Table II—Enzymatic Analysis of Skin Decontamination Water and Skin Scrub Solution

Chemical	Condition	Active Agent, %[a] Decontamination Water[b]	Active Agent, %[a] Scrub Solution[c]
Phosphonofluoridate	No shower	—	13 ± 12
	Shower	15 ± 6	17 ± 6
Thickened phosphonofluoridate	No shower	—	8 ± 7
	Shower	16 ± 13	15 ± 15

[a] Percent of recovered radioactivity associated with active agent as determined by enzymatic assays. Numbers are mean ± *SD*. [b] Water collected in chamber B after shower decontamination of the skin. [c] One percent aqueous surfactant solution used to scrub and rinse the skin.

course of the study, and after the completion of the experiments. Purity was essentially 100% at the initial and midpoint, but fell to 70% after the completion of the experiments. However, no significant differences were found between results from initial and final samples.

The inactivation of 1,2,2-trimethylpropyl methylphosphonofluoridate in contact with skin is consistent with the observations of Fredricksson (3) who demonstrated the presence of organophosphate ester hydrolases in the skin of rats. Other investigators (4) who compared skin penetration of the phosphonofluoridate activity with radiolabel penetration reported that human skin decomposed at least 80% of the applied phosphonofluoridate as contrasted with 30% inactivation of its penetration of guinea pig skin.

Diethyl malonate was selected as a simulant for the phosphonofluoridate in skin-surface removal studies on the basis of similar physical properties. For both compounds, the majority of the applied dose evaporated. After 15 min, most of the radiolabel that remained (Table I) was recovered from the skin surface (by showering or scrubbing). The lack of agreement between radioactivity and enzyme inhibition assays of the shower and scrub water would suggest that some of the phosphonofluoridate hydrolyzed after it contacted the skin. In the case of diethyl malonate, hydrolysis on the skin is certainly possible. It is, therefore, possible that the shower removed a mixture of the original compounds as well as hydrolysis products, and it is no longer clear what is being compared. Presumably, the intact compound would have a different affinity for the skin than its hydrolysis products. The fact that similar skin decontamination efficiencies (as judged by the percent removal of radioactivity still on the skin after evaporation) were obtained by showering after application of the radiolabeled diethyl malonate and phosphonofluoridate may be fortuitous. However, if the shower removed the upper layers of skin, and thus removed contaminants in these layers indirectly, decontamination of surface residues would be independent of chemical structure, and similar decontamination efficiencies would be obtained as in this study.

Under the conditions of this study, skin penetration of 1,2,2-trimethylpropyl methylphosphonofluoridate and diethyl malonate was limited by the dose, short interval between application and decontamination, and the free evaporation of the chemicals during this interval. The skin penetration of the phosphonofluoridate is complicated by the possibilities of chemical degradation during its passage through the skin. Attempts to compare the percutaneous penetration of this phosphonofluoridate (and other highly toxic organophosphorous compounds) to analogues or simulants must await knowledge of the fate and distribution of these compounds in skin.

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Stereospecific Fluorescence High-Performance Liquid Chromatographic Analysis of Warfarin and Its Metabolites in Plasma and Urine

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Abstract □ A stereospecific assay for the simultaneous determination of the enantiomers of warfarin and its major metabolites, 6- and 7-hydroxywarfarin and warfarin alcohols, in plasma and urine was developed. Involved in this determination was the formation of diastereoisomeric esters with carbobenzyloxy-L-proline, separation by normal-phase high-performance liquid chromatography, and detection by fluorescence after postcolumn aminolysis with *n*-butylamine. The determination limit for any enantiomer is in the order of 50–100 ng. The method was applied to the analysis of the enantiomers of warfarin and metabolites in plasma and urine of human subjects receiving racemic drug. The results for warfarin enantiomers are comparable with those obtained by an MS method, involving administration of a synthetic pseudoracemate [$^{12}C(R)$,$^{13}C(S)$]warfarin. In addition to all known metabolites, the detection of 7-*R*-hydroxywarfarin indicates that 7-hydroxylation is stereoselective rather than stereospecific.

Keyphrases □ Fluorescence, HPLC—stereospecific analysis of warfarin and its metabolites □ Warfarin—stereospecific fluorescence HPLC, metabolites □ Isomers—stereospecific fluorescence HPLC, warfarin and its metabolites

Warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin] contains an asymmetric center. In humans, the *R*- and *S*-isomers show differences in pharmacological activity (1), hepatic clearance, and metabolism (2–4). Drugs have been shown to interact differently with the isomers; for example, when coadministered with phenylbutazone, the hepatic clearance

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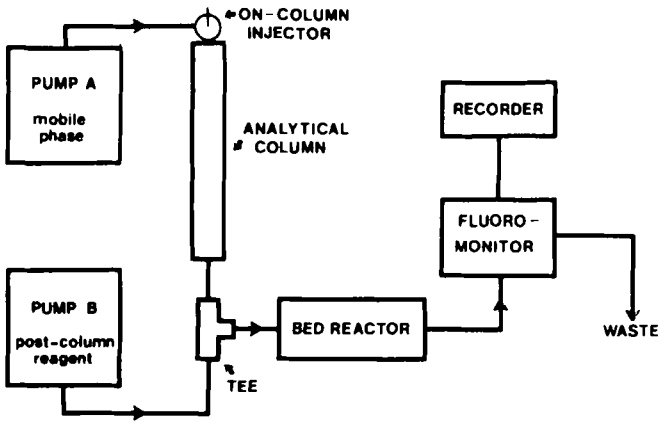

Figure 1—*Flow diagram of HPLC system used for separation and fluorescence detection of warfarin isomers and metabolites.*

of the *S*-isomer is decreased, whereas that of the *R*-isomer is, if anything, hastened (3, 5).

To explore the pharmacokinetics of the individual isomers and drug interactions with warfarin, several stereospecific assays were developed for the determination of warfarin enantiomers in plasma. One assay involved the synthesis of a stable isotope of one of the enantiomers, administration of a pseudoracemate, [^{12}C(*R*),^{13}C(*S*)]warfarin, and determination of the isomers in plasma by MS (6, 7). Another assay used was a stereospecific radioimmunoassay (8). Recently, we reported a high-performance liquid chromatographic (HPLC) technique involving UV detection (9). We now report an extension of the HPLC technique for determination of not only the enantiomers of warfarin, but also its enantiomeric metabolites in plasma and urine after administration of *RS*-warfarin to humans. The method is more sensitive and specific than the UV method.

EXPERIMENTAL SECTION

Reagents and Materials—Racemic warfarin was obtained from its sodium salt[1] by precipitation with 0.1 M HCl. The dried material was recrystallized from absolute ethanol. *R*(+)- and *S*(−)-warfarin[2]; warfarin metabolites[3] [6-hydroxywarfarin, 7-hydroxywarfarin, alcohol 1 (*RS* and *SR* alcohols)]; the selected internal standard[4], 3-α-[(4′-fluorophenyl)-β-acetylethyl]-4-hydroxycoumarin and 4′-fluorowarfarin; imidazole[5]; dicyclohexylcarbodiimide[6]; carbobenzyloxy-L-proline[6]; *n*-butylamine[6]; hexane (HPLC grade)[7]; methanol[8]; and ethyl acetate (HPLC grade)[7] were used as supplied. Peroxide-free ether[9] was prepared by passage through a column of activated alumina[10] (45 g).

Extraction from Plasma—To culture tubes[11] containing citrated plasma were added the internal standard (4′-fluorowarfarin), ranging from 0.85 to 6.8 μg/100 μL of water, sodium hydroxide (0.2 mL), and peroxide-free ether (4 mL). The mixture was shaken mechanically for 5 min and centrifuged[12] at 3000 rpm for 5 min; the organic phase was removed by aspiration. The aqueous phase was then acidified with 3 M HCl (0.5 mL), shaken with peroxide-free ether (8 mL) for 2 min, and centrifuged at 3000 rpm for 10 min; the ethereal layer was transferred to a clean culture tube. The ether was washed free of any citrate by shaking with pH 4 phosphate buffer (5 mL) for 5 min. (If the anticoagulant used is heparin, the pH 4 phosphate buffer

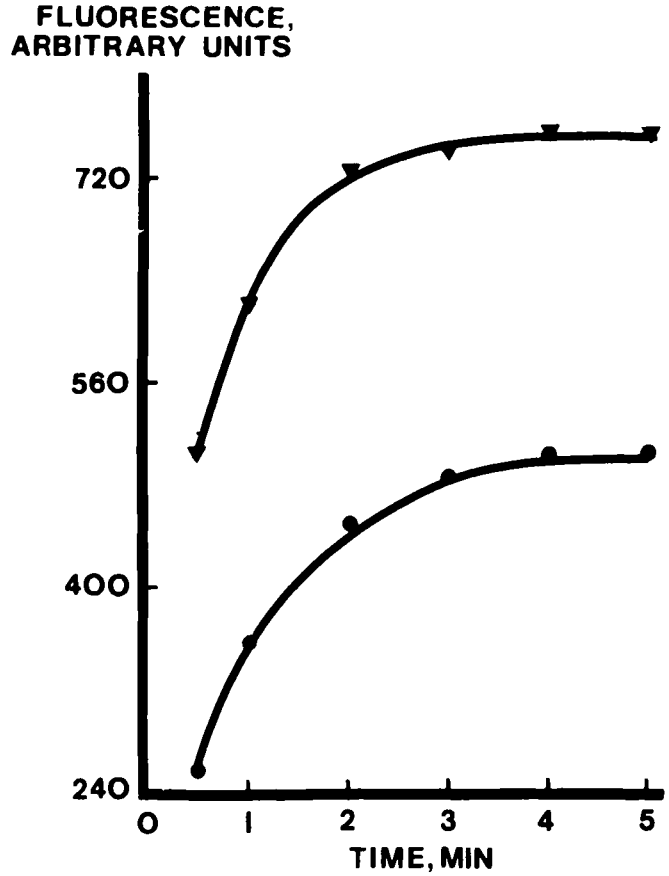

Figure 2—*Fluorescence intensity on incubation of the carbobenzyloxy-L-prolyldiastereoisomeric esters of* RS*-warfarin with* n*-butylamine (●) and 1,3-diaminopropane (▼).*

washing step can be omitted from the procedure.) After allowing the phases to separate, the ethereal layer was dried over calcium chloride (~1 g), centrifuged (3000 rpm for 3 min), and transferred to a culture tube, the tip of which was drawn out to a capacity of 0.2 mL. An antibumping granule was added to each tube, and the ether was evaporated on a heating block at 45°C under a stream of nitrogen. The tubes were washed three times with small volumes of ether, with evaporation of the ether carried out between each addition. Finally, a small volume of ether (100 μL) was added, vortexed, and evaporated to dryness.

Extraction from Urine—To silanized culture tubes containing internal standard (0.8–7 μg of 4′-fluorowarfarin in 100 μL of water) were added a urine sample (0.5 mL), 3 M HCl (0.5 mL), and chloroform (8 mL). The tubes were capped with screw-caps with polytef liners; the mixture was shaken for ~45 min by rotating gently and centrifuged at 3000 rpm for 10 min. The aqueous layer was removed by aspiration, and to the organic phase was added 0.1 M NaOH (1.7 mL). The process of extraction and phase separation was then repeated. The sodium hydroxide layer was transferred to a clean culture tube, acidified with 3 M HCl (0.5 mL), and shaken with chloroform (8 mL) for ~45 min. After centrifugation (10 min), the aqueous layer was removed by aspiration, and the chloroform layer was dried by vortexing for ~10 s over calcium chloride (~1 g/tube), followed by centrifugation at 3000 rpm for 5 min. As much as possible of the chloroform layer was then transferred to a culture tube, the tip of which was drawn out to a capacity of 0.2 mL and evaporated to dryness with a heating block at 65°C under a stream of nitrogen.

Derivatization—To the extracts in the tapered culture tubes were added carbobenzyloxy-L-proline (200 mg/mL of acetonitrile; 10 μL), imidazole (1 mg/ml of acetonitrile; 10 μL), and dicyclohexylcarbodiimine (200 mg/mL of acetonitrile; 10 μL), and the mixture was vortexed for ~10 s. After 2 h at ambient temperature a small volume of ethyl acetate (10 μL) was added to the mixture, and aliquots were analyzed by HPLC.

High-Performance Liquid Chromatography—A flow diagram of the HPLC system used for separation and fluorescence detection of the diastereoisomeric esters after aminolysis is shown in Fig. 1. The mobile phase [ethyl acetate-hexane-methanol-acetic acid (25:74.75:0.25:0.3)] was pumped[13] at a flow

[1] Sorex, U.K.
[2] Gifts from Endo Laboratories, Inc.
[3] Gifts from Dr. William Trager, School of Pharmacy, University of Washington, Seattle, Wash.
[4] Gifts from Ciba Geigy Ltd., Switzerland.
[5] Sigma Chemical Co.
[6] Aldrich Chemical Co.
[7] Rathburn, Scotland.
[8] Analar grade; Fisons, U.K.
[9] May and Baker, Dagenham, U.K.
[10] Alkaline, Brokman type 1; B.D.H., U.K.
[11] Corning Glass Works.
[12] Super Minor Centrifuge, no. 533A; MSE, U.K.
[13] Model 100A; Altex, Anachem Ltd., U.K.

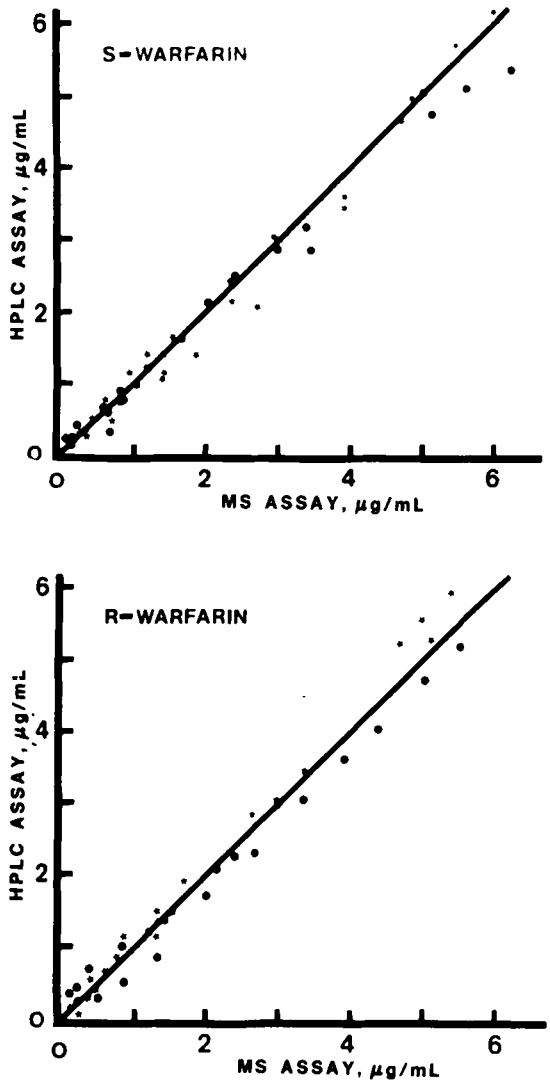

Figure 3—*Comparison between the HPLC assay carried out in this study and an MS assay (16) for the determination of warfarin enantiomers in plasma in subjects receiving racemic warfarin either alone (●) or during phenylbutazone administration (★).*

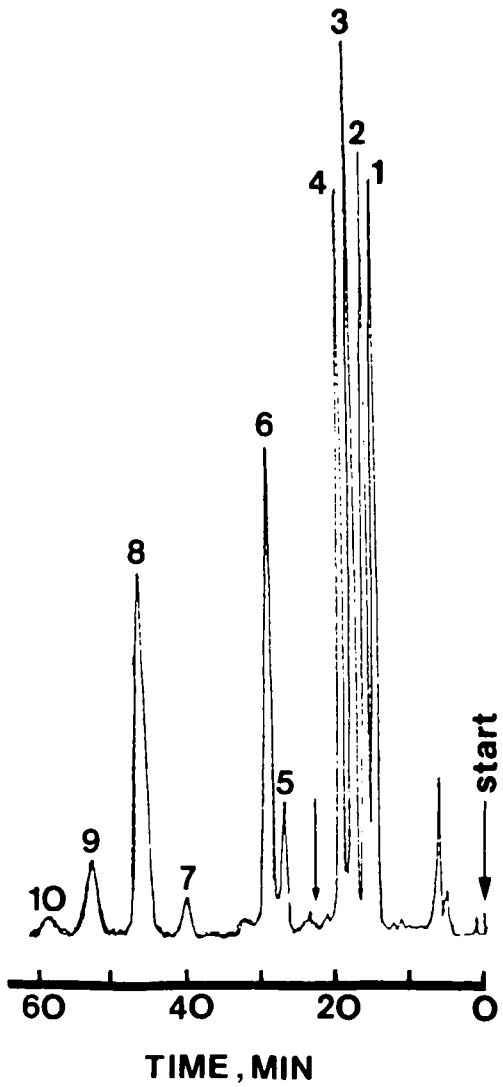

Figure 4—*Chromatogram resulting from extraction and derivatization with carbobenzyloxy-L-proline with subsequent postcolumn aminolysis of plasma (1 mL) obtained from a subject 24 h after oral administration of* RS-*warfarin (1.5 mg/kg). Key: (1)* S-*warfarin (3.2 μg/mL); (2) first eluting peak of internal standard (0.42 μg); (3)* R-*warfarin (4.0 μg/mL); (4) second eluting peak of internal standard (0.42 μg); (5)* SS-*alcohol (not quantitated); (6)* RS-*alcohol (0.53 μg/mL); (7) 6-*S-*hydroxywarfarin (0.11 μg/mL); (8) 7-*S-*hydroxywarfarin (0.77 μg/mL); (9) 6-*R-*hydroxywarfarin (0.18 μg/mL); (10) 7-*R-*hydroxywarfarin (not quantitated). Arrow indicates a change of sensitivity setting from 1 to 0.2 μA.*

rate of 0.8 mL/min through a stainless steel column (250 mm × 5 mm i.d.) packed with silica[14]. The analytical column outlet was fitted through the shortest possible length of polytef tubing (0.030 mm i.d.) to a stainless steel tee[15] (~0.16 cm i.d.), through which the postcolumn reagent [*n*-butylamine–methanol (1:1), pumped[16] at a flow rate 0.4 mL/min] was mixed with the mobile phase. The outlet of the tee was connected to a bed reactor (stainless steel column 3.0 mm i.d. × 25 cm) packed with glass beads[17] (40 μm). The entire system was at ambient temperature. The effluent from the bed reactor was analyzed fluorometrically with excitation at 313 nm, and the emission was measured in the presence of a filter[18] with a sensitivity setting of 6.16 and a time constant of 6 s. For the simultaneous determination of warfarin and metabolites, a range setting of 1 μA was used for between 5 and 1.0 μg per isomer of warfarin. When either the last internal standard ester or *SR*-alcohol had eluted, the range was switched to 0.2 μA to quantitate the other metabolites. At 0.2 μg per isomer of warfarin, the range setting was constant at 0.2 μA throughout the analysis.

The concentrations of enantiomers of warfarin and its oxidation products (6-hydroxywarfarin, 7-hydroxywarfarin) and reduction products (warfarin alcohols) in the unknown samples of plasma and urine were calculated by reference to appropriate calibration curves. These curves were constructed by taking known quantities of the reference materials in warfarin-free plasma and urine through the assay procedure and plotting the peak height ratios obtained, using either isomer of the internal standard, *versus* the known amount of isomeric material per sample.

Elution Order of Warfarin and Metabolites—The individual isomer elution order was established from the administration of either the *R*- or the *S*-warfarin in an isolated perfused rat liver system in a manner similar to that described by Pang and Rowland (10). *R*(+)- or *S*(−)-warfarin (20 μg/mL in Kreb's bicarbonate buffer) was added to the perfusate in a recirculating experiment. After 90 min, the experiment was stopped, and a sample of the reservoir was subjected to analysis for warfarin and metabolites as described above.

RESULTS AND DISCUSSION

Previously, we successfully developed a stereospecific HPLC method for the determination of *R*(+)- and *S*(−)-warfarin in plasma (9). The method involves the reaction of the 4-hydroxy group with carbobenzyloxy-L-proline with formation of diastereoisomeric esters, separation of the esters on silica as the stationary phase, and UV detection at 313 nm. The method has sufficient sensitivity (~0.1 μg per isomer) to measure the plasma concentrations of warfarin enantiomers after chronic maintenance therapy with racemic war-

[14] Si 5-μm; Spherisorb, Phase Separation, U.K.
[15] HETP, U.K.
[16] Model 600DA; Waters Associates.
[17] Whatman 4338-015; Whatman, Inc.
[18] No. 370.

farin and for up to 5 d after a large single oral dose (1.5 mg/kg) of the racemic drug. However, the method is insufficiently sensitive to measure in detail the pharmacokinetics after lower single doses and to measure the concentration of the warfarin metabolites (6-hydroxywarfarin, 7-hydroxywarfarin, warfarin alcohols), which are generally much lower than that of the parent drug (3). These limitations prompted us to develop a more sensitive stereospecific analytical procedure which was still based on the formation of diastereoisomeric esters with carbobenzyloxy-L-proline. Preliminary work showed that this reaction can readily be extended to warfarin metabolites. Use was made of the native fluorescence of warfarin and its metabolites (3).

Postcolumn Aminolysis—Fluorescence techniques often offer greater specificity, as well as enhanced sensitivity, over UV methods. However, by forming an ester at the 4-hydroxyl function, the fluorescent property of warfarin and its metabolites is lost, since only the ionized form of these species fluoresces (3). Therefore, to produce a fluorescent species, the chromatographed esters must be cleaved post column.

The optimal HPLC conditions for separation of the diastereoisomeric esters is the normal-phase mode, with silica used as the stationary phase. The mobile phase [ethylacetate–hexane–methanol–acetic acid (25:74.75:0.25:0.3)] is immiscible with water, so the frequently employed aqueous alkaline hydrolysis of esters could not be employed. The possibility of nonaqueous aminolysis of the carbobenzyloxy-L-proline esters of warfarin enantiomers with organic amines was explored by monitoring the liberated warfarin fluorometrically.

Successful nonaqueous aminolysis and development of a fluorescent species was achieved with *n*-butylamine and 1,3-diaminopropane in methanol. Triethylamine, *N*,*N*-dimethylaniline, and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine were also investigated. However, not only did the warfarin carbobenzyloxy-L-prolyl esters aminolyze more slowly with these organic bases, but fluorescence of the liberated warfarin was also markedly reduced in the presence of these amines, as compared with that achieved in the presence of either *n*-butylamine or 1,3-diaminopropane. TLC analysis [ethyl acetate–hexane–acetic acid (30:70:0.2), silica gel] of the butylamine reaction mixture at the end of the reaction, as judged by the maximum fluorescence reading, confirmed that aminolysis was complete. In place of the two diastereoisomeric esters (R_f 0.27 and 0.32) there was now only one spot corresponding to warfarin (R_f 0.35). Phenyl acetate undergoes aminolysis by *n*-butylamine to form *n*-butylacetamide (11) and, probably, the warfarin esters react similarly. A factor favoring butylamine and 1,3-diaminopropane is that alone, neither reagent produces significant fluorescence.

The fluorescent intensity of a methanolic solution containing the warfarin esters, in the presence of 16.7% base, reached an asymptote within ~4 min (Fig. 2). The data could be described by the first-order expression:

$$\ln (F_{max} - F_t) = \ln F_{max} - kt$$

where F_{max} is the asymptotic value of the fluorescent intensity, F_t is the fluorescent intensity at time t, and k is the first-order rate constant. From such a plot of the data, rate constants of 1.0 ($r^2 > 0.99$) and 1.3 min^{-1} ($r^2 > 0.99$), corresponding to half-lives of 42 and 32 s were obtained for the *n*-butylamine- and 1,3-diaminopropane-catalyzed reactions, respectively. These very short half-lives indicate that the kinetics of aminolysis and production of a fluorescent species is sufficiently rapid for use in an analytical procedure involving a postcolumn reaction step.

Careful selection of the design of the postcolumn reactor is required if chromatographic resolution is to be maintained. Open tubes do not allow effective mixing of the mobile phase and reagent (12). For reaction times greater than 1 min, the long tube required to create sufficient residence time leads to considerable dispersion of sample (13) with resultant loss of resolution and sensitivity. In addition, open-tube reactors are sensitive to changes in flow rate [decreasing flow rate leads to increased residence time resulting in decreased peak height (12)]. In contrast, bed reactors (tubes packed with large-diameter glass beads) provide micromixing of sample and reagents by increasing radial dispersion (14); they are suitable for reactions of up to ~5 min and are less sensitive than open-tube reactors to changes in the flow rate (13). Therefore, based on the reported better performance of bed reactors over tube reactors and the relatively rapid kinetics of aminolysis and development of a fluorescent species, this system was employed in the fluorometric assay (Fig. 1). The residence time in the bed reactor was ~1.5 min (given by the difference in retention of the unretained solute in the presence and absence of the bed reactor). Although it is not critical that the reaction goes to completion, as long as it is reproducible, the less complete the reaction the lower is the overall sensitivity of the assay. Therefore, with the postcolumn reagent [*n*-butylamine–methanol (1:1)] in the HPLC system, which was used at an identical dilution to that in the kinetic study (16.7%), the estimate of half-life computed from the kinetic study was used in conjunction with the residence time in the bed reactor to estimate the percentage of maximum aminolysis

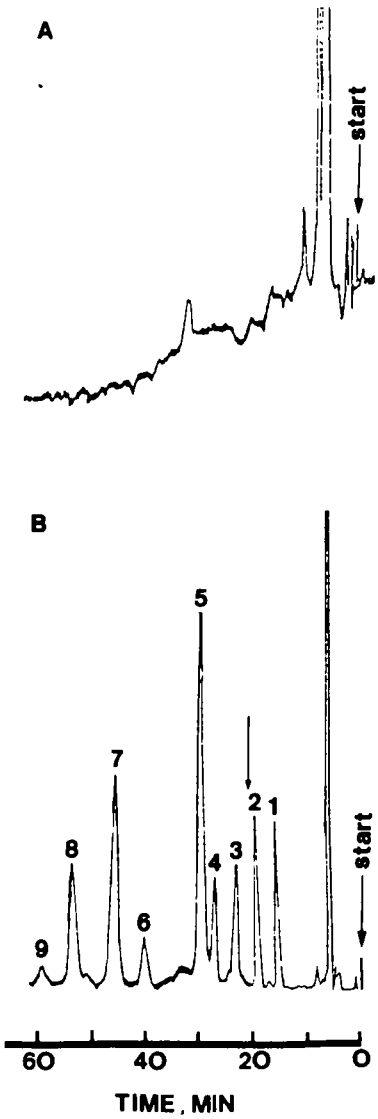

Figure 5—*Chromatogram resulting from extraction and derivatization with carbobenzyloxy-L-proline of a control urine sample (A) and a urine sample (0.5 mL) obtained from a subject between 4 and 5 d after oral administration of* RS-*warfarin (1.5 mg/kg) (B). Key: (1) first eluting peak of internal standard (0.42 μg); (2) second eluting peak of internal standard (0.42 μg); (3)* SR-*alcohol?; (4)* SS-*alcohol (not quantitated); (5)* RS-*alcohol (0.8 μg/mL); (6) 6-*S-*hydroxywarfarin (0.28 μg/mL); (7) 7-*S-*hydroxywarfarin (1.1 μg/mL); (8) 6-*R-*hydroxywarfarin (0.92 μg/mL); (9) 7-*R-*hydroxywarfarin (0.15 μg/mL). For (A), a sensitivity setting of 0.2 μA was used throughout; for (B), the arrow indicates a change of sensitivity setting from 1 to 0.2 μA.*

and fluorescence developed. Under these conditions, at least 78% of the maximum value is obtained. Although 1,3-diaminopropane gives superior results to those of *n*-butylamine, its choice for further investigation was rejected because of the greater toxicity of diamines.

The use of a racemic internal standard which forms diastereoisomeric esters requires a chromatographic system with high resolution to separate the internal standard esters from those of warfarin. Our situation was acute since under optimal chromatographic conditions, the ester of *S*(−)-warfarin elution was followed by the first eluting ester of the racemic internal standard, which preceded the ester of *R*(+)-warfarin. The lack of significant band broadening of the peaks when the postcolumn reactor was used (Fig. 1) is an important feature of the assay technique.

Elution Order of Warfarin and Metabolites—By using the isolated perfused rat liver preparation as a metabolite generator, in all cases, the metabolite derived from the parent molecule with the *S*(−)-configuration eluted first. Thus, 7-*S*-hydroxywarfarin eluted before 7-*R*-hydroxywarfarin, and 6-*S*-hydroxywarfarin eluted before the corresponding *R*-isomer. With the alcohols, the availability of (*R* and *S*)-alcohols (a mixture of all four alcohols), alcohol

1 (*SR* and *RS*), and pure *RS*-alcohol, permitted assignment of the order of elution as *SR*-, *SS*-, *RR*-, and *RS*-alcohol.

Analysis of Plasma—By varying both the concentration of the internal standard (0.85–6.8 μg) and the sensitivity setting on the fluorescence detector, warfarin and its major metabolites can be simultaneously quantitated from plasma (1 mL). The complete analysis time for warfarin and metabolites is 1 h. No major peaks occurred in the chromatogram of control citrated plasma obtained from a healthy volunteer. Similar results were observed for plasma obtained from other subjects. Warfarin and its metabolites had to be separated from citric acid with pH 4 phosphate buffer. In high concentration in citrated plasma, this acid consumes the activating reagent (*N,N*-dicyclohexylcarbodiimide) and also forms esters in place of carbobenzyloxy-L-proline with warfarin and metabolites, leading to nonreproducible results. There were no such problems with heparin, so the pH 4 phosphate buffer wash is not needed when this anticoagulant is used.

Calibration plots for racemic warfarin and the separate metabolites (*RS*-alcohol, 7-*S*- and 7-*R*-hydroxywarfarin, and 6-*S*- and 6-*R*-hydroxywarfarin) in plasma are linear ($r^2 > 0.99$), as are the corresponding plots when these species are analyzed as a multicomponent mixture. Once formed, the respective carbobenzyloxy-L-propyl esters are stable for at least 24 h. The *RSD* over the range 0.1 to 1.0 μg per isomer for the *S*- and *R*-warfarin isomers varied between 8.7 and 1.7% (first eluting esters of the internal standard). By using the second eluting peak of the internal standard, the *CV* ranged between 8.7 and 2.6%. By varying both the concentration of the internal standard (0.85–6.8 μg) and the sensitivity setting on the fluorescence detector, warfarin and its major metabolites could be simultaneously quantitated from plasma (1 mL). The determination limit (with a 10% *CV*) of the metabolites in plasma varied from 0.045 μg for 7-*R*-hydroxywarfarin to 0.15 μg for 6-*R*-hydroxywarfarin, when the first eluting ester of the internal standard was used to compute the peak height ratios. Similar results were obtained when the more slowly eluting ester of the internal standard was used. The noise level of the fluorescence detector was one of the major factors limiting sensitivity.

Analysis from Urine—Preliminary investigations of metabolite extraction from urine by the assay procedure for plasma and detection by the UV (313 nm) procedure were discouraging, because compounds present in metabolite-free urine were UV absorbent and chromatographed with retention times similar to those for the internal standard and the warfarin alcohols.

In contrast, fewer interferences were seen in chromatograms when the extraction procedure described above was used and when fluorescence was the mode of detection. Linear calibration plots were obtained for all the metabolites over their respective ranges (*RS*-alcohol, 0.106–0.85 μg; 7-*R*- and 7-*S*-hydroxywarfarin, 0.1–1.0 μg; 6-*R*- and 6-*S*-hydroxywarfarin, 0.14–1.1 μg). Owing to the lack of a good analytical standard for *SS*-alcohol (alcohol 2), no calibration plots were obtained. The determination limit (with a 10% *CV*) of the metabolites in urine were similar to those in plasma, ranging from 0.042 μg for the 7-*R*-hydroxywarfarin to 0.17 μg for 6-*S*-hydroxywarfarin.

Comparison with a Mass Spectroscopic Assay—As part of a collaborative program, plasma samples[19] from subjects who had participated in a phenylbutazone–warfarin study 2 years previously were received for analysis of *R*(+)- and *S*(−)-warfarin. In the study, subjects received a pseudoracemate of warfarin [^{12}C(*R*),^{13}C(*S*)]warfarin before and during administration of phenylbutazone, and the concentration of the individual enantiomers was determined by an MS method (15). The samples were stored frozen until they were analyzed by us, and the results of the previous analysis were not made available to the analyst until after the results by the present fluorescence method were obtained.

Rigorous statistical evaluation of the performance of the HPLC and the MS methods cannot be shown since both assays were not performed by the same laboratory. The concentrations of the isomers when *RS*-warfarin was administered alone or with phenylbutazone are presented in Fig. 3. The slopes of the warfarin concentrations in the absence or during phenylbutazone treatment are close to 1, suggesting that the two methods give comparable results. These graphs also illustrate both the good stability of warfarin in plasma, since these analyses were performed ~2 years apart, and the lack of interference of phenylbutazone and its metabolites in the analysis of warfarin. This lack of interference in the fluorescence assay was further demonstrated by the absence of interfering peaks and identity of the slopes of the calibration curves for the enantiomers of warfarin when assayed from plasma that was spiked with therapeutic concentrations of phenylbutazone and its major oxidation metabolites, oxyphenbutazone and γ-hydroxyphenylbutazone (16).

Presence of Other Drugs—Interference in the plasma or urine assay by other drug substances such as cimetidine and salicylate drugs that may be administered concomitantly with warfarin was also studied. Cimetidine produced no spurious peaks in the chromatograms in the presence or absence of *RS*-warfarin. As an imidazole, it is a base and presumably was extracted by the acid–base extraction step.

A large peak in the chromatogram, which interferes with the analysis of warfarin metabolites, was observed when urine spiked with salicylate was taken through the assay procedure. Salicylate fluoresces at the maximum wavelength of excitation of warfarin, is activated by dicyclohexylcarbodiimide, and forms an ester with warfarin or consumes the chiral reagent by formation of an ester at the phenolic group. Hence, the quantitation of the metabolites of warfarin in urine in the presence of salicylate is likely to be severely limited by some or all of the factors described above.

***In Vivo* Study**—Figure 4 shows a chromatogram of a plasma sample obtained 24 h after the administration of *RS*-warfarin (1.5 mg/kg) to a healthy volunteer. The metabolic pattern observed is consistent with the stereoselectivity of metabolism shown by other workers after the administration of the individual isomers (3, 4). In addition, a small quantity of 7-*R*-hydroxywarfarin was seen. A similar metabolic pattern can be seen in urine 123 h after ingestion of *RS*-warfarin (1.5 mg/kg) (Fig. 5). In addition, there is a peak which chromatographs with a retention time similar to that of the *SR*-alcohol. This peak is absent in a metabolite-free urine sample taken from the same subject. The presence of this peak suggests that the reduction of the *S*-isomer may not be stereospecific for the *S*-configuration.

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