Stereoselective 4\(\infty\)-hydroxylation of phenytoin: relationship to (S)-mephenytoin polymorphism in Japanese


1 Division of Pharmaceutical Sciences, Kyushu University, 2 Kyushu Pharmacology Research Clinic, 3 SRL Inc., 4 Department of Clinical Pharmacology and Therapeutics, Oita Medical University, and 5 Department of Psychiatry, Faculty of Medicine, Kyushu University, Japan

Aim: The aim of this study was to clarify whether phenytoin (PHT) stereoselective hydroxylation cosegregates with (S)-mephenytoin phenotype.

Methods: A single dose of PHT (100 mg) was administered orally to six healthy Japanese subjects in whom the genotype and phenotype of CYP2C19 had been determined previously. The urinary excretion profiles of the metabolites of PHT, (R)- and (S)-p-HPPH (5-(4-hydroxyphenyl)-5-phenylhydantoin) up to 36 h postdose were compared between the two groups of poor metabolizers (PMs, \(n=3\)) and extensive metabolizers (EMs, \(n=3\)) with respect to CYP2C19. CYP2C9 genotype was also determined.

Results: All the alleles were found to be wild type (Arg144Tyr358Ile359Gly417) in each subject. The mean value for cumulative urinary excretion of unchanged PHT was not significantly different between the PMs and the EMs. However, recovery of (R)-p-HPPH at 36 h was 3.5-fold lower and that of (S)-p-HPPH 1.3-fold lower in PMs than in EMs. Although the mean urinary excretion values for both metabolites were significantly lower in the PMs than in the EMs, the difference between the two groups was larger for (R)-p-HPPH. A significant negative correlation was observed between the hydroxylation index of omeprazole (the ratio between the serum concentrations of omeprazole and hydroxyomeprazole in blood samples drawn 3 h after drug intake) and the log10 0–12 h urinary recovery of (R)-p-HPPH.

Conclusions: In humans, the 4\(\infty\)-hydroxylation of PHT is highly stereoselective towards formation of the (S)-enantiomer. Thus, (S)-hydroxylation by CYP2C9 might be the major determinant of the disposition of PHT. However, these results support the hypothesis that the stereoselective hydroxylation pathway of PHT to form (R)-p-HPPH cosegregates with the CYP2C19 metabolic polymorphism.

Keywords: phenytoin, stereoselective hydroxylation, CYP2C19, CYP2C9, omeprazole

Introduction

Cytochrome P450 (CYP) 2C19 plays an important role in the metabolism of a number of drugs, such as omeprazole [1], diazepam [2], proguanil [3] and several antidepressants [4]. Phenytoin (PHT) is a prochiral compound with a chemical structure similar to mephenytoin. 5-(4-Hydroxyphenyl)-5-phenylhydantoin (p-HPPH) is the major metabolite of PHT, and 4\(\infty\)-hydroxylation gives rise to two enantiomers, (S)-p-HPPH and (R)-p-HPPH. p-HPPH has not been shown to possess anticonvulsant properties. The pharmacokinetic profile of PHT has been well characterized in humans, with large interindividual variability in plasma drug concentrations being observed. There is evidence that the metabolic disposition of PHT may be determined genetically. Thus, Fritz et al. [5] reported that formation of the (R)-enantiomer of p-HPPH was clearly deficient in some Caucasian subjects, and was associated with the mephenytin oxidation polymorphism. Furthermore, we recently reported that the (R)/(S)-p-HPPH concentration ratio in serum samples appeared to be bimodally distributed. In view of a suspected role for (R)-p-HPPH in the development of gingival hyperplasia, a chronic side effect of long-term PHT therapy [6], genetic variation in the stereoselective 4\(\infty\)-hydroxylation of PHT may have clinical significance. Therefore the aim of present study was to clarify whether PHT stereoselective hydroxylation cosegregates with (S)-mephenytoin phenotype.

Methods

Subjects

Six healthy Japanese volunteers were recruited from among pharmaceutical staff and students. They were considered to be healthy as assessed by their medical histories. Their genotype and phenotype with respect to CYP2C19 were determined in a previous study [7]. The ratios of the concentrations of omeprazole and hydroxyomeprazole in serum samples drawn 3 h after drug intake, and the CYP2C19 phenotype in the three PMs were 6.6, 7.1, and
The urinary excretion profile of unchanged PHT displayed no differences. All subjects were informed both verbally and in writing of the experimental procedure and the purpose of the study. Each subject gave written consent before the study, the protocol of which was approved by the Institutional Review Board of the Clinical Pharmacology Center, Medical Co. Ltd.

Study protocol

The participants came to the clinic after an overnight fast. After emptying the bladder each subject received an oral dose of 100 mg of PHT (Aleviatin, Dainippon Co. Ltd, Osaka, Japan) with 150 ml water. Lunch was served 4 h after the ingestion of the drug. To replace fluid loss each subject drank the same volume of water as the volume of urine voided after the drug ingestion. Tuned urine collections were made at 2 h intervals for the first 4 h, followed by collections at 8, 12, 24 and 36 h. Immediately after the collection of each urine sample, its volume was measured. Aliquots (10 ml) were stored frozen at −80 °C until analysed.

Genotyping of CYP2C9

Venous blood (10 ml) was obtained from all subjects, and deoxyribonucleic acid (DNA) was isolated from peripheral leukocytes. Genotyping procedures for the identification of the mutations were performed by PCR amplification using primers specific for exon 3, exon 7 and exon 8 of CYP2C9 as described by Wang et al. [8]. The PCR products were sequenced with a DNA cycle sequencing kit (Takara, Shiga, Japan) according to the manufacturer’s instructions (DSQ-1, Shimadzu, Kyoto, Japan).

Assay of phenytoin and its two metabolites

The concentrations of PHT, (R)- and (S)-p-HPPH, in urine samples were measured in duplicate by high performance liquid chromatography (h.p.l.c.) according to the methods of Enomoto et al. [9] with minor modifications. 100 mM Phosphate buffer (0.6 ml, pH 6.8) containing allobarbital as an internal standard was added to 0.1–0.5 ml urine. Since p-HPPH is excreted rapidly after glucuronidation, the p-HPPH conjugate was hydrolysed prior to assay. After treatment of p-HPPH glucuronide with 400 U glucurono-l-saltase at 37 °C for 6 h, 1.0 ml of the mixture was poured into an Extrelut-1 column (Merck, Darmstadt, Germany). After 10 min, the column was eluted with 3.0 ml tert-butil methyl ether. The eluate was dried and dissolved in 100 μl methanol, and 20 μl aliquots were injected into the chromatograph. A Shimadzu LC-10AS system (Shimadzu, Kyoto, Japan) equipped with an LC-10AS pump and a u.v. detector (SPD-10A) was employed. The column was LiChroCART Superphil 100 RP-18(e) (12.5 cm × 4 mm) with 4 μm particle diameter (Merck). The mobile phase was a mixture of 11.2 mM β-cyclodextrin in 20 mM KH₂PO₄ and 3% acetonitrile. The column temperature was ambient. The flow rate was 0.85 ml min⁻¹, and the eluate was monitored at 210 nm.

The sensitivity of the PHT assay in the urine samples was 10 ng ml⁻¹, the coefficient of the intra-assay variation was 3.5%, and that of the inter-assay variation was 4.0%. The corresponding values were 10 ng ml⁻¹, 1.9 and 3.5% for the (S)-p-HPPH assay, and 10 ng ml⁻¹, 3.1 and 4.3% for the (R)-p-HPPH assay. Recoveries of PHT, (S)-p-HPPH and (R)-p-HPPH ranged from 90 to 100%.

Statistical analysis

Differences in urine parameters between the EM and PM groups were evaluated statistically using the Student’s t-test. Spearman’s rank correlation (rₛ) was also used where appropriate. All data are expressed as the mean (± s.d.), and a P value of <0.05 was considered significant.

Results

No clinically undesirable signs or symptoms that could be attributed to the administration of PHT were recognizable throughout the study period. All subjects were homozygous for the Arg980, Tyr989, Bcl10 and Gly107 alleles of the CYP2C9 gene. The time courses of the urinary excretion of PHT, (R)- and (S)-p-HPPH in the two phenotypes are shown in Figure 1. Two hours after the dose of PHT, the mean excretion of (R)-p-HPPH was 0.006±0.012% in the PM group, and 0.036±0.009% in the EM group. This interphenotypic difference was observed throughout the urine collection period and was statistically significant (P<0.05) except after 2 h. The mean cumulative excretion of (R)-p-HPPH at 36 h was 0.52±0.19% in the PMs and 1.83±0.52% in the EMs. The urinary excretion profile of (S)-p-HPPH showed significant differences between the two groups similar to those observed with (R)-p-HPPH. The mean cumulative excretion of this metabolite at 12, 24 and 36 h was 12.3±1.6, 28.3±4.9 and 38.3±7.4% in the PMs, and 19.9±4.8, 39.2±4.3 and 49.6±4.4%, respectively, in the EMs (P<0.05). Thus, the interphenotypic differences between (R)- and (S)-p-HPPH increased at the later collection times. The mean value for the cumulative urinary excretion of (R)-p-HPPH at 36 h after drug intake was 3.5-fold lower, whereas that of (S)-p-HPPH was 1.3-fold lower in the PMs than in the EMs. In this study, interphenotypic difference in urine recovery of (R)-p-HPPH at 36 h differed by power of 0.82 (R-p-HPPH) and of 0.72 (S-p-HPPH) at an α of 0.05 (two-tailed test), even though the power is limited by the minimum sample size. The mean urinary PHT/(R)-p-HPPH and PHT/(R)-p-HPPH ratios at 36 h were 0.088±0.002 and 0.65±0.14 in the PMs, and 0.005±0.002 and 0.17±0.10 in the EMs. A significant phenotypic difference (P<0.05) in the PHT/(R)-p-HPPH ratio (0.5±0.3, mean difference ±95% C.I.) but not in the PHT/(S)-p-HPPH ratio was observed. The urinary excretion profile of unchanged PHT displayed no interphenotype differences.

There was a significant negative correlation between the serum concentration ratio of omeprazole and hydroxyomeprazole determined 3 h after drug intake, and the log₁₀ 12 h urinary recovery of (R)-p-HPPH (Figure 2, rₛ=−0.96, P<0.05).
Cumulative urinary excretion-time data of (R), (S)-p-HPPH and unchanged PHT after an oral dose of 100 mg phenytoin administered to three EMs (◆) and three PMs (●) of CYP2C19. The mean differences (95% CI) between PMs and EMs in (R), (S)-p-HPPH and unchanged PHT at each sampling point are also indicated. The data are mean ± s.d. *P<0.05 compared with PMs.

Figure 2 The relationship between the omeprazole hydroxylation index and the log_{10} of the percentage of dose excreted as (a) (S)-p-HPPH and (b) (R)-p-HPPH in the urine samples collected from 0 to 12 h after a p.o. dose of 100 mg phenytoin administered to three EMs (◆) and three PMs (●) of CYP2C19. Individual CYP2C19 genotypes also indicated: m1, CYP2C19*1; m2, CYP2C19*2; wt, normal wild-type allele (CYP2C19*3). *P<0.05.
Discussion

Wide interindividual variability in the 4'-hydroxylation of PHT, the major determinant of plasma drug concentration, has been well documented. Defective hydroxylation of PHT in a patient who developed severe PHT intoxication was reported by Kutt et al. [10], and this was suggested to be an inherited trait [11, 12]. In respect to the debrisoquine polymorphism, however, no cosegregation between debrisoquine oxidation phenotype and phenotype metabolism was found [13], and phenytoin did not competitively inhibit the same enzyme in human liver microsomes [14]. Our present results have shown that the stereoselective metabolism of phenytoin cosegregates with the polymorphic 4'-hydroxylation of (S)-mephenytoin in Japanese. The lower urinary excretion of (R)-p-HPPH and higher PHT/(R)-p-HPPH ratio [15] in the PMs than in the EMs suggest that (R)-p-HPPH is catalysed by CYP2C19. This is supported by the negative correlation between the metabolic ratio of omeprazole, used as an index of CYP2C19 activity [16–18], and the log_{10}/% urinary excretion of (R)-p-HPPH. The data presented in this report are in agreement with the findings of Fritz et al. [5] who showed that the formation of the (R)-enantiomer of p-HPPH was significantly decreased in Caucasian PMs of mephenytoin, that there was a bimodal distribution of the urinary p-HPPH S/R-ratio in humans, and that ratio was correlated closely with the mephenytoin hydroxylation index.

Although the mean urinary excretion of both metabolites was lower in the PMs than in the EMs, the difference between the two groups was smaller for (S)-p-HPPH. This suggests that the contributory role of CYP2C19 to the formation of (S)-p-HPPH is less than that of (R)-p-HPPH. The results of recent in vitro studies suggest strongly that the 4'-hydroxylation of PHT to form total (R + S)-p-HPPH is catalysed predominantly by CYP2C9 [19, 20], an isoform distinct from CYP2C19. In humans, (R)-p-HPPH is the minor and (S)-p-HPPH the major enantiomer of p-HPPH formed. Thus, the formation of (S)-p-HPPH by CYP2C9 might be the dominant determinant of the disposition of PHT.

Small differences in amino acid composition of the CYP2C9 gene can alter its catalytic activities toward phenytoin metabolism by more than 5-fold. Allelic CYP2C9 variants with the following amino acid substitutions at Cys144→Arg, Tyr358→Cys, Leu359→Ile, and Gly417→Asp have been identified [8, 21]. Heterologously expressed enzymes containing the Cys144→Arg and the Leu359→Ile substitutions increased the rate of PHT hydroxylation by up to 3-fold and 5-fold, respectively [20]. All our subjects were homozygous for the wild type CYP2C9 gene in Orientals [8, 21] and thus any influence of these known polymorphisms of CYP2C9 on PHT 4'-hydroxylation can be ruled out. The present findings of a decrease in the urinary excretion of (S)-p-HPPH in the PMs of CYP2C19 are difficult to interpret, but we speculate that CYP2C19 may also contribute in part to the formation of (S)-p-HPPH, even though CYP2C9 is the predominating enzyme. In addition, we cannot rule out the occurrence of additional inactivating CYP2C9 mutations that are not detected by the known PCR/restriction enzyme procedures [8]. In relation to (R)-p-HPPH, although less was recovered in the urine of PMs, it was still present, indicating a role for CYP2C9 or other form of cytochrome P450 in this reaction. More detailed studies are necessary to elucidate further the cytochrome P450 specificity of PHT metabolite but our present results and previous observations suggest that at least two enzymes, CYP2C19 and CYP2C9, are involved in the 4'-hydroxylation of PHT.

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References


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