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Human Liver Cytochrome P450 Enzymes Involved in the 7-Hydroxylation of *R*- and *S*-Warfarin Enantiomers

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ABSTRACT. Human liver microsomes had about 8-fold higher 7-hydroxylation activities for *S*-warfarin than for *R*-warfarin. Activities of racemic warfarin 7-hydroxylation by liver microsomes of 35 human samples correlated more closely with those of *S*-warfarin 7-hydroxylation ($r = 0.95$) than with those of *R*-warfarin 7-hydroxylation ($r = 0.69$). The correlation coefficient between *R*-warfarin 7-hydroxylation and 7-ethoxyresorufin *O*-deethylation activities was 0.73 in these human samples, suggesting that *R*- and *S*-warfarin enantiomers are catalyzed by different forms of human cytochrome P450 (P450 or CYP) enzymes. Anti-CYP2C9 antibodies inhibited completely the 7-hydroxylation of *S*-warfarin, but not *R*-warfarin, catalyzed by human liver microsomes, while anti-CYP1A2 inhibited *R*-warfarin 7-hydroxylation by about 70%. Interestingly, the racemic warfarin 7-hydroxylation activities (turnover numbers of 1.6 ± 1.0 pmol/min/mg protein in 35 human samples) were found to be low compared with the *S*-warfarin 7-hydroxylation activities (4.1 ± 2.5 pmol/min/mg protein), indicating that *R*-warfarin may have affected the CYP2C9-dependent *S*-warfarin 7-hydroxylation activities when racemic warfarin was used as a substrate. Several P450 inhibitors, as well as *R*-warfarin, were examined for their abilities to inhibit *S*-warfarin 7-hydroxylation; we found that *R*-warfarin was a non-competitive inhibitor with a K_i value of about 150 μM, whereas both tolbutamide and sulfaphenazole were competitive inhibitors with K_i values of about 100 and 0.5 μM, respectively, for *S*-warfarin 7-hydroxylation activities. These results suggest that *R*- and *S*-warfarin enantiomers are catalyzed principally by CYP1A2 and CYP2C9, respectively, in human liver microsomes, and that the pharmacokinetic properties of *S*-warfarin may be altered by *R*-warfarin *in vivo* when racemic warfarin is administered clinically to humans. BIOCHEM PHARMACOL **54**;11:1195–1203, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. CYP2C9; CYP1A2; *S*-warfarin; *R*-warfarin; inhibition; drug interaction

P450 or CYP† comprises a superfamily of enzymes that catalyze oxidation of a number of xenobiotic and endobiotic chemicals, and individual forms of P450 have considerable, but overlapping, substrate specificities [1–3]. P450 enzymes belonging to the CYP2C subfamily have been shown to constitute the major forms of P450 in mammalian livers and play important roles in the oxidation of clinically used drugs and other xenobiotic chemicals [4, 5]. In humans, at least four CYP2C gene products (CYP2C8, CYP2C9, CYP2C18, and CYP2C19) have been shown to be expressed in livers and, in some instances, in extrahepatic organs [4, 6]. CYP2C9 is one of the major CYP2C gene products that catalyzes oxidation of a variety of clinically used drugs such as tolbutamide, phenytoin, diclofenac, piroxicam, tienilic acid, tetrahydrocannabiol, torsemide, diazepam, and *S*-warfarin in human liver microsomes [4, 6].

Racemic warfarin has been used clinically as an oral anticoagulant drug with a narrow therapeutic index in humans and as an environmental rodenticide. The *S*-warfarin enantiomer has been determined to have the major pharmacological activities of racemic warfarin, while the *R*-enantiomer is essentially inactive [7]. Stereoselective hydroxylations of warfarin enantiomers have been reported in human P450 enzymes where *S*-warfarin 7-hydroxylation is catalyzed mainly by CYP2C9, whereas *R*-warfarin 7-hydroxylation appears to be catalyzed by several P450 enzymes [8–13]. However, there is some controversy as to which P450 enzyme(s) is (are) the major enzyme(s) involved in *R*-warfarin 7-hydroxylation in human liver microsomes.

It has been reported recently that there are a variety of chemicals that inhibit selectively the drug oxidation reactions catalyzed by specific forms of P450 in humans and that these phenomena are thought to be one of the important mechanisms for understanding the basis for drug–drug interactions [14–19]. Sulfaphenazole is reported to be a potent inhibitor of CYP2C9-dependent drug oxidations, and some other chemicals such as substrates for CYP2C9 have been shown to inhibit drug oxidations catalyzed by this particular P450 form in humans [12, 20–23].

The aim of the present study was to determine (a) whether *R*-warfarin inhibits *S*-warfarin metabolism by hu-

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† *Abbreviations:* P450 or CYP, cytochrome P450; b_5, cytochrome b_5; αNF, α-naphthoflavone; and DDC, diethyldithiocarbamate.

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man liver microsomes when racemic warfarin is used as a substrate *in vitro* and (b) which P450 form(s) is (are) the major enzyme(s) involved in the oxidation of *R*-warfarin in human liver microsomes. Liver microsomes from 35 human samples and recombinant human P450 enzymes expressed in human lymphoblast cells and in *Escherichia coli* were used as enzyme sources. In some experiments, liver microsomes from untreated male rats were also used.

MATERIALS AND METHODS

Chemicals

Tolbutamide and racemic warfarin were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). *S*- and *R*-Warfarin and their 4′-, 6-, 7-, 8-, and 10-hydroxylated metabolites and S-mephenytoin were obtained from the Ultra Fine Chemicals Co. (Manchester, U.K.). Other drug substrates, their metabolites, and the reagents used in this study were obtained from sources as described previously or were of the highest quality commercially available [5, 24, 25].

Enzyme Preparation

Human liver samples were obtained from organ donors or patients undergoing liver resection as described previously [5, 24, 25]. Male Sprague–Dawley rats (weighing about 200 g) were obtained from the Nihon Clea Co. (Osaka). Liver microsomes were prepared as described and suspended in 10 mM Tris–Cl buffer (pH 7.4) containing 1.0 mM EDTA and 20% glycerol (v/v) [26].

Microsomes of human lymphoblast cells expressing CYP1A1, 1A2, 2A6, 2B6, 2C9(Arg144) (wild-type of CYP2C9), 2C9(Cys144) (Cys144 variant of CYP2C9), 2C19, 2D6, 2E1, and 3A4 were purchased from the Gentest Co. (Woburn, MA, U.S.A.). Recombinant human CYP1A1, 1A2, 2C10(9), 2E1, and 3A4 [27–31], which were purified from membranes of *E. coli* in which respective cDNA clones have been introduced, were donated by Dr. F. P. Guengerich of Vanderbilt University School of Medicine. Liver microsomal CYP2C9 was purified to electrophoretic homogeneity by a method described previously [32]. NADPH-P450 reductase and b_5 were purified from liver microsomes of phenobarbital-treated rabbits by the method of Yasukochi and Masters [33] as modified by Taniguchi *et al.* [34]. Rabbit anti-P450 antibodies raised against human CYP1A2 and CYP2C9 were prepared and the IgG fractions were obtained as described [32, 35, 36].

Enzyme Assays

Microsomal incubations included liver microsomes (0.1 mg protein/mL) or microsomes (0.2 mg/mL) of recombinant P450 enzymes in human lymphoblast cells in 50 mM potassium phosphate buffer (pH 7.4) containing an NADPH-generating system consisting of 0.5 mM $NADP^+$, 5 mM glucose-6-phosphate, and 0.5 U of glucose-6-phosphate dehydrogenase/mL, and various concentrations of drug substrates [5, 24, 25]. Methyl hydroxylation of tolbutamide (substrate concentration of 2.5 mM) and 4′-hydroxylation of S-mephenytoin (0.4 mM) were determined using HPLC as described [20, 37–39]. 6- and 7-Hydroxylations of racemic and *R*- and *S*-warfarin were determined according to the original method [40]. The standard incubation mixture consisted of microsomal protein with substrates (0.10 mM) in a final volume of 0.20 mL of 50 mM potassium phosphate buffer (pH 7.4) containing the NADPH-generating system. Incubations were carried out at 37° for 15 min and were terminated by adding 10 μL of 60% $HClO_4$ (w/v). The separation of hydroxylated warfarin metabolites by HPLC was carried out with a C_{18} 5-μm analytical column (4.6 × 150 mm, Kanto Chemical, Tokyo) eluted with a mixture of 36% CH_3CN (v/v) containing 0.04% aqueous H_3PO_4 [40].

Reconstitution of warfarin hydroxylation activities (final volume of 0.20 mL) by recombinant CYP2C10 was carried out in systems containing P450 (5 pmol), NADPH-P450 reductase (10 pmol), b_5 (10 pmol), sodium cholate (100 nmol), and the lipid mixture (4 μg) in 50 mM potassium phosphate buffer (pH 7.4) containing the NADPH-generating system and warfarin (20 nmol). In the case of CYP3A4-dependent reconstitution, $MgCl_2$ (30 mM) and GSH (3 mM) were included, and 50 mM potassium phosphate buffer was replaced by 50 mM potassium HEPES buffer (pH 7.4). For the reconstitution with CYP1A1 and 1A2, the concentration of potassium phosphate buffer was raised to 100 mM, and b_5 was omitted from the incubation mixture.

7-Ethoxyresorufin O-deethylation activities were determined by a method described previously [41]. P450 contents were estimated spectrally by the method of Omura and Sato [42]. Protein concentrations were estimated by the method of Lowry *et al.* [43].

Statistical Analysis

Kinetic parameters for warfarin 7-hydroxylation by recombinant human P450 enzymes and by liver microsomes were estimated using a computer program (KaleidaGraph program from Synergy Software, Reading, PA, U.S.A.) designed for nonlinear regression analysis. The correlations between P450-dependent drug oxidation activities in different human liver microsomal preparations were analyzed using a linear regression analysis program (InStat program from GraphPad Software, San Diego, CA, U.S.A.).

RESULTS

R- and S-Warfarin 7-Hydroxylations by Liver Microsomes of Untreated Male Rats and of a Human Sample, HL-C15

R- and *S*-Warfarin were incubated with liver microsomes of untreated male rats and of a human sample (HL-C15), and their metabolites formed were detected by HPLC (Fig. 1). Rat liver microsomes catalyzed the 7-hydroxylation of *R*-warfarin more preferentially than that of *S*-warfarin,

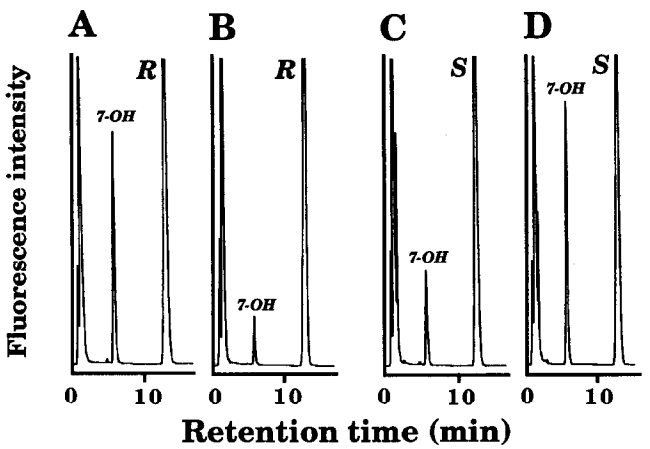

FIG. 1. HPLC profile of R-warfarin hydroxylation (A and B) and S-warfarin hydroxylation (C and D) by liver microsomes of untreated male rats (A and C) and of a human sample, HL-C15 (B and D). Abbreviations: 7-OH, 7-hydroxylated metabolite of R- and S-warfarin; R, R-warfarin; and S, S-warfarin.

while human liver microsomes had higher activities for S-warfarin. Other metabolites such as 6-hydroxylated metabolites of R- and S-warfarin were also detected by HPLC analysis, although the response was weak.

Correlation between 7-Hydroxylations of Warfarin Enantiomers and other Drug Oxidation Activities by Liver Microsomes of 35 Human Samples

Racemic and R- and S-warfarin 7-hydroxylation activities were determined in liver microsomes of 35 human samples and compared with those of tolbutamide methyl hydroxylation, S-mephenytoin 4′-hydroxylation, and 7-ethoxyresorufin O-deethylation (Fig. 2, Table 1). There was excellent correlation (r = 0.95) between racemic and S-warfarin 7-hydroxylation activities in 35 human samples examined, although the turnover numbers were lower in racemic warfarin 7-hydroxylation (1.6 ± 1.0 pmol/min/mg protein) than in S-warfarin 7-hydroxylation (4.1 ± 2.5 pmol/min/mg protein). Correlation coefficients between R-warfarin 7-hydroxylation versus racemic warfarin 7-hydroxylation and S-warfarin 7-hydroxylation were 0.69 and 0.55, respectively. We also found that there was good correlation between R-warfarin 7-hydroxylation and 7-ethoxyresorufin O-deethylation (r = 0.73) activities in these human samples. Correlation coefficients between tolbutamide methyl hydroxylation versus racemic and R- and S-warfarin 7-hydroxylation were 0.73, 0.66, and 0.70, respectively.

R- and S-Warfarin 7-Hydroxylations by Recombinant Human P450 Enzymes

Racemic and R- and S-warfarin 7-hydroxylation activities were determined in recombinant human P450 enzymes in microsomes of human lymphoblastoid cells (Table 2). The 7-hydroxylation activities of warfarin enantiomers by liver microsomes of 35 human samples were also presented in this table. S-Warfarin 7-hydroxylation was highly catalyzed by CYP2C9(Arg) followed by CYP2C9(Cys); these activities were higher than those catalyzed by human liver microsomes. Other recombinant P450 enzymes, except for CYP2C19, had no measurable activities for S-warfarin 7-hydroxylation activities. Of these recombinant P450 enzymes examined, CYP2C9(Arg), CYP2C19, CYP2C9(Cys), and CYP1A2 had substantial activities for R-warfarin 7-hydroxylation, while the activities of CYP1A1, 2A6, 2B6, 2D6, 2E1, and 3A4 were low. The turnover numbers for racemic warfarin 7-hydroxylation activities by recombinant CYP2C9 enzymes were lower than those of S-warfarin 7-hydroxylation activities, but higher than those of R-warfarin 7-hydroxylation activities.

Kinetic analysis of the 7-hydroxylations of R- and S-warfarin were determined in reconstituted systems containing recombinant CYP1A1, 1A2, 2C10, 2E1, and 3A4 and in human liver microsomes (Table 3). Of the recombinant P450 enzymes examined, CYP1A1 had the highest V_{max} value for R-warfarin 7-hydroxylation with a high K_m value. CYP1A2 and 2C10 gave similar rates for R-warfarin 7-hydroxylation activities having similar K_m values; the V_{max} and K_m values of these two P450 enzymes were comparable to those of liver microsomes of two human samples, HL-C15 and HL-C19. CYP2C10 had the highest V_{max} value for S-warfarin 7-hydroxylation with the lowest K_m value among the recombinant P450s examined. The K_m values with liver microsomes of two human samples were about the same as those of CYP2C10 in the reconstituted system. CYP2E1 and 3A4 had negligible activities for R- and S-warfarin 7-hydroxylations.

Effects of anti-CYP1A2 and anti-CYP2C9 on R- and S-Warfarin 7-Hydroxylation Activities by Human Liver Microsomes

Specific antibodies raised against CYP2C9 and CYP1A2 were examined to determine whether or not these antibodies inhibit R- and S-warfarin 7-hydroxylations catalyzed by human liver microsomes (Fig. 3). Anti-CYP2C9 IgG was found to inhibit completely S-warfarin 7-hydroxylation by human liver microsomes (human sample HL-C15), but did not affect R-warfarin 7-hydroxylation. On the other hand, anti-CYP1A2 IgG inhibited by about 70% R-warfarin 7-hydroxylation by human liver microsomes, although it did not affect the activities of S-warfarin 7-hydroxylation.

Effects of several P450 Inhibitors and R-Warfarin on S-Warfarin 7-Hydroxylation by Human Liver Microsomes

As described above, warfarin 7-hydroxylation activities were lower when racemic warfarin, rather than S-warfarin, was used as a substrate, indicating that R-warfarin may inhibit S-warfarin 7-hydroxylation activities in human liver microsomes. Several P450 inhibitors and CYP2C9 substrates as well as R-warfarin were used to examine their effects on tolbutamide methyl hydroxylation, S-mephenytoin 4′-hydroxylation, S-warfarin 7-hydroxylation, and R-warfarin 7-hydroxylation activities catalyzed by human

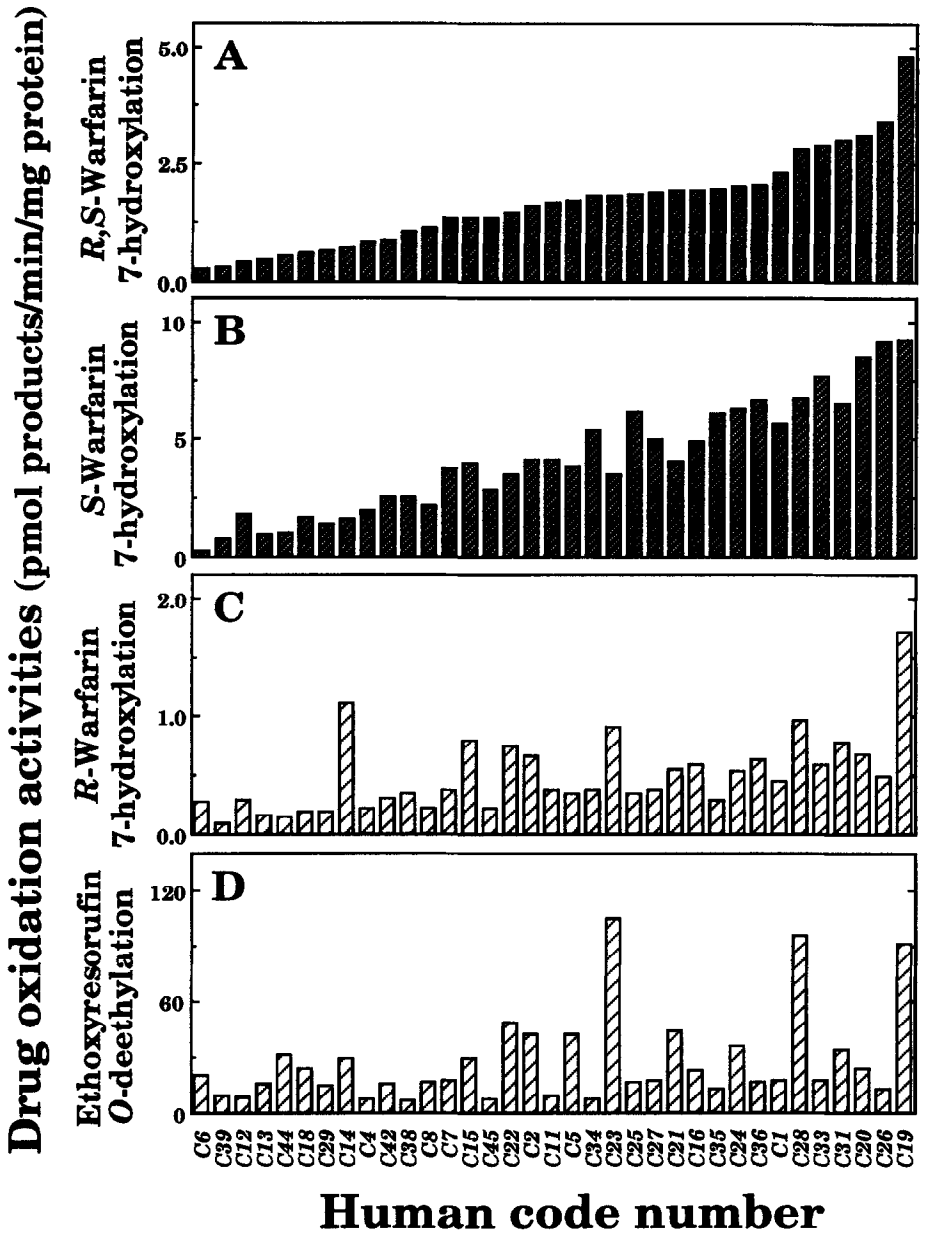

FIG. 2. Activities of *R,S*-warfarin 7-hydroxylation (A), *S*-warfarin 7-hydroxylation (B), *R*-warfarin 7-hydroxylation (C), and 7-ethoxyresorufin *O*-deethylation (D) by liver microsomes of 35 human samples. The order of human samples was plotted with increasing *R*, *S*-warfarin 7-hydroxylation activities from left to right, as shown in part A.

liver microsomes (sample HL-C15) (Fig. 4). αNF, fluvoxamine, and DDC were found to inhibit all of the microsomal activities examined with varying extents. There were differences in inhibition of tolbutamide methyl hydroxylation and *S*-warfarin 7-hydroxylation with those of *S*-mephenytoin 4′-hydroxylation by several chemicals in which the latter reaction was not inhibited by sulfaphenazole, diazepam, diclofenac, *R*-warfarin, and tolbut-

TABLE 1. Correlation between liver microsomal drug oxidation activities in 35 human samples

	Correlation coefficient, *r*				
	R-Warfarin	*S*-Warfarin	Tolbutamide	*S*-Mephenytoin	7-Ethoxyresorufin
R,S-Warfarin	0.69	0.95	0.73	0.30	0.45
R-Warfarin		0.55	0.66	0.34	0.73
S-Warfarin			0.70	0.29	0.27
Tolbutamide				0.44	0.41
S-Mephenytoin					0.15

Key: *R,S*-Warfarin, *R,S*-warfarin 7-hydroxylation; *R*-Warfarin, *R*-warfarin 7-hydroxylation; *S*-Warfarin, *S*-warfarin 7-hydroxylation; Tolbutamide, tolbutamide methyl hydroxylation; *S*-Mephenytoin, *S*-mephenytoin 4′-hydroxylation; 7-Ethoxyresorufin, 7-ethoxyresorufin O-deethylation.

TABLE 2. Warfarin 7-hydroxylation by recombinant human P450s and by human liver microsomes

	Warfarin 7-hydroxylation (pmol/min/nmol P450)		
	R,S-Warfarin	R-Warfarin	S-Warfarin
CYP1A1	0.3 ± 0.1	0.4 ± 0.3	< 0.1
CYP1A2	1.3 ± 0.1	3.2 ± 0.1	< 0.1
CYP2A6	< 0.1	< 0.1	< 0.1
CYP2B6	< 0.1	0.8 ± 0.8	< 0.1
CYP2C9(Arg)	219 ± 2.8	20 ± 1.9	392 ± 13
CYP2C9(Cys)	35 ± 3.5	4.8 ± 0.2	55 ± 1.3
CYP2C19	14 ± 2.1	15 ± 2.6	2.5 ± 0.2
CYP2D6	0.2 ± 0.2	0.9 ± 0.1	< 0.1
CYP2E1	< 0.1	1.0 ± 0.1	< 0.1
CYP3A4	< 0.1	1.0 ± 0.1	< 0.1
Liver microsomes	5.0 ± 2.7	1.5 ± 0.7	12.9 ± 7.8

Data are means ± range of duplicate determinations for the recombinant P450s and means ± SD of 35 human samples for liver microsomal activities.

amide, while the former two reactions were inhibited by these chemicals with varying extents. Differences were also noted in inhibition of S- and R-warfarin 7-hydroxylations by several chemicals, particularly in the effects of sulfaphenazole, which was a potent inhibitor of S-warfarin 7-hydroxylation, but not R-warfarin 7-hydroxylation, catalyzed by human liver microsomes. Ketoconazole, an inhibitor of CYP3A4 activities [44, 45], was found to inhibit S-warfarin 7-hydroxylation, but not other reactions, in human liver microsomes.

Inhibition of S-Warfarin 7-Hydroxylation by Sulfaphenazole, Tolbutamide, and R-Warfarin in Human Liver Microsomes

The effects of R-warfarin, tolbutamide, and sulfaphenazole on S-warfarin 7-hydroxylation were determined kinetically in liver microsomes of human sample HL-C19, in microsomes of human lymphoblast cells expressing CYP2C9, and in reconstituted systems containing CYP2C10 (Fig. 5). Tolbutamide and sulfaphenazole were found to be competitive inhibitors of S-warfarin 7-hydroxylation in the three P450 systems examined, while R-warfarin inhibited S-warfarin 7-hydroxylation non-competitively (Figs. 5 and 6). The K_i values for the inhibition of S-warfarin 7-hydroxylation by R-warfarin, sulfaphenazole, and tolbutamide were determined to be approximately 150, 0.5, and 100 μM, respectively, in human liver microsomes (Table 4). The K_i values by R-warfarin and sulfaphenazole of the inhibition of S-warfarin 7-hydroxylation in recombinant CYP2C9 and 2C10 enzymes were determined to be approximately 60 and 0.3 μM, respectively.

TABLE 3. Kinetic analysis of warfarin 7-hydroxylation by reconstituted systems containing recombinant human P450 enzymes

	R-Warfarin		S-Warfarin	
Enzyme	K_m (μM)	V_{max} (pmol/min/nmol P450)	K_m (μM)	V_{max} (pmol/min/nmol P450)
(Reconstitution)				
CYP1A1	186	26	33	3
CYP1A2	84	15	304	11
CYP2C10	80	9	31	135
CYP2E1		0.8		0.4
CYP3A4		1.0		0.7
(Human liver microsomes)				
HL-C15	107	2.4	32	14.0
HL-C19	161	3.9	22	15.6

Data are means of duplicate determinations.

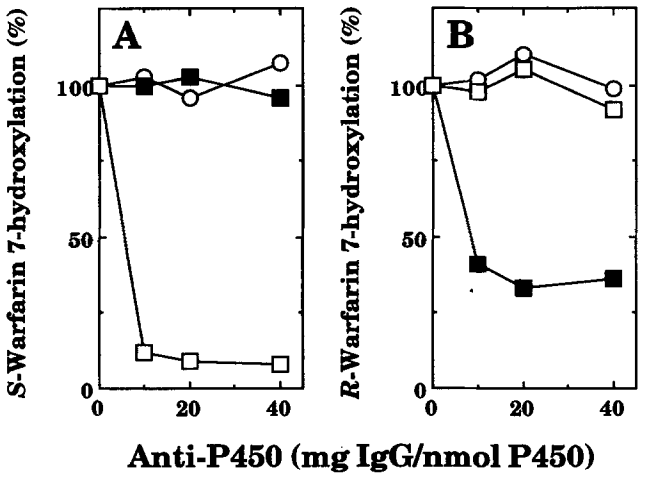

FIG. 3. Effects of preimmune IgG (○), anti-CYP1A2 IgG (■), and anti-CYP2C9 IgG (□) on S-warfarin 7-hydroxylation (A) and R-warfarin 7-hydroxylation (B) activities catalyzed by liver microsomes of HL-C15. Control activities without antibodies were 21 and 3.8 pmol/min/nmol P450 for S-warfarin 7-hydroxylation and R-warfarin 7-hydroxylation, respectively.

DISCUSSION

A number of studies have suggested that S-warfarin enantiomer is catalyzed principally by CYP2C9 to a 7-hydroxylated product, which is one of the major metabolites of S-warfarin, in humans [8–10, 12, 46]. However, which P450 forms are the principal ones involved in the R-warfarin 7-hydroxylation in human liver microsomes was not precisely identified [7, 13]. The present findings that there was good correlation between R-warfarin 7-hydroxylation and 7-ethoxyresorufin O-deethylation activities in liver microsomes of 35 human samples and that anti-CYP1A2 inhibited R-warfarin 7-hydroxylation by human liver microsomes supported the view that CYP1A2 is a major P450 enzyme involved in R-warfarin 7-hydroxylation by human liver microsomes. However, among recombinant P450s in the human lymphoblast cells examined, CYP2C9(Arg), CYP2C19, and CYP2C9(Cys) were found to have higher activities for R-warfarin 7-hydroxylation than those catalyzed by CYP1A2. The possible role of CYP2C9 in R-warfarin 7-hydroxylation seemed to be minor, because anti-CYP2C9 antibodies and sulfaphenazole did not cause inhibition of R-warfarin 7-hydroxylation

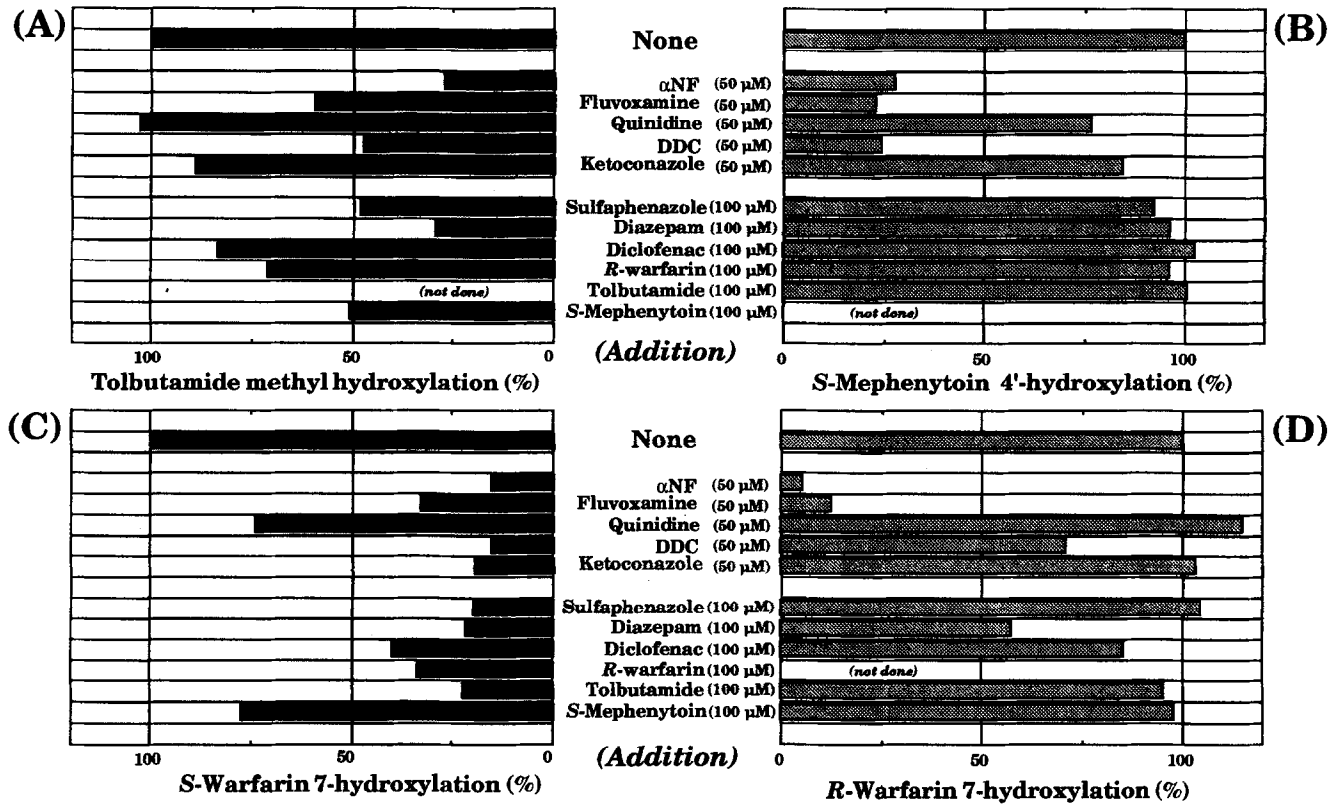

FIG. 4. Effects of several chemicals on tolbutamide methyl hydroxylation (A), S-mephenytoin 4'-hydroxylation (B), S-warfarin 7-hydroxylation (C), and R-warfarin 7-hydroxylation (D) catalyzed by liver microsomes of a human sample, HL-C15. The concentrations of chemicals included in the incubation mixtures are shown in the figure. The substrate concentration used was 100 μM in these cases. The control activities (pmol/min/mg protein) without addition of chemicals were 404 for tolbutamide methyl hydroxylation, 140 for S-mephenytoin 4'-hydroxylation, 9.6 for S-warfarin 7-hydroxylation, and 1.2 for R-warfarin 7-hydroxylation. Abbreviations used in this figure are: αNF, α-naphthoflavone; and DDC, diethyldithiocarbamate.

activities catalyzed by human liver microsomes. In addition, the finding that anti-CYP2C9, which inhibits CYP2C19-dependent S-mephenytoin 4'-hydroxylation [32] as well as CYP2C9-dependent S-warfarin 7-hydroxylation in humans, did not affect R-warfarin 7-hydroxylation activities suggested that the role of CYP2C19 in the R-warfarin 7-hydroxylation reaction may also be minor. These results suggest that R-warfarin 7-hydroxylation is catalyzed princi-

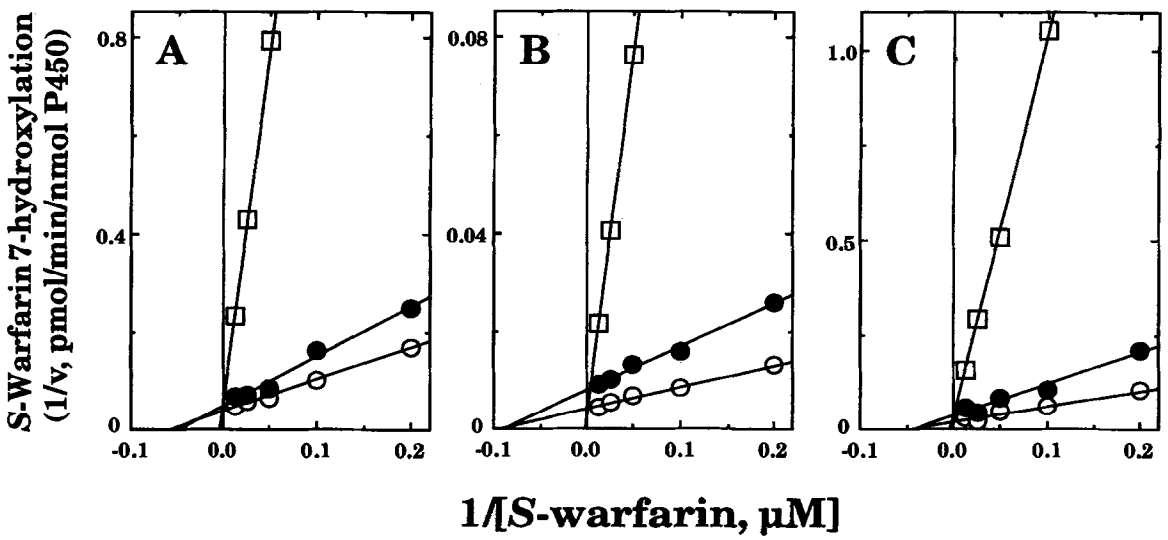

FIG. 5. Effects of R-warfarin (50 μM, ●) and sulfaphenazole (10 μM, □) on the kinetics of S-arfarin 7-hydroxylation catalyzed by liver microsomes of a human sample, HL-C19 (A), by microsomes expressing CYP2C9 (B), and by a reconstituted system containing CYP2C10 (C). Control incubations in the absence of R-warfarin and sulfaphenazole are shown by an open circle (○).

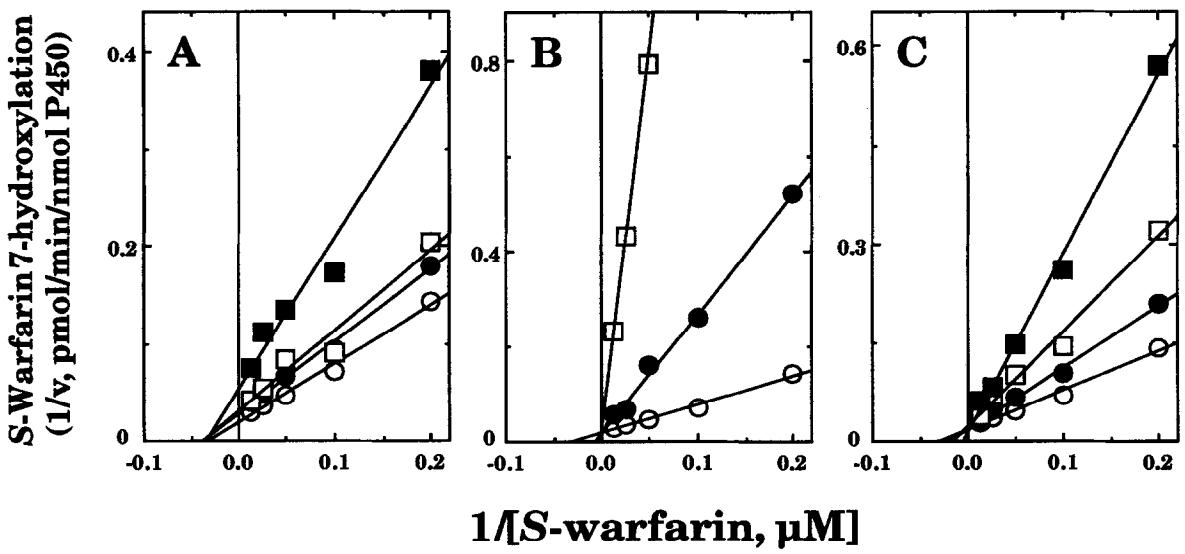

FIG. 6. Effects of concentrations of R-warfarin (A), sulfaphenazole (B), and tolbutamide (C) on S-warfarin 7-hydroxylation catalyzed by liver microsomes of a human sample, HL-C15. Concentrations of R-warfarin were 0 (○), 50 (●), 100 (□), and 200 μM (■) in panel A. Concentrations of sulfaphenazole were 0 (○), 1 (●), and 10 μM (□) in panel B. Concentrations of tolbutamide were 0 (○), 50 (●), 100 (□), and 200 μM (■) in panel C.

pally by CYP1A2 rather than by the CYP2C and other P450 enzymes in human liver microsomes [7, 10, 13].

Drug–drug interactions associated with inhibition of specific P450 enzymes by co-administered drugs have been shown to affect the bioavailabilities of drugs when these drugs are administered simultaneously [16, 18, 19, 22, 47, 48]. Terfenadine/ketoconazole interaction is one of the cases that caused serious drug interactions in humans [14, 49, 50]. The inhibitors for human P450 enzymes reported to date include furafylline, fluvoxamine, and αNF for CYP1A2, sulfaphenazole for CYP2C9, quinidine for CYP2D6, DDC for CYP2E1, and ketoconazole, troleandomycin, and gestodene for CYP3A4 [44, 51–53]. DDC has also been shown to inhibit CYP2A6-coumarin 7-hydroxylation by human liver microsomes [54].

In the present study, we found that warfarin 7-hydroxylation activities by human liver microsomes were lower when racemic warfarin, rather than S-warfarin, was used as a substrate. The turnover numbers obtained for racemic warfarin 7-hydroxylation were 1.6 ± 1.0 pmol/min/mg protein and those for S-warfarin 7-hydroxylation were 4.1 ± 2.5 pmol/min/mg protein in 35 human samples examined. These results suggest that R-warfarin may be an inhibitor of S-warfarin 7-hydroxylation activities in human liver microsomes. In fact, when R-warfarin was added to the incubation mixture for the analysis of S-warfarin 7-hydroxylation by human liver microsomes, the formation of 7-hydroxylated metabolite was suppressed. Kinetic analysis suggested that R-warfarin was a non-competitive inhibitor of S-warfarin 7-hydroxylation by liver microsomes with a K_i value of about 100 μM. The non-competitive nature of R-warfarin inhibition of S-warfarin 7-hydroxylation was also supported in systems containing CYP2C9 in microsomes of human lymphoblast cells and in reconstituted systems containing recombinant CYP2C10; the results are inconsistent with those reported by Kunze *et al.* [55], who showed that R-warfarin inhibits S-warfarin 7-hydroxylation in a competitive manner with a K_i value of 5–10 μM in human liver microsomes. The reasons for the discrepancies between their and our results are unclear at present; however, it should be mentioned that R-warfarin may not be a preferred substrate for CYP2C9 in human liver microsomes, as discussed above [7, 13].

Present results supported the previous view that sulfaphenazole is a very potent and competitive inhibitor of S-warfarin 7-hydroxylation activities by recombinant human CYP2C9 [9]. The K_i values obtained for the inhibition of S-warfarin 7-hydroxylation were estimated to be 0.2 to 1.0 μM in human liver microsomes, in microsomes of human lymphoblast cells expressing CYP2C9, and in reconstituted systems containing recombinant CYP2C10; the

TABLE 4. Inhibition of S-warfarin 7-hydroxylation by R-warfarin, sulfaphenazole, and tolbutamide in human liver microsomes and in recombinant CYP2C9 and 2C10 enzymes

Enzyme	Inhibition of S-warfarin 7-hydroxylation Inhibition constant (K_i, μM)		
	R-Warfarin	Sulfaphenazole	Tolbutamide
(Liver microsomes)			
HL-C15	167 ± 31	0.53 ± 0.04	90 ± 36
HL-C19	145	0.39	
HL-C31	149	0.78	98
(Recombinant P450s)			
CYP2C9*	52	0.23	
CYP2C10†	69	0.39	

K_i values were obtained from the data set using a non-linear kinetic analysis of means in single (without ± SEM) and two (with ± range) or three (with ± SEM) concentrations of inhibitors with different concentrations of substrate as indicated.

* Microsomal system containing CYP2C9 in human lymphoblast cells.

† Reconstituted system containing CYP2C10 expressed in *E. coli*.

results are consistent with the results of Rettie *et al.* [9]. Tolbutamide, a known substrate for CYP2C9 [20], has also been shown to be a competitive inhibitor of S-warfarin 7-hydroxylation, though not as potent (K_i value of about 100 μM) as sulfaphenazole. Other CYP2C9 substrates including diazepam and diclofenac also inhibited S-warfarin 7-hydroxylation by human liver microsomes.

S-Mephenytoin 4′-hydroxylation was found to be unaffected by CYP2C9 inhibitors and substrates such as sulfaphenazole, tolbutamide, diazepam, and diclofenac, but was inhibited by fluvoxamine and DDC at a concentration of 50 μM. It has been reported recently that fluvoxamine, which has been shown to be a potent inhibitor of CYP1A2 [56–58], also inhibits CYP2C19-dependent activities [59]. In addition, fluvoxamine was found to inhibit CYP2C9-dependent S-warfarin 7-hydroxylation in human liver microsomes, and we also found that DDC was an inhibitor of CYP2C9 and CYP2C19.

In conclusion, the present results showed that *R*- and S-warfarin 7-hydroxylations are catalyzed principally by CYP1A2 and CYP2C9, respectively, in human liver microsomes and that *R*-warfarin inhibits S-warfarin 7-hydroxylation by human liver microsomes. The mechanisms underlying inhibition of CYP2C9 between *R*-warfarin and sulfaphenazole may be different since the former chemical inhibits S-warfarin 7-hydroxylation non-competitively while the latter inhibits it competitively. These results suggest that S-warfarin pharmacokinetics may be altered when racemic warfarin is administered to humans in a clinical situation.

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