CYTOCHROME P4502C9 IS THE PRINCIPAL CATALYST OF RACEMIC ACENOCOUMAROL HYDROXYLATION REACTIONS IN HUMAN LIVER MICROSONES

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ABSTRACT:
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\text{m} values and a ratio of 0.9:1:0.1 for V\text{max}. CYP2C9 also mediated the 6-, 7-, and 8-hydroxylations of (R)-acenocoumarol with K\text{m} values three to four times and V\text{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\text{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 Thürmann, 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)\textsuperscript{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\textsubscript{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\text{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 acenocoumarol...
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 (Bellow 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′-cyano-warfarin 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 methylhydroxylaton 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 ammonium; 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 K_m and V_max 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 Implot, 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)-Acenocoumarol was found to be hydroxylated principally by CYP2C9 at the 6-, 7-, and 8-position. Minor hydroxylization 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 (K_m = 3 μM) to hydroxylate (S)-acenocoumarol preferentially at the 6- and 7-positions with equal V_max values. The hydroxylase kinetics of (R)-acenocoumarol proceeded with higher K_m (three times and lower V_max values (10–20%). (S)-Warfarin hydroxylation was regioselective for the 7-position. Compared with (S)-acenocoumarol, higher K_m (three to four times) and lower V_max values were obtained. The total intrinsic clearances (V_max/K_m) of the CYP2C9-mediated hydroxylations related to 26/3.5/1 for (S)-acenocoumarol, (S)-warfarin, and (R)-acenocoumarol, respectively. CYP2C19 catalyzed the hydroxylations of (R)-acenocoumarol with moderate affinity, K_m = 70 to 100 μM. The hydroxylation reactions of (R)-warfarin occurred with low affinity (K_m > 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 K_m value) and equal V_max. Hydroxylations of (R)-acenocoumarol proceeded with high K_m values. Furthermore, (R)-acenocoumarol was preferentially

### Table 1

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Acenocoumarol</th>
<th>Warfarin</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-OH</td>
<td>0.75</td>
<td>0.45</td>
</tr>
<tr>
<td>7-OH</td>
<td>1.24</td>
<td>1.38</td>
</tr>
<tr>
<td>8-OH</td>
<td>0.89</td>
<td>0.96</td>
</tr>
<tr>
<td>10-OH</td>
<td>ND*</td>
<td>0.82</td>
</tr>
<tr>
<td>4′-OH</td>
<td>0.63</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*ND, not detected. *IS, internal standard = 4′-cyano-warfarin.

The results did not differ by more than 4%.

### Data Analysis

Eadie-Hofstee plots were constructed of the enzyme kinetic parameters...
Acenocoumarol was preferentially hydroxylated at the R-droxylation reactions were studied in one human liver sample (HD10) -acenocoumarol. Intrinsic clearance of (S)-values varied 10-fold between the samples. On the average, the high. The 7-hydroxylation of (R)-warfarin (data not shown).

The 7-hydroxylation of (R)- and (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 μM (Fig. 4). Furafylline (CYP1A2) was found to inhibit the 6-hydroxylation of (R)-acenocoumarol, but not for the hydroxylations of (R)-warfarin. Troleandomycin (CYP3A4) was without effect on the hydroxylation of (R)-acenocoumarol.

6-hydroxylated. Biphasic Eadie-Hofstee plots were obtained for the 6- (four of six samples) and the 7-hydroxylations (two of six samples) of (R)-acenocoumarol, indicating that at least two enzymes were involved. Typical Eadie-Hofstee plots of (R)-acenocoumarol hydroxylations are shown in Fig. 2. The overall intrinsic clearance (V_{max}/K_m) values varied 10-fold between the samples. On the average, the intrinsic clearance of (S)-acenocoumarol was 33-fold higher than of (R)-acenocoumarol.

Comparative enzyme kinetics of acenocoumarol and warfarin hydroxylation reactions were studied in one human liver sample (HD10) (Table 4). (R)-Acenocoumarol was preferentially hydroxylated at the 6-position. The K_m value of the 6-hydroxylation rate was relatively high. The 7-hydroxylation of (R)-acenocoumarol was promoted by a single enzyme with moderate affinity. The hydroxylations of (S)-acenocoumarol proceeded with high affinity, K_m value ~ 0.4 μM, and with equal preference for the 6- and 7-position. The (S)-warfarin hydroxylation was regioselective for the 7 position and was mediated by a single enzyme. 6-Hydroxylation of (S)-warfarin was catalyzed by (at least) two enzymes. Generally, the acenocoumarol enantiomers were more active substrates (higher affinity and higher V_{max} values) than the corresponding warfarin enantiomers. Next to the hydroxylations of the coumarin ring structure, the sample HD10 also catalyzed the 4′-hydroxylation of (R)- and (S)-warfarin and the 10-hydroxylation of (R)-warfarin (data not shown).

Correlation between the 6- and 7-Hydroxylation Activities in Human Liver Microsomes. Hydroxylation activities of the acenocoumarol and warfarin enantiomers of eight human liver microsomal samples were compared. With 10 μM substrate concentrations significant mutual correlations (all: r^2 > 0.92, P < .01) were obtained between the hydroxylations (6-, 7-, and 8-) of (S)-acenocoumarol, between the (S)-acenocoumarol hydroxylations and the 7-hydroxylation of (R)-acenocoumarol, and between the (S)-acenocoumarol hydroxylations and the 7-hydroxylation of (S)-warfarin. No mutual correlations were found for the other hydroxylation reactions. When tested at higher substrate concentrations, 60 μM for the (S)- and 300 μM for the (R)-enantiomers, the 7-hydroxylation of (R)-acenocoumarol no longer correlated with any of the other hydroxylation reactions.

Tolbutamide methylhydroxylation was estimated in six human liver microsomal preparations. Significant (P < .001) correlations were obtained between tolbutamide hydroxylation and the 7-hydroxylation of (R)-acenocoumarol (r^2 = 0.98), the hydroxylations of (S)-acenocoumarol (r^2 > 0.92), and the 7-hydroxylation of (S)-warfarin (r^2 = 0.96). The hydroxylation rates of (S)-acenocoumarol 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 μM (Fig. 4). Furafylline (CYP1A2) was found to inhibit the 6-hydroxylation of the (R)-substrates by 20 to 30% maximally. Minor inhibition (10−20%) was observed with 200 μM (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 hydroxylation 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 $K_m$ reported before (Hermans and Thijssen, 1993). Our observed and Zhang, 1997; He et al., 1999).

In marked contrast, (S)-warfarin agrees with previously -warfarin agrees with previously

$Eadie-Hofstee$ plots were always monophasic. (R)-AC and CYP2C9 (Newton et al., 1995; Miners and Birkett, 1998); 4)

Kinetic parameters of acenocoumarol hydroxylation reactions in human liver microsomes

The data are the mean ± S.D. of six human liver microsomal samples. Incubations were done in duplicate.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Metabolite</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
<th>$V_{max}/K_m$</th>
<th>CL $\gamma$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(R)-AC $^a$</td>
<td>6-OH</td>
<td>36.6 ± 17.4</td>
<td>28.4 ± 33.9</td>
<td>0.696 ± 0.766</td>
<td>1.20 ± 0.84</td>
</tr>
<tr>
<td></td>
<td>6-OH</td>
<td>270 ± 207</td>
<td>47.0 ± 31.8</td>
<td>0.285 ± 0.330</td>
<td>0.250 Range: 0.04–0.85</td>
</tr>
<tr>
<td></td>
<td>7-OH</td>
<td>15.1 ± 10.0</td>
<td>5.4 ± 4.3</td>
<td>0.398 ± 0.250</td>
<td>0.726 Range: 0.10–0.86</td>
</tr>
<tr>
<td>(S)-AC</td>
<td>6-OH</td>
<td>0.89 ± 0.32</td>
<td>14.3 ± 10.3</td>
<td>18.9 ± 16.7</td>
<td>41.0 ± 37.7</td>
</tr>
<tr>
<td></td>
<td>7-OH</td>
<td>0.83 ± 0.30</td>
<td>13.8 ± 9.1</td>
<td>22.1 ± 21.4</td>
<td>5.4–46.6 Median: 24.0</td>
</tr>
</tbody>
</table>

$^a$ Intrinsic clearance CL, 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) 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. (S)-Acenocoumarol appears to be a high affinity substrate with $K_m$ values one fourth to one fifth of the $K_m$ of (S)-warfarin 7-hydroxylation (Tables 2 and 4). The difference in $K_m$ between acenocoumarol and warfarin has been reported before (Hermans and Thijssen, 1993). Our observed $K_m$ value of the 7-hydroxylation of (S)-warfarin agrees with previously reported data (Rettie et al., 1992; Kaminsky and Zhang, 1997; He et al., 1999). A π-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 electronegative 4'-nitro group on the phenyl ring enhances the interaction forces by about 1 kcal mol$^{-1}$. The binding appears to give equal probability to the 6- and 7-position of (S)-acenocoumarol to be positioned over the reactive oxygen species. In marked contrast, (S)-warfarin is regioselectively hydroxylated at the 7-position by CYP2C9 (this study, Rettie et al., 1992; Kaminsky and Zhang, 1997; He et al., 1999).

(R)-Acenocoumarol. The (R)-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)-warfarin, which is hardly metabolized (Fig. 1; Rettie et al., 1992; Kaminsky and Zhang, 1997; He et al., 1999). Furthermore, (R)-acenocoumarol was found to be hydroxylated by recombinant CYP1A2 and CYP2C19. Considering the more favorable kinetics of the CYP2C9 mediated (R)-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)-acenocoumarol. Clearly, this holds for the 7-hydroxylation. At low substrate concentrations the 7-hydroxylation of (R)-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

$^2$ The clinical plasma concentrations of acenocoumarol range between 50 and 150 ng/ml and are mainly (R)-acenocoumarol. Assuming that the free hepatic concentration equals free plasma concentration (bound 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 equiselective 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)-

### Table 4

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Metabolite</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/mg/min)</th>
<th>$V_{max}/K_m$ (pmol/min/μM)</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>117</td>
<td>37.0</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>7-OH</td>
<td>16.9</td>
<td>8.4</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>8-OH 1</td>
<td>14</td>
<td>4.1</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>8-OH 2</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>
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<td>5.3</td>
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<tr>
<td>(S)-W</td>
<td>6-OH 1</td>
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<td>0.79</td>
<td>0.32</td>
</tr>
<tr>
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<td>7-OH</td>
<td>2.1</td>
<td>1.8</td>
<td>0.86</td>
</tr>
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</table>

a AC, acenocoumarol; W, warfarin.

b Kinetic constants of the low affinity (high $K_m$) reaction.

CYP2C9 content was estimated by immunoprobing after Western blotting. A pooled preparation of the six microsomal samples served as reference (relative amount = 1).
acenocoumarol may be less sensitive, because it is only partly (approximately 50%) metabolized by CYP2C9. On the other hand, interaction with (R)-acenocoumarol is clinically of more importance. Thus, piroxicam was found to reduce (R)- but not the (S)-acenocoumarol clearance (Bonnabry et al., 1996). Lornoxicam, on the other hand, was found to reduce only (S)-acenocoumarol clearance, without any observable pharmacodynamic effect (Masche et al., 1999). Potent inhibitors (low \( K_i \)) that reach high hepatic concentrations, on the other hand, may completely suppress the body clearance of (S)-acenocoumarol, converting it from a clinically inactive drug into a potent anticoagulant. Azole fungicides (Kunze et al., 1996), some serotonin 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)-acenocoumarol such as CYP1A2 and CYP2C19 substrates or inhibitors. However, omeprazole, a CYP2C19 substrate (Andersson et al., 1994), was not found to interact with acenocoumarol (De Hoon et al., 1997; Vreeburg et al., 1997).

In conclusion, CYP2C9 is the main and probably sole enzyme for the hydroxylation of (S)-acenocoumarol. 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)-acenocoumarol. The 6-hydroxylation is partly mediated by CYP2C9. Other enzymes involved in the 6-hydroxylation of (R)-acenocoumarol are CYP1A2 and CYP2C19. 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 acenocoumarol and may be at enhanced risk of drug interactions.

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References


