Relationship between mephenytoin oxidation polymorphism and phenytoin, methylphenytoin and phenobarbitone hydroxylation assessed in a phenotyped panel of healthy subjects

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1 In a phenotyped panel of healthy subjects correlations were studied between the oxidation of mephenytoin, phenytoin, methylphenytoin and phenobarbitone, with respect to the formation of their 4-hydroxy metabolites (OH-).

2 On different occasions phenotyped extensive metabolizers (EM; $n = 16$) and poor metabolizers (PM; $n = 4$) of mephenytoin received phenytoin (100 mg), methylphenytoin (100 mg) and phenobarbitone (50 mg) and urine was collected up to 24 h. The excreted 4-hydroxy metabolites of all compounds were measured by h.p.l.c.

3 Urinary recovery of OH-phenytoin was $31.0 \pm 11.7\%$, of OH-methylphenytoin $3.4 \pm 2.7\%$ and of OH-phenobarbitone $1.4 \pm 1.2\%$. No correlation was found between the recovery of OH-mephenytoin and OH-phenytoin. A subject who produced virtually no OH-phenytoin was an EM of mephenytoin, confirming a dissociation of mephenytoin polymorphism and phenytoin hydroxylation.

4 The correlation coefficient for OH-mephenytoin and OH-methylphenytoin recovery was 0.71 (Spearman rank, $P = 0.002$). The PMs of mephenytoin excreted the least amount of OH-methylphenytoin, suggesting a cosegregation of the 4-hydroxylation pathways. No correlation was found between the urinary recovery of OH-phenobarbitone and that of the other 4-hydroxy metabolites.

Keywords mephenytoin polymorphism phenytoin methylphenytoin and phenobarbitone hydroxylation cytochrome P-450

Introduction

The oxidation of mephenytoin is subject to genetic polymorphism in man (Küpfer *et al.*, 1979; Küpfer & Preisig, 1984). The S-enantiomer of mephenytoin is almost exclusively hydroxylated and rapidly eliminated into the urine after conjugation whereas the R-enantiomer is demethylated at a much slower rate (Küpfer *et al.*, 1980, 1982; Wedlund *et al.*, 1985). The polymorphism is confined to S-mephenytoin (Küpfer

et al., 1980, 1981; Wedlund *et al.*, 1985) and the poor metabolizer (PM) incidence is approximately 2–5% in Caucasian populations (Inaba *et al.*, 1984; Küpfer & Preisig, 1984). In a recently phenotyped Dutch population it was 2.3% (Schellens *et al.*, 1986), whereas it is considerably higher in Japanese (23%) (Jurima *et al.*, 1985). The latter population is therefore potentially more at risk of toxicity when treated with

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mephenytoin and other compounds which are predominantly oxidized by the same enzyme(s). At least two similar forms of human cytochrome P-450 seem to be involved in S-mephenytoin 4-hydroxylation and the molecular basis of the polymorphism has been partly elucidated (Guengerich *et al.*, 1988; Meier *et al.*, 1985; Shimada *et al.*, 1986). Polymorphisms in the oxidation of sparteine/debrisoquine and mephenytoin have been found to be independent entities (Inaba *et al.*, 1984; Jurima *et al.*, 1984). In contrast to the relatively large number of drugs the oxidation of which cosegregates with sparteine/debrisoquine only that of 5-phenyl-5-ethylhydantoin (Nirvanol) and mephobarbitone are known to cosegregate with mephenytoin oxidation *in vivo* (Jacqz *et al.*, 1986; Kpfer & Branch, 1985; Kpfer *et al.*, 1984). The oxidation of Nirvanol and mephobarbitone is stereoselective and the polymorphism is, like that of mephenytoin, confined to hydroxylation of one of the enantiomers (Jacqz *et al.*, 1986; Kpfer & Branch, 1985; Kpfer *et al.*, 1984). Results of a study by Bertilsson *et al.* (1989) indicate that the oxidation of diazepam might be related to the hydroxylation of S-mephenytoin. Also the metabolism of the antimalarial drug proguanil to its principal metabolite cycloguanil is suggested to cosegregate with that of the S-mephenytoin (Ward *et al.*, 1989).

It has also been suggested that the oxidation of phenytoin is subject to polymorphism in man (Kutt *et al.*, 1964; Vasko *et al.*, 1980; Vermeij *et al.*, 1985), the affected route of oxidation being the 4-hydroxylation pathway (Hvidberg & Dam, 1976; Richens, 1979). This pathway is stereoselective for the formation of the S-enantiomer and seems to be preserved in poor metabolizers of mephenytoin (Fritz *et al.*, 1987). The urinary recovery of OH-phenytoin and/or the ratio of phenytoin/OH-phenytoin in plasma have been used to define oxidation status (Vasko *et al.*, 1980; de Wolff *et al.*, 1983). Methylphenytoin closely resembles phenytoin in structure, but its oxidation is not known to be polymorphic. Phenobarbitone has a close structural relationship with mephobarbitone, but is eliminated at a much slower rate (Eadie, 1976). Phenytoin, methylphenytoin and phenobarbitone all lack a centre of asymmetry (Figure 1).

The aim of the present study was to establish in a panel of healthy subjects, phenotyped for mephenytoin and sparteine, whether there exist any correlations between the 4-hydroxylation pathways of mephenytoin, phenytoin, methylphenytoin and phenobarbitone and whether mephenytoin polymorphism and phenytoin hydroxylation cosegregate.

Figure 1 Chemical structures of mephenytoin (1), phenobarbitone (2), phenytoin (3) and methylphenytoin (4). * Marks the chiral centre of mephenytoin.

Methods

Compounds

Mephenytoin was a gift from Sandoz (Uden, The Netherlands). Methylphenytoin was kindly supplied by Katwijk Farma (Katwijk, The Netherlands). OH-mephenytoin was a gift from Dr A. Kpfer (Bern, Switzerland) and OH-phenytoin was obtained from Aldrich (Milwaukee, Wis., USA). OH-methylphenytoin was synthesized according to Henze & Isbell

(1954) and Wedlund *et al.* (1984) with minor modifications. Thus, alkylation was stopped after 1 h of incubation at room temperature in order to prevent formation of further methylated compounds. Limpet acetone powder (LAP) was obtained from Sigma (St Louis, MO, USA).

Human experiments

The protocol for the study was approved by the Committee for Medical Ethics of the University Hospital of Leiden. Twenty volunteers (14 males, six females) participated after having given their written informed consent. All were healthy according to clinical history, physical examination and routine blood analysis, especially with respect to renal function. Except for three females, who were using contraceptive steroids, all participants were non-medicated. Their mean age was 24 ± 3 years and three were moderate to heavy smokers. Of the volunteers, four had been phenotyped as PMs of mephenytoin and 16 as extensive metabolizers (EM) (Schellens *et al.*, 1986). All PMs of mephenytoin were EMs of sparteine, whereas there were two PMs of sparteine among the EMs for mephenytoin. The PM group comprised three males and one female, who was using contraceptive steroids, and none was smoking. Phenotyping with mephenytoin was done by measuring the amount of OH-mephenytoin excreted in the 0–8 h urine after administration of 100 mg racemic mephenytoin. The recovery of OH-mephenytoin in all PMs was less than 0.5% and in EMs it was $28.4 \pm 6.1\%$. The reproducibility of the phenotype was evaluated in a separate study (Schellens *et al.*, 1989). Phenotyping with sparteine was done by estimating the log metabolic ratio of sparteine and dehydrospartheine in a 0–8 h urine collection after administration of 25 mg sparteine sulphate. Subjects with a log metabolic ratio greater than 2 were characterized as PMs (Eichelbaum *et al.*, 1979, 1986; Schellens *et al.*, 1986).

Prior to the present study all PMs had been characterized for their rate of antipyrine (AP) oxidation and metabolite formation. They were given 250 mg AP in aqueous solution after an overnight fast and plasma samples were collected up to 32 h and urine up to 48 h. The clearance of AP and the formation clearance of its metabolites were calculated according to Breimer *et al.* (1984). All PMs had normal values of AP clearance ($37.0\text{--}56.9 \text{ ml min}^{-1}$) and formation clearances of the main metabolites of AP (3-hydroxymethylantipyrine, norantipyrine and 4-hydroxyantipyrine).

In the present study all participants received randomly, at intervals of at least 8 days, 100 mg

phenytoin (tablet as sodium phenytoin), 100 mg methylphenytoin (capsule) and 50 mg phenobarbitone (tablet). The drugs were taken after an overnight fast and at 2 h after drug intake the subjects were allowed food and beverages. All urine was collected in two fractions (0–8, 8–24 h).

Analytical procedures

All drugs and metabolites were assayed by h.p.l.c. The equipment (Waters Ass., Milford, MA, USA) comprised a M-45 pumping device, a WISP 710B automatic sample injector and a u.v. detector (model Lambda Max 480) set at 214 nm. A Novopack 8C18 cartridge column (8 mm internal diameter, C18 coating material) was used and all data were processed with a Shimadzu CR3A reporting integrator (Kyoto, Japan).

OH-mephenytoin was measured according to the method of Küpfer *et al.* (1981), with modifications as described by Schellens *et al.* (1989). The eluent for the analysis of OH-mephenytoin was a distilled water/methanol (65/35%) mixture adjusted to pH 5.2 by addition of hydrochloric acid. For OH-phenobarbitone, OH-phenytoin and OH-methylphenytoin acetonitrile was used as a moderator (OH-phenobarbitone: 25%, OH-phenytoin and OH-methylphenytoin: 30%).

To deconjugate metabolites limpet acetone powder (10 mg) was added to 1 ml urine and incubated at 37° C. Deconjugation was found to be complete for OH-mephenytoin after 18 h, according to Küpfer *et al.* (1981), and for the other compounds it was complete after 3 h. OH-phenobarbitone was also measured without prior deconjugation. Extraction was carried out with diethylether (5 ml), after addition of aprobarbitone as an internal standard.

The synthetic OH-mephenytoin contained some impurities. Therefore, this compound was used solely for the identification of OH-mephenytoin in urine calculations being done using the calibration curve for OH-phenytoin and assuming identical u.v. extinction. For the identification of OH-methylphenytoin chromatograms from blank urine and from urine samples from each volunteer after ingestion of methylphenytoin were compared with chromatograms from samples spiked with the reference compound. Also, the reference compound was added in different concentrations to urine samples containing OH-methylphenytoin produced after ingestion of methylphenytoin. A single peak was present at the retention time of the reference compound.

Table 1 Urinary recoveries (% dose) of 4-hydroxy metabolites after administration of mephenytoin (100 mg), phenytoin (100 mg), methylphenytoin (100 mg) and phenobarbitone (50 mg)

	EM (n = 16)	PM (n = 4)
OH-mephenytoin	28.4 ± 6.1	0.1 ± 0.1*
OH-phenytoin	29.4 ± 12.5	37.3 ± 4.2
OH-methylphenytoin	4.2 ± 2.4	0.5 ± 0.4*
OH-phenobarbitone	1.6 ± 1.3	0.8 ± 0.2

* $P \leq 0.001$ Wilcoxon rank sum test.

EM and PM refer to phenotypes for mephenytoin hydroxylation. Data are mean and s.d. The urine collection time for OH-mephenytoin was 0–8 h and for the other drugs it was 0–24 h.

Data analysis

The urinary recoveries of mephenytoin, phenytoin, methylphenytoin, phenobarbitone and their metabolites are expressed as a % of dose. It was assumed that absorption of all drugs was complete. Differences between the EMs and PMs of mephenytoin in excretion of the compounds were assessed by Wilcoxon's rank sum test. Correlations between the excretion of the different compounds were assessed using Spearman rank correlation coefficients.

Results

Drug analysis

All calibration graphs for the measurement of metabolites in urine were linear in the concentration range studied and assay limits were approximately 50 ng ml⁻¹. The coefficients of variation of the assays were less than 5%.

Excretion of 4-hydroxy metabolites in urine

All excretion data for OH-phenytoin, OH-methylphenytoin and OH-phenobarbitone refer to a 0–24 h urine collection period. The recovery of OH-phenytoin in EMs of mephenytoin

ranged from 0.7 to 46.2% and in PMs from 33.2 to 42.7%. The recovery of OH-methylphenytoin in EMs of mephenytoin ranged from 1.5 to 9.3% and in PMs from 0.08 to 0.8%. The recovery of OH-phenobarbitone in EMs of mephenytoin ranged from 0.6% to 4.5% and in PMs of mephenytoin from 0.7% to 0.9%. Negligible amounts of phenytoin and methylphenytoin were excreted unchanged, in contrast to phenobarbitone, the recovery of which was 1.6 to 8.2% (EMs + PMs of mephenytoin). The mean excretion data are summarized in Table 1 and the individual data are shown in Figure 2. Only in the case of methylphenytoin did there appear to be a marked dissociation in excretion of the 4-hydroxy metabolite in EMs and PMs of mephenytoin. The recovery of OH-methylphenytoin in PMs of mephenytoin was significantly different from that in EMs (Wilcoxon, $P = 0.001$). The Spearman rank correlation coefficient for OH-mephenytoin and OH-methylphenytoin recovery was 0.71 ($P = 0.002$). No correlation was found between the recoveries of OH-mephenytoin, OH-phenytoin or OH-phenobarbitone, nor between those of OH-methylphenytoin, OH-phenytoin or OH-phenobarbitone (Table 2). In one male subject no OH-phenytoin was detectable in the urine. On a second occasion this subject was redosed with 200 mg phenytoin, urine was collected up to 24 h and a plasma sample was taken at 6 h. On this occasion the urinary recovery of OH-phenytoin was 0.7%, but it was not found in the plasma. Therefore, this subject may be a poor metabolizer of phenytoin, although a pedigree study would be necessary to confirm this. He was an EM of mephenytoin and a relatively rapid metabolizer of methylphenytoin (Figure 2).

Discussion

The primary aim of the present study was to establish any correlations between the 4-hydroxylation of mephenytoin, phenytoin, methylphenytoin and phenobarbitone. A phenotyped panel approach was applied, which

Table 2 Spearman rank correlation coefficients between urinary recoveries (% dose) of the 4-hydroxy metabolites of mephenytoin, phenytoin, methylphenytoin and phenobarbitone

	OH-mephenytoin	OH-phenytoin	OH-methylphenytoin
OH-phenytoin	-0.02		
OH-methylphenytoin	0.71*	0.14	
OH-phenobarbitone	0.14	0.17	0.25

* $P = 0.002$.

Figure 2 Urinary recoveries (% of dose) of the 4-hydroxy metabolites of mephenytoin (MP), phenytoin (PH), methylphenytoin (MPH) and phenobarbitone (PB). The urine collection time was 0–8 h for mephenytoin and 0–24 h for the other compounds. The open circles (○) denote the extensive metabolizers of mephenytoin, the asterisks (*) denote the poor metabolizers of mephenytoin. The arrow (▲) denotes the extensive metabolizer of mephenytoin showing a negligible urinary recovery of OH-phenytoin.

has the advantage that overlap in substrate selectivity of relatively specific isoenzymes can be established. EMs and PMs can be characterized relatively simply on the basis of urinary excretion data (Küpfer & Branch, 1985). However, some drawbacks have to be appreciated. Thus, the formation clearance to metabolites is not calculated directly because plasma data are not collected.

No correlation was found between the recoveries of OH-mephenytoin and OH-phenytoin (Table 2). Also one subject with negligible formation of OH-phenytoin was characterized as an EM of mephenytoin. Thus it is unlikely that the hydroxylation of the two compounds cosegregates. This result is not consistent with an *in vitro* study using purified cytochrome P-450 isoenzymes with high affinity for mephenytoin 4-hydroxylation (designated P-450_{MP-1} and P-450_{MP-2}) which were found to catalyze both mephenytoin and phenytoin hydroxylation

(Shimada *et al.*, 1986). However it is in agreement with *in vivo* results obtained by Fritz *et al.* (1987).

The formation of OH-methylphenytoin and OH-phenytoin also seem to be unrelated (Table 2). In contrast, the correlation coefficient for the recoveries of OH-mephenytoin and OH-methylphenytoin was 0.71 ($P = 0.002$), all PMs of mephenytoin also being the lowest excretors of OH-methylphenytoin (Table 1, Figure 2). The urinary recovery of OH-methylphenytoin was much lower than that of OH-mephenytoin over the same time interval and the magnitude of the difference between EMs and PMs was less than in the case of mephenytoin. However, the results in the present study suggest that a cosegregation of both 4-hydroxylation pathways and formation of OH-mephenytoin and OH-methylphenytoin seem to be mediated, at least partly, by the same isoenzyme(s). Interestingly, a cosegregation between the hydroxylations of methylphenytoin and mephenytoin was not expected based on the *in vitro* experiments with P-450_{MP-1} and P-450_{MP-2}, in which these isoenzymes did not have catalytic activity for methylphenytoin hydroxylation (Shimada *et al.*, 1986). A relatively high K_m value of these isoenzymes for the hydroxylation of methylphenytoin may contribute to the observed differences in outcome of the *in vivo* and *in vitro* studies.

No correlation was found between the urinary recovery of OH-phenobarbitone and those of any of the other 4-hydroxylation products (Table 2). Hence different isoenzymes seem to be involved in the oxidation of phenobarbitone.

The hydroxylation of mephobarbitone, Nirvanol, diazepam and of methylphenytoin seems to cosegregate with mephenytoin polymorphism. Also the formation of cycloguanil from proguanil seems to be mediated by the isoenzyme(s) responsible for polymorphic mephenytoin hydroxylation (Ward *et al.*, 1989). Mephobarbitone, Nirvanol, methylphenytoin and phenytoin have the closest structural resemblance to mephenytoin. Mephenytoin, mephobarbitone and methylphenytoin have a *N*-methyl group in proximity to the aromatic ring. However, this *N*-methyl group is not a prerequisite with regard to cosegregation with mephenytoin polymorphism, because Nirvanol does not possess one. Diazepam does have a *N*-methyl group, but the hydroxylation is not aromatic as in the case of the other substrates. The *N*-methyl group appears to be of some importance, because methylphenytoin does and phenytoin does not seem to cosegregate with mephenytoin polymorphism.

Among the 20 participants of this study two PMs of sparteine were identified. Both belonged to the EM group of mephenytoin, thus confirming the independent character of the polymorphisms. The formation clearance of AP metabolites were all in the normal range, indicating that AP metabolism is not predictive of the metabolism of mephenytoin and allied substrates.

Based on the results of the present study it seems likely that the oxidation of mephenytoin and methylphenytoin cosegregates with respect

to their 4-hydroxylation pathways. Polymorphic oxidation of mephenytoin and formation of the major metabolite of phenytoin are under different genetic control. Isoenzymes responsible for the formation of OH-phenobarbitone seem to be independent of those responsible for the formation of OH-mephenytoin, OH-phenytoin and OH-methylphenytoin. AP oxidation and metabolite formation is unimpaired in PMs of mephenytoin.

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