Influence of the \textit{CYP2C9} \& \textit{CYP2C19} polymorphisms on phenytoin hydroxylation in healthy individuals from south India

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\textit{Background \& objectives:} Phenytoin, a widely used anti-epileptic drug, is metabolized mainly by \textit{CYP2C9} (90\%) and partly by \textit{CYP2C19} (10\%) to its major metabolite 5-(para-hydroxyphenyl)-5-phenylhydantoin (p-HPPH). The \textit{CYP2C9} and \textit{CYP2C19} genes encoding these enzymes are polymorphically expressed and most of the variants result in decreased metabolism of the respective substrates. The present study was undertaken to investigate the influence of the \textit{CYP2C9*2} and *3 as well as \textit{CYP2C19*2} and *3 variant genotypes on phenytoin hydroxylation in healthy subjects from south India.

\textit{Methods:} A total of 27 healthy, unrelated, subjects were administered a single oral dose of 300 mg phenytoin. Four hours later, 5 ml of blood was collected and genotyped for \textit{CYP2C9*1}, *2, *3, \textit{CYP2C19*1}, *2 and *3 by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Phenytoin and the major metabolite p-HPPH were estimated by reverse phase HPLC. The metabolic ratio was calculated as concentration of phenytoin/p-HPPH.

\textit{Results:} A significant correlation was observed between the \textit{CYP2C9} genotype and metabolic ratio of phenytoin/p-HPPH (r = 0.472, 95\% CI 0.100 to 0.728; \(P = 0.01\)). While no association was found with \textit{CYP2C19} alone, a significant correlation was observed between the combined \textit{CYP2C9} and \textit{CYP2C19} genotypes and phenytoin metabolic ratio (r = 0.507, 95\% CI 0.146 to 0.749; \(P < 0.01\)).

\textit{Interpretation \& conclusion:} \textit{CYP2C9*2} and *3 mutant alleles caused decreased hydroxylation of phenytoin \textit{in vivo}, whereas the mutant alleles of \textit{CYP2C19} played only a minor role in the metabolism of phenytoin in subjects of our study. The results of present preliminary study needs to be confirmed with a larger sample.

\textit{Key words} \textit{CYP2C9} - \textit{CYP2C19} - metabolism - phenytoin - south Indians
Phenytoin, a widely used anti-epileptic drug, is known to exhibit marked inter-individual variation in pharmacokinetics, which has, to some extent, been attributed to genetic factors such as the CYP2C9 polymorphism. Phenytoin is metabolized almost extensively by the cytochrome P450 (CYP)2C9 enzyme and to a small extent by CYP2C19 to its major metabolite 5-(para-hydroxyphenyl)-phenylhydantoin (p-HPPH). CYP2C9, the gene encoding this enzyme, is polymorphically expressed with about 20 variant alleles. Of these, CYP2C9*2, characterized by a C430T transversion on exon 3 producing amino acid change Arg144Cys, and CYP2C9*3, resulting from an A1075C mutation on exon 7 causing Ile359Leu amino acid substitution are the most common variants. In vitro studies have suggested a significant reduction in CYP2C9 activity with the *3 allele, but only a slight reduction with *2. A study by Aynacioglu et al. in the Turkish population has shown significantly increased phenytoin levels and significantly reduced p-HPPH/phenytoin ratios in individuals with CYP2C9*1/*2, *1/*3 and *3/*3 compared to *1/*1 genotypes.

Previous genotyping analysis in our laboratory has revealed significantly different frequencies of the CYP2C9 mutations in Tamilians (a group of south Indians) when compared to Caucasians and Chinese. The clinical relevance of the CYP2C9 polymorphism in the Indian population of different regions of the country has not been studied.

The present preliminary study was aimed to evaluate the influence of CYP2C9 and CYP2C19 polymorphisms on phenytoin hydroxylation in healthy subjects from south India.

Material & Methods

Subjects: Twenty seven unrelated healthy individuals (17 males and 10 females, with a mean age ± SEM of 26.1 ± 2.2 yr) were enrolled in the study during May 2003 to April 2004. Individuals were not screened particularly for this study. The frequency of CYP2C9 alleles was established in an earlier study in 453 individuals which included 135 individuals from Tamil Nadu and Pondicherry. Of these 135 individuals, those who were available in Pondicherry and gave consent to participate in this study, were included. The study was approved by the Institute Ethics Committee and written informed consent was obtained from all the study participants. Based on their CYP2C9 genotype, they were divided into 4 groups viz., CYP2C9*1/*1 (n=10), *1/*2 (n=5), *1/*3 (n=11) and *3/*3 (n=1).

Study protocol: After an overnight fast, 300 mg phenytoin (Dilantin, Parke-Davis, Mumbai, India) was administered at 0700 to each individual followed by a standardized regular south Indian low fat breakfast at 0900 (2 h after the administration of phenytoin). This consisted of 4 idlis (steamed rice cakes), chutney, sambhar and 1 cup (100 ml) of coffee. At 1100 (4 h after phenytoin administration), 5 ml of venous blood was collected from the antecubital vein into ethylenediaminetetraacetic acid (EDTA) tubes. Plasma was separated by centrifugation and stored at -20°C until the assay.

Genotyping of CYP2C9 and CYP2C19: After separation of plasma, the cellular fraction was subjected to DNA extraction from leukocytes by the standard phenol:chloroform procedure. CYP2C9*2 and CYP2C9*3 were identified by the polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) protocol described by Sullivan-Klose et al. Genotyping of CYP2C19*1, *2 and *3 was done according to the method of Sviri et al.

Analytical procedure: Pure powders of phenytoin and its metabolite p-HPPH were procured from Aldrich Chem Co., (Milwaukee Wisconsin, USA). The levels of phenytoin and p-HPPH were estimated by reverse phase liquid chromatography by the method described by Gerson et al., with minor modifications.
The HPLC system consisted of a Shimadzu LC-10ADvp solvent delivery module, Shimadzu SPD-10Avp UV-VIS detector, a 200 µl injection loop (Rheodyne LLC, CA, USA) and a Hypersil ODS column 250 x 4.6 mm, 5 µm particle size. Flow rate was maintained at 2.0 ml/min, the analytes were detected at 194 nm and absorbance was set at 0.01 Aufs. The metabolite p-HPPH and parent drug phenytoin were eluted at 4.5 min and 12.0 min, respectively. Carbamazepine was used as an internal standard and it eluted at approximately 13.0 min. The mean recovery of compounds was consistent, being 100 per cent for phenytoin and carbamazepine and 70 per cent for p-HPPH. The limit of quantification was 20 ng/ml for phenytoin and 50 ng/ml for p-HPPH. The intra-day and inter-day coefficients of variation were less than 3.1 and 12.7 per cent for phenytoin and less than 7.6 and 13.7 per cent for p-HPPH, respectively.

Statistical analysis was done using the GraphPad InStat statistical software (GraphPad Software Inc., San Diego, CA, USA). Mean values of metabolic ratios (phenytoin/p-HPPH) among the CYP2C9 genotype groups were tested for deviation from normality using the Kolmogorov-Smirnov test. The Spearman rank (nonparametric) correlation was used to assess the relationship between phenytoin hydroxylation and the CYP2C9 as well as CYP2C19 genotype. Values of metabolic ratios among the genotype groups were compared using the Kruskal-Wallis test. The same tests were applied to compare mean metabolic ratios based on CYP2C19 genotype.

**Results**

After 4 h of drug intake, mean plasma levels of phenytoin were 4.11 µg/ml (95% CI 3.69 to 4.54) and of p-HPPH was 0.11 µg/ml (95% CI 0.08 to 0.14). Values of metabolic ratios among the study groups did not deviate from a normal distribution.

A significant correlation was observed between the CYP2C9 genotype and metabolic

<table>
<thead>
<tr>
<th>Group</th>
<th>CYP2C9 genotype</th>
<th>n</th>
<th>Metabolic ratio</th>
<th>95% Confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>*1/*1</td>
<td>10</td>
<td>34.8 ± 21.8</td>
<td>19.2 - 50.3</td>
</tr>
<tr>
<td>II</td>
<td>*1/*2</td>
<td>5</td>
<td>68.2 ± 45.2</td>
<td>12.0 - 124.3</td>
</tr>
<tr>
<td>III</td>
<td>*1/*3</td>
<td>11</td>
<td>69.8 ± 47.9</td>
<td>37.6 - 102.0</td>
</tr>
<tr>
<td>IV</td>
<td>*3/*3</td>
<td>1</td>
<td>109.9 a</td>
<td>–</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SD; a not statistically analysis since n = 1; Metabolic ratio = Concentration of phenytoin/p-HPPH

**Table II.** Influence of the combined CYP2C9 and CYP2C19 genotype on phenytoin metabolic ratio

<table>
<thead>
<tr>
<th>Group</th>
<th>Genotype</th>
<th>n</th>
<th>Metabolic ratio</th>
<th>95% Confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>CYP2C9: wt</td>
<td>10</td>
<td>34.8 ± 21.8</td>
<td>19.2 - 50.3</td>
</tr>
<tr>
<td>II</td>
<td>CYP2C9: ht, CYP2C19: wt</td>
<td>7</td>
<td>62.6 ± 50.3</td>
<td>16.2 - 109.2</td>
</tr>
<tr>
<td>III</td>
<td>CYP2C9: ht, CYP2C19: ht</td>
<td>9</td>
<td>74.4 ± 43.9</td>
<td>40.6 - 108.2</td>
</tr>
<tr>
<td>IV</td>
<td>CYP2C9: hm, CYP2C19: wt</td>
<td>1</td>
<td>109.9 a</td>
<td>–</td>
</tr>
</tbody>
</table>

Values expressed as mean ± SD; a not statistically analysis since n = 1; metabolic ratio = concentration of phenytoin/p-HPPH; wt: wild-type (*1/*1), ht: heterozygous (*1/*2, *1/*3), hm: homozygous mutant (*3/*3)
ratio of phenytoin/p-HPPH \((r = 0.472, \text{95\% CI 0.100 to 0.728}; P=0.01)\). The mean metabolic ratio was two-fold higher in carriers of the CYP2C9*2 or *3 allele when compared to the wild-type genotype. This was, however, not statistically significant probably owing to wide intragroup variation (Table I).

On comparing the metabolic ratios based on the CYP2C19 genotype, no significant difference was observed among the groups (data not given).

The individuals were grouped based on their combined CYP2C9 and CYP2C19 genotypes to assess the combined influence of these polymorphisms. Ten subjects were wild-type for CYP2C9 and of any CYP2C19 genotype, 7 were heterozygous for CYP2C9 and wild for CYP2C19, 9 were heterozygous for both CYP2C9 and CYP2C19, and 1 was homozygous mutant for CYP2C9 and wild-type for CYP2C19. A significant correlation was observed between the combined CYP2C9 and CYP2C19 genotypes and phenytoin metabolic ratio \((r = 0.507, \text{95\% CI 0.146 to 0.749}; P<0.01)\). The difference in metabolic ratio values among the groups were not significantly different (Table II).

**Discussion**

Phenytoin is one of the most-widely prescribed anti-epileptic agents in the Indian population. This, combined with the fact that the CYP2C9 enzyme which metabolizes phenytoin is polymorphically expressed and that phenytoin has a narrow safety margin makes it important to study the effect of the CYP2C9 polymorphism with regard to phenytoin metabolism. Phenytoin has been found to be safe and suitable for use as a probe to study the pharmacogenetics of CYP2C913.

Phenytoin follows non-linear pharmacokinetics. At a concentration of \(\leq 10 \mu g/ml\), it follows first order (linear) kinetics, and beyond this, zero-order kinetics14. Since the influence of enzymes can only be studied on the drugs that follow first-order kinetics, only a single dose of phenytoin was administered to the subjects, although epileptic patients receive long-term therapy in the clinical setting.

The present study is probably the first attempt to establish the genotype-phenotype relationship of CYP2C9 in Indians. A significant association was found between the CYP2C9 genotype and phenytoin metabolism. Carriers of either the CYP2C9*2 or *3 variant allele were found to have a two-fold higher metabolic ratio than the wild-type individuals. This is in agreement with findings from other world populations7,15. This difference, however, failed to attain statistical significance. This could be due to a relatively low sample size \((n=5)\) in *1/*2 group, which led to a low statistical power in this study. A low sample size could not be avoided since the frequency of the CYP2C9*2 allele in the entire south Indian population was found to be only 3.7 per cent9. The means of metabolic ratio in individuals with *1/*2 and *1/*3 were similar. Another reason for lack of significance could be the wide variation observed in phenytoin metabolic ratio values within the genotype groups.

In the single individual who was homozygous for CYP2C9 *3, the metabolic ratio was more than 3 times than that in normal individuals and the plasma level of phenytoin was 6.05 \(\mu g/ml\), as compared to 3.9 \(\mu g/ml\) in *1/*2 and 4.2 \(\mu g/ml\) in *1/*3 individuals. In the clinical setting, CYP2C9 *3 carriers are prone to a higher incidence of adverse effects owing to impaired phenytoin metabolism. A CYP2C9 *3 variant has been shown to be associated with a higher proportion of patients with phenytoin-induced cutaneous adverse drug reactions16. In another study, although the CYP2C9 polymorphisms did not directly correlate with gingival hyperplasia, patients with gingival overgrowth exhibited significantly higher serum phenytoin levels owing to the presence of the CYP2C9 *3 allele17.
On combining CYP2C9 and CYP2C19 genotypes, no significant difference was seen in the means of metabolic ratio between those who were heterozygous for CYP2C9 but wild-type for CYP2C19 and those who were heterozygous for both. This is in accordance with an earlier study, which has shown that while both CYP2C9 *2 and *3 heterozygotes have significantly different metabolic ratios and plasma phenytoin levels compared to normal individuals there was no association between the CYP2C19 genotype and phenytoin metabolism. This suggests that CYP2C19 may play only a minor role, if any, in the in vivo hydroxylation of phenytoin. The metabolic ratio of phenytoin/p-HPPH depends predominantly on the CYP2C9 genotype.

The major limitation of this study was a small sample size that could not determine whether there was a statistically significant difference in phenytoin metabolic ratio, based on the CYP2C9 genotype. The results of this study therefore need to be confirmed using a larger sample. A significant correlation between the CYP2C9 genotype and phenytoin metabolic ratio indicated that CYP2C9 variants *2 and *3 might have importance in phenytoin metabolism in the south Indian population.

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References


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