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Short communication

Highly sensitive and specific high-performance liquid chromatographic analysis of 7-hydroxywarfarin, a marker for human cytochrome P-4502C9 activity

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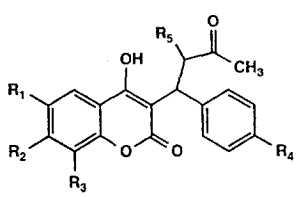
Abstract

The formation of 7-hydroxywarfarin in incubations of (*S*)-warfarin with human liver microsomes reflects their cytochrome P-4502C9 activity. This paper describes a rapid high-performance liquid chromatographic method for the determination of 7-hydroxywarfarin with high sensitivity, selectivity, and a simple sample clean-up procedure. Separation was achieved with a C_{18} reversed-phase column and quantification by fluorometric detection. The method employs an internal standard resulting in good accuracy and precision. The limit of detection is 150 fmol for 7-hydroxywarfarin.

1. Introduction

Warfarin, a chiral coumarin derivative (Fig. 1), is widely used as an anticoagulant and rodenticide. In vivo and in vitro studies with different species on the metabolism of warfarin showed the formation of several metabolites [1–7]. Some investigations on the regio- and stereoselectivity demonstrated significant differences towards the metabolism of (*R*)- and (*S*)-warfarin [1,3–6]. (*S*)-Warfarin, the more potent antipode, is metabolized by human microsomes predominantly to 7-hydroxywarfarin, 6-hydroxywarfarin and 4′-hydroxywarfarin and to a lower extent to 8-hydroxywarfarin and 10-hydroxywarfarin [5–7]. Kinetic studies have identified a high-affinity (*S*)-warfarin 7-hydroxylase present in human liver microsomes. Experiments with eleven cDNA-expressed human cytochrome P-450 enzymes and concomitant inhibition studies exhibited the 7-hydroxylation of (*S*)-warfarin as a reaction near-



	R_1	R_2	R_3	R_4	R_5
Warfarin	H	H	H	H	H
6-Hydroxywarfarin	OH	H	H	H	H
7-Hydroxywarfarin	H	OH	H	H	H
8-Hydroxywarfarin	H	H	OH	H	H
4′-Hydroxywarfarin	H	H	H	OH	H
10-Hydroxywarfarin	H	H	H	H	OH

Fig. 1. Structures of warfarin and its metabolites.

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ly exclusively catalyzed by cytochrome P-4502C9 (CYP2C9) [6].

A number of analytical procedures for the quantification of warfarin and its metabolites from biological fluids are described [8–10]. For the determination and quantification of warfarin and its metabolites from in vitro studies with P-450 enzymes a GC–MS method [10] can be used. This method is more sensitive than HPLC techniques involving UV detection.

Due to the native fluorescence of warfarin and its metabolites [1] this paper describes a simple and rapid HPLC method using fluorimetric detection after a simple clean-up procedure of the incubation mixture. 7-Hydroxywarfarin was detected under specific conditions of the excitation and emission wavelengths. Inhibition by sulphaphenazole and kinetic studies on five human liver samples exhibited good agreement of K_m and V_{max} values compared to those described previously [6] obtained by GC–MS analysis. As HPLC is widely used and more available for everyone than GC–MS, this method offers a highly sensitive and specific alternative tool for screening CYP2C9 activity in human liver microsomes.

2. Experimental

2.1. Chemicals

Racemic warfarin was purchased from Aldrich (Steinheim, Germany). Optically pure (*R*)-(+)- and (*S*)-(−)-warfarin were obtained by the method of West et al. [11]. The enantiomers were analysed by a chiral-phase HPLC technique to prove the enantiomeric purity. The analysis showed no detectable impurity of the other antipode, of either the (*S*)- or the (*R*)-warfarin fractions. 4′-Hydroxywarfarin, 7-hydroxywarfarin and 6-hydroxywarfarin were synthesized as described in the literature [12,13]. 7-Ethoxycoumarin was from Sigma (Deisenhofen, Germany). Sulfaphenazole was kindly provided by Ciba-Geigy (Basel, Switzerland). Glucose-6-phosphate, NADP and glucose-6-phosphate dehydrogenase were purchased from Boehringer (Mannheim, Germany). Acetonitrile was from Fisons (Loughborough, UK). All other chemicals were of the highest purity available.

2.2. Organ material

Human liver samples were provided by the Department of Clinical Pathology of the University of Erlangen–Nürnberg (Professor Dr. C. Wittekind). The samples were from three female (CH41, CH43, CH45) and two male (CH40, CH44) patients undergoing liver resection for various clinical reasons. After histomorphological investigation of the residual tissue samples, histomorphologically normal tissue was frozen in liquid nitrogen and stored at −70°C until preparation of the microsomes. The P-450 enzymes were prepared and quantitated according to standard procedures [14–16].

2.3. Incubations

(*S*)-Warfarin (1–16 μM) was incubated at 37°C in 0.1 *M* potassium phosphate buffer (pH 7.4) with human liver microsomes (containing 100 pmol of P-450 and a NADPH-generating system consisting of 13.7 m*M* glucose 6-phosphate, 0.66 m*M* $NADP^+$ and 2.8 I.U. of glucose 6-phosphate dehydrogenase) in a total volume of 0.5 ml. Incubations were started after a 3-min preincubation (37°C) by addition of the NADPH-generating system. After 60 min the reaction was quenched with 10 μl of perchloric acid (70%), and 10 ng of 7-ethoxycoumarin were added as an internal standard. Denaturated protein was precipitated by centrifugation at 3000 *g* for 5 min (4°C) and an aliquot of the supernatant was injected for HPLC analysis or stored at −20°C until analysis. For the preparation of a stock solution (*S*)-warfarin was dissolved in a small volume of 1 *M* potassium hydroxide and diluted with water to a final concentration of 5 m*M* potassium hydroxide.

2.4. Chromatographic system

HPLC analysis was performed at room temperature using two LC-pumps (Model T414), a

variable-wavelength UV detector (Uvicon 720 LC micro), a spectrofluorometer (SFM 25) and an Anacomp 220 integrating controller unit (all apparatus from Kontron Neufahrn, Germany).

Aliquots of the samples were injected by a Perkin Elmer ISS 101 autosampler (Überlingen, Germany) and analyzed on a Nucleosil C_{18} 5-μm analytical column (125 × 4 mm; Macherey–Nagel, Düren, Germany) equipped with a Nucleosil C_8 10-μm guard column (11 × 4 mm; Macherey–Nagel). The eluent consisted of acetonitrile–0.5% phosphoric acid (38:62, v/v). The flow-rate was 1.3 ml/min. UV detection was performed at 205 nm and fluorimetric detection at an excitation wavelength of 320 nm and an emission wavelength of 415 nm.

To determine the enantiomeric purity of (*S*)- and (*R*)-warfarin a HPLC equipment from TSP (Darmstadt, Germany) with a Spectra-Physics SP8800 ternary HPLC pump and a Spectra Focus optical scanning detector was used. Enantioselective separation was achieved with a Chiral-AGP column (5 μm, 100 × 4 mm; ChromTech, Norsborg, Sweden) and a mobile phase of 0.02 *M* potassium phosphate buffer (pH 6.0)–2-propanol (85:15, v/v) at a flow-rate of 1 ml/min. UV detection was controlled in the range from 200 to 360 nm, and the enantiomers were identified according to their spectral characteristics.

2.5. Quantification

Aliquots of 100 μl of the samples were injected to the HPLC column. Standard curves were obtained from blank samples spiked with different concentrations of 7-hydroxywarfarin. For the quantification of the incubation samples peak-area ratios relative to the internal standard were used.

3. Results and discussion

Analysis of warfarin and three synthesized metabolites, by UV detection at 205 nm, exhibits a good baseline separation (Fig. 2A). To obtain information on the chromatographic characteris-

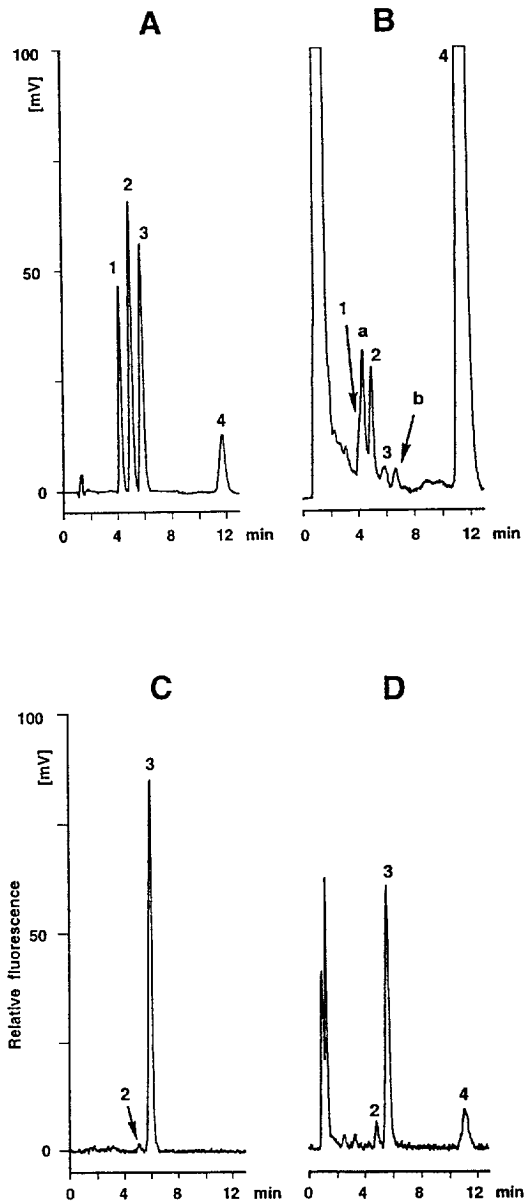

Fig. 2. Analysis of warfarin and its metabolites. (A) Reference mixture of warfarin and three synthesized metabolites, UV detection at 205 nm (0.7 nmol each compound). (B) Incubation of (*R*)-warfarin (0.5 m*M*) with human liver microsomes (2 nmol P450/ml), UV detection at 205 nm. (C) Fluorimetric analysis (excitation at 320 nm and emission at 415 nm) of a 1:100 dilution of the sample mentioned in A. (D) Fluorometric analysis of a 1:50 dilution of the sample mentioned in B. Peaks: 1 = 4′-hydroxywarfarin (4.24 min); 2 = 6-hydroxywarfarin (5.05 min); 3 = 7-hydroxywarfarin (5.95 min); 4 = warfarin (11.70 min); a = 10-hydroxywarfarin (4.45 min); b = 8-hydroxywarfarin (6.70 min).

tics of 8-hydroxywarfarin and 10-hydroxywarfarin, incubations of (*R*)-warfarin and (*S*)-warfarin in human liver microsomes were compared. Referring to the known different metabolic patterns of the two enantiomers [5], 8- and 10-hydroxywarfarin were identified based on their UV characteristics at retention times of 6.6 and 4.3 min. When (*R*)-warfarin was incubated with liver microsomes from a dexamethasone-treated rat and compared to an incubation with microsomes of a control rat, the peak at 4.3 min increased about fifteen-fold and was therefore identified as 10-hydroxywarfarin, which is known to be formed by cytochrome P-4503A enzymes [7] (data not shown). The analysis of 7-hydroxywarfarin after incubation of (*R*)-warfarin does not interfere with the other metabolites (Fig. 2B). The limit of UV detection at 205 nm was about 30–50 pmol for the synthesized compounds.

To optimize the fluorometric analysis, excitation and emission spectra were recorded for each compound. Excitation at 320 nm and emission at 415 nm were the best conditions to analyse 7-hydroxywarfarin with much less sensitivity for the other metabolites. Nearly exclusively, 7-hydroxywarfarin was detected under these fluorometric conditions (Fig. 2C and D) from dilutions of the reference mixture (1:100) (Fig. 2A) and of the (*R*)-warfarin incubation mixture (1:50) (Fig. 2B).

The detection limit for 7-hydroxywarfarin is 150 fmol, corresponding to ca. 0.5 ng/ml (injection volume 100 μl, signal-to-noise ratio 4:1; the noise was determined within 2 min as the electronical oscillation of the detector signal). The reproducibility of the chromatographic assay was $\pm 1.11\%$ (S.D.) at 6.2 pmol 7-hydroxywarfarin ($n = 10$). The recovery of 7-hydroxywarfarin was $92.7 \pm 0.8\%$ ($n = 6$) in a concentration range of 30.8–2.5 μM at a fixed protein concentration of 3 mg/ml. The corresponding detection limit for 6-hydroxywarfarin is 5 pmol and about 3 nmol for 4'-hydroxywarfarin. Therefore, 7-hydroxywarfarin was exclusively the fluorometrically detectable metabolite of (*S*)-warfarin after incubations at low concentrations with human liver microsomes (Fig. 3).

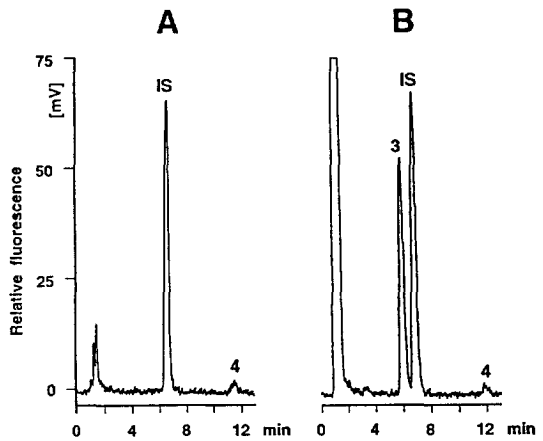

Fig. 3. Fluorometric analysis of a (*S*)-warfarin incubation (8 μM) with human liver microsomes (CH40) and internal standard I.S. (20 ng/ml). (A) Microsomal incubation mixture without NADPH-generating system. (B) Microsomes plus NADPH-generating system. Peaks: 3 = 7-hydroxywarfarin (5.95 min); I.S. = 7-ethoxycoumarin (internal standard) (6.80 min); 4 = (*S*)-warfarin (11.80 min).

Microsomal preparations of five human liver samples were used to obtain the kinetic parameters V_{max} and apparent K_m for (*S*)-warfarin-7-hydroxylation (Table 1). Formation of this metabolite was linear up to at least 90 min. V_{max} varied between 7.5 and 12.3 pmol of 7-hydroxywarfarin formed (per nmol of P-450 per min). Calculation of K_m from Eadie–Hofstee plots (V versus V/S) resulted in an apparent K_m of 4.1 ± 0.9 μM. These data were in good agreement with those described in the literature ($K_m = 3.9 \pm 0.1$ μM, $V_{max} = 5.6$–14 pmol 7-hydroxywarfarin per nmol of P-450 per min [6]).

In incubations of (*S*)-warfarin at a concen-

Table 1
Kinetic parameters V_{max} (pmol of 7-hydroxywarfarin per nmol of P-450 per min), apparent K_m (μM) and correlation coefficients (r^2) from Eadie–Hofstee plots of (S)-warfarin 7-hydroxylation on human liver microsomes

Subject	K_m	V_{max}	r^2
CH40	4.8	12.3	0.95
CH41	3.8	9.0	0.94
CH43	3.8	8.3	0.90
CH44	5.2	7.5	0.97
CH45	2.9	9.1	0.94

tration of 16 μM, sulphaphenazole inhibits the formation of 7-hydroxywarfarin (by 10% at 0.02 μM, 25% at 0.2 μM and 75% at 2 μM sulphaphenazole, respectively)

4. Conclusion

To determine exclusively 7-hydroxywarfarin formation as a marker reaction for CYP2C9 activity the HPLC assay described in this paper offers an excellent method. The advantages are short time of analysis, a simple sample clean-up and a higher sensitivity compared to multiple extraction steps, derivatizations and a longer analysis time of the generally used GC–MS method [10].

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