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Abstract

OBJECTIVES: (S)-Mephenytoin is selectively metabolised to (S)-4'-hydroxymephenytoin by CYP2C19. The urinary excretion of 4'-hydroxymephenytoin reflects the activity of individual enzymes. We evaluated fractioned urinary collection and beta-glucuronidase pre-treatment in order to determine the optimal CYP2C19 metrics. We also assessed whether urinary excretion of N-desmethylmephenytoin (nirvanol) might be a useful CYP2B6 metric in in vivo studies. METHODS: A 50-mg dose of mephenytoin was administered to 52 volunteers as a component of phenotyping cocktails in four separate studies. Urine was collected up to 166 h post-dose. Urinary excretion of 4'-hydroxymephenytoin and nirvanol was quantified by liquid chromatography-tandem mass spectrometry, and common CYP2C19 and CYP2B6 genotypes were determined. RESULTS: Cumulative excretion of 4'-hydroxymephenytoin in urine with beta-glucuronidase treatment collected from before mephenytoin administration up to 12-16 h thereafter showed the greatest difference between CYP2C19 genotypes and the lowest intra-individual variability (7%). Renal elimination of nirvanol was highest for a *4/*4 individual and lowest for individuals carrying the *5/*5 and *1/*7 genotype, but lasted for several weeks, thus making its use in cross-over studies difficult. CONCLUSION: Cumulative urinary excretion of 4'-hydroxymephenytoin 0-12 h post-administration is a sensitive and reproducible metric of CYP2C19 activity, enabling the effect of a drug on CYP2C19 to be assessed in a small sample size of n=6 volunteers. While nirvanol excretion may reflect CYP2B6 activity in vivo, it is not useful for CYP2B6 phenotyping.
Assessment of urinary mephenytoin metrics to phenotype for CYP2C19 and CYP2B6 activity

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ABSTRACT

Objectives: (S)-Mephenytoin is selectively metabolised to (S)-4’-hydroxymephenytoin by CYP2C19. Urinary excretion of 4’-hydroxymephenytoin reflects individual enzyme activity. We evaluated fractioned urinary collection and β-glucuronidase pre-treatment to assess the optimal CYP2C19 metric. Furthermore, we addressed whether urinary excretion of N-desmethylmephenytoin (nirvanol) might be a useful CYP2B6 metric in vivo.

Methods: 50 mg of mephenytoin was administered to 52 volunteers as part of phenotyping cocktails in four separate studies. Urine was collected up to 166 hours postdose. Urinary excretion of 4’-hydroxymephenytoin and nirvanol was quantified by LC-MS/MS, and common CYP2C19 and CYP2B6 genotypes were determined.

Results: Cumulative excretion of 4’-hydroxymephenytoin in urine with β-glucuronidase treatment collected from before mephenytoin administration up to 12-16 hours thereafter showed the greatest difference between CYP2C19 genotypes and the lowest intraindividual variability (7 %). Renal elimination of nirvanol was highest for a *4/*4 individual and lowest for individuals carrying the *5/*5 and *1/*7 genotype, but lasted for several weeks, thus making its use in cross-over studies difficult.

Conclusion: Cumulative urinary excretion of 4’-hydroxymephenytoin 0-12 hours is a sensitive and reproducible metric of CYP2C19 activity, allowing a small sample size of n=6 volunteers to assess the effect of a drug on CYP2C19. While nirvanol excretion may reflect CYP2B6 activity in vivo, it is not useful for CYP2B6 phenotyping.
INTRODUCTION

The genetically polymorphic human cytochrome P450 enzyme CYP2C19 is involved in the metabolism of several frequently prescribed drugs like omeprazole\(^1\) and diazepam\(^2\).

While CYP2C19*1 represents the wild type allele, 26 variant alleles have been described to date.\(^3\) There are pronounced differences in the frequency of polymorphisms between ethnic groups. The CYP2C19 alleles *2\(^4\) and *3\(^5\) which are almost exclusively responsible for truncated CYP2C19 proteins with absent functional activity are present in 30% and 5% of the Asian, 17% and 0.4% of the African-American, and 15% and 0.04% of the Caucasian population, respectively. The remaining defective alleles are rarely encountered throughout all ethnicities.\(^6\)

Fast and reliable genotyping assays have been developed, enabling a clear assignment to the poor or extensive metabolizer phenotype.\(^7\) Individual enzyme activity, however, is also influenced by various non-genetic factors like the intake of drugs that inhibit or induce CYP2C19, age or different disease states.\(^8, 9\) In contrast to a genetic approach, phenotyping determines actual enzyme activity in vivo, and thus can take these factors into account and quantify their effects. Hence, there is an essential demand for reliable, selective, and reproducible CYP2C19 phenotyping metrics.

Mephenytoin is the standard probe drug for the assessment of CYP2C19 activity in humans\(^10\) and has successfully been employed in several cocktail phenotyping studies to evaluate the effects of drugs or disease states on CYP2C19 activity in vivo.\(^11, 12\) Omeprazole and proguanil are used as alternative probe drugs for CYP2C19 phenotyping. But, in addition to CYP2C19 mediated hydroxylation, CYP3A4 mediated sulfoxidation of both omeprazole and 5-hydroxyomeprazole, the metabolite formed by CYP2C19, occurs.\(^1\) Interactions with CYP3A4 (phenotyping) substrates and or CYP3A4 inhibitors could be the consequence when applied concomitantly, which may be one reason why CYP3A4 substrates were not administered simultaneously with omeprazole in published phenotyping cocktails.\(^13, 14\) Additionally, due to
the short half-life of omeprazole, plasma sampling has to take place early after administration and results may therefore be influenced by dissolution or enteral absorption of the probe drug. The within-subject variability of omeprazole metrics thus is substantial (35~40%)\textsuperscript{15} and could not be attributed to variation in CYP2C19 activity only. Hence, omeprazole does not appear to be an optimal alternative CYP2C19 probe. Proguanil metabolism seems to be affected significantly by CYP3A4 and is not recommended for CYP2C19 phenotyping anymore.\textsuperscript{10} Mephenytoin is administered as a racemic mixture and undergoes a complex metabolism (see Figure I). Only extensive metabolizers (EM), expressing functional CYP2C19, are able to hydroxylate (S)-mephenytoin to (S)-4′-hydroxymephenytoin, which is subsequently conjugated almost completely to the (S)-4′-hydroxymephenytoin glucuronide and excreted renally.\textsuperscript{16, 17} In CYP2C19 deficient poor metabolizers (PM), (S)-mephenytoin is biotransformed via slow N-demethylation, which in vitro is preferentially mediated by CYP2B6 (with minor contribution of CYP2C9), resulting in the formation of (S)-nirvanol.\textsuperscript{18-20} (R)-mephenytoin is preferably demethylated to (R)-nirvanol and hydroxylated only to a small extent.\textsuperscript{16} Based on this highly stereoselective metabolism, either the enantiomeric S/R ratio of unchanged mephenytoin in 0-8 hour urine\textsuperscript{21} or the amount of 4′-hydroxymephenytoin recovered in urine\textsuperscript{22} are used as CYP2C19 phenotyping metrics. Shortcomings of the S/R ratio have been reported.\textsuperscript{23, 24} Therefore we chose the molar urinary recovery of 4′-hydroxymephenytoin for our investigations. The urinary recovery of 4′-hydroxymephenytoin was widely used to phenotype for CYP2C19 activity during the last twenty years, but validation of this metric is incomplete in the literature. The optimal duration of urine collection remains to be defined. In addition, it is unknown whether cumulative or fractioned urine collections yield better results. Finally, it is unclear whether enzymatic hydrolysis of 4′-hydroxymephenytoin glucuronide prior to analysis is inevitable, or whether the small fraction of free 4′-hydroxymephenytoin found in
untreated urine satisfactorily reflects individual CYP2C19 enzyme activity when a sensitive LC-MS/MS assay is used.

An increasing number of compounds, e.g. bupropion, cyclophosphamide or efavirenz, have been identified as substrates of the highly polymorphic enzyme CYP2B6. As (S)-mephenytoin demethylation to (S)-nirvanol is mediated mainly by CYP2B6 \textit{in vitro}, it is tempting to use mephenytoin as a dual phenotyping drug for CYP2C19 and CYP2B6. However, it is unknown whether urinary excretion of nirvanol represents an appropriate metric for CYP2B6 \textit{in vivo}.

The objective of our present work was to identify the optimal CYP2C19 phenotyping metric of urinary 4′-hydroxymephenytoin excretion. To decrease the risk of sedation particularly in poor metabolizers, we further examined the feasibility of phenotyping with 50 mg of mephenytoin in a Caucasian population. Additionally, we wanted to explore whether phenotyping of CYP2B6 can be accomplished with mephenytoin \textit{in vivo}. 
METHODS

Volunteers and general procedures

A total number of 52 male, healthy Caucasians participated in four phenotyping cocktail studies (A, B, C and D) which included mephenytoin as a CYP2C19 probe. Two of the volunteers took part in two studies; to avoid confounding, for these subjects only the data from the first study in which they participated was used for evaluation. The demographics of study participants is shown in Table I.

All studies were approved by the Ethics Committee of the Medical Faculty of the University of Cologne, Germany, and were conducted in accordance with the laws of Germany. Written informed consent was obtained from each subject before participating in the studies. All subjects were in good health as indicated by pre- and post-study screening examinations.

To rule out possible interactions with the cytochrome P450 system, all study participants were advised to avoid the consumption of food or beverages containing methylxanthines, grapefruit or alcohol from at least 48 hours prior to the drug administration until 48 hours thereafter. In case of study C and D the dietary restrictions were extended on beverages containing quinine and maintained until the end of the post study examinations. Therapeutic or illicit drug intake two weeks preceding the trials was an exclusion criteria. All subjects were regularly asked for the occurrence of adverse events, their well being was continuously surveyed, and blood pressure and pulse rate were recorded regularly.

In all four studies the subjects had to empty their bladder completely before administration of the phenotyping cocktail. Half a tablet of Epilan Gerot (Gerot Pharmazeutika, Vienna, Austria), corresponding to 50 mg racemic mephenytoin, was administered to each study participant as part of a phenotyping cocktail for important drug-metabolizing enzymes and transporters. The individual dose was determined by weighing the tablet halves. Food and fluid intake as well as body position (supine position until 6 hours postdose) during the studies was standardized.
**Individual study designs and objectives**

Study A was a one period, single dose cocktail phenotyping pilot study. Studies B, C and D were cocktail drug interaction studies in which the effect of an investigational drug in a Test period was compared to a Reference period. These three studies had a randomized, placebo-controlled cross-over design. The phenotyping drugs used in each of the four studies are shown in Table II.

Studies A and B were primarily used to assess \( \beta \)-glucuronidase pretreatment of the samples and fractioned vs. cumulative urine collection. The procedures of these studies has already been described in more detail.\(^{11,28}\)

The primary phenotyping objectives of study C with regard to mephenytoin were the generation of confirmatory data for variability and selectivity evaluations of the CYP2C19 metrics as well as a more thorough characterization of the slow nirvanol excretion. In this study, urine was collected in-house until 48 hours postdose and completed by two additional overnight in-house urine collection periods from 86-94 and 158-166 hours postdose. There was a washout phase of 4 weeks between both study periods.

The objective of study D for this evaluation was further confirmation of intraindividual variability data with respect to CYP2C19 phenotyping. In this study, a washout phase of 2 weeks was used between study periods.

**Urine and blood sampling**

In all studies urine was collected in 2 L plastic containers. The containers were cooled at +4°C during the collection. Exact duration of urine collection (for scheduled periods in the individual studies see Table I) and the pH value were recorded for each sample. The volume of urine was determined by weighing the containers assuming a mean density of 1 g/mL for urine. Aliquots of 10 mL were stored in plastic tubes at −80°C until analysis.
Whole blood samples for genotyping were drawn into tubes containing ethylene diamine tetraacetic acid (EDTA tubes, Sarstedt, Nümbrecht, Germany) before the first drug administration in all studies.

**Urine analysis by LC-MS/MS**

Mephenytoin, nirvanol and 4’-hydroxymephenytoin were analyzed by a validated assay for liquid chromatography tandem mass spectrometry as described earlier. Both the fraction of free, unconjugated 4’-hydroxymephenytoin and total 4’-hydroxymephenytoin after β–glucuronidase pre-treatment were measured in samples of study A and B. For study C and D only total 4’-hydroxymephenytoin concentrations up to 12 hours postdose were determined, while nirvanol concentrations were determined for all collection periods of study A, B and C.

**Genotyping**

Genotyping for CYP2C19 was performed for all subjects participating in studies A, B and C. DNA was extracted from blood samples using a standard phenol-chloroform extraction method. Analysis for allele CYP2C19*2 was performed by a polymerase chain reaction / restriction fragment length polymorphism method as described by de Morais et al. The volunteers of studies A, B and C were also genotyped for CYP2B6. Single nucleotide polymorphisms (SNPs) accounting for the CYP2B6 alleles *2, *3, *4, *6 and *7 were determined as described by Lang et al.
Data Analysis

*Calculation of urinary recovery.* For each cumulative and each fractioned collection period, the urinary recovery of metabolites was calculated as the molar excretion of 4′-hydroxymephenytoin or nirvanol in urine expressed as a percentage of the accurate dose of mephenytoin administered. Recovery for cumulative collection periods was calculated by summing up the results of the fractioned collection periods. Urinary excretion rates were calculated as molar excretion divided by sampling time.

To estimate the amount of nirvanol excreted during periods without urine collection, the following formula was used in study C:

\[ A_{e(t_{1-2})} = \sqrt{R_1 \cdot R_2} \cdot (t_1 - t_2) \]

In this formula, \( A_e \) reflects the amount excreted, \( R \) is the mean excretion rate in the collection interval before and after the period in which no urine was collected, respectively, and \( t \) is the corresponding time where the adjacent collection intervals ended or started.

*CYP2C19 genotype and 4′-hydroxymephenytoin metrics.* To identify the CYP2C19 metric which reflects the CYP2C19 genotype best, urinary recovery of 4′-hydroxymephenytoin [%] was calculated for fractioned and cumulative collection intervals of the respective Reference periods, each with and without previous deconjugation of glucuronic acid, in studies A, B and C. Kruskal-Wallis one way analysis of variance (ANOVA) with CYP2C19 genotype as the independent parameter and the various metrics as dependent parameters was used for statistical analysis.

*CYP2B6 genotype and nirvanol metrics.* For a comparison of nirvanol excretion between CYP2B6 genotypes, fractioned and cumulative urinary recovery of nirvanol [%] was calculated for study A, for the respective first study periods of Study B (assuming that propiverine, the drug characterized in this interaction study, had no effect on nirvanol excretion) and for the respective Reference periods of study C. For nirvanol metrics in study B, it was not possible to use the Reference period only because after two weeks of wash-out,
in the second period there was still a pronounced excretion of nirvanol prior to administration of the mephenytoin test dose. Comparison of nirvanol excretion to CYP2B6 genotype is descriptive only.

**Intra-subject variability of CYP2C19 and CYP2B6 metrics.** To determine the intra-subject variability of 4’-hydroxymephtooin and nirvanol excretion parameters, respectively, the intraindividual coefficients of variation were calculated after logarithmic transformation of urinary recovery data from the residual variance obtained by the standard ANOVA approach used for bioavailability assessment (software BIAV, Stephan Rietbrock, 1994). For 4’-hydroxymephtooin metrics, data from both Test and Reference periods of the studies B, C, and D were used, and all calculations were performed for fractioned and cumulative collection periods, each with and without previous deconjugation of glucuronic acid. In the case of nirvanol metrics, intraindividual coefficients of variation for fractioned and cumulative excretion could only be calculated for study C because the wash-out period was too short in studies B and D. Based on the intraindividual coefficients of variation, the sample size needed for a cross-over drug interaction study with the CYP2C19 and CYP2B6 metrics identified was assessed assuming a power of 80%, a range of “no drug interaction“ from 0.8 to 1.25 and a significance level of 5% by means of confidence intervals as described by Diletti et al..31

All other analyses were performed with SPSS 12.00 (SPSS Inc., Chicago, IL, USA) and Excel 2000 (Microsoft Corp., Redmond, WA, USA). For all statistical procedures P < .05 was considered significant. Results are given as arithmetic means ± standard deviations (SD) unless stated otherwise.
RESULTS

In general, all phenotyping cocktails were well tolerated. No severe adverse effects related to mephenytoin were observed throughout all studies. Since analytical selectivity had been validated previously, no interfering peaks were observed in any of the chromatograms.

4′-hydroxymephenytoin metrics for CYP2C19 activity. Analysis of CYP2C19 genotype for studies A, B, and C resulted in 24 homozygous CYP2C19*1/*1 carriers, 12 heterozygous CYP2C19*1/*2 extensive metabolizers and one poor CYP2C19*2/*2 metabolizer. (Volunteer B11, who was a carrier of the CYP2C19*1/*2 genotype, also took part in study C. Therefore, the results obtained for this subject in study C were excluded from further evaluation.) Respective numbers for individual studies A, B and C were 5, 5, 0; 12, 3, 1; and 7, 4, 0. The effect of CYP2C19 genotype on urinary excretion of 4′-hydroxymephenytoin was statistically significant for cumulative urine collections from the administration of mephenytoin to 6 or more hours postdose when combined with β-glucuronidase pre-treatment of the samples (see Table III). A significant genotype effect was also seen for fractioned collection of (deglucuronidated) urine from 2 to 8 hours (data available for studies A and B; n=26) with a p-value of 0.036. Apart from that, neither cumulative collection without previous deconjugation of glucuronic acid, nor all other fractioned urine sampling periods provided significant results (data not shown). The one CYP2C19*2/*2 homozygote poor metabolizer was, however, identified in all cumulative and fractioned collection intervals, because excretion of 4′-hydroxymephenytoin was roughly 100-fold lower than in carriers of at least one *1 allele. This is also indicated by comparison of urinary excretion rates as depicted in Figure II. Without deglucuronidation, 4′-hydroxymephenytoin concentrations were below the lower limit of quantification in all samples of the poor metabolizer, whereas all postdose concentrations were above the lower limit of quantification in the group of extensive metabolizers.
In the drug-drug interaction studies where we used the residual error to estimate intraindividual variability (studies B, C, D), there was no relevant effect of the potentially interacting drugs on CYP2C19 metrics (data not shown). As it becomes evident from Figure III, the lowest intraindividual variability of 4′-hydroxymephenytoin recovery was achieved by cumulative collection periods combined with β-glucuronidase pre-treatment. Because of the findings on deglucuronidation in studies A and B, only β-glucuronidase pretreated samples were analysed for studies C and D. Overall intra-subject variability from these samples was 67%, 57%, 22%, 11% and 7% for urine collection periods 0-2 hours (study included: B), 0-4 hours (B/D), 0-6 hours (B/D), 0-8 hours (B/C) and 0-12 hours (B/C/D) after oral administration of 50 mg mephenytoin, respectively. As expected, for the longer sampling periods these values were clearly lower than intersubject variation (CV 72%, 45%, 32%, 25% and 23% for the respective sampling periods). Further extension of collection periods up to 0-16 hours (B) or 0-24 hours (B) hours postdose led to coefficients of variation of 6 % and 11%, respectively, and thus did not provide clearly better results. On the basis of these values for intraindividual variability, sample sizes of 24, 8, or 6 subjects were calculated for cross-over studies to assess an effect on CYP2C19 phenotype, if collection periods of 0 to 6 hours, 0 to 8 hours, or 0-12 or more hours postdose, respectively, are used.

**Nirvanol metrics for CYP2B6 activity.** Nirvanol excretion was very slow, with elimination rates being almost unchanged during the first week after mephenytoin administration. Even after the 4 weeks wash-out period in study C, concentrations above the lower limit of quantification (30 ng/ml) were found in 7 of 12 samples. Mean ± SD urinary excretion of nirvanol within 166 hours after cocktail administration in the reference periods of study C was 3.2 ± 1.4 mg and thus reached 6.4 ± 2.7% of molar mephenytoin dose.

Excretion of nirvanol depending on the CYP2B6 genotype in studies A, B and C is shown in Figure IV. In this three studies, the following CYP2B6 genotypes were determined: *1/*1, n=10; *1/*2, n=2; *4/*4, n=1; *1/*5, n=6 (Subject B11 excluded from further evaluation, thus
*1/*5, n=5); *5/*5, n=2; *1/*6, n=8; *6/*6, n=3 and *1/*7, n=2. For these genotypes mean urinary excretion up to 24 hours postdose was 0.72–, 1.94–, 1.07–, 0.63–, 0.82–, 1.64–, and 0.63-fold compared to homozygote carriers of the wild type allele. Four volunteers could not be assigned clearly to one of the genotypes examined.

For studies B and D, a wash out phase of 2 weeks between both study periods resulted in a considerable carry-over of nirvanol into the second period; therefore, intraindividual variability of nirvanol excretion could only be calculated for study C. Figure VI shows that intraindividual variability for cumulative urine collections declined with increasing collection time reaching 27%, 23%, 22%, 22% and 19% for collection periods from mephenytoin administration up to 24 hours, 36 hours, 48 hours, 94 hours and 166 hours thereafter. On the basis of these intraindividual coefficients of variation, sample sizes of 28, 24, 24, 20 or 14 individuals were calculated to be required for cross-over studies to assess the effect of CYP2B6 phenotype, if collection periods of 0-24, 0-36, 0-48, 0-94 or up to 166 hours, respectively, are used.
DISCUSSION

Our results indicate that the quantification of 4′-hydroxymephenytoin with previous deglucuronidation of samples in a cumulated urine collection 0-12 h after administration of 50 mg mephenytoin is the most suitable mephenytoin-based phenotyping metric for CYP2C19. Under these conditions, an intraindividual coefficient of variation as low as 7% and a significant relationship between CYP2C19 genotype and phenotyping metric was seen. A small sample size of n=6 would be sufficient to assess a potential inhibitory or inductive effect of a drug on CYP2C19 activity, if this phenotyping metric is used in a crossover study. In contrast, mephenytoin was not useful to assess CYP2B6 activity in vivo.

4′-hydroxymephenytoin metrics for CYP2C19 activity. Characteristics of a probe drug for metabolic phenotyping should include good tolerability and availability, lack of drug-drug interaction with other probes as well as high sensitivity and reproducibility. The use of mephenytoin as a phenotyping probe for CYP2C19 activity in man meets most of these criteria. Among the advantages of mephenytoin, a high reproducibility between two or more study periods (Table IV), a significant gene-dose relationship, and a validated lack of drug-drug interaction between mephenytoin and numerous other phenotyping probes, including the other components of the cocktails used here, have to be appreciated. However, also a number of reservations have been expressed against mephenytoin. Severe sedation after administration of 100 mg mephenytoin has been reported in poor metabolizers and in subjects with low body mass index. Additionally, the urinary recovery of 4′-hydroxymephenytoin is, as any urine-based metric, susceptible to incomplete or deficient urine collection. Finally, mephenytoin has lost its clinical relevance and is no longer commercially available in the United States and most European countries. Nevertheless, for CYP2C19 phenotyping purposes, if available mephenytoin seems to date to be the best choice.
In comparison to the S/R ratio the application of the urinary recovery of 4′- hydroxymephenytoin appears to be favorable as the reliability of the S/R ratio is compromised by the presence of a pH and storage labile (S)-mephenytoin cysteine conjugate which leads to an increased risk of false phenotype classification by 1.5%.\textsuperscript{23, 24, 38}; such problems do not occur with 4′-OH-mephenytoin\textsuperscript{39}.

However, no systematic efforts concerning the optimization of 4′-hydroxymephenytoin recovery in urine as a CYP2C19 metric have been reported since this metric was introduced more than twenty years ago.\textsuperscript{22} Only few studies with urinary collection intervals deviating from the “traditional” 8 hours were conducted.\textsuperscript{33, 39} Therefore, we tested various urinary collection periods of different duration to assess an optimal collection interval.

To facilitate the analytical sample processing method we firstly examined whether the small fraction of free 4′-hydroxymephenytoin present in untreated urine would satisfactorily reflect individual CYP2C19 activity. But, in spite of the applied sensitive LC-MS/MS assay our investigations clearly support the demand for the deglucuronidation of urine before analysis since the omission of β-glucuronidase pre-treatment resulted in an overall loss of reproducibility and of phenotype-genotype relationship. Hence, the well recognized intra- and interindividual variability of UDP-glucuronosyltransferase activity seems to be an important confounder.\textsuperscript{40}

Urinary recovery of 4′-hydroxymephenytoin allowed a clear identification of a poor metabolizer phenotype in all fractioned and cumulative collection periods. The quantification of 4′-hydroxymephenytoin in cumulated urine collections of at least 6 hours after dosing was statistically significantly affected by CYP2C19 genotype (table III). Regardless of this fact, the overlapping of the 95% confidence intervals (see Table III) for 4′-hydroxymephenytoin in homozygous and heterozygous extensive metabolizers indicate that no clear-cut differentiation between these genotypes can be made with this phenotyping metric. This
finding is in accordance with published results for 4’-hydroxymephenytoin urinary recovery.\textsuperscript{34}

Furthermore, our investigations demonstrate that optimal reproducibility is reached after 12 hours of cumulative urine collection. A generally smaller susceptibility towards minor sampling errors in longer collection intervals as well as a minor interference of 4’-hydroxymephenytoin excretion with mephenytoin absorption and distribution are likely reasons. In consequence, a lower sample size of n=6 is needed when urine is collected during 12 h in comparison to a sample size of n=8 when the “traditional” 8 h sampling interval is chosen. There is remarkable concordance between literature data and our results (Table IV). Hence, an intraindividual coefficient of variation of below 10 % appears to be consistent for 4’-hydroxymephenytoin urinary recovery for both the 100 mg and the 50 mg mephenytoin dose.

We compared our results to published data on other established CYP2C19 metrics. In analogy to our results, a clear-cut discrimination between CYP2C19*1/*1 homozygote and *1/*2 heterozygote extensive metabolizers was achieved neither with the S/R-Ratio of mephenytoin\textsuperscript{33, 41} nor with the omeprazole metabolic ratio.\textsuperscript{42, 43} Similar finding for several metrics indicate that although homozygous extensive metabolizers may have a slightly higher CYP2C19 activity than heterozygous extensive metabolizers, this difference is probably not relevant for any drug metabolized by CYP2C19. As presented in Table IV, urinary recovery of 4’-hydroxymephenytoin yields the lowest intraindividual coefficients of variation throughout all phenotyping metrics. This was not achieved by a generally low variability of this metric (as compared to metabolic ratios); indeed, intersubject variability remained 3-fold higher for the 0-12 hours sampling period. The principal reason for this superior performance seems to be caused by the analytical assay. Mass spectrometric quantification of 4’-hydroxymephenytoin following deglucuronidation takes place at concentrations that are more
than 200 times above the lower limit of quantification. In contrast, many of the samples used for the determination of omeprazole metabolic ratio and the S/R ratio of mephenytoin, respectively, exhibit concentrations close to or below the lower limit of quantification of the HPLC-based analytical method used in the respective studies.

In conclusion, we propose to carry out CYP2C19 phenotyping with a low dose of 50 mg mephenytoin and cumulative urinary collection of 12 hours, instead of 8 hours, combined with previous deglucuronidation and LC-MS/MS detection of 4′-hydroxymephenytoin.

**Nirvanol metrics for CYP2B6 activity.** Although (S)-nirvanol formation from (S)-mephenytoin has been proved by in vitro studies, respective parameters are not established as an in vivo phenotyping metric. Currently, in the only study available, the autoinduction by artemisinin has tentatively been assessed using the (S)-nirvanol to (S)-mephenytoin ratio. In the present study, we carried out only a limited evaluation of the suitability of nirvanol metrics to estimate CYP2B6 activity. Because of the diversity of the CYP2B6 gene, much higher sample sizes would be required to assess effects of CYP2B6 genotypes on nirvanol excretion. With this limitation, indeed the high urinary excretion of nirvanol of the single homozygous carrier of the CYP2B6*4 allele is in accordance with previous findings for CYP2B6 mediated bupropion hydroxylation, and the low excretion rates for carriers of the *5 or *7 allele correspond to in vitro findings. However, the relative high nirvanol excretion of homozygote carriers of the *6 allele does not reflect previous findings in vivo. For those subjects a lower CYP2B6 mediated clearance of efavirenz and methadone was reported.

Nevertheless, our investigations clearly show that mephenytoin is not optimal to phenotype for CYP2B6 activity, at least for cross-over studies. First of all, adequate intraindividual variability with coefficients of variation of 23 % resulting in a samples size of 24 subjects were achieved not earlier than 36 hours after cocktail administration. While cumulative...
urinary collection of 36 hours is still feasible, a wash-out phase of more than six weeks between two consecutive study periods seems necessary to rule out carry-over of nirvanol. Phenotyping under such premises is not practicable. Besides, in addition to CYP2B6 as low affinity/high capacity component of (S)-mephenytoin demethylation the influence of CYP2C9 as high affinity/low capacity component cannot be dismissed especially at low plasma concentrations that occur after a single phenotyping dosage. For this reasons, it seems more reasonable to validate other metrics such as buproprion hydroxylation as a phenotyping tool for CYP2B6 in vivo as it has been proposed.

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**LEGEND OF FIGURES**

**Figure I:** Supposed stereoselective metabolism of mephenytoin (MEP) to nirvanol (NIR) and 4´-hydroxymephenytoin (4OHM). Phase I enzymes mediating the metabolism of (S)-mephenytoin are indicated; those for (R)-mephenytoin are unknown in vivo.\textsuperscript{16-20}

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**Figure V:** Intraindividual variability of nirvanol urinary recovery for fractioned and cumulative collection of urine in study C (n=12). Data points at midpoint sampling time of each collection period; duration of collection periods indicated in the lower part of the figure. The figure includes extrapolated collection intervals 48 - 86 hours and 94 - 158 hour postdose.
### Table I. Demographic characteristic and urinary collection periods of all studies

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of subjects</th>
<th>Age* (years)</th>
<th>Height* [cm]</th>
<th>Body weight* [kg]</th>
<th>Urinary collection periods [h]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10</td>
<td>31 (25-40)</td>
<td>185 (177-199)</td>
<td>85 (74-98)</td>
<td>Predose, 0-2, 2-4, 4-6, 6-8, 8-12, 12-24</td>
</tr>
<tr>
<td>B</td>
<td>16</td>
<td>29 (23-42)</td>
<td>182 (171-195)</td>
<td>78 (71-101)</td>
<td>Predose, 0-2, 2-4, 4-6, 6-8, 8-12, 12-16, 16-24</td>
</tr>
<tr>
<td>C</td>
<td>12 (11)**</td>
<td>36 (24-49)</td>
<td>181 (172-198)</td>
<td>80 (60-103)</td>
<td>Predose, 0-4, 4-8, 8-12, 12-16, 16-24, 24-36, 36-48, 86-94, 158-166</td>
</tr>
<tr>
<td>D</td>
<td>16 (15)**</td>
<td>29 (21-43)</td>
<td>182 (173-194)</td>
<td>80 (66-99)</td>
<td>Predose, 0-6, 6-12</td>
</tr>
</tbody>
</table>

* Mean values (range) of pre-study examinations  
** Only 52 different subjects took part in all studies. Subjects B09 and B11 are identical with subjects D16 and C10, respectively.
### Table II. Other phenotyping drugs coadministered with mephenytoin in studies A to D

<table>
<thead>
<tr>
<th>Phenotyping drug</th>
<th>Study A</th>
<th>Study B</th>
<th>Study C</th>
<th>Study D</th>
</tr>
</thead>
<tbody>
<tr>
<td>amoxicillin</td>
<td>100 mg</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>caffeine</td>
<td>150 mg</td>
<td>150 mg</td>
<td>150 mg</td>
<td>150 mg</td>
</tr>
<tr>
<td>chlorzoxazone</td>
<td>250 mg</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>dextromethorphan</td>
<td>30 mg</td>
<td>30 mg</td>
<td>30 mg</td>
<td>30 mg</td>
</tr>
<tr>
<td>digoxin</td>
<td>0.5 mg</td>
<td>-</td>
<td>0.5 mg</td>
<td>-</td>
</tr>
<tr>
<td>midazolam p.o.</td>
<td>2 mg*</td>
<td>2 mg**</td>
<td>2 mg**</td>
<td>2 mg**</td>
</tr>
<tr>
<td>midazolam i.v.</td>
<td>1 mg*</td>
<td>1 mg**</td>
<td>1 mg**</td>
<td>1 mg**</td>
</tr>
<tr>
<td>nicotine</td>
<td>0.5 mg</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>tolbutamide</td>
<td>125 mg</td>
<td>125 mg</td>
<td>125 mg</td>
<td>125 mg</td>
</tr>
</tbody>
</table>

* Study A: 1 mg of midazolam i.v. with all other phenotyping drugs, followed by 2 mg of oral midazolam 90 minutes later
** Study B, C and D: 2 mg of oral midazolam with all other phenotyping drugs, followed by 1 mg midazolam i.v. 240 minutes later
Table III. Urinary recovery of 4'-hydroxymephenytoin relative to the mephenytoin dose for individual genotypes groups. Cumulative collection intervals were used, and samples were pretreated with β-glucuronidase.

<table>
<thead>
<tr>
<th>Collection interval</th>
<th>Studies</th>
<th>No. of subjects*</th>
<th>*1/*1 urinary recovery [%]</th>
<th>95% CI</th>
<th>*1/*2 urinary recovery [%]</th>
<th>95% CI</th>
<th>*2/*2 urinary recovery [%]</th>
<th>95% CI</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-2 h</td>
<td>A/B</td>
<td>26 (17/9/1)</td>
<td>3.1±2.1</td>
<td>2.1-4.2</td>
<td>2.6±2.0</td>
<td>0.9-4.2</td>
<td>0.02</td>
<td>-</td>
<td>0.199</td>
</tr>
<tr>
<td>0-4 h</td>
<td>A/B/C</td>
<td>37 (24/12/1)</td>
<td>14.1±5.7</td>
<td>11.7-16.5</td>
<td>12.9±5.0</td>
<td>9.7-16.1</td>
<td>0.06</td>
<td>-</td>
<td>0.189</td>
</tr>
<tr>
<td>0-6 h</td>
<td>A/B</td>
<td>26 (17/9/1)</td>
<td>20.6±3.8</td>
<td>18.7-22.6</td>
<td>17.1±3.4</td>
<td>14.3-20.0</td>
<td>0.10</td>
<td>-</td>
<td>0.029</td>
</tr>
<tr>
<td>0-8 h</td>
<td>A/B/C</td>
<td>37** (24/12/1)</td>
<td>26.3±3.8</td>
<td>24.7-27.9</td>
<td>23.3±4.6</td>
<td>20.4-26.2</td>
<td>0.15</td>
<td>-</td>
<td>0.040</td>
</tr>
<tr>
<td>0-12 h</td>
<td>A/B/C</td>
<td>37 (24/12/1)</td>
<td>29.7±3.7</td>
<td>28.2-31.3</td>
<td>26.9±4.2</td>
<td>24.2-29.6</td>
<td>0.24</td>
<td>-</td>
<td>0.048</td>
</tr>
<tr>
<td>0-16 h</td>
<td>B</td>
<td>16 (12/3/1)</td>
<td>31.8±3.0</td>
<td>29.9-33.7</td>
<td>25.6±2.6</td>
<td>19.2-32.0</td>
<td>0.32</td>
<td>-</td>
<td>0.025</td>
</tr>
<tr>
<td>0-24 h</td>
<td>A/B</td>
<td>26 (12/3/1)</td>
<td>31.9±3.7</td>
<td>30.0-33.9</td>
<td>27.9±3.3</td>
<td>25.1-30.7</td>
<td>0.49</td>
<td>-</td>
<td>0.028</td>
</tr>
</tbody>
</table>

* Numbers in brackets: Number of subjects subdivided into genotype groups (CYP2C19*1/*1 / CYP2C19*1/*2 / CYP2C19*2/*2)
Table IV. Intraindividual variability for different CYP2C19 phenotyping metrics

<table>
<thead>
<tr>
<th>author</th>
<th>metric</th>
<th>dose of probe drug [mg]</th>
<th>number of subjects</th>
<th>number of study periods</th>
<th>intraindividual CV [%]</th>
<th>calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Christensen et al.13</td>
<td>OMEP-MR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20</td>
<td>24</td>
<td>2</td>
<td>25</td>
<td>“coefficient of variation”</td>
</tr>
<tr>
<td>Kim et al.49</td>
<td>OMEP-MR&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30&lt;sup&gt;g&lt;/sup&gt;/40&lt;sup&gt;h&lt;/sup&gt;</td>
<td>24</td>
<td>6</td>
<td>18.5 6.3-51.3</td>
<td>“median CV%” and range</td>
</tr>
<tr>
<td>Balian et al.50</td>
<td>OMEP-MR&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20</td>
<td>5</td>
<td>3</td>
<td>5.8-64.3</td>
<td>“range of variability after repeated determinations”</td>
</tr>
<tr>
<td>Yin et al.42</td>
<td>OMEP-MR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>40</td>
<td>27</td>
<td>3</td>
<td>18.2 4.5-37.7</td>
<td>“mean coefficient of variation” and range</td>
</tr>
<tr>
<td>Tamminga et al.51</td>
<td>S/R&lt;sup&gt;d&lt;/sup&gt;</td>
<td>100</td>
<td>129</td>
<td>2</td>
<td>28</td>
<td>“mean of all individual coefficients of variation”</td>
</tr>
<tr>
<td>Frye et al.36</td>
<td>4OHM recovery&lt;sup&gt;g&lt;/sup&gt;</td>
<td>100</td>
<td>14</td>
<td>3</td>
<td>7.4</td>
<td>“median within-subject coefficient of variation”</td>
</tr>
<tr>
<td>Zhu et al.35</td>
<td>4OHM recovery&lt;sup&gt;g&lt;/sup&gt;</td>
<td>100</td>
<td>14</td>
<td>4</td>
<td>10.3</td>
<td>“median within-subject coefficient of variation”</td>
</tr>
<tr>
<td>This study</td>
<td>4OHM recovery&lt;sup&gt;g&lt;/sup&gt;</td>
<td>50</td>
<td>40 (27)</td>
<td>2</td>
<td>7 (11)</td>
<td>intra-subject variability calculated by ANOVA for log-transformed urinary recovery data</td>
</tr>
</tbody>
</table>

OMEP MR = omeprazole metabolic ratio, S/R = mephenytoin S/R ratio, 4OHM = urinary recovery of 4’-hydroxymephenytoin

a: calculated as plasma ratio omeprazole/5’-hydroxyomeprazole 3 hours postdose
b: calculated as plasma ratio omeprazole/5’-hydroxyomeprazole 2 hours postdose
c: calculated as plasma ratio AUC(omeprazole)/AUC(5’-hydroxyomeprazole) up to 12 hours postdose
d: calculated as concentration ratio (S)-mephenytoin/(R)-mephenytoin in 8h urine
e: calculated as total urinary recovery of 4’-hydroxymephenytoin in 8h urine
f: calculated as molar urinary recovery of 4’-hydroxymephenytoin in 12h urine (data for 8 hour collection period in brackets)
g: body weight 45-66 kg
h: body weight 67-90 kg
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