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Phase II Metabolism of Warfarin in Primary Culture of Adult Rat Hepatocytes

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SUMMARY

We used adult rat hepatocytes in primary culture (HPC) as a model system to study the hepatic phase II metabolism of the anticoagulant warfarin. Hepatocytes were isolated by a collagenase perfusion technique and maintained for 24 hr in Waymouth's medium containing 0.1 mM (*R*)-warfarin. When HPC medium was analyzed by reverse phase high performance liquid chromatography with diode-array detection, 4′-, 6-, and 7-hydroxywarfarin were identified. Several putative conjugates were observed eluting between 13 and 18 min. Treatment of hepatocyte medium with β-glucuronidase and sulfatase resulted in the loss of five putative conjugates and concomitant increases in 4′-, 6-, and 7-hydroxywarfarin and warfarin, suggesting that these metabolites and warfarin were conjugated. Use of the β-glucuronidase inhibitor saccharic acid 1,4-lactone enabled the determination of the relative extents of conjugation of each metabolite by glucuronic acid and sulfate. Glucuronidation was the predominant pathway for 4′-hydroxywarfarin, whereas 6-hydroxywarfarin and warfarin occurred mainly as sulfate conjugates. In contrast, 7-hydroxywarfarin was converted to both glucuronide and sulfate conjugates. Exposure of HPC to phenobarbital resulted in a decrease in cytochrome P-450-mediated production of hydroxylated warfarin metabolites; however, an increase in the production of 8-hydroxywarfarin was observed when HPC were exposed to β-naphthoflavone. Unique conjugation patterns were found when hydroxylated warfarins were substituted for warfarin in HPC medium. Both 7- and 8-hydroxywarfarin were converted to one sulfate and two glucuronide conjugates, whereas 4′-hydroxywarfarin was converted to a single glucuronide conjugate. A spectral library of these conjugates was used to identify the major conjugates of warfarin formed by rat HPC.

Warfarin (Fig. 1) is widely used as a safe and effective drug for the treatment of a variety of human coagulation disorders. The anticoagulant properties of warfarin and related 4-hydroxycoumarins are attributed to the inhibition of vitamin K-epoxide reductase and vitamin K reductase, key enzymes in the vitamin K-dependent synthesis of clotting factors II, VII, IX, and X (1, 2). The efficacy of warfarin as an anticoagulant can be strongly influenced by the coadministration of other therapeutic agents (3, 4) or by its conversion to relatively inactive products by the microsomal monooxygenase system, which has as its terminal oxidase cytochrome P-450 (5). A number of forms of purified rat liver cytochromes P-450 metabolize warfarin to distinct profiles of hydroxylated metabolites and dehydrowarfarin (6). The hydroxylated warfarins are further metabolized via phase II enzyme systems to conjugates of glucuronic acid and sulfate (7, 8). Although phase II reactions are not as well characterized as those mediated by cytochromes P-450, in recent years much has been learned about the structure/activity requirements, inducibility, and substrate specificities of these conjugating enzymes.

UDPGTs exist as a family of enzymes (9, 10) that catalyze the transfer of glucuronic acid from uridine 5′-diphosphoglucuronic acid to a broad range of xenobiotic and endogenous substrates. UDPGT activities are classified according to their substrate specificities (11, 12), developmental activities (12), and inducibility by various xenobiotics (13, 14). Group 1 or 3-methylcholanthrene-inducible UDPGTs display activities toward small planar molecules such as phenols, whereas group 2 or PB-inducible UDPGTs are active toward more complex substrates like bilirubin and steroid hormones (10, 14). Other known inducers of UDPGT activity include DDT (14), BNF (14), and Aroclor 1254 (14). STs catalyze the sulfate conjugation of numerous endogenous compounds, drugs, and other xenobiotics. Multiple forms of STs have been found in human (15) and rat liver (16) and, like UDPGTs, STs display different but overlapping substrate specificities (17). Although some STs appear to be under hormonal control (18, 19), the inducibility of ST activity by xenobiotics or pharmacological agents has not been clearly established.

Warfarin metabolites generated by cytochrome P-450-mediated reactions are well characterized (20, 21); however, few studies have attempted to elucidate warfarin metabolite con-

ABBREVIATIONS: UDPGT, uridine diphosphoglucuronsyltransferase; ST, sulfotransferase; PB, phenobarbital; BNF, β-naphthoflavone; SAL, saccharic acid 1,4-lactone; DAD, diode-array detector (detection); HPC, hepatocytes in primary culture; HPLC, high performance liquid chromatography; DDT, dichlorodiphenyltrichloroethane.

jugation. The development of methods for isolating and maintaining functional rat HPC (22, 23) has led to their extensive use for studying the biotransformation of drugs and other xenobiotics by hepatic phase I and phase II enzyme systems (11, 24, 25). In this investigation we used rat liver HPC as a model system for studying hepatic cytochrome P-450-mediated and conjugative metabolism of warfarin. By providing a system with functioning phase I and phase II enzyme activities, HPC afford a unique approach for investigating how multiple products of a single substrate, generated by a number of phase I enzymes, are conjugated.

Materials and Methods

Hepatocyte preparation. Hepatocytes were isolated from sodium pentothal-anesthetized rats by the collagenase perfusion technique, essentially as described by Bissell and Guzelian (26). After the collagenase perfusion the softened liver was carefully excised, transferred to a sterile 100-ml bottle containing 40 ml of diluted (1/8) digestion medium, and allowed to digest for an additional 10 min at 37°. The digest was filtered through 253-μm mesh, and cells were pelleted at low speed and washed twice in culture medium. The final pellet was resuspended in modified Waymouth MB-752/1 medium and counted with a hemocytometer. Cell viability, determined by trypan blue exclusion, was typically >90%. Collagen-coated dishes (100 mm) were equilibrated with Waymouth medium (5 ml/plate) before plating. Hepatocytes were seeded at 7×10^6 cells/dish and incubated under 95% O_2/5% CO_2 and 95% humidity.

Treatment of cells. Before each experiment, stock solutions of the test chemical in dimethyl sulfoxide were diluted to the appropriate concentration with Waymouth medium. Freshly isolated hepatocytes were allowed to attach for 3 hr at 37°, after which cultures received Waymouth medium containing either dimethyl sulfoxide, BNF (10 μM), or PB (1 mM). After 24 hr, the medium was removed and cultures were fed either native warfarin (100 μM) or the appropriate hydroxylated warfarin. Cultures were incubated for 24 hr, and the medium was removed, centrifuged at low speed to remove debris, and stored at −70°.

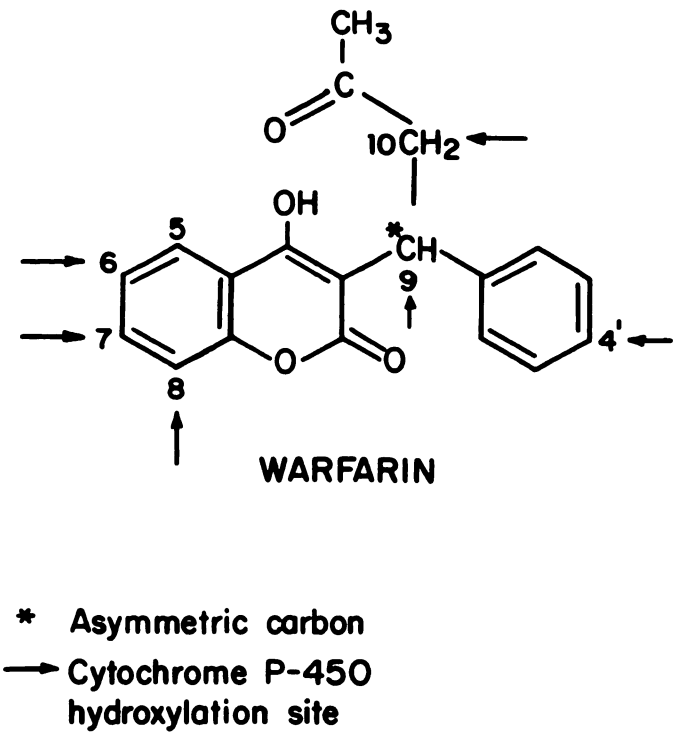

Fig. 1. Structure of warfarin.

Monolayers were washed twice with Hanks' balanced salt solution, harvested by scraping into 5 ml of this solution, centrifuged, and stored at −70°. Cell pellets were later thawed and protein determinations were made by the method of Bradford (27), with bovine serum albumin as a standard.

Metabolite isolation and identification. Aliquots of HPC medium were adjusted to pH 4.5 with 50% acetic acid and extracted twice with 2 ml of chloroform. Aliquots of the aqueous extract containing warfarin conjugates were analyzed essentially as previously described (28), on a Beckman 344 gradient HPLC system equipped with a Waters Nova-Pak C18 column (10 cm × 8 mm) and a Hewlett-Packard 1040M DAD. The mobile phase was composed of 1.5% ammonium acetate, pH 4.5 (buffer A), and 50% acetonitrile/50% buffer A (buffer B). The separation of warfarin and metabolites was achieved using the following elution gradient: from 0 to 9 min buffer B was increased linearly from 20% to 60%, and this condition was maintained for 20 min; buffer B was then increased to 100% over 1 min and maintained at 100% for 9 min. The column was reequilibrated at initial conditions of 80% A/20% B. The flow rate was 2 ml/min. Metabolites were identified by retention time and by comparison of spectra with spectra of known standards, as previously described (28). Warfarin conjugates were hydrolyzed by incubation of extracts with a mixture of β-glucuronidase (1000 units) and sulfatase (10–50 units), alone or in the presence of 50 μg of SAL, an inhibitor of β-glucuronidase activity. Because shifts in the retention times of conjugates were sometimes noted when HPLC profiles of control and enzyme-treated preparations were compared, conjugates were also identified by UV spectra and sensitivity to β-glucuronidase and sulfatase.

Biochemicals and chemicals. Collagenase (type I), β-glucuronidase sulfatase (type H2-S; β-glucuronidase, 100,000 units/ml; sulfatase, 1000–5000 units/ml), SAL, BNF, and PB were purchased from Sigma Chemical Company. (*R*)-Warfarin was prepared from the racemate as previously described (28).

Results

Phase I and phase II metabolites formed by HPC exposed to (*R*)-warfarin were analyzed by HPLC-DAD, along with a standard mixture containing authentic 4′-, 6-, 7-, 8-, and 10-hydroxy- and dehydrowarfarins. Lack of purified standards precluded precise identification of warfarin conjugates that eluted between 13 and 18 min (Fig. 2). The phase I metabolites in the

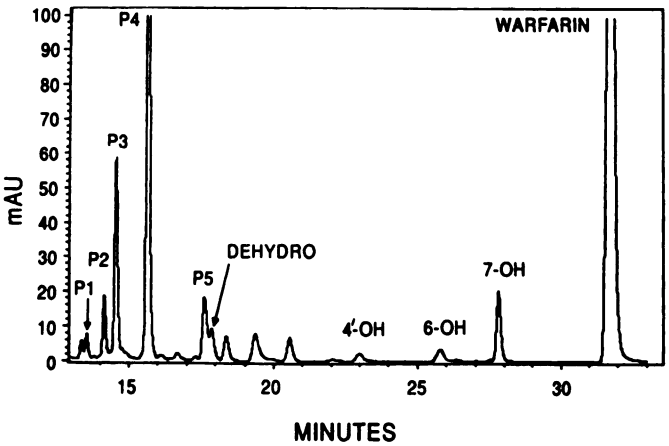

Fig. 2. Reverse phase HPLC elution profile of culture medium harvested from HPC (7×10^6 cells) fed 0.1 mM (*R*)-warfarin for 24 hr. Hydrophilic putative conjugates eluted between 13 and 18 min, and hydroxylated metabolites eluted between 18 and 29 min. Hydroxylated metabolites were identified by retention time and spectra were obtained by UV DAD. Elution of metabolites was monitored at 313 nm. Other experimental details are described in Materials and Methods.

HPLC profile shown in Fig. 2 are dehydrowarfarin and 4′-, 6-, and 7-hydroxywarfarins.

A series of experiments utilizing a mixture of β-glucuronidase and sulfatase was carried out to determine whether the putative conjugates were either glucuronides or sulfates. Medium from cells exposed to 0.1 mM (*R*)-warfarin for 24 hr was harvested and extracted with chloroform to remove hydroxylated metabolites. The resulting solution, containing the putative conjugates, was treated with a mixture of β-glucuronidase and sulfatase, and the liberated hydroxylated warfarins were analyzed by HPLC-DAD. After hydrolysis of the five major peaks eluting between 13 and 20 min, there was an increase in 4′-, 6-, and 7-hydroxywarfarin and warfarin concentrations (Fig. 3).

Addition of PB to HPC cultures resulted in a decrease in the total free plus hydroxylated warfarins formed (Table 1), but the proportion of free to conjugated hydroxylated metabolites was not significantly different. When cultures were exposed to BNF, there was a significant increase in warfarin 8-hydroxylase activity, whereas 4′- and 6-hydroxywarfarin were not detected. These results revealed that 7-hydroxywarfarin was the major phase I product in control as well as PB- and BNF-treated cultures and that at least 50% of the hydroxylated metabolites formed in control cultures were subsequently conjugated.

The use of SAL as a β-glucuronidase inhibitor permits determination of the relative proportions of glucuronide or sulfate conjugates of each hydroxylated metabolite found. Because

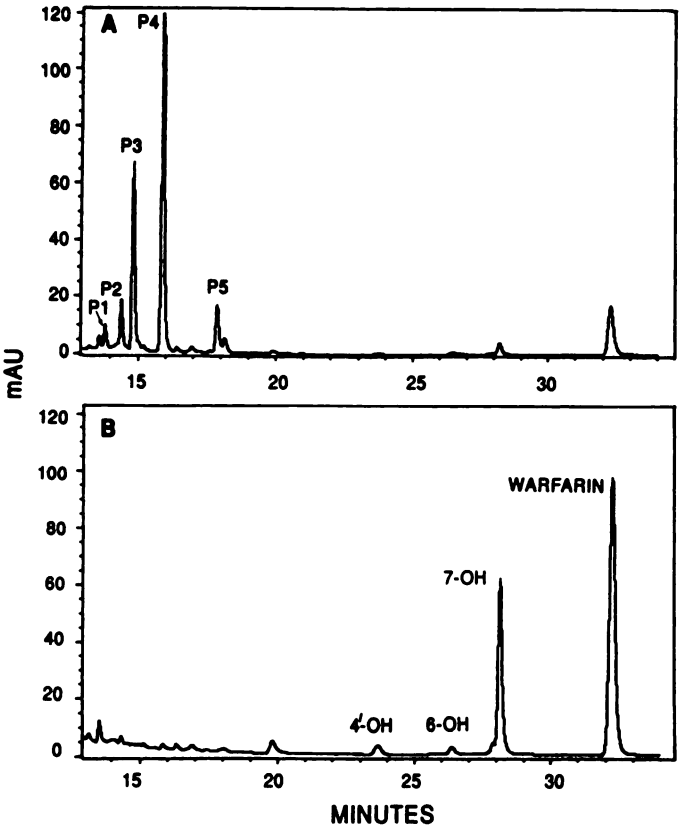

Fig. 3. β-Glucuronidase/sulfatase hydrolysis of putative warfarin conjugates and the resultant hydroxylated metabolites. A, HPC medium was acidified to pH 4.5, extracted twice with chloroform to remove hydroxylated warfarins, and analyzed by reverse phase HPLC with UV DAD at 313 nm. B, HPC medium extracted as described in A was then hydrolyzed with β-glucuronidase/sulfatase. Metabolites were identified by retention time and UV-DAD spectra.

TABLE 1

Effect of PB and BNF on the proportion of hydroxylated warfarin metabolites converted to conjugates

Adult rat hepatocytes were exposed to 0.1 mM (*R*)-warfarin for 24 hr, and the levels of hydroxylated warfarin metabolites present in the medium were determined (unconjugated). Aliquots of culture medium were also extracted with chloroform and treated with β-glucuronidase/sulfatase. The amounts of hydroxylated warfarin metabolites liberated by enzymatic hydrolysis were then determined (conjugated). Metabolites were analyzed by reverse phase HPLC with UV DAD. Data represent averages of duplicate determinations.

	Warfarin metabolites formed		
	DMSO[a]	PB	BNF
	nmol/mg of protein/24 hr		
4′-OH-Warfarin			
Unconjugated	10	10	ND[b]
Conjugated	13	9	ND
Total	23	19	
6-OH-Warfarin			
Unconjugated	20	13	ND
Conjugated	11	6	ND
Total	31	19	
7-OH-Warfarin			
Unconjugated	31	30	37
Conjugated	98	71	26
Total	129	101	63
8-OH-Warfarin			
Unconjugated	ND	ND	11
Conjugated	ND	ND	33
Total			44
Warfarin			
Unconjugated	854	750	862
Conjugated	242	200	65
Total	1096	950	927

[a] DMSO, dimethyl sulfoxide.
[b] ND, not detected.

SAL does not inhibit sulfatases, any SAL-mediated decrease in conjugate hydrolysis is indicative of the extent of glucuronidation. Based on the appearance of warfarin and its metabolites on hydrolysis of the conjugates, the data presented in Fig. 4 suggest that 4′-hydroxywarfarin is found primarily as a glucuronide conjugate, whereas sulfate conjugation appears to be the preferred pathway for 6-hydroxywarfarin and warfarin. In contrast, approximately 47% of 7-hydroxywarfarin is conjugated with sulfate, with the remainder found as the glucuronide conjugate.

A comparison of HPLC profiles of HPC medium hydrolyzed with β-glucuronidase/sulfatase in the presence of SAL permits identification of (*R*)-warfarin conjugate species as sulfates or glucuronides (Fig. 5). In the absence of SAL, P1, P3, P4, and P5 were completely hydrolyzed, whereas P2 was digested by >85% (Fig. 5A). Inhibition of β-glucuronidase activity with SAL resulted in P1 and P3 being retained to >70% of control values and P5 being retained to 92% of control values. In contrast, P2 was only 30% of control values and P4 was still completely hydrolyzed (Fig. 5B).

We examined the capacity of HPC phase II enzymes to conjugate administered purified hydroxylated warfarins, in order to explore possible structure/activity relationships that may govern warfarin metabolite conjugation and to identify the conjugates. HPC were maintained in the presence of either 4′-, 7-, or 8-hydroxywarfarin for 24 hr, at which time the medium was removed and processed as described above. The HPLC profiles in Fig. 6A show that 7-hydroxywarfarin was converted to three major conjugates, which were hydrolyzed >95% by β-glucuronidase/sulfatase. In the presence of SAL, two of the conjugates were retained to 95% and 86% of control

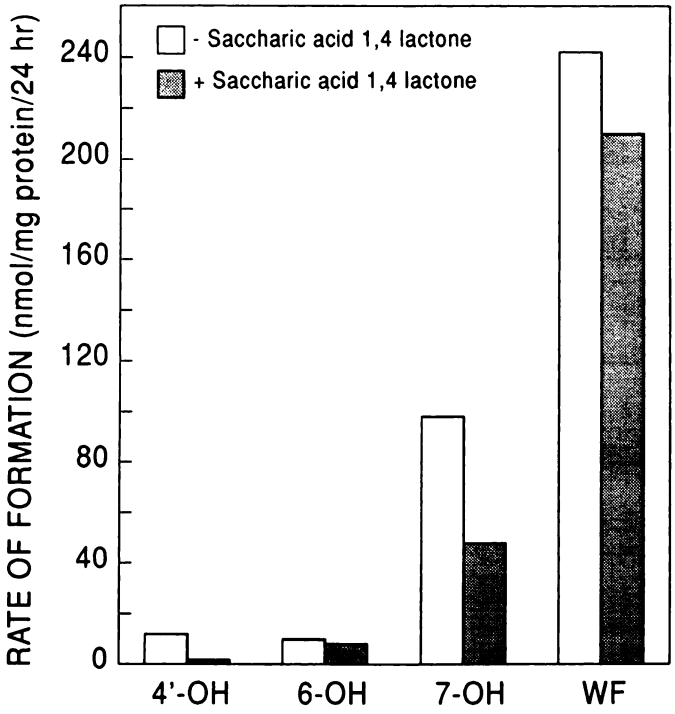

Fig. 4. Proportion of hydroxylated warfarins and warfarin (*WF*) converted to glucuronide or sulfate conjugates. *–SAL*, hydroxylated warfarins released when putative conjugates were enzymatically hydrolyzed without β-glucuronidase inhibitor; both glucuronide and sulfate conjugates were hydrolyzed. *+ SAL*, hydroxylated warfarins released when putative conjugates were enzymatically hydrolyzed in the presence of β-glucuronidase inhibitor; only sulfate conjugates were hydrolyzed. Metabolites were analyzed by reverse phase HPLC with UV DAD at 313 nm.

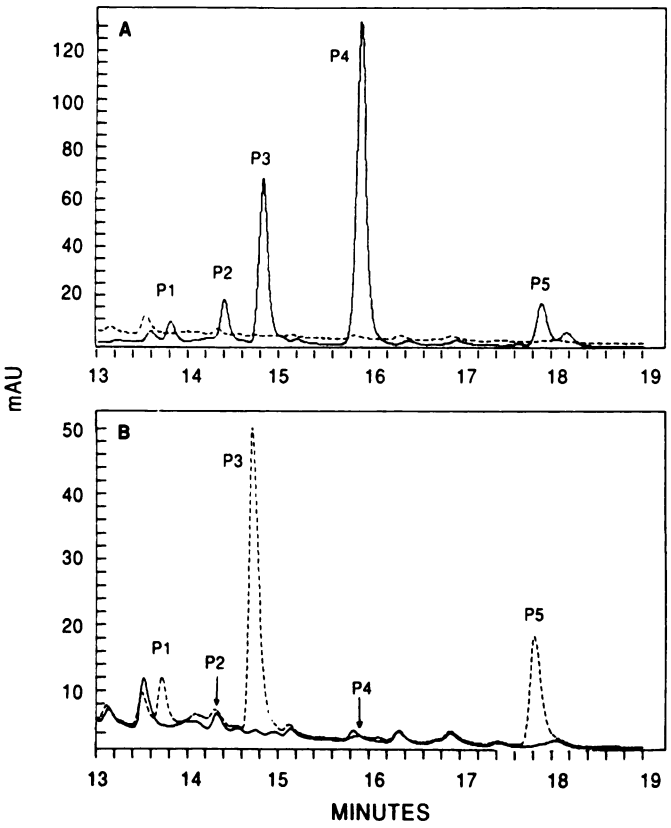

Fig. 5. Hydrolysis of five putative warfarin and metabolite conjugates by β-glucuronidase/sulfatase, with and without β-glucuronidase inhibitor. Reverse phase HPLC elution profile of putative (*R*)-warfarin conjugates (P1–P5). A, Control (——) and β-glucuronidase/sulfatase-hydrolyzed (– – –); B, β-glucuronidase/sulfatase hydrolyzed with (——) and without (– – –) SAL. Conjugates hydrolyzed in A and not in B were identified as glucuronide conjugates. Conjugates hydrolyzed in both A and B were identified as sulfate conjugates.

values, whereas the third conjugate was still completely hydrolyzed by the sulfatase activity (Fig. 6B). The elution profile of putative conjugates formed from 8-hydroxywarfarin is shown in Fig. 7A. These conjugates were susceptible to β-glucuronidase/sulfatase hydrolysis; however, only the conjugate eluting at 18 min was hydrolyzed in the presence of SAL, whereas the conjugates eluting between 14 and 15 min were retained to 84% and 92%, respectively, of control values (Fig. 7B). In contrast to the conjugation patterns obtained for 7- and 8-hydroxywarfarin, 4′-hydroxywarfarin appeared to be metabolized to one major conjugate of glucuronic acid, which was 99% hydrolyzed (Fig. 8A) by β-glucuronidase/sulfatase and was retained to 85% of the control value when β-glucuronidase was inhibited (Fig. 8B).

Experiments were carried out to examine warfarin conjugates formed when HPC were exposed to combinations of purified hydroxylated warfarins. HPLC profiles of warfarin conjugates formed by HPC exposed to equimolar concentrations of 4′- and 7-hydroxywarfarin are shown in Fig. 9. The four conjugates seen in this HPLC profile were identified by HPLC-DAD spectra and sensitivity to β-glucuronidase/sulfatase as the single conjugate formed from 4′-hydroxywarfarin (Fig. 8) and the three conjugates formed from 7-hydroxywarfarin (Fig. 6). Moreover, the total amount of conjugate formed by the HPC under these conditions was essentially equal (107%) to the amount of conjugate formed when the hydroxylated metabolites were added individually to HPC.

A spectral library of each of the major conjugates formed from 4′-, 7-, and 8-hydroxywarfarin was used to identify the conjugates of (*R*)-warfarin. Four of the five warfarin conjugates gave very high quality matches with four of the library spectra. Thus, from Fig. 2, P1 is a glucuronide conjugate of 4′-hydroxywarfarin, P2 is a sulfate conjugate of 7-hydroxywarfarin, P3 is a glucuronide conjugate of 7-hydroxywarfarin, and P4 is a sulfate conjugate of warfarin. The spectrum obtained for conjugate P5 closely matches the spectrum obtained for warfarin.

Discussion

Glucuronidation and sulfation are the most widely utilized conjugation pathways for converting environmental chemicals, drugs, and endogenous substrates to water-soluble metabolites. Experimental evidence indicates that some activities of the phase I and phase II enzyme systems are coordinately expressed and that the balance of these activities is an important determinant in preventing the accumulation of harmful levels of drugs or toxic intermediates (29).

In the present study, we used HPC as the experimental system to study how multiple phase I products of (*R*)-warfarin are conjugated. Using HPLC-DAD, we identified multiple hydroxylated warfarins and characterized four major warfarin metabolite conjugates.

When warfarin metabolite conjugates were enzymatically hydrolyzed, the liberated hydroxylated warfarins accounted for

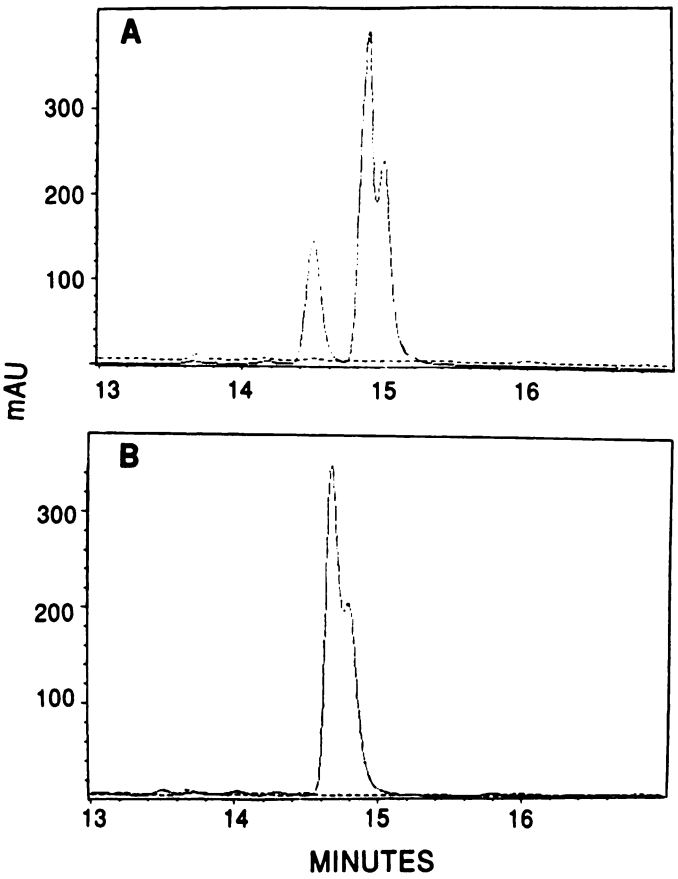

Fig. 6. Reverse phase HPLC chromatograms of conjugates formed by HPC fed 7-hydroxywarfarin. A, Control (——) and β-glucuronidase/sulfatase-hydrolyzed (– – –); B, β-glucuronidase/sulfatase-hydrolyzed with (——) and without (– – –) SAL. Conjugates not hydrolyzed in the presence of SAL were identified as glucuronide conjugates. Conjugates hydrolyzed in the presence of SAL were identified as sulfate conjugates. Conjugates were analyzed by reverse phase HPLC with UV DAD at 313 nm.

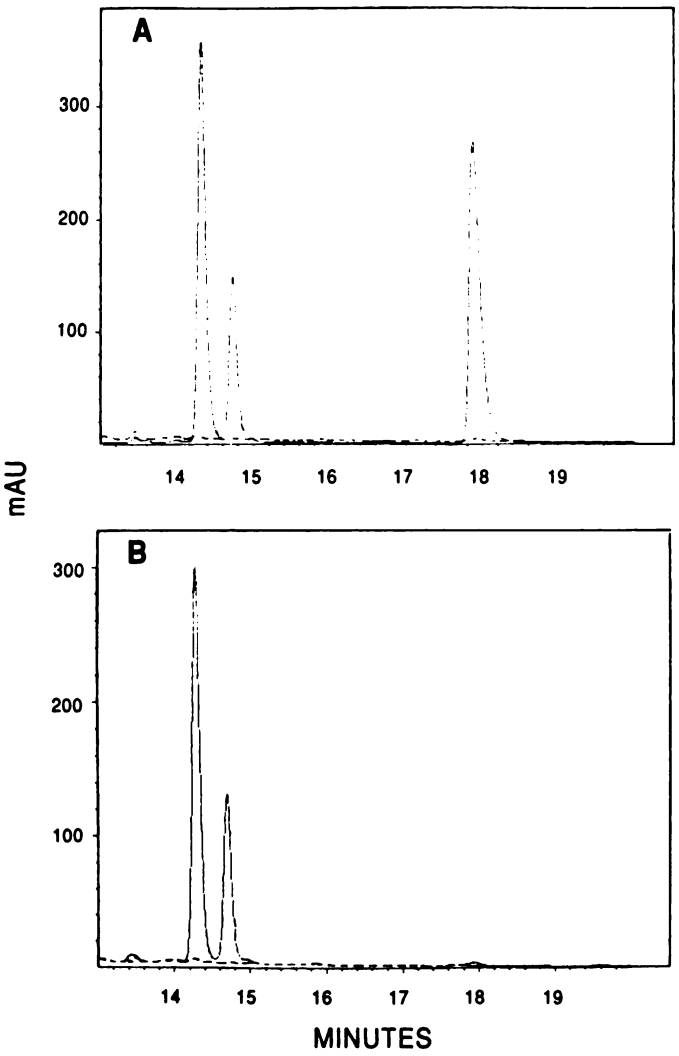

Fig. 7. Reverse phase HPLC chromatogram of conjugates formed by HPC fed 8-hydroxywarfarin. A, Control (——) and β-glucuronidase/sulfatase-hydrolyzed (– – –); B, β-glucuronidase/sulfatase-hydrolyzed in the presence (——) and absence (– – –) of SAL. Conjugates not hydrolyzed in the presence of SAL were identified as glucuronide conjugates. Conjugates hydrolyzed in the presence of SAL were identified as sulfate conjugates. Conjugates were analyzed by reverse phase HPLC with UV DAD at 313 nm.

>50% of the total hydroxylated warfarins produced by HPC. Cytochromes P-450 metabolize warfarin to a specific pattern of hydroxylated metabolites, and the rates of formation of these metabolites can be used to assess the functional concentration of cytochrome P-450 forms present *in vivo* and in cultured cells or microsomal preparations. However, accurate determinations of warfarin hydroxylation rates can be complicated if metabolites are preferentially utilized as substrates by conjugating enzymes. Although each hydroxylated metabolite was found to serve as an effective substrate for conjugation (Table 1), 7-hydroxywarfarin, the major hydroxylated warfarin formed, was the preferred substrate for phase II enzymes, with 76% of the phase I product being conjugated in control hepatocytes.

Enzymatic hydrolysis of (*R*)-warfarin and metabolite conjugates in the presence and absence of SAL permitted determination of whether the conjugates were glucuronides or sulfates. The data presented in Fig. 4 provide evidence that glucuronidation was the preferred pathway for 4′-hydroxywarfarin, whereas 6-hydroxywarfarin and warfarin were found mainly as sulfate conjugates. In contrast, 7-hydroxywarfarin was converted to both sulfate (48%) and glucuronide (52%) conjugates.

In general, the conjugative pathway any phase I metabolite follows appears to be dependent upon its concentration and on the availability of cofactors required by phase II enzymes. Reactions catalyzed by STs, characterized as a high affinity, low capacity pathway, predominate at low substrate concentration and are dependent upon availability of inorganic sulfate (30, 31). Glucuronidation, characterized as a low affinity, high capacity pathway, predominates at high substrate concentrations and is limited by the availability of a key cofactor, uridine diphosphoglucuronic acid (30, 32). It appears that enzymes of each pathway have the ability to increase in activity in order to compensate for a decrease in the activity of the other (33).

Unique conjugation patterns were found when hydroxylated warfarins were presented directly as substrates for conjugating enzymes. Both 7- and 8-hydroxywarfarin were converted to one sulfate and two glucuronide conjugates, whereas 4′-hydroxywarfarin was converted to a single conjugate of glucuronic acid (Figs. 6–8). The hydroxylated warfarin metabolites have two sites for conjugation, the site of hydroxylation and C4 (Fig. 1). In this study we have not resolved the specific sites of conjugation but, at least in the case of 7- and 8-hydroxywarfarin,

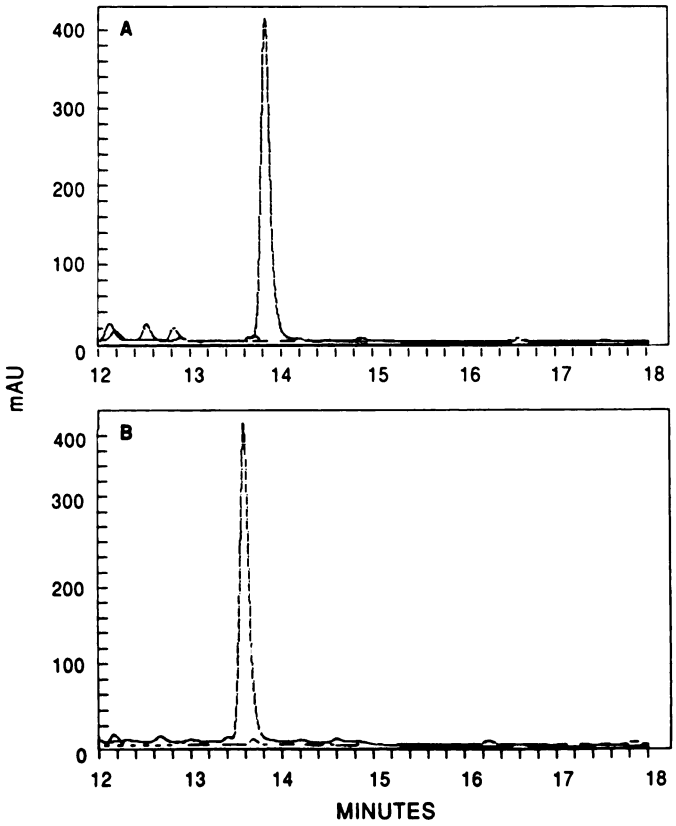

Fig. 8. Reverse phase HPLC chromatogram of conjugates formed by HPC fed 4′-hydroxywarfarin. A, Control (——) and β-glucuronidase/sulfatase-hydrolyzed (– – –); B, 4′-hydroxywarfarin conjugate hydrolyzed in the presence (——) and absence (– – –) of SAL. Conjugate not hydrolyzed in the presence of SAL was identified as a glucuronide conjugate. Conjugates were analyzed by reverse phase HPLC with UV DAD at 313 nm.

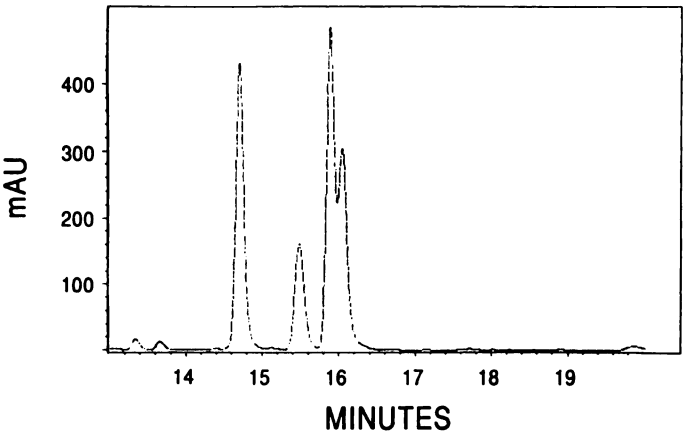

Fig. 9. Reverse phase HPLC chromatogram of conjugates formed by HPC fed equimolar concentrations of 4′- and 7-hydroxywarfarin. Conjugates were identified by their susceptibility to β-glucuronidase/sulfatase and by their UV-DAD spectra.

both sites are utilized for glucuronidation and one is also a site for sulfation. With 4′-hydroxywarfarin, only one of the sites is used, presumably C4, because that is clearly available for conjugation in the other warfarin metabolites. This suggests that the 4′-hydroxyl group in this warfarin metabolite is not available for conjugation. Under these experimental conditions there were much higher substrate concentrations (0.1 mM) than when phase II enzymes utilized phase I products as substrates. This resulted in the formation of at least one major glucuronide conjugate (formed from 7-hydroxywarfarin) (compare Figs. 2A and 6A) that was undetected when native warfarin was metabolized by HPC, which is consistent with the general concentration effects noted above. The results presented in Figs. 6–9 suggest that, although structure/activity relationships may exist for the formation of multiple conjugates of warfarin, HPC conjugating enzymes did not preferentially utilize either 4′- or 7-hydroxywarfarin when presented with a mixture of these substrates. Studies investigating xenobiotic metabolism in rodents provide strong evidence to support a model whereby various xenobiotics, such as polynuclear aromatic hydrocarbons or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, induce the coordinate expression of genes associated with the *Ah* locus that code for phase I and phase II enzyme activities (34). The conversion of a xenobiotic to its ultimate hydrophilic form would, therefore, involve a series of reactions catalyzed by closely associated membrane-bound activities of the oxidative and conjugative systems. Bypassing these coupled reactions by the introduction of hydroxylated metabolites could be another factor contributing to a conjugation pattern that is different from that seen when the compound is metabolized by sequential phase I and phase II reactions. These differences in conjugation patterns may also reflect a regioselectivity on the part of UDPGTs and STs, because multiple conjugates were formed only when hydroxyl substituents were present on the 4-hydroxycoumarin moiety of warfarin.

PB and BNF are known to enhance the activities of particular rat liver microsomal cytochromes P-450, as well as various forms of UDPGTs (8, 14). In the present study, exposure of HPC to PB resulted in a decrease in cytochrome P-450-mediated production of hydroxylated warfarin metabolites. Two rat liver cytochrome P-450 isozymes, P-450-PB-C and P-450-PB-B (P4502C6 and P4502B1), are inducible by PB and are regioselective for the 7- and 4′-positions of warfarin. Our inability to demonstrate these activities may be due to the reported rapid loss of PB-inducible P-450 isozymes in HPC (35). When HPC were exposed to BNF, there was a significant increase in warfarin 8-hydroxylase activity and a decrease in the amount of 7-hydroxywarfarin and native warfarin conjugated.

In summary, the use of HPC, which provide coupled phase I and phase II enzyme systems, and warfarin, which is regio- and stereoselectively metabolized at several aromatic and aliphatic sites by a number of identified rat liver cytochromes P-450, have provided several important insights into phase I/phase II coupling. Notwithstanding the relatively high polarity of the warfarin metabolites, they were conjugated >50% overall, but to varying extents. The warfarin metabolites and warfarin exhibited species selectivity and regioselectivity in their susceptibility to conjugation; 4′-hydroxywarfarin is glucuronidated, 6-hydroxywarfarin is sulfated, and 7-hydroxywarfarin is conjugated to approximately the same extent by the two systems, and warfarin is sulfated to a much greater extent than it is glucuronidated. Presumably, these conjugation patterns are governed to some extent by cytochrome P-450/conjugating enzyme transferral of products/substrates, because, when the hydroxywarfarins were added to HPC, different conjugate profiles were detected. However, the concentration of substrates for conjugating enzymes is also a factor in this effect.

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