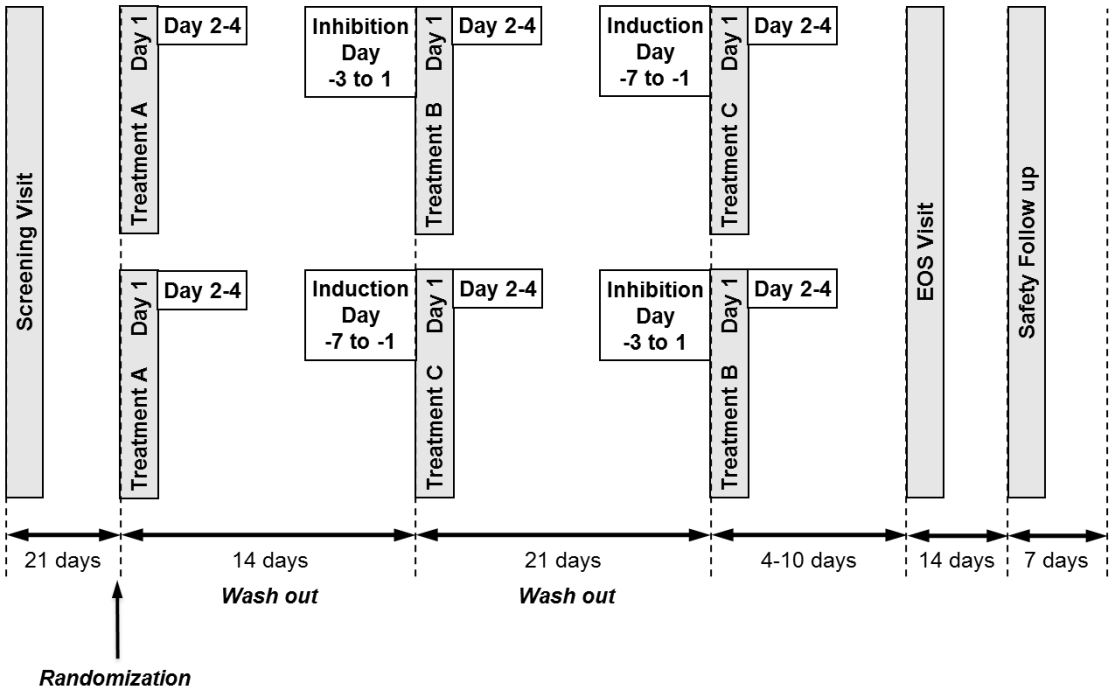


Figure 26. Study Design

Table 17. Study drug administration plan

Study Day			- 7		- 6		- 5		- 4		- 3		- 2		- 1		1	
Time (hh:mm)			08:00	20:00	08:00	20:00	08:00	20:00	08:00	20:00	08:00	20:00	08:00	20:00	08:00	20:00	07:00	08:00
Treatment	Drug	Dose																
A = Baseline	Caffeine	200 mg																½
	Efavirenz	50 mg																1
	Losartan	12.5 mg																1
	Omeprazole	10 mg																1
	Metoprolol	25 mg																½
	Midazolam	2 mg																1
B = Inhibition	Fluconazole	200 mg									2		1		1		1	
	Ciprofloxacin	750 mg													1	1	1	
	Paroxetine	20 mg									1		1		1		1	
	Caffeine	200 mg																½
	Efavirenz	50 mg																1
	Losartan	12.5 mg																1
	Omeprazole	10 mg																1
	Metoprolol	25 mg																½
	Midazolam	2 mg																1
C = Induction	Rifampicin	600 mg	1		1		1		1		1		1		1			
	Caffeine	200 mg																½
	Efavirenz	50 mg																1
	Losartan	12.5 mg																1
	Omeprazole	10 mg																1
	Metoprolol	25 mg																½
	Midazolam	2 mg																1

All the subjects received the Basel Cocktail (treatment A) to establish the baseline pharmacokinetic profile:

- Treatment A: single oral dose of 100 mg caffeine, 50 mg efavirenz, 12.5 mg losartan, 10 mg omeprazole, 12.5 mg metoprolol and 2 mg midazolam.

After treatment A, one group received treatment B and then treatment C, while the other group received first treatment C and then treatment B:

- Treatment B (Inhibitors and Basel cocktail):
  - Day -3, morning: fluconazole 400 mg and paroxetine 20 mg.
  - Day -2, morning: fluconazole 100 mg and paroxetine 20 mg.
  - Day -1, morning: fluconazole 100 mg, paroxetine 20 mg, ciprofloxacin 750 mg.
  - Day -1, evening: ciprofloxacin 750 mg.
  - Day 1, 1 h before administration of the Basel Cocktail: fluconazole 100 mg, paroxetine 20 mg, ciprofloxacin 750.

- Treatment C (Inducer and Basel cocktail):
  - Day -7 to -1, daily: rifampicin 600 mg.
  - Day 1 Basel Cocktail.

7.2.2. Pharmacokinetic sampling

Pharmacokinetic sampling was performed as described for the 1st Basel cocktail (see 6.2.3).

7.2.3. Pharmacodynamic measurements and adverse events

Pharmacodynamic assessment (heart rate, blood pressure, sedation) was performed as described for the 1st Basel cocktail study (see 6.2.4).

7.2.4. Study drugs

The Basel cocktail drugs (see paragraph 6.2.5), ciprofloxacin (Ciproxin®, 750 mg, Bayer), fluconazole (Diflucan®,200 mg, Pfizer), paroxetine (Deroxat®, 20 mg, GlaxoSmithKline), and rifampicin (Rimactan®, 600 mg, Sandoz) were purchased through the University Hospital Pharmacy, Basel, Switzerland.

7.2.5. Bioanalytical analysis

The bioanalytical method used for the analysis of the plasma, saliva, blood and DBS samples is described in chapter 5.

7.2.6. Genotype analysis

DNA was isolated from 400 µL EDTA-blood on an m2000sp instrument (Abbott Molecular, De Plaines, IL, USA) following the manufacturer's protocol. Final elution volume was 100 µL. Specific single nucleotide polymorphisms in the genes encoding CYP1A2 (alleles *1A, *1F), CYP2B6 (alleles *1, *6), CYP2C9 (alleles *1, *2, *3), CYP2C19 (alleles *1, *2, *3) and CYP2D6 (*1, *2, *3, *4, *5, *6, *XN) were detected using primer/probe real time PCR reagent (Lightmix, TIB MOLBIOL, Berlin, Germany) on a Lightcycler 1.5 instrument (Roche Diagnostics, Rotkreuz, Switzerland). Detection of hybridization of amplified products was performed using the Infiniti chip technology (Autogenomics, Carlsbad, USA) according to the manufacturer's instructions.

Subjects with SNPs responsible for relevant altered CYP activity were not included in the phenotyping data evaluation.

7.2.7. Data analysis

The pharmacokinetics parameters (AUC, half-life, Cmax, and tmax) were calculated as already described in paragraph 6.2.7. Analysis of variance (ANOVA) was performed to test for overall differences between the MRs in the different treatments. Post-hoc analysis using the Dunnet test was used to test for between group differences. All statistical analyses were done using GraphPad Prism version 6.02 (GraphPad Software Inc., La Jolla, CA).

7.3.Results

7.3.1. Pharmacodynamic effects and adverse events

Sixteen healthy male volunteers (mean age 23.8 years, range 20-35 years, mean BMI 23.5 kg/m2, range 20.6-27.5 kg/m^2) completed the study according to the protocol. The simultaneous administration of all probe drugs, inhibitors as well as the inducer was well tolerated. No serious adverse events (CTC grade 3 or higher) were observed and no relevant changes in heart rate (Figure 27 A), blood pressure (Figure 27 B and C) or sedation score (Figure 27 D) were observed.

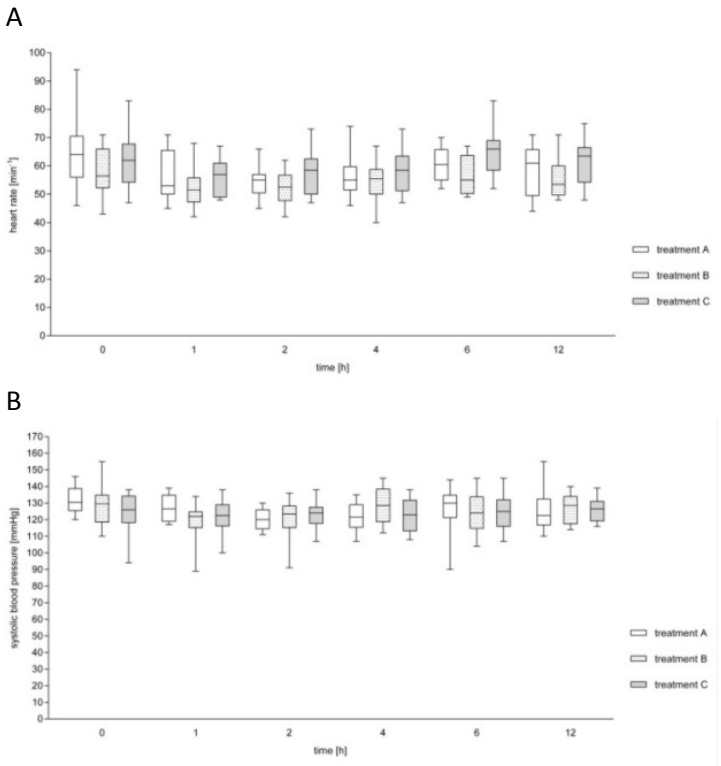


Figure 27 (A, B). Changes of heart rate (A) and systolic blood pressure (B) were compared after administration of Basel cocktail (treatment A, white boxes), after administration of Basel cocktail and inhibitors (treatment B, dotted boxes) and after administration of Basel cocktail and inducer (treatment C, grey boxes). The whiskers represent the range with minimum and maximum. The boxes represent the 25.-75. percentile and the median.

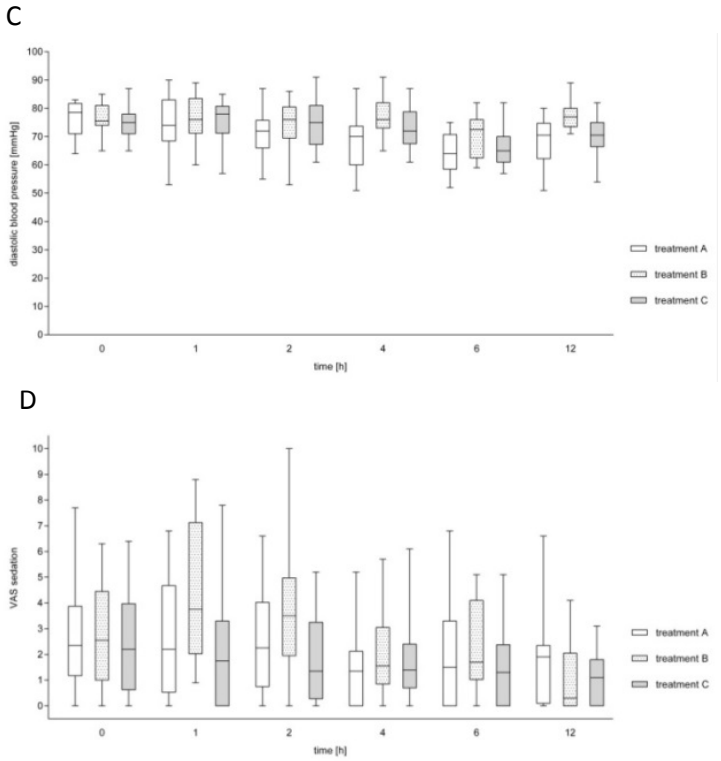


Figure 27 (C, D). Changes of diastolic blood pressure (C) and sedation scores (D) were compared after administration of Basel cocktail (treatment A, white boxes), after administration of Basel cocktail and inhibitors (treatment B, dotted boxes) and after administration of Basel cocktail and inducer (treatment C, grey boxes). The whiskers represent the range with minimum and maximum. The boxes represent the 25.-75. percentile and the median.

7.3.2. Effects of induction and inhibition on the pharmacokinetics of the cocktail probe drugs

Each subject received the Basel cocktail, the Basel cocktail in concomitance with specific inhibitors (ciprofloxacin, fluconazole and paroxetine) and the Basel cocktail after one week pretreatment with the CYP inducer rifampicin. The pharmacokinetic profiles in plasma (Figure 28), saliva, blood and DBS of the probe drugs and of their respective metabolites were measured for the three treatments. 1'-OH-midazolam glucuronide and 8-OH-efavirenz glucuronide were indirectly quantified in plasma and saliva after incubating the samples with β-glucuronidase. The pharmacokinetic profiles of the metabolites before and after treatment of the plasma samples with the enzyme are reported in Figure 29 and Figure 30.

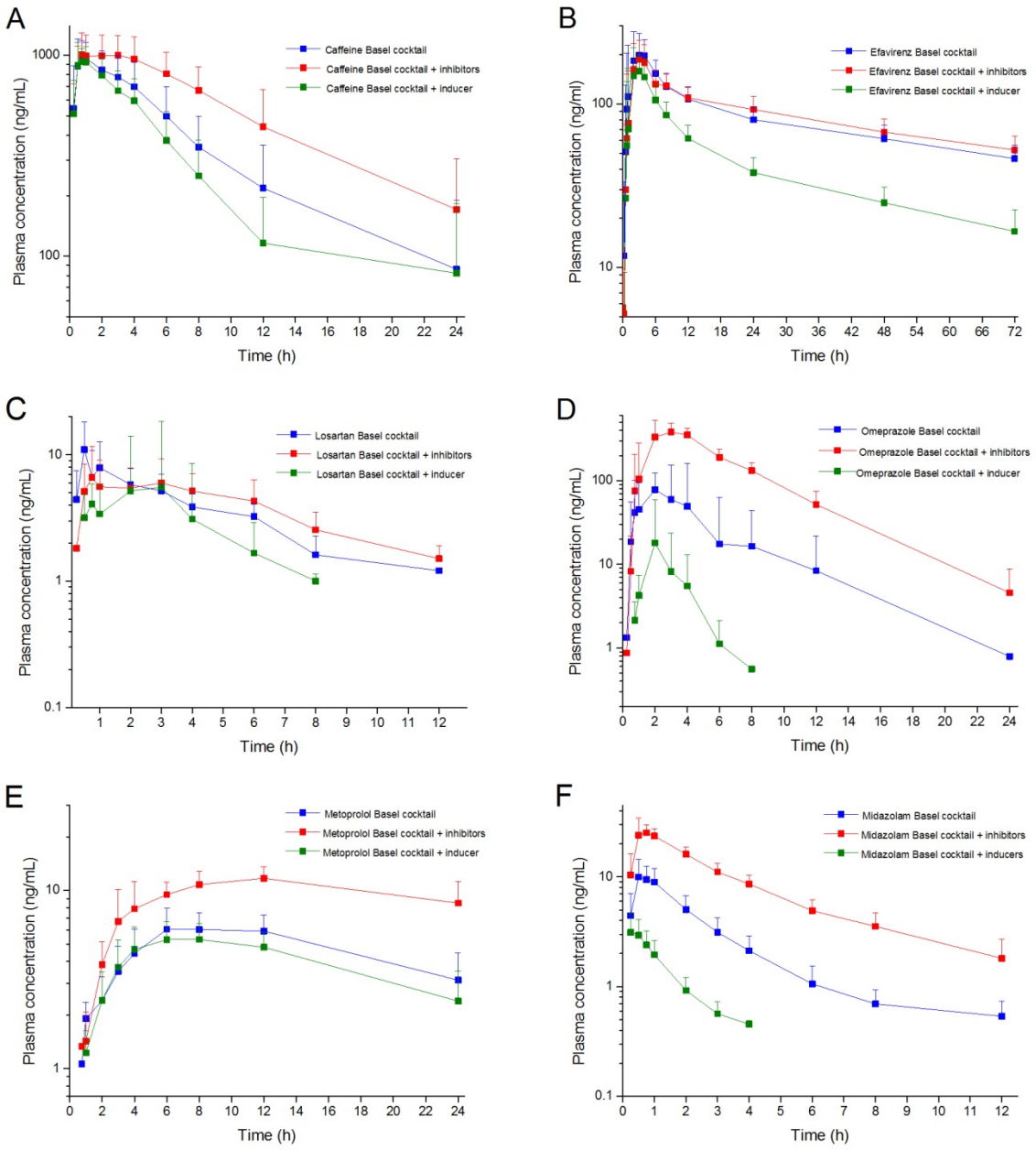


Figure 28. Comparison of plasma concentration time-profiles of cocktail probe drugs obtained after application of the 6-drug cocktail alone (blue curves), in presence of the inhibitors paroxetine, ciprofloxacin, and fluconazole (red curves) as well as in presence of the inducer rifampicin (green curves). Concentration-time profiles are shown for parent drugs and their corresponding metabolites for CYP1A2 (A), CYP2B6 (B), CYP2C9 (C), CYP2C19 (D), CYP2D6 (E) and CYP3A4 (F).

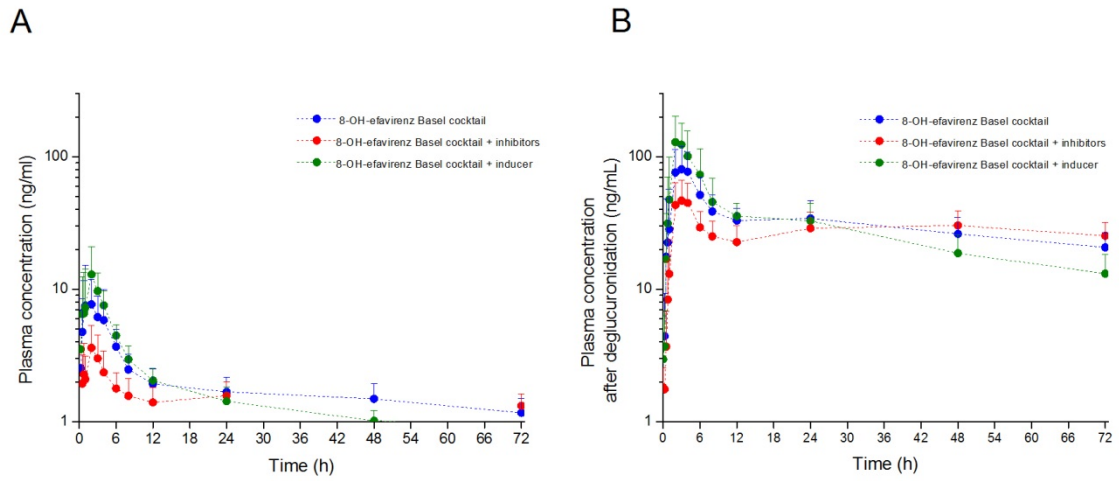


Figure 29. Comparison of plasma concentration time-profiles of 8-OH-efavirenz obtained after application of the 6-drug cocktail alone (blue curves), in presence of the inhibitors paroxetine, ciprofloxacin, and fluconazole (red curves) as well as in presence of the inducer rifampicin (green curves). Concentration-time profiles before (A) and after (B) treatment with β-glucuronidase.

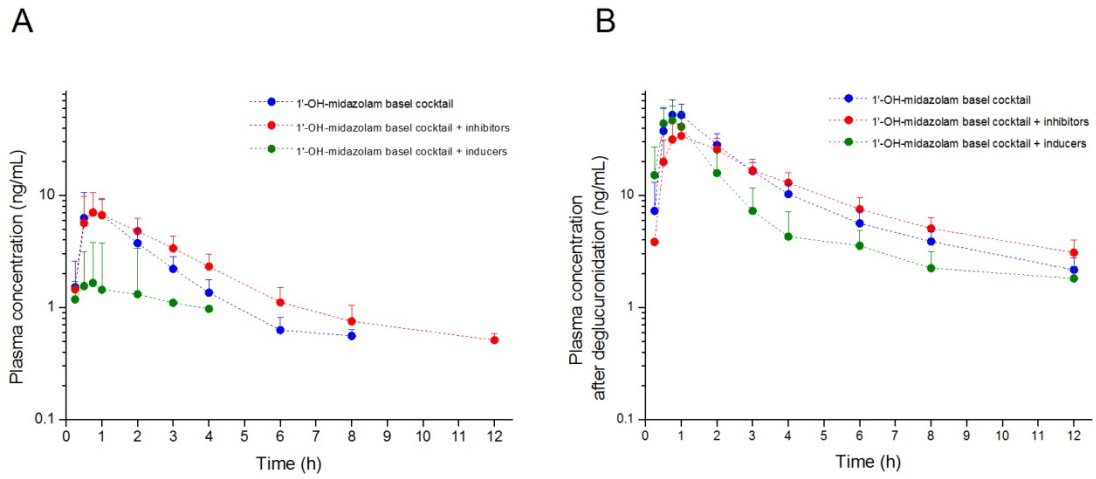


Figure 30. Comparison of plasma concentration time-profiles of 1'-OH-midazolam obtained after application of the 6-drug cocktail alone (blue curves), in presence of the inhibitors paroxetine, ciprofloxacin, and fluconazole (red curves) as well as in presence of the inducer rifampicin (green curves). Concentration-time profiles before (A) and after (B) treatment with β-glucuronidase.

The exposure to inhibitors and the inducer was checked by determining the AUCs of paroxetine, ciprofloxacin, fluconazole and rifampicin in each subject (Figure 31). Subject number 1 was found to be non-compliant with rifampicin pre-treatment and was excluded from subsequent analyses of induction data.

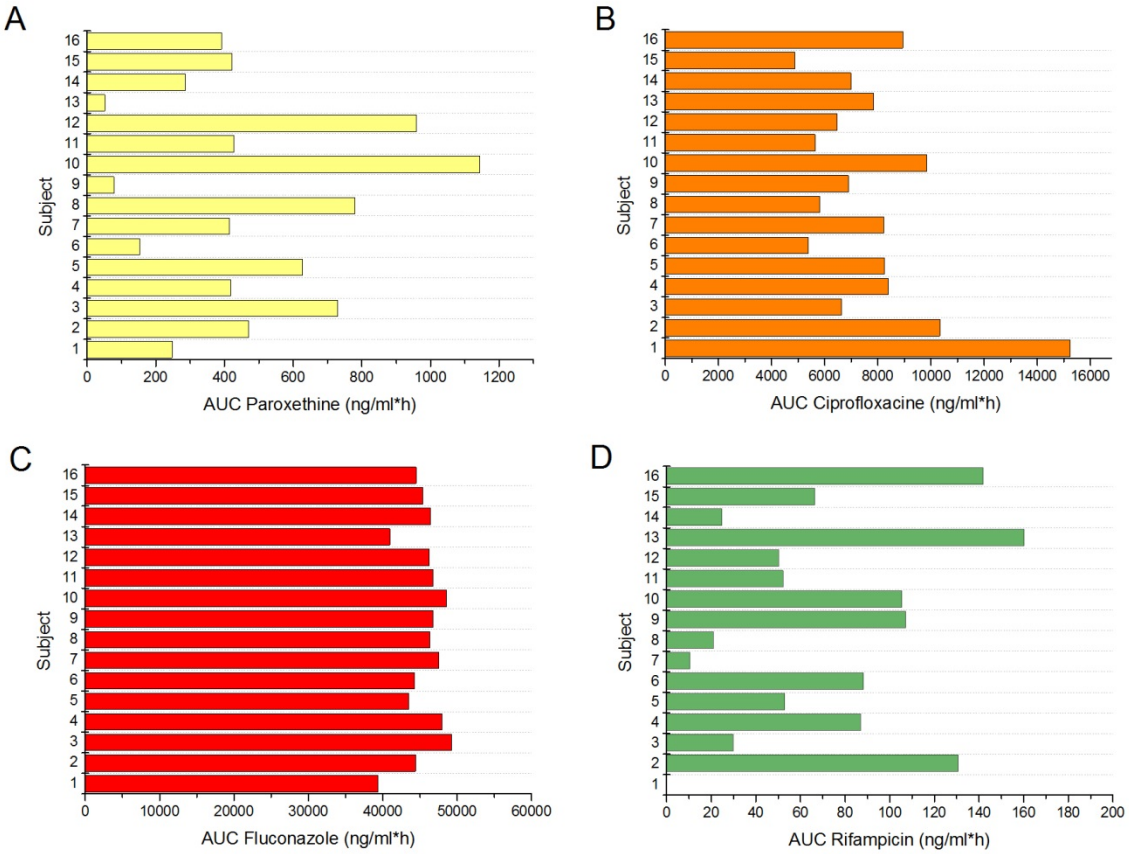


Figure 31. Exposure of study subjects to inhibitors and inducers. Plasma $AUC_{0\text{-}24h}$ of Paroxetine (A), Ciprofloxacin (B), Fluconazole (C), and Rifampicin (D).

The extent of inhibition and induction was graded following FDA guidelines. A drug was considered a weak, moderate or strong inhibitor of a CYP isoform when a 1.25 to 2-fold, 2 to 5-fold or more than 5-fold increase in the AUC of the CYP specific substrate was observed, respectively. It was considered a weak, moderate or strong inducer of a CYP isoform when a 20-50%, 50-80% or more than 80% decrease in AUC of the CYP specific substrate was observed, respectively.

In our study varying degrees of inhibition (Table 18, Figure 32) and induction (Table 19, Figure 33) of the different CYPs involved were observed for each individual study subject. Despite using three different inhibitors we were not able to achieve adequate inhibition of CYP 2B6 in all subjects and in up to one third of the subjects no inhibition was achieved for CYP1A2 and CYP2C9. For CYP2C19 in contrast, most subjects showed a strong level of inhibition, while for the remaining CYP isoforms weak to moderate inhibition was reached. Using rifampicin, weak to moderate induction was seen for CYP2B6 and CYP2C9. The highest effect was observed for CYP2C19 and CYP3A4. For CYP1A2 and CYP2D6 on the other hand, no induction was observed in more than half of the subjects.

Table 18. Degree of inhibition (n=16) estimated using plasma $AUC_{0\text{-}24h}$ of parent drugs. $AUC_{0\text{-}24h}$ fold change ($AUC_{0\text{-}24h}$ after administration of inhibitors / $AUC_{0\text{-}24h}$ baseline) for CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4.

INHIBITION	no inhibition	weak	moderate	strong
CYP1A2	4 (25%)	9 (56%)	3 (19%)	-
CYP2B6	16 (100%)	-	-	-
CYP2C9	6 (37%)	10 (63%)	-	-
CYP2C19	-	1 (6%)		15 (94%)
CYP2D6	-	9 (56%)	7 (64%)	-
CYP3A4	-	-	13 (81%)	3 (19%)

Table 19. Degree of induction (n=15, subject 1 excluded from the evaluation) estimated using plasma $AUC_{0\text{-}24h}$ of parent drugs. $AUC_{0\text{-}24h}$ % decrease (100-$AUC_{0\text{-}24h}$ after administration of inducer / $AUC_{0\text{-}24h}$ baseline x 100) for CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4.

INDUCTION	no induction	weak	moderate	strong
CYP1A2	8 (53%)	7 (47%)	-	-
CYP2B6	-	14 (93%)	1 (7%)	-
CYP2C9	-	6 (40%)	9 (60%)	-
CYP2C19	-	-	1 (7%)	14 (93%)
CYP2D6	10 (67%)	5 (33%)		
CYP3A4	-	-	8 (53%)	7 (47%)

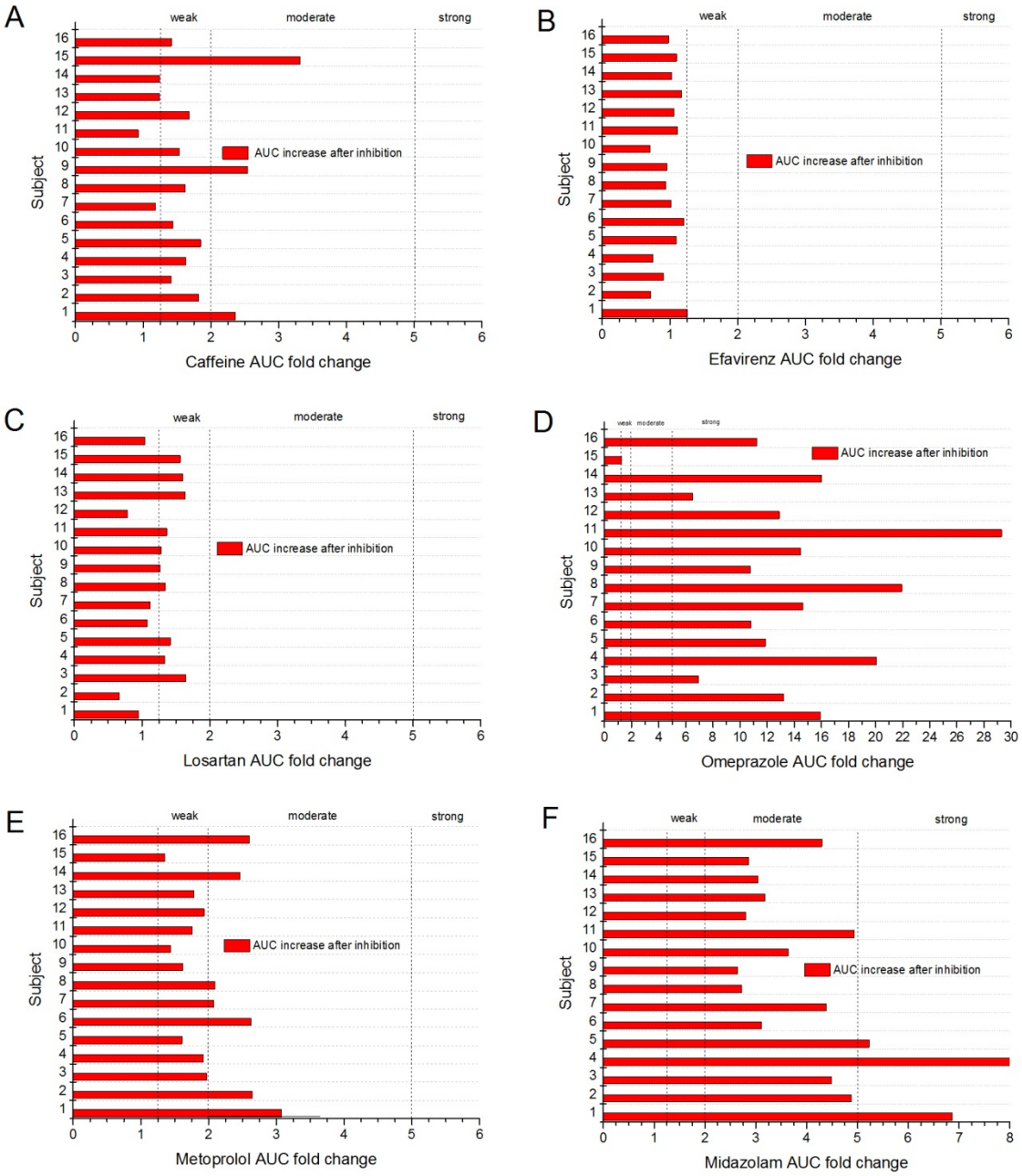


Figure 32. Effect of inhibitors on plasma $AUC_{0\text{-}24h}$ of parent drugs. $AUC_{0\text{-}24h}$ fold change ($AUC_{0\text{-}24h}$ after administration of inhibitors / $AUC_{0\text{-}24h}$) for CYP1A2 (A), CYP2B6 (B), CYP2C9 (C), CYP2C19 (D), CYP2D6 (E), and CYP3A4 (F).

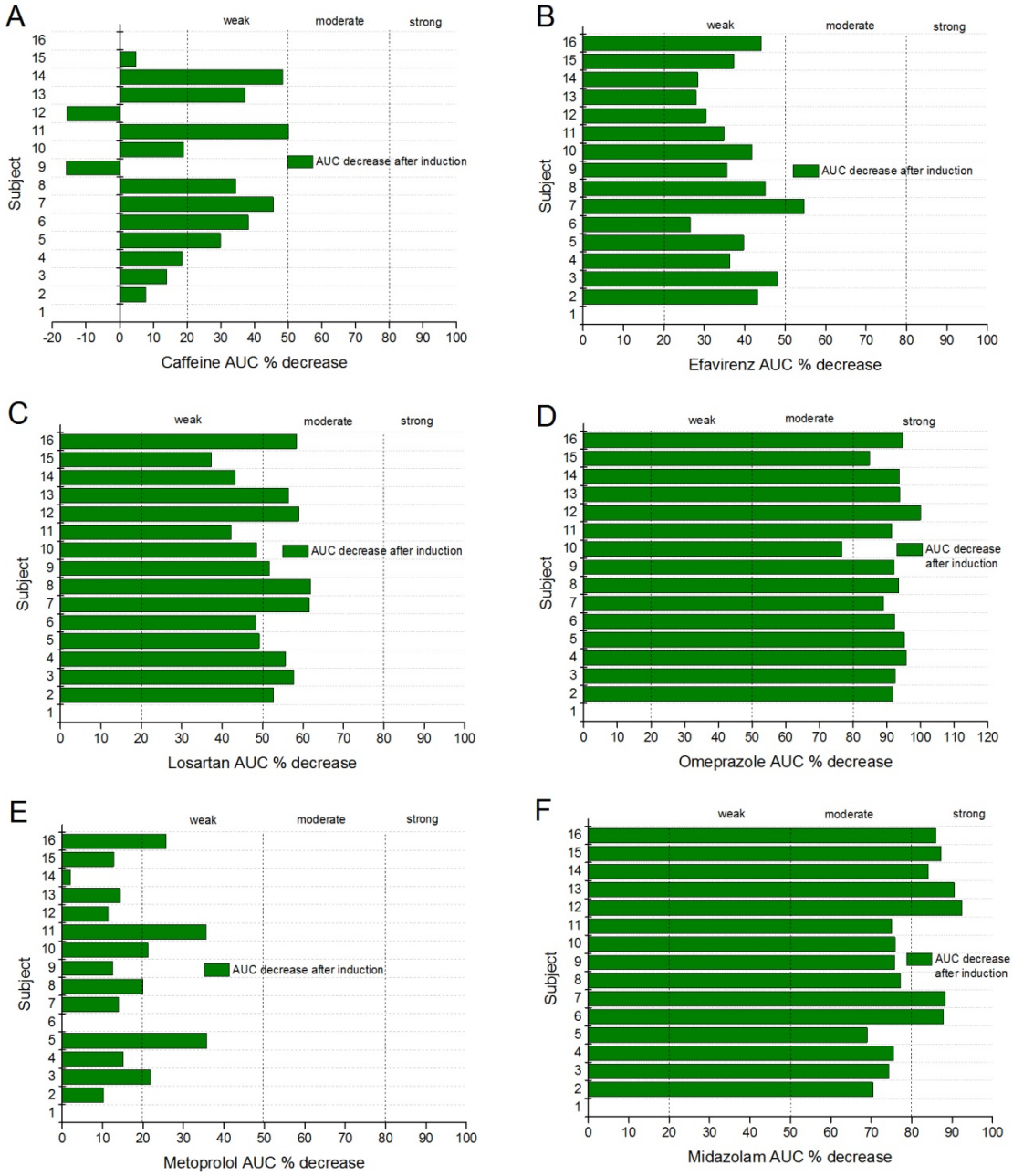


Figure 33. Effect of rifampicin on plasma $AUC_{0\text{-}24h}$ of parent drugs. $AUC_{0\text{-}24h}$ % decrease (100-$AUC_{0\text{-}24h}$ after administration of inducer / $AUC_{0\text{-}24h}$ X100) for CYP1A2 (A), CYP2B6 (B), CYP2C9 (C), CYP2C19 (D), CYP2D6 (E), and CYP3A4 (F).

7.3.3. Genotyping

Fifteen study participants gave their consent for genotype testing for altered function alleles (**Fehler! Verweisquelle konnte nicht gefunden werden.**). CYP2D6 genotyping identified one intermediate (IM), 14 extensive (EM) and no ultrarapid metabolizers. The IM subject (n. 10) showed the highest metabolic ratio at 8h after treatment A and C in plasma (9.0 and 8.0, respectively) and saliva (18.1 and 17.6, respectively). Therefore additional genotype testing was performed and a combination of a loss-of-function and a diminished-function allele (CYP2D6*4/*41) was observed.

For CYP2B6 and CYP2C9 no poor metabolizers were identified. Nine subjects were heterozygous for diminished-function alleles of CYP2B6 (CYP2B6*6, n=6) and/or CYP2C9 (CYP2C9*2, n=1 and CYP2C9*3, n=5). CYP2C19 genotyping identified four heterozygous subjects for a diminished-function allele (CYP2C19*2) and one poor metabolizer (CYP2C19 *2/*2). This subject (n. 15) showed the highest metabolic ratio at 2h in plasma (5.8) after treatment with rifampicin.
Genotyping of CYP1A2 identified four homozygous and ten heterozygous subjects for the *1F allele, which is associated with increased inducibility.

Data obtained from the CYP2D6 IM (subject 10) and the CYP2C19 PM (subject 15) were not included in the statistical analysis of metabolic ratios for CYP2D6 and CYP2C19, respectively.

Table 20: Results of genotyping analysis (Basel cocktail study II). WT, wildtype; HT, heterozygous; HO, homozygous; neg, gene deletion or duplication not observed. Subject 8 did not give consent for genotype testing. CYP2D6*41 was only tested in subject 10.

CYP	*Allele*	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7	Subject 9
1A2	*CYP1A2*1F*	***HT***	**HT**	***HO***	***HT***	***WT***	***HT***	***HT***	***HO***
2B6	*CYP2B6*6*	HT	WT	WT	HT	WT	HT	HT	HT
2C9	*CYP2C9*2*	WT	**WT**	WT	WT	**WT**	WT	WT	WT
	*CYP2C9*3*	WT	WT	WT	HT	HT	WT	HT	WT
2C19	*CYP2C19*2*	WT	WT	WT	WT	WT	**HT**	WT	HT
	*CYP2C19*3*	WT	WT	WT	WT	WT	WT	**WT**	WT
2D6	*CYP2D6*3*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2D6*4*	WT	**HT**	HT	HT	HT	WT	WT	WT
	*CYP2D6*5*	neg	neg	neg	neg	neg	neg	neg	neg
	*CYP2D6*6*	WT	WT	WT	WT	WT	WT	WT	WT
	*CYP2D6*XN*	neg	neg	neg	neg	neg	neg	neg	neg
	*CYP2D6*41*								

CYP	*Allele*	Subject 10	Subject 11	Subject 12	Subject 13	Subject 14	Subject 15	Subject 16	
1A2	*CYP1A2*1F*	***HO***	**HT**	***HT***	***HT***	***HT***	**HT**	**HO**	
2B6	*CYP2B6*6*	WT	HT	HT	HT	HT	WT	WT	
2C9	*CYP2C9*2*	**WT**	WT	WT	**WT**	**HT**	**WT**	WT	
	*CYP2C9*3*	WT	WT	HT	HT	WT	WT	WT	
2C19	*CYP2C19*2*	HT	WT	WT	**HT**	WT	HO	WT	
	*CYP2C19*3*	**WT**	WT	WT	WT	WT	**WT**	**WT**	
2D6	*CYP2D6*3*	WT	WT	WT	WT	WT	WT	WT	
	*CYP2D6*4*	HT	WT	HT	WT	WT	WT	HT	
	*CYP2D6*5*	neg	neg	neg	neg	neg	neg	neg	
	*CYP2D6*6*	WT	WT	WT	WT	WT	WT	WT	
	*CYP2D6*XN*	neg	neg	neg	neg	neg	neg	neg	
	*CYP2D6*41*	HT							

7.3.4. Effects of induction and inhibition on single point metabolic ratios in plasma

In the first Basel cocktail study metabolic ratios were proposed 2 h after dosing for CYP2C19 and CYP3A4, and 8 h after dosing for CYP1A2, CYP2B6, CYP2C9, and CYP2D6. We determined metabolic ratios at the same time-points in the second study. All study samples were analyzed using sensitive high performance liquid chromatography tandem mass spectrometry. Despite the high sensitivity of our analytical method, concentrations of metabolite or parent compound were not detectable in a number of subjects after pre-treatment with inhibitors or the inducer. Since non-detectability of the parent or of the metabolite is a consequence of strong inhibition or induction, we decided to substitute a concentration corresponding to the lower limit of quantification (e.g. 0.5 or 1 ng/mL) of the bioanalytical method for undetectable parent or metabolite concentrations. Substituting the LLOQ for non-detectable parent or metabolite concentrations allowed us to calculate an "artificial" metabolic ratio, representing the most cautious estimate of the true metabolic ratio that could be obtained if a more sensitive analytical method would be available.

The correlations of individual single point metabolic ratios with their corresponding AUC ratios for the 2 hour and 8 hour time-points are shown in Figure 34. In the first study the coefficients of determination were >0.8 for all correlations except for losartan, which had an R^2 of 0.59. For the second study the coefficients of determination after administration of the Basel cocktail were also >0.8 for all correlations except for omeprazole, which had an R^2 of 0.698.

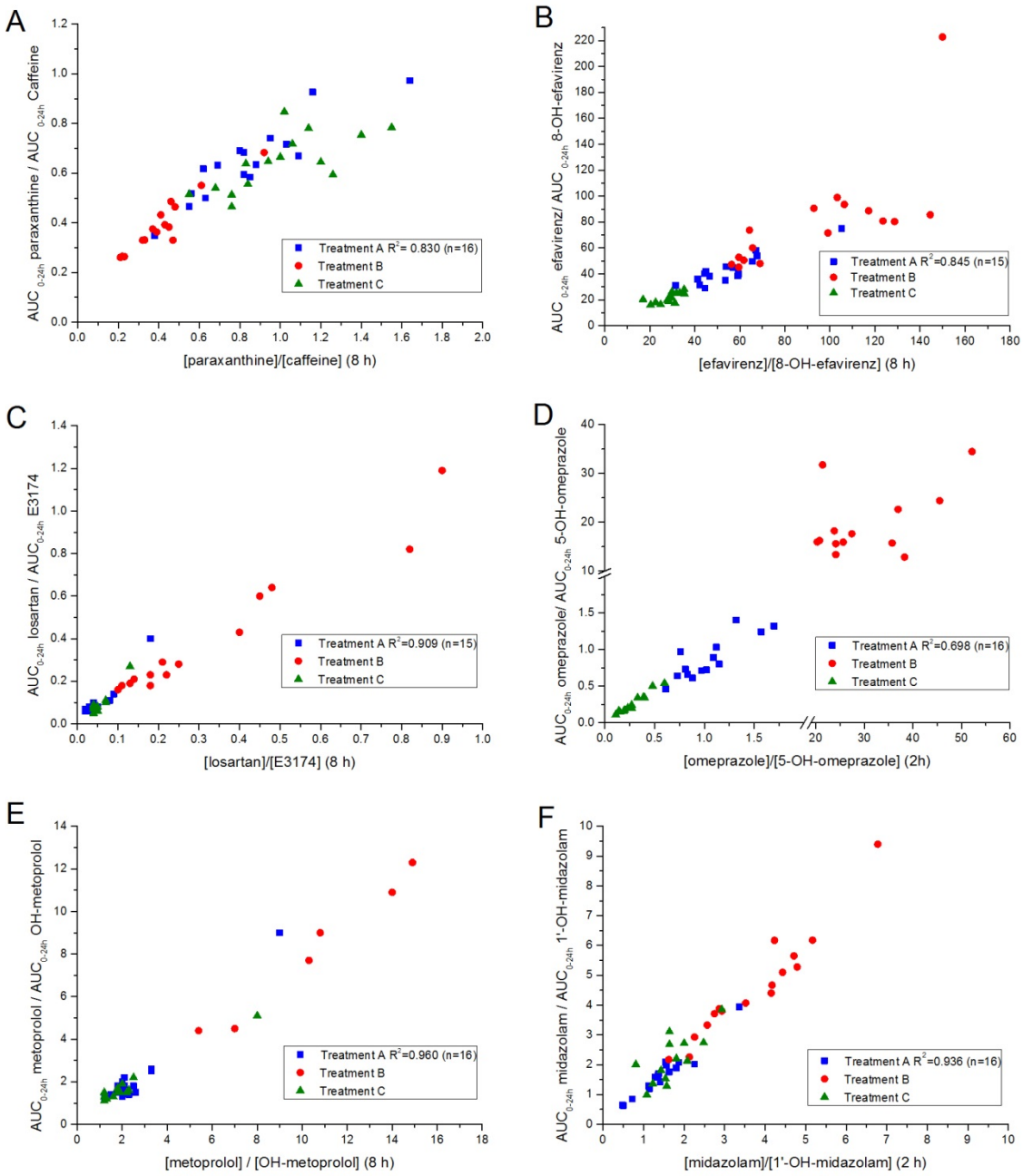


Figure 34. Effect of CYP-inhibitors (treatment B) and the CYP-inducer rifampicin (treatment C) on the correlation between AUC_{0-24h} ratios and single time-point concentration ratios of specific CYP substrates and their main metabolites in plasma for CYP1A2 (paraxanthine/caffeine at 8 hours, Figure 34A) , CYP2B6 (efavirenz/8-OH-efavirenz at 8 hours,Figure 34B), CYP2C9 (losartan / E3174 at 8 hours,Figure 34C), CYP2C19 (omeprazole /5-OH-omeprazole at 2 hours, Figure 34D), CYP2D6 (metoprolol/OH-metoprolol at 8 hours, Figure 34E), and CYP3A4 (midazolam/1'-OH-midazolam at 2 hours, Figure 34F).

Rifampicin is known to induce enzymes involved not only in phase I but also in phase II reactions. Considering only the concentration of the phase I metabolite (e.g. 1'-OH-midazolam) in cases where it is further metabolized by an inducible phase II reaction (e.g. glucuronidation), will result in metabolic ratios that do not adequately reflect the extent of induction. For a reasonable estimate of induction, all

induction products, i.e. phase I and phase II metabolites have to be taken into account. This can be done either by direct determination of the concentrations of the phase II metabolites (i.e. glucuronides) or indirectly by reverting the phase II reaction e.g. by incubating the samples with glucuronidase. For the cocktail probe drugs this indirect procedure was used for the metabolic ratios of CYP2B6 and CYP3A4 resulting in an improved separation of the metabolic ratios obtained after induction compared to baseline conditions (Figure 35). No glucuronidation products of other metabolites, indirectly assessed by a lacking increase of the phase I metabolite after treatment with β-glucuronidase, were observed.

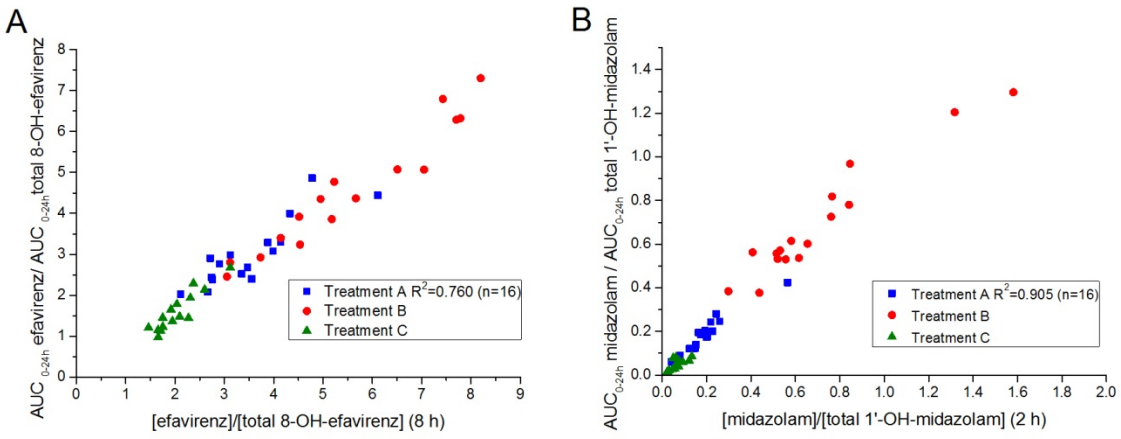


Figure 35. Effect of inhibitors (treatment B) and inducer (treatment C) on the correlation between $AUC_{0\text{-}24h}$ ratios and single time-point concentration ratios of specific CYP substrates and metabolites in plasma after treatment with glucuronidase for CYP2B6 (efavirenz/total 8-OH-efavirenz at 8 hours,Figure 35 A) and CYP3A4 (midazolam/total 1'-OH-midazolam at 2 hours,Figure 35 B).

Considering the total 8-OH-efavirenz (CYP2B6) and 1'-OH-midazolam (CYP3A4) results in a better separation of the metabolic ratios between treatment A and treatment C.

The effect of inhibition and induction on metabolic ratios was visualized using box plots (Figure 36 and Figure 37) and individual changes of metabolic ratios using line series graphs (Figure 38-Figure 44).

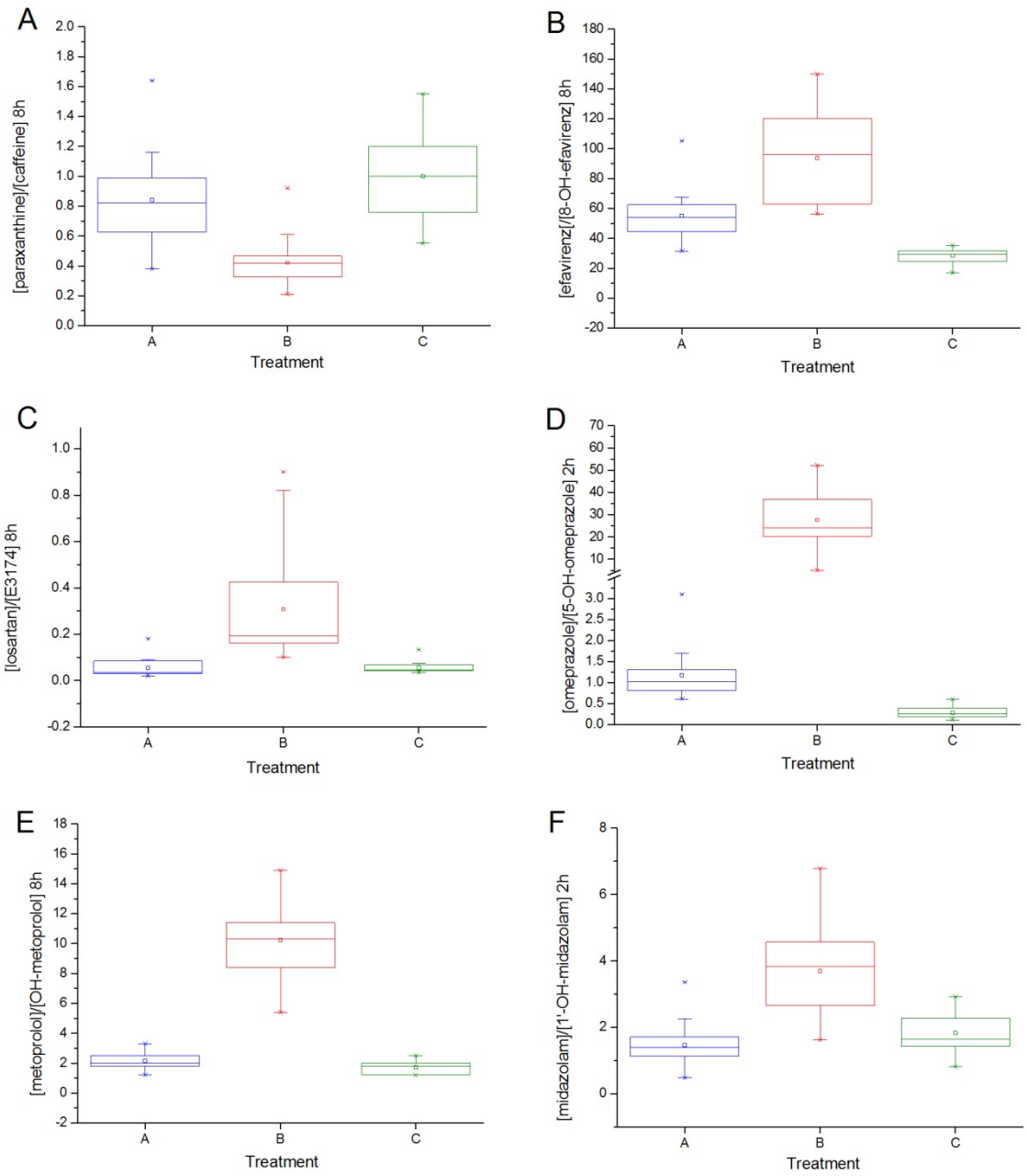


Figure 36. Box plot comparing the effect of inhibitors (treatment B) and inducer (treatment C) on the metabolic plasma ratio for CYP1A2 (Figure 36A) , CYP2B6 (Figure 36B), CYP2C9 (Figure 36C), CYP2C19 (Figure 36D), CYP2D6 (Figure 36E), and CYP3A4 (Figure 36F).

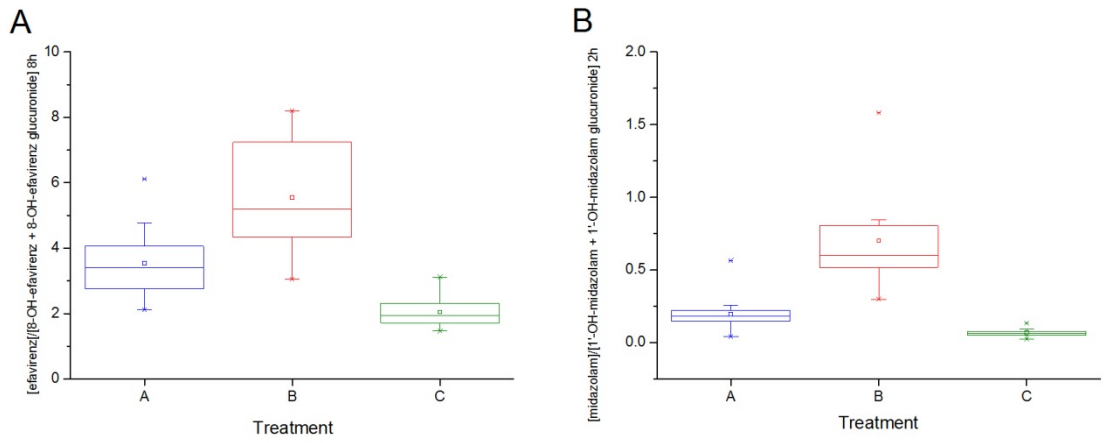


Figure 37. Box plot comparing the metabolic plasma ratios for CYP2B6 (A) and CYP3A4 (B) in the different treatments after enzymatic hydrolysis with glucuronidase.

The individual metabolic ratios of all subjects treated with inhibitors increased compared to baseline. Post hoc analysis confirmed a statistically significant difference between A and B for all CYPs. Comparison between treatment A and C, showed a non-significant difference between A and C for CYP1A2, CYP2C9 and CYP3A4. However, for CYP3A4 the difference between A and C treatment did become significant when the total deglucuronized OH-midazolam was used to calculate the metabolic ratio.

Table 21. Mean plasma metabolic ratios (MR) of the different CYP isoforms under study. Evaluation of the MR difference between treatment A and B and between treatment A and C by post hoc analysis.

CYP	Treatment A (baseline)			Treatment B (inhibition)			Treatment C (induction)		
	n	MR mean (range)	SD	n	MR mean (range)	SD	n	MR mean (range)	SD
1A2	16	**0.842** (0.38-1.64)	0.300	16	**0.421****** (0.21-0.92)	0.171	15	**1.00** (0.55-1.55)	0.277
2B6	16	**55.2** (31.5-105)	16.9	16	**93.8***** (56.3-150)	32.4	15	**28.4****** (17.0-35.4)	5.24
2B6 (deglucuronidation)	16	**3.54** (2.1-6.1)	0.994	16	**5.55***** (3.05-8.19)	1.70	15	**2.04****** (1.4-2.6)	0.434
2C9	16	**0.0543** (0.02-0.18)	0.0425	16	**0.308***** (0.1-0.9)	0.245	14	**0.0550** (0.04-0.13)	0.0247
2C19	15	**1.18** (0.61-3.1)	0.613	15	**28.0****** (10-52.2)	11.5	14	**0.284****** (0.11-0.6)	0.141
2D6	15	**2.16** (1.2-3.3)	0.579	15	**10.2****** (5.4-14.9)	2.55	14	**1.71*** (1.2-2.5)	0.445
3A4	16	**1.46** (0.48-3.36)	0.697	16	**3.69****** (1.62-6.78)	1.35	15	**1.83** (0.81-2.93)	0.625
3A4 (deglucuronidation)	16	**0.196** (0.04-0.56)	0.114	16	**0.702****** (0.29-1.3)	0.333	15	**0.0689***** (0.024-0.13)	0.0299

*, P<0.05 ***, P<0.001 ****, P<0.0001

7.3.5. Intra-individual changes of metabolic ratios under inhibition or induction

Line series graphs were used to visualize the intra-individual changes of the metabolic ratios in the three different conditions (CYP1A2 Figure 38, CYP2B6 Figure 39, CYP2C9 Figure 40, CYP2C19 Figure 41, CYP2D6 Figure 42, CYP3A4 Figure 43, CYP2B6 and CYP3A4 after treatment with glucuronidase Figure 44). The treatment with inhibitors resulted in a clear increase of MR (or decrease in the case of CYP1A2 due to the "inverse" metabolic ratio) in each subject. The pretreatment with the inducer did not change the CYP1A2, CYP2C9 or the CYP2D6 metabolic ratios. However, a clear reduction of the metabolic ratio in each subject was observed for CYP2B6 and for CYP2C19. CYP3A4 line series graphs showed a clear trend when the total OH-midazolam (i.e. after de-glucuronidation) was considered.

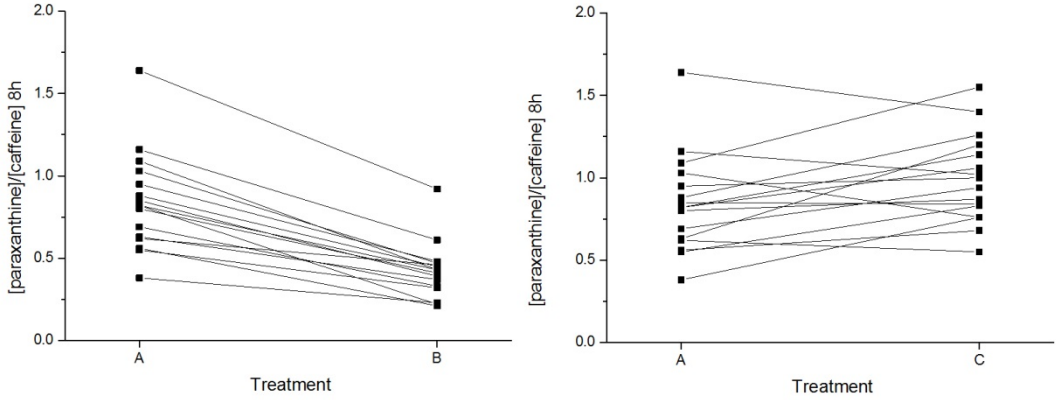


Figure 38. Comparison of the plasma MR of CYP1A2 after administration of the cocktail alone (treatment A) versus the cocktail co-administered with inhibitors (treatment B) or co-administered with inducer (treatment C).

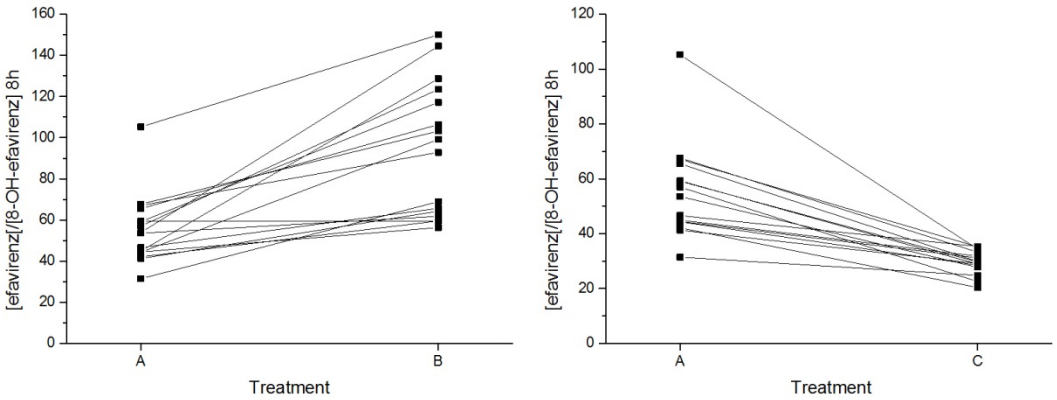


Figure 39. Comparison of the plasma MR of CYP2B6 after administration of the cocktail alone (treatment A) versus the cocktail co-administered with inhibitors (treatment B) or co-administered with inducer (treatment C).

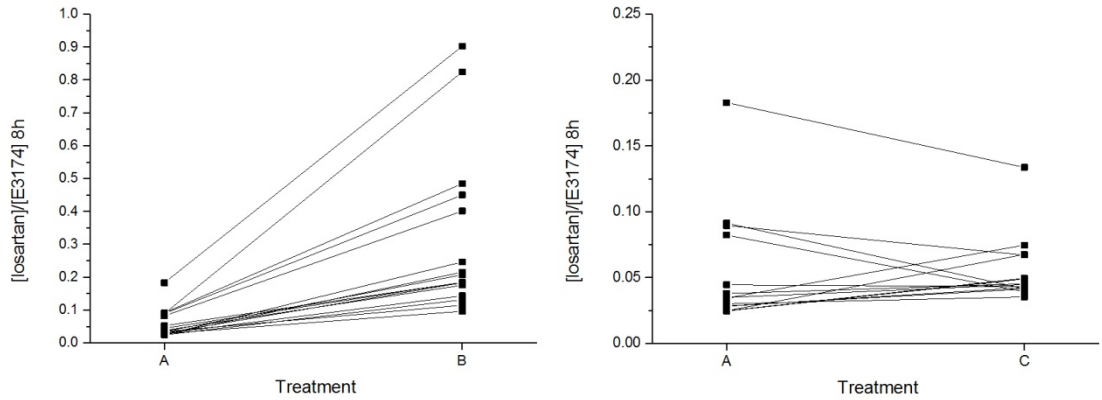


Figure 40. Comparison of the plasma MR of CYP2C9 after administration of the cocktail alone (treatment A) versus the cocktail co-administered with inhibitors (treatment B) or co-administered with inducer (treatment C).

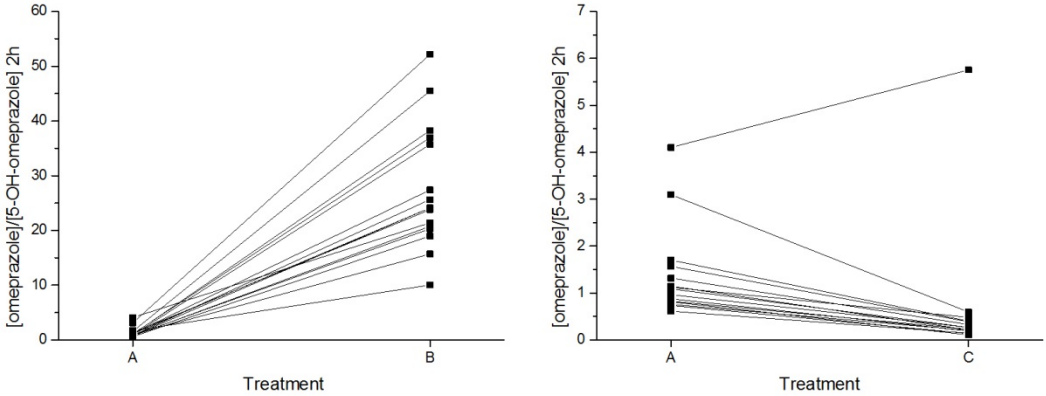


Figure 41. Comparison of the plasma MR of CYP2C19 after administration of the cocktail alone (treatment A) versus the cocktail co-administered with inhibitors (treatment B) or co-administered with inducer (treatment C). The subject with the highest MR after treatment A and C had a poor metabolizer genotype.

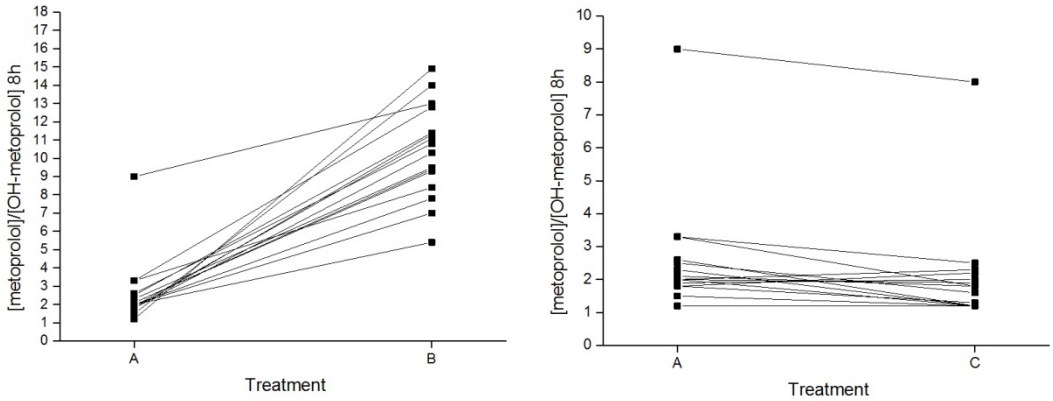


Figure 42. Comparison of the plasma MR of CYP2D6 after administration of the cocktail alone (treatment A) versus the cocktail co-administered with inhibitors (treatment B) or co-administered with inducer (treatment C). The subject with the highest MR after treatment A and C had an intermediate metabolizer genotype.

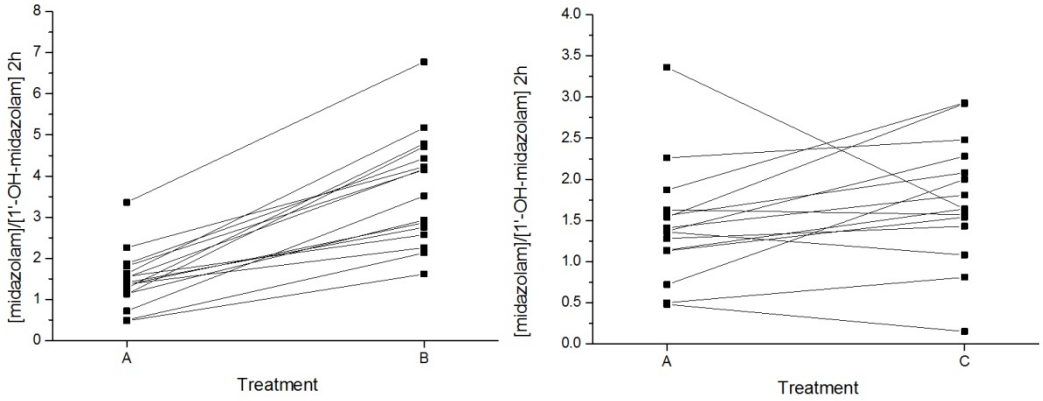


Figure 43. Comparison of the plasma MR of CYP3A4 after administration of the cocktail alone (treatment A) versus the cocktail co-administered with inhibitors (treatment B) or co-administered with inducer (treatment C).

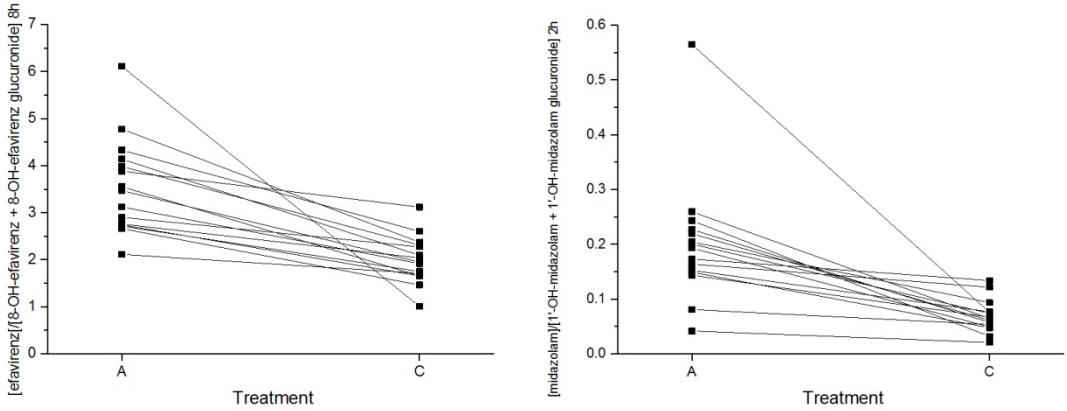


Figure 44. Comparison of the plasma MR of CYP2B6 and CYP3A4 after administration of the cocktail alone (treatment A) versus the cocktail co-administered with inducer (treatment C) after treatment with glucuronidase.

7.3.6. Effect of induction and inhibition on single point metabolic ratios in oral fluid and DBS

In saliva, the concentrations of all analytes except caffeine, paraxanthine, omeprazole, 5-hydroxy-omeprazole, metoprolol and hydroxy-metoprolol were too low to allow reliable quantification (LLOQ between 0.5 and 5 ng/ml, Table 8). Non-detectable concentrations of metabolite or parent compound were therefore replaced with the appropriate LLOQs to estimate the metabolic ratios in saliva.
In saliva the glucuronidation products present in plasma were not detected.

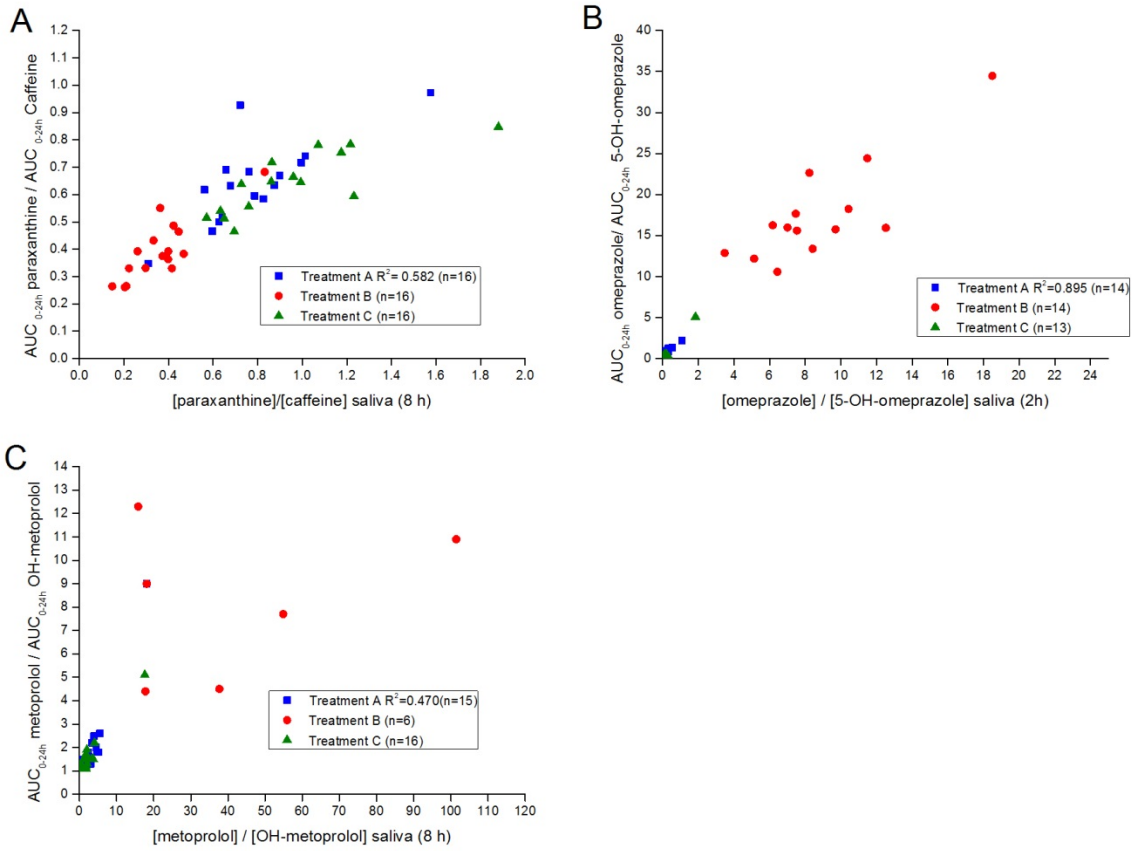


Figure 45. Effect of inhibitors (treatment B) and inducer (treatment C) on the correlation between $AUC_{0\text{-}24h}$ ratios and single time-point concentration ratios of specific CYP substrates and metabolites in saliva for CYP1A2 (paraxanthine/caffeine at 8 hours, Figure 45A) , CYP2C19 (omeprazole /5-OH-omeprazole at 2 hours, Figure 45B), and CYP2D6 (metoprolol/OH-metoprolol at 8 hours, Figure 45C).

Box plot and line series graphs of saliva metabolic ratios showed the same trend as observed in plasma (Figure 46, Figure 47, Figure 48, and Figure 49). In saliva the difference between the metabolic ratio A and C for CYP1A2 and CYP2C19 was not significant (Table 22).

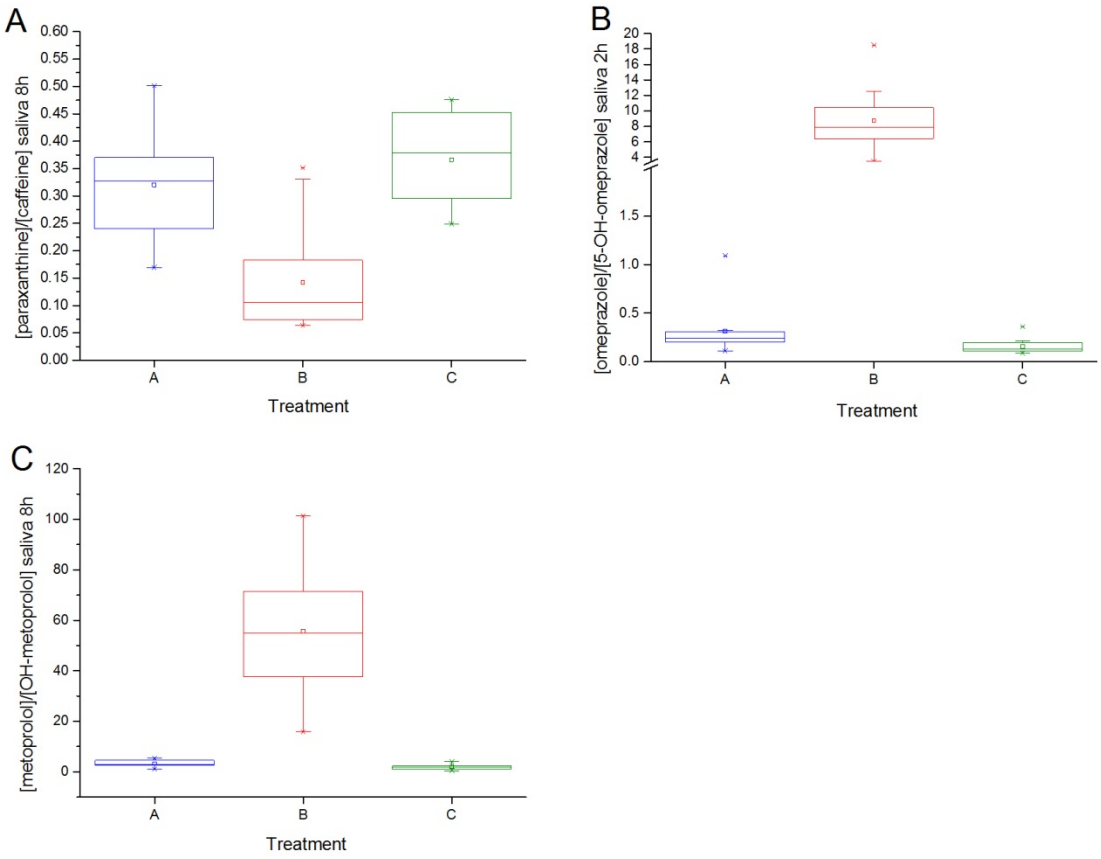


Figure 46. Box plot comparing the effect of inhibitors (treatment B) and inducer (treatment C) on the metabolic saliva ratio for CYP1A2 (Figure 46A), CYP2C19 (Figure 46B), and CYP2D6 (Figure 46C).

Table 22. Mean saliva metabolic ratios (MR) of the CYPs under study. Evaluation of the MR difference between treatment A and B and between treatment A and C by post hoc analysis.

CYP	Treatment A (baseline)			Treatment B (inhibition)			Treatment C (induction)		
	n	MR mean	SD	n	MR mean	SD	n	MR mean	SD
1A2	16	**0.783**	0.275	16	**0.362******	0.158	15	**0.953**	0.336
2C19	14	**0.315**	0.245	14	**8.75******	3.72	13	**0.173**	0.0954
2D6	15	**3.25**	1.34	15	**55.8******	26.6	14	**2.043****	1.06

**, P<0.01 ****, P<0.0001

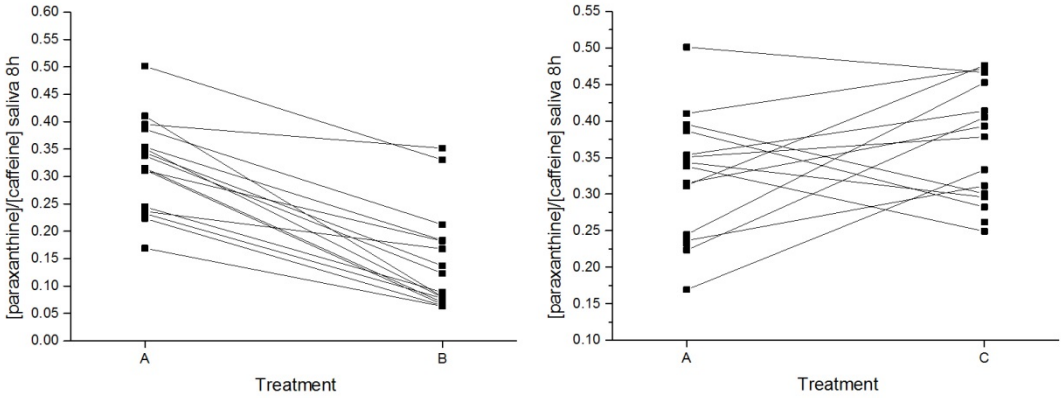


Figure 47. Comparison of the saliva MR of CYP1A2 after administration of the cocktail alone (treatment A) versus the cocktail co-administered with inhibitors (treatment B) or co-administered with inducer (treatment C).

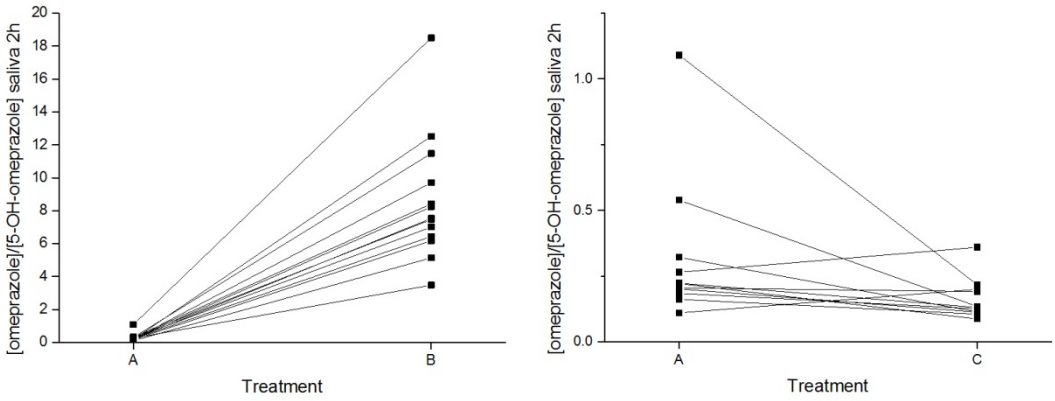


Figure 48. Comparison of the saliva MR of CYP2C19 after administration of the cocktail alone (treatment A) versus the cocktail co-administered with inhibitors (treatment B) or co-administered with inducer (treatment C). The subject with the poor metabolizer genotype showed non-detectable concentrations of omeprazole and OH-omeprazole in saliva 2h after administration of treatment A and B.

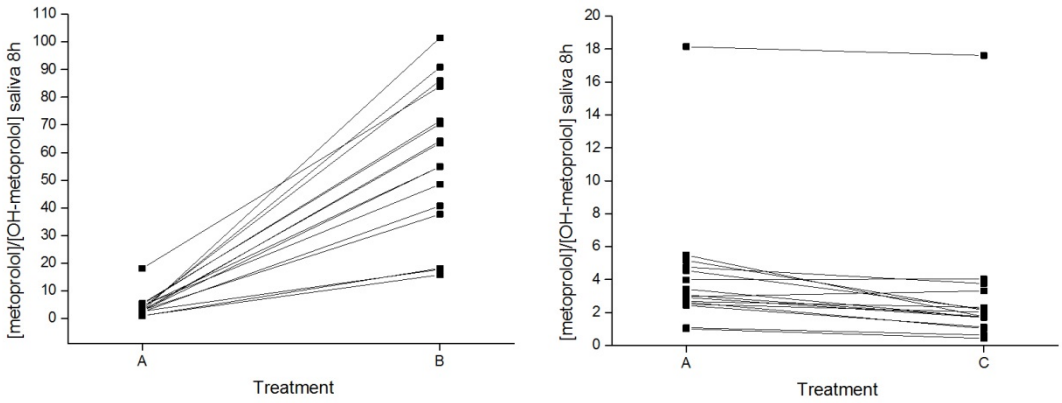


Figure 49. Comparison of the saliva MR of CYP2D6 after administration of the cocktail alone (treatment A) versus the cocktail co-administered with inhibitors (treatment B) or co-administered with inducer (treatment C). The subject with the highest MR after treatment A and C had an intermediate metabolizer genotype.

DBS samples were also analysed. The LLOQ for losartan, E-3174, and OH-efavirenz was not low enough to quantify these analytes in DBS. Analysis will need to be repeated with a more sensitive method specifically adapted to DBS applications.

7.3.7. Reproducibility of single point plasma metabolic ratios measured in the two Basel cocktail studies

The metabolic ratios measured in the first and the second Basel cocktail studies are displayed in a box-plot (Figure 50). For the first cocktail study not only the metabolic ratios measured in treatment E were considered, but also the MRs from the safe 3-drug combination. For the second study, only the MRs after treatment A are reported. Four subjects participated in both studies. Their MRs measured in the first (October-December 2010) and in the second study (October-December 2011) are reported in Figure 51.

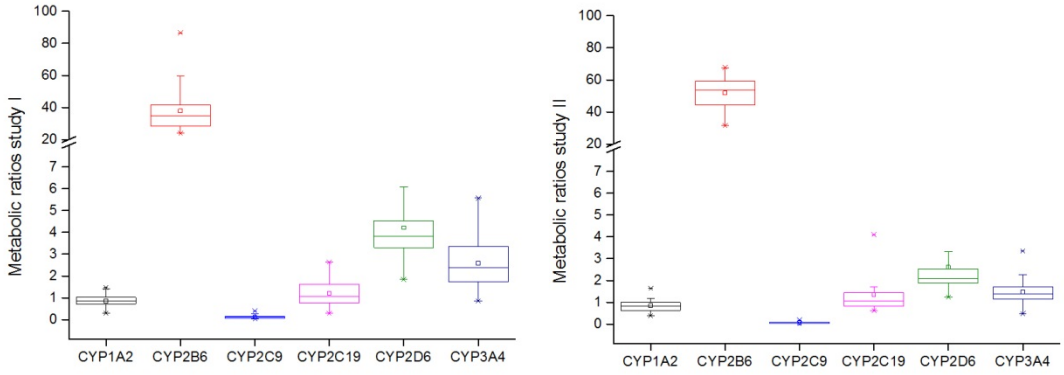


Figure 50. Metabolic ratios measured in the first and the second Basel cocktail study

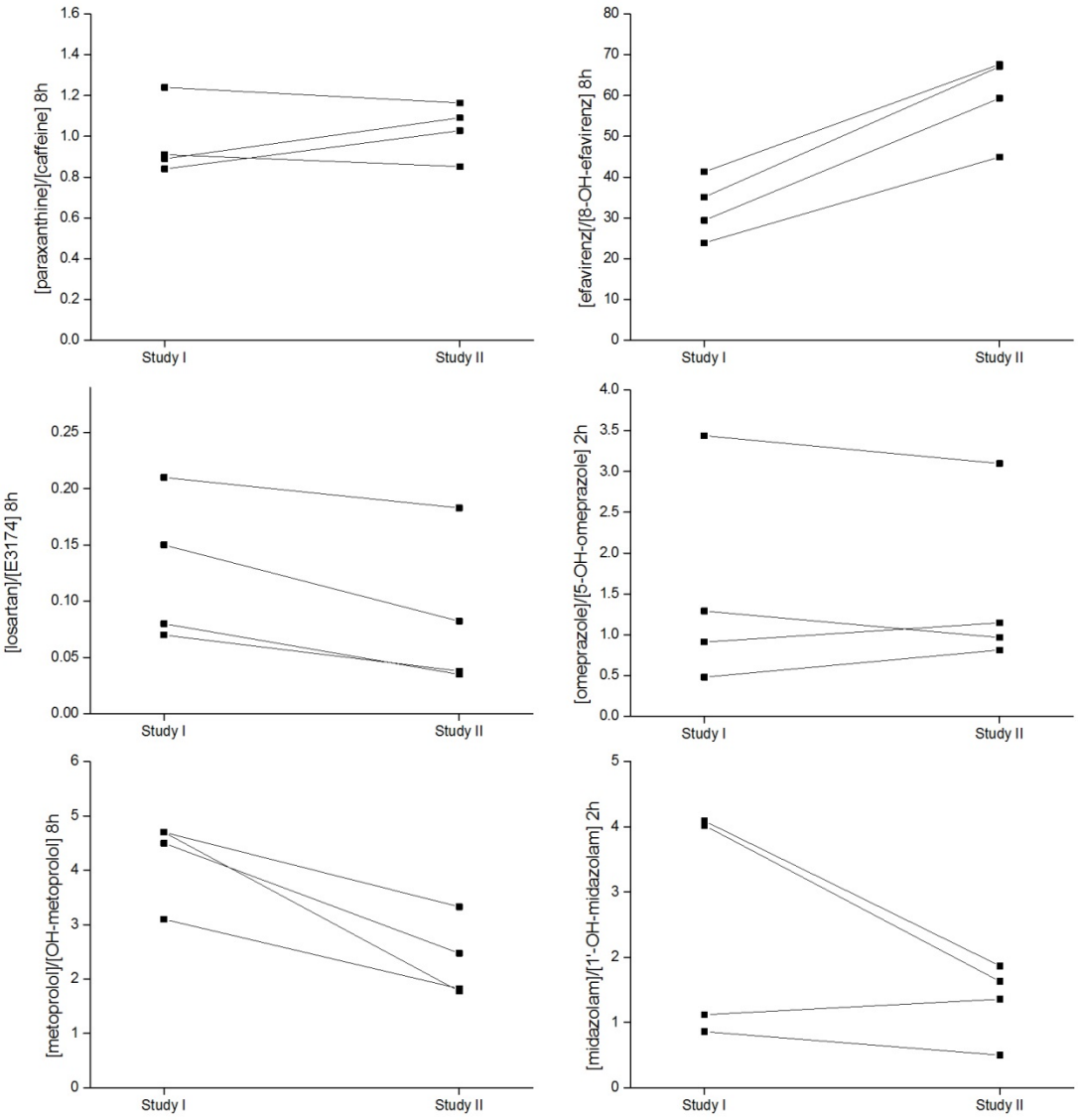


Figure 51. Metabolic ratios of the four subjects that took part in both studies.

7.4.Discussion

In the first Basel cocktail study we showed that a new probe drug combination can be used simultaneously without mutual interactions. We demonstrated that single point metabolic ratios (MRs) collected 2 and 8h after administration of the phenotyping drugs correlate with the AUC ratios and therefore we proposed these single timepoint determinations to simplify the sampling procedure. In the second Basel cocktail study we have performed additional validation steps and evaluated the effects of inducers and inhibitors. According to FDA guidelines the increase or decrease of AUC of the phenotyping drug before and after the administration of the potential inhibitor or inducer of a drug metabolizing enzyme should be measured to determine whether a drug acts as an inducer or inhibitor. The choice of inhibitors and inducers for the second study was based on the criteria mentioned in paragraphs 3.5. According to literature data, paroxetine is a potent inhibitor of CYP2D6 and a weak inhibitor of CYP2B6, whereas fluconazole inhibits CYP2C9, CYP2C19, and CYP3A4, while ciprofloxacin is a weak inhibitor of CYP1A2. Rifampicin is a well-documented inducer of CYP2B6, CYP2C9, CYP2C19, and CYP3A4. In our study, when considering AUC fold increase, we observed no inhibition of CYP2B6 and weak to strong inhibition of the others CYPs (Table 18, Figure 32), while when considering AUC decrease after administration of rifampicin, we measured a weak induction of CYP1A2 and CYP2B6 and a weak to strong induction of the other CYPs (Table 19, Figure 33). For most of the cocktails published so far no data about the change of the phenotyping metric under conditions of induction or inhibition is available. The only exception is the Karolinska cocktail which has been tested after pretreatment with rifampicin [69]. Using the same probe substrates and MRs as in our study for CYP1A2, CYP2C9 and CYP2C19, the authors showed the MRs to be sensitive to rifampicin induction (500mg during 14 days). Unlike that study, we used plasma to quantify the CYP2C9 MR instead of urine. Moreover the induction of CYP3A4 was already observed after pretreatment with a low dose of rifampicin (20 mg) using both quinine/3'-hydroxyquinine and 4β-hydroxycholesterol MRs as phenotyping indexes. Despite the CYP2C9 MR variation after induction being statistically significant, a relevant intra-subject change was only observed in a few subjects.

The use of MRs instead of full pharmacokinetic profiles considerably simplifies sample collection for phenotyping, e.g. for clinical applications, and has already been extensively documented in previous studies [16,104]. In the present study we confirmed the high correlation between plasma MRs and AUC ratios of the phenotyping drugs under baseline conditions. In the first study the R^2 for all correlations were higher than 0.592, while in the second study all R^2 were higher than 0.698.

One of the objectives of the second Basel cocktail study was to evaluate whether the effect of inducers and inhibitor on AUC, the reference procedure suggested by FDA, could be observed to a same extent using MRs. After pre-treatment with a specific CYP inhibitor, a clear separation of the MRs compared to baseline was observed for CYP2C19 and CYP2D6. This becomes apparent by the non-overlapping boxplots and the clearly different ranges of MRs (Table 21). Subjects with a CYP2C19 EM genotype had baseline MRs between 0.61 and 3.1, while after pre-treatment with the inhibitor their MRs were between 10.0 and 52.2. Subjects with a CYP2D6 EM genotype showed baseline MRs between 1.2 and 3.3, while after inhibition their MRs moved to a range between 5.4 and 14.9. Considering intra-subject variations, MRs were just as sensitive indicators of inhibition as the extent of AUC increase, the reference approach. In all subjects an increase of MR (decrease for CYP1A2) was observed between

basal concentrations and post-inhibition concentrations with statistically significant differences for all the CYPs.

Compared to inhibition, the effects of induction were less evident in changes of AUCs and MRs. A significant within-subject decrease of MRs was observed for CYP2B6, CYP2C19, and CYP3A4 ($p<0.001$ when total OH-midazolam was considered). For CYP1A2, metabolic ratios after pre-treatment with rifampicin did not show significant differences compared to baseline. This can be explained by the fact that rifampicin is not an ideal inducer of CYP1A2. Potent inducers of CYP1A2 are halogenated or polycyclic aromatic hydrocarbons (e.g. present in cigarette smoke) that act via the aryl hydrocarbon receptor, which is not activated by rifampicin [105]. Therefore, induction studies on CYP1A2 are generally performed by comparing cigarette smokers with non-smokers, a strategy that was not feasible for our cocktail study due to logistical reasons.

We introduced the use of LLOQs to calculate the MRs for cases where the concentrations of parent compound or metabolite were below the limit of quantification of the analytical method. This approach takes into consideration that the non-detectability of the parent or the metabolite also provides relevant information regarding induction or inhibition.

CYP2D6 is regarded as a non-inducible CYP isoform. In this study, five subjects showed an AUC decrease between 20 and 50%, formally corresponding to weak induction while the other subjects showed a non-relevant AUC decrease lower than 20%. A small but statistically significant decrease of MRs ($p<0.05$) was observed. In 1982 Bennet et al [106] reported a decrease of 30% in metoprolol AUC after the administration of 600 mg of rifampicin for 13 days. Our findings show a less pronounced decrease of AUC. Induction of other metabolic pathways of metoprolol could explain such an effect. However, a search of the literature did not reveal any studies confirming Bennet's findings or supporting the hypothesis of alternative metabolic pathways. Metoprolol appears to be a good probe drug for phenotyping itself and in the presence of inhibitors, but is probably not the best choice when performing induction studies. The hypothesis of alternative CYP isoforms involved in metoprolol metabolism after induction with rifampicin should be followed up e.g. with in vitro experiments using supersomes expressing specific CYP isoforms.

Induction of CYP2C9 using rifampicin was weak to moderate when estimated by extent of AUC decrease, while decrease of losartan/E3174 plasma ratios was not significant. In 2012, during completion of our study, Daali et al suggested the use of flurbiprofen to phenotype CYP2C9 measuring 4'-hydroxy-flurbiprofen/flurbiprofen metabolic ratio in plasma and dried blood spots 2h after administration of 50 mg of flurbiprofen [17]. The authors showed that the proposed metabolic ratios were sensitive to fluconazole inhibition and to rifampicin induction. Flurbiprofen seems to be a valuable alternative to the use of losartan to perform the phenotyping CYP2C9.

During the evaluation of our data, Jiang et al [39] published a study proposing the use 8,14-dihydroxy-efavirenz and efavirenz $AUC_{0\text{-}120h}$ ratio for CYP2B6 phenotyping. We adapted the analytical method to include 8,14-dihydroxy-efavirenz and repeated the analysis of plasma and saliva samples. The concentrations of 8,14-dihydroxy-efavirenz 8 hours after cocktail administration were, however, below the limit of quantification before enzymatic hydrolysis, and lower than 6 ng/ml after treatment with

glucuronidase. Overall, the observed concentrations of 8,14-dihydroxy-efavirenz were negligible compared to the measured concentrations of 8-hydroxy-efavirenz. We therefore decided to not further consider the di-hydroxy metabolite in our data analysis.

MRs appear to be less suited than AUCs to monitor induction, if sequential enzymes of the metabolic cascade are involved. If induction affects enzymes responsible for the decrease of both parent compound and phase I metabolite concentration, the ratio may not change. This phenomenon was observed with midazolam and efavirenz. Rifampicin is an inducer of phase I but also of phase II UGT mediated metabolism. After treatment with β-glucuronidase, we observed an increase of the concentrations of 1'- OH-midazolam and 8-OH-efavirenz, showing that phase II metabolism concerns only the isoforms CYP3A4 and CYP2B6 in our cocktail. The total amount of metabolites (i.e. the sum of phase I and phase II metabolites) has to be taken into account for the calculation of MRs of these two CYPisoforms [16].

While the AUC fold increase or % decrease is a quantitative measure of inhibition and induction (weak, moderate, or strong), MRs can only give qualitative information. A quantitative evaluation of MR change as for AUC was explored but the correlation results were not satisfactory. AUCs that depend on parent compound should not be compared with MR ratios, which consider parent and metabolite.

The metabolic ratios measured for baseline treatment in the first and in the second Basel cocktail study were collected and compared. The mean MR values for all the CYPs were comparable. In four subjects that participated in both studies, an intra-subject comparison was performed. Reproducibility of MRs is fundamental for clinical applications of phenotyping, that requires MRs to be stable if no external factors cause a change of the phenotype. Despite these limited set of data, we observed a systematic MR decrease after one year, that was more pronounced for CYP2B6 and CYP2D6. Attributing such a decrease to external causes for all subjects to a same extent does not seem likely. The more sensitive analytical method used for the analysis of the second study may be the cause of the observed discrepancies.

The possibility of performing less invasive samplings by measuring the phenotyping drugs in oral fluid and DBS was also investigated in this study. In saliva, MRs could be calculated for CYP1A2, CYP2C19 and CYP2D6. The correlation of saliva metabolic ratios for CYP1A2 at 8 hours with the corresponding plasma AUC ratios was lower than previously observed (previous study R^2=0.812, actual study R^2=0.582) whereas the saliva metabolic ratios at 2 hours for CYP2C19 were good (previous study R^2=0.473, actual study R^2=0.895). For CYP2D6 the correlation between the MRs in saliva and plasma AUC ratio was 0.470. Comparison of the saliva MR between treatment A and B by post hoc analysis confirmed the findings observed in plasma. Comparison of the saliva MR between treatment A and C by post hoc analysis produced the same results observed in plasma only for CYP1A2 and CYP2D6. In saliva CYP2C19 metabolic ratio did not decrease significantly after rifampicin treatment.

A considerable effort was devoted to improve the analytical method used in the first study for DBS analysis. The analytical method used for the second study had improved sensitivity but still not enough to quantify the low metabolite concentrations after inhibition or the low parent concentrations after

induction. Before being able to perform phenotyping using DBS for all major CYP isoforms and different levels of CYP activity the sensitivity needs be considerably improved.

7.5.Conclusions

We compared the pharmacokinetics profiles and metabolic ratios of the Basel phenotyping drugs at baseline levels, after co-administration of inhibitors, and a broad CYP inducer. We showed that plasma metabolic ratios provide valuable qualitative information on the activity of relevant drug metabolizing CYP isoforms and constitute a valuable alternative to the reference method based on AUC measurements. So far, for the majority of the published phenotyping cocktail, no validation data under conditions of CYP inhibition or induction has been published. Induction of CYP1A2 was not effective, therefore we were not able to estimate if the MR for this CYP is suitable to detect induction. Losartan, the drug used to phenotype CYP2C9, produced metabolic ratios that did not reflect induction. For all the other CYPs plasma MRs were highly correlated with changes of AUC ratios. Ranges of the chosen phenotyping metrics under conditions of induction and inhibition have been presented. As more data will be collected in the future, these ranges can be expected to gain sufficient validity to allow sufficiently accurate phenotype determination. Suitability of saliva sampling has been shown for phenotyping of CYP1A2, CYP2C19, and CYP2D6. However, also for this non-invasive sampling method additional validation data will have to be collected.

8. Ongoing projects

8.1.Currently running applications: the Combi-pill

8.1.1. Introduction

In both Basel cocktail studies, marketed formulations of caffeine, efavirenz, losartan, omeprazole, metoprolol and a low-dose midazolam formulation, prepared by the hospital pharmacy, were administered. The lowest available doses were selected and applied. The dosing as well as the release mechanisms of commercially available formulations were developed and approved for a specific therapeutic application, not for phenotyping applications. For example, we used a 12.5 mg metoprolol prolonged release formulation, which is the only one available in Switzerland or in the EU, to perform the phenotyping of CYP2D6. An oral low- dose oral formulation of midazolam was not available, therefore a formulation provided by the Basel hospital pharmacy was used for the cocktail studies. It becomes apparent that a single tablet containing all the Basel cocktail components at a dose and in a formulation adapted to be used for phenotyping, would be an important step forward in the development of a phenotyping cocktail which could be used routinely as a diagnostic tool in clinical practice.

Several drugs included in the same formulation are known as combination drugs and are already used in clinical practice medicine. Typical examples are combinations of anti-infective drugs (e.g. amoxicillin+ clavulanic acid, antiretroviral drug combinations) or antihypertensive drugs (e.g. ACE-inhibitors + thiazide). Augmentin is a preparation of clavulanic acid and amoxicillin, HIV treatment Truvada, a combination of tenofovir and emtricitabine or Atripla (efavirenz, tenofovir and emtricitabine), just to mention the most important.

The main advantages related with combi-pills are improved medication compliance in subjects that already received several medications, a modulated and adapted pharmacokinetic profile, an improved pharmacodynamic effects and consequently ideally a reduction of side effects of the drugs combination.

The Basel University Pharmaceutical Technology Department has developed, to make these advantages available for phenotyping, a prototype of a single pill containing the six Basel cocktail substrates.

After preliminary testing (dissolution tests, content uniformity and drug-drug compatibility tests) the combi-pill prototype was administered in a pilot clinical trial to one healthy volunteer. The plasma PK profiles generated with the new formulation were compared with the profiles measured after intake of the individual probe drug formulations used in the two Basel phenotyping cocktail studies.

8.1.2. Results and discussion

An immediate release formulation containing metoprolol (10 mg, Sigma), S-omeprazole (10 mg, NEXUIM pellets), caffeine (10 mg, Sigma), efavirenz (50 mg, Stocrin tablets), losartan (12.5 mg, Cosaar tablets) and midazolam (2 mg, Dormicum tablets) was prepared by Puckov et al (Figure 52).

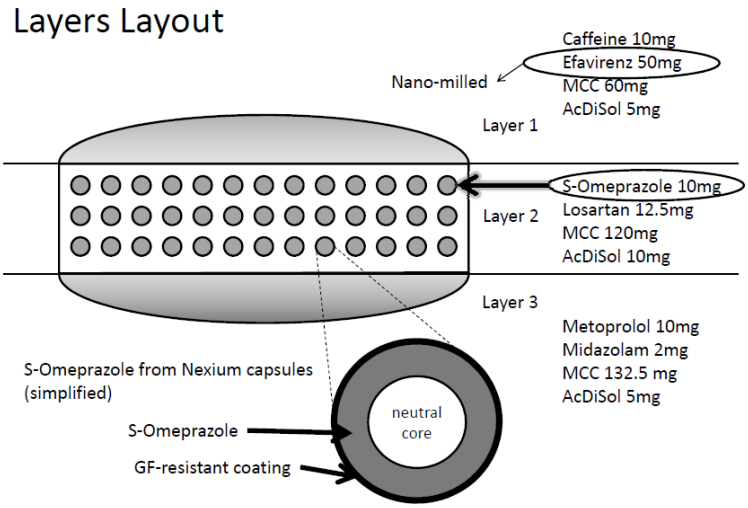
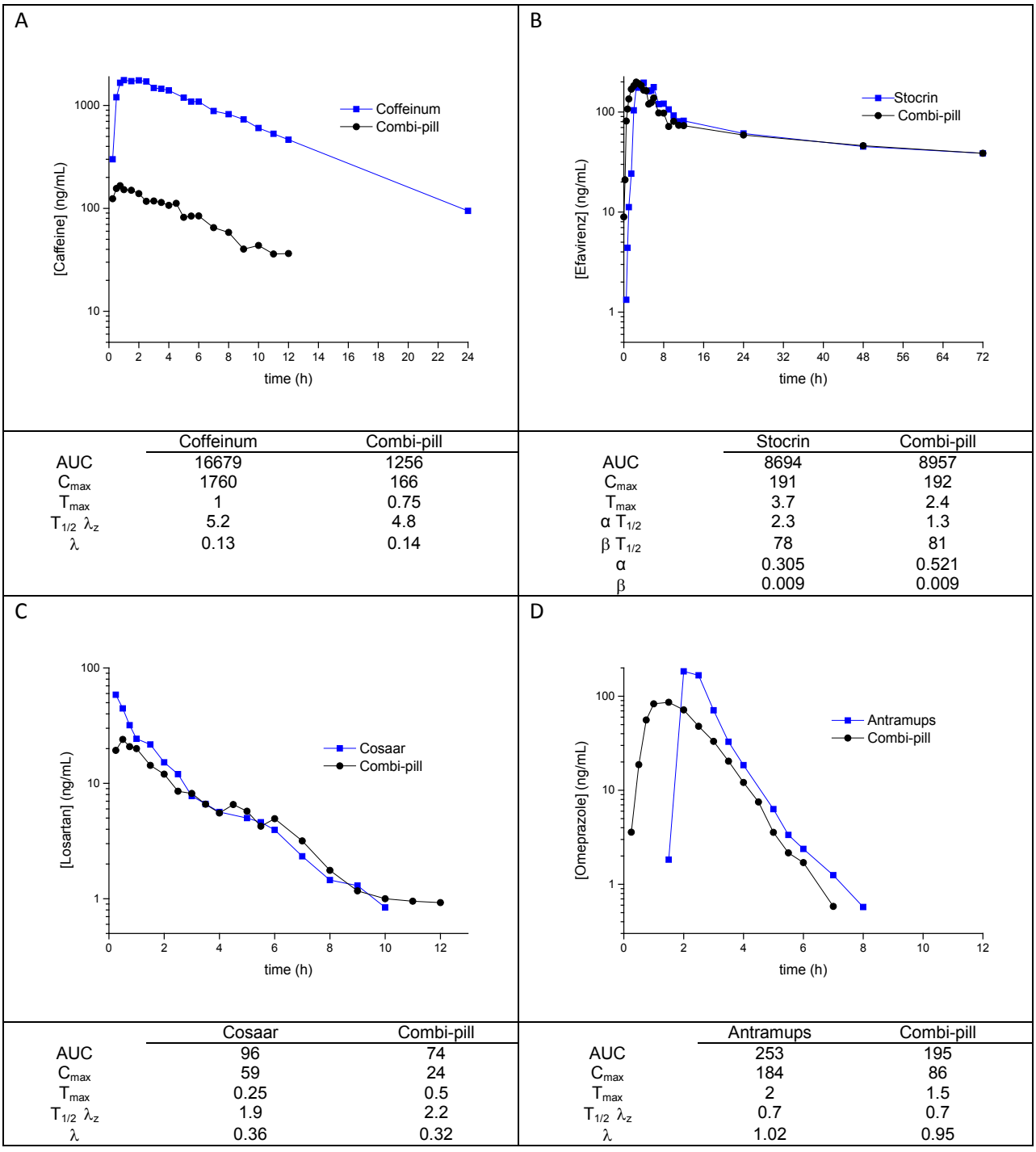


Figure 52. Layering and composition of the Combi-pill prototype

Prior to the *in vivo* study, an in vitro comparative dissolution and content uniformity test was performed. The dissolution patterns of the new and the reference formulation were comparable and the results of the content uniformity test fulfilled the requirements (data not shown).

The combi-pill prototype was tested in one healthy volunteer in an exploratory *in vivo* bioequivalence study.



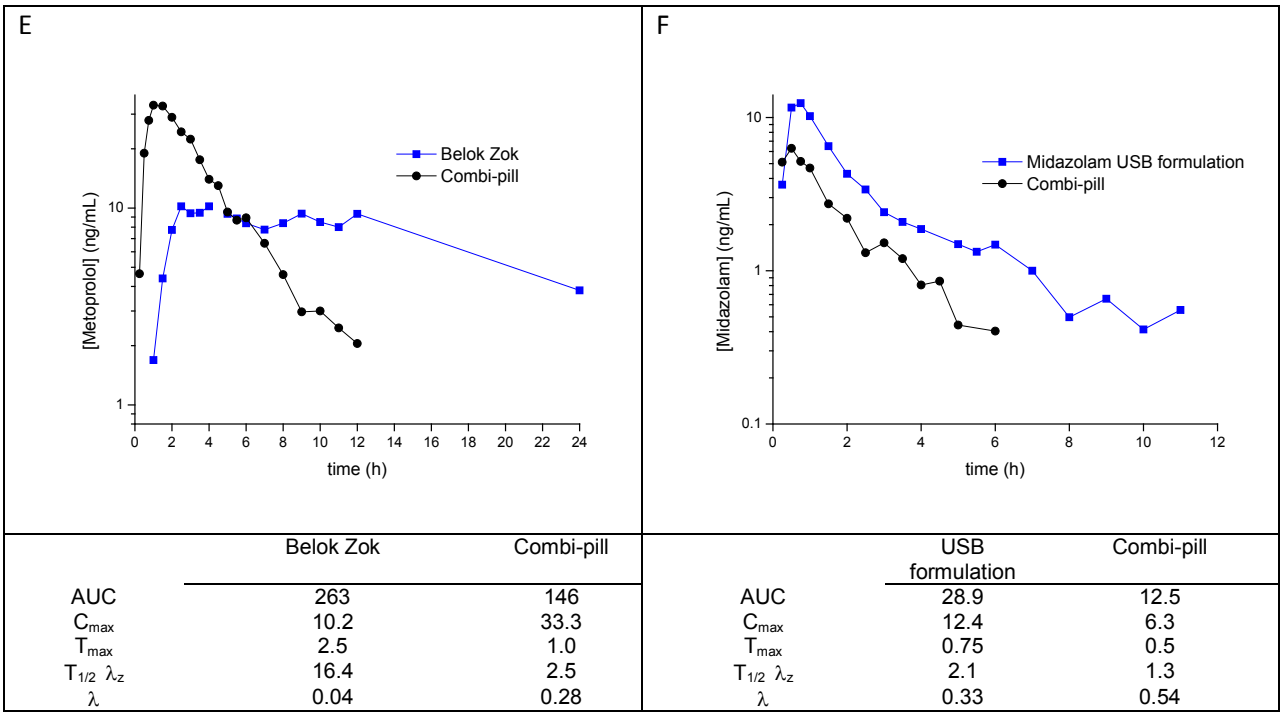
	Coffeinum	Combi-pill
AUC	16679	1256
C_{max}	1760	166
T_{max}	1	0.75
$T_{1/2}$ λ_z	5.2	4.8
λ	0.13	0.14

	Stocrin	Combi-pill
AUC	8694	8957
C_{max}	191	192
T_{max}	3.7	2.4
α $T_{1/2}$	2.3	1.3
β $T_{1/2}$	78	81
α	0.305	0.521
β	0.009	0.009

	Cosaar	Combi-pill
AUC	96	74
C_{max}	59	24
T_{max}	0.25	0.5
$T_{1/2}$ λ_z	1.9	2.2
λ	0.36	0.32

	Antramups	Combi-pill
AUC	253	195
C_{max}	184	86
T_{max}	2	1.5
$T_{1/2}$ λ_z	0.7	0.7
λ	1.02	0.95

Figure 53 (A-D). Plasma concentration time-profiles and PK parameters of the cocktail probe drugs obtained after application of marketed formulations of caffeine (A), efavirenz (B), losartan (C), omeprazole (D), metoprolol (E), midazolam (F, formulation prepared by the hospital pharmacy) and the same phenotyping drugs in the combi-pill.



	Belok Zok	Combi-pill
AUC	263	146
C_{max}	10.2	33.3
T_{max}	2.5	1.0
$T_{1/2}$ λ_z	16.4	2.5
λ	0.04	0.28

	USB formulation	Combi-pill
AUC	28.9	12.5
C_{max}	12.4	6.3
T_{max}	0.75	0.5
$T_{1/2}$ λ_z	2.1	1.3
λ	0.33	0.54

Figure 53 (E-F). Plasma concentration time-profiles and PK parameters of the cocktail probe drugs obtained after application of marketed formulations of caffeine (A), efavirenz (B), losartan (C), omeprazole (D), metoprolol (E) , midazolam (F, formulation prepared by the hospital pharmacy) and the same phenotyping drugs in the combi-pill.

To determine whether a new formulation has the same rate and extent of absorption as a reference drug, an *in-vitro* dissolution test followed by an *in-vivo* bioequivalence study is required. According to FDA guidelines, peak drug concentration (Cmax) is used to estimate drug rate absorption while the area under the curve (AUC) reflects the extent of absorption. Two treatments or formulations are equivalent if the 90% confidence interval of the geometric mean test/reference ratio for Cmax and AUC fall within the standard bioequivalence limits of 0.8 to 1.25. If the intra-subject variability of the PK parameters is higher than 30%, the drug is considered as highly variable and wider acceptance limits, e.g. 0.7 to 1.43 may be applied. However, as shown in the first Basel cocktail study, none of the probe drugs showed high variability and thus the acceptance limits for the planned bioequivalence study with the combi-pill will also be the standard 0.8-1.25.

Pharmacokinetic profiles and PK parameters after administration of Coffeinum (caffeine, 100 mg) and the combi-pill (caffeine, 10 mg) were comparable (Figure 53 A). AUC and Cmax were as expected 10 fold lower. Efavirenz administered as Stocrin® and as combi-pill showed comparable AUC and Cmax. Tmax was shorter for the combi-pill (Figure 53 B). Losartan AUC and particularly Cmax were higher after Cosaar® compared to the combi-pill, indicating a slower release from the combi-pill (Figure 53 C).

The combi-pill was formulated using only (S)-omeprazole, while Antramups administered in the Basel cocktail studies is a 1:1 mixture of (R)- and (S)-omeprazole. The two isomers have different metabolic pathways, therefore the omeprazole and OH-omeprazole PK profiles of the combi-pill differ from the PK

profile of the racemate. (S)-omeprazole is metabolized by CYP2C19 to 5-O-desmethyl omeprazole (46%) and 5-hydroxy-omeprazole (27%), but an important role is also played by CYP3A4 that is responsible for the formation of the sulphone (27%). The (R)-isomer is a specific substrate for CYP2C19, responsible for the production of the main metabolite 5-hydroxy-omeprazole (94%). Only negligible amount of 5-O-desmethyl omeprazole and sulphone are produced. As expected, the OH-omeprazole AUC of the combi-pill was lower than the OH-omeprazole AUC measured when the racemate was administered (Figure 53 D and Figure 54). Therefore, the combi-pill that will be used for the third Basel cocktail study has been reformulated, replacing (S)-omeprazole with the racemate to obtain PK profiles comparable to the ones after intake of the racemate.

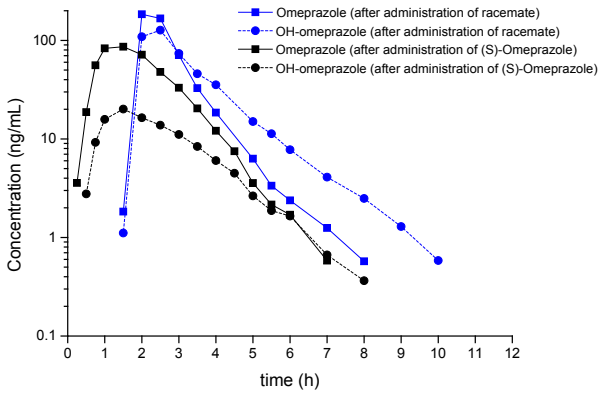


Figure 54. Plasma concentration time-profiles of omeprazole and 5-OH-omeprazole after administration of omeprazole racemate and S-omeprazole.

The PK profiles of metoprolol are not directly comparable because in the combi-pill immediate release metoprolol was used which is not available as a commercial formulation for clinical use (Figure 53 E). For midazolam a liquid low-dose formulation produced by the local hospital pharmacy was used for the Basel cocktail studies while the combi-pill contained the same dose as solid formulation, which was less well absorbed than the liquid formulation (Figure 53 F).

8.1.3. Conclusions

These results showed that combination of six low-dose phenotyping probe drugs with diverse physico-chemical properties into one single combi-pill is feasible and that comparable PK characteristics can be observed when the formulation of the compound is not altered. The presence of enantiomeric forms or modified release mechanisms will cause differences that have to be taken into account when planning the Basel cocktail III bioequivalence study. On the other hand, the combi-pill also offers the opportunity

to optimize PK characteristics for the specific purpose of phenotyping. These aspects will be explored in future clinical projects.

8.2. Phenotyping of CYP1A2, CYP3A4 and quantification of erlotinib, OSI-420, sunitinib, SU-12662, and pazopanib in plasma, blood and DBS in cancer patients

8.2.1. Introduction

Tyrosine kinases (TK) are enzymes that catalyze the transfer of phosphate groups from adenosine-triphosphates (ATP) to tyrosine residues of target proteins and activate them. TKs control various processes in normal cells, and are also involved in oncogenesis [107, 108]. Thus, the inhibition of TKs by TK inhibitors (TKI) is used in targeted cancer treatment. TKIs such as sunitinib, erlotinib, and pazopanib are used to treat non-small-cell lung cancer and renal cell carcinoma. Due to their more selective nature, the respective side effects of TKI are less pronounced in comparison to adverse reactions caused by conventional unspecific cytotoxic chemotherapies. Nevertheless, therapeutic effect and toxicity are determined by patient exposure, which shows considerable variation after standard doses. Sunitinib and pazopanib are metabolized by CYP3A4 while erlotinib is additionally metabolized by CYP1A2. Activity of these metabolizing enzymes shows large inter-individual variability due to multiple factors (genetic and environmental), therefore adapted dosing according to the phenotype of the patient could result in improved clinical effect and/or tolerability.

The main objective of the ongoing clinical study conducted together with the department of oncology at the Kantonsspital St. Gallen is to show that the individual CYP3A4 and CYP1A2 phenotype predicts drug exposure of sunitinib, pazopanib or erlotinib.

A secondary objective is to test the feasibility of using dried blood spots (DBS) instead of conventional venous blood samples for drug analysis of these TKIs. Based on these results, an integrated covariate model on sunitinib, pazopanib, and erlotinib pharmacokinetics will be built and a dosing algorithm based on the individual CYP-phenotype will be proposed.

Since the clinical study is still recruiting patients, only a small number of samples have so far been available for phenotyping and determination of drug concentrations. The following chapters describe the development and optimization of the analytical tools used to reach the objectives mentioned above, with special attention to DBS, a technique that facilitates blood sampling for both patients and medical staff, and could increase acceptance of therapeutic drug monitoring and phenotype testing.

DBS collection is a micro-sampling technique. After a finger or heel prick, drops of blood are transferred onto blood collection cards and dried for 2 hours at room temperature. While conventional blood or plasma sampling requires volumes of at least 0.5 mL [109, 110], only 10-15 µL of capillary blood are sufficient for DBS. DBS has the potential to be a useful tool for TDM, as for instance the samples can be collected by the patients at home, without the support of medical personnel and be shipped by regular mail to the analytical laboratory. However, before replacing conventional wet sampling with DBS, the feasibility of reliable quantification of the drug of interest within the DBS, as well as the correlation

between concentrations measured in DBS and plasma or whole blood has to be assessed. Additional validation steps are required, to evaluate effects of hematocrit and blood volume.

Although DBS sample collection is easier, the manual processing of DBS is more time consuming and laborious compared to conventional wet sampling. Thus the sample work-up automation and the use of a sensitive detection system were fundamental requirements to accomplish this project. We therefore established a collaboration with industry and academic partners. Camag, a Swiss company and world leader in thin-layer chromatography, supplied a newly developed autosampler which can be used for on-line DBS extraction. Shimadzu Switzerland, a key-player in chromatography and more recently also in MS equipment, supplied a UHPLC system coupled with a high end triple quadrupole mass spectrometer. The Division of Pharmaceutical Technology of Prof. Jörg Huwyler and Dr. Maxim Puchkov agreed to house the equipment in one of their laboratories, whilst providing logistical as well as scientific support.

8.2.2. Results and discussion

A UHPLC-MS/MS method for the simultaneous quantitative analysis in plasma, blood and DBS of caffeine, paraxanthine, midazolam, 1'-OH-midazolam, sunitinib, SU12662, erlotinib, and OSI-420 was initially validated. Online DBS analysis was performed using a CAMAG DBS 500 autosampler coupled to a Shimadzu LCMS 8040 (Figure 55).

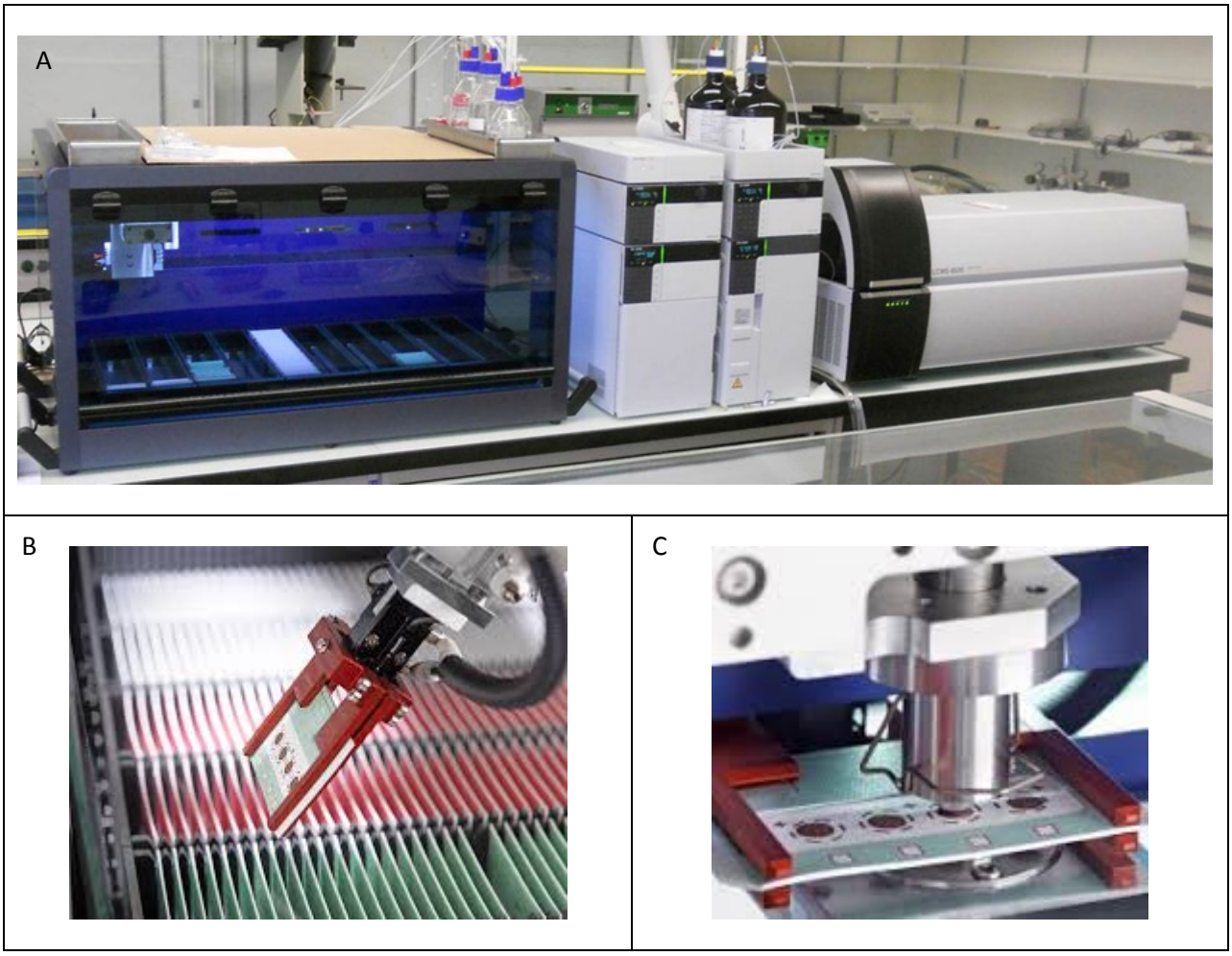


Figure 55. Camag DBS-MS 500 and Shimadzu UHPLC and MS8040 (A). Camag DBS-MS 500, detail view of the automatized cards handler (B) and of the online extraction head (C).

In plasma the method was specific, selective and linear in the range of 5-1250 ng/mL for sunitinib, 12.5-5000 ng/mL for erlotinib, 1-250 ng/mL for midazolam and 1'OH-midazolam, and 50-5000 ng/mL for caffeine and paraxanthine. Intra assay CV% was lower than 8.1%, while inter assay CV% was lower than 6.7% for all analytes. The total run time was 2.3 min.

An additional TKI, pazopanib, was subsequently introduced in the study. A second UHPLC-MS/MS method was developed and validated for the quantification of pazopanib, sunitinib, SU12662, erlotinib,

and OSI-420 in plasma, blood and DBS. The DBS method was specific, selective and linear in the range of 350-70'000 ng/mL for pazopanib, 25-5000 ng/mL for erlotinib, 2.5-500 ng/mL for sunitinib, 5-1000 ng/mL for OSI-420, and 1.5-100 ng/mL for SU12662. Intra assay CV% was lower than 9.7% (11.9% for LLOQ), while inter assay CV% was lower than 7.6% (10.5% for LLOQ) for all analytes. Total run time was 2 min.

A representative chromatogram is reported in Figure 56.

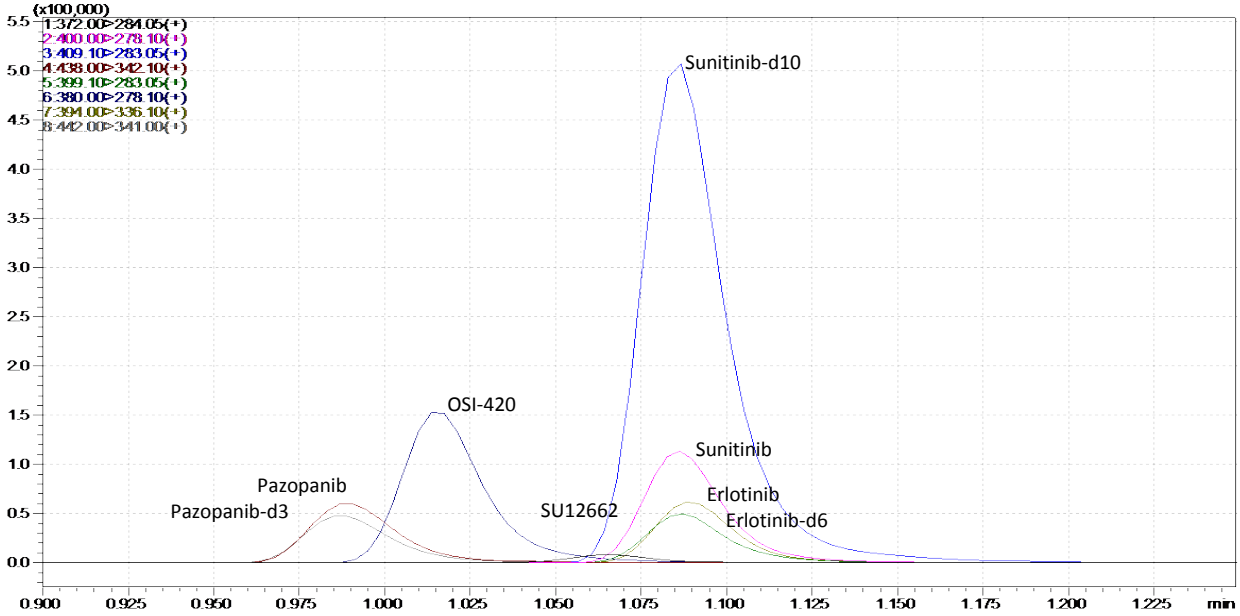


Figure 56. Chromatogram of a DBS sample containing 50 ng/mL of pazopanib, erlotinib, sunitinib, and 10 ng/mL SU12662, and OSI-420.

DBS analysis was performed online by means of a DBS autosampler developed and produced by Camag. The entire process was automated and did not require any manual intervention. Up to 500 DBS cards can be stored, sequentially transferred to the extraction head, sprayed with the internal standard and extracted.

Several additional parameters need to be validated when DBS rather than traditional blood samples are analyzed. Factors that need to be evaluated are: inter/intra-card variability, hematocrit, venous versus capillary blood sampling, anticoagulant, sample and spot homogeneity, internal standard addition, extraction recovery, sample dilution procedures, spotting technique and volume, punched spot size, sample stability and storage of cards (pre- and post-spotting) [111].

Of these parameters the effect of hematocrit, sample stability, and extraction recovery were considered to be of greatest importance and were therefore evaluated in this study.

Hematocrit effect on TKIs and metabolites was investigated for a hematocrit range between 20 and 60%. No significant changes of accuracy were observed for spots made by spiking 15 µL of blood.

Accuracy deviation compared to the reference hematocrit value of 40% was between -21.8% and 15.1% (Table 23). For the phenotyping drugs and metabolites, the hematocrit effect was not evaluated. We assumed that if there was to be a hematocrit effect, its extent would be the same for parent compounds and their metabolites. Since metabolic ratios are used as phenotyping metric, a possible hematocrit effect would be cancelled out of the calculation.

Table 23. Accuracy deviation compared to the haematocrit 40%

Analyte	Concentration	Hematocrit				
		20	30	40	50	60
		Deviation compared to hematocrit 40%				
	ng/mL	*%*	*%*	*%*	*%*	*%*
Pazopanib	1050	-10.9	-8.4	reference	6.3	15.1
	5200	-9.9	11.6		7.0	12.4
Erlotinib	75	-10.3	-0.6	reference	7.4	7.9
	3750	-8.0	3.7		6.4	1.8
Sunitinib	7.5	-21.8	-8.2	reference	-2.3	-17.2
	375	-7.8	3.0		1.0	-1.4
OSI-420	15	-9.2	-2.5	reference	-0.8	5.3
	750	-4.6	5.0		7.3	0.9
SU12662	1.5	-15.6	-10.0	reference	-13.2	-14.4
	75	-3.8	9.3		2.6	0.5

An alternative procedure to avoid hematocrit effects is to analyze the entire spot. This implies to exactly measure the volume of blood used to create the spot. Accurate pipetting and spotting of small blood volume constitutes a routine procedure for a lab technician or a nurse but if the sample has to be collected directly by the patient or by non-trained personnel an acceptable reproducibility could not be assured.

DBS stability in different conditions was evaluated. All analytes were stable for at least 24 days at -20°C, +4°C, and at room temperature (Table 24).

Table 24. DBS stability for at 24 days at -20°C, +4°C, and at room temperature

Analyte	Concentration	Change of concentration after 24 days			
		RT protected from light	RT exposed to light	+4°C	-20°C
	ng/mL	*%*	*%*	*%*	*%*
Pazopanib	1050	5.0	5.7	0.5	-5
	5250	8.7	1.8	1.4	-0.4
	52200	-0.5	2.8	-0.4	-0.6
Erlotinib	75	2.0	0.9	2.6	-2.2
	375	3.8	-2.5	-0.8	-1.1
	3750	0.7	-0.5	1.8	0.5
Sunitinib	7.5	2.2	-2.0	8.0	-0.6
	37.5	-2.7	-3.5	-2.3	3.4
	375	1.7	0.0	0.5	5.1
OSI-420	15	6.4	9.8	5.2	-3.3
	75	11.3	10.9	2.5	-1.7
	750	3.4	3.9	1.8	-0.2
SU12662	1.5	5.8	3.6	9.1	-9.8
	7.5	12.3	6.6	10.9	6.9
	75	-0.5	-0.8	-0.8	7.3

The addition of the internal standard through an automated spraying process greatly improves accuracy and precision, as already shown by Zimmer et al [112].

Quantification of substances in different concentration ranges within the same sample could be a challenge, which is especially of interest in the analysis of DBS. Whilst low abundant compounds are difficult to detect in a small blood volume, too high concentrations samples have to be diluted. For this study, a sensitive triple quadrupole MS detector enabled us to balance out the sensitivity issue. DBS samples with a too high concentration can be diluted with DBS blank extract. However, with the DBS MS-500 on-line extraction system this approach is not possible. Therefore MS parameters, in particular collision energy, were optimized to reduce too high signals and avoid overloading of the photomultiplier of the mass spectrometer.

The correlation between venous and capillary concentrations (DBS) of TKIs is currently under investigation in patient samples. These results will be used to evaluate the application of DBS for therapeutic drug monitoring of TKIs.

8.2.3. Conclusions

Before a meaningful analysis of the correlation between individual CYP1A2 and CYP3A4 phenotypes and TKI exposure can be performed, a sufficient number of patients have to be recruited. Patient recruitment is expected to be completed by the end of 2013. The developed method will then facilitate the fast and reliable analysis of all patient samples of this clinical trial.

9. Final Conclusions and Outlook

At the beginning of this project, our main goal was to develop and validate a new diagnostic tool for phenotyping of phase I drug metabolizing enzymes that would be easy to use and thus suited also for clinical applications. We imagined a phenotyping kit containing a pill, a collection tool for saliva or blood, a simple patient instruction sheet, and an envelope for shipping the sample to the phenotyping laboratory.

During the last years several cocktails have been described. In general, they were well characterized in terms of mutual interactions between the probe drugs used and for few cocktails the analytical methods proposed were designed and validated for phenotype testing. Sampling simplification has been considered by several authors that proposed saliva or DBS sample collection for phenotyping of few CYP isoforms. However, despite some improvement, none of the already proposed cocktails has received general acceptance in clinical practice so far.

With our studies, we showed that low doses of the phenotyping drugs of the Basel cocktail did not interfere with each other and did not provoke adverse effects. We proposed a simplified sampling procedure using metabolic ratios in plasma 2 hours after administration of omeprazole and midazolam for CYP2C19 and CYP3A4 or after 8 hours administration of caffeine, efavirenz, losartan, and metoprolol for CYP1A2, CYP2B6, CYP2C9, and CYP2D6 respectively. Although not generally accepted for all CYP isoforms (e.g. midazolam), our data showed that MRs obtained at a single time point showed high correlations with AUC ratios both under baseline conditions as in conditions with induced or inhibited CYP activity.

We compared the pharmacokinetics profiles and metabolic ratios of the Basel phenotyping drugs at baseline levels, after co-administration of inhibitors, and a broad CYP inducer. We showed that plasma metabolic ratios provide valuable qualitative information on the activity of relevant drug metabolizing CYP isoforms and can be considered as a valuable alternative to the reference method based on AUC measurements. Induction of CYP1A2 was not effective, therefore we were not able to estimate if the MR for this CYP was responsive to induction. Losartan, the drug used to phenotype CYP2C9, produced metabolic ratios that were not sensitive to induction. For all the other CYPs plasma MRs could replace AUC estimation for induction monitoring.

Alternative sampling procedures such as saliva and DBS would facilitate phenotyping and were explored in both clinical studies. The MRs in saliva and DBS for CYP1A2, and CYP2C19 are potential alternatives to MRs determined in plasma as they reproduced the results observed in plasma.

The six low-dose phenotyping probe drugs used in the Basel cocktail were formulated into one single, three-layered combi-pill and tested in one healthy volunteer in a pilot study. Comparable PK characteristics were observed after intake of the combi-pill compared to the simultaneous intake of the probe drugs as separate formulations. The combi-pill offers the opportunity to optimize dose,

absorption, and release properties for the specific purpose of phenotyping. These possibilities will be explored in future clinical projects.

The possibility of using phenotype information of CYP1A2 and CYP3A4 for dose individualization of TKIs is currently investigated in an ongoing a clinical study in collaboration with PD Dr. Jörger (department of oncology at the Kantonsspital St. Gallen, Switzerland) with a special focus on minimally invasive DBS sampling. The development of on-line DBS extraction for quantification of phenotyping drugs and TKIs has been performed. This method will facilitate the fast and reliable analysis of all patient samples. A meaningful analysis of the correlation between individual CYP1A2 and CYP3A4 phenotypes and TKI exposure will be performed as soon as the clinical study is completed (end of 2013).

Development and validation of sensitive analytical LC-MS methods were fundamental for the evaluation of the considerable amount of samples generated in the first and second Basel cocktail study, in the combi-pill pilot and TKIs studies. In addition to high sensitivity, a short run time and the capability to measure the phenotyping drugs and their metabolites within the same analytical run using the same conditions, were fundamental requirements that are fulfilled by the method presented.

The Basel cocktail is a promising tool for phenotyping of CYP1A2, CYP2B6, CYP2C9 and CYP3A4 using single point plasma measurement and for phenotyping of relevant polymorphic CYPs such as CYP2C19 and CYP2D6 using both single point plasma or non-invasive oral fluid measurements. Beside the “solo” phenotyping application, administration of our cocktail in concomitance with a new drug in drug-interaction studies, would simultaneously evaluate inhibitory or inducing potential on CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4.

The phenotyping procedure proposed in this dissertation needs further validation and has not yet the format of a commercial kit. The acquisition of more reference data and the development of the combi-pill, already planned, will be the next steps in this direction.

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12. Curriculum vitae

Current position

- PhD candidate / Head of Laboratory - Phenotyping and Biomarkers, Basel University Hospital since 2009

✓ Method development and validation of LC-MS/MS analytical methods for routine clinical analysis (therapeutic drug monitoring)
✓ PhD project: "*in vivo* phenotyping of six human cytochrome P450 enzymes using a new cocktail approach". Design of the new substrates combination, writing of the clinical study protocols, analysis of the clinical samples (plasma, saliva, blood, dried blood spot), data analysis and writing of the two manuscripts that summarize the PhD project.
✓ Online analysis of dried blood spot (DBS) – Initiated partnership with CAMAG
Project funded by the Swiss commission for technology and innovation (CTI) - DMS/DBS–MS: Extension of the capabilities to suit the hospital market for therapeutic drug monitoring.
✓ Development of DBS for phenotyping and for therapeutic drug monitoring
✓ Analytical support to research projects of the clinical pharmacology and toxicology department of Prof. S. Krähenbühl.
✓ Analysis of MDMA and metabolites in several clinical studies
✓ Line management of a laboratory technician, instruction and assistance of master and PhD students

Professional experience

- LC-MS/MS Senior Scientist, Basilea Pharmaceutica Ltd, Basel (Switzerland) 2001-2009

Development and validation of quantitative analytical methods
Improvement of automated solutions for samples preparation and analysis

- Drug Metabolism Assistant – Actelion Ltd, Allschwill (Switzerland) 2000-2001

In vitro pharmacokinetic studies

- Drug Metabolism Assistant - Hoffmann-La Roche, Basel (Switzerland) 1999-2000

Development of high throughput methods for drug metabolism studies

- Degree thesis - Faculty of Pharmacy, University of Milano (Italy) 1998-1999

Experimental work in the group of Prof G. D'Alfonso

- Internship – Hoffmann-La Roche, Basel (Switzerland) 1997-1998

Development of *in vitro* models for drug metabolism studies

Education

- Ph.D. in Clinical Pharmacology (CYP450 Phenotyping) 2009-2013
  Basel University, Switzerland

- M.Sc. in Chemistry and Pharmaceutical Technologies 1991-1999
  University of Milano, Italy

Donzelli M. et al.
The Basel cocktail for simultaneous in vivo phenotyping of six human cytochrome P450 enzymes
Paper submitted for publication

Derungs A., Donzelli M. et al.
Effects of inhibition and induction on the Basel cocktail for simultaneous in vivo phenotyping of six human cytochrome P450 enzymes
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13. Poster presentations

Poster 1, presented at the EBF conference (Barcelona, December 2009)

University Hospital Basel

Simultaneous quantification of Posaconazole, Voriconazole and Fluconazole in human serum by liquid chromatography–tandem mass spectrometry using on-line extraction

Donzelli M, Vetter B, Krähenbühl S , Haschke M

Analytical Unit, Division of Clinical Pharmacology and Toxicology, University Hospital Basel, Switzerland

Introduction

Posaconazole and voriconazole are extended-spectrum triazoles used for prophylaxis and treatment of invasive fungal infections.

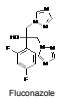
Increasing evidence suggests a relevant correlation of drug-exposure with clinical effect and toxicity.

Dosing is complicated by large interindividual pharmacokinetic variability and/or non-linear pharmaco-kinetics (voriconazole).

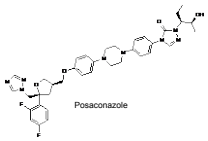
Although there are no generally accepted target concentration ranges, therapeutic drug monitoring represents an important tool to optimize drug therapy, especially in immunocompromised patients.

We describe a highly sensitive analytical method for the simultaneous analysis of three frequently used triazoles in human serum. The short run-time and online-sample extraction make this method useful for routine application.

Voriconazole

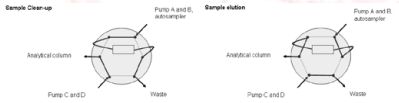


Fluconazole



Posaconazole

Methods



Time [min]	Flow [ml/min]	B Concentration [%]	Valve position
0	0.6	0	Loading
1.49	1.2		
1.5			
1.51	0.6	60	Elution
1.52			
4		95	
5			
5.01		0	Loading
6			

Mass Spectrometry Conditions
A triple quadrupole mass spectron
with a ESI source with the followin
Ion Spray voltage: 5500 eV. Temp
The selected mass-to-charge (*m/z*
standard ions [M +H]+ used in the
Posaconazole: *m/z* 701–*m/z* 683
Voriconazole: *m/z* 350–*m/z* 281
Fluconazole: *m/z* 307–*m/z* 238
The dwell times are set at 50 ms f

Results

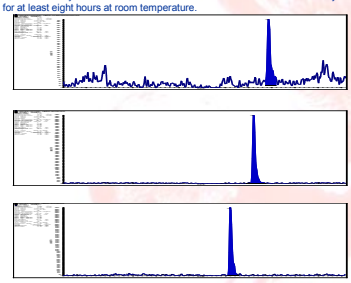
Our method fulfills all pre-specified validation criteria. It is specific, selective and linear over the concentration range of 10 to 5000 ng/mL for posaconazole (R^2 >0.998), voriconazole (R^2 >0.997) and fluconazole (R^2 >0.998).

Inter-assay precision for calibration samples were ≤7.1% for Posaconazole, ≤9.8% for Voriconazole and ≤6.0% for Fluconazole.

Inter-assay precision for quality control samples were ≤7.9% for Posaconazole, ≤7.0% for Voriconazole and ≤6.4% for Fluconazole.

Recovery rates at concentrations of 50 ng/ml and 500 ng/ml were 76.7% and 70.0% for Posaconazole, 96.5% and 92.2% for Voriconazole, and 101.6% and 94.5% for Fluconazole, respectively.

The three analytes were stable in human serum after three freeze/thaw cycles and for at least eight hours at room temperature.



Poster 2, presented at the EBF conference (Barcelona, December 2010)

Departement Biomedizin Basel

University Hospital Basel

New applications for an old technique: Clinical Validation of Dried Blood Spot (DBS) sampling for CYP1A2 phenotyping

***M Donzelli*, B Berger, S Krähenbühl, M Haschke**

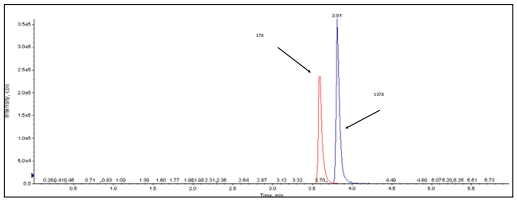
Division of Clinical Pharmacology and Toxicology, University Hospital Basel, Switzerland

Background

Extensive efforts have been directed towards developing genotyping methods for detecting specific DNA mutations that determine individual drug metabolizing capacity. However, genotyping is not able to monitor changes of phenotype caused by non-genetic factors, e.g. drug-drug or drug-food interactions. Phenotyping provides a measure of the activity of a given drug-metabolizing enzyme taking into account not only genetic but also non-genetic and environmental factors.
Phenotyping involves the administration of an appropriate substrate that is selectively metabolized to a known metabolite by a given CYP isoform. Caffeine (CAF) is the probe substrate for CYP1A2 phenotyping and the ratio between CAF and paraxanthine (PAR) is commonly used as phenotype measure.
Methods using dried blood spot sampling require formal testing of specific validation parameters (effect of blood spot sampling location, the sort of sampling paper, and the effect of blood volume spotted). Before replacing traditional venous sampling by DBS sampling cross validation using a sufficient number of subjects should be performed. We have investigated feasibility of DBS sampling for CYP1A2 phenotyping compared to venous blood sampling.

Methods

In a clinical study that involved 46 healthy volunteers, we compared concentrations of CAF and PAR in DBS samples and venous blood samples after dietary caffeine intake. After 2h of drying, blood spots were ready for further manual work-up and analysis by LC-MS/MS or storage.



Results

The method applied for caffeine and paraxanthine quantitation was sensitive (LLOQ 50 ng/mL), linear within the range 50-20000 ng/mL and inter-run precision and accuracy where within the recommended specifications. The correlation between caffeine and paraxanthine concentrations measured in DBS samples with the corresponding concentrations measured in venous blood evaluated by linear regression analysis showed a significant correlation between concentrations in DBS and venous blood samples.

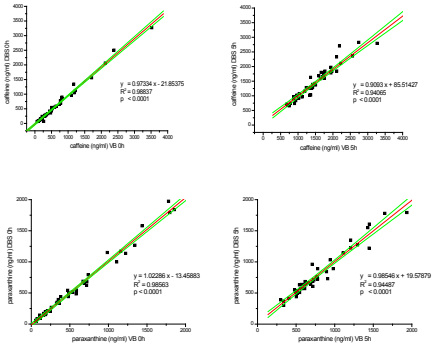


Figure 2. Linear regression analysis: DBS versus venous blood paraxanthine levels before and after caffeine administration.

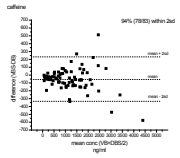


Figure3 . Bland Altman analysis: caffeine venous blood minus DBS level versus mean caffeine level

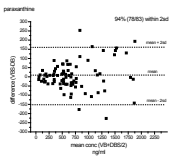


Figure 4. Bland Altman analysis: paraxanthine venous blood minus DBS level versus mean paraxanthine level

Conclusions

Concentrations of caffeine and paraxanthine measured in DBS are highly correlated with concentrations in venous blood. The correlation between the concentration of an analyte in venous blood and DBS can not be assumed a priori as already shown by several authors and by our group for Efavirenz. The reported procedure is an indirect clinical validation of DBS using analysis of caffeine and paraxanthine in venous blood as reference.

DBS seems to be suited as minimally invasive sample collection technique for CYP1A2 phenotyping. Suitability of DBS for phenotyping probes of other CYP isoforms remains to be shown.

Poster 3, presented at the EBF conference (Barcelona, December 2010)

Pharmacokinetic profile of efavirenz in dried blood spot (DBS) samples. Comparison of manual sample preparation and automated on-line extraction with the CAMAG DBS-MS device

M Donzelli[1], M Loppacher[2], L Nezic[1], S Krähenbühl[1], M Haschke[1]

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[2] *CAMAG, Sonnenmattstrasse 11, 4132 Muttenz, Switzerland*

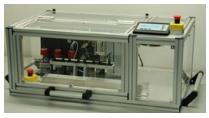
Background

To optimize therapeutic effect of certain drugs, therapy has to be individualized based on a patient's genotype and/or phenotype. Efavirenz (EFV) is a non-nucleoside reverse transcriptase inhibitor for the treatment of human HIV type 1 infection. Genetic polymorphism of CYP2B6 markedly influences metabolism of EFV and pharmacokinetic monitoring can help to avoid treatment failure or CNS toxicity. Monitoring of EFV concentrations in DBS samples would facilitate outpatient management. Our goal was to test feasibility of analyzing EFV in DBS samples using the automated CAMAG DBS-MS extraction device.

Methods

After a subtherapeutic oral dose of 50mg, venous blood and DBS samples were collected from a healthy male volunteer for 96 hours. DBS samples were dried for 2h before conventional manual work-up or automated extraction on a CAMAG DBS-MS autosampler. All samples were analyzed with a fully validated LC-MS/MS method using online-solid phase extraction, either using an external switchvalve or by integrating an extraction column into the CAMAG DBS-MS autosampler.

DBS analysis – manual sample preparation
A disc with a diameter of 3 mm was cut out of the centre of the dried blood spot using a manual hole punching device. Each disc was mixed with 150 µl ISTD working solution (Efavirenz 100 ng/mL in MeOH). After vortexing and centrifugation, aliquots of 20 µl were injected into the LC-MS/MS system.
Three discs per sample were punched, manually extracted and analyzed.



DBS-CAMAG analysis – automated sample work-up
Four collection cards were positioned in the autosampler.
Each DBS was loaded on a trapping column integrated in the autosampler with extraction eluent (water/MeOH 1/1, Efavirenz 100 ng/mL).
After 1.5 min the valve was switched and the analytes were eluted in back flush mode to the analytical column. Three spots per sample were analyzed.

Results

Linear regression analysis showed a highly significant correlation for EFV concentrations obtained by the manual and the automated approach (R^2=0.996). Bland-Altman correlation analysis did not show a difference between the two methods (bias :-1.21 ng/ml, all data points within the acceptance limits of -13.1 to 10.7 ng/ml). The mean precision estimated as CV% of triplicate analysis was 9.2% (range 1.7-28.7) for the manual work-up and 4.4% (range 0.2-6.6 %) for the CAMAG DBS-MS autosampler.

Sample name	time (h)	DBS Camag measured ng/mL	DBS Camag mean ng/mL	DBS Camag STD	DBS Camag CV%	DBS measured ng/mL	DBS mean ng/mL	DBS STD	DBS CV%
md_1h_01	1	233	228	13.9	6.1	238	235	4.2	1.8
md_1h_02		210				236			
md_1h_03		235				230			
md_2h_01	2	281	281	0.6	0.2	285	284	16.0	5.6
md_2h_02		281				300			
md_2h_03		282				268			
md_4h_01	4	160	170	9.1	5.3	197	165	12.2	7.4
md_4h_02		174				179			
md_4h_03		177				159			
md_6h_01	6	118	119	2.1	1.8	116	120	4.5	3.7
md_6h_02		121				120			
md_6h_03		117				125			
md_8h_01	8	91.3	89	4.7	5.3	99.8	99	1.7	1.7
md_8h_02		83.9				101			
md_8h_03		92.7				97.6			
md_10h_01	10	73.1	76	2.4	3.2	71.2	78	7.7	9.9
md_10h_02		77				86.5			
md_10h_03		77.6				76.8			
md_13.5h_01	13	57.2	54	3.6	6.6	50.6	50	4.3	8.6
md_13.5h_02		54.3				45.2			
md_13.5h_03		50.1				53.7			
md_24h_01	24	46.7	46	2.2	4.8	46.3	48	2.7	5.7
md_24h_02		47.7				47.4			
md_24h_03		43.5				51.5			
md_37h_01	37	37.6	37	0.9	2.6	28.4	30	2.6	8.5
md_37h_02		35.8				29.4			
md_37h_03		36.2				33.3			
md_48h_01	48	29	31	1.6	5.1	32.9	28	8.1	28.7
md_48h_02		31.8				18.9			
md_48h_03		31.8				33			
md_61h_01	61	32.3	32	1.5	4.5	26.7	24	2.7	11.0
md_61h_02		30.9				24			
md_61h_03		33.8				21.4			
md_72h_01	72	29.7	29	1.4	4.7	22.3	25	2.1	8.5
md_72h_02		30				25.4			
md_72h_03		27.5				26.3			
md_85h_01	85	29.8	28	1.8	6.4	20.2	20	2.1	10.3
md_85h_02		27.1				21.5			
md_85h_03		29.9				17.6			
md_96h_01	96	28.6	27	1.3	4.8	19.5	21	3.7	17.7
md_96h_02		27.2				18			
md_96h_03		26				25			

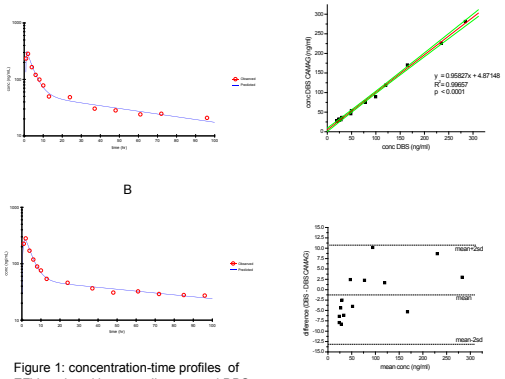


Figure 1: concentration-time profiles of EFV analyzed in manually prepared DBS samples (A) and DBS samples extracted with the CAMAG autosampler (B).

Conclusions

EFV concentrations can be monitored even after a subtherapeutic dose using DBS samples. Use of the CAMAG DBS-MS autosampler increased precision compared to the manual DBS sample work-up. Four collection cards (4 DBS per card, totally 16 samples) can be analyzed without manual handling. More than 130 DBS were analyzed without any system failure. The optimization of the extraction conditions, method qualification, and sample analysis was performed in approx. four workdays. Carry-over and inter-day accuracy were not systematically evaluated but preliminary results do not suggest presence of any systematic challenges.

Poster 4, presented at the Biozentrum Symposium (Basel, February 2012)

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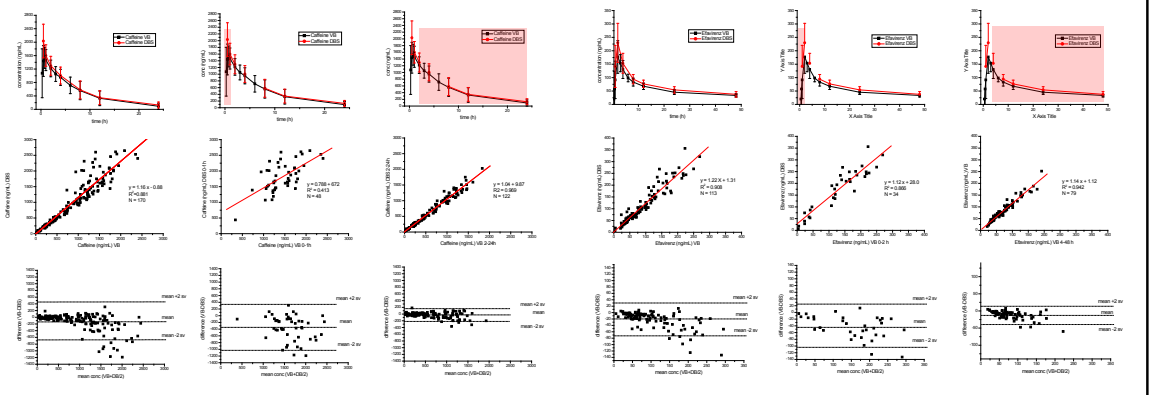
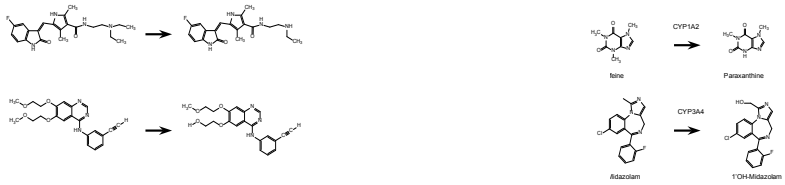
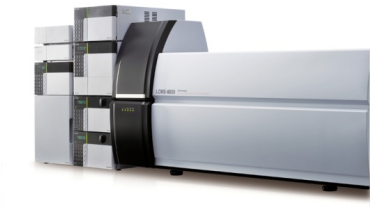
University Hospital Basel



Poster 5, presented at the EBF2012 conference (Barcelona, November 2012)





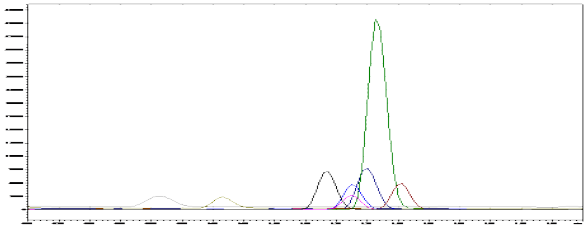
ıd LCMS-8030
ɔectrometer

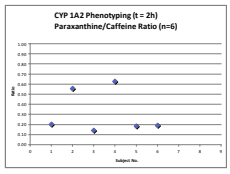
Liquid chromatography

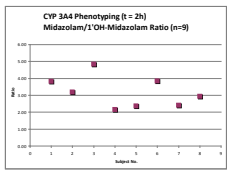
An Acquity UPLC HSS T3 column (2.1x50 mm, 1.8 µm, Waters, Switzerland) was used for the separation of the analytes. Eluent A (0.1% formic acid in water) and eluent B (0.1% formic acid in methanol) were used with the following gradient: 100% A from 0 to 0.1 min, 15% B from 0.1 to 0.3 min, 50% to 98% B from 0.3 to 1.4, 98% B from 1.4 to 1.75 min, 100% A from 1.75 to 2.3 min. The mobile phases were delivered at a constant flow of 1 mL/min. Total run time was 2.3 min. The thermostated column oven was set to 55°C.

Mass spectrometry conditions

Mass spectrometric detection was performed using a triple quadrupole mass spectrometer operating in electrospray-ionization positive-ion mode. Samples were quantified using peak area ratios.







henotyping ratio at 2 hours for CYP1A2 and CYP3A4

Conclusions

ʰe method fulfils the requested conditions concerning speed and reliability. It is suitable for TDM and will be expanded to analyze other tyrosine kinase inhibitors. Before a meaningful ınalysis of the correlation between individual CYP1A2 and CYP3A4 phenotypes and TKI exposure can be performed, a sufficient number of patients have to be recruited. Patient ecruitment is expected to be complete by the end of 2013. The developed method will then be used for fast and reliable analysis of all patient samples of this clinical trial.

Poster 6, presented at the HPLC 2013 conference (Amsterdam, June 2013)

SHIMADZU Excellence in Science

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University Hospital Basel

Fast UHPLC-MS/MS method to simultaneously quantify erlotinib, OSI-420, sunitinib, SU-12662, and pazopanib in dried blood spots (DBS)

D. Winogradova [1], B. Berger [1], B. Erxleben [2], U. Burger [3], M. Swiderska [4], M. Loppacher [4], M. Joerger [5], S. Krähenbühl [1], M. Haschke [1], M. Donzelli [1]

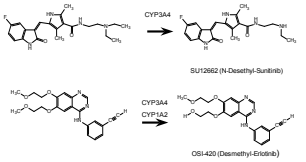
armacology and Toxicology, University Hospital Basel, Switzerland

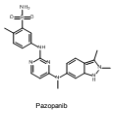
id

[4]CAMAG, Switzerland

[5]Department of Medical Oncology, Cantonal Hospital

Background





Sunitinib, erlotinib and pazopanib are tyrosine kinase inhibitors (TKI) used to treat non-small-cell lung cancer and renal cell carcinoma. Individualizing TKI doses may optimize drug treatment by avoiding subtherapeutic levels or toxicity. The method, previously validated according to the EMEA guidelines for plasma samples, was also validated for blood and dried blood spots (DBS). Additional DBS validation steps such as stability and the effect of the hematocrit were also evaluated.
DBS sample collection can facilitate blood sampling for both patients and medical staff.
From a technical perspective, conventional analysis of DBS is a challenging task. Each DBS spot is manually processed using a manual puncher, transferred into a test tube and extracted with a suitable solvent. Handling of a high number of DBS cards following the manual procedure is time consuming and error prone. In this study, automated on-line DBS extraction was performed using an on-line extraction system.

hod for the simultaneous
b, erlotinib, SU12662, OSI-
BS was validated. After on-
nalysis was performed on a
LC system coupled to a
e quadrupole MS, supplied
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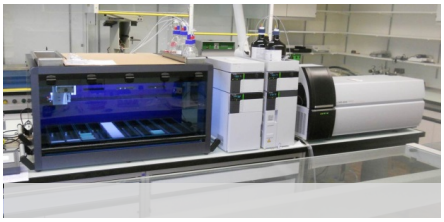
as fully automated. Sample
d with a CAMAG DBS-MS
ater 80/20 solution at a flow
ample was trapped in a 5 µl
red to the LC system.



Liquid chromatogr

An Acquity UHPL
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acid in water) ar
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20% B from 0 to
0.75 min, 98% B
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Results

The method was specific, selective and linear in the range of 350-70'000 ng/mL for pazopanib (R^2>0.989), 25-5000 ng/mL for erlotinib (R^2>0.998), 2.5-500 ng/mL for sunitinib (R^2>0.998), 5-1000 ng/mL for OSI-420 (R^2>0.998), and 1.5-100 for SU12662 (R^2>0.994). Intra assay CV% was lower than 9.7% (11.9% for LLOQ), while inter assay CV% was lower than 7.6% (10.5% for LLOQ) for all analytes. Total run time was 2 min.
DBS stability at three different concentrations up to 24 days at room temperature (exposed and protected from light), at +4 ° C and at -20° C was tested (Table 1).
The effect of different hematocrit values was also estimated (Table 2).

Analyte	Concentration	Change of concentration after 24 days			
		RT protected from light	RT exposed to light	+4°C	-20°C
	ng/mL	%	%	%	%
Pazopanib	1050	5.0	5.7	0.5	-5
	5250	8.7	1.8	1.4	-0.4
	52200	-0.5	2.8	-0.4	-0.6
Erlotinib	75	2.0	0.9	2.6	-2.2
	375	3.8	-2.5	-0.8	-1.1
	3750	0.7	-0.5	1.8	0.5
Sunitinib	7.5	2.2	-2.0	8.0	-0.6
	37.5	-2.7	-3.5	-2.3	3.4
	375	1.7	0.0	0.5	5.1
OSI-420	15	6.4	9.8	5.2	-3.3
	75	11.3	10.9	2.5	-1.7
	750	3.4	3.9	1.8	-0.2
SU12662	1.5	5.8	3.6	9.1	-9.8
	7.5	12.3	6.6	10.9	6.9
	75	-0.5	-0.8	-0.8	7.3

Analyte	Concentration	Hematocrit				
		20	30	40	50	60
		Deviation to hematocrit 40				
	ng/mL	%	%	%	%	%
Pazopanib	1050	-10.9	-8.4	0.0	6.3	15.1
	5200	-9.9	11.6	0.0	7.0	12.4
Erlotinib	75	-10.3	-0.6	0.0	7.4	7.9
	3750	-8.0	3.7	0.0	6.4	1.8
Sunitinib	7.5	-21.8	-8.2	0.0	-2.3	-17.2
	375	-7.8	3.0	0.0	1.0	-1.4
OSI-420	15	-9.2	-2.5	0.0	-0.8	5.3
	750	-4.6	5.0	0.0	7.3	0.9
SU12662	1.5	-15.6	-10.0	0.0	-13.2	-14.4
	75	-3.8	9.3	0.0	2.6	0.5

Table 2. Effect of different hematocrit values on D

All analytes were stable for 24 days at room temperature and were not sensitive to light.
For all analytes accuracy was not affected by hematocrit values between 20% and 60%.
The method is currently being used in a clinical study investigating the correlation between DBS and plasma concentrations of pazopanib, sunitinib, and erlotinib in cancer patients.

Conclusions

Our method fulfills the requirements concerning speed and reliability. Online DBS analysis considerably reduces sample work up compared to manual punching. DBS samples were stable for 24 days at room temperature, at 4° C and -20° C. Hematocrit values between 20 and 60 % did not influence accuracy. The correlation between venous and capillary concentrations (DBS) of TKIs is currently under investigation in patient samples. These results will be used to evaluate the use of DBS for therapeutic drug monitoring of TKIs.