The oral anticoagulant acenocoumarol is given as a racemic mixture. The (S)-enantiomer is rapidly cleared and is the reason why only (R)-acenocoumarol contributes to the pharmacological effect. The objective of the study was to establish the cytochrome P450 (CYP) enzymes catalyzing the hydroxylations of the acenocoumarol enantiomers. Of various cDNA-expressed human CYPs, only CYP2C9 hydroxylated (S)-acenocoumarol. Hydroxylation occurred at the 6-, 7-, and 8-position with equal \( K_m \) values and a ratio of 0.9:1:0.1 for \( V_{max} \). CYP2C9 also mediated the 6-, 7-, and 8-hydroxylations of (R)-acenocoumarol with \( K_m \) values three to four times and \( V_{max} \) values one-sixth times those of (S)-acenocoumarol. (R)-Acenocoumarol was also metabolized by CYP1A2 (6-hydroxylation) and CYP2C19 (6-, 7-, and 8-hydroxylation). In human liver microsomes one enzyme only catalyzed (S)-acenocoumarol hydroxylations with \( K_m \) values < 1 \( \mu \)M. In most of the samples tested the 7-hydroxylation of (R)-acenocoumarol was also catalyzed by one enzyme only. The 6-hydroxylation was catalyzed by at least two enzymes. Sulfaphenazole could completely inhibit in a competitive way the hydroxylations of (S)-acenocoumarol and the 7-hydroxylation of (R)-acenocoumarol. The 6-hydroxylation of (R)-acenocoumarol could be partially inhibited by sulfaphenazole, 40 to 50%, and by furafylline, 20 to 30%. Significant mutual correlations were obtained between the hydroxylations of (S)-acenocoumarol, the 7-hydroxylation of (R)-acenocoumarol, the 7-hydroxylation of (S)-warfarin, and the methylhydroxylation of tolbutamide. The results demonstrate that (S)-acenocoumarol is hydroxylated by a single enzyme, namely CYP2C9. CYP2C9 is also the main enzyme in the 7-hydroxylation of (R)-acenocoumarol. Other enzymes involved in (R)-acenocoumarol hydroxylation reactions are CYP1A2 and CYP2C19. Drug interactions must be expected, particularly for drugs interfering with CYP2C9. Also, drugs interfering with CYP1A2 and CYP2C19 may potentiate acenocoumarol anticoagulant therapy.

The clinical use of oral anticoagulant drugs in the management of thromboembolic disorders is widely expanding (Hylek et al., 1996; Smith, 1999). Generally, oral anticoagulation is characterized by a narrow therapeutic index, which needs careful effect monitoring. In this respect drug-drug interactions are notorious for the disturbance of the delicate dose-effect relationship of oral anticoagulant therapy (Hirsh, 1991; Harder and Thir mann, 1996).

Racemic warfarin is the most widely prescribed oral anticoagulant drug. The pharmacological activity resides mainly in the (S)-enantiomer of warfarin. Warfarin is eliminated almost completely by biotransformation. The predominant route of biotransformation of (S)-warfarin is 7-hydroxylation of the coumarin ring, which is catalyzed particularly by the cytochrome P450 (CYP) \(^1\) enzyme CYP2C9 (Rettie et al., 1992; Kaminsky and Zhang, 1997). Drug interactions that potentiate the effect of warfarin can be mostly ascribed to interference with the metabolism of (S)-warfarin. The stereoselective reduction of the metabolic clearance of (S)-warfarin by interacting drugs has been demonstrated in several human studies (Toon et al., 1986; O’Reilly et al., 1992; Chan et al., 1994). In continental Europe acenocoumarol and phenprocoumon are preferentially used as oral anticoagulants. Acenocoumarol is the 4’-nitro analog of warfarin. It is a short-acting compound having a plasma half-life of about 8 h, about one-fourth the half-life of racemic warfarin. Like warfarin, the main route of elimination of racemic acenocoumarol is by biotransformation, 6- and 7-hydroxylation mainly (Dieterle et al., 1977). The metabolic clearance of (S)-acenocoumarol is high (plasma \( t_{1/2} \) < 2 h), therefore the pharmacological effect lies almost exclusively with the (R)-enantiomer (Godbillion et al., 1981; Thijssen et al., 1986). In vitro experiments with human liver microsomes showed that the high intrinsic clearance of (S)-compared with (R)-acenocoumarol is mainly due to the low \( K_m \) values of the 6- and 7-hydroxylation reactions (Hermans and Thijssen, 1993). Furthermore, a role of CYP2C9 in the hydroxylation reactions of (R)- and (S)-acenocoumarol was suggested. However, no correlation between acenocoumarol and warfarin hydroxylations in a set of human liver microsomes was found (Hermans and Thijssen, 1993). To anticipate potential drug interactions with acenocoumarol therapy, knowledge of the enzymes involved in the biotransformation of, particularly, (R)-acenocoumarol is needed.

The aim of the present study was to characterize the cytochrome P450 enzymes that mediate the hydroxylation reactions of the aceno-
coumarol enantiomers. The enzyme sources used were human liver microsomes and human recombinant P450 enzymes expressed in yeast cells. Results show that CYP2C9 is the main enzyme involved in (S)- and (R)-acenocoumarol metabolism.

Materials and Methods

Chemicals. Acenocoumarol and the 6- and 7-hydroxy metabolites (for reference purpose) were a gift of Ciba-Geigy (Basel, Switzerland). The 6- and 7-hydroxy metabolites of warfarin (for reference purpose) were a kind gift of Dr. J. de Vries (University of Heidelberg, Germany). Sulfaphenazole was kindly given by Dr. T. B. Vree (University of Nijmegen, The Netherlands), and furafylline was a gift of Dr. E. Groene (RITOX, University of Utrecht, The Netherlands). Other drugs were purchased from Sigma Chemical Co. (St. Louis, MO). Chemicals were of the purest grade and solvents were of HPLC grade. The enantiomers of acenocoumarol and warfarin were isolated according to West et al. (1961).

cDNA-Expressed Human CYPs. Human CYP1A2, CYP3A4, CYP2C9, and CYP2C19 were expressed in yeast, and microsomes were prepared as described (Lemoine et al., 1993).

Human Liver Microsomes. Two sets of human livers were used, eight samples of a bank of the Paris laboratory (P.H.B.), and six samples of the Maastricht laboratory (H.H.T.). Human livers were obtained from kidney donors. Liver microsomes were prepared by standard techniques (Hermans and Thijssen, 1993; Lemoine et al., 1993). Microsomes were stored at -70°C. Microsomal protein was assayed by the Lowry method using bovine serum albumin as standard.

Relative microsomal content of CYP2C9 was assayed by Western blotting and probing with a polyclonal antibody raised in rabbits (Belloc et al., 1996).

Incubation Conditions. Yeast microsomes (0.05 nmol of cytochrome P450) or human liver microsomes (0.25 mg of microsomal protein) were mixed with Tris buffer (0.15 M potassium chloride in 0.050 M Tris-HCl, pH 7.4) and substrate in Tris buffer to give a volume of 0.27 ml. The mixtures were preincubated for 5 min at the reaction temperature. Reactions were started by adding 0.030 ml of preincubated (5 min at 37°C) NADPH-generating system (final concentrations: NADPH, 1 mM, glucose 6-phosphate, 8 mM, MgCl₂, 2.5 mM, glucose-6-phosphate dehydrogenase, 0.1 U). Reaction temperature for yeast microsomes was 28°C (the activity of some of the yeast-expressed CYPs appeared to deteriorate at higher temperatures); for human liver microsomes it was 37°C. Incubation times for reactions with (S)-acenocoumarol were 5 to 20 min. For the other substrates a 40-min incubation time was taken. Incubations were run in duplicate. At the chosen conditions, reactions were linear in time and linear with microsomal protein. Reactions were stopped by adding 0.5 ml of ice-cold acetonitrile containing 100 ng of 4'-cyanowarfarin as the internal standard.

Enzyme kinetic parameters were established from reactions with 6 to 10 different substrate concentrations, ranging 0.2 to 60, 5 to 500, 1 to 100, and 10 to 600 μM for (S)-acenocoumarol, (R)-acenocoumarol, (S)-warfarin, and (R)-warfarin, respectively.

For inhibition studies the incubation system was preincubated with inhibitor in the presence of NADPH for 10 min. The reaction was started by the addition of substrate. Inhibition studies were performed with 10 μM substrate concentration except for sulfaphenazole inhibition kinetics (see Results for details). Tolbutamide methylhydroxylation activity in human liver microsomes was estimated as described using 400 μM substrate concentration (Miners et al., 1988).

Sample Analysis. After stopping the reaction, the mixture was centrifuged and the supernatant was evaporated to dryness. The residue was taken up in 0.1 ml of mobile phase, 20 μl was analyzed by HPLC. Conditions: column, ChromSpherC18 5 μm (200 × 3 mm); mobile phase, 0.1% acetic acid in acetonitrile (71.5/28.5, v/v) brought to pH 4.67 with 4 M ammonia; flow, 0.8 ml/min; UV detection at 303 nm. Calibration factors of the metabolites were established as follows: liver microsomes of a phenobarbital-induced rat were incubated with racemic [14C]acenocoumarol and racemic [14C]warfarin. The calibration factors that were obtained from the peak areas at 303 nm of the HPLC analysis and the counts (in becquerels) under the peaks are listed in Table 1.

Data Analysis. Eadie-Hofstee plots were constructed of the enzyme kinetic data to decide if one or two (or more) enzymes were involved in the reaction (monophasic or biphasic Eadie-Hofstee plots, respectively). The kinetic parameters Kₘ and Vₘₐₓ were obtained by fitting a single Michaelis-Menten equation (one enzyme reaction) or the summation of two Michaelis-Menten equations (two enzyme reaction) to the data (software package Imploq, GraphPad, San Diego, CA).

Results

Recombinant Human Cytochrome P450 Enzymes. The activities of cDNA-expressed human CYP enzymes to hydroxylate the coumarin ring of the acenocoumarol and warfarin enantiomers are depicted in Fig. 1. (S)-A cenocoumarol was found to be hydroxylated principally by CYP2C9 at the 6-, 7-, and 8-position. Minor hydroxylase activity was also found with CYP2C19 and CYP2C18 (the latter is not shown). No activity was observed for CYP1A2, CYP3A4, CYP2B6, CYP2C8, and CYP2D6. (R)-Acenocoumarol was hydroxylated by CYP2C9 and CYP2C19 (6-, 7-, and 8-hydroxylation) and by CYP1A2 (6-hydroxylation). Warfarin hydroxylation followed the same pattern of sensitivity. CYP2C9 was the principal enzyme involved in the 6- and 7-hydroxylations of the (S)-enantiomer. Hydroxylation of (R)-warfarin was observed with CYP1A2 (6-hydroxylation) and 2C19 (6-, 7-, and 8-hydroxylation). Not shown in Fig. 1, hydroxylation at the 4'-position of warfarin was observed for CYP2C9 (both the enantiomers) and 2C19 (both the enantiomers). Incubations of (R)-warfarin with CYP3A4 resulted in the formation of 10-hydroxywarfarin. No reactions were found with CYP2B6 and CYP2D6.

The kinetics of the hydroxylation reactions are summarized in Table 2. CYP2C9 appeared to be a high affinity enzyme (Kₘ = 3 μM) to hydroxylate (S)-acenocoumarol preferentially at the 6- and 7-position with equal Vₘₐₓ. The hydroxylation kinetics of (R)-acenocoumarol proceeded with higher Kₘ (three times) and lower Vₘₐₓ values (10–20%). (S)-Warfarin hydroxylation was regioselective for the 7-position. Compared with (S)-acenocoumarol, higher Kₘ (three to four times) and lower Vₘₐₓ values were obtained. The total intrinsic clearances (Vₘₐₓ/Kₘ) of the CYP2C9-mediated hydroxylations related as 26/3.5/1 for (S)-acenocoumarol, (S)-warfarin, and (R)-acenocoumarol, respectively. CYP2C19 catalyzed the hydroxylations of (R)-acenocoumarol with moderate affinity, Kₘ = 70 to 100 μM. The hydroxylation reactions of (R)-warfarin occurred with low affinity (Kₘ > 200 μM).

Human Liver Microsomes. The kinetics of the acenocoumarol hydroxylation reactions were studied in six human liver microsomes (Table 3). Because 8-hydroxylase activities were generally low, their kinetics is not included. The 6- and 7-hydroxylations of (S)-acenocoumarol appeared to be mediated by a single enzyme. Both the hydroxylations proceeded with equal high affinity (low Kₘ value) and equal Vₘₐₓ. Hydroxylations of (R)-acenocoumarol proceeded with high Kₘ values. Furthermore, (R)-acenocoumarol was preferentially

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>10-OH</th>
<th>4'-OH</th>
<th>8-OH</th>
<th>6-OH</th>
<th>IS¹</th>
<th>7-OH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acenocoumarol</td>
<td>ND²</td>
<td>0.63</td>
<td>0.89</td>
<td>0.82</td>
<td>1.00</td>
<td>0.75</td>
</tr>
<tr>
<td>Warfarin</td>
<td>0.45</td>
<td>1.00</td>
<td>0.96</td>
<td>0.82</td>
<td>1.00</td>
<td>1.38</td>
</tr>
</tbody>
</table>

¹ IS, internal standard = 4'-cyanowarfarin.
droxylation reactions were studied in one human liver sample (HD10) of (S)-acenocoumarol was 33-fold higher than of the corresponding warfarin enantiomers. Next to the hydroxylation of (S)-warfarin (data not shown). The 7-hydroxylation of (S)-warfarin was catalyzed by a single enzyme with moderate affinity. The hydroxylations of (S)-acenocoumarol, the 7-hydroxylation of (S)-warfarin, and the 7-hydroxylation of (R)-acenocoumarol were found to correlate significantly (P ≤ .01) with the (relative) microsomal CYP2C9 content (Fig. 3).

Inhibition of Microsomal Acenocoumarol and Warfarin Hydroxylations. The results so far strongly indicate that in human liver microsomes the hydroxylations of (S)-acenocoumarol and the 7-hydroxylation of (R)-acenocoumarol, like the 7-hydroxylation of (S)-warfarin, are mainly mediated by CYP2C9. Additional proof was obtained from in vitro inhibition studies with some selected inhibitors (Newton et al., 1995) in three microsomal samples. Sulfaphenazole almost completely inhibited (more than 88%) the 6-, 7-, and 8-hydroxylation of (S)-acenocoumarol, the 7-hydroxylation of (R)-acenocoumarol, and the 7-hydroxylation of (S)-warfarin. Partial inhibition, 40 to 50%, was observed for the 6-hydroxylation of (R)-acenocoumarol. The kinetics of the sulfaphenazole inhibition of acenocoumarol hydroxylation was found to be competitive, $K_i = 0.55 ± 0.26 \mu M$ (Fig. 4). Furafylline (CYP1A2) was found to inhibit the 6-hydroxylation of (R)-substrates by 20 to 30% maximally. Minor inhibition (10–20%) was observed with 200 $\mu M$ (S)-mephenytoin (CYP2C19 substrate; Goldstein and de Morais, 1994) for the 6- and 7-hydroxylation of (R)-acenocoumarol, but not for the hydroxylations of (R)-warfarin. Troleandomycin (CYP3A4) was without effect on the hydroxylations of acenocoumarol. The 10-hydroxylation of (R)-warfarin, however, was completely inhibited.

Discussion

Acenocoumarol, like warfarin, is mainly (>95%) eliminated by biotransformation. The reactions involved are keto reduction and hydroxylation of the coumarin structure, mainly by the latter reaction (Dieterle et al., 1977). The reported reduction of the aromatic nitro group is mediated by the gut flora, but does not occur at normal therapeutic use (Thijssen et al., 1984). The objective of the study was to characterize the cytochrome P450 enzymes involved in the hydroxylation reactions of the acenocoumarol enantiomers. Such insight is of importance to anticipate possible interactions in case of coadministration of medications.

(S)-Acenocoumarol. The hydroxylations appear to be mainly, if not exclusively, mediated by CYP2C9: 1) from the set of yeast-expressed human cytochrome P450 enzymes, only CYP2C9 catalyzed the hydroxylations with high (intrinsic) activity; 2) the hydroxylation reaction rates in human liver microsomes were highly correlated with the rates of two well documented CYP2C9 reactions, the 7-hydroxylation of (S)-warfarin (Rettie et al., 1992; Kaminsky and Zhang, 1997) and the methyl hydroxylation of tolbutamide (Veronese et al., 1991);
The difference in reported before (Hermans and Thijssen, 1993). Our observed
In marked contrast, \((S)-\text{warfarin}\) is regioselectively hydroxylated at \((S)-\text{acenocoumarol}\) to be positioned over the reactive oxygen species. \((S)^2\) ring enhances the interaction forces by about 1 kcal mol\(^{-1}\) warfarin phenyl group (Mancy et al., 1995; Jones et al., 1996; He et al., 1997). A pocket is believed to be of primary importance for the binding of the 6-hydroxylation reaction with high \(K_m\) value was observed in four samples. The \((R),(S)^2\)-acenocoumarol correlated highly (\(r^2 > 0.90, P < .01\)) with the other CYP2C9-mediated reactions and was inhibited competitively by sulfaphenazole. A second, low affinity enzyme catalyzing the 7-hydroxylation reaction with high \(K_m\) value was observed in two samples.

3) hydroxylation activities in human liver microsomes were almost completely suppressed by sulfaphenazole, a potent selective inhibitor of CYP2C9 (Newton et al., 1995; Miners and Birkett, 1998); 4) Eadie-Hofstee plots were always monophasic. \((R)-\text{warfarin}\) agrees with previously reported data (Rettie et al., 1992; Kaminsky and Zhang, 1997). A \(\pi\)-stacking substrate-binding domain within the CYP2C9 protein pocket is believed to be of primary importance for the binding of the warfarin phenyl group (Maney et al., 1995; Jones et al., 1996; He et al., 1999). Clearly, the electronnegative 4'-nitro group on the phenyl ring enhances the interaction forces by about 1 kcal mol\(^{-1}\).

The \((R)-\text{Acenocoumarol}\). \((R)-\text{Acenocoumarol}\) is the \(\pi\)-enantiomer of acenocoumarol is the therapeutic relevant compound. The drug appeared to be a substrate of cDNA-expressed human CYP2C9. This is in contrast with \((R)-\text{warfarin}\), which is hardly metabolized (Fig. 1; Rettie et al., 1992; Kaminsky and Zhang, 1997; He et al., 1999). Furthermore, \((R)-\text{acenocoumarol}\) was found to be hydroxylated by recombinant CYP1A2 and CYP2C19. Considering the more favorable kinetics of the CYP2C9 mediated \((R)-\text{acenocoumarol}\) hydroxylation (Table 2) and the higher CYP2C9 hepatic content compared with CYP2C19, it is to be expected that CYP2C9 is of importance in the in vivo clearance of \((R)-\text{acenocoumarol}\). Clearly, this holds for the 7-hydroxylation. At low substrate concentrations\(^2\) the 7-hydroxylation of \((R)-\text{acenocoumarol}\) correlated highly (\(r^2 > 0.90, P < .01\)) with the other CYP2C9-mediated reactions and was inhibited competitively by sulfaphenazole. A second, low affinity enzyme catalyzing the 7-hydroxylation was found in some of the human liver samples. The nature of this enzyme could not be established, but CYP2C19 may be a candidate. The 6-hydroxylation, even at low substrate concentrations, appeared to be catalyzed by at least two enzymes, one of which

<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Kinetic parameters of acenocoumarol and warfarin hydroxylations catalyzed by recombinant human cytochrome P450 enzymes</th>
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</thead>
<tbody>
<tr>
<td>P450</td>
<td>Substrate Metabolite K_m [(\mu M)] V_max [(\text{pmol} \cdot \text{min}^{-1} \cdot \text{nmol}^{-1} \text{CYP})] V_max/K_m [(\mu M^{-1} \cdot \text{min}^{-1} \cdot \text{nmol}^{-1} \text{CYP})]</td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>((R)-\text{AC}) 6-OH 9.2 ± 0.6 40.5 ± 7.4 3.7 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>7-OH 12.0 ± 0.7 59.0 ± 2.5 4.2 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>8-OH 10.7 ± 2.0 6.5 ± 1.0 0.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>((S)-\text{W}) 6-OH 4'-OH 7.1 ± 1.1 13.8 ± 2.2 2.2 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>7-OH 9.9 ± 0.6 190 ± 12 18.7 ± 3.2</td>
</tr>
</tbody>
</table>

\(^{a}\) AC, acenocoumarol; W, warfarin.

<table>
<thead>
<tr>
<th>TABLE 3</th>
<th>Kinetic parameters of acenocoumarol hydroxylation reactions in human liver microsomes</th>
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<tbody>
<tr>
<td>Substrate Metabolite K_m [(\mu M)] V_max [(\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1})] V_max/K_m [(\mu M^{-1} \cdot \text{min}^{-1} \cdot \text{mg}^{-1})] CL_i [(\text{mg} \cdot \text{min}^{-1} \cdot \text{mg}^{-1})]</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>((R)-\text{AC}) 6-OH 1 36.6 ± 17.4 28.4 ± 33.9 0.696 ± 0.766 (range: 0.14–2.17) 1.20 ± 0.84 Range: 0.23–2.65 Median: 1.053</td>
<td></td>
</tr>
<tr>
<td>6-OH 2' 270 ± 207 47.0 ± 31.8 0.285 ± 0.330 (range: 0.04–0.85) 0.325 ± 0.40 Range: 0.10–0.86</td>
<td></td>
</tr>
<tr>
<td>7-OH 1 15.1 ± 10.0 5.4 ± 4.3 0.398 ± 0.250 (range: 0.10–0.86) 0.45 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>((S)-\text{AC}) 6-OH 0.89 ± 0.32 14.3 ± 10.3 18.9 ± 16.7 (range: 4.5–53.0) 41.0 ± 37.4 Range: 9.9–117.6 Median: 24.0</td>
<td></td>
</tr>
<tr>
<td>7-OH 0.83 ± 0.30 13.8 ± 9.1 22.1 ± 21.4 (range: 5.4–64.6)</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) Intrinsic clearance CL_i is the sum of V_max/K_m values of the 6- and 7-hydroxylations.\(^{b}\) AC, acenocoumarol. \(^{c}\) 6-hydroxylation reaction with high K_m value was observed in four samples. \(^{d}\) 7-hydroxylation reaction with high K_m value was observed in two samples.

3. The clinical plasma concentrations of acenocoumarol range between 50 and 150 ng/ml and are mainly \((R)-\text{acenocoumarol}\). Assuming that the free hepatic concentration equals free plasma concentration (unbound fraction, 1–2%), the substrate concentration would be 2 to 10 nmol/l.
could be CYP2C9, at 40 to 50% of the total 6-hydroxylation activity. The other enzymes may be CYP1A2 (20–30%) and CYP2C19 (10–20%).

The overall intrinsic activity of human liver microsomes was regioselective for the 6-hydroxylation of (R)-acenocoumarol and equi-selective for the 6- and 7-hydroxylation of (S)-acenocoumarol (Table 3). However, urinary recoveries of the metabolites after single oral doses of racemic acenocoumarol or of the enantiomers showed 1.5- to 2-fold more 7-hydroxy metabolite excretion (Dieterle et al., 1977; Thijssen et al., 1986). The reason is unclear for the moment.

In a previous study (Hermans and Thijssen, 1993), no correlation was found between the 7-hydroxylation of (S)-warfarin and any of the hydroxylations of (S)- or (R)-acenocoumarol in five human liver samples. Two of the five human liver samples in that study were postmortem autopsy samples. Whether this could have contributed to the results different from the present study is not clear.

Evidently, drugs being substrates or inhibitors of CYP2C9 will form the main source of interactions with acenocoumarol therapy. The clearance of both the enantiomers will be reduced, although (R)-

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### FIG. 2.
Eadie-Hofstee plots of the 6- and 7-hydroxylation kinetics of (R)-acenocoumarol in human liver microsomal sample HD22–4.

The estimated constants $K_m$ (μM) and $V_{max}$ (pmol/mg/min) were: $K_{m1} = 12, V_{max1} = 5.8, K_{m2} = 370, V_{max2} = 16$ of the 6-hydroxylation activity; $K_{m1} = 6.3, V_{max1} = 1.5, K_{m2} = 68, V_{max2} = 2.3$ of the 7-hydroxylation activity.

### FIG. 3.
Relationship between the 7-hydroxylation rate of (R)-acenocoumarol ($\bullet$) and (S)-acenocoumarol (■) and the relative amount of CYP2C9 in six human liver microsomes.

CYP2C9 content was estimated by immunoprobing after Western blotting. A pooled preparation of the six microsomal samples served as reference (relative amount = 1).

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### TABLE 4

Kinetic parameters of acenocoumarol and warfarin hydroxylations in human liver microsomes (sample HD10)
The data are the mean of incubations in triplicate.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Metabolite</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol·min$^{-1}$·mg$^{-1}$)</th>
<th>$V_{max}/K_m$ (pmol·min$^{-1}$·mg$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R)-AC</td>
<td>6-OH 1</td>
<td>30.8</td>
<td>16.8</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>6-OH 2</td>
<td>17</td>
<td>8.4</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>7-OH</td>
<td>16.9</td>
<td>4.1</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>8-OH 1</td>
<td>50</td>
<td>7.3</td>
<td>0.15</td>
</tr>
<tr>
<td>(S)-AC</td>
<td>6-OH</td>
<td>0.47</td>
<td>10.4</td>
<td>21.2</td>
</tr>
<tr>
<td></td>
<td>7-OH</td>
<td>0.35</td>
<td>12.6</td>
<td>36.0</td>
</tr>
<tr>
<td></td>
<td>8-OH</td>
<td>0.60</td>
<td>1.2</td>
<td>2.0</td>
</tr>
<tr>
<td>(R)-W</td>
<td>6-OH 1</td>
<td>64.0</td>
<td>4.8</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>6-OH 2</td>
<td>380</td>
<td>22.6</td>
<td>0.06</td>
</tr>
<tr>
<td>(S)-W</td>
<td>6-OH 1</td>
<td>2.5</td>
<td>0.79</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>6-OH 2</td>
<td>196</td>
<td>7.2</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>7-OH</td>
<td>2.1</td>
<td>1.8</td>
<td>0.86</td>
</tr>
</tbody>
</table>

a AC, acenocoumarol; W, warfarin.
b Kinetic constants of the low affinity (high $K_m$) reaction.
acenoconurol may be less sensitive, because it is only partly (approximately 50%) metabolized by CYP2C9. On the other hand, interaction with (R)-acenoconurol is clinically of more importance. Thus, piroxicam was found to reduce (R)- but not the (S) -acenoconurol clearance (Bonnabry et al., 1996). Lornoxicam, on the other hand, was found to reduce only (S) -acenoconurol clearance, without any observable pharmacodynamic effect (Masche et al., 1999). Potent inhibitors (low KM) that reach high hepatic concentrations, on the other hand, may completely suppress the body clearance of (S)-acenoconurol, converting it from a clinically inactive drug into a potent anticoagulant. Azole fungicides (Kunze et al., 1996), some serotonine reuptake inhibitors (Hemeryck et al., 1999), and statines (Transon et al., 1996) may be candidates for such an interaction. Furthermore, clinical interactions can be expected for drugs interfering with the non-CYP2C9-dependent 6-hydroxylation of (R)-acenoconurol such as CYP1A2 and CYP2C19 substrates or inhibitors. However, omeprazole, a CYP2C19 substrate (Andersson et al., 1994), was not found to interact with acenoconurol (De Hoon et al., 1997; Vreeburg et al., 1997).

In conclusion, CYP2C9 is the main and probably sole enzyme for the hydroxylation of (S)-acenoconurol. Hydroxylation proceeds with high activity, explaining the high body clearance of the enantiomer. CYP2C9 is also the main enzyme involved in the 7-hydroxylation of (R)-acenoconurol. The 6-hydroxylation is partly mediated by CYP2C9. Other enzymes involved in the 6-hydroxylation of (R)-acenoconurol are CYP1A2 and CYP2C9. The importance of CYP2C9 implies that clinically relevant interactions have to be expected for drugs that are inhibitors or substrates of CYP2C9. In addition, reduced clearance can be expected for carriers, hetero- or homozygous, of the allelic variants CYP2C9*2(Cys-144) and CYP2C9*3(Leu-359) (Goldstein and de Morais, 1994; Rettie et al., 1994; Steward et al., 1997). These subjects will be more sensitive for acenoconurol and may be at enhanced risk of drug interactions.

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References


