The effect of ethanol on the metabolism of S-warfarin and diclofenac by recombinant cytochrome P450 2C9.1 (CYP2C9.1) was studied. The 7-hydroxylation metabolism of S-warfarin was inhibited by as low as 0.1 vol% (17 mM) ethanol. Ethanol decreased the $V_{max} / K_m$ and $V_{max}$ values of S-warfarin metabolism in a concentration-dependent manner, but the $K_m$ value was unchanged by ethanol. The inhibitory effect of ethanol on the 4'-hydroxylation metabolism of diclofenac was not observed even at 1.0 vol% (170 mM) ethanol. Ethanol at a concentration of 3.0 vol% (510 mM) increased the $K_m$ value of diclofenac metabolism without changes in the $V_{max}$, which indicates that diclofenac 4'-hydroxylation by CYP2C9.1 was competitively inhibited by ethanol. S-Warfarin metabolism by CYP2C9.1 was more sensitive to ethanol than diclofenac metabolism. These results suggest that ethanol inhibits the metabolism by CYP2C9.1 in a substrate-dependent manner.

**Key words** warfarin; ethanol; cytochrome P450 2C9.1 microsome; diclofenac; drug interaction; metabolism

Warfarin, which consists of a racemic mixture of S- and R-enantiomer, has been used as an anticoagulant agent. The anticoagulant activity of S-warfarin is 3—5 times greater than that of R-warfarin. This drug has a narrow therapeutic index and shows marked drug-drug interactions when coadministered with other agents that alter warfarin metabolism. The stereoselective metabolism of warfarin enantiomers in humans has been shown to be catalyzed by cytochrome P450 (CYP) such as CYP1A1/2, 2C9, and 3A4. The 7-hydroxylation of S-warfarin is exclusively catalyzed by CYP2C9. R-Warfarin is metabolized primarily by CYP1A2 to 6- and 8-hydroxywarfarin, and by CYP3A4 to 10-hydroxywarfarin. Diclofenac, a non-steroidal anti-inflammatory drug (NSAID), is also metabolized to 4'-hydroxydiclofenac by CYP2C9.6,7

Ethanol is widely used as a pharmaceutical excipient for the solubilization of many hydrophobic drugs. Various drugs can pharmacokinetically or pharmacodynamically interact with alcohol. Our previous study suggested that ethanol changes the binding of warfarin enantiomer to human serum albumin stereoselectively. Acute intoxication by alcohol reduces the metabolism of warfarin by CYPs, causing increased anticoagulant effects, and leading to a risk of hemorrhage. Conversely, chronic alcohol ingestion might enhance metabolic enzyme activity, leading to a decrease in the anticoagulant effects of warfarin. Hamitouche et al. showed that CYP2C9, as well as other CYPs such as CYP2E1, CYP1A2, and CYP3A4, is also able to metabolize ethanol, and indicated that these CYPs have a low affinity for ethanol for its metabolization. The apparent $K_m$ value determined for ethanol oxidation by various CYPs was around 10 mM. Therefore, it is possible that ethanol affects the metabolism of other substrates by CYP2C9. Few studies on the effect of ethanol on warfarin metabolism by human CYP2C9 in vitro have been reported. The non-significant metabolic interaction of diclofenac (15 μM) with ethanol by CYP2C9 has been reported. However, the effect of ethanol on the metabolism of diclofenac at lower concentrations by CYP2C9 has not been examined. In this paper, we have examined the effect of ethanol on the metabolism of S-warfarin and diclofenac by recombinant CYP2C9.1 microsomes (CYP2C9.1).

**MATERIALS AND METHODS**

**Materials** $S$-(-)-Warfarin, diclofenac sodium, ethanol, naproxen, NADP+ glucose-6-phosphate, MgCl2·6H2O, and glucose-6-phosphate dehydrogenase were purchased from Wako Pure Chemical Ind. (Osaka, Japan). 4'-Hydroxydiclofenac was purchased from Sigma-Aldrich (MO, U.S.A.). 7-Hydroxywarfarin and human CYP2C9.1+P450 reductase microsomes derived from baculovirus expression systems were purchased from BD Gentest (MA, U.S.A.). All other chemicals and solvents were of analytical grade or higher.

**Inhibition Study** According to the method of Iwakawa et al., a metabolic inhibition study was performed. The time of incubation and concentration of microsomal protein in the study were determined to be in a linear range for the rate of formation of 7-hydroxywarfarin or 4'-hydroxydiclofenac. Initially, S-warfarin or diclofenac (final concentration: 1—10 μM) in 50 mM Tris–HCl (pH 7.4) with or without ethanol (0.05—3.0 vol%; 8.5—510 mM) and an NADPH-regenerating system (NADP+: 0.5 mM, glucose-6-phosphate: 2 mM, MgCl2: 4 mM, glucose-6-phosphate dehydrogenase: 1 U/ml) were preincubated for 10 min at 37°C, and then CYP2C9.1 (6.5 mM) was added to the preincubated mixture at 37°C. The total incubation volume was 0.25 ml. After 30 min, the incubation was terminated by the addition of acetonitrile (0.25 ml) containing 2.7 μM naproxen as an internal standard. The mixture was centrifuged for 5 min at 18620×g at 4°C. An aliquot of the supernatant was used for the analysis of warfarin metabolites or diclofenac metabolites by the HPLC system. Incubations were performed in duplicate.

**HPLC Analysis** According to a slightly modified method reported by Iwakawa et al. the contents of 7-hydroxywarfarin enantiomers and 4'-hydroxydiclofenac were...
analyzed by HPLC using a chiral column. HPLC analyses were performed using a Shimadzu SCL-10Avp system controller, a Shimadzu LC-10Adp pump, a Shimadzu SIL-10Advp auto-injector, a Shimadzu SPD-10Avp variable wavelength UV detector, a Shimadzu RF-10AXL fluorescence detector (Kyoto, Japan), and a Daicel Chiralcel OD-RH column (4.6 mm i.d. ×150 mm, 5 μm) (Tokyo, Japan). The mobile phase consisted of 0.1 M sodium dihydrogen phosphate pH 2.0/acetonitrile (65/35 by volume). The flow rate was 0.5 ml/min. The column temperature was maintained at 40°C. The injected volume for the S-warfarin or diclofenac sample was 25 μl or 50 μl, respectively. For detecting 7-hydroxywarfarin, the effluent was monitored at 312 nm with a UV detector and at an excitation wavelength of 320 nm and an emission wavelength of 415 nm with a fluorescence detector. For detecting 4’-diclofenac, the UV detector was set at 280 nm.14)

Statistical Analysis Data are expressed as the mean values and standard deviation. Analysis of variance (ANOVA) was used to test the significance of differences among groups. Significance regarding differences in the means among groups was determined by Tukey’s test. Significance was defined as p<0.05.

RESULTS

The ethanol concentration to inhibit S-warfarin metabolism was lower than that to inhibit diclofenac metabolism (Fig. 1). The inhibitory effect of ethanol on the metabolism of 1 or 10 μM S-warfarin was observed by increasing the ethanol concentration. The metabolism of 1 μM diclofenac was also decreased by increasing the ethanol concentration. However, ethanol only slightly inhibited the rate of metabolism of 10 μM diclofenac.

Ethanol non-significantly affected the Km value of S-warfarin metabolism (Table 1). On the contrary, the Vmax and Vmax/Km (apparent intrinsic clearance) values of S-warfarin metabolism decreased linearly with an increasing ethanol concentration. The Vmax and Vmax/Km values in the presence of 1 vol% (170 mM) ethanol were about 75 and 85%, respectively, being lower than those of the control. These results suggest that the inhibition profile of ethanol on 7-hydroxylation metabolism of the S-warfarin enantiomer by CYP2C9.1 shows an allosteric phenomenon.

Non-significant changes in the Vmax value of diclofenac metabolism were observed up to 3.0 vol% (510 mM) of ethanol. The Km value of diclofenac metabolism was increased from 3.3±0.6 to 5.5±0.7 μM in the presence of 3.0 vol% (510 mM) ethanol (p<0.05). The Vmax/Km value of diclofenac metabolism was decreased with an increasing ethanol concentration. The Vmax/Km value (1.2±0.1 ml/min/nmol P450) with 3.0 vol% (510 mM) ethanol was about 40% lower than the Vmax/Km value (2.0±0.1 ml/min/nmol P450) of the control (Table 2). These results suggest that ethanol inhibits 4’-hydroxylation of diclofenac by CYP2C9.1 in a competitive manner.

DISCUSSION

Acute intoxication by alcohol reduces the metabolism of warfarin by cytochrome P450, leading to increased anticoagulant effects and the risk of hemorrhage.10) Practically, an increased antithrombotic effect of warfarin by ethanol was induced by protein-binding interactions and decreased warfarin metabolism through the cytochrome P450 enzyme system.15) Our previous study suggested that ethanol changes the binding of warfarin enantiomer to human serum albumin stereoselectively.15) However, that study was performed 2.9% (500 mM) of ethanol. The present study indicated that ethanol affected warfarin metabolism by CYP2C9.1. The 7-hydroxylation metabolism of S-warfarin was inhibited by as low as 0.1 vol% (17 mM) ethanol, which suggest the inhibition of S-warfarin metabolism by CYP2C9.1 is more sensitive to ethanol than that of S-warfarin binding to human serum albumin.

Our findings showed that S-warfarin 7-hydroxylation and diclofenac 4’-hydroxylation by Recombinant Human CYP2C9.1 were inhibited by ethanol, whereas Busby et al.12) reported that CYP2C9 (diclofenac 4’-hydroxylase activity) was not inhibited by ethanol (0.3—3 vol%; 51—510 mM). Their study was performed using a high concentration of diclofenac (15 μM). In our study performed with a relatively low concentration of diclofenac (1—10 μM), which is comparable to the blood concentration during medication, ethanol inhibited diclofenac 4’-hydroxylation by CYP2C9.1, which may be ex-

| Table 1. Kinetic Parameters for 7-Hydroxylation of S-Warfarin by Recombinant Human CYP2C9.1 |
|-----------------------------|-------------|---------|------|
| Ethanol (vol%) | Vmax (pmol/min/nmol P450) | Km (μM) | Vmax/Km (pmol/min/nmol P450) |
| Control | 64.1±13.5 | 2.5±1.1 | 28.2±7.1 |
| 0.05% | 48.5±14.4 | 2.5±1.0 | 20.4±5.0 |
| 0.10% | 50.9±6.2 | 3.9±0.5 | 13.0±0.7 |
| 0.30% | 33.0±6.1 | 4.5±1.1 | 7.5±0.7 |
| 1.00% | 16.1±3.8 | 4.6±1.9 | 4.1±2.3 |

Each value represents the mean±S.D. (n=3). *p<0.05, **p<0.01 (Tukey’s t-test).

| Table 2. Kinetic Parameters for 4’-Hydroxylation of Diclofenac by Recombinant Human CYP2C9.1 |
|-----------------------------|-------------|---------|------|
| Ethanol (vol%) | Vmax (nmol/min/nmol P450) | Km (μM) | Vmax/Km (nmol/min/nmol P450) |
| Control | 6.5±1.0 | 3.3±0.6 | 2.0±0.1 |
| 1.0% | 5.8±0.5 | 3.6±0.1 | 1.6±0.2 |
| 3.0% | 6.3±0.6 | 5.5±0.7 | 1.2±0.1 |

Each value represents the mean±S.D. (n=3). *p<0.05, **p<0.01 (Tukey’s t-test).
plained by the differences in experimental conditions.

The inhibitory effect of ethanol on S-warfarin metabolism by CYP2C9 differed from that on diclofenac metabolism. S-Warfarin 7-hydroxylation by CYP2C9.1 was inhibited by ethanol at as low as 0.1 vol% (17 mM) allosterically. The $V_{\text{max}}$ value of S-warfarin 7-hydroxylation decreased linearly with an increasing ethanol concentration, although the $K_m$ value was unaffected. On the contrary, diclofenac 4'-hydroxylation by CYP2C9.1 was inhibited by 3 vol% (510 mM) ethanol competitively. The $K_m$ value of diclofenac 4'-hydroxylation was increased in the presence of 3.0 vol% (510 mM) ethanol and non-significant changes in the $V_{\text{max}}$ value were observed up to 3.0 vol% (510 mM) of ethanol. Since it was reported that ethanol denatures globular proteins, the allosteric inhibition of S-warfarin metabolism by ethanol may result in structural changes in the binding site of CYP2C9.1. In contrast, structural changes of the binding site brought about by ethanol at a low concentration did not alter the metabolism of diclofenac, a planar structure compound. Busby et al. suggested that the inhibitory effects of solvents are substrate-dependent for a given CYP. The substrate-dependent inhibitory effect of acetonitrile was also observed in the metabolism of various drugs by CYP2C9. The present study suggests that the inhibitory effects of ethanol on drug metabolism are substrate-dependent for CYP2C9.1. Further experiments are required to define the extent of this substrate selectivity.

REFERENCES