Polymorphic drug metabolising enzymes
Tamminga, Willem Jan

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PART II METHODOLOGY

2.1 MEPHENYTOIN AS A PROBE FOR THE CYP2C19 ACTIVITY: A CRITICAL EVALUATION OF ITS APPLICABILITY IN EPIDEMIOLOGY AND CLINICAL PHARMACOLOGY

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Keywords: CYP2C19, mephenytoin, phenotyping, clinical applicability

2.1.1 Abstract

Aims: To evaluate mephenytoin as a probe for CYP2C19 activity on its applicability in epidemiology and clinical pharmacological research.

Methods: Two thousand six hundred and thirty-eight (2638) subjects were evaluated for CYP2C19 expression using mephenytoin in a cocktail method. The urinary (S)-mephenytoin to (R)-mephenytoin excretion ratio was analysed by gas chromatography. The poor metabolizer (PM) status was confirmed by the acidification procedure. The data were analysed on reproducibility, sample stability and the influence of the acidification procedure. Drug safety was assessed by evaluation of the recorded adverse experiences.

Results: In spiked urine samples (S) and (R)-mephenytoin were stable after repeated freezing and thawing and after storage for about two years at -20 °C. In in-vivo samples of extensive metabolizers (EMs) initially analysed within 30 days and reanalysed after prolonged storage for five months the S/R-ratio significantly increased (18 - 27%) and after storage for more than five months it increased even further (85%). A significant increase for both EMs and false positive PMs of 2497% and 137% respectively was observed after acid pre-treatment. For true PMs the mean S/R-ratio did not significantly change after long-term storage or acid pre-treatment of samples. There was no significant influence of metabolizing status on the number of adverse experiences (p = 0.385).

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Conclusions: The robustness of mephenytoin as a probe for CYP2C19 activity was good. The acidification confirmation procedure is an absolutely necessity for an adequate classification of the CYP2C19 metabolizing status. If the enzyme activity should be measured quantitatively samples should be analysed as soon as possible. Mephenytoin has shown to be a safe probe drug.

2.1.2 Introduction
The anticonvulsant drug mephenytoin is the drug that led to the discovery of the polymorphic character of the Cytochrome P450 2C19 (CYP2C19) by Küpfer and Preisig in 1984.[2] In a family study they observed an autosomal inherited deficiency in the 4'-hydroxylation of (S)-mephenytoin.[2] In subjects with deficient 4'-hydroxylation, so-called poor metabolizers (PMs), plasma levels of S-mephenytoin were increased significantly as compared to extensive metabolizers and the metabolism was no longer stereoselective.[3] In 1993 it was discovered that CYP2C19 was strongly correlated with the (S)-mephenytoin hydroxylation[4] and in 1994 the molecular basis for the impaired metabolism was found[5]. Currently two wild type alleles and seven defective alleles have been identified accounting for more than 96% of all PMs.[6] The CYP2C19 genetic polymorphism displays large interracial differences with a poor metabolizers’ incidence of 2 to 5% in Caucasians and of 13-23% in Oriental populations.[7] The current knowledge of the biotransformation of mephenytoin is summarized in Figure 1.
Figure 1: Metabolism of (S) and (R)-Mephenytoin [8-11].

In extensive metabolizers for CYP2C19 the route of metabolism strongly differs between (S) and (R)-mephenytoin, whereas in poor metabolizers the routes of elimination are comparable. Mephenytoin is widely used as a probe drug for the assessment of CYP2C19 activity. Other, more recently implemented probes for CYP2C19 are the proton pump inhibitor omeprazole[12] and the anti-malarial drug proguanil[13]. Although especially omeprazole is being increasingly used, the majority of the studies on CYP2C19 activity are still performed using mephenytoin. Extensive and poor metabolizers can be characterized using racemic mephenytoin by measurement of the S/R-ratio[3] or the 4'-hydroxylation index, i.e. the amount of (S)-mephenytoin administered (50% of racemic dose) divided by the molar amount of 4'-hydroxymephenytoin eliminated over 8 hours[2]. Several aspects are important in selecting a probe drug: (i) it should
give a good indication of the enzyme activity for both poor and extensive metabolizer, (ii) it should be safe, (iii) the phenotyping procedure should be as less invasive as possible. Furthermore, analytical aspects and sample stability should be evaluated in selecting a probe drug. Finally the requirements of the study for which the probe drug should be used plays a role: is it applied in an epidemiological study, is it used for screening in phase I or II studies or is it applied in a phase I study to explain pharmacokinetic or pharmacodynamic observations.

Some disadvantages of mephenytoin as probe drug have been reported: The difficulty to obtain mephenytoin medication and analytical reference materials due to reduced clinical use [14], stability problems of urine samples of extensive metabolizers due to an acid-labile component [15] and the possibility of a sedative side effect as reported in a study in South-eastern Oriental subjects [16].

In this study we evaluate the applicability of mephenytoin as probe for CYP2C19 activity by analysing a large data set of volunteers phenotyped with a cocktail method. The data being assessed towards analytical aspects, the discriminative power in metabolic ratio and clinical side effects.

2.1.3 Methods

Subjects and study protocol

Two thousand six hundred and thirty-eight (2638) unrelated subjects (1830 males and 808 females) were evaluated for CYP2C19 expression. This group was also phenotyped for CYP2D6 and N-acetyltransferase, using dextromethorphan and caffeine as substrates, respectively. This cocktail approach has been validated for co-administration of mephenytoin and dextromethorphan [17,18] and mephenytoin and caffeine [19] and it was proven that the metabolic ratios were not influenced by these co-administrated drugs. All subjects were healthy volunteers and were phenotyped in the context of various clinical pharmacology studies. Most of them were recruited from the northern part of The Netherlands. Health assessment was made by recording medical history, which was without major pathology, and by an eligibility screening including clinical chemistry, haematology and urinalysis before drug administration. None of the volunteers had a history of alcohol abuse, drug addiction or a smoking habit of more than 15 cigarettes daily. The participants were not allowed to take any medication (with the exception of oral contraceptives or paracetamol) during 14 days before phenotyping. Subjects gave their written informed consent and the phenotyping protocol was approved by the independent Medical Ethics Committee (‘Stichting Beoordeling Ethiek Bio-Medisch Onderzoek’, Assen, The Netherlands).

In-Vivo Phenotyping

Subjects emptied their bladders and each took their medication: 10 mL of dextromethorphan hydrobromide monohydrate syrup containing 2.2 mg.mL\(^{-1}\) dextromethorphan (Samenwerkende Apothekers B.V., Utrecht, The
Netherlands), 200 mg caffeine powder (Genfarma, Maarssen, The Netherlands) and a tablet containing 100 mg of racemic mephenytoin (Epilanex®; Gerot Pharmazeutika, Vienna, Austria). All urine was collected for 8 hours after intake of the medication, after completion the urine was homogenized and an aliquot of about 20 mL was stored at –20 °C until analysis.

Bioanalysis

(S)-mephenytoin and (R)-mephenytoin were analysed by a modified enantioselective capillary gas chromatography assay described by Wedlund.[20] In short, 500 µL of urine was extracted after adding 200 µL of sodium acetate buffer (0.1 M, pH = 5.0) and 5 mL of diethyl ether by mixing on a tumble mixer for 10 minutes. The organic layer was separated and washed twice with 1 mL 0.25 M NaOH solution. The organic layer was evaporated to dryness at 37 °C under a gentle stream of nitrogen and reconstituted in 250 µL of ethyl acetate.

Of the final extract 2 µL was injected into the gas chromatograph by a splitless injection at an injector temperature of 225 °C. Separation was performed by a Chirasil-Val capillary column (length: 25 m; id: 0.32 mm; thickness 0.2 µm; Alltech Nederland BV, Breda, The Netherlands) with helium as carrier gas at a flow of 1.4 mL.min⁻¹. For elution the temperature was raised from 100 °C to 200 °C in 5.5 minutes and this temperature was held for another 9 minutes to finalize the chromatographic separation. The temperature of the detector was 260 °C and the flow rates of the detector gases were 3.3 mL.min⁻¹ for hydrogen, 27 mL.min⁻¹ for nitrogen and 100 mL.min⁻¹ for air. The urinary (R)-mephenytoin and (S)-mephenytoin excretion ratio based on peak heights was used as metabolic ratio. Subjects with S/R-ratios ≥ 0.8 were classified as poor metabolizer and subjects with ratios < 0.8 were classified as extensive metabolizer.[17] In addition, all CYP2C19 PMs, except for three subjects, were reanalysed after acidification of the urine before extraction with HCl (12M) to confirm the PM status.[21] The analysis for the 4'-hydroxylation index (4-hydroxymephenytoin) using a capillary GC method were performed by the pharmacy laboratory of the University Hospital 'Vrije Universiteit' Amsterdam, The Netherlands.[22] A hydroxylation index (HI) above 50 indicates a poor metabolizer.

Method Validation with Spiked Samples

The analytical methodology was validated on linearity, selectivity, precision, accuracy, storage stability, freeze-thaw stability and stability in sample compartment of the auto injector. All these experiments were performed using blank urine samples spiked with racemic mephenytoin and therefore all S/R-ratio levels during validation should be approximately 1. Furthermore, these spiked samples were stored at –20 °C before they were analysed. Linearity was assessed by analysing 8 concentration levels (10 - 500 µg.L⁻¹) in triplicate on the same day. These data was evaluated by ANOVA. Selectivity was checked by analysing six independent blank urine samples. Chromatograms of these samples were compared with a chromatogram of a test solution containing racemic mephenytoin. For the assessment of precision and accuracy samples at
four concentration levels (10, 25, 100 and 400 µg.L⁻¹) were analysed for five days in triplicate. These data were subjected to one-way ANOVA to estimate the within-run and between-run reproducibility. Freeze-thaw stability was assessed by analysing samples at two concentration levels (25 and 400 µg.L⁻¹) at five different days (freezing and thawing each day). The mean measurements were compared with the corresponding mean measurements from the precision and accuracy assessment. For the measurement of the stability in the sample compartment in the auto injector pooled extracts of spiked urine samples (25 and 400 µg.L⁻¹) were injected every two hours for a period of 30 hours. For in-process quality control spiked urine samples at four concentration levels (25, 100, 200 and 400 µg.L⁻¹) were analysed in duplicate each analytical run. This data was used to assess the influence of sample storage at -20 °C for a storage period of nearly two years by analysing the data by linear regression.

Method Validation with Samples Obtained from In-Vivo Screening
The data of the in-vivo screening were analysed on reproducibility, short-term sample storage stability, long-term storage stability and the influence of the acidification procedure on both PM and EM samples. All urine samples were stored at −20 °C before analysis or re-analysis. The effect of short-term sample storage up to approximately seven months was investigated in a selection of 40 samples, initially analysed as extensive metabolizer. Three groups were selected: samples that were initially analysed within 15 days and re-analysed after at least 4 months (n = 15), samples that were initially analysed after storage for 15 to 30 days and re-analysed after at least 4 months (n = 15) and samples that were initially analysed within 30 days and re-analysed after 2 days of storage (n = 10, reference group). The effect of long-term storage was investigated in a selection of EM samples and in all samples originally identified as PMs. For the PM samples no selection criteria were used, all available PM samples (n = 51) were re-analysed. For EM subjects samples with an S/R-ratio above 0.10, that were initially analysed within 30 days and re-analysed after one to two years, randomly selected and covering this whole storage period were used (n = 66). Finally, all samples that were phenotyped as EM (n = 2584) were divided per week of storage and statistical analysis was performed on the mean S/R-ratio. To study the effect of the acid sample pre-treatment 47 EM and 51 PM samples were selected. For PMs all available samples were used and for the EMs random selection of samples with an S/R-ratio above 0.1 was made.

Evaluation of Phenotyping Procedure
Due to recruitment requirements some volunteers were phenotyped more than once (n = 129). These data were used to assess the intra-individual variability (robustness) of the phenotyping procedure for mephentoin as CYP2C19 selective probe. Furthermore, in a small number of samples (n = 14) the S/R-ratio was compared with the 4'-hydroxylation index. Finally, drug safety was assessed by evaluation of a random sample (n = 797) of the recorded adverse experiences. The adverse experiences were queried using non-leading questions (‘How do you feel’). All adverse events were recorded and interpreted
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by a medical investigator according to the WHO dictionary for adverse events.[23]

Statistical Analysis
All data was collected in Microsoft Excel (Microsoft, Redmond, WA USA) in which all database handlings and statistical analyses (descriptive statistics, t-tests, regression and ANOVA) were performed. SAS version 6.12 (SAS Institute Inc., Cary, NC USA) was used for the Chi-square test.

2.1.4 Results

Method Validation with Spiked Samples
The selectivity of the method was evaluated by comparing the chromatograms of a test solution of the pure compounds with the chromatograms of the six independent blank urine samples of female and male subjects. The peaks of S-mephenytoin and R-mephenytoin were well resolved and showed no interferences with metabolites of mephenytoin or dextromethorphan / caffeine or endogenous materials. The lower and upper limits of quantitation, for both enantiomers, were 10.0 µg.L⁻¹ and 500 µg.L⁻¹, respectively. For (R)-mephenytoin the lack of fit (F-value) was found to be 1.19 and for (S)-mephenytoin it was 0.97, which were both below the critical F-value (F-table = 2.74; α = 0.05; df = 6) indicating a linear relationship between peak height and concentration for 10.0 µg.L⁻¹ to 500 µg.L⁻¹. The results of the accuracy and precision experiments are summarized in Table 1.

Table 1: Accuracy and precision of the S/R ratio of mephenytoin in spiked urine samples.

<table>
<thead>
<tr>
<th>Concentration of S-mephenytoin and R-mephenytoin (µg.L⁻¹)</th>
<th>S/R-Ratio</th>
<th>Bias (%)</th>
<th>Within-Run CV (n = 15) (%)</th>
<th>Between-Run CV (n = 5) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0</td>
<td>1.000</td>
<td>1.018</td>
<td>1.8</td>
<td>3.5</td>
</tr>
<tr>
<td>25.0</td>
<td>1.000</td>
<td>0.998</td>
<td>-0.2</td>
<td>2.9</td>
</tr>
<tr>
<td>100</td>
<td>1.000</td>
<td>0.989</td>
<td>-1.1</td>
<td>1.6</td>
</tr>
<tr>
<td>400</td>
<td>1.000</td>
<td>0.984</td>
<td>-1.6</td>
<td>2.3</td>
</tr>
</tbody>
</table>

The within-run coefficients of variation were below 3.6% at all concentration levels and the between-run coefficients of variation were below 11.5% at all concentration levels. The bias observed in these experiments was between -1.6% and +1.8%.

During a storage period of 30 hours S/R-ratios of prepared samples varied between 0.942 (t = 2 h) and 1.045 (t = 0 h) for the 25 µg.L⁻¹ level and between 0.942 (t = 8 h) and 1.037 (t = 26 h) for the 400 µg.L⁻¹ level indicating no degradation of the prepared samples during the observed period. The results of the freeze/thaw experiments are summarized in Table 2, from which can be
seen that racemic mephenytoin spiked in urine showed no notable deterioration after repeated freezing and thawing (*n* = 5).

**Table 2:** Stability of the S/R-ratio of mephenytoin during freezing and thawing cycles in spiked urine samples.

<table>
<thead>
<tr>
<th>Cycle number</th>
<th>Mean S/R-ratio (25 µg.L(^{-1}); <em>n</em> = 2)</th>
<th>Mean S/R-ratio (400 µg.L(^{-1}); <em>n</em> = 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nominal</td>
<td>Measured</td>
</tr>
<tr>
<td>1</td>
<td>1.000</td>
<td>1.030</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>0.996</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>1.037</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
<td>0.964</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>0.983</td>
</tr>
</tbody>
</table>

The results of long-term storage at -20 °C of urine samples spiked with racemic mephenytoin are given in Figure 2.

**Figure 2:** Effect of long-term storage at -20 °C on the S/R-ratio of urine samples spiked with racemic mephenytoin and stored for a period of approximately two years.

The minimum observed ratio was 0.86 (25 µg.L\(^{-1}\) level) and the maximum observed level was 1.10 (25 µg.L\(^{-1}\) level). There was no significant trend observed as can be concluded from linear regression analysis of the data (*R^2* = 0.0124), so that *(S)* and *(R)*-mephenytoin can be considered stable in spiked samples for about two years when stored at -20 °C.
Method Validation with Samples obtained from In-Vivo screening

The results of short-term storage of urine samples obtained from in-vivo phenotyping are given in Table 3.

**Table 3:** Short-term stability of the S/R-ratio of mephenytoin in urine samples obtained from volunteers that were identified as extensive metabolizers by in-vivo phenotyping.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean S/R-Ratio Initial</th>
<th>Mean S/R-ratio Reanalysis</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S/R-ratio   Variance n</td>
<td>S/R-ratio   Variance n</td>
<td>(%)  p-value</td>
</tr>
<tr>
<td>A</td>
<td>0.1779 0.0117 15</td>
<td>0.2260 0.0292 15</td>
<td>+27 0.07</td>
</tr>
<tr>
<td>B</td>
<td>0.2386 0.0174 15</td>
<td>0.2807 0.0212 15</td>
<td>+18 0.01</td>
</tr>
<tr>
<td>Reference</td>
<td>0.1691 0.0035 11</td>
<td>0.1627 0.0032 11</td>
<td>-3.4 0.79</td>
</tr>
</tbody>
</table>

Group A: initial analysis within fifteen days of storage at -20 °C, reanalysis after 129 to 203 days; Group B: initial analysis after fifteen days but before 30 days of storage at -20 °C, reanalysis after 203 days; Reference group: initially analysed within 30 days of storage at -20 °C and reanalysed after two days. p-value: two tailed p-value as obtained by a paired t-test.

In group A the samples were initially analysed within 4 to 14 days and reanalysed after prolonged storage for 129 to 203 days. In group B samples were initially analysed within 15 to 29 days and reanalysed after prolonged storage for 203 days. The reference group samples were initially analysed within 13 to 29 days and reanalysed after storage for 2 days. From Table 3 it can be seen that the mean S/R-ratio increased 27% (p = 0.07) in group A, it increased 18% (p = 0.01) in group B and it decreased 3.4% (p = 0.79) for the reference group.

The result of long-term storage in both PM samples and EM samples are summarized in Table 4 and Figure 3.

**Table 4:** Long-term stability of the S/R-ratio of mephenytoin in urine samples obtained from volunteers that were characterized by in-vivo phenotyping.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Mean S/R-Ratio Initial</th>
<th>Mean S/R-ratio Reanalysis</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S/R-ratio Variance n</td>
<td>S/R-ratio Variance n</td>
<td>(%)  p-value</td>
</tr>
<tr>
<td>PM</td>
<td>1.058 0.005 51</td>
<td>1.047 0.005 51</td>
<td>-1.2 0.03</td>
</tr>
<tr>
<td>EM</td>
<td>0.177 0.005 66</td>
<td>0.327 0.031 66</td>
<td>+84.9 &lt;0.05</td>
</tr>
</tbody>
</table>

PM: initial analysis within 2 to 129 days of storage at -20 °C, reanalysis after 190 to 1010 days; EM: initial analysis within 4 to 30 days of storage at -20 °C, reanalysis after 434 to 1176 days; p-value: two tailed p-value as obtained by a paired t-test.

For PM samples initial analysis was performed between 2 and 129 days of storage at -20 °C, while reanalysis was performed within 190 to 1010 days of storage. For EM samples initial analysis was performed between 4 and 30 days of storage.
of storage at −20 °C, whereas reanalysis was performed within 343 to 1176 days of storage. It can be seen that due to long term storage the mean metabolic ratio decreased by 1.2% (p = 0.03) for PM subjects and increased by 84.9% (p < 0.05) for EMs. This is further illustrated by Figure 3 in which can be seen that almost all EM S/R-ratios increased after reanalysis, whereas for PMs no such increase is observed. Furthermore, it can be seen that in one case a sample shifted from EM to PM (initial ratio: 0.122; storage 15 days and reanalysis ratio: 1.016; storage 1086 days). For PM samples no classification shifts were observed.

![Figure 3](image)

**Figure 3**: Long-term stability of the S/R-ratio of mephenytoin in urine samples obtained from volunteers that were characterized by in-vivo phenotyping. —: no difference between initial and reanalysis (Y = X); - - -: Antimode between EM and PM (S/R-ratio = 0.8)

In Figure 4 the results of the S/R-ratio stability in a large group of extensive metabolizers (n = 2586) was retrospectively evaluated. The S/R-ratio measured was grouped per week of storage before analysis. It can be seen that the mean S/R-ratio is stable for two weeks of storage (mean S/R-ratio = 0.138) and that prolonged storage significantly increased the S/R-ratio with about 14% (p-value 2 to 3 weeks = 0.02). After three weeks of storage the mean S/R-ratio varied between 0.158 (8 weeks) and 0.185 (10 weeks) but no significant difference was observed.
Figure 4: Changes in S/R-ratio as a function of sample storage: data obtained from all volunteers that were phenotyped as EM divided per period (weeks) of storage.

The results of acid sample pre-treatment on the S/R-ratio in both PMs and EMs are given in Table 5 and Figure 5. In Table 5 the PM subjects were subclassified in true and false PMs: true PMs are those subjects that did not show a significant increase of the S/R-ratio after the acidification procedure, whereas false PMs were initially classified as PMs but who showed an increased S/R-ratio (S/R-ratio ≥ 1.40) after acidification. A significant increase for both EMs and false PMs of 2497% (p < 0.01) and 137% (p = 0.02) respectively was observed. For true PMs the mean S/R-ratio did not change significantly (p = 0.15). For subjects with an initial S/R-ratio above 0.80, 6 (11.8%) of them showed a significant increase in ratio (S/R-ratio ≥ 1.40) and were therefore finally characterized as EM (Figure 5). It can also be seen that all EM samples gave an increase in S/R-ratio after acidification, which varied from 1.08 (4.5 times the initial S/R-ratio) to 12.3 (82 times the initial ratio).
Table 5: Influence of acidification on the S/R-ratio of mephenytoin in urine samples obtained from volunteers that were characterized by in-vivo phenotyping.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Mean S/R-Ratio Initial S/R-ratio</th>
<th>Mean S/R-ratio Reanalysis S/R-ratio</th>
<th>Difference (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM all</td>
<td>1.059 0.003 51</td>
<td>1.222 0.229 51</td>
<td>+15 0.03</td>
<td></td>
</tr>
<tr>
<td>PM false</td>
<td>0.949 0.005 6</td>
<td>2.251 0.839 6</td>
<td>+137 0.02</td>
<td></td>
</tr>
<tr>
<td>PM true</td>
<td>1.074 0.002 45</td>
<td>1.085 0.002 45</td>
<td>+1 0.15</td>
<td></td>
</tr>
<tr>
<td>EM</td>
<td>0.195 0.019 47</td>
<td>5.056 5.023 47</td>
<td>+2497 &lt;0.01</td>
<td></td>
</tr>
</tbody>
</table>

PM all: all initially phenotyped with a S/R-ratio above the antimode (0.80), PM false: S/R-ratio initially above 0.80, but above 1.4 after acidification pre-treatment; PM true: S/R-ratio initially above 0.80 and below 1.4 after acidification pre-treatment; p-value: two tailed p-value obtained by a paired t-test.

Figure 5: Influence of acidification on the S/R-ratio of mephenytoin in urine samples obtained from volunteers that were characterized by in-vivo phenotyping. - - -: Antimode between EM and PM (S/R-ratio = 0.8); — --- —: acidification limit (S/R-ratio = 1.4).

Evaluation of Phenotyping Procedure
The repeatability of the phenotyping procedure is summarized in Table 6 and Figure 6. The subjects (n = 129) involved were all initially classified as EM. It can be seen that there was no significant difference between the mean S/R-ratio value of the first and the second phenotyping (p = 0.92). However, sometimes large intra-individual differences exist; in one case it led to a misclassification (initially EM, but PM at second phenotyping; Figure 6).
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**Table 6:** Repeatability of CYP2C19 phenotyping procedure using mephenytoin as probe assessed by repeating the phenotyping procedure.

<table>
<thead>
<tr>
<th>Source</th>
<th>Phenotyping Result (S/R-ratio)</th>
<th>p-value</th>
<th>Repeatability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>n</td>
</tr>
<tr>
<td>Initial</td>
<td>0.1508</td>
<td>0.1187</td>
<td>129</td>
</tr>
<tr>
<td>Second</td>
<td>0.1499</td>
<td>0.1260</td>
<td>129</td>
</tr>
</tbody>
</table>

p-value: two tailed p-value as obtained by a paired t-test; repeatability: mean variation between result of first and second phenotyping.

![Graph showing linear regression between first and second S/R-ratio](image)

**Figure 6:** Repeatability of CYP2C19 phenotyping procedure using mephenytoin as probe assessed by repeating the phenotyping procedure. —: linear regression between first and second S/R-ratio (slope ± 95% interval: 0.6748 ± 0.1439 \((p < 0.05)\); intercept ± 95% interval: 0.0482 ± 0.0276 \((p < 0.05)\)).

The intra-individual repeatability of the mephenytoin CYP2C19 phenotyping test is estimated at 28% (Table 6). A comparison between the S/R-ratio and the hydroxylation index is given in Figure 7. There was a significant correlation between the hydroxylation index and the S/R-ratio (correlation coefficient: 0.556) but this correlation was rather poor as can be concluded from the large variation in the slope \((9.73 ± 9.65; \text{p-value: } 0.048)\) and intercept \((2.31 ± 1.92; \text{p-value: } 0.02)\).
Figure 7: Correlation between S/R-ratio and hydroxylation index in urine samples obtained from volunteers that were characterized by in-vivo phenotyping.

- hydroxylation index; —: linear regression between hydroxylation index and S/R-ratio (slope ± 95% interval: 9.73 ± 9.65; p-value: 0.048 and intercept 2.31 ± 1.92; p-value: 0.02) ---: Antimode between EM and PM (S/R-ratio = 0.8; HI = 50).

The results on the assessment of the safety of the drug are summarized in Table 7. In 22% (n = 175) of all cases an adverse drug experience was recorded. The majority of the recorded adverse experiences were of mild intensity (98.9%), no interventions were necessary and 73% had a possible relationship with medication while 27% had no relation with the medication. Adverse experiences recorded where: sleeplessness (50%), abdominal discomfort and nausea (23%), drowsiness and vertigo (20%) and headache (9%). Application of the chi-square test showed no significant influence of the phenotype on the number of adverse experiences (p = 0.358).

Table 7: Drug safety assessment by evaluation of the recorded adverse experiences of a randomly selected subpopulation (n = 797).

<table>
<thead>
<tr>
<th>Group</th>
<th>Adverse Drug Experiences</th>
<th>No Adverse Drug Experiences</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Expected</td>
</tr>
<tr>
<td>Extensive</td>
<td>174</td>
<td>172.8</td>
</tr>
<tr>
<td>Poor</td>
<td>1</td>
<td>2.2</td>
</tr>
<tr>
<td>Total</td>
<td>175</td>
<td>-</td>
</tr>
</tbody>
</table>

Chi square test on possible relationship between phenotype and adverse experiences resulted in a p-value of 0.358, which is not significant.
2.1.5 Discussion

Mephenytoin is the most common probe for the assessment of CYP2C19 activity in both epidemiological studies and clinical pharmacological research. In addition, mephenytoin has shown to be a potential indicator of hepatic pathologic condition with an ability to discriminate between contributory alternative mechanisms.[24] A search on the Internet (Medline) in the period 1990 until 1999 on keywords CYP2C19 and phenotype gave 60 hits: in 87% mephenytoin was mentioned, in 28% omeprazole and proguanil in 7% of the hits. A survey through twenty-nine population studies from 1984 to 1998 indicated similar results: in 23 studies (79%) mephenytoin was used, in 5 studies (17%) omeprazole was used and in one study (3%) CYP2C19 was probed with proguanil. In the studies using mephenytoin the CYP2C19 activity was assessed by the S/R-ratio (64%) or by the hydroxylation index (20%) or by both ratios (16%). Bioanalysis was performed by GC (88%) or by HPLC (12%) and in 45% of the studies the acidification procedure was used to confirm the metabolizer status. The quality of the analytical methodology to assess the metabolic activity is of great importance for clinical pharmacological research. A poor procedure may lead incorrect assignment of phenotype in Phase I studies which may lead to false conclusions with respect to enzyme activity or may lead to an increased risk for the volunteers. A good validation showing the appropriateness of the technique to achieve the ultimate goal is therefore needed.[25] Therefore, a comprehensive and correct validation is necessary to show the appropriateness of the techniques and to make sure that the conclusions drawn are warranted. From the validation experiments in this study using the spiked samples it can be concluded that the selectivity, sensitivity, accuracy and precision are all appropriate for phenotyping the CYP2C19 activity (Table 1). Recently, we estimated the risk that samples may be misclassified due to analytical errors at 0.3% using an antimode of 0.80.[26] Furthermore, analyte stability for (S) and (R)-mephenytoin was shown good in spiked urine samples during sample processing and storage for approximately 2 years (Table 2 and Figure 2). In contrast with this, analyte stability was poor in urine samples obtained from in-vivo screening. This poor analyte stability was only observed in extensive metabolizers (Table 4 and Figure 3) and was most pronounced during the first weeks of storage (Table 3 and Figure 4). This phenomenon may incidentally lead to misclassification (Figure 3). These analyte stability problems could be demonstrated even more extensively by acidification of the in-vivo urine samples. Treatment with concentrated HCl resulted in significant increase in the S/R-ratios of EMs and it was also observed from this procedure that subjects (n = 6) have been falsely classified as PM initially (Table 5 and Figure 5). These analyte stability problems have been known for several years and are due to an acid-labile metabolite of S-mephenytoin excreted in urine of EMs but not PMs (Figure 1).[8,15] This acid-labile metabolite has been identified as an S-mephenytoin cysteine conjugate, which is easily hydrolysed back to S-mephenytoin, resulting in an increased S/R-ratio.[10] This is also the underlying principle for the acidification confirmation procedure in which a significant increase in S/R-ratio will be obtained in EMs and not in PMs after
acidification of an urine sample.[21] Our data support these findings: in spiked urine samples the S/R-ratio was stable but in in-vivo EM samples it was not, storage for more than two weeks increases the S/R-ratio significantly in EM subjects and acidification of in-vivo samples increases in all cases the S/R-ratio of EM samples while the ratio is stable in real PMs. This finding has consequences for the appropriateness of using mephenytoin as probe for CYP2C19 activity. Firstly the acidification confirmation procedure is absolute necessary for a good classification of the metabolizer status: all PMs should be confirmed by this procedure and should be classified as EM in case the S/R-ratio increases above 1.4. Neglecting this procure may lead to misclassification in 12% of all identified PMs. Secondly if the in-vivo enzyme activity should be measured quantitatively, e.g. in drug interaction studies, it is of great importance to analyse the samples as soon as possible, ideally within two weeks.

The correlation between the hydroxylation index and the S/R-ratio was shown to be significant (Figure 7). Both indices have advantages and disadvantages as a measure for enzyme activity: the hydroxylation index is sensitive for non-compliance and incomplete urine collection, whereas the S/R-ratio may suffer from instability problems if not properly handled.[27] For routine phenotyping however we prefer the S/R-ratio, since the stability problems can be easily overcome by the measures described above. Furthermore, non-compliance and incomplete urine collection are easily detected, since (R)-mephenytoin can be regarded as an ‘in-vivo internal standard’. The robustness (intra-individual repeatability) of mephenytoin as probe for CYP2C19 activity was shown to be adequate (28%) but caution should be taken when the S/R-ratio is above 0.3. Mephenytoin at the 100 mg dose level administered in combination with dextromethorphan (22 mg) and caffeine (100 mg) has shown to be a safe probe drug with no serious adverse drug reactions in a group of both poor (n = 10) and extensive metabolizers (n = 787). No relationship between adverse experiences and the CYP2C19 phenotype was observed.

Recently, we reported on oral contraceptive-related gender differences for CYP2C19.[26] In that study we found a CYP2C19 activity significantly decreased by 68% in oral contraceptive using females. Similar results were reported from a study using proguanil as probe for CYP2C19 activity.[28] This illustrates the usefulness of mephenytoin in epidemiological studies in which environmental effects on CYP2C19 activity can be detected. In addition, it emphasizes an advantage of phenotyping over genotyping in such studies. Compared to phenotyping genotyping is a more simple procedure: no probe drugs are necessary, a blood sample or sometimes even a swab sample taken from the mucosa of the cheek is convenient, the analytical equipment is more simple and less expensive than for phenotyping. Furthermore, genotyping bypasses some confounding factors involved in phenotyping and directly identifies the possibility to express the iso-enzyme of interest.[27] For these reasons genotyping is rapidly replacing phenotyping as the tool to describe populations. Recently, we showed a good correlation between phenotype on CYP2C19 using mephenytoin and genotyping on CYP2C19*2 and CYP2C19*3
mutant alleles.[29] Nevertheless, we still recommend phenotyping for the ultimate confirmation of the actual metabolic capacity in the context of clinical pharmacology studies.

Acknowledgements
The authors would like to thank Dr. F. Sollie for his assistance in the statistical analysis of the data. This project was supported by grants of ‘Senter voor technologie, energie en milieu’ (BIO96052) and ‘Stichting voor Technische Wetenschappen (STW)’ (GPR66.4084).

2.1.6 References
Methodology

2.2 EVALUATION OF ANALYTICAL AND CLINICAL PERFORMANCE OF A DUAL-PROBE PHENOTYPING METHOD FOR CYP2D6 POLYMORPHISM AND CYP3A4 ACTIVITY SCREENING#

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KEY WORDS: CYP2D6; CYP3A4; Dextromethorphan and metabolites; Validation; HPLC; Error Propagation

2.2.1 Abstract
A bioanalytical method for the determination of dextromethorphan and its metabolites dextrorphan, 3-methoxymorphinan and 3-hydroxymorphinan in human urine was developed. The purpose of the method was CYP2D6 phenotyping and CYP3A4 activity measurements for clinical pharmacology studies using dextromethorphan administered in a drinking solution as substrate. Sample cleanup was performed using liquid-liquid extraction after hydrolysis at 100 °C to release conjugated dextrorphan. Separation was performed by reversed-phase high performance liquid chromatography using a 5 µm Chromsphere® 5B C18 column (150 × 4.6 mm) and a mobile phase consisting of a mixture of 0.05 M phosphate buffer (pH = 3.0), triethylamine and acetonitrile (75.3 : 0.7 : 24, v/v). Quantitation was performed using fluorescence detection (excitation wavelength: 230 nm; emission wavelength 310 nm). The performance of the method was evaluated through thorough conventional validation and by a cross-validation of the method with a previously applied method for dextromethorphan and dextrorphan only (CYP2D6 phenotyping). The accuracy and precision were within the acceptance criteria of 85-115%. The analytes were stable during the sample preparation and instrumental analysis steps. Cross-validation with the former method showed no significant differences in measured concentrations of volunteer samples. This guaranteed the consistency of epidemiological data in our database collected from two methods. For the CYP2D6 and CYP3A4 evaluations, the clinical parameters are ratios of concentrations. It appeared that severe variance in individual concentrations did,

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in general, not influence the variance of ratios significantly, since experimental errors in concentrations of two analytes proved to correlate considerably. For CYP2D6 values around the antimode, the chance of a misclassification is very small. The chance of classifying an extensive metabolizer as a poor metabolizer or vice versa is negligible. For CYP3A4 activity determinations it was concluded that in general a change in DEX/3MM ratios, detected with the current method, of 10% or more, is a significant increase or decrease in the activity of CYP3A4. We concluded that we had obtained an analytically valid and clinically reliable bioanalytical method for the determination of dextromethorphan and its metabolites dextrophan, 3-methoxymorphinan and 3-hydroxymorphinan in human urine for CYP2D6 phenotyping and CYP3A4 activity measurements for clinical pharmacology studies.

2.2.2 Introduction

CYP isoenzymes are known to be important catalysts for oxidative biotransformation of both endogenous and exogenous compounds [1]. Drugs are often metabolised by a limited number of isoenzymes of cytochrome P450 (CYP). Due to genetic polymorphism of some of these isoenzymes and due to exogenous factors influencing the activity of these isoenzymes (inhibition, induction), standard medication may result in large interindividual variation in drug levels causing a large variability in therapeutic response and enhancement of adverse drug effects. Although the recognition of extensive metabolizers (EMs) and poor metabolizers (PMs) with respect to many cardiovascular and psychotropic agents already occurred 15 years ago, routine phenotyping and/or genotyping for these isoenzymes is rare in current clinical practice [2]. Today, genotyping is rapidly replacing phenotyping as the tool to describe populations, mainly through recent and current developments in molecular biology. Genotyping is a more simple procedure compared to phenotyping: no probe drugs are necessary, a blood sample or even a cheek mucosa swab sample is convenient and the analytical equipment is simple and cheap. Furthermore, genotyping bypasses the confounding factors involved in phenotyping and directly identify the possibility to express the iso-enzyme of interest. However, we still recognize the value of phenotyping instead of genotyping in studies in which environmental effects on cytochrome P450 activity can be evaluated. Although we generally find good correlations between genotype and phenotype, we still use phenotyping for the ultimate confirmation of the actual metabolic capacity in the context of clinical pharmacology studies. CYP2D6, debrisoquine 4-hydroxylase, a well-studied isoform of CYP450, is involved in the metabolism of many widely prescribed drugs, including beta-adrenergic receptor antagonists, antiarrhythmics, antidepressants and neuroleptics [3]. The individual status of CYP2D6 activity can be probed with several drugs such as debrisoquine, sparteine, metoprolol and dextromethorphan. Poor activity of the enzyme (PM phenotype) is an autosomal recessive trait that affects approximately 7% of Caucasians and 1% of Orientals. The opposite phenomenon called ultrarapid metabolism also exists and is
caused by gene amplification [4]. With standard medication this phenotype will suffer from sub therapeutic plasma concentrations and thus from decreased drug response.

A widely used method for CYP2D6 phenotyping is the use of dextromethorphan as substrate. Extensive dextromethorphan metabolizers excrete more than 30% of the administered dose in urine as the O-demethylated metabolite dextrorphan within eight hours, whereas in poor metabolizers (PM) this amount is less than 5%.

When the metabolic ratio is calculated, i.e. the ratio of urinary excreted dextromethorphan/dextrorphan excretion, a bimodal distribution is found with a gap between the metabolic ratios of 0.2 and 0.6 and an antimode of 0.3 separating the EMs from the PMs. Subjects with a metabolic ratio > 0.3 are classified as PM. They constitute about 10% of the Caucasian population.

For phenotyping, a urine sample is collected 8 hours after administration of dextromethorphan. Although in general a distinction is made between poor and extensive metabolizers for CYP2D6, we have defined three classes of metabolizers (although not supported by genotyping databases) as follows: if the ratio of dextromethorphan over dextrorphan is ≥ 0.3, the subject is a poor metabolizer; if the ratio is less than 0.02, the subject is an extensive metabolizer. All other subjects are classified as intermediate metabolizer. During ten years of clinical pharmacology studies, we have collected phenotyping results on CYP2D6 (dextromethorphan) activity of 4301 Dutch volunteers in this way [5]. From an evaluation of the results it was concluded that the PM incidence (8.0%) is in accordance with literature data and that gender differences exist.

Cytochrome P450 3A4 (CYP3A4) is an iso-enzyme involved in Phase I oxidative metabolism of many endogenous and exogenous substances. From a quantitative point of view it is the most important hepatic CYP-enzyme accounting for approximately 25% of all liver cytochrome P450s [6]. Since CYP3A4 is also present in the small intestine, it has a significant effect on the first-pass effect of the CYP3A4 substrates. The gene coding for CYP3A4 is situated on chromosome 7 and at this moment no genetic polymorphisms have been described that are associated with changed enzyme activity [7]. Nevertheless, the variability of CYP3A4 activity has appeared to be quite severe. The intrinsic clearance for CYP3A4 metabolised substances can vary dramatically amongst individuals, with inter-individual differences of factors of 10 or higher [8]. The iso-enzyme is quite sensitive to inhibition and induction [9,10], which is perhaps the largest cause for the high variability. Inhibition of CYP3A4 in the intestines by, amongst others, grapefruit juice may lead to a substantial increase of bioavailability. Such interactions are clinically relevant for CYP3A4 substrates with a low bioavailability, such as terfenadine and saquinavir [11]. Amongst these substances, the CYP3A4 enzyme is involved in the metabolism of about 50 well-known drug substances, such as erythromycin, cyclosporin, ketoconazole, nifedipine, midazolam, sertindole and verapamil [9,10].

Several methods have been published to establish the CYP3A4 activity. The gold standard is the erythromycin breath test [12], which is based on
n-demethylation of erythromycin (ERM) exclusively catalysed by CYP3A4. The metabolite formaldehyde is mainly excreted as CO\textsubscript{2} in expired air. For performing this test, a sub-therapeutic dosage of 0.06 mg 14C-ERM (3 µCi) is administered intravenously. Subsequently, 14C\textsubscript{2} in expired air is collected (usually 20 minutes after dosing) and measured with a conventional breath test and liquid scintillation counting. The measured radioactivity is a measure for the CYP3A4 activity. This test only provides the hepatic CYP3A4 activity. If intestinal CYP3A4 activity has to be measured, an alternative method must be used, such as orally administered midazolam clearance or the orally administered dextromethorphan/3-methoxymorphinan clearance establishment. From a study subject’s point of view, the establishment of the endogenous ratio of 6-β-hydroxycortisol over cortisol in a 24-hour urine fraction would be the easiest way to establish CYP3A4 activity. This latter test has many practical advantages, but it has little correlation with the gold standard (14C-ERM, midazolam).

We have selected dextromethorphan as a substrate for both CYP2D6 phenotyping and CYP3A4 activity measurements. Dextromethorphan is a well-validated probe for CYP2D6. Studies have been published on the applicability of dextromethorphan as CYP3A4 probe, where the authors studied inhibition detection with dextromethorphan with regard to correlation with verapamil and tamoxifen [13, 14]. Using dextromethorphan as a probe for CYP2A4 activity also has a constraint with respect to a conclusion by Kashuba et al [15]. They state that there is a large intraindividual variability of the DEX/3MM urinary ratio and that using this ratio does not enable investigators to discriminate moderate CYP3 inhibition.

The present paper describes the bioanalytical method that we have implemented for the analysis of dextromethorphan (CYP2D6 and CYP3A4), dextrorphan (CYP2D6) and 3-methoxymorphinan (CYP3A4). Figure 1 shows the metabolism of dextromethorphan. The secondary metabolite 3-hydroxymorphinan is thought to be formed from dextrorphan via CYP3A4 and from 3-methoxymorphinan via CYP2D6. Although this compound is currently not used by us for CYP2D6 phenotyping or CYP3A4 activity determinations, we have included the compound in the method for possible future work. The objective of the research described was to evaluate the developed methodology from an analytical and a clinical point of view.

The method was validated thoroughly, including a cross-validation with a method previously used for CYP2D6 only (dextromethorphan over dextrorphan ratio), to guarantee equal quality of data from two different methods, and including an investigation of the influence of analytical errors on the clinical outcome (poor or extensive metabolizer). The next theory section discusses the background and consequences of analytical errors in bioanalyses of multiple components, when ratios are used for clinical evaluations. It must be noted that this paper does not include discussions on all potential errors occurring from clinical operations.
2.2.3 Theory

The processing and analysing of samples for clinical evaluations is subject to many error sources. In cases where two compounds are analysed simultaneously in the same sample or sub-sample, and the outcomes for both compounds are reported as their ratio, many of these error sources, such as sampling amount or chromatographic injection volume, do not influence the outcome, since they affect both compounds identically. However, a compound and its metabolite possess different physico-chemical properties; therefore, they may behave differently during sample clean-up (extraction) and during the instrumental analysis (separation and detection). This may result in random errors that may be different for both compounds. A broad discussion on dissimilar analytical errors of simultaneously analysed compounds originating from chromatographic analysis and/or sample preparation was given by Wieling et al [16]. Similar to what they concluded for liquid-liquid extraction of an analyte simultaneously with an internal standard, a robust concentration ratio of two compounds (robust with respect to varying random conditions) for phenotyping highly determines the precision and accuracy of the clinical method. In cases where several analytes are analysed in one phenotyping assay, the robustness of all targeted concentration ratios must be optimal in order not to predict a false phenotype.

Figure 1: Metabolic pathway for dextromethorphan
The ratio of the concentrations of two simultaneously analysed compounds (i.e. analysed in the same clean-up and instrumental analytical step and not in two separate assays) is expressed by the quotient of both concentrations:

\[ Q = \frac{C_a}{C_b} \]  

(1)

The rules for error propagation give an expression for the variance of the ratio of the concentrations:

\[ S_Q^2 = S_a^2 \left( \frac{\partial Q}{\partial C_a} \right)^2 + S_b^2 \left( \frac{\partial Q}{\partial C_b} \right)^2 + 2 \cdot S_{ab} \cdot \frac{\partial Q}{\partial C_a} \cdot \frac{\partial Q}{\partial C_b} = Q^2 \left[ \left( \frac{S_a}{C_a} \right)^2 + \left( \frac{S_b}{C_b} \right)^2 \right] + 2 \cdot \frac{S_{ab}}{C_a \cdot C_b} \]  

(2)

The partial derivatives provide an estimate of the change of the overall response variable Q with a change in one of the component variables (C_a and C_b) while the other component variable is held constant. When the variables C_a and C_b are completely independent of each other (i.e. uncorrelated) the covariance \( S_{ab} \) is zero and the variance of the ratio is:

\[ S_Q^2 = Q^2 \left[ \left( \frac{S_a}{C_a} \right)^2 + \left( \frac{S_b}{C_b} \right)^2 \right] = S_a^2 + Q^2 \cdot \frac{S_b^2}{C_b^2} \]  

(3)

The covariance can be calculated from n replicate measurements of the concentrations of both compounds:

\[ S_{a,b} = \frac{\sum_{j=1}^{n} C_{a,j} \cdot C_{b,j} - \left( \sum_{j=1}^{n} C_{a,j} \right) \cdot \left( \sum_{j=1}^{n} C_{b,j} \right)}{n-1} \]  

(4)

The correlation coefficient \( \rho \) is a measure of the correlation between the two variances of the concentrations of each compound:

\[ \rho = \frac{S_{ab}^2}{S_a \cdot S_b} \]  

(5)

A detailed discussion on error propagation is given by Ku [17] and Balke [18]. In conclusion, the variance of the ratio of the concentrations of two compounds is a function of the ratio Q, the concentrations of the compounds C_a and C_b, the variances of the concentrations of both compounds \( S_a^2 \) and \( S_b^2 \) and the correlation between those variances \( \rho \).
2.2.4 Methods

**Instruments and Instrumental Conditions**
Separation and quantitation were performed using an HPLC system consisting of the following components: a Model M 510 pump (flow rate 0.8 mL/min) and an Autosampler Model 717plus (injection volume 75 µL) from Waters Associates (Milford, MA, USA). The analytical column was a 5 µm Chromsphere® 5B C18 cartridge column (150 × 4.6 mm), temperature-conditioned at 30 °C. An R2 guard column was supplied by Chrompack (Middelburg, The Netherlands). The Model 821 FP fluorescence detector was obtained from Jasco (Hachioji City, Japan) (excitation wavelength: 230 nm; emission wavelength: 310 nm). Data collection and reduction was performed with Waters Millennium™ 2020 Version 2.15.1 Chromatography Manager. A Millipore HVLP 0.45 µm filter (Millipore Corp., Bedford, MA, USA) was used to filter the mobile phase.

**Chemicals and Reagents**
The reference materials used during the study (dextromethorphan hydrobromide, dextrorphan tartrate, 3-hydroxymorphinan hydrochloride and 3-methoxymorphinan hydrobromide) were supplied by Roche, Basel, Switzerland. The internal standard (levallorphan) was supplied by USPC Inc., USA. Acetonitrile, glacial acetic acid, n-hexane, triethylamine and ethyl acetate were supplied by Merck KGaA, Darmstadt, Germany. Hydrochloric acid, phosphoric acid and sodium carbonate were supplied by J.T. Baker, Phillipsburg, NJ, USA. Water was purified using a Milli-Ro-10 and a Milli-Q water purification system (Millipore Corp., Bedford, USA). Unless otherwise stated, water of Milli-Q quality was used.

A phosphate buffer (0.05 M; pH = 3.0) was prepared by dissolving 6.8 g of potassium dihydrogen phosphate and 1.0 mL of triethylamine in 1000 mL water. The pH was adjusted with phosphoric acid (85%). Acetic acid (3%) was prepared by dilution of 3.0 mL of glacial acetic acid (100%) to 100 mL with water. Hydrochloric acid (10 M) was prepared by dilution of 83 mL of hydrochloric acid (37%) to 100 mL with water. Saturated sodium carbonate was prepared by dissolving 29.6 g of sodium carbonate in 200 mL water. The mobile phase was prepared by mixing 760 mL buffer and 240 mL acetonitrile. Before use, the mobile phase was degassed for 20 minutes. Separate stock solutions of dextromethorphan, dextrorphan, 3-hydroxymorphinan and 3-methoxymorphinan (1.0 mg/mL) were prepared by dissolving 25 mg of pure substance in 25 mL of water containing 25 µL of triethylamine. A few drops of acetic acid (3%) were added for dissolving the substance. Diluted stock solutions (10 µg/mL) were prepared by dilution of 100 µL stock solution to 10.0 mL with Milli-Q water. A stock solution of levallorphan (internal standard) (0.5 mg/mL) was prepared by dissolving 25 mg of levallorphan in 50 mL of Milli-Q containing 50 µL triethylamine. A few drops of acetic acid (3%) were added for dissolving. An internal standard solution (12.5 µg/mL) was prepared by diluting 250 µL of the stock solution of levallorphan to 10.0 mL with Milli-Q water. All stock and standard solutions were stored at +4 °C.
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From the stock and diluted stock solution calibration and validation samples were prepared. The stock solutions or diluted stock solutions were added to blank human urine to obtain the nominal urine concentrations as given in Tables 1 and 2. The spiked urine pools were stored at -20 °C.

**Table 1:** Calibration curve concentrations

<table>
<thead>
<tr>
<th></th>
<th>DEX (ng/mL)</th>
<th>DTX (ng/mL)</th>
<th>3MM (ng/mL)</th>
<th>3HM (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10.0</td>
<td>25.0</td>
<td>2.50</td>
<td>50.0</td>
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</tr>
<tr>
<td>25.0</td>
<td>50.0</td>
<td>5.00</td>
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<td>75.0</td>
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<td>20.0</td>
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</tr>
<tr>
<td>250</td>
<td>250</td>
<td>75.0</td>
<td>500</td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>750</td>
<td>250</td>
<td>1000</td>
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<td>10000</td>
<td>10000</td>
<td>5000</td>
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<td></td>
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</table>

**Table 2:** Validated concentrations and accompanying ratios

<table>
<thead>
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<th></th>
<th>Concentrations (ng/mL)</th>
<th>Concentration Ratios</th>
</tr>
</thead>
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<tr>
<td></td>
<td>DEX</td>
<td>DTX</td>
</tr>
<tr>
<td>QC1 *</td>
<td>15.0</td>
<td>9000</td>
</tr>
<tr>
<td>QC2</td>
<td>100</td>
<td>2500</td>
</tr>
<tr>
<td>QC3</td>
<td>300</td>
<td>9000</td>
</tr>
<tr>
<td>QC4 *</td>
<td>800</td>
<td>25.0</td>
</tr>
<tr>
<td>QC5</td>
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<td>2500</td>
</tr>
<tr>
<td>QC6</td>
<td>1200</td>
<td>9000</td>
</tr>
<tr>
<td>QC7 *</td>
<td>9000</td>
<td>1000</td>
</tr>
</tbody>
</table>

* Concentrations validated for recovery and freeze/thaw stability

**Sample Preparation**

Samples, collected 8 hours after dosing were taken from the -20 °C freezer and thawed at room temperature for 30 minutes. Subsequently, the samples were homogenized five times. An aliquot of 500 µL of the urine sample to be analysed was transferred into a polypropylene tube. Next, 100 µL of the working internal standard solution and 150 µL of 10 M hydrochloric acid were added. After mixing for approximately five seconds by means of a vortex mixer, the sample was centrifuged for two minutes at 3200 × g (4000 rpm), followed by incubation for 90 minutes at 100 °C. After cooling down to room temperature, four times 250 µL of saturated sodium carbonate were added to the mixture. Next, 6.0 mL of the extraction solvent (a mixture of n-hexane and ethyl acetate (1 : 1, v/v)) was added and the mixture was extracted for 15 minutes using a Heidolph tumble mixer at 45 rpm. After centrifugation of the samples for five minutes at 3200 × g (4000 rpm), the aqueous layer was discarded and the organic layer was transferred into a clean polypropylene tube. The organic layer was re-extracted for...
ten minutes on a Heidolph tumble mixer at 60 rpm with 150 µL of acetic acid (3%). After centrifugation at 3200 × g (4000 rpm) for two minutes, the aqueous layer was transferred into a vial. In order to ensure the complete removal of the organic solvent, the vial was left in the dark for at least another 15 min. Finally, 75 µL were injected into the HPLC system.

**Conventional Validation Study**

In five validation runs, accuracy and precision, recovery, stability after repeated freezing and thawing and over-curve control were assessed. Each run contained a calibration curve, twenty-one precision and accuracy samples (seven concentrations in triplicate); three over-curve control samples (one concentration at 5 × HLQ diluted ten times in triplicate, to obtain a nominal diluted concentration of 0.5 × HLQ), nine samples for stability assessment after repeated freezing and thawing (three concentrations (QC1, QC4 and QC7 from table 2) in triplicate (sub samples were taken from samples which were prepared from the validation pools and which were frozen and thawed before each next run), nine direct injections of test solutions for recovery determination (these direct injections contained such amounts of the analytes as would correspond with 100% recovery from validation samples at three different concentrations (QC1, QC4 and QC7) in triplicate). An additional run was used for testing the stability during storage conditions of extracted samples in the sample compartment of the auto injector; pooled extracts of spiked urine samples at three concentrations (QC1, QC4 and QC7) were injected every two hours for a total period of 30 hours, during which the extracts were kept in the sample compartment of the injector (protected from light and at a temperature of 10 °C).

The specificity of the assay method was checked by analysing six independent blank urine samples. The chromatograms of these blank urine samples were compared with chromatograms obtained by analysing a test solution of the pure compounds and with chromatograms obtained by analysing urine samples taken from subjects after administration of a dextromethorphan-containing dosage form. The specificity was also tested for compounds (and their metabolites) that we generally use simultaneously with dextromethorphan in phenotyping studies, such as racemic mephentoin, omeprazole and 5-hydroxyomeprazole and caffeine metabolites AAMU and 1MX.

**Calculation of Assay Method Performance Characteristics**

The peak height ratio of dextromethorphan (over internal standard) was taken as the response for a given sample. Calibration curves for all compounds were calculated by quadratic linear regression ($W = X^{-1}$) on the responses ($y$) of a series of calibration samples versus the corresponding nominal concentrations ($x$). The measured concentration in a sample was calculated by substituting the response for that sample in the equation of the corresponding calibration curve ($y = ax^2 + bx + c$).

Calculations for the evaluation of the performance characteristics were performed using spreadsheets programmed in Microsoft Excel. For the autosampler stability, the measured peak heights were plotted versus time.
Linear regression of this response versus time was performed to assess any increase or decrease in dextromethorphan concentrations during 30 hours of storage in the autosampler. For assessment of the precision and accuracy, the fifteen measured concentrations per concentration level (triplicates from five runs) as obtained by analysing the validation samples were subjected to analysis of variance to estimate overall, between-run and within-run precision. The accuracy of the method was determined from the same results that were used for the determination of the precision; the bias was calculated by comparing the mean (x) of the fifteen measured concentrations per concentration level to the nominal concentration (µ) (bias (%) = (x-µ)/µ × 100%).

For the determination of the recovery of the analytes, the mean peak heights obtained for the triplicate measurements from run 1 to run 5 were compared with the mean peak heights obtained from triplicate direct injections performed in the same run.

The three measured concentrations at each concentration level after each freezing and thawing cycle were used to calculate a mean value. These mean values were compared to the corresponding mean value obtained from the fifteen precision and accuracy measurements. A deviation of ± 15% from this mean value was considered acceptable. The fifteen measured concentrations for the over-curve control samples were subjected to analysis of variance. The overall mean was compared with the results for the precision and accuracy samples at the corresponding concentration level.

Cross-validation

A cross-validation was performed with a former method for dextromethorphan and dextrorphan using measured concentrations of volunteer samples using both methods. The main reason for this cross-validation was to guarantee the similarity of epidemiological data in our database collected from two methods (the former method, already used for phenotyping over 5000 volunteers, and the current method, used for future phenotyping).

The methods differed mainly in the composition of the extraction liquid and in the composition of the mobile phase. The old method used a mixture of n-hexane and 1-butanol (90:10, v/v) as the extraction liquid and a mixture of 0.05 M phosphate buffer pH 4.0 (containing 0.1% triethylamine) and acetonitrile (66:34, v/v). More over, the old method, which was developed for measuring dextromethorphan and dextrorphan only, had limited concentrations ranges (100-7000 ng/mL for dextromethorphan and 2500-60000 ng/mL for dextrorphan).

Error Propagation for CYP2D6 Phenotyping and CYP3A4 Activity

The effect of analytical errors on the determination of the CYP2D6 phenotype and the CYP3A activity was investigated using the precision and accuracy results. At each ratio, indicated in Table 2, the standard deviations in the individually measured concentrations of dextromethorphan, dextrorphan and 3-methoxymorphinan and the standard deviation in the ratios obtained after the five runs for each ratio were calculated, including covariances and correlations.
between analytical errors for individual components, according to the algorithms discussed in the ‘Theory’ section. The values obtained were also used for an evaluation of the error propagation of analytical errors in the phenotype for CYP2D6 and the activity for CYP3A.

2.2.5 Results and Discussion

Conventional Assay Validation

The assay as implemented above had a chromatography time for a sample of approximately 21 minutes, with retention times of dextromethorphan, dextrorphan, 3-methoxymorphinan and 3-hydroxymorphinan and internal standard being 18, 6, 15, 5 and 9.5 minutes, respectively. No interfering peaks were detected at the retention times of the compounds of interest (see Figure 2 for a chromatogram of an extensive metabolizer, urine collected 8 hours after dosing). The peaks of dextromethorphan and metabolites and the internal standard were well resolved and showed no interferences with endogenous materials and potential co-medication and their metabolites (racemic mephenytoin, omeprazole, 5-hydroxyomeprazole and caffeine metabolites AAMU and 1MX).

Figure 2: Chromatogram of a subject sample (extensive metabolizer) obtained from a 0-8 hour urine fraction collected after administration of 40 mg of dextromethorphan in a drinking solution.

The lowest concentrations of the calibration curve were 10.0, 25.0, 2.50 and 50.0 ng/mL for dextromethorphan, dextrorphan, 3-methoxymorphinan and 3-hydroxymorphinan, respectively, which were therefore the practical lower limits of quantitation. The accuracy and precision at this level meet the criteria established for this purpose (Shah et al [20]). The autosampler stability experiments showed that for all compounds at all tested concentrations, peak ratios of dextromethorphan and metabolites were between 90% and 110% after
Part II

30 hours of storage in the sample compartment of the autosampler (based on regression) as compared to zero hours of storage. No significant deterioration was observed. The results on precision and accuracy as derived from the measured concentrations for the validation samples are listed in Table 3. In summary: the overall precision (CV%) of the method was better than 14.6% at all concentrations for all compounds (n = 15); within-run and between-run precision (CV%) were better than 14.2% and 20.3%, respectively; the bias varied between -1.7% and +14.7% at all concentration levels. The results for overall precision and bias meet the criteria established during the Washington Meeting on Analytical Methods Validation (Shah et al [20]). Figure 3 gives the imprecision profiles of dextromethorphan, dextrorphan and 3-methoxymorphinan versus the concentrations. The data are taken from Table 3. It is clear from the profiles that imprecision increases with decreasing concentrations, with maximum coefficient of variation values around the lower limits of quantitation of approximately 10-15%. For the continuing part of this paper this will prove to be important; two different samples may provide equal concentration ratios, although their individual concentrations may vary dramatically. This will have consequences for the accuracy and precision of the established ratio and hence the reported phenotype or enzyme activity.
Figure 3: Error profiles (coefficients of variation versus concentrations) for dextromethorphan (DEX), dextrorphan (DTX) and 3-methoxymorphinan (3MM)
Table 3: Summary statistics for each validated concentration for all compounds.

<table>
<thead>
<tr>
<th>QC</th>
<th>DEX</th>
<th>DTX</th>
<th>3MM</th>
<th>3HM</th>
</tr>
</thead>
<tbody>
<tr>
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<td>3.00</td>
<td>50.0</td>
</tr>
<tr>
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<td>9520</td>
<td>3.42</td>
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</tr>
<tr>
<td>CV%</td>
<td>6.5</td>
<td>4.7</td>
<td>14.5</td>
<td>6.5</td>
</tr>
<tr>
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<td>14.1</td>
<td>8.9</td>
</tr>
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<td>DTX</td>
<td>3MM</td>
<td>3HM</td>
</tr>
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<td>10.0</td>
<td>9000</td>
</tr>
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<td>10.9</td>
<td>9171</td>
</tr>
<tr>
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<td>1.8</td>
<td>4.9</td>
<td>3.7</td>
</tr>
<tr>
<td>Bias%</td>
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<td>-0.2</td>
<td>9.3</td>
<td>1.9</td>
</tr>
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<td>DTX</td>
<td>3MM</td>
<td>3HM</td>
</tr>
<tr>
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<td>9000</td>
<td>25.0</td>
<td>5000</td>
</tr>
<tr>
<td>Mean</td>
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<td>25.3</td>
<td>5201</td>
</tr>
<tr>
<td>CV%</td>
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<td>4.2</td>
<td>4.2</td>
<td>4.9</td>
</tr>
<tr>
<td>Bias%</td>
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<td>1.2</td>
<td>4.0</td>
</tr>
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<td>DTX</td>
<td>3MM</td>
<td>3HM</td>
</tr>
<tr>
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<td>1000</td>
<td>5000</td>
</tr>
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<td>Mean</td>
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<td>1013</td>
<td>5201</td>
</tr>
<tr>
<td>CV%</td>
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<td>12.8</td>
<td>1.8</td>
<td>4.9</td>
</tr>
<tr>
<td>Bias%</td>
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<td>3.0</td>
<td>1.3</td>
<td>4.0</td>
</tr>
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<td>3MM</td>
<td>3HM</td>
</tr>
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<td>2500</td>
<td>4500</td>
<td>5000</td>
</tr>
<tr>
<td>Mean</td>
<td>1034</td>
<td>2576</td>
<td>4307</td>
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</tr>
<tr>
<td>CV%</td>
<td>1.4</td>
<td>1.6</td>
<td>2.3</td>
<td>4.3</td>
</tr>
<tr>
<td>Bias%</td>
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<td>3.0</td>
<td>-4.3</td>
<td>3.7</td>
</tr>
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<td>DEX</td>
<td>DTX</td>
<td>3MM</td>
<td>3HM</td>
</tr>
<tr>
<td>Nominal</td>
<td>1200</td>
<td>9000</td>
<td>50.0</td>
<td>50.0</td>
</tr>
<tr>
<td>Mean</td>
<td>1212</td>
<td>9540</td>
<td>50.8</td>
<td>54.8</td>
</tr>
<tr>
<td>CV%</td>
<td>2.5</td>
<td>2.6</td>
<td>3.1</td>
<td>11.3</td>
</tr>
<tr>
<td>Bias%</td>
<td>1.0</td>
<td>6.0</td>
<td>1.6</td>
<td>9.7</td>
</tr>
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<td>QC7</td>
<td>DEX</td>
<td>DTX</td>
<td>3MM</td>
<td>3HM</td>
</tr>
<tr>
<td>Nominal</td>
<td>9000</td>
<td>1000</td>
<td>4500</td>
<td>9000</td>
</tr>
<tr>
<td>Mean</td>
<td>8871</td>
<td>1047</td>
<td>4422</td>
<td>9318</td>
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<tr>
<td>CV%</td>
<td>3.0</td>
<td>2.7</td>
<td>3.3</td>
<td>4.8</td>
</tr>
<tr>
<td>Bias%</td>
<td>-1.4</td>
<td>4.7</td>
<td>-1.7</td>
<td>3.5</td>
</tr>
</tbody>
</table>

The mean recoveries of dextromethorphan, dextrorphan, 3-methoxymorphinan and 3-hydroxymorphinan were consistent over the evaluated concentration ranges, and were 104%, 97%, 99% and 78%, respectively. The recovery of the internal standard at the concentration used during the actual analysis of the urine...
samples (8333 ng/mL) was on average 99.5%. The results of the experiments to investigate the stability after repeated freezing and thawing did not consistently exceed the 85% limits. Incidentally, bias values or CV% values exceed 15%, but this was the result of experimental errors rather than instability. No significant deterioration was observed. The bias values obtained from the over-curve control experiments were below 10%, for a tenfold dilution, which indicated that dilution of samples is allowed.

Cross-Validation

For the cross-validation with the former method for dextromethorphan and dextrorphan, samples were re-analysed with the new method, two years after they had been originally analysed with the old method. The old method, which was developed for measuring dextromethorphan and dextrorphan only, had limited concentrations ranges (100-7000 ng/mL for dextromethorphan and 2500-60000 ng/mL for dextrorphan), which did not entirely overlap with the calibration ranges for the new method. During application of the old method for routine phenotyping, not in all cases exact results were reported; in some cases results were reported as ‘> 7000 ng/mL’ or ‘> 60000 ng/mL’, depending on the phenotype. The same is true for results from the new method. In some other cases no results could be reported, since the measured concentration was below the lower limit of quantitation (the lowest calibrator). Therefore, not for all samples and both analytes results were available for both methods for a cross-comparison. The resulting plots from the cross-validation are given in Figure 4, which is a correlation analysis of the new method result versus the old method result, including slope and intercept values and a correlation coefficient (for dextromethorphan, dextrorphan and for the DEX/DTX ratio). For all plots, the correlation coefficient was good (≥ 0.9881). The plots show no significant differences in measured concentrations of volunteer samples for dextromethorphan; for dextrorphan, however, there was a significant decrease of the concentration measured with the new method versus the concentration measured with the old method (slope = 0.8976). This also affected the slope of the plot of the DEX/DTX ratio from both methods. The main cause for this slope value is that the concentration ranges differ for the old (2500-60000 ng/mL) and the new method (25-10000 ng/mL) and that the corresponding precision and accuracy profiles at the measured concentrations for the cross-comparison samples are therefore quite different as well. Another plausible reason for this deviation is the fact that samples were stored at −20 ºC for two years before they were analysed with the new method, which influences dextrorphan quantitation. In general, we concluded good correlation between both methods, which guaranteed the similarity of epidemiological data in our database collected from these two methods.
Figure 4: Cross-validation results of the new method results versus the old method results (in parenthesis the standard errors)

Evaluation of CYP2D6 Classification and CYP3A4 Activity Determination using Error Propagation Rules

The accuracy and precision results were calculated from triplicates during 5 runs; these data were also used for the evaluation of the propagation of experimental errors (established concentrations for dextromethorphan (DEX), dextorphan (DTX) and 3-methoxymorphinan (3MM) and the ratio of their concentrations for CYP2D6 phenotyping and CYP3A4 activity. Table 3 (for statistics on concentrations) and Table 4 (for statistics on ratios) give the nominal values and the measured values from the 15 replicates, including bias of measured versus nominal and coefficient of variation. Furthermore, correlations between the experimental errors of two compounds are provided ($r_{\text{dex/dtx}}$ and $r_{\text{dex/3mm}}$) in Table 4. The higher this value (limits: -1.0 and +1.0), the more correlated experimental errors are. Higher correlations of individual concentration errors result in less variance of their ratios. Coefficients of variations in concentration ratios are calculated from the 15 replicates and also by using the rules of error propagation.

**Table 4**: Summary statistics for the ratios DEX/DTX and DEX/3MM, including values predicted from the rules of error propagation.

<table>
<thead>
<tr>
<th></th>
<th>QC1</th>
<th>QC2</th>
<th>QC3</th>
<th>QC4</th>
<th>QC5</th>
<th>QC6</th>
<th>QC7</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEX/DTX</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nominal</td>
<td>0.0017</td>
<td>0.0400</td>
<td>0.0333</td>
<td>32.00</td>
<td>0.4000</td>
<td>0.1333</td>
<td>9.000</td>
</tr>
<tr>
<td>Mean</td>
<td>0.0018</td>
<td>0.0402</td>
<td>0.0314</td>
<td>33.69</td>
<td>0.4015</td>
<td>0.1272</td>
<td>8.482</td>
</tr>
<tr>
<td>CV% measured</td>
<td>8.6</td>
<td>2.2</td>
<td>5.2</td>
<td>11.7</td>
<td>1.8</td>
<td>3.8</td>
<td>4.5</td>
</tr>
<tr>
<td>Correlation</td>
<td>-0.1543</td>
<td>0.5469</td>
<td>0.0375</td>
<td>0.0119</td>
<td>0.2462</td>
<td>-0.1745</td>
<td>-0.2162</td>
</tr>
<tr>
<td>CV% predicted</td>
<td>8.6</td>
<td>2.2</td>
<td>5.0</td>
<td>12.9</td>
<td>1.8</td>
<td>3.9</td>
<td>4.4</td>
</tr>
<tr>
<td>Bias%</td>
<td>8.7</td>
<td>0.4</td>
<td>-5.9</td>
<td>5.3</td>
<td>0.4</td>
<td>-4.6</td>
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<tr>
<td>DEX/3MM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nominal</td>
<td>5.000</td>
<td>10.00</td>
<td>12.00</td>
<td>0.8000</td>
<td>0.2222</td>
<td>24.00</td>
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<tr>
<td>Mean</td>
<td>5.103</td>
<td>9.191</td>
<td>11.84</td>
<td>0.8451</td>
<td>0.2402</td>
<td>23.87</td>
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</tr>
<tr>
<td>CV% measured</td>
<td>12.6</td>
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<td>1.7</td>
<td>2.2</td>
<td>2.3</td>
<td>2.3</td>
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<tr>
<td>Correlation</td>
<td>0.2964</td>
<td>0.1503</td>
<td>0.7587</td>
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<td>0.6794</td>
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<td>CV% predicted</td>
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</tr>
<tr>
<td>Bias%</td>
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<td>8.1</td>
<td>-0.6</td>
<td>0.3</td>
</tr>
</tbody>
</table>
2.2.6 Summary statistics
All statistics in Tables 3 and 4 are generated from values obtained on several days (from several validation runs), which means that they form a good representation of actual studies for the assessment of CYP2D6 phenotypes and CYP3A4 activity measurements.

Measured coefficients of variations in ratios vary between 1.8% and 11.7% for DEX/DTX and between 1.7% and 12.6% for DEX/3MM. The difference between the predicted coefficient of variation using error propagation rules and the measured values are between 0.0% and 1.2% for DEX/DTX and between 0.0% and 1.4% for DEX/3MM, indicating that the rules for error propagation are applicable to these types of problems. The bias values (the deviations of the mean measured concentration ratios versus the nominal concentration ratio) vary between $-5.9\%$ and $+8.7\%$ for DEX/DTX and between $-8.1\%$ and $+8.1\%$ for DEX/3MM. The correlation coefficients between the concentrations for two compounds vary between $-0.22$ and $+0.55$ for DEX and DTX and between $+0.15$ and $+0.76$ for DEX and 3MM. The latter results show that the correlations between the experimental errors in measured concentration is much higher for DEX and 3MM than it is for DEX and DTX, although this cannot directly be expressed in the previously mentioned coefficient of variation and bias values.

2.2.7 Evaluation of the results
The results in Table 3 and 4 show that, in general, experimental errors in individual concentrations of the analytes in the same experiment are correlated, although not in the same magnitude for the two ratios and at the different concentration levels. This results in less relative variance in concentration ratios than the sum of individual relative variances in concentrations. Despite the small number of experiments ($n = 15$) good estimates of variances in ratios can be obtained using the rules for error propagation (as compared to measured variances). As assays were repeated 15 times (five runs in three-fold), there is some uncertainty in the resulting variance and covariance estimates. A discussion on confidence limits for population variance is given in [19]. It is assumed that the concentrations measured ($C_1$, $C_2$, ..., $C_n$) are independent, normally distributed random variables having mean $\mu$ and variance $\sigma^2$. The standardised sum of squares $\sum z_j^2$ of deviations from the population mean has a chi-square distribution with $n$ degrees of freedom:

$$\sum z_j^2 = \frac{\sum (C_j - \mu)^2}{\sigma^2} \sim \chi^2$$

For very precise estimation of a population variance many observations are needed. This is beyond the scope of this paper; here, it was important to obtain a first estimate of variances and covariances. The experimental errors in concentrations of dextromethorphan and its metabolites in urine contribute significantly to the variance in the established concentration ratios used for CYP2D6 phenotyping and for CYP3A4 activity determinations. However, the
coefficients of variation in the ratios are less to much less than the sum of the coefficients of variation in the individual concentration, suggesting a significant correlation of the experimental errors in measured concentration. The absolute concentrations of the analytes highly affect the correlation of the errors in the concentrations.

For some cases, the correlation between the concentrations of two compounds (r) have negative values. This significantly influences the variance of the ratio. For some other cases, relatively high values of the bias of the measured concentrations do not result in high values of the bias of the concentration ratios (e.g.: see DEX 15 ng/mL and 3MM 3 ng/mL; concentration bias values 14.7% and 14.1%; ratio bias value: 2.1%.

2.2.8 Impact on CYP2D6 classification

For QC5 and QC6, nominal CYP2D6 ratios of 0.4000 and 0.1333 are obtained. These values are relatively close to the antimode of 0.3. The coefficients of variation and bias values for these two validation samples values obtained are relatively low (CV%: 1.8% and 3.8%; bias%: +0.4 and −4.6%; total errors in ratios: 2.2% and 7.4%, respectively). This means that for actual values around the antimode of 0.3, the chance of a misclassification is relatively small. In fact the chance of classifying a poor metabolizer (ratio ≥ 0.3) as an extensive metabolizer (ratio ≤ 0.02) is negligible, so in the worst case one can classify a poor metabolizer as an intermediate metabolizer.

For QC2 and QC3, nominal CYP2D6 ratios of 0.0400 and 0.0333 are obtained. These values are relatively close to the antimode of 0.02. The coefficients of variation and bias values for these two validation samples values obtained are relatively low as well, although they are clearly lower for QC2 than for QC3 (CV%: 2.2% and 5.2%; bias%: +0.4 and −5.9%; total errors in ratios: 2.6% and 10.9%, respectively). This means that for actual values around the antimode of 0.02, the chance of a misclassification is relatively small as well. In fact the chance of classifying an extensive metabolizer (ratio ≤ 0.02) as a poor metabolizer (ratio ≥ 0.3) is negligible, so in the worst case one can classify a poor metabolizer as an intermediate metabolizer.

2.2.9 Consequences for CYP3A4 activity measurements

For DEX/3MM ratios (used for evaluation of the CYP3A4 activity), total errors vary between 2.6% and 16.1% (absolute sum of bias% and CV%), with a mean value of 8.1%. This means that the overall uncertainty in measured ratios is approximately 8%. QC1 and QC2 give relatively high total error values (16.1% and 13.3%), which can be explained by the relatively low concentrations for these samples for both dextromethorphan and 3-methoxymorphinan.

The above indicates that in general a change in DEX/3MM ratios of 10% or more is a significant increase or decrease in the activity of CYP3A4 (under the condition that no other factors are involved, of course).
2.2.10 Conclusions

An analytically valid and clinically reliable bioanalytical method for the determination of dextromethorphan and its metabolites dextrorphan, 3-methoxymorphinan and 3-hydroxymorphinan in human urine for CYP2D6 phenotyping and CYP3A4 activity measurements for clinical pharmacology studies was developed.

For CYP2D6 the validation results show that dextromethorphan/dextrorphan ratios are determined with better precision and accuracy than individual concentration, leading to reduced misclassification of phenotypes. For cases far away from antimodes (CYP2D6 ratios <<0.02 or >>0.3) relatively large experimental errors in individual concentrations or ratios do not lead to false classifications for CYP2D6. Poor metabolizers cannot be classified as extensive metabolizers and vice versa. The chance of other misclassifications is relatively small (in general there is less than 10% error in measured ratios).

For CYP3A4 the method has an overall error of approximately 8%, which indicates that in general a change in DEX/3MM ratios of 10% or more is a significant increase or decrease in the activity of CYP3A4.

In general we concluded that the method is suitable for our purposes.

2.2.11 References

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2.3 ASSESSMENT OF CYP2D6 AND CYP2C19 ACTIVITY IN THE CONTEXT OF CLINICAL DRUG RESEARCH BY SIMULTANEOUS PHENOTYPING USING A COCKTAIL AND GENOTYPING#

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2.3.1 Abstract

Objectives: The aim of this study was to investigate which probe on CYP2D6 and CYP2C19 activity yields the best information. Furthermore, it was investigated whether plasma is an appropriate matrix in order to combine genotyping and phenotyping by a simple one-sample approach.

Methods: The study was single combined dose pharmacokinetic study with six healthy male volunteers. The subjects received 22 mg dextromethorphan, 40 mg omeprazole and 100 mg metoprolol. During study blood pressure, pulse rates and a standard 12-lead electrocardiogram were recorded. Blood samples were taken urine was collected at intervals and saliva samples were collected. Plasma samples were analysed on dextromethorphan, dextrorphan, metoprolol and α-hydroxymetoprol, omeprazole and 5-hydroxyomeprazole levels by high performance liquid chromatography (HPLC). Saliva samples were analysed on dextromethorphan, dextrorphan, omeprazole and α-hydroxymetoprol using HPLC. Urine samples were analysed on dextromethorphan on dextrorphan by reversed phase HPLC. Genotyping was performed on both CYP2D6 and CYP2C19 using DNA isolated from whole blood samples. CYP2D6 was investigated on the five mutations using a long-range polymerase chain reaction (PCR) followed by a multiplex allele-specific PCR. CYP2C19 was investigated on two mutations (CYP2C19*2 and CYP2C19*3) using a PCR with restriction analysis.

# Paper has been accepted for publication in the European Journal of Clinical Pharmacology in reduced form (short paper)
Results: All predicted phenotypes were in accordance with the actual phenotype. A clear difference between extensive and poor metabolizers was observed. An increase of AUC\(_{0-12}\) between extensive and poor metabolizers was observed for both dextromethorphan and metoprolol was observed. An increase of AUC\(_{0-12}\) between extensive and poor metabolizers was observed for both dextromethorphan (17.5 to 204 ng.h.mL\(^{-1}\)) and metoprolol (615 to 2126 ng.h.mL\(^{-1}\)) whereas both metabolites decreased: dextrorphan (1287 to 63.5 ng.h.mL\(^{-1}\)) and α-hydroxy metoprolol (640 ng.h.mL\(^{-1}\) to not quantifiable). In EMs excretion of dextromethorphan was nearly quantifiable whereas in PMs excretion was about 3.5%; for dextrophan in EMs excretion was about 35% in poor metabolizers it was about 1.5%. The saliva AUC\(_{0-12}\) values for dextromethorphan were 2522 ng.h.mL\(^{-1}\) in EMs and 4490 ng.h.mL\(^{-1}\) in PMs and for dextrorphan they were 17.0 ng.h.mL\(^{-1}\) in EMs and 11.3 ng.h.mL\(^{-1}\) in PMs. The plasma AUC\(_{0-12}\) for omeprazole was 446 ng.h.mL\(^{-1}\) and 664 ng.h.mL\(^{-1}\) for 5-OH-omeprazole. In saliva omeprazole and 5-OH-omeprazole levels were all below 3 ng.mL\(^{-1}\). The pulse rate decreased with 7.6 min\(^{-1}\) (n = 3) for EMs and with 20.3 min\(^{-1}\) (n = 3) for PMs but this difference was not significant (p = 0.12).

Conclusions: If dextromethorphan saliva metabolic ratios are to be used for phenotyping purposes dextromethorphan should be administered in a capsule. Omeprazole is a preferable probe drug compared to mephenytoin to assess CYP2C19 activity. A three hours plasma sample is a good measure for the enzyme activity of both CYP2D6 when probed with dextromethorphan or with metoprolol and CYP2C19 when probed with omeprazole. Metoprolol is the preferred substrate above dextromethorphan when used in combination with genotyping and is applied to estimate actual enzyme activity.

2.3.2 Introduction
Pharmacokinetic characteristics of drugs often vary considerably between individuals and therefore pharmacodynamics are often better related to actual blood levels rather than to the administered dose.\(^1\) In clinics this variation may lead to altered drug response and drug toxicity and in clinical drug development this may lead to problems with interpretation of pharmacokinetics and possible unexpected adverse drug reactions. This inter-individual variation is often caused by variation of the enzyme activity or levels of expression of various drug metabolizing enzymes from which the cytochrome P450 enzymes are the most important.\(^2\) Some of the cytochrome P450s like CYP2A6, CYP2C9, CYP2C19 and CYP2D6 are polymorphically expressed due to variation in the genes coding for these enzymes\(^3\) others like CYP1A2 and CYP3A4 are easily inhibited or induced by environmental factors\(^4,5\). Cytochrome P450 2D6 (CYP2D6) catalyses the oxidative biotransformation of more than 40 clinical important drugs such as several CNS drugs and cardiovascular agents.\(^3\) The inter-individual in-vivo activity may vary between ultra rapid metabolism (UM), normal metabolism (EM), intermediate metabolism (IM) and poor metabolism (PM). This variability may have clinical consequences and is associated with more than 50 mutations in the highly polymorphic CYP2D6 gene locus\(^6\). A homozygous combination of non-coding alleles leads to the PM phenotype, whereas heterozygous wild type
or combinations with alleles with diminished enzyme activity may lead to reduced CYP2D6 activity and in addition, gene duplication may lead to the ultra-rapid phenotype. In Caucasians the most common non-coding alleles are CYP2D6*4 (about 75% of null alleles), CYP2D6*5 (about 15% of null alleles) and CYP2D19*3 (about 5% of null alleles). All the other non-coding alleles are rare and have an incidence of 1% or lower of the non-coding alleles.\textsuperscript{7} In-vivo enzyme activity can be assessed by measurement of the metabolic ratio of an enzyme specific probe; for CYP2D6 dextromethorphan, sparteine, debrisoquine and metoprolol have been described as probe drugs\textsuperscript{8,9}. The incidence of CYP2D6 PM phenotype differs per race and is reported to be 5 to 10% in white populations and 1 to 2% in Asians.\textsuperscript{3} Deficiency of cytochrome P450 2C19 (CYP2C19) occurs with an incidence of PMs of 1-3% among white Europeans and about 20% among Orientals.\textsuperscript{10} The anticonvulsant drug mephenytoin is the drug that led to the discovery of the polymorphic character of CYP2C19.\textsuperscript{11} The S-enantiomer of mephenytoin (MEP) is a substrate for CYP2C19 and the urinary ratio of (S)-MEP over (R)-MEP (S/R-ratio) after administration of racemic MEP can be used to identify PMs. Other, more recently implemented probes for CYP2C19 are the proton pump inhibitor omeprazole\textsuperscript{12} and the anti-malarial drug proguanil\textsuperscript{13}. Two null-alleles in the CYP2C19 gene have been described to account for approximately 83% of all PMs in Caucasians and 100% of all PMs in Orientals.\textsuperscript{14,15} There are several reasons to assess activity of polymorphic metabolizing enzymes and the application determines the test procedure specifications needed. In drug development the main question is which iso-enzyme is involved in drug metabolism and what is its contribution to the wanted and unwanted effects of a drug.\textsuperscript{16} In Phase I studies information on drug metabolizing status of volunteers is often needed for inclusion or to explain pharmacokinetic or pharmacodynamic observations. In further drug development stages information might be helpful in interpreting adverse drug reactions and/or absence of drug efficacy in certain sub-populations. In clinical practice information about drug metabolizing enzyme capacities might be helpful to optimise pharmacotherapy and may reduce risk on unwanted drug reactions in e.g. psychiatry or cardiology\textsuperscript{17}. In general there are three ways to get information on enzyme activity: (i) study the genes that code for the enzyme, (ii) study the level of enzyme expression in a certain tissue or (iii) assess actual enzyme activity using an enzyme specific probe. Some relations are obvious e.g. individuals, which lack wild type alleles, cannot produce the enzyme and therefore lack enzyme expression resulting in absence of the enzyme specific metabolites. Other relations however are more complex e.g. an individual genotyped as EM does not necessarily express enzymes or possess enzyme activity. Furthermore, the enzyme activity range within a certain group might have a large variation. This is especially the case for CYP2D6, which in nicely illustrated by a German study in which extensively genotyping in a large population only poorly predicts actual enzyme activity.\textsuperscript{7} Thus genomic information only predicts enzyme activity and on the other hand information on actual enzyme activity only makes a certain
Methodology

genotype likely. Therefore, a test that may result in both information on the individual possibility (genotyping) and the actual activity (phenotyping) without difficult clinical and analytical procedures might be helpful, especially in phase I research. In this study we incorporated two CYPD6 probes (dextromethorphan and metoprolol) and one CYP2C19 probe (omeprazole) as alternative for mephenytoin which has some disadvantages. The aim of this study was to investigate which probe yields the best information on actual enzyme activity of CYP2D6 and CYP2C19. Furthermore, it was investigated whether plasma is an appropriate matrix in order to combine genotyping and phenotyping by a simple one-sample approach. Finally, saliva was investigated as an alternative matrix for phenotyping using dextromethorphan and omeprazole.

2.3.3 Methods

Study design and Subjects
The study was an open-label, single combined dose, pharmacokinetic study with six healthy male volunteers. The volunteers were selected from a database with volunteers phenotyped in the context of clinical pharmacology studies on CYP2D6 (probed with dextromethorphan), CYP2C19 (probed with mephenytoin) and N-acetyltransferase 2 (NAT-2; probed with caffeine). The volunteers were selected on their CYP2D6 activity: three extensive metabolizers (metabolic ratio below 0.30) and three poor metabolizers (metabolic ratio above 0.30) were selected. Health assessment was made by recording medical history, which was without major pathology, and by an eligibility screening including clinical chemistry, haematology and urinalysis before drug administration. None of the volunteers had a history of alcohol abuse, drug addiction or a smoking habit of more than 15 cigarettes daily. The participants were not allowed to take any medication (with the exception of oral contraceptives or paracetamol) during 14 days before the study. Subjects gave their written informed consent and the phenotyping protocol was approved by the independent Medical Ethics Committee [Stichting Beoordeling Ethiek Bio-Medisch Onderzoek’, Assen, The Netherlands]. Furthermore, the study was carried out in accordance with the rules for good clinical practice (GCP) and good laboratory practice (GLP).

Pre-study Phenotyping
The pre-study phenotyping procedure was described earlier. In short, the subjects were given 22 mg dextromethorphan, 100 mg mephenytoin and 200 mg caffeine. For CYP2D6, the dextromethorphan /dextrorphan metabolic ratios in urine samples taken for a subsequent 8 hours were used. Dextromethorphan and dextrorphan were quantified by reversed phase high performance liquid chromatography. Subjects with metabolic ratios ≥ 0.3 were classified as poor metabolizer (PM) and ratios < 0.3 were classified as extensive metabolizers (EM). For CYP2C19 similarly obtained (R)-mephenytoin and (S)-mephenytoin ratios were used. (S)-mephenytoin and (R)-mephenytoin were analysed and quantified by enantioselective capillary gas chromatography. Subjects with S/R-ratios ≥ 0.8 were classified as poor metabolizer whilst subjects with
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ratios < 0.8 were classified as extensive metabolizer\(^{23}\). In addition, CYP2C19 PM subjects were reanalysed after acidic pre-treatment of urine samples to confirm the PM status.

Clinical procedures

The clinical part of was conducted in the clinical research centre of Pharma Bio-Research International BV, Zuidlaren, The Netherlands. The subjects were domiciled from the afternoon preceding the day of drug administration until 22:00 h on day 1. At 8 h on day 1 the subjects received their single combined dose in the upright position and after a 10 hours fast: 10 mL dextromethorphan syrup containing 22 mg dextromethorphan hydrobromide monohydrate (Samenwerkende Apothekers BV, Utrecht, The Netherlands), two capsules of 20 mg Losec\(^{\circledR}\) containing 40 mg omeprazole (Astra Pharmaceutica BV) and one tablet of Selokeen\(^{\circledR}\) containing 100 mg metoprolol (Astra Pharmaceutica BV). The medication was administered with 200 mL water without chewing the dosage forms. The volunteers were queried just before drug administration and at 12 h after drug administration using non-leading questions to determine the occurrence of adverse experiences. During study blood pressure, pulse rates and a standard 12-lead electrocardiogram were recorded just before and at 3, 6 and 12 h after drug intake. Blood samples were taken via an indwelling Venflon\(^{\circledR}\) catheter or by direct venepuncture in EDTA-containing polypropylene or Vacutainer\(^{\circledR}\) tubes. Blood samples of 20 mL each were collected just before and at 1, 2, 3, 4, 6, 8, and 12 after dosing on day 1. Blood samples were processed to plasma within one hour after collection by centrifugation for 10 minutes 1500 x g at 0 °C. Of the pre-dose sample 1 mL of whole blood was taken for genotyping. Whole blood and plasma samples were stored at -20 °C until analysis. Urine was collected quantitatively at intervals: pre-dose (-12 - 0), 0 - 4, 4 - 8, 8 - 12 h after dosing in polypropylene containers which were stored at +4 °C during collection. A 40 mL aliquot from each homogenized fraction was stored at -20 °C until analysis. Saliva samples were obtained by chewing on parafilm for the time it takes to obtain 5 mL of saliva. Saliva samples were collected in polypropylene tubes and stored at -20 °C until analysis.

Bioanalysis

Plasma samples were analysed on dextromethorphan, dextrorphan, metoprolol and α-hydroxymetoprol, omeprazole and 5-hydroxyomeprazole levels. The dextromethorphan and dextrorphan concentrations were determined by reversed phase high performance liquid chromatography (HPLC). In short, plasma samples were deconjugated by acid hydrolysis with 10 M HCl and incubation at 100 °C for 90 minutes. After neutralizing with saturated sodiumcarbonate the samples were further prepared by a liquid/liquid extraction with butanol and hexane and back extracted with a phosphate buffer (pH = 4.0). Separation was performed by a 5 µm Chromosphere\(^{TM}\) 5B column (150 x 4.6 mm; Chrompack, Middelburg, The Netherlands) and a mobile phase consisting of phosphate buffer (pH = 3.0): methanol: acetonitrile (100: 46: 75, v/v). Levallorphan was used as internal standard. Detection was performed with fluorescence detection.
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A calibration curve with concentrations varying from 1 ng.mL\(^{-1}\) to 500 ng.mL\(^{-1}\) for both dextromethorphan and dextrorphan was used to calculate plasma concentrations. Metoprolol and α-hydroxymetoprol were analysed by a reversed phase HPLC method. Plasma sample cleanup was performed by liquid-liquid extraction with diethyl ether, no internal standard was used. Separation was performed by a 3 µm Microsphere® column (100 x 4.6 mm; Chrompack, Middelburg, The Netherlands) and a mobile phase consisting of phosphoric acid, triethylamine and acetonitrile (1800 : 20 : 200, v/v). Quantitation was performed using fluorescence detection (\(\lambda_{\text{excitation}} = 228 \text{ nm}; \lambda_{\text{emission}} = 306 \text{nm}\)). A calibration curve with concentrations varying from 5 ng.mL\(^{-1}\) to 500 ng.mL\(^{-1}\) for both metoprolol and α-hydroxymetoprol was used to calculate plasma concentrations. Omeprazole and 5-hydroxy-omeprazole were analysed by a modified straight phase HPLC as described earlier\(^{12}\). In short, sample cleanup was performed by liquid extraction with tert-butyl methyl ether and dichloromethane (8 : 2, v/v) and separation was performed with a 3 µm Chromspher Si column (100 x 4.6 mm; Chrompack, Middelburg, The Netherlands). Quantitation was performed by measurement of the UV-absorption at a wavelength of 302 nm. Acetaminophen (paracetamol) was used as internal standard. A calibration curve with concentrations varying from 5 ng.mL\(^{-1}\) to 1000 ng.mL\(^{-1}\) for both omeprazole and 5-hydroxy-omeprazole was used to calculate plasma concentrations. Saliva samples were analysed on dextromethorphan, dextrorphan, omeprazole and α-hydroxymetoprol using the similar assays as described for plasma samples.

Urine samples were analysed on dextromethorphan on dextrorphan. Dextromethorphan and dextrorphan were analysed by reversed phase high performance liquid chromatography as described earlier\(^{21}\). Sample preparation was performed by liquid-liquid extraction with a mixture of n-hexane and n-butanol (90 : 10, v/v) after hydrolysis with 12 M hydrochloric acid (100 °C for 90 minutes) to release conjugated dextrorphan. Separation was performed on a C\(_8\) analytical column and a mobile phase containing phosphate buffer (pH = 4), triethylamine and acetonitrile. Quantitation was performed by fluorescence detection (\(\lambda_{\text{excitation}} = 230 \text{ nm}; \lambda_{\text{emission}} = 310 \text{ nm}\)). The concentration range varied from 0.1 to 7.00 mg.L\(^{-1}\) for dextromethorphan and 2.5 to 60 mg.L\(^{-1}\) for dextrorphan.

The analytical methodologies were validated on precision, accuracy, reproducibility, recovery and sample stability. The analytical methodologies for salivary drug level concentrations were not re-validated in the saliva matrix.

Genotyping

Genotyping was performed on both CYP2D6 and CYP2C19 using DNA isolated from whole blood samples. DNA was isolated using the QIAamp® DNA mini kit (Westburg BV, Leusden, The Netherlands). CYP2D6 was investigated on the five most common allelic variants using a long range polymerase chain reaction (PCR) amplifying the whole CYP2D6 gene followed by a multiplex allele-specific PCR\(^{24}\). The following allelic variants were investigated: CYP2D6*3 (A), CYP2D6*4 (B), CYP2D6*6 (T), CYP2D6*7 (E), CYP2D6*8 (G). Unless otherwise
stated all reagents were obtained from Amersham Pharmacia Biotech. In short, the long amplicon of 4666 basepairs was amplified using 100 ng genomic DNA, primers P1-5 and n at a concentration of 0.300 µM each, 5 µL of 10 x Taq extender buffer (Stratagene), 400 µM of each dNTP and 2.5 units of each Taq Polymerase and Taq Extender (Stratagene) in a final volume of 50 µL. Amplification was performed in a Progene thermocycler (Techne Cambridge Ltd., Duxford, UK). Initial denaturation (94 °C, 2 min) was followed by 30 cycles of denaturation (94 °C, 0.5 min), annealing (62 °C, 0.5 min) and extension (72 °C, 4.5 min) followed by a final extension step (72 °C, 7 min). The pre-amplicon was 1 : 3 diluted with water and 2 µL was used as template for two separate PCR reactions for the multiplex allele-specific PCR. Primers were added either wild type (A1[72 nM], B1[104 nM], E3[88 nM], T1[480 nM], G1[80 nM], M[800 nM]) or mutation-specific primers (A2[72 nM], B2[800 nM], E4[88 nM], T2[36 nM], G2[360 nM], M[800 nM]). Furthermore, the reaction mixture (50 µL) contained 10x PCR buffer without MgCl₂, 1.5 mM MgCl₂, 250 µM of each dNTP and 1.3 units Taq polymerase (Promega). The PCR mix was amplified as follows: pre-heating (40 °C, 2 min), initial denaturation (94 °C, 5 min) was followed by 32 cycles of denaturation (94 °C, 1 min), annealing (54 °C, 1 min) and extension (72 °C, 1 min) followed by a final extension step (72 °C, 5 min). The products were restricted with SmaI (CYP2C19*2) and BamHI (CYP2C19*3) by overnight incubation at 25°C and 37°C respectively. The products were analysed by a 2.5% agarose NA and Gelstar® nucleic acid staining (FMC Bioproducts).

The genotyping assays were validated on sensitivity, specificity, reproducibility and storage stability of whole blood and DNA extracts. For both CYP2D6 and CYP2C19 in case of homozygous mutant genotypes a poor metabolizer phenotype was predicted in all other cases a subject was assumed to have normal enzyme activity.
Pharmacokinetic data and Statistics
The pharmacokinetic parameters calculated were AUC_{0-t}, AUC_{0-12} for plasma and saliva data and metabolic ratios per time point for plasma, saliva and urine samples. The AUC_{0-t}, defined as the area under the concentration-time curve up to time t, where t is the last time point at which all subjects show concentrations above the lower limit of quantitation for each pair of parent compound and its metabolite, was calculated by the log-linear trapezoidal rule. The AUC_{0-12} defined as the area under the concentration-time curve up to 12 hours post dose was calculated similarly. Pharmacokinetic parameters were calculated using SAS version 6.12 (SAS Institute Inc., Cary, NC USA). Other statistics were performed in SPSS release 9.0.0 (SPSS Inc., Chicago, IL USA).

2.3.4 Results

Subjects
The demographic data and the results of pre-study phenotyping and genotyping of the subjects are given in Table 1. For CYP2D6 three poor metabolizers were included (02, 03 and 06) and for CYP2C19 none of the subjects was poor metabolizer. For CYP2D6 three genotypes were found homozygous wild type (*wt/*wt; 04), heterozygous wild type (*wt/*4; 01 and 05) and homozygous mutant (*4/*4; 02, 03 and 06). For CYP2C19 all subjects were homozygous wild type (*1/*1). All predicted phenotypes were in accordance with the actual phenotype. Two adverse experiences were observed: subject 02 experienced light-headedness starting 2.5 h post dose which lasted for 2.5 hours and subject 05 was nauseous for a few minutes 5 hours post dose. The first adverse experience was possible related to medication whereas the second adverse experience is not likely to be related with medication. Vital signs, ECGs and clinical laboratory data revealed no clinical significant abnormalities.

Table 1: Individual characteristics and results of pre-study phenotyping and genotyping of subjects.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Gender</th>
<th>Age</th>
<th>CYP2D6 Phenotype</th>
<th>CYP2C19 Phenotype</th>
<th>CYP2D6 Ratio</th>
<th>CYP2D6 Genotype</th>
<th>CYP2C19 Ratio</th>
<th>CYP2C19 Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>Male</td>
<td>47</td>
<td>EM</td>
<td>EM</td>
<td>0.0035</td>
<td>*wt/*4</td>
<td>0.070</td>
<td>*1/*1</td>
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<tr>
<td>02</td>
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<td>26</td>
<td>PM</td>
<td>EM</td>
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<td>*4/*4</td>
<td>0.044</td>
<td>*1/*1</td>
</tr>
<tr>
<td>03</td>
<td>Male</td>
<td>19</td>
<td>PM</td>
<td>EM</td>
<td>1.08</td>
<td>*4/*4</td>
<td>0.020</td>
<td>*1/*1</td>
</tr>
<tr>
<td>04</td>
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<td>26</td>
<td>EM</td>
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<tr>
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<tr>
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<td>PM</td>
<td>EM</td>
<td>3.45</td>
<td>*4/*4</td>
<td>0.097</td>
<td>*1/*1</td>
</tr>
</tbody>
</table>

EM. Extensive metabolizer; PM. Poor metabolizer; *wt. CYP2D6*wt allele (wild type); *4. CYP2D6*4 allele (mutant); *1. CYP2C19*1 allele (wild type).
Part II

Phenotyping on CYP2D6

The plasma levels measured for dextromethorphan, dextrophan, metoprolol and α-hydroxymetoprolol are given in figure 1 and figure 2. A clear distinguish between extensive and poor metabolizers for both dextromethorphan and metoprolol was observed. CYP2D6 probing with dextromethorphan results in low levels for dextromethorphan in extensive metabolizers ($C_{\text{mean}; 2\,h} = 2.1\,\text{ng.mL}^{-1}; n = 3$) and normal levels for dextrorphan ($C_{\text{mean}; 2\,h} = 247\,\text{ng.mL}^{-1}; n = 3$) and therefore low metabolic ratio values ($\text{MR}_{2\,h} = 0.011; n = 3$). In poor metabolizers dextromethorphan levels increased ($C_{\text{mean}; 2\,h} = 14.2\,\text{ng.mL}^{-1}; n = 3$) whereas dextrophan levels decreased ($C_{\text{mean}; 2\,h} = 7.9\,\text{ng.mL}^{-1}; n = 3$) and therefore metabolic ratio increased ($\text{MR}_{2\,h} = 1.81; n = 3$). CYP2D6 probing with metoprolol results in normal levels for metoprolol in extensive metabolizers ($C_{\text{mean}; 2\,h} = 128\,\text{ng.mL}^{-1}; n = 3$) and normal levels for α-hydroxymetoprolol ($C_{\text{mean}; 2\,h} = 88.1\,\text{ng.mL}^{-1}; n = 3$) and good measurable metabolic ratio values ($\text{MR}_{2\,h} = 1.73; n = 3$). In poor metabolizers metoprolol levels increased ($C_{\text{mean}; 2\,h} = 305\,\text{ng.mL}^{-1}; n = 3$) whereas α-hydroxymetoprolol levels could not be measured and therefore no metabolic ratio could be calculated.
Figure 1: Left panel: mean plasma concentration for dextromethorphan and dextrorphan and mean metabolic ratio (dextromethorphan over dextrorphan) for extensive metabolizers (n = 3) after a dose of 22 mg dextromethorphan; right panel: mean plasma concentration for dextromethorphan and dextrorphan and mean metabolic ratio (dextromethorphan over dextrorphan) for poor metabolizers (n = 3) after a dose of 22 mg dextromethorphan; Error bars show mean ± 1 standard error of mean.
Figure 2: Left panel: mean plasma concentration for metoprolol and \( \alpha \)-hydroxymetoprolol and mean metabolic ratio (metoprolol over \( \alpha \)-hydroxymetoprolol) for extensive metabolizers (\( n = 3 \)) after a dose of 100 mg metoprolol; right panel: mean plasma concentration for metoprolol and \( \alpha \)-hydroxymetoprolol and mean metabolic ratio (metoprolol over \( \alpha \)-hydroxymetoprolol) for poor metabolizers (\( n = 3 \)) after a dose of 100 mg metoprolol; Error bars show mean ± 1 standard error of mean.
This is furthermore clearly illustrated by the data on the AUC (Table 2). An increase of AUC_{0-12} between extensive and poor metabolizers was observed for both dextromethorphan (17.5 to 204 ng.h.mL^{-1}) and metoprolol (615 to 2126 ng.h.mL^{-1}) whereas both metabolites decreased: dextrorphan (1287 to 63.5 ng.h.mL^{-1}) and \( \alpha \)-hydroxymetoprolol (640 ng.h.mL^{-1} to not quantifiable). There was a good correlation between the AUCs of dextromethorphan and metoprolol \( (R^2 = 0.9142; n = 6) \) and between dextrorphan and \( \alpha \)-hydroxymetoprolol \( (R^2 = 0.964; n = 3) \) although the latter value is based on a small number of observations. The results on the renal excretion measured for dextromethorphan and dextrorphan and are summarized in Figure 3. In extensive metabolizers renal excretion of dextromethorphan was nearly quantifiable whereas in poor metabolizers excretion is about 1% in the time intervals up to 8 hours and about 2% in the 8-12 hours interval. For dextrophan in extensive metabolizers renal excretion varied from 20% (0 - 4 hours interval) to 4.5% (8 - 12 h interval) and in poor metabolizers it was about 0.5% in all intervals.
Figure 3: Mean renal excretion for dextromethorphan and dextrorphan after a 22 mg dose in time intervals of 4 hours (0-4, 4-8 and 8-12 h post dose). Excretion is expressed as a percentage the administered dose.

Saliva dextromethorphan and dextrorphan levels are summarized in Figure 4 and Table 2. Dextromethorphan levels in saliva were high for the first hours after dose in both EMs ($C_{\text{mean}}$: 1 h: 1532 ng.mL$^{-1}$; $n = 3$) and PMs ($C_{\text{mean}}$: 1 h: 1905 ng.mL$^{-1}$; $n = 3$) but steeply decreased for EMs ($C_{\text{mean}}$: 12 h: 0.65 ng.mL$^{-1}$; $n = 3$) and PMs ($C_{\text{mean}}$: 12 h: 74.3 ng.mL$^{-1}$; $n = 3$). Dextrorphan levels in saliva increased for the first hours after dose for EMs to $C_{\text{mean}}$: 3 h: 2.90 ng.mL$^{-1}$ ($n = 3$) and for PMs to $C_{\text{mean}}$: 6 h: 1.41 ng.mL$^{-1}$ ($n = 3$) and decreased for EMs to $C_{\text{mean}}$: 12 h: 0.22 ng.mL$^{-1}$ ($n = 3$) and for PMs to $C_{\text{mean}}$: 12 h: 0.38 ng.mL$^{-1}$ ($n = 3$). The mean metabolic ratios ($n = 3$) at 3 and
6 hours post dose were 41.9 and 8.0 for EMs respectively and 204 and 96.8 for PMs respectively. The AUC\textsubscript{0-12} values for dextromethorphan were 2522 ng.h.mL\textsuperscript{-1} in EMs and 4490 ng.h.mL\textsuperscript{-1} in PMs and for dextrorphan they were 17.0 ng.h.mL\textsuperscript{-1} in EMs and 11.3 ng.h.mL\textsuperscript{-1} in PMs (Table 2). In none of the cases there was a statistical significant difference between EMs and PMs for the salivary AUC values (p > 0.05).

**Figure 4**: Left panel: mean saliva concentration for dextromethorphan and dextrorphan and mean metabolic ratio (dextromethorphan over dextrorphan) for extensive metabolizers (n = 3) after a dose of 22 mg dextromethorphan; right panel: mean saliva concentration for dextromethorphan and dextrorphan and mean metabolic ratio (dextromethorphan over dextrorphan) for poor metabolizers (n = 3) after a dose of 22 mg dextromethorphan; Error bars show mean ± 1 standard error of mean.
Correlation between plasma and saliva levels was not significant for dextromethorphan (Pearson correlation = –0.105; p = 0.54; n = 36) but significant for dextrorphan (Pearson correlation = 0.835; p < 0.01; n = 36). The correlation between the metabolic ratio based on individual measurements per time point post dose and the metabolic ratio based on the AUC<sub>0-12</sub> value of dextromethorphan and dextrorphan and metoprolol and α-hydroxymetoprolol are given in Table 3. For dextromethorphan a significant correlation was observed for all time-points in plasma sample whereas in saliva samples at 3, 6 and 12 hours post dose metabolic ratios were significantly correlated with the AUC. Plasma metabolic ratios of metoprolol were significantly correlated at 3, 4 and 6 hours post dose whereas at the other time-point p values exceeded 5%.

**Table 2: AUC<sub>0-12</sub> values for dextromethorphan, dextrorphan, metoprolol and α-hydroxymetoprolol.**

<table>
<thead>
<tr>
<th></th>
<th>Dextromethorphan</th>
<th>Dextrorphan</th>
<th>Metoprolol</th>
<th>α-hydroxy- metoprolol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extensive</td>
<td>Poor</td>
<td>Extensive</td>
<td>Poor</td>
</tr>
<tr>
<td><strong>Plasma</strong> AUC&lt;sub&gt;0-12&lt;/sub&gt;</td>
<td>17.5</td>
<td>204</td>
<td>1287</td>
<td>63.5</td>
</tr>
<tr>
<td></td>
<td>SEM#</td>
<td>3.9</td>
<td>11</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Ratio</td>
<td>0.0014</td>
<td>3.22</td>
<td>-</td>
</tr>
<tr>
<td><strong>Saliva</strong> AUC&lt;sub&gt;0-12&lt;/sub&gt;</td>
<td>2522*</td>
<td>4490*</td>
<td>17.0*</td>
<td>11.3*</td>
</tr>
<tr>
<td></td>
<td>SEM#</td>
<td>435</td>
<td>1338</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>N</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Ratio</td>
<td>149*</td>
<td>422*</td>
<td>-</td>
</tr>
</tbody>
</table>

*: units ng.h.mL<sup>-1</sup>; §: all concentrations below limit of detection (<2.0 µg.mL<sup>-1</sup>); N.C.: no calculation possible; N.M.: not measured; SEM: standard error of mean; ratio: ratio between parent and metabolite. +*: difference between extensive and poor metabolizer not significant (p > 0.05) using an unpaired t-test, for all not marked AUC values difference between extensive and poor is significant (p < 0.02).

**Phenotyping on CYP2C19**

The plasma levels measured for omeprazole and 5-OH-omeprazole given in figure 5. CYP2C19 probing with omeprazole results in increasing plasma levels up to 3 hours post dose low levels for omeprazole (C<sub>mean: 3 h</sub>: 169 ng.mL<sup>-1</sup>; n = 6) and for 5-OH-omeprazole (C<sub>mean: 3 h</sub>: 199 ng.mL<sup>-1</sup>; n = 6) resulting in a metabolic ratio values MR<sub>3 h</sub>: 0.82 (n = 6). Omeprazole levels decreased to C<sub>mean: 8 h</sub>: 2.57 ng.mL<sup>-1</sup> (n = 6) and 5-OH-Omeprazole levels decreased to C<sub>mean: 8 h</sub>: 8.50 ng.mL<sup>-1</sup> (n = 6) and finally below the limit of detection (3.0 ng.mL<sup>-1</sup>) for both compounds at 12 hours post dose. The AUC<sub>0-12</sub> for omeprazole was 446 ng.h.mL<sup>-1</sup> and 664 ng.h.mL<sup>-1</sup> for 5-OH-omeprazole (Table 4). There was as expected no significant difference between the observed AUCs in CYP2D6 EMs.
and PMs for both omeprazole (p = 0.93) and 5-OH-omeprazole (p = 0.56). The correlation between the metabolic ratio based on individual measurements per time point post dose and the metabolic ratio based on the AUC$_{0-12}$ value of omeprazole and 5-OH-omeprazole is given in Table 3. A significant correlation was observed at 2 hours (R = 0.815 p = 0.048) and 3 hours (R = 0.862 p = 0.027) whereas the correlation was not significant at 4 hours (R = 0.782 p = 0.066) and 6 hours (R = 0.280 p = 0.59). In saliva samples omeprazole and 5-OH-omeprazole levels were all below the limit of detection (3 ng.mL$^{-1}$).

Figure 5: Mean plasma concentration for omeprazole, 5-OH-omeprazole and mean metabolic ratio (omeprazole over 5-OH-omeprazole) for extensive metabolizers (n = 3) after a dose of 40 mg omeprazole; Error bars show mean ± 1 standard error of mean.
Table 3: Pearson correlation analysis between metabolic ratios at a certain timepoint post dose and the metabolic ratio based on the AUC values for dextromethorphan (plasma and saliva) and metoprolol (plasma).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Dextromethorphan Plasma Correlationp (n = 6)</th>
<th>Dextromethorphan Saliva Correlationp (n = 6)</th>
<th>Metoprolol Plasma Correlationp (n = 3)</th>
<th>Omeprazole Plasma Correlationp (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.937</td>
<td>0.006</td>
<td>0.359*</td>
<td>0.485</td>
</tr>
<tr>
<td>2</td>
<td>0.974</td>
<td>0.001</td>
<td>0.501*</td>
<td>0.312</td>
</tr>
<tr>
<td>3</td>
<td>0.999</td>
<td>0.000</td>
<td>0.930</td>
<td>0.007</td>
</tr>
<tr>
<td>4</td>
<td>0.997</td>
<td>0.000</td>
<td>N.M.</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>0.973</td>
<td>0.001</td>
<td>0.982</td>
<td>0.000</td>
</tr>
<tr>
<td>8</td>
<td>0.958</td>
<td>0.003</td>
<td>N.M.</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>0.979</td>
<td>0.001</td>
<td>N.M.</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>0.964</td>
<td>0.002</td>
<td>0.917</td>
<td>0.010</td>
</tr>
</tbody>
</table>

N.C.: no calculation possible; N.M.: not measured; *: not significant (p > 0.05)

Table 4: Plasma AUC$_{0-12}$ values for omeprazole and 5-OH-omeprazole.

<table>
<thead>
<tr>
<th></th>
<th>Omeprazole</th>
<th>5-OH-Omeprazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC$_{0-12}$ (ng.h.mL$^{-1}$)</td>
<td>446</td>
<td>664</td>
</tr>
<tr>
<td>SEM (ng.h.mL$^{-1}$)</td>
<td>72</td>
<td>91</td>
</tr>
<tr>
<td>N</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.67</td>
<td>-</td>
</tr>
</tbody>
</table>

Pharmacodynamics of the Cocktail
The pharmacodynamic effects of intake of the phenotyping cocktail on blood pressure and pulse rate are shown in Figure 6. It can be seen that both the diastolic and systolic blood pressure decreased post dose to reach a minimum blood pressure about 8 hours post dose for the diastolic blood pressure and 5 to 7 hours post dose for the systolic blood pressure. No significant differences were observed between EMs and PMs for both diastolic and systolic blood pressure although the duration of blood pressure decrease tended to be longer in PMs. The pulse rate decreased to a minimum at 3 hours post dose for EMs with 7.6 min$^{-1}$ (n = 3) and for PMs it decreased with 20.3 min$^{-1}$ (n = 3). The difference between EMs and PMs on the pulse rate at 3 hour post dose however was not significant (p = 0.12).
Figure 6: Pharmacodynamic effects of the administration of the phenotyping cocktail on the blood pressure and pulse rate. 0; ____ : mean difference on blood pressure (diastolic and systolic) and pulse rate for extensive metabolizers after a dose of 22 mg dextromethorphan, 100 mg metoprolol and 40 mg omeprazole; Δ; −−: mean difference on blood pressure (diastolic and systolic) and pulse rate for poor metabolizers (n = 3) after a dose of 22 mg dextromethorphan, 100 mg metoprolol and 40 mg omeprazole; Error bars show mean ± 1 standard error of mean.
2.3.5 Discussion

For both probes phenotype results were in accordance with the predicted phenotype based on genotyping. This was expected since both the O-demethylation of dextromethorphan and the hydroxylation of metoprolol does reflect mainly CYP2D6 activity\textsuperscript{22,25} and Furthermore, the genotyping assay does detect approximately 99\% of all non-coding alleles\textsuperscript{24}. In EMs plasma metabolic ratios were difficult to measure for dextromethorphan due to low concentrations of the parent compound (Figure 1). Therefore, the discriminative power is rather poor for the subgroups in the EM phenotypes e.g. heterozygous mutant, homozygous mutant and gene-duplication (ultra rapid phenotype) and it seems difficult to detect inhibition of CYP2D6 in plasma samples of EMs. Metoprolol on the other hand showed difficulties in measuring metabolic ratios in PMs due to non detectable levels of the metabolite whereas the metabolic ratios in plasma samples of EMs could be measured with a high precision (Figure 2). Urinary excretion ratios of dextromethorphan over dextrorphan shows the same as plasma low renal excretion of dextromethorphan in EMs and therefore difficulties with measuring the metabolic ratio (Figure 3). Saliva levels were high for dextromethorphan irrespective of their phenotype whereas dextrorphan levels were low but good measurable in EMs and difficult to measure in PMs (Figure 4). Metabolic ratios in plasma samples were comparable with other studies for both dextromethorphan\textsuperscript{26} and metoprolol\textsuperscript{27}. Saliva levels of dextromethorphan were not comparable with other data whereas dextrorphan levels were comparable.\textsuperscript{28} When compared with the study of Hou et al. dextromethorphan levels were found to be extremely increased at the first time-points post dose: 1 hour 400 times, 2 hours 80 times whereas at later time-points levels were comparable or slightly increased when corrected for the increased dose given in that study (50 mg instead of 22 mg). The main difference between the studies was the dosage form of dextromethorphan used: syrup in the present study and a capsule in the study of Hou et al. From these observations it is obvious that when dextromethorphan is administered as syrup the drug remains in the mouth for a long time after administration even if the mouth is rinsed with water and therefore will interfere in metabolic ratios leading to wrong conclusions. From this we conclude that if dextromethorphan saliva metabolic ratios are to be used for phenotyping purposes dextromethorphan should be administered in a capsule. Urinary excretion and urinary metabolic ratios were comparable with other studies\textsuperscript{22,26} and our own observations\textsuperscript{21}. The pharmacodynamic effects of the cocktail (Figure 6) are most likely due to metoprolol since the effects a decreased blood pressure and hart frequency are highly associated with β receptor antagonists whereas the effects are not associated with proton-pump inhibitors (omeprazole) or antitussive drugs (dextromethorphan). No significant difference on the dynamic effect could be observed between EMs and PMs, although especially pulse rates decreases is doubled in PMs as compared to EMs. This is likely related to the small numbers since others good indeed find pharmacodynamic differences between EMs and PMs.\textsuperscript{9,29} Omeprazole plasma levels and metabolic ratios were in accordance
with other observation\textsuperscript{12,30} but it should be noted that no CYP2C19 PMs were included and all volunteers were found to be homozygous wild type CYP2C19. Omeprazole could not be detected in saliva and therefore this matrix cannot be used for phenotyping. There was no significant influence of CYP2D6 genotype on omeprazole or 5-OH-omeprazole levels and it therefore can be concluded that omeprazole can be used in a cocktail approach either with dextromethorphan or metoprolol or with both of them. Mephenytoin is being the most frequent used probe for CYP2C19 but as earlier described it has some disadvantages with respect to sample stability restricted availability and adverse drug reactions (sedation).\textsuperscript{20,31} Furthermore, we and others reported that in about 10% S/R-ratios in EMs cannot be measured due to S-mephenytoin levels below the limit of detection and therefore discrimination with the EM group is limited.\textsuperscript{21,30} Omeprazole has shown to be a reliable and safe alternative\textsuperscript{30} although recently some disconcordances between phenotype and genotype were reported in Japanese patients\textsuperscript{32} but in our opinion this observation is likely to be independent of the type of probe drug and is rather a plea for a combination of genotyping and phenotyping. We therefore conclude that omeprazole is the preferable probe drug compared to mephenytoin and that plasma level ratio between omeprazole and 5-OH-omeprazole are a reliable reflection of actual CYP2C19 activity. Although the best measure for enzyme activity is the metabolic ratio based on the AUC this only occasionally used due the practical limitations: several blood samples are needed and therefore it is a rather invasive and expensive procedure. A good and more practical alternative is a one time-point measurement that significantly correlates with the AUC. In our study, we observed that at three hours post dose all correlations between individual metabolic ratios and the AUC were significant and from this we conclude that a three hours plasma sample is a good measure for the enzyme activity of both CYP2D6 when probed with dextromethorphan or with metoprolol and CYP2C19 when probed with omeprazole. The discussion, which is the best CYP2D6 probe, is not an easy one: besides metoprolol and dextromethorphan, sparteine and debrisoquine are frequently used and each of the probes has advantages and disadvantages. We used metoprolol and dextromethorphan since these drugs are available in the Netherlands and in many other countries whereas the obsolete sparteine and debrisoquine are not registered in the Netherlands and are of small distribution worldwide. For safety reasons dextromethorphan would be preferable above metoprolol, although a single dose of 100 mg of metoprolol in healthy volunteers has a low risk on serious adverse effects. On the other hand the use of metoprolol in patients may be limited. A disadvantage of dextromethorphan is that the formation of dextrorphan is not under monogenic control (CYP2D6) but is catalysed by other cytochromes as well\textsuperscript{33} which is also illustrated by our data in which dextrorphan is still quantifiable in genetic poor metabolizers (Figure 1 and Table 2). Furthermore, as described above due to the low plasma levels of the dextromethorphan in EMs its use may be limited in detecting UM or small changes in CYP2D6 activity. An advantage of dextromethorphan over metoprolol might be that it can
be used as dual probe drug since it was shown that the \( \text{n-demetylation} \) to 3-methoxymorphinan was primarily catalysed by \( \text{CYP3A4} \) and to lesser extent by \( \text{CYP3A5} \). Detection of inhibition or induction of the non polymorphic \( \text{CYP3A4} \) is also of great importance in clinical drug research and several reports have been published using dextromethorphan for this purpose\(^{35,36,37} \) although some doubts on the specificity have also been reported\(^{38} \). A good non-invasive alternative however is the measurement of the ratio cortisol over 6\( \text{g-hydroxycortisol} \) in a 24-hour urine interval or in a morning spot\(^{39} \). Although metoprolol is not often used in probing \( \text{CYP2D6} \) it has been shown an adequate measure for \( \text{CYP2D6} \) activity\(^{27,9} \) and our data supports these findings. Limitations with respect to its applicability in detection of bimodality in African populations have been reported\(^{9,40} \) but seems not to be exclusive for metoprolol and possibly related with allelic variants with altered substrate specificity\(^{41} \). Overseeing these arguments we tend to prefer metoprolol as substrate above dextromethorphan when used in combination with genotyping and is applied to estimate actual enzyme activity. In conclusion, the following procedure is suggested when the activity of \( \text{CYP2D6} \) and \( \text{CYP2C19} \) have to be established in the context of clinical drug research: probe \( \text{CYP2D6} \) with metoprolol (100 mg) and probe \( \text{CYP2C19} \) with omeprazole (40 mg), take a blood sample (EDTA) at 3 hours post dose, before preparation of plasma take a 1 mL whole blood sample for genotyping purposes, prepare the remaining blood to plasma to assess metoprolol, \( \alpha \)-hydroxymetoprolol, omeprazole and 5\( \text{-hydroxyomeprazole} \). Using this procedure all possible information is obtained and further genetic analysis can be performed in case genotype does not correctly predicts phenotype.

2.3.6 References

Methodology

for the in-vivo catalytic function of CYP2D6 in German population. Pharmacogenetics 1998; 8:15-26


