Journal of Chromatography, 131 (1977) 365–373

CHROM. 9638

BIOCHEMICAL APPLICATIONS OF A QUANTITATIVE HIGH-PRESSURE LIQUID CHROMATOGRAPHIC ASSAY OF WARFARIN AND ITS METABOLITES

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(Received June 21st, 1976)

SUMMARY

A quantitative high-pressure liquid chromatographic assay of warfarin and its diastereoisomeric alcohols and 4'-, 6-, 7-, 8- and benzylic hydroxylated metabolites was accomplished using a reversed-phase, microparticle column (μBondapak/C_{18}) employing 1.5% acetic acid (pH 4.7)–acetonitrile (69:31) as solvent. Concentration of these compounds on the column prior to their elution permitted the loading of 0.01–0.5 ml solutions of warfarin and metabolites without consequent alterations in peak heights and resolution. Details of the use of this technique in the study of *R* and *S* warfarin metabolism by hepatic mixed function oxidases and in the determination of warfarin and its metabolites in blood are presented.

INTRODUCTION

Warfarin, 3-(α-acetonylbenzyl)-4-hydroxycoumarin (I), an extensively used oral anticoagulant and rodenticide, functions as a vitamin K_1 antagonist via (an) unknown mechanism(s). The importance of warfarin in clinical medicine and as a means for investigating vitamin K_1-dependent biochemical pathways and, more recently, of its *R* and *S* enantiomers as probes of hepatic mixed function oxidases has prompted the publication of a number of quantitative analytical procedures for warfarin and its metabolites in biological fluids[1–5]. These currently available analytical techniques for the determination of warfarin are, however, extremely tedious and/or are not capable of the simultaneous assay of individual metabolites. We have consequently developed a simple high-pressure liquid chromatographic (HPLC) assay of warfarin and all its known metabolites which will facilitate pharmacological and biochemical investigations of these compounds.

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EXPERIMENTAL

Apparatus

A Waters Assoc. (Milford, Mass., U.S.A.) liquid chromatograph was used, which comprised a Model 6000A three channel solvent delivery system, a U6K injector, a Model 440 absorbance detector equipped with a 313-nm filter, and an Omniscribe recorder. The column (30 cm × 4 mm) packing was reversed-phase, microparticle silica, 10 μm (μBondapak/C_{18}, Waters Assoc.). Aqueous buffer solutions were filtered through a 0.22-μm membrane (Millipore, Bedford, Mass., U.S.A.) before chromatographic use. Glass-distilled acetonitrile was purchased from Burdick and Jackson (Muskegon, Mich., U.S.A.).

All other reagents and chemicals were of the highest purity grade commercially available.

*Compounds**

Racemic warfarin was purchased from Calbiochem (La Jolla, Calif., U.S.A.). The *R* and *S* enantiomers were resolved by the method of West *et al.*[6] to optical purities of 100 and 101%, respectively, based on published optical rotation values[6].

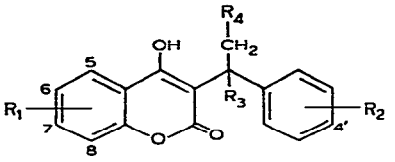
4'-Hydroxywarfarin (V) was prepared by a modification of the method of Hermodson *et al.*[7]. 4-Hydroxycoumarin (4.4 g, 0.027 mole) and *p*-hydroxybenzalacetone (6.6 g, 0.045 mole) were heated to reflux for 20 h in 20 ml dioxan containing a catalytic amount of piperidine. The reaction mixture was cooled, diluted with 250 ml of 0.1 *M* sodium hydroxide and extracted twice with dichloromethane. The aqueous phase was acidified (concentrated HCl), and extracted twice with ethyl acetate. The extracts were combined, dried with sodium sulfate and evaporated to dryness *in vacuo*. The residue was then dissolved in a minimum of acetone, six volumes of chloroform were added, and, after concentration to 25% of the original volume, the solution was allowed to crystallize at 5° overnight. Filtration of the product followed by recrystallization from diethyl ether yielded the desired product. The appropriate benzyloxy-2-hydroxyacetophenone intermediates of the 6- (II), 7- (III) and 8-hydroxywarfarin (IV) metabolites were prepared as previously described[8]. Ring closure to the corresponding benzyloxy-4-hydroxycoumarins was accomplished by the method of Buckle *et al.*[9] and side chain addition of benzalacetone was performed as described for 4'-hydroxywarfarin. Removal of the benzyl-protecting group was accomplished by catalytic hydrogenation[8]. Final purification of each of these compounds was accomplished by crystallization from diethyl ether. The *R,R*; *R,S* and *S,S*; *S,R* warfarin alcohols (VII) were respectively prepared from the *R* and *S* warfarin enantiomers by the method of Trager *et al.*[10] and were resolved as described by Chan *et al.*[11]. The melting points of all synthesized compounds were in good agreement with reported values[7]. Benzylic hydroxywarfarin (VI) was isolated as a product of the *in vitro* hepatic microsomal metabolism of *R* warfarin as described by Pohl *et al.*[12].

Chromatographic technique

The ability of the μBondapak/C_{18} column to resolve all of the metabolites of warfarin from each other and the parent compound was critically dependent upon

* See Table I for structures.

TABLE I
STRUCTURES OF WARFARIN AND ITS METABOLITES



No.	Compound	Structure			
		R_1	R_2	R_3	R_4*
I	Warfarin	H	H	H	$-\overset{\overset{O}{\|\|}}{C}-CH_3$
II	6-Hydroxywarfarin	6 = –OH	H	H	$-\overset{\overset{O}{\|\|}}{C}-CH_3$
III	7-Hydroxywarfarin	7 = –OH	H	H	$-\overset{\overset{O}{\|\|}}{C}-CH_3$
IV	8-Hydroxywarfarin	8 = –OH	H	H	$-\overset{\overset{O}{\|\|}}{C}-CH_3$
V	4'-Hydroxywarfarin	H	4' = –OH	H	$-\overset{\overset{O}{\|\|}}{C}-CH_3$
VI	Benzylic hydroxywarfarin	H	H	–OH	$-\overset{\overset{O}{\|\|}}{C}-CH_3$
VII	*R,R*; *R,S*, and *S,S*; *S,R* warfarin alcohols	H	H	H	$-\overset{\overset{OH}{\|}}{\overset{*}{C}}-CH_3$

* Asymmetric center indicated by *.

the choice of solvent, ionic strength, flow-rate and pH. The optimum separation was obtained in 1.5% acetic acid (pH 4.7 with concentrated NH_4OH)–acetonitrile (69:31) (solvent 2) at a flow-rate of 2.0 ml/min. For very dilute solutions of warfarin or any one of its metabolites (of the order of 0.04 μg/ml), large volumes containing sufficient material to be detected were injected onto the column and concentrated prior to elution to avoid peak spreading. This operation was performed by concentrating the sample on the column in 1.5% acetic acid (pH 4.7 with concentrated NH_4OH)–acetonitrile (9:1) (solvent 1) at a flow-rate of 2.0 ml/min. After 4 min, warfarin and its metabolites were eluted by passage of solvent 2 through the column at the same flow-rate. Re-equilibration of the column was readily accomplished by washing at 2.0 ml/min with solvent 1 for 5 min.

Calibration plots

Only a single peak on the chromatogram was observed for each synthesized

compound. Quantities of warfarin and its hydroxylated metabolites ranging from 0.02–0.4 μg were concentrated on the column and eluted as described. The peak heights of each hydroxylated metabolite of warfarin over the concentration range investigated were plotted against the corresponding concentration of that metabolite, and the best straight lines were fitted to the points by least squares regression analysis. The independence of the technique from load volume was determined by a comparison of the peak heights obtained when volumes of methanol–water (1:9) ranging from 0.01–0.5 ml and containing 0.2 μg of each warfarin metabolite were injected and chromatographed as described.

Quantitation of warfarin metabolites in plasma

Male Wistar rats were injected intraperitoneally (i.p.) with the sodium salt of either *R* or *S* warfarin, 10 mg/kg, dissolved in 0.9% saline. After 16 h, animals were anesthetized with 0.5 ml Diabutal (Diamond Labs., Des Moines, Iowa, U.S.A.), i.p., and 4.5 ml of blood was removed from the posterior vena cava with a plastic syringe containing 0.5 ml of 3.8% trisodium citrate. The blood was then centrifuged at 10,000 *g* for 20 min and the plasma separated by decantation.

For the assay of warfarin and its metabolites, 1.3 ml of plasma was mixed with 0.2 ml of a saturated solution of sodium borate, vigorously shaken with 3 ml of chloroform and centrifuged at 20,000 *g* for 30 min. An appropriate amount of the aqueous phase was chromatographed as described.

Quantitation of warfarin metabolites from liver microsomes

Preparation of liver microsomes. Groups of two or three male Wistar rats were used in the preparation of each batch of microsomes. The rats were killed by cervical dislocation and the livers excised, pooled and washed with 0.02 *M* Tris–HCl/0.15 *M* KCl buffer, pH 7.4. The livers were minced and homogenized in 1.5 volumes of cold 0.02 *M* Tris–HCl/0.15 *M* KCl buffer using a Potter–Elvehjem homogenizer fitted with a PTFE pestle. Microsomes were then prepared by slight modification of the method of Tangen *et al.*[13]. A column of Sepharose 2B (6.0 cm × 8 cm) was used to separate the microsomes from cytoplasmic contaminants. The protein concentration of the microsomal suspension was determined by the method of Schacterle and Pollack[14] and the cytochrome P-450 concentration by the method of Omura and Sato[15].

Incubation of warfarin with microsomes and quantitation of metabolites. Sodium warfarin (2.0 mg, 1.5 m*M*) in 1.0 ml of 0.01 *M* TES [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] and 0.001 *M* $MgCl_2$, pH 7.4, was placed in a 25-ml Erlenmeyer flask cooled in ice. To this was added a NADPH generating system which comprised NADP (1.0 mg sodium salt, 1.25 μmoles), glucose-6-phosphate (20.0 mg disodium salt monohydrate, 62.1 μmoles), and glucose-6-phosphate dehydrogenase (yeast, 5.0 units) in 1.0 ml of the same TES·$MgCl_2$ solution. The contents were then incubated at 37° for 2 min, 2.0 ml of microsomal suspension containing 4.0 mg protein/ml were added, and the mixture was incubated for 10 min. The cytochrome P-450 concentration was 1.12 nmoles per mg microsomal protein. Shortly before the end of the incubation, approximately 2 ml of the mixture were removed with a plastic syringe and at exactly 10 min were rapidly filtered through a 13-mm Swinnex filter unit (Millipore) containing a pad of a 1 × 1.2 μm filter and 2 × 0.22 μm filters. A suitable quan-

tity of the filtrate was injected into the liquid chromatograph, concentrated on the column and eluted as described under Chromatographic technique.

Recovery experiments

Aliquots of methanol–water (1:9) solutions of warfarin metabolites were added to microsomes in TES buffer, pH 7.4, or rat plasma or water (controls) and incubated for 10 min at 37°. The solutions were then assayed as described to determine the recoveries of warfarin and its metabolites.

RESULTS AND DISCUSSION

A typical HPLC separation, in solvent 2, of the synthetic metabolites of warfarin is illustrated in Fig. 1. All of the hydroxylated metabolites were clearly separated and readily quantitated from their respective peak heights. The *R* or *S* warfarin alcohol diastereoisomers were not completely separated from each other and tended to spread relative to the other peaks notwithstanding their elution near the solvent front. *R* and *S* warfarin have been shown, however, to be primarily reduced at the ketonic carbon to the *S* configuration in man[11] and in *in vitro* investigations with rat liver homogenates[12], and the relatively low levels of the *R* configuration alcohols thus permit quantitation in metabolic investigations.

The products of *R* and *S* warfarin arising as a consequence of incubation with rat liver microsomes in the presence of an NADPH generating system and analyzed

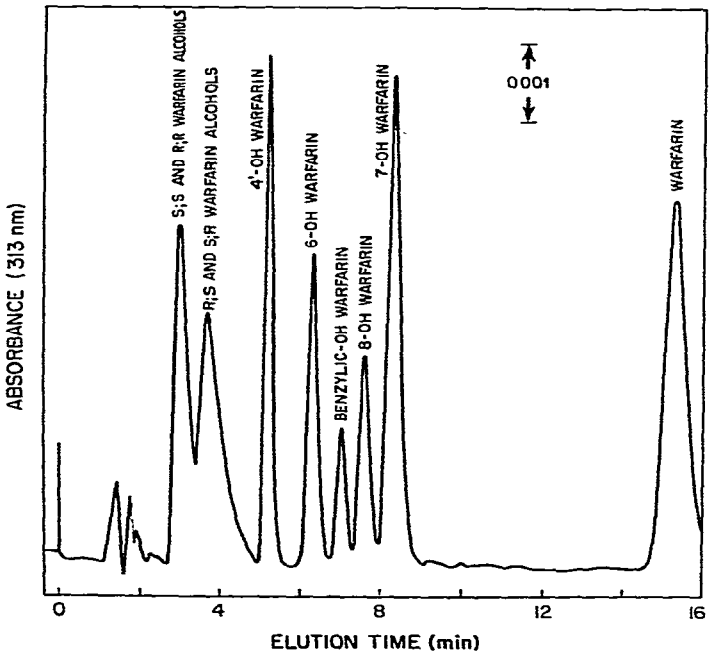


Fig. 1. HPLC separation of warfarin and its synthetic metabolites on a μBondapak/C_{18} column employing 1.5% acetic acid (pH 4.7 with concentrated NH_4OH)–acetonitrile (69:31) at a flow-rate of 2.0 ml/min and a chart speed of 0.5 in./min.

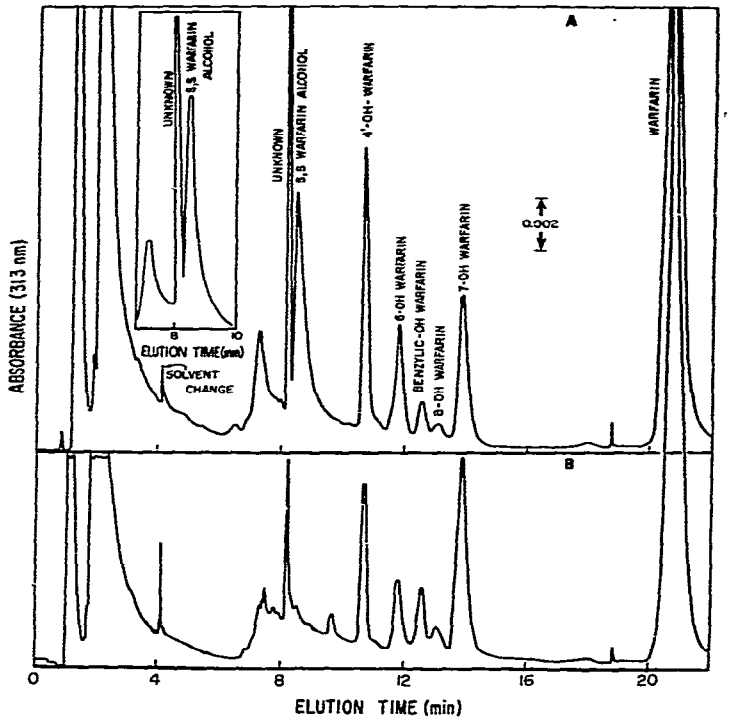


Fig. 2. HPLC analysis of the products of (A) *S* warfarin and (B) *R* warfarin metabolism by rat liver microsomes (2.0 mg protein/ml; 1.12 nmoles cytochrome P-450 per mg protein). The filtered microsomal suspension was loaded and concentrated on the μBondapak/C_{18} column with 1.5% acetic acid (pH 4.7 with concentrated NH_4OH)–acetonitrile (9:1) and eluted with 1.5% acetic acid (pH 4.7 with concentrated NH_4OH)–acetonitrile (69:31) at a flow-rate of 2.0 ml/min and a chart speed of 0.5 in./min. Insert: Effect of acidification and extraction with dichloromethane on the quantities of the previously undetected metabolite of warfarin.

by the described technique are shown in Fig. 2. The metabolic patterns are in good agreement with those previously reported[12], in particular, the stereoselectivity of 7-hydroxylation and benzylic hydroxylation of *R* warfarin and the stereoselectivity of 4′-hydroxylation and reduction to the *S,S* alcohol of *S* warfarin. The baseline absorbance change at 6 min was due to refractive index changes between solvents 1 and 2. Control microsomes were free of 313-nm absorbing compounds in the region of warfarin and its metabolites. Chemically synthesized metabolites added to the products of warfarin produced by incubation with liver microsomes cochromatographed as homogeneous peaks.

R and *S* warfarin and their metabolites present in the plasma of rats which had received the individual enantiomers were assayed as described. The pharmacologically more active *S* enantiomer of warfarin[16–19] was present in greater concentrations than the *R* isomer, and this is consistent with the reports of others[17,18]. Interestingly, the 4′-hydroxy metabolite (V) of *S* warfarin was present in a significant concentration and nearly twice that of *R* 4′-hydroxywarfarin. Since 4′-hydroxywarfarin has been reported to possess antivitamin K_1 activity in the rat[20], the high plasma levels of this metabolite may explain, at least in part, the increased pharmacological activity

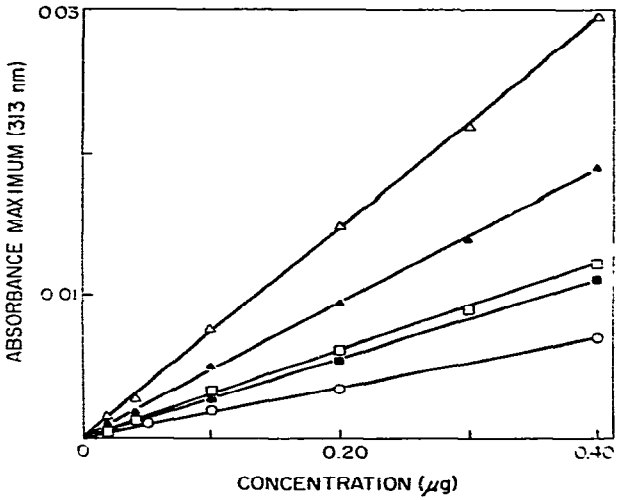


Fig. 3. HPLC calibration plots of warfarin and its metabolites over the concentration range of 0.02–0.4 μg. Peak heights were measured after separation of the compounds as described in Fig. 2. △, 4′-Hydroxy and 7-hydroxywarfarin; ▲, *S,S* and *R,R* warfarin alcohols and 6-hydroxywarfarin; □, warfarin; ■, *S,R* and *R,S* warfarin alcohols; ○, 8-hydroxywarfarin.

of the *S* enantiomer of warfarin in these animals. Control plasma was free of 313-nm absorbing materials in the region of warfarin and its hydroxylated metabolites.

Standard solutions of synthesized warfarin metabolites were quantitated by the use of the same technique as employed in the isolation of these products from liver microsomes. Calibration plots of each metabolite, based on peak heights, were linear throughout a concentration range of 0.02–0.4 μg and were independent of such variables as concentrations of other metabolites and load volumes over a range of 0.01–0.5 ml. Straight lines obtained by least squares analysis of the data obtained for each metabolite are shown in Fig. 3. The correlation coefficient of each line was near unity, and the intercepts were essentially at the origin.

Recoveries of warfarin and its metabolites from liver microsome preparations and plasma were quantitative over the range of 0.04–0.8 μg/ml of solution. The microsomal protein concentration used for these studies was 2.0 mg/ml.

The optimal conditions for the separation of the warfarin metabolites were determined by trial and error. For example, the use of a water–acetonitrile mixture containing 1 % acetic acid as eluting solvent completely resolved the warfarin alcohols (VII) from each other but the *S* configuration alcohols did not separate from 7-hydroxywarfarin (III), and both diastereoisomers migrated as nonpolar compounds relative to the majority of the hydroxylated metabolites. Moreover, 7- and 8-hydroxywarfarin were not resolved in this system and changes in solvent composition did not achieve separation. Buffering the acetic acid to pH 4.0 with either sodium or ammonium hydroxide caused the warfarin alcohols to migrate as very polar compounds, which now eluted ahead of the hydroxylated metabolites. The increase in pH to 4.0 did not affect the separation of 7- and 8-hydroxywarfarin, but a further increase in pH to values of 4.7 or greater achieved their resolution. At pH 4.8, however, benzylic and 8-hydroxywarfarin were very poorly resolved, while at pH 4.6 these two metab-

olites were well separated from each other but were not clearly resolved from 6- and 7-hydroxywarfarin, respectively. The migration of benzylic and 8-hydroxywarfarin is therefore critically dependent on the pH of the eluting solvent, and a variation from pH 4.7 of as little as 0.1 of a pH unit will dramatically affect the quality of the metabolite elution profile.

A comparison of the HPLC elution profile of the synthetic warfarin metabolites (Fig. 1) with that of the products of the microsomal metabolism of warfarin (Fig. 2) indicates the formation of a previously undetected metabolite. This compound elutes at approximately 8 min and prior to the *S,S* alcohol (Fig. 2). The formation of this metabolite is stereoselective for *S* warfarin and its concentration approximates that of the major monohydroxylated metabolites produced from this enantiomer (assuming similar extinction coefficients at 313 nm). A markedly lower concentration of this metabolite was, however, observed when the products of *S* warfarin metabolism were solvent extracted from acidified microsomal suspensions (see insert Fig. 2), although the concentrations of the other metabolites were not affected by this treatment. Attempts are currently under way to identify this warfarin metabolite and determine its role in warfarin function.

Vesell and Shively[4] were the first to describe the increased precision, sensitivity and speed of liquid chromatography compared to thin-layer and spectrophotometric methods previously employed for the quantitation of warfarin in plasma. Recently, O'Reilly and Motley[5] reported the separation of warfarin, its alcohol diastereoisomers, and certain of its hydroxylated products on a Sperisorb silica column and demonstrated the presence of warfarin metabolites in plasma extracts. Neither of these liquid chromatographic techniques have demonstrated the separation of all the warfarin metabolites from one another, however, and thus errors in quantitation may be introduced as a consequence of compound overlap. Moreover, both these procedures require the loading of small quantities of concentrated samples in order to achieve reproducible peak heights and avoid losses in resolution due to peak spreading. The presently reported HPLC assay of warfarin and all its known metabolites readily and rapidly permits their simultaneous determination, uniformly concentrates small quantities of products on the column prior to their elution, and allows for accurate quantitation of the less prominent warfarin metabolites, since they are eluted nearer the solvent front. Further, in the case of the microsomal metabolism of warfarin, the direct analysis of metabolites without prior acidification and extraction overcomes the potential problems of product instability[8] and/or abnormally high aqueous solubilities of metabolites.

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