Biochemical Pharmacology, Vol. 57, pp. 1297–1303, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00034-9

Human CYP2C-Mediated Stereoselective Phenytoin Hydroxylation in Japanese: Difference in Chiral Preference of CYP2C9 and CYP2C19

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ABSTRACT. Regio- and stereoselective hydroxylation of phenytoin was determined in liver microsomes of nine extensive (EM) and three poor metabolizers (PM) of mephenytoin. Hydroxyphenytoins (HPPH) were isolated and quantified after separation into four regio- and stereoisomers. The total rates of microsomal phenytoin 4′- hydroxylation were approximately 3-fold higher than those of 3′-hydroxylation, and not significantly different in EM and PM. Formation of 4′-(*R*)-HPPH was 4.4-fold higher in EM than in PM, whereas no clear differences between EM and PM were detected in the formation of 4′-(*S*)-, 3′- (*R*)-, and 3′- (*S*)-HPPH. Cytochrome P450 (CYP)2C9, expressed in a fission yeast, *Schizosaccharomyces pombe*, catalyzed the formation of 4′-(*R*)- and 4′-(*S*)-HPPH stereoselectively, as observed with EM, in which predominantly 4′- (*S*)-HPPH was formed. Recombinant CYP2C19 was more stereoselective for 4′-(*R*)-HPPH formation. These results, in addition to inhibition experiments with anti-human CYP2C antibody, indicate that phenytoin hydroxylation is mainly catalyzed by CYP2C9. Furthermore, CYP2C19 showed limited contribution to phenytoin 4′-hydroxylation with a different chiral preference from CYP2C9. BIOCHEM PHARMACOL **57**;11:1297–1303, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. phenytoin; cytochrome P450; human liver; drug metabolism; expression in yeast; polymorphism; pharmacogenetics

Genetic polymorphism of drug-metabolizing enzymes often resulted in large interindividual variations in the rate and pathways of drug metabolism [1]. An antiepileptic drug, phenytoin, undergoes aromatic hydroxylation regio- and stereoselectively (Fig. 1) and shows large interindividual differences in metabolism in humans [2–5]. The hydroxylated metabolites of phenytoin have been isolated and identified by Butler *et al.* [2]. Enzymatically released 3′-HPPH¶ and 4′-HPPH were mixtures of optical isomers. Mephenytoin also undergoes aromatic hydroxylation, and exhibits a genetic polymorphism [6–8]. Although phenytoin is structurally related to mephenytoin, dissociation has been reported in their polymorphic metabolism in humans [9–11]. Poor metabolizers (PM) of mephenytoin are detected with low frequencies in Caucasians (2 to 5%), but with considerably higher frequencies (approximately 20%) in Japanese subjects [12, 13]. Slow metabolizers of phenytoin are also detected, but with extremely low frequencies as compared to the incidence of PM in mephenytoin 4′-hydroxylation [9, 10]. However, Fritz *et al.* [9] reported that the formation of 4′-(*R*)-HPPH was significantly decreased in PM of mephenytoin, leading to a bimodal distribution of the urinary R/S ratio of HPPH, which was correlated closely with the mephenytoin hydroxylation index. Microsomal phenytoin hydroxylation is shown to correlate significantly with tolbutamide hydroxylation *in vitro* [14]. However, the reason for the poor relationship between phenytoin and mephenytoin hydroxylation in livers remains unclear. As for the metabolism of phenytoin by cytochrome P450, CYP2C9 has been reported to oxidize phenytoin [15]. To better understand the metabolism of phenytoin, HPPH metabolites were separated into the four regio- and stereoisomers and quantified by a chiral HPLC. In our earlier studies, clear shifts in the metabolic pathway and enzyme form involved were observed *in vitro*, depending on the drug concentrations [16, 17]. Thus, we examined the concentration dependency using two different substrate concentrations (0.2 and 0.02 mM) in the present study. Furthermore, the CYP2C9 and CYP2C19 expressed in yeasts were used to unequivocally verify the relationship between phenytoin and mephenytoin hydroxylations [18–21]. The results ob-

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¶ *Abbreviations:* EM, extensive metabolizer; PM, poor metabolizer; HPPH, hydroxyphenytoin; and IgG, immunoglobulin G.

Received 2 June 1998; accepted 12 November 1998.

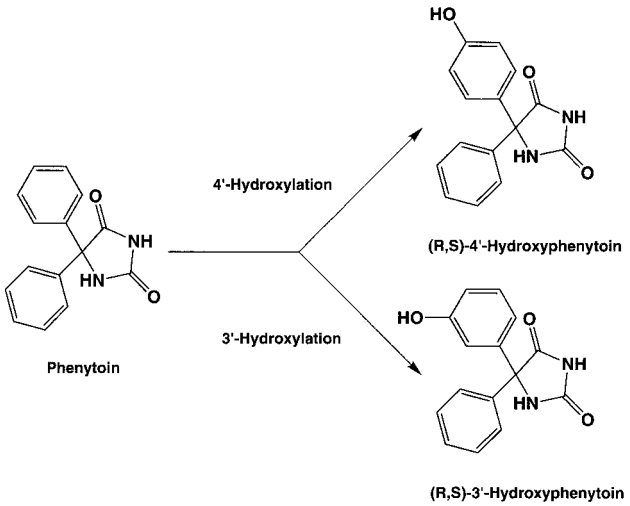

FIG. 1. Microsomal oxidation pathway of phenytoin.

tained in the present study show a clear difference in the chiral preference of phenytoin hydroxylation in CYP2C9 and CYP2C19, and also demonstrate that a minor, but significant, metabolic pathway of phenytoin, microsomal 4′-(*R*)-HPPH formation (approximately 20% of the total phenolic phenytoin hydroxylation), is closely related to (*S*)-mephenytoin 4′-hydroxylation in human livers.

MATERIALS AND METHODS

Chemicals

Phenytoin was obtained from Dainippon Pharmaceuticals Co. Racemic hydroxymetabolites of phenytoin, 5-(3′-hydroxyphenyl)-5-phenylhydantoin (3′-HPPH) and 5-(4′-hydroxyphenyl)-5-phenyhydantoin (4′-HPPH) were obtained from Aldrich Chemical Co., Inc. Racemic 3′- and 4′-HPPHs were separated to their stereoisomers by a method described below. Racemic mephenytoin was kindly provided by Drs. D. Römer and H. Stähelin (Sandoz Ltd., Basel, Switzerland). The racemic mixture was separated to the enantiomers on a Chiralcel OJ column (10 μm, 4.6 × 250 mm, Daicel Chemical Co., Ltd.) with ethanol as an eluent [22]. The standard metabolites of (*S*)-mephenytoin were from Salford Ultrafine Chemicals and Research Ltd. Other chemicals and reagents were of the highest grade commercially available.

Liver Samples

Seven liver samples (H24, H25, H26, H27, H28, H29, and H30) were obtained in surgical operations from visually normal portions of livers from Japanese patients with cancer. Liver samples were frozen in liquid nitrogen within 30 min of removal and stored at −80° until use. Five human liver samples from Japanese subjects (H10, H12, H14, H16, and H23) were obtained within 3 hr of clinical death. The source and case histories of liver samples are described in Table 1. After microsomal preparation, protein and cytochrome P450 concentrations were determined, and microsomal suspensions were stored at −80° until use, following approval by the local Ethics Committee (Keio University). The stereoselective metabolism of (*R*)- and (*S*)-mephenytoins to their 4′-hydroxymetabolites was determined with these liver microsomes. Nine subjects who showed (R/S) hydroxylation ratios of less than 0.3 were judged EM, the other three subjects being PM according to the standard described in the previous studies [22]. The data were also supported by the genotyping method previously reported [21]. The mean ratio (R/S) of EM was 0.09 ± 0.04 (mean ± SD), and the value of PM was 0.44 ± 0.06. The incidence of PM was similar to that reported in previous *in vivo* and *in vitro* studies of Japanese subjects [12, 13, 22, 23]. The amounts of P450 in EM and PM microsomes were 0.47 ± 0.18 and 0.41 ± 0.21 nmol/mg protein (mean ± SD), respectively.

Yeasts

Microsomes of yeasts expressing CYP2C9 or CYP2C19 as well as of control yeasts were prepared as described elsewhere, with minor modifications [19, 24].

Assay

Phenytoin hydroxylation was determined by the assay of mephenytoin hydroxylation, with some modifications [22]. The incubation mixture consisted of 0.3 mg protein of liver microsomes, 100 mM sodium phosphate buffer (pH 7.4), 0.1 mM EDTA, an NADPH-generating system (6 mM $MgCl_2$, 0.8 mM NADP, 8 mM glucose 6-phosphate, 1 unit/mL of glucose-6-phosphate dehydrogenase), and 0.2 mM of a substrate in a final volume of 0.25 mL. Anti-human CYP2C antibody (anti-CYP2C9 IgG) was added to the incubation mixture of liver microsomes when necessary. The incubation mixture (0.25 mL) for yeast microsomes consisted of 0.2 nmol of expressed P450, 0.2 units of NADPH–cytochrome P450 reductase, 0.1 nmol of cytochrome b_5, 0.03 mg of microsomal lipids, 0.1 M sodium phosphate buffer (pH 7.4), an NADPH-generating system, and 0.2 mM of a substrate. All determinations were performed in duplicate with liver samples, and in triplicate with yeasts. The reaction was started by the addition of the NADPH-generating system and terminated 30 min after the incubation with 0.1 mL of a 2% sodium azide solution containing 2 μM of 5-(4-methylphenyl)-5-phenylhydantoin (internal standard). The mixture was extracted with 5 mL of diethylether. The extract was evaporated under a gentle stream of nitrogen. The residue was dissolved in 0.1 mL of water and an aliquot (0.05 mL) was subjected to an HPLC analysis. The HPLC system consisted of a PU-980 pump, a UV/VIS-970 spectrophotometer (Japan Spectroscopic Co.) set at 204 nm, and a Nucleosil $7C_{18}$ column (4.6 × 300 mm). The metabolites were eluted with a mixture of 25% acetonitrile/75% 20 mM sodium perchlorate (pH 4.0, adjusted with perchloric acid) at a flow rate of 1 mL/min. The 3′- and 4′-hydroxy metabolites were col-

TABLE 1. Phenytoin hydroxylase activities and the metabolic ratios of hydroxylation (3′/4′ and R/S) in liver microsomes

Hydroxylation	EM	PM
Total phenolic hydroxylation	5.87 ± 0.85 (2.04 ± 0.39)	5.01 ± 0.71 (1.74 ± 0.26)
3′-Hydroxylation	1.36 ± 0.52 (0.24 ± 0.08)	1.33 ± 0.23 (0.21 ± 0.04)
4′-Hydroxylation	4.50 ± 0.77 (1.80 ± 0.36)	3.69 ± 0.74 (1.53 ± 0.26)
3′/4′ Ratio	0.31 ± 0.14 (0.14 ± 0.06)	0.37 ± 0.11 (0.14 ± 0.04)
3′-(*R*)-Hydroxyphenytoin	0.76 ± 0.27 (0.12 ± 0.04)	0.69 ± 0.13 (0.11 ± 0.02)
3′-(*S*)-Hydroxyphenytoin	0.71 ± 0.26 (0.30 ± 0.10)	0.64 ± 0.10 (0.10 ± 0.02)
R/S Ratio (3′-hydroxylation)	1.07 ± 0.06 (1.15 ± 0.17)	1.09 ± 0.06 (1.06 ± 0.08)
4′-(*R*)-Hydroxyphenytoin	1.45 ± 0.37 (0.53 ± 0.18)	0.37 ± 0.08* (0.15 ± 0.02*)
4′-(*S*)-Hydroxyphenytoin	3.05 ± 0.49 (1.27 ± 0.21)	3.32 ± 0.67 (1.38 ± 0.29)
R/S Ratio (4′-hydroxylation)	0.48 ± 0.10 (0.41 ± 0.09)	0.11 ± 0.01* (0.11 ± 0.01*)

Hydroxylase activities (formation of stereoisomers) are expressed as pmol/min/mg protein. Substrate concentrations are 0.2 and 0.02 mM. Values in parentheses are data from the experiments with substrate concentrations at 0.02 mM. Data are means ± SD of EM (N = 9) and PM (N = 3).

*$P < 0.001$ compared with EM.

lected separately and re-extracted with 2 mL of dichloromethane. The extract was evaporated and the residue then dissolved in 0.1 mL of water. An aliquot (0.05 mL) was subjected to HPLC analysis for separation of the stereoisomers. The UV detector was set at 204 nm, and an Ultron ES-OVM column (4.6 × 150 mm, Shinwa Chemical Industries Ltd.) was used. The metabolites were eluted with a mixture of 7.4% ethanol/92.6% 20 mM potassium phosphate buffer (pH 7.0) at a flow rate of 1 mL/min. The amount formed was determined by the peak area of the metabolite using a standard curve generated by the authentic standard. Absolute configurations of HPPHs were determined by their specific rotation, measured by the Shodex OR-1 optical rotation detector (Showadenko Co.). Assay of microsomal mephenytoin 4′-hydroxylation was performed as described previously [22].

Other Methods and Materials

Protein content was determined by the method using BSA as the standard [25]. The content of CYP in microsomes was determined according to the method of Omura and Sato [26], except for the inclusion of 0.2% Emulgen 913 (Kao Co.) in a solubilizing buffer. Microsomal lipids were prepared according to the conventional method [27]. Anti-human CYP2C antibody (anti-CYP2C9 IgG) and NADPH–cytochrome P450 reductase were prepared as described previously [28]. Anti-CYP2C9 IgG cross-reacts with CYP2C19 and inhibits human microsomal mephenytoin 4′-hydroxylation mediated by CYP2C19. The contents of CYP2C9 in the microsomes were determined by immunoblot analysis using anti-CYP2C9 IgG [28]. Statistical analysis was performed with the StatView software package (Abacus Concepts, Inc.) on a Macintosh computer system (Apple Computer Inc.).

RESULTS

Regio- and Stereoselective Hydroxylations of Phenytoin and 3′/4′ and R/S Ratios

Phenytoin hydroxylation was measured using liver microsomes of EM and PM for (*S*)-mephenytoin 4′-hydroxylation of Japanese subjects. The metabolites of the four regio- and stereoisomers of HPPH were isolated and quantified (Table 1). At a high substrate concentration (0.2 mM), the rates of microsomal phenytoin 4′-hydroxylation were approximately 3-fold higher than those of 3′-hydroxylation in all subjects. The ratios of 3′/4′-hydroxylation were not significantly different in the two phenotypes. The ratios of 3′/4′-hydroxylation were decreased 3-fold at a low substrate concentration (0.02 mM). Although the mean rates of total phenolic (3′-plus 4′-) 3′- and 4′-hydroxylations (racemic) in EM were slightly higher than those in PM, no significant difference was observed between EM and PM at 0.2 and 0.02 mM substrate concentrations.

These regioisomers (3′- and 4′-HPPH) were further separated into the (*R*)- and (*S*)-enantiomers. The rate of microsomal formation of 4′-(*R*)-HPPH was approximately 4-fold higher in EM than in PM, whereas no clear difference was observed in the formation of 4′-(*S*)-HPPH in EM and PM at 0.2 and 0.02 mM substrate concentrations (Table 1). Thus, the R/S ratio of microsomal 4′-hydroxylation was significantly different in the two phenotypes. Rates of microsomal formation of 3′-(*R*)- and 3′-(*S*)-HPPH did not significantly differ in EM and PM, although the rates of 3′-(*R*)- and 3′-(*S*)-HPPH formation decreased at a 0.02 mM substrate concentration.

Kinetic Parameters for Phenytoin Hydroxylation in Liver Microsomes

Kinetic parameters for phenytoin hydroxylation were assessed in liver microsomes to verify the P450 forms responsible for enantioselective HPPH formation. The formation of HPPHs in liver microsomes from EM and PM appeared to be mediated by plural enzymes with similar K_m and V_{max} values, except for 4′-(*R*)-HPPH formation, as described in Table 2. Apparent K_m and V_{max} values for 4′-(*R*)-HPPH formation were significantly different in liver microsomes from EM and PM; however, Lineweaver–Burk and Eadie–Hofstee plots for all HPPH formations were nearly monophasic in the liver microsomes derived from three EMs and three PMs (data not shown). As described in

TABLE 2. Michaelis–Menten parameters for phenytoin hydroxylation in human liver microsomes

Phenotype	K_m (V_{max}) [V_{max}/K_m]			
	3′-(*R*)-HPPH	3′-(*S*)-HPPH	4′-(*R*)-HPPH	4′(*S*)-HPPH
EM	314 ± 15	261 ± 18	38.8 ± 1.07	33.2 ± 0.42
	(2.35 ± 0.42)	(1.88 ± 0.35)	(2.10 ± 0.45)	(4.09 ± 0.53)
	[7.44 ± 1.39]	[7.17 ± 1.08]	[54.5 ± 15.5]	[120 ± 20]
PM	310 ± 35	286 ± 38	31.7 ± 0.54	33.5 ± 0.96
	(1.72 ± 0.18)	(1.55 ± 0.11)	(0.44 ± 0.08)	(3.94 ± 0.64)
	[5.66 ± 1.42]	[5.53 ± 1.16]	[13.9 ± 3.2]	[116 ± 23]

Values are expressed as μM, pmol/min/mg protein, and μL/min/mg protein ($\times 10^{-3}$) for K_m, V_{max}, and V_{max}/K_m, respectively. Data are means ± SD of three EM (H23, H24, and H26) and three PM (H14, H29, and H30). The range of substrate concentration is from 0.005 to 0.2 mM.

Table 2, the V_{max}/K_m values for 4′-(*S*)-HPPH formation were more than 2- and 8-fold higher than those for 4′-(*R*)-HPPH formation in EM and PM, respectively, while those for 3′-(*R*)- and 3′-(*S*)-HPPH formation were very small and almost equivalent.

Immunoinhibition of Microsomal Phenytoin Hydroxylation by Anti-human CYP2C Antibody

The effect of the anti-human CYP2C antibody was examined to ascertain the role of human CYP2C forms in microsomal phenytoin hydroxylation. As shown in Fig. 2, racemic phenytoin 4′-hydroxylation was inhibited approximately 50–70% by the addition of anti-human CYP2C antibody in EM and PM at 0.2 and 0.02 mM substrate concentrations. However, racemic phenytoin 3′-hydroxylation was not effectively inhibited by anti-human CYP2C antibody in either phenotype at 0.2 mM (less than 30%) and 0.02 mM (less than 40%) substrate concentrations (data not shown). Microsomal formation of 4′-(*R*)-HPPH in PM was clearly less than in EM, but unaffected by the addition of anti-human CYP2C antibody at 0.02 mM substrate concentration (Fig. 3). Formation of 4′-(*S*)-HPPH was also inhibited by 50% in both EM and PM. At 0.2 mM substrate concentration, similar profiles of inhibition by anti-human CYP2C antibody were also observed on formation of 4′-(*R*)- and 4′-(*S*)-HPPH (data not shown).

Correlations between Microsomal Formations of HPPH Enantiomers and Relation to (S)-Mephenytoin 4′-Hydroxylation

Among four microsomal enantioselective hydroxylations of phenytoin at 0.02 mM substrate concentration, a significant correlation ($r = 0.95$; $P < 0.001$) was observed between microsomal formation of 3′-(*R*)-HPPH and 3′-(*S*)-HPPH. However, no clear correlation was obtained between other reactions (3′-(*R*)-HPPH vs 4′-(*R*)-HPPH, $r = 0.33$; 3′-(*R*)-HPPH vs 4′-(*S*)-HPPH, $r = 0.10$; 3′-(*S*)-HPPH vs 4′-(*R*)-HPPH, $r = 0.37$; 3′-(*S*)-HPPH vs 4′-(*S*)-HPPH, $r < 0.01$; and 4′-(*R*)-HPPH vs 4′-(*S*)-HPPH, $r = 0.27$) (data not shown). Despite no clear correlation between total phenolic phenytoin hydroxylation and (*S*)-mephenytoin 4′-hydroxylation (0.2 mM, $r = 0.55$; 0.02 mM, $r = 0.60$), a clear correlation (0.2 mM, $r = 0.87$; 0.02 mM, $r = 0.89$; $P < 0.001$) was observed between

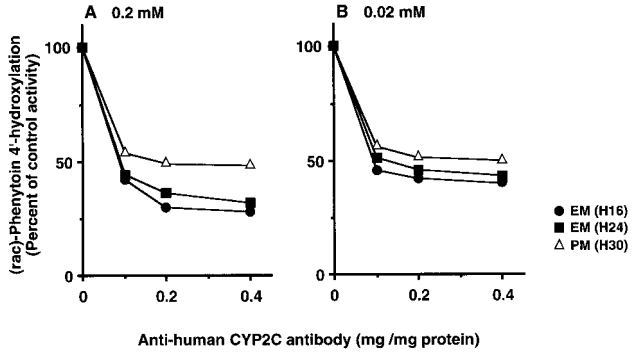

FIG. 2. Immunoinhibition of 4′-HPPH formation (racemic) in liver microsomes of two EM (H16 and H24) and one PM (H30) by anti-human CYP2C antibody at 0.2 (A) and 0.02 mM (B) substrate concentrations. Activities are expressed as relative percentages of the controls (added nonimmune antibody). The control activities for 4′-HPPH formation were 4.66, 4.89, and 4.06 pmol/min/mg protein for H16, H24, and H30, respectively, at 0.2 mM substrate concentration, and 1.85, 2.11, and 1.73 pmol/min/mg protein for H16, H24, and H30, respectively, at 0.02 mM substrate concentration.

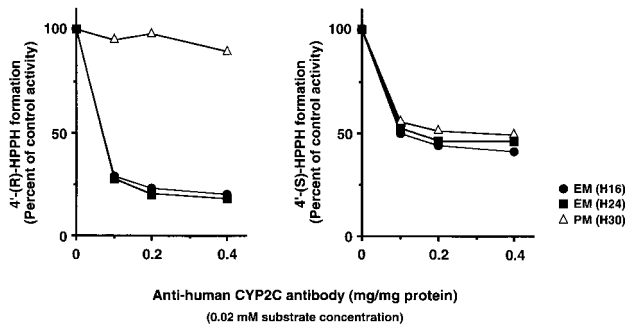

FIG. 3. Immunoinhibition of 4′-(*R*)- and 4′-(*S*)-HPPH formation in liver microsomes of two EM (H16 and H24) and one PM (H30) by anti-human CYP2C antibody at a high substrate concentration (0.2 mM). Activities are expressed as relative percentages of the controls (added non-immune antibody). The control activities for 4′-(R)-HPPH formation were 1.42, 1.80, and 0.37 pmol/min/mg protein for H16, H24, and H30, respectively. The control activities for 4′-(S)-HPPH formation were 3.24, 3.09, and 3.69 pmol/min/mg protein for H16, H24, and H30, respectively.

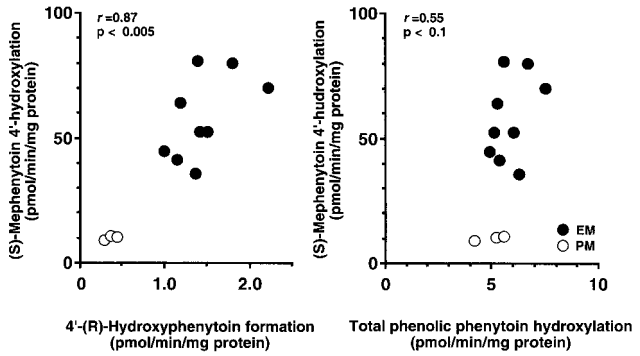

FIG. 4. Correlation between microsomal HPPH and 4′-hydroxymephenytoin formation in livers. Symbols represent different individual livers of EM and PM.

microsomal 4′-(*R*)-HPPH formation and (*S*)-mephenytoin 4′-hydroxylation (Fig. 4).

Regio- and Stereoselective Hydroxylations of Phenytoin by CYP2C9 and CYP2C19-Producing Yeast

Phenytoin hydroxylase activities of *leu1-32h*$^-$[pTLCYP2C9] and *leu1-32h*$^-$[pTLCYP2C19] (the microsomes of a fission yeast, *Schizosaccharomyces pombe*, in which CYP2C9 and CYP2C19 cDNA are expressed, respectively) were determined; their K_m and V_{max} values are described in Table 3. The specific contents of CYP2C9 and CYP2C19 expressed in yeast microsomes ranged from 0.8 to 1.3 and from 0.2 to 0.3 nmol/mg protein, respectively. Yeast-expressed P450 catalyzed phenytoin hydroxylation regio- and stereoselectively, whereas *leu1-32h*$^-$[pTL2M1] microsomes (the yeast microsomes that contain the control expression vector pTL2M1 only) showed no detectable activity for phenytoin hydroxylation. Both *leu1-32h*$^-$[pTLCYP2C9] and *leu1-32h*$^-$[pTLCYP2C19] microsomes mediated 4′-HPPH formation, but not that of 3′-HPPH. The *leu1-32h*$^-$[pTLCYP2C9] and *leu1-32h*$^-$[pTLCYP2C19] expressed in yeasts showed different chiral preference for 4′-HPPH formation. The K_m values for 4′-(*R*)- and 4′-(*S*)-HPPH formation by CYP2C9 were 5-fold lower than those of CYP2C19, indicating that these CYP2C forms had different affinities for phenytoin (Table 3). Phenytoin hydroxylation of *leu1-32h*$^-$[pTLCYP2C9] and *leu1-32h*$^-$[pTLCYP2C19] was almost completely inhibited by the addition of anti-human CYP2C antibody to the reaction mixture (data not shown). As described in Table 3, the V_{max}/K_m values for 4′-(*S*)-HPPH formation by CYP2C9 were approximately 8-fold higher than those for 4′-(*R*)-HPPH formation, while the V_{max}/K_m values for 4′-(*R*)- and 4′-(*S*)-HPPH formation by CYP2C19 were not significantly different.

DISCUSSION

The notion that the major metabolic pathway of phenytoin, 4′-(*S*)-HPPH formation, is predominantly catalyzed by CYP2C9 is confirmed in the present study. Microsomal 4′-HPPH formation accounted for approximately 70% of total phenolic phenytoin hydroxylation, in which 4′-(*S*)-HPPH constituted approximately 60% of total 4′-HPPH metabolites. Although no clear difference was observed in 4′-(*S*)-HPPH formation in the EM and PM samples, apparent K_m and V_{max} values for 4′-(*R*)-HPPH formation were significantly different in liver microsomes of EM and PM. The V_{max}/K_m values for 4′-(*R*)-HPPH formation in EM were approximately 4-fold higher than those in PM (Table 2). The V_{max}/K_m for 4′-(*S*)-HPPH formation by recombinant CYP2C forms indicated a major contribution of CYP2C9 to microsomal 4′-(*S*)-HPPH formation (Table 3). Although microsomal 4′-(*R*)- and 4′-(*S*)-HPPH formation was clearly inhibited by anti-human CYP2C antibody, no clear effect was observed on microsomal 3′-HPPH formation. The human CYP form(s) responsible for microsomal 3′-HPPH formation is still unclear. Other human CYP forms, namely CYP1A1, 1A2, 2B6, 2E1, and 3A4, were reported to show no activity in phenytoin hydroxylations [29]. The *in vitro* results obtained in this study were consistent with those of the *in vivo* study by Fritz *et al.* [9], in which a bimodal distribution of the urinary ratio of 4′-(*R*)-HPPH was correlated closely with the mephenytoin

TABLE 3. Michaelis–Menten parameters for phenytoin hydroxylase activities in yeast microsomes

	K_m (V_{max}) [V_{max}/K_m]	
Formation	*leu1-32h*$^-$*[pTLCYP2C9]† (CYP2C9)	*leu1-32h*$^-$[pTLCYP2C19]† (CYP2C19)
4′-(*R*)-Hydroxyphenytoin	10.9 ± 2.6	56.6 ± 8.5
	(2.33 ± 0.36)	(5.71 ± 0.52)
	[22.6 ± 8.5]	[10.3 ± 2.1]
4′-(*S*)-Hydroxyphenytoin	9.8 ± 1.5	53.4 ± 7.8
	(17.4 ± 1.45)	(4.09 ± 0.36)
	[177 ± 35]	[7.81 ± 1.70]
R/S Ratio (V_{max})	0.14 ± 0.04	1.40 ± 0.28

Values are expressed as μM, pmol/min/mg protein, and μL/min/mg protein ($\times 10^{-2}$) for K_m, V_{max}, and V_{max}/K_m, respectively. The range of substrate concentration is from 0.005 to 0.2 mM. Data are mean values of triplicate experiments. Hydroxylase activities were not detected (<0.01) in control yeast microsomes (*leu1-32h*$^-$[pTL2M1]).

**Schizosaccharomyces pombe* [genotype: *leu1-32h*$^-$ (ATCC 38399)].

†Plasmid for expression of CYP2C9 or CYP2C19 cDNA containing hCMV promoter and terminator.

hydroxylation index. Recently, Ieiri *et al.* [11] reported a similar low recovery of 4′-(*R*)-HPPH in urinary excretion. In addition, a slightly lower recovery of 4′-(*S*)-HPPH, which was possibly due to CYP2C19, was also observed in PM [11]. However, the difference in microsomal formation of 4′-(*S*)-HPPH in EM and PM was not clear in this study.

Interestingly, CYP2C9 and 2C19 expressed in yeasts showed different chiral preference in 4′-HPPH formation. The enantio ratio of 4′-HPPH formed by CYP2C9 was similar to the enantiomeric composition observed in human liver microsomes, where 4′-(*S*)-HPPH was dominant. In contrast, CYP2C19 was more stereoselective for 4′-(*R*)-HPPH formation, this difference in stereoselectivity possibly being due to the structural differences in the reactive sites of CYP2C9 and CYP2C19. Both forms show more than 90% amino acid sequence similarity, while the opposite preference of CYP2C9 and CYP2C19 may reflect the difference in their substrate binding sites. When a ureid group of the substrate is defined as being located in the horizontal position in a three-dimensional model, the 4′-position of the phenyl ring (oxidized site) is located under the ureido surface. In addition, the 4′-positions of 4′-(*R*)-HPPH and 4′-(*S*)-hydroxymephenytoin are located in similar specific positions. Therefore, we suggest that CYP2C19 recognizes the carbonyl moiety in the ureid group of (*S*)-mephenytoin and phenytoin precisely in the substrate binding site. On the other hand, steric recognition of the substrates by CYP2C9 is comparatively generous. CYP2C9 presumably recognizes the acidic group of the hydantoins, and oxidation occurs at the 5′-substituted side chain located at a certain distance. The low rates of phenytoin hydroxylation were possibly due to the steric hindrance of the two phenyl groups. These ideas are consistent with the earlier observations that CYP2C9 oxidized both (*R*)- and (*S*)-hexobarbital at the 3′-position to predominantly form 3′α- and 3′β-hydroxymetabolites, respectively [22]. In either case, preferential oxidation occurred at the back site of the ureid plain. CYP2C9 also oxidized the alkyl chain in substrates such as tolbutamide and omeprazole, suggesting that a similar special relationship between the key functional moiety and the site of oxidation is also applicable [24, 30–32]. These results support the notion that a minor metabolic pathway of phenytoin, microsomal 4′-(*R*)-HPPH formatin, is closely related to (*S*)-mephenytoin 4′-hydroxylation (Fig. 4).

Large interindividual differences in phenytoin hydroxylation have been observed in humans [2–5]. Thus, a low rate of microsomal 4′-(*R*)-HPPH formation had no clear effect on the rates of total phenolic phenytoin hydroxylation, and the total rates of phenolic phenytoin hydroxylation in PM were not significantly different from those in EM. These results are corroborated by the inhibition experiments with anti-human CYP2C antibody (Fig. 3) and the correlation studies (Fig. 4). The apparent K_m and V_{max} values for 4′-(*R*)-HPPH formation were significantly different in liver microsomes of EM and PM (Table 2). Recently, Bajpai *et al.* reported that a liver microsomal sample which had a low content of CYP2C19 showed K_m and V_{max} values for phenytoin hydroxylation similar to those of PM in the present study [33]. Interindividual differences in phenytoin hydroxylation were partially due to acid exchanges of Leu359 to Ile in the amino acid sequence of CYP2C9 [34]. Several amino acid exchanges were observed in the sequence of CYP2C9 [35], and the change in catalytic activities in relation to these mutations will be the object of further investigation. The results indicate that phenytoin hydroxylation is mainly catalyzed by CYP2C9 and also suggest the limited contribution of CYP2C19 to the major pathway of phenytoin hydroxylation. CYP2C19 possibly plays a major role in microsomal 4′-(*R*)-HPPH formation. On the contrary, in (*S*)-mephenytoin 4′-hydroxylation, the contributions of CYP2C9 and CYP2C19 were reversed.

In conclusion, it has become clear that phenytoin hydroxylation is related to mephenytoin hydroxylation, with this being partly revealed through the analysis of their stereoselective profiles of hydroxylation. Further investigation of drug metabolism, analyzing the stereoselective profiles reported in this study, will elucidate the complicated correspondence between specific drugs and the enzymes responsible for the metabolism.

This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Science and Culture, and grants from the Japan Research Foundation for Clinical Pharmacology and the Yokoyama Research Foundation for Clinical Pharmacology. We would like to express our gratitude to Dr. H. Fujima (Shinwachemical Industries Ltd., Kyoto, Japan) for a large scale separation of 3′- and 4′-hydroxyphenytoin to their (R)- and (S)-enantiomers, respectively.

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