Nutritional conditioning
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Quantitative Method for Simultaneous Analysis of a Five-Probe Cocktail for Cytochrome P450 enzymes


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Chapter 3

Abstract

Background
The metabolic activity of P450 enzymes in vivo can be determined using selective probe drugs. The simultaneous administration of multiple CYP-specific probe drugs is commonly known as the “cocktail approach.” Disadvantages of a cocktail are large volumes of samples required for analysis and time consuming analyses. The aim of this study was to develop and validate a simplified but sensitive method for the simultaneous quantification of 5 probe drugs [caffeine (CYP1A2), metoprolol (CYP2D6), midazolam (CYP3A4), omeprazole (CYP2C19), and S-warfarin (CYP2C9)] in a previously validated cocktail using a liquid chromatography–tandem mass spectrometry (LC-MS/MS) method.

Methods
The method entailed a single method for sample preparation that enables quick processing of the samples containing all 5 probe drugs in a small volume of blood (≥10 μL) followed by a chiral and nonchiral LC-MS/MS method. The method was validated for selectivity, specificity, resolution of racemic warfarin, linearity, accuracy, imprecision, recovery, process efficiency, ionization efficiency, and carryover effect.

Results
The method showed good selectivity without matrix interferences and differentiated S- and R-warfarin enantiomers with adequate resolution (Rs = 1.55). For all analytes, the mean process efficiency was >95%, and the mean ionization efficiency was >97%. Furthermore, the accuracy was between 94.9% and 108% for all analytes, and the within- and between run imprecision were <11.7% for the lower limit of quantification and <12.6% for the middle level and upper limit of quantification.

Conclusions
The method presented here enables the simultaneous quantification of the 5 probes in a very small blood volume (≥10 μL). Furthermore, it is less time consuming than previously reported methods because it requires only 1 simple method for sample preparation followed by a nonchiral and chiral LC-MS/MS method that can be performed sequentially.
Introduction

Cytochrome P450 (P450) enzymes play an important role in drug metabolism. There is considerable variability in the activity of P450 enzymes between and within subjects, due to induction or inhibition of these enzymes.¹ This can result in different treatment outcomes such as treatment failure or unwanted side effects. Approximately 20-40% of this variability can be explained by genetic factors whereas more than 50% is explained by physiological or environmental circumstances, also including nutritional factors.¹ The combination of these factors results in specific phenotypes of drug metabolism.

Phenotyping of drug metabolism by P450 enzymes in vivo can be performed by using selective probe drugs. This is commonly used in drug development to study drug-drug interactions but can also be used for pharmacological research purposes or as an approach to individualize pharmacotherapy. In addition to the administration of a single CYP-specific probe drug at a time, it is possible to simultaneously administer multiple types of CYP-specific probe drugs, which is commonly referred to as “cocktail approach”. Preferably, the probe drugs used in such a cocktail should be selective for the individual CYP isoforms and should not interact with each other. In 2008, Turpault et al. studied the pharmacokinetics (PK) of selective substrates of five CYP isoforms that play an important role in human drug metabolism: caffeine (CYP1A2), S-warfarin (CYP2C9), omeprazole (CYP2C19), metoprolol (CYP2D6) and midazolam (CYP3A4).² They observed no PK interaction between the probe drugs when administered as a cocktail, relative to the probes administered alone. The lack of interaction between the probe drugs indicates that this cocktail can be used in vivo.

For the quantification of the probes, Turpault et al. used three different LC-MS/MS methods.² Caffeine, metoprolol and omeprazole plasma samples were analyzed together whereas midazolam and R- and S-warfarin plasma concentrations were analyzed separately. In addition to these three different LC methods three different approaches for sample preparation were used: 1) solid-phase extraction for caffeine, metoprolol and omeprazole, 2) extraction with an organic solvent followed by evaporation of this solvent under nitrogen for midazolam and 3) extraction with another organic solvent followed by evaporation under nitrogen for R- and S-warfarin. This can be time-consuming and costly when analyzing multiple samples or using the cocktail in clinical practice. The different methods for sample preparation also require enough blood volume per sample, which may not always be feasible in certain groups of patients or, for ethical reasons, in combination with the number of samples to be drawn. Therefore, the aim of our study was to develop and validate a simplified, but sensitive, method for the simultaneous quantification of the five probe drugs using a single LC-MS/MS method.
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Materials and Methods

Reagents and chemicals
The reference standards S-warfarin, R-warfarin, omeprazole and caffeine were obtained from Sigma-Aldrich (Steinheim, Germany). Metoprolol and midazolam reference standards were obtained from BUFA (Spruyt-Hillen, Ijsselstein, The Netherlands). The internal standards (IS) caffeine-D9 metoprolol-D7, midazolam-D5, warfarin-D5 and omeprazole-D3 were obtained from Toronto Research Chemicals (TRC, Toronto, Canada). The reagents acetonitril and methanol were obtained in HPLC supra-gradient quality from Biosolve (Lexington, MA, USA). Formic acid and ammonium formate, used for HPLC, were obtained from Sigma-Aldrich (Steinheim, Germany). Water was purified and deionized using an ELGA purelab Optron Q (Veolia Water; Saint Maurice, France). Drug free, human plasma, used for preparation of the calibration and QC samples, was obtained from pooled blood of healthy volunteers in the Academic Medical Centre, University of Amsterdam, The Netherlands.

Instrumentation
The LC-MS/MS setup comprised a LC-30 Nexera (Shimadzu, Kyoto, Japan) system with CT020AC column oven coupled to a Qtrap 5500 system (ABSciex, Concord, Canada). Analyst™ 1.6.x (ABSciex) was used as software package for controlling the LC-MS/MS system and for data processing.

LC-MS/MS conditions
To differentiate between the chiral components S- and R- warfarin, a chiral method was used, whereas the other non-chiral drugs in the cocktail were analyzed using a non-chiral method.

For the chiral method, an Astec Chirobiotic V (150x2.1 mm, 5μm) from Sigma-Aldrich was used as LC column and a ternary gradient was applied for chromatographic separation using three eluents; A: purified H₂O, B: acetonitrile 100%, and C: formic acid 2% and ammonium formate 2% in purified H₂O. Eluent C was set at 5% throughout the run. At the start of the gradient A was set at 90% and B at 5%. A 3.5 min linear gradient was applied to 55% A and 40% B. In 0.01 min the gradient was turned to 5% A and 90% B for 1 min after which it was brought back to the starting conditions in 0.01 min for another minute giving a total chromatographic run time of 5 min. The flow rate was 0.6 ml min⁻¹. The auto-sampler temperature was maintained at 10 °C, the column oven temperature at 40 °C. The analytes were detected in the negative ion mode. Mass transitions of R- and S-warfarin and collision energy are summarized in Table 1. The ion spray voltage was -4000 V.
Table 1: Mass transitions and collision energy of the drugs in the cocktail

<table>
<thead>
<tr>
<th>Component</th>
<th>Mass transition</th>
<th>Collision energy (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Precursor</td>
<td>Fragment</td>
</tr>
<tr>
<td>Caffeine</td>
<td>194.85</td>
<td>138.00</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>268.05</td>
<td>191.00</td>
</tr>
<tr>
<td>Midazolam</td>
<td>326.10</td>
<td>291.10</td>
</tr>
<tr>
<td>Omeprazole</td>
<td>345.85</td>
<td>197.95</td>
</tr>
<tr>
<td>R/S-Warfarin</td>
<td>307.00</td>
<td>160.85</td>
</tr>
</tbody>
</table>

For the non-chiral method, a Thermo Scientific Hypersil Gold (50x2.1 mm, 1.9 μm) from ThermoFisher Scientific (Waltham, MA, USA) was used as LC column, and a two-phase gradient was applied for chromatographic separation using A: purified H₂O, B: acetonitrile 100%, and C: formic acid 2% and ammonium formate 1% in purified H₂O. Eluent C was set at 10% throughout the run. At the start of the gradient A was set at 88% and B at 2%. A 4-minute linear gradient was applied to 90% B and 0% A. The gradient was brought back to the starting conditions in the next 0.01 min and re-equilibrated for 0.99 minutes giving a total chromatographic run time of 5 minutes. The flow rate was 0.4 ml min⁻¹. The auto-sampler temperature was maintained at 10 °C, and the column oven temperature at 40 °C. The analytes were detected in the positive ion mode. Mass transitions of the drugs in the cocktail and collision energies are shown in Table 1. The ion spray voltage was 2000 V.

Analytical procedures

**Preparation of stock solutions, internal standard, quality control samples and patient samples**

The stock solutions were prepared in H₂O:MeOH 1:1 (vol/vol) at a concentration of 0.5 mg L⁻¹ for midazolam, 50 mg L⁻¹ for caffeine, 2 mg L⁻¹ for omeprazole, 1 mg L⁻¹ for metoprolol and 4 mg L⁻¹ for R- and S-warfarin. The internal standards were prepared in ACN:MeOH 420/80 (vol/vol) at a concentration of 5 μg L⁻¹ for midazolam-D5, 500 μg L⁻¹ for caffeine-D9, 40 μg L⁻¹ for warfarin-D5 and 10 μg L⁻¹ for omeprazole-D3 and metoprolol-D7. Both stock solutions and internal standards were stored at -80 °C until use. Eight calibration standards per component of the drug cocktail were prepared by diluting the respective stocks with blank human plasma, as shown in Table 2.

For each component of the drug cocktail, QC samples were prepared at three concentrations: the predefined lower limit of quantification (LLOQ), the middle level of quantification (MLQ) and the upper limit of quantification (ULOQ) (Table 2). The QC samples were prepared in the same manner as the calibration standards and stored at -80 °C. Calibration standards, QC samples and patient samples were thawed and vortexed prior
to analysis. Samples were protein precipitated by adding 750 μL of internal standard (I.S.) to 100 μL of sample while vortexing. The samples were then cooled at -20 °C for 30 minutes, vortexed again and centrifuged for 5 minutes at 2750 g (4000 rpm) after which 5 μL of the supernatant was injected.

**Table 2:** Calibration and Quality Control (QC) standards for the drugs in the cocktail

<table>
<thead>
<tr>
<th>Calibration standard</th>
<th>QC standard</th>
<th>Concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Caffeine</td>
</tr>
<tr>
<td>1</td>
<td>LLOQ</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>250</td>
</tr>
<tr>
<td>4</td>
<td>MLQ</td>
<td>500</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>1000</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>2000</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>5000*</td>
</tr>
<tr>
<td>8</td>
<td>ULOQ</td>
<td>-</td>
</tr>
</tbody>
</table>

LLOQ: lower limit of quantification, MLQ: middle level of quantification, ULOQ: upper limit of quantification.

*Ad * The limits for the preset ULOQ of 10,000 ng ml⁻¹ of caffeine were not met during validation. Therefore, the ULOQ was set to 5,000 ng ml⁻¹ and seven instead of eight calibration standards were used.

**Quantification**

MS response was expressed as integrated area of the chromatographic peak. For calibration, the concentrations of prepared calibration standards were the known variable (x). The ratio of analyte MS response divided by internal standard MS response per calibration level was the unknown variable (y). Patient samples were back-calculated using the calibration line by their respective area ratio of analyte/IS MS response.

**Method validation**

**General**

The following parameters of each of the six analytes were validated: selectivity, specificity and resolution of racemic warfarin, linearity, accuracy, imprecision, recovery, process efficiency (PE), ionization efficiency (IE), carryover effect and stability in the auto-sampler.

**Selectivity**

Human pooled plasma together with plasma samples from five different clinical patients not receiving any of the tested analytes were tested for interference. Samples were protein-precipitated using ACN:MeOH 420/80 (vol/vol) without IS. Data of the chromatograms were processed, and the integrated response should not exceed 10% of the average integrated response of the LLOQs and 5% of the IS.
Specificity and resolution of racemic warfarin
The specificity and resolution measure the extent of separation between the two adjacent peaks of S- and R- warfarin. Specificity of the analyses of S-warfarin and R-warfarin was tested by separately injecting both compounds in order to determine the retention time. Furthermore, the contribution of S-warfarin to the R-warfarin component (area/area) and vice versa should not exceed 0.1%. For the resolution, the difference between the retention times of the two peaks relative to their width \( R_s = \frac{\Delta t_{\text{retention}}}{W_{\text{average}}} \) should be more than 1.5 as given in the European Pharmacopeia (Ph. Eur.).

Linearity
For each analyte, a total of six calibration lines, consisting of eight different concentrations, were prepared in blank human plasma and measured during six separate runs. For caffeine, the calibration lines consisted of seven different concentrations. The calibration model was quadratic, with y-intercept and \( 1/x^2 \) weighting, for caffeine, omeprazole and \( R-/S \)-warfarin and linear for metoprolol and midazolam \( (1/x) \). The linearity of the calibration lines should have coefficients of determination \( (r^2) \) of more than 0.990.³

Accuracy and imprecision
Accuracy and imprecision were calculated following the measurement of LLOQ, MLQ and ULOQ samples of each analyte during six consecutive runs. During the first run, six replicates for all levels were determined; during the other five runs a single sample of each level was determined. Mean accuracy was calculated from the results of the first run and should be within 80-120% for the LLOQ level and within 85-115% for the MLQ and ULOQ levels. The within-run imprecision was also calculated from the results of the first run and should not exceed 20% for the LLOQ level and 15% for the MLQ and ULOQ levels. The between-run imprecision was calculated from the results of run one through six and should not exceed the same levels for LLOQ, MLQ and ULOQ as for the within-run imprecision.⁴

Recovery, process- and ionization efficiency
In terms of LC-MS/MS, PE is a combination of extraction recovery of the analyte during sample pretreatment and the IE in the MS source. These parameters were quantified based on the strategies proposed by Matuszewski et al.⁵ PE was determined for all analytes, including the ISs, by spiking six samples at the MLQ level prior to sample pretreatment: five samples were prepared in plasma from five different clinical patients not receiving any of the analytes and one sample was prepared in blank human plasma. Furthermore, six samples at the MLQ level were prepared in water. Recovery (RE) was defined as the relative signal of post-precipitation versus pre-precipitation spiked samples. IE was defined as the relative signal of post-precipitation spiked plasma samples versus spiked aqueous samples. PE was defined as the product of RE and IE: the overall signal
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of spiked plasma versus an aqueous standard solution. For each analyte the mean values and coefficients of variation were calculated for RE, IE and PE.

**Carry-over effect**

Carry-over effect was evaluated by sequentially injecting the ULOQ followed by blank plasma for six times. The peak area of analytes should not be more than 20% of the LLOQ.6

**Stability**

The stability in the auto-sampler (10°C) was assessed by comparing freshly prepared samples with samples that were stored for 96 hours in the auto-sampler. The mean accuracy of stability tested LLOQ samples should be between 80% and 120%. MLQ and ULOQ samples should have a mean accuracy of 85%-115%.

**Method applicability**

To show applicability of the method, we present the plasma concentration-versus-time curves of the five probe-drugs within the cocktail when orally administered to a healthy male subject. The study design was approved by the institutional ethics review board (ABRnr: NL40834.018.12). The subject provided written informed consent before study entry and the study was conducted in accordance with the ethical standard of the responsible committee on human experimentation and with the Helsinki Declaration of 1975 (as revised in 2008). At 8:00 in the morning the drug cocktail was administered orally consisting of 100 mg caffeine (CYP1A2) (10 mg ml\(^{-1}\) ampoules, VUMC, Amsterdam, The Netherlands), 5 mg racemic warfarin (CYP2C9) (5 mg tablet, Crescent Pharma Ltd, Hampshire, United Kingdom), 20 mg omeprazole (CYP2C19) (20 mg capsule, Teva Pharmachemie, Haarlem, The Netherlands), 100 mg metoprolol (CYP2D6) (100 mg tablet, Teva Pharmachemie, Haarlem, The Netherlands) and 0.03 mg kg\(^{-1}\)midazolam (CYP3A4) (1 mg ml\(^{-1}\) oral solution, UMCG, Groningen, The Netherlands).2,7 Serial blood samples were collected after administration at t=0, t=1, t=2, t=3, t=4, t=5, t=6, t=7, t=8 and t=10 hours. Furthermore, PK samples at day 2, 3, 8 and 15 were obtained, of which the latter two due to the long elimination half-life of warfarin. Plasma was separated by centrifugation and stored at -80 °C until analysis.7
Results

Chromatography
The chromatographic results after injection of drug free human plasma and LLOQ of the six analytes are shown in Figure 1. Retention times for caffeine, omeprazole, metoprolol, midazolam, S-warfarin and R-warfarin were 1.57, 2.25, 1.97, 2.5, 2.74 and 2.39 minutes, respectively. The reproducibility of the retention times was good, showing only a few seconds of variation during the period of method development, validation and running patient samples (data not shown).

Validation
A summary of all performance indicating parameters is given in Table 3. The method showed good selectivity, because no significant chromatographic matrix interferences were observed as shown by the chromatogram of drug free human plasma (Figure 1). Furthermore, the method was able to differentiate S- and R-warfarin enantiomers with adequate resolution ($R_s = 1.55$), and there was no significant contribution of S-warfarin to the R-warfarin component (area/area) or vice versa. The linearity of the calibration lines showed coefficients of determination ($r^2$) of more than 0.996. For S-warfarin, the coefficient of determination was $r^2=0.995$ (Table 3).

The accuracy, within and between run imprecision were all within the preset limits of <20% for the LLOQ level and <15% for the MLQ and ULOQ levels. For all compounds, accuracy ranged from 100.6% to 107.5% at the LLOQ level and from 97.6% to 107.3% and from 102.1% to 110.4% at the MLQ and ULOQ levels, respectively. For caffeine, the predefined upper limit of quantification was 10,000 ng/ml. During validation, the limits for this ULOQ have not been met (data not shown). Therefore, the ULOQ for caffeine was lowered to 5000 ng ml$^{-1}$, after which all limits regarding accuracy, within and between run imprecision met the requirements stated in the Materials and Methods section (Table 3).

For all analytes, the mean PE was more than 95%. The mean IE was more than 97%, and the mean RE exceeded 95% (Table 3). This indicates that the analytes are not prone to any significant matrix effects in varying plasma samples during sample pretreatment or MS source ionization.

After injection of the ULOQ samples, the peak area of all analytes was much less than 20% of the LLOQ for all analytes ($\leq 0.02\%$, Table 3) which indicates that it is feasible to determine a low concentration sample after injecting a high concentration sample.

Samples showed no significant degradation during storage for 96 hours in the auto-sampler ($10^\circ$C) (Table 3).
### Table 3: Summary of the validation results

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Level</th>
<th>Caffeine</th>
<th>Metoprolol</th>
<th>Midazolam</th>
<th>Omeprazole</th>
<th>R-Warfarin</th>
<th>S-Warfarin</th>
<th>Warfarin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity (mean $r^2$)</td>
<td></td>
<td>0.999</td>
<td>0.999</td>
<td>0.999</td>
<td>0.999</td>
<td>0.998</td>
<td>0.995</td>
<td></td>
</tr>
<tr>
<td>Accuracy</td>
<td>LLOQ</td>
<td>103.4</td>
<td>100.6</td>
<td>100.6</td>
<td>104.4</td>
<td>106.3</td>
<td>107.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MLQ</td>
<td>101.8</td>
<td>108.0</td>
<td>99.6</td>
<td>107.3</td>
<td>97.6</td>
<td>94.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ULOQ</td>
<td>102.9</td>
<td>106.3</td>
<td>106.2</td>
<td>110.4</td>
<td>104.7</td>
<td>102.1</td>
<td></td>
</tr>
<tr>
<td>Between-run imprecision</td>
<td>LLOQ</td>
<td>7.4%</td>
<td>5.0%</td>
<td>11.7%</td>
<td>6.0%</td>
<td>1.6%</td>
<td>2.3%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MLQ</td>
<td>4.3%</td>
<td>3.8%</td>
<td>3.3%</td>
<td>4.9%</td>
<td>2.6%</td>
<td>2.0%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ULOQ</td>
<td>7.6%</td>
<td>7.5%</td>
<td>8.1%</td>
<td>8.6%</td>
<td>8.2%</td>
<td>12.6%</td>
<td></td>
</tr>
<tr>
<td>Within-run imprecision</td>
<td>LLOQ</td>
<td>2.8%</td>
<td>7.3%</td>
<td>7.3%</td>
<td>6.7%</td>
<td>1.8%</td>
<td>1.6%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MLQ</td>
<td>2.5%</td>
<td>3.6%</td>
<td>4.8%</td>
<td>5.5%</td>
<td>1.7%</td>
<td>1.7%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ULOQ</td>
<td>2.7%</td>
<td>1.9%</td>
<td>4.4%</td>
<td>7.2%</td>
<td>3.1%</td>
<td>3.5%</td>
<td></td>
</tr>
<tr>
<td>Process efficiency*</td>
<td>MLQ</td>
<td>106.2 ±11.4</td>
<td>99.7 ±6.9</td>
<td>98.1 ±3.2</td>
<td>96.8 ±6.3</td>
<td>98.9 ±6.2</td>
<td>98.9 ±4.7</td>
<td></td>
</tr>
<tr>
<td>Ionization efficiency*</td>
<td>MLQ</td>
<td>105.7 ±11.1</td>
<td>98.4 ±7.7</td>
<td>98.3 ±4.1</td>
<td>97.6 ±7.2</td>
<td>97.4 ±6.5</td>
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<td></td>
</tr>
<tr>
<td>Recovery*</td>
<td>MLQ</td>
<td>101.0 ±11.4</td>
<td>101.6 ±6.8</td>
<td>100.0 ±4.9</td>
<td>99.3 ±4.4</td>
<td>98.6 ±5.8</td>
<td>97.6 ±4.8</td>
<td></td>
</tr>
<tr>
<td>Stability in autosampler(^\d)</td>
<td>LLOQ</td>
<td>96.9%</td>
<td>102.5%</td>
<td>97.6%</td>
<td>92.5%</td>
<td>110.4%</td>
<td>110.4%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MLQ</td>
<td>107.6%</td>
<td>107.5%</td>
<td>96.9%</td>
<td>94.8%</td>
<td>96.9%</td>
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<tr>
<td></td>
<td>ULOQ</td>
<td>100.0%</td>
<td>101.1%</td>
<td>93.2%</td>
<td>103.5%</td>
<td>103.5%</td>
<td>103.5%</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) Data are presented as mean ± CV (%) (n=6)

\(^\d\) For the stability of R- and S-warfarin, only racemic warfarin was tested
Figure 1: Chromatographic results after injection of drug free human plasma and LLOQ of the six analytes

Caffeine - Blanc

Caffeine – LLOQ

Metoprolol – blanc

Metoprolol – LLOQ
Figure 1: (continued)

Midazolam – blanc

Midazolam – LLOQ

Omeprazole – blanc

Omeprazole – LLOQ
Quantitative Method for Analysis of 5-Probe Cocktail

R-Warfarin – blanc

S-Warfarin – blanc

R-Warfarin – LLOQ

S-Warfarin - LLOQ
Method applicability
Figure 2 contains the plasma concentration-versus-time curves of the five drugs in the cocktail after administration to a healthy male subject. For an elaborate analysis of the pharmacokinetics of the drug cocktail after administration to healthy subjects, we refer to our previously published work.

Discussion
Based on three previously published methods for the quantification of a five-probe cocktail for P450 enzymes, we developed a simple, accurate and precise assay for the simultaneous quantification of a five-probe cocktail for CYPs 1A2, 2C9, 2C19, 2D6 and 3A4. The probe drugs for these enzymes are caffeine, S-warfarin, omeprazole, metoprolol and midazolam, respectively. As a probe drug, warfarin can only be administered as a racemic compound. Because the S-enantiomer of warfarin is a specific probe for CYP2C9, a chiral method was implemented to distinguish between R- and S-warfarin. Therefore, we used a single and simple method for sample preparation and developed a non-chiral method to quantify caffeine, warfarin, omeprazole, metoprolol and midazolam followed by a chiral method to further quantify R- and S-warfarin. The single method for sample preparation enables quick processing of the samples. Each prepared sample can first be analyzed by the non-chiral method and then immediately, or at least within 4 days as indicated by our stability data, by the chiral method (or vice versa). Furthermore, this allows the quantification of all probe drugs in a very small volume of blood (≥ 10 μL). Thus, compared to the method described by Turpault et al., our method requires a single (1) and simplified (2) sample preparation step, two LC-systems instead of three (3) and a small volume (10 μL) of plasma for the quantification of the cocktail probe-drugs (4).

The limits for the preset ULOQ of 10,000 ng ml\(^{-1}\) of caffeine were not met during validation. Therefore, the ULOQ was set to 5000 ng ml\(^{-1}\), and seven instead of eight calibration standards were used. Conform the guideline on analytical method validation of the European Medicines Agency (EMA) and the FDA guidance for industry on bioanalytical method validation, the number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the analyte/response relationship. Concentrations of standards should be chosen on the basis of the concentration range expected in a particular study and should consist of six (EMA guideline) or six to eight (FDA guidance) non-zero samples covering the expected range. Therefore, with seven calibration standards for caffeine, the EMA and FDA criteria are still met. Moreover, after oral administration of a relevant dose of caffeine (100 mg) to healthy subjects, concentrations less than the revised ULOQ of 5000 ng ml\(^{-1}\) were measured, which means that an upper limit of qualification of 10,000 ng ml\(^{-1}\) is much too high. The calibration range for caffeine with an ULOQ of 10,000 ng ml\(^{-1}\) was initially based on the method validated by Turpault et al. Since the \(C_{\text{max}}\) of caffeine did not
exceed the adjusted ULOQ of 5000 ng ml\(^{-1}\) after administration of 100 mg caffeine in healthy volunteers, there was no need to further evaluate the dilution integrity. Furthermore, most of the concentrations measured after administration of 100mg caffeine were far above the lower limit of quantification (50 ng ml\(^{-1}\)).\(^7\) This indicates that even lower doses than 100 mg of caffeine can be used in future studies.

Although the drugs in the cocktail are therapeutically low-dosed and no side effects have been reported, it is possible to further decrease the doses to the level of microdosing.\(^7\) Microdosing (maximum dose of 100 μg) has recently been introduced in clinical studies to minimize the risk of harmful events to human subjects.\(^10\) As can be seen in Figure 2, the maximum concentrations (C\(_{\text{max}}\)) measured after administration of the cocktail are far above the lower limits of quantification (Table 2). Therefore, our highly sensitive and quantitative method allows even 10-100 times lower doses than typical therapeutic which approaches microdosing.

Previously, Oh et al. developed a high-sensitive LC-MS/MS method for the simultaneous quantitation of five low dosed CYP isoform specific substrates and their metabolites: CYP1A2 (caffeine), CYP2C9 (losartan), CYP2C19 (omeprazole), CYP2D6 (dextromethorphan) and CYP3A (midazolam).\(^11\) To quantify the low doses of the drugs in the cocktail administered, a relatively high sensitivity of LC–MS/MS for these analytes is necessary. Therefore, the authors used two different solvent extractions: a basic media extraction followed by an extraction under acidic conditions. Although this resulted in high extraction recoveries, it hampers quick sample processing and required relatively large plasma volumes of 500 μl. Compared to the method of Oh et al., the drug cocktail in our study was higher dosed, but no adverse events were experienced and the small volume of blood required (≥10 μl) allows testing the drug cocktail in a broader group of patients (eg. children) because of ethical reasons. And, although not validated in our study, this could make our method suitable for the quantification of the probe drugs by using blood obtained from dried blood spots.\(^12\)

Our method does not include the analysis of all metabolites of the five probe drugs. By quantification of metabolites it is possible to determine metabolite-to-parent drug ratios. However, when the objective of the study is to quantify the effect of interventions on different enzymes, the EMA has recommended to determine complete AUCs (area under the plasma concentration time curves) of the probe drugs in order to estimate effects on (oral) clearance.\(^13\) In that case, simpler ratios such as metabolite to parent drug ratios in urine are usually not a satisfactory parameter as results may have more confounding factors and as the magnitude of an effect is difficult to translate into inhibition or induction potency and to treatment recommendations.\(^13\) However, when using the drug cocktail in clinical practice, the determination of a complete AUC will be too invasive and time consuming. Therefore a limited sampling strategy should be developed to be able to characterize the pharmacokinetics of a typical patient using only one or two blood samples after administration of the cocktail.\(^14\) Determination of metabolite
to parent drug ratios may provide additional insight in the pharmacokinetics of a drug within and between patients, but this strategy may also have its limitations. For example, omeprazole is metabolized to 5-hydroxyomeprazole by CYP2C19 for about 80% and to hydroxysulfon by CYP3A4 for approximately 20%. Therefore, some authors include 5-hydroxyomeprazole in their analysis of CYP2C19 activity. However, 5-hydroxyomeprazole itself is also metabolized to hydroxysulfon by CYP3A4. The addition of 5-hydroxyomeprazole to the analysis would therefore not make omeprazole a better probe for CYP2C19.

**Figure 2:** Plasma concentration-versus-time curves of the five probe-drugs in the cocktail after oral administration to a healthy male subject.
Conclusion

Drug cocktails can be used to characterize the effect of a compound on the metabolic activity of multiple P450 enzymes in a single clinical study. In the literature, several cocktails have been described, each with its own advantages and limitations. Frequently stated disadvantages include side-effects of, and interaction between, probe drugs, large volumes of samples required for analysis and time-consuming analysis. The cocktail described in this study has been validated by Turpault et al. and can be administered safely. Furthermore, our method for the simultaneous quantification of the five probes requires a very small blood volume (≥10 μL) and is less time-consuming than previously reported methods because it requires only one simple method for sample preparation followed by a nonchiral and chiral LC-MS/MS method that can be performed sequentially.

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Chapter 3

References

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