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ANALYSIS OF WARFARIN AND ITS METABOLITES BY REVERSED-PHASE ION-PAIR LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A high-performance liquid chromatographic method was developed for the determination of warfarin and its metabolites (diastereomeric warfarin alcohols and 6-, 7-, 8-, 4′- and 3′-hydroxywarfarin) in microbial cultures. Ion-pair chromatography with tetrabutylammonium ion as the counter ion allowed for the complete resolution of all compounds at pH 7.5 on a reversed-phase (C_{18}) column, thus permitting direct fluorescence detection without the use of post-column pH switching techniques. Analysis of cell suspension cultures of the fungus *Cunninghamella elegans* (ATCC 36112) indicated that this organism metabolizes warfarin to all known mammalian metabolites, plus the previously unreported 3′-hydroxywarfarin. Detection limits for all compounds were in the low nanogram range.

INTRODUCTION

Warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin, **1**; Fig. 1] has been used extensively both as a rodenticide[1] and as a clinically effective oral anticoagulant[2]. More recently, this compound has been exploited as a probe to study the multiplicity of cytochrome P-450 isozymes from a variety of mammalian sources[3–6]. Because of its clinical, pharmacological and biochemical importance, a number of sophisticated chromatographic methods have been developed to quantitate warfarin (**1**) and its metabolites (**2**–**11**) in various biological matrices[7–11].

Most notable of these analytical methods are the high-performance liquid chromatographic (HPLC) assay of Fasco and co-workers[7,8] and the gas chromatographic–mass spectrometric (GC–MS) assay of Bush *et al.*[11]. The HPLC method of Fasco utilizes either an isocratic[7] or gradient[8] elution technique with UV absorption detection at 313 nm. While offering high selectivity and complete resolution of all metabolites, this assay suffers from the inherent sensitivity limitation of UV photometric

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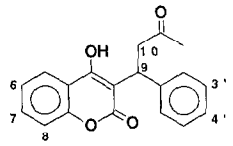
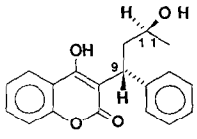


1. Warfarin
2. 6-Hydroxywarfarin
3. 7-Hydroxywarfarin
4. 8-Hydroxywarfarin
5. 4'-Hydroxywarfarin
6. 3'-Hydroxywarfarin
7. 9-Hydroxywarfarin
8. 10-Hydroxywarfarin



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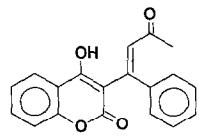

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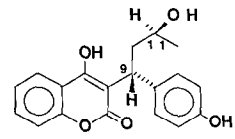
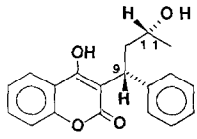
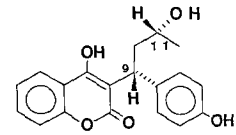


12. RS/SR-Int. Std. A (9R,11S- Shown)



10. RR/SS-Alcohol (9R,11R- Shown)



13. RR/SS-Int. Std. B (9R,11R- Shown)

Fig. 1. Structures of warfarin (**1**), warfarin metabolites (**2**–**11**) and internal standards (**12**, **13**).

detection. The GC–MS assay of Bush *et al.*[11] employs a high-resolution capillary column to achieve separation of all compounds. Although excellent sensitivity is afforded by MS selected ion detection, specialized instrumentation and deuterated internal standards are required for warfarin and each metabolite.

Warfarin exhibits a strong native fluorescence[12]; thus, HPLC separation with fluorescence detection should make possible the sensitive analysis of warfarin and its metabolites. In most reports, however, full exploitation of warfarin's fluorescence has been precluded by the use of acidic mobile phases which quench the fluorescence[9]. This problem can be resolved by using post-column pH-switching techniques which alter the pH of the mobile phase with post-column reagents as it enters the detector, and has been described for the normal-phase HPLC determination of warfarin and its metabolites[9,10]. However, such methods are not suitable for the analysis of microbial cultures with high concentrations of residual warfarin in comparison to levels of warfarin metabolites. Since warfarin elutes before its metabolites in normal-phase chromatography, the large amount of warfarin introduced would overload the HPLC column and interfere with the quantitation of the metabolites.

Ion-suppression techniques with acidic mobile phases have been used for reversed-phase HPLC of warfarin and its analogues which are 4-enols and thus act as

weak acids. At neutral pH these compounds are totally ionized and do not chromatograph efficiently on reversed-phase columns. Ion-pair chromatography with a cationic counter ion and a mobile phase buffered at pH 7.5 to afford complete ionization of all analytes offers an alternative approach for the chromatography of these compounds. The use of mobile phases at a pH above 6.5 also permits direct fluorescence detection without the experimental complexity of post-column pH-switching techniques. This method has been described previously for the analysis of coumarin rodenticide residues in animal tissue[13] and rodenticide concentrates[14]. The present report describes the development of a highly sensitive reversed-phase ion-pair HPLC method with direct fluorescence detection for the analysis of warfarin (**1**) and its major metabolites (**2**–**6**, **9**, **10**) in microbial culture.

EXPERIMENTAL

Chemicals and reagents

All reagents are analytical reagent grade or higher in quality. Solvents for HPLC were HPLC grade (OmniSolv, MCB Manufacturing Chemists, Cincinnati, OH, U.S.A.). Racemic warfarin was obtained from Sigma (St. Louis, MO, U.S.A.). Tetrabutylammonium phosphate (1.0 *M* solution) was purchased from Aldrich (Milwaukee, WI, U.S.A.). Phenolic warfarin metabolites [6-, 7-, 8-, 4′- and 3′-hydroxywarfarin (**2**–**6**)] were synthesized by the methods of Hermodson *et al.*[15] or Bush and Trager[16]. Dehydrowarfarin (**11**) was prepared by cuprous chloride oxidation of warfarin[8]. Warfarin alcohols (**9**, **10**) and the internal standard 4′-hydroxywarfarin alcohols (**12**, **13**) were synthesized by sodium borohydride reduction of warfarin (**1**) and 4′-hydroxywarfarin (**5**) respectively in water[17]. It should be noted that warfarin is racemic, based upon the presence of a chiral center at C-9; thus, reduction at C-11 (with the generation of a second chiral center) yields two chromatographically resolvable diastereomeric sets of enantiomers. Consequently, compounds **9** and **12** (Fig. 1) depict their respective 9*R*,11*S*/9*S*,11*R*-racemates (only one enantiomer is shown for convenience), while compounds **10** and **13** depict their respective 9*R*,11*R*/9*S*,11*S*-racemates (again, only one enantiomer is shown in each case for convenience). The mass spectrum of the synthetic 4′-hydroxywarfarin alcohol (**12**/**13**) was consistent with that expected for this compound[18], and was used as the internal standard without further purification.

Preparation of cell suspensions of Cunninghamella elegans (ATCC 36112)

The fungus *Cunninghamella elegans* (ATCC 36112) was maintained on refrigerated (4°C) slants of Sabouraud-maltose agar (Difco, Detroit, MI, U.S.A.) and transferred to fresh slants every six months to maintain viability. This fungus was grown according to a two-stage fermentation procedure. The surface growth of a slant was used to inoculate one Stage-1 Bellco-Delong flask (1 l) containing 200 ml of growth medium. The medium used in these experiments consisted of the following: dextrose, 20 g; soybean meal (20 mesh; Capitol Feeds, Austin, TX, U.S.A.), 5 g; sodium chloride, 5 g; potassium phosphate (dibasic), 5 g; yeast extract (Difco), 5 g; distilled water, 1000 ml; pH adjusted to 7.0 with 6 *M* hydrochloric acid. The medium was sterilized in individual flasks at 121°C for 15 min. After inoculation, the Stage-1 flask was incubated for 72 h at 27°C and 250 rpm in a G-25 Environmental Shaker (New Bruns-

wick Scientific Co., Edison, NJ, U.S.A.) at which time 10 ml of growth was used to inoculate each Stage-2 flask (1 l) containing 200 ml of fresh medium. After incubation for 24–48 h, the fungal cells were harvested by filtration, rinsed by resuspension in distilled water followed by filtration. This process was repeated three times. Each cell suspension culture was prepared by suspending 4 g of cell pellets in 20 ml of pH 6.7 phosphate buffer (0.5 *M*) in a 125-ml Bellco-Delong flask. After pre-incubation for 1 h under the conditions specified above, 6 mg of warfarin as the potassium salt in 200 μl sterile water was added to each flask to give a final substrate concentration of 0.3 mg/ml of buffer, and the incubation was resumed. One whole flask of cell suspension was harvested at 2, 6, 12, 24 and 48 h after substrate addition. Samples were filtered to remove fungal cells, and filtrates were stored at −20°C until analysis.

Instrumentation and HPLC conditions

HPLC was conducted using a Beckman Model 110A pump with a Schoeffel Model FS 970 fluorometric detector. The excitation wavelength was 290 nm and emission was measured in the presence of an emission filter (No. 389). A Hitachi model 100-10 variable-wavelength UV detector was also used for certain experiments, as specified (wavelength 310 nm). Chromatograms were recorded with a Hewlett-Packard Model 3390A reporting integrator. The mobile phase consisted of methanol–tetrahydrofuran–1.0 *M* aqueous TBA^+–5 m*M* aqueous ammonium phosphate (pH 7.5) (30:7:1:62) and was prepared by filtering individual components through glass filter pads, GF/F grade (Whatman, Clifton, NJ, U.S.A.), mixing and degassing ultrasonically before use. The 5 m*M* ammonium phosphate buffer was prepared by adjusting the pH of a 5 m*M* potassium phosphate solution to pH 7.5 with ammonium hydroxide. The column was a Beckman ODS 5-μm (C_{18}), 250 × 4.5 mm I.D., eluted at a solvent flow-rate of 1.0 ml/min.

Excitation and emission spectra of warfarin were generated on an Aminco-Bowman spectrophotofluorimeter (American Instrument Co., Silver City, MD, U.S.A.).

Extraction of warfarin and metabolites

The extraction method was designed for pH 6.7 microbial cell suspensions. The sample filtrate (4 ml), distilled water (200 μl), and the internal standard (4′-hydroxy-warfarin alcohols, 400 ng as the potassium salt in 100 μl of water) were added to an extraction tube (125 × 16 mm). The solution was extracted with 10% dichloromethane in cyclohexane (2 × 4 ml). The organic layer was removed by an aspirator and discarded. The residual aqueous phase was then extracted with ethyl acetate (5 ml). The organic layer was then back extracted with 0.1 *M* potassium hydroxide (2 ml), and the organic layer discarded. The aqueous phase was acidified with 5 *M* hydrochloric acid (1 ml) and extracted with diethyl ether (5 ml). The ethereal layer was taken to dryness under nitrogen, and reconstituted with 250 μl of mobile phase. A total of 50–200 μl of this solution was injected into the chromatograph.

Preparation of standard curves

A standard solution containing all alcohol and phenolic warfarin metabolites (**2**–**6**, **9**, **10**) was prepared by dissolving 5 mg of each metabolite in 1000 ml of water with the aid of a minimum amount of 0.1 *M* potassium hydroxide. This solution

(1000 ng/200 μl) was further diluted to give four additional solutions containing 400, 200, 80 and 40 ng of each metabolite in 200 μl. Standard curves were constructed using five blank cell suspension filtrate samples of 4.0 ml each spiked with 200 μl of one of the five standard solutions. These samples were subjected to the extraction and HPLC analysis described above. The resultant peak area ratios (metabolite/internal standard) were plotted *versus* metabolite concentration in ng/ml (Fig. 3).

Selection of excitation wavelength

A solution of warfarin, its phenolic metabolites and warfarin alcohols at a concentration of 100 ng/ml in mobile phase was prepared. A total of 250 μl of this solution was injected into the HPLC system. The peak area as recorded on the integrator was taken as the detector response for each compound at a specific excitation wavelength. The excitation wavelengths investigated ranged from 280 to 325 nm. The detector response for each compound then was normalized to the optimal excitation wavelength at 290 nm (Table I).

TABLE I

RELATIVE FLUORESCENCE INTENSITY OF WARFARIN AND METABOLITES AS A FUNCTION OF EXCITATION WAVELENGTHS

Compound	*Normalized fluorescence intensity*[a]							
	Excitation wavelength (nm)							
	280	*285*	*290*	*295*	*300*	*310*	*320*	*325*
Warfarin (**1**)	64	93	100	91	78	47	21	16
6-Hydroxywarfarin (**2**)	61	92	100	94	83	49	25	20
7-Hydroxywarfarin (**3**)	78	104	100	88	82	63	33	25
8-Hydroxywarfarin (**4**)	61	92	100	93	81	42	17	11
4'-Hydroxywarfarin (**5**)	73	111	100	91	76	48	22	17
3'-Hydroxywarfarin (**6**)	78	103	100	88	74	45	22	15
RS/SR-Warfarin alcohol (**9**)	67	97	100	95	80	49	23	17
RR/SS-Warfarin alcohol (**10**)	54	80	100	79	68	44	19	15

[a] Each value was normalized for the fluorescence response at the excitation and emission wavelength of 290 and >389 nm, respectively.

RESULTS AND DISCUSSION

Ion-pair chromatography

Preliminary experiments using methanol, pH 7.5 phosphate buffer, and a reversed-phase C_{18} column showed that tetrabutylammonium ion (TBA^+) could be used as a counter ion for the ion-pair chromatography of warfarin and its metabolites. With the exception of 6- and 7-hydroxywarfarin which co-eluted, baseline resolution of all compounds was achieved with excellent peak shape. The selectivity of an ion-pair chromatographic system is controlled by the stationary phase, the pH of the mobile phase, the organic modifiers, and the nature of the counter ion. In an attempt to resolve the 6- and 7-hydroxywarfarin, these parameters were systematically al-

tered while monitoring the selectivity of the chromatographic system. No selectivity effect was observed as the concentration of the TBA^+ was increased, but the capacity factor of all compounds increased accordingly. In addition, very little improvement was obtained with either a C_8 column as the stationary phase, or with tetramethylammonium ion (TMA^+) as the counter ion. Increasing the pH beyond 8.0 yielded a gradual improvement in resolution; at pH 10, an almost baseline resolution of 6- and 7-hydroxywarfarin was achieved. The use of a pH above 7.5 is, however, not feasible because of silica dissolution and column deterioration.

Variation in the organic modifiers yielded the desired resolution. Among the organic modifiers investigated, tetrahydrofuran provided unusual selectivity, and a mixture of methanol and tetrahydrofuran ultimately afforded complete resolution of all analytes. The final chromatographic system chosen consists of a 5-μm C_{18} stationary phase, with a mobile phase consisting of methanol–tetrahydrofuran–1.0 *M* tetrabutylammonium phosphate–5 m*M* ammonium phosphate (pH 7.5) (30:7:1:62) (Fig. 2). The aliphatic hydroxywarfarin metabolites (**7** and/or **8**) were not included because analytical standards were not available. Dehydrowarfarin (**11**) was also not included in the final assay because this metabolite does not exhibit fluorescence in the mobile phase.

An anticipated difficulty encountered with this chromatographic system was the gradual deterioration of column performance over a two-week period. As described by Gloor and Johnson[19], a mobile phase pH of 7.5 (representing the pH limit of column stability) in combination with TBA^+ as the counter ion (although at a concentration much higher than that used in the current study) accelerate column degeneration. The problem was overcome by using a guard column (75 × 4.5 mm I.D.) packed with silica (40 μm), installed between the HPLC pump and injector to presaturate the mobile phase with silica.

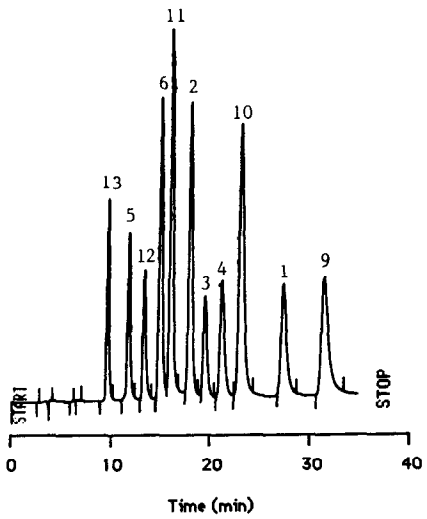


Fig. 2. Reversed-phase ion-pair HPLC separation of warfarin (**1**), metabolite standards (**2–6, 9, 10**), and internal standard (**11, 12**). See text for chromatographic conditions. Structure numbers are as specified in Fig. 1.

Selection of excitation wavelength

In order to exploit the sensitivity of fluorimetric detection, careful determination of the optimum excitation and emission wavelengths was undertaken. Analysis of the excitation/emission spectrum of warfarin generated with the Aminco-Bowman spectrofluorimeter indicated maximum emission at 395 nm when excited at 325 nm in the mobile phase, in close agreement with the wavelengths selected by Hunter[20] for the fluorescent detection of warfarin. However, preliminary HPLC investigations with these excitation and emission wavelengths showed that fluorimetric detection did not produce superior sensitivity over UV photometric detection, probably because the fluorimetric detector and spectrofluorimeter employed different lamp sources to provide energy of excitation. While the spectrofluorimeter was equipped with an xenon arc lamp, the HPLC fluorimetric detector relied on a deuterium lamp which provided very little excitation energy beyond 300 nm. The optimal wavelength of excitation for the HPLC fluorescence detector in use was subsequently determined experimentally. This was carried out by monitoring the fluorescence response of the detector for each compound at different excitation wavelengths between 280 and 325 nm. The results shown in Table I indicate that the best overall sensitivity was obtained with excitation at 290 nm. An even greater sensitivity would be obtainable on a detector equipped with a different lamp source because the emission spectrum of warfarin generated with the spectrofluorimeter indicates that the quantum yield can be doubled by excitation at 325 nm.

Extraction procedures

While offering excellent resolution of all compounds, this HPLC method did not allow for the quantitation of *RS*/*SR*-warfarin alcohol (**9**) in some microbial cultures with concentrations of residual warfarin substantially higher than those of its metabolites. This is because warfarin eluted just prior to this metabolite and the two chromatographic peaks tended to overlap at high warfarin concentration. Consequently, an extraction scheme was developed which preferentially removed warfarin from the microbial cultures, but allowed the metabolites to be recovered during a subsequent extraction step.

The use of cyclohexane to selectively extract warfarin from microsomal mixtures at pH 5.8 was reported by Bush *et al.*[11]. Preliminary investigations showed that at pH 6.7 (the pH at which microbial metabolic studies were conducted), warfarin could be selectively extracted using cyclohexane containing a small amount of methylene chloride, and several experiments were carried out to optimize this extraction. Table II lists the protocols examined for extracting buffer solutions spiked with warfarin and metabolites (which would mimic the microbial cell suspension mileau). The results shown in Table II indicate that the most successful treatment developed for the removal of the majority of residual warfarin is to initially extract samples with 10% methylene chloride in cyclohexane. The metabolites could then be recovered quantitatively from the aqueous phase by a single extraction with ethyl acetate. Since over 80% of warfarin is removed before final analysis by HPLC, *SR*/*RS*-warfarin alcohol could then be accurately quantitated.

Calibration, accuracy and precision

Calibration plots of individual metabolites were constructed by the method of

TABLE II

EFFICIENCY OF WARFARIN REMOVAL AND METABOLITE EXTRACTION *VERSUS* EXTRACTION PROTOCOL

Extraction conditions[a]	*Percentage of theoretical remaining in aqueous phase*						
	*Warfarin (**1**)*	*6-Hydroxy-warfarin (**2**)*	*7-Hydroxy-warfarin (**3**)*	*8-Hydroxy-warfarin (**4**)*	*4'-Hydroxy-warfarin (**5**)*	*3'-Hydroxy-warfarin (**6**)*	*SS/RR-Warfarin alcohol (**10**)*
1	72	99	99	100	98	102	100
2	46	99	98	97	106	101	98
3	15	101	104	98	105	102	104
4	1	0	0	0	0	0	0

[a] Extraction conditions: (1) 1 × 4 ml 5% dichloromethane in cyclohexane; (2) 2 × 4 ml 5% dichloromethane in cyclohexane; (3) 2 × 4 ml 10% dichloromethane in cyclohexane; (4) 1 × 4 ml ethyl acetate.

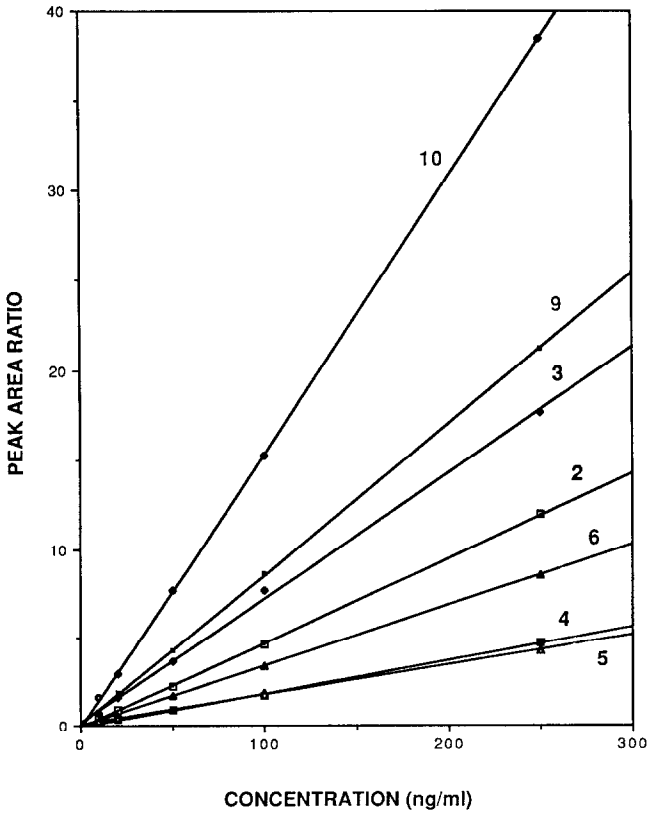


Fig. 3. Calibration plots for warfarin metabolites over the concentration range of 10–250 ng/ml. Numbering as in Fig. 1.

TABLE III

STATISTICAL ANALYSIS OF STANDARD CURVES GENERATED FOR WARFARIN METABOLITES (n = 5)

Compound	r^2	*Slope ± S.D.*	*Intercept ± S.D.*
6-Hydroxywarfarin (**2**)	0.9971	0.0506 ± 0.0026	−0.2358 ± 0.0949
7-Hydroxywarfarin (**3**)	0.9958	0.0778 ± 0.0046	−0.0110 ± 0.1945
8-Hydroxywarfarin (**4**)	0.9976	0.0195 ± 0.0015	−0.0331 ± 0.0625
4′-Hydroxywarfarin (**5**)	0.9941	0.0177 ± 0.0009	0.0673 ± 0.0865
3′-Hydroxywarfarin (**6**)	0.9987	0.0365 ± 0.0024	−0.0465 ± 0.0641
RS/SR-Warfarin alcohol (**9**)	0.9974	0.0911 ± 0.0051	−0.0937 ± 0.1828
RR/SS-Warfarin alcohol (**10**)	0.9986	0.1628 ± 0.0076	−0.3891 ± 0.2269

internal standardization with 4′-hydroxywarfarin alcohol as the internal standard (tentatively assigned as the *RS/SR*-4′-hydroxywarfarin alcohol (**12**) by reference to the elution order of *SS/RR*- and *SR/RS*-warfarin alcohols). Linear calibration plots were obtained for all metabolites (Fig. 3), and the calculated linear regression parameters for each metabolite are shown in Table III. The regression coefficients (r^2) for all compounds were better than 0.99 indicating good linearity. The coefficient of variation (C.V.) (n = 5) of the peak area ratio at each calibration point was less than 10% for all compounds indicating good precision. The accuracy of the assay was determined by duplicate analyses of samples containing standard amounts of metabolites. Deviation from the true value at all concentration levels was less than 10% for each metabolite (Table IV). Although the lowest calibration point of this assay was 10 ng/ml for all metabolites, levels as low as 1 ng/ml were detectable.

Analysis of microbial cell suspension of Cunninghamella elegans (ATCC 36112)

Fig. 4 shows typical chromatograms obtained from the analysis of cell suspension cultures of the fungus *Cunninghamella elegans* (ATCC 36112) (72 h incubation, sample and blank). It is evident that this organism metabolized warfarin to all known mammalian metabolites of this agent plus the previously unreported 3′-hydroxywar-

TABLE IV

RECOVERY OF WARFARIN METABOLITES FROM CELL SUSPENSION CULTURES OF *CUNNINGHAMELLA ELEGANS*

Concentration (ng/ml)	*Metabolite recovered (theoretical percentage)*						
	*6-Hydroxywarfarin (**2**)*	*7-Hydroxywarfarin (**3**)*	*8-Hydroxywarfarin (**4**)*	*4′-Hydroxywarfarin (**5**)*	*3′-Hydroxywarfarin (**6**)*	*RR/SS-Warfarin alcohol (**10**)*	*RS/SR-Warfarin alcohol (**9**)*
250	102, 103	98, 103	102, 98	100, 99	99, 100	97, 101	102, 97
150	108, 94	104, 94	106, 94	106, 97	107, 95	106, 101	100, 100
100	97, 106	97, 106	99, 98	95, 101	98, 99	101, 103	100, 101
50	103, 106	96, 106	99, 101	99, 101	101, 100	101, 95	100, 105
25	93, 104	90, 96	104, 97	102, 101	101, 112	92, 90	92, 94
12.5	93, 104	90, 96	99, 104	109, 104	101, 112	90, 96	90, 104

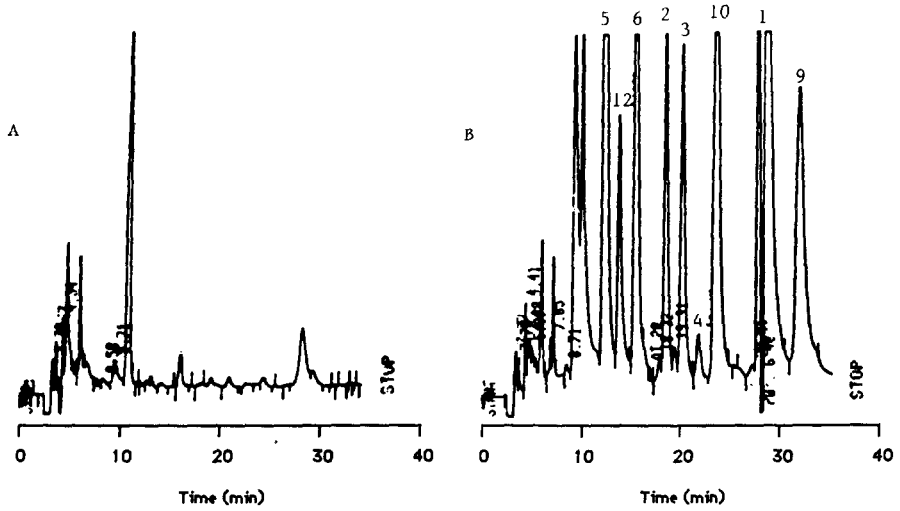


Fig. 4. HPLC analysis of warfarin metabolism by cell suspension cultures of *Cunninghamella elegans* (ATCC 36112). (A) Culture blank; (B) culture extract after 72 h incubation. Numbering as in Fig. 1.

farin. This suggests that this particular fungus possesses a drug metabolizing system quite similar to that found in mammalian species in terms of the metabolism of this important therapeutic agent and metabolic probe. The time course for the production of warfarin metabolites by *Cunninghamella elegans* (ATCC 36112) as monitored by this assay is shown in Fig. 5. The isolation and full structural characterization of each metabolite present will be the subject of a separate publication.

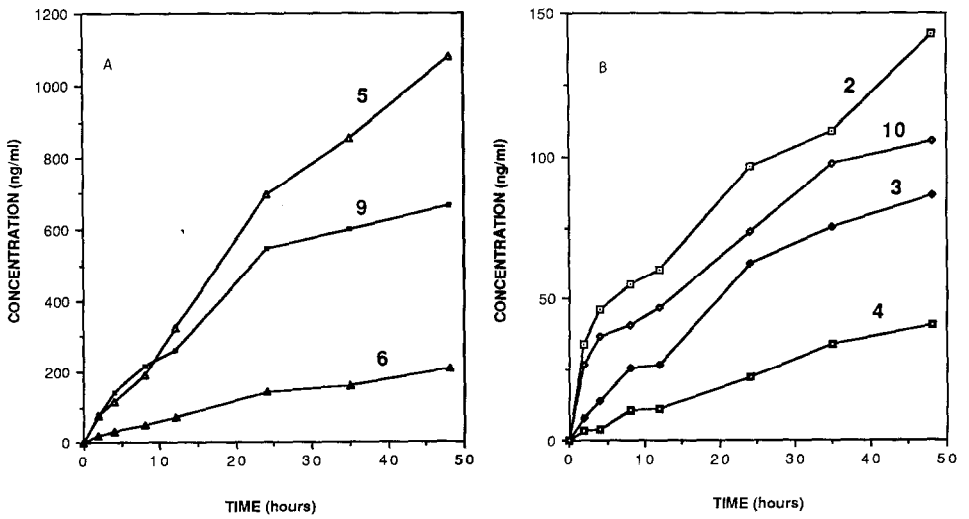


Fig. 5. Time-course profiles for the generation of warfarin metabolites using cell suspension cultures of *Cunninghamella elegans*. (A) Major metabolites (200–1000 ng/ml); (B) minor metabolites (25–150 ng/ml). Numbering as in Fig. 1.

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