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Determination of unbound warfarin enantiomers in human plasma and 7-hydroxywarfarin in human urine by chiral stationary-phase liquid chromatography with ultraviolet or fluorescence and on-line circular dichroism detection

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Abstract

Enantiomers of warfarin and 7-hydroxywarfarin in human plasma and urine, respectively, were determined by high-performance liquid chromatography using a cellulose-derivative column with UV or fluorescent detection, and their absolute configuration was determined simultaneously by a circular dichroism spectropolarimeter connected in series. Enantiomers of warfarin and its major metabolites [i.e., (*R*)-6-hydroxywarfarin, (*S*)-7-hydroxywarfarin and (*RS*)-warfarin alcohol] were well resolved. The method was precise and sensitive: within- and between-day coefficients of variation were <9.6% for warfarin enantiomers in plasma and <7.1% for 7-hydroxywarfarin enantiomers in urine, respectively, and the lower detection limits were 20 ng/ml for (*R*)-warfarin, 40 ng/ml for (*S*)-warfarin, 2.5 ng/ml for (*R*)-7-hydroxywarfarin and 4.5 ng/ml for (*S*)-7-hydroxywarfarin in 0.5 ml of both plasma and urine. The ultrafiltration technique was used for determining unbound concentrations of warfarin enantiomers in plasma using [^{14}C]warfarin enantiomers resolved by the present HPLC system. Clinical applicability of the method was evaluated by determining unbound concentrations of warfarin enantiomers in five consecutive plasma samples obtained from a patient exhibiting an unstable anticoagulant response to warfarin (4 mg/day, p.o.). Results indicated that the present method would be useful in clarifying factors responsible for a large intra- and inter-patient variability in warfarin effects with regard to unbound plasma enantiomer pharmacokinetics. © 1997 Elsevier Science B.V.

Keywords: Enantiomer separation; Warfarin; 7-Hydroxywarfarin

1. Introduction

Warfarin, a chiral coumarin derivative, is widely

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prescribed for the treatment and prophylaxis of various thromboembolic diseases [1]. However, the risk of warfarin therapy is substantial, and appropriate dosage titration is often difficult because of considerable inter- and intra-patient variability in anticoagulant response [2]. While attempts have been made to account for the principal sources of such variability or fluctuation in warfarin effect, the interpretation of previous studies has been hampered by the complicated pharmacokinetic properties of this anticoagulant. Clinically available warfarin is a racemic mixture of (*R*)- and (*S*)-enantiomers in which pharmacokinetic and dynamic properties differ considerably: (*S*)-warfarin not only has about 1.5 times greater systemic clearance (CL) but also has 3–5 times greater anticoagulant potency than its (*R*)-congener [3,4]. Furthermore, warfarin is highly bound to plasma protein [3]. Because only unbound drug can reach the site of action, the variability in warfarin response should be related to the unbound (C_u) rather than total concentration of the pharmacologically more relevant (*S*)-enantiomer [5].

Warfarin is extensively metabolized by the liver and its hepatic metabolism has been shown to be enantioselective and regioselective in humans: the pharmacologically more relevant (*S*)-warfarin is metabolized mainly to (*S*)-7-hydroxywarfarin, whereas (*R*)-warfarin is metabolized to (*RS*)-warfarin alcohol and (*R*)-6, 8 and 10-hydroxywarfarin [6]. Because the hydroxylated warfarin metabolites are eliminated mainly into urine, a simultaneous determination of (*S*)-7-hydroxywarfarin in urine and its parent enantiomer in plasma would provide a means of understanding intra- and inter-patient variability in warfarin anticoagulant effects in light of the hepatic metabolism of (*S*)-warfarin.

There have been two distinct strategies for determining warfarin enantiomers in human biological fluids by HPLC: chiral derivatization [7,8] of the drug followed by an achiral HPLC separation or direct chiral separation of the drug by an α_1-acid glycoprotein immobilized column [9]. While the former method is laborious, time-consuming and prone to greater assay variability because of complex preparative procedures, the latter has the disadvantage of a relatively short column life. In previous reports [10–12] different types of chiral cellulose-derivative HPLC columns were successfully applied to develop enantioselective assays for propranolol, disopyramide, flecainide and verapamil in human biological fluids. The columns showed excellent stability. In addition, plasma C_u values for the respective enantiomers were determined by the ultrafiltration technique using radiolabeled enantiomers of each compound resolved from the commercially available racemates by the above HPLC systems [13,14]. Here, we present a sensitive and accurate assay method for determining C_u of warfarin enantiomers in plasma and (*S*)-7-hydroxywarfarin in urine using a chiral cellulose-derived column combined with UV and fluorescent detection, respectively. We also present a novel application of a circular dichroism (CD) detector connected to the chiral HPLC system in series for simultaneously determining the absolute configuration of warfarin and its metabolites as they elute from the column.

2. Experimental

2.1. Chemicals

Racemic warfarin, β-glucuronidase and sulphatase were purchased from Sigma (St. Louis, MO, USA), [^{14}C]warfarin (specific activity, 2.11 GBq/mmol) was from Amersham (Buckinghamshire, UK), and diclofenac and naproxen were from Wako (Osaka, Japan). (*R*)- and (*S*)-Warfarin, (*R/S*)-6-hydroxywarfarin, (*R/S*)-7-hydroxywarfarin and (*RR/RS*)-warfarin alcohol were donated by Eisai (Tokyo, Japan). (*SS/SR*)-Warfarin alcohol and (*R*)-7-hydroxywarfarin were kindly provided by Dr. William F. Trager (University of Washington, Seattle, WA, USA). (*R/S*)-8-Hydroxywarfarin and (*R/S*)-10-hydroxywarfarin were from Funakoshi (Tokyo, Japan). All other chemicals were of reagent grade from Wako.

2.2. Chromatographic system

The chromatographic system consisted of a L-7100 pump (Hitachi, Tokyo, Japan), a L-7200 automatic sample-injector, a SPD-10AV variable wavelength UV detector (Shimadzu, Kyoto, Japan) set at

312 nm with a range of 0.01 a.u.f.s. for detecting warfarin enantiomers in plasma, a F-1050 fluorescence detector (Hitachi) set at excitation and emission wavelengths of 320 and 415 nm, respectively, for detecting 7-hydroxywarfarin enantiomers in urine. All chromatograms were recorded and the peak heights of the respective analytes were calculated with a D-7500 chromato-integrator (Hitachi). A Chiralcel OD analytical column (10 μm, 250×4.6 mm I.D., Daicel, Tokyo, Japan) coupled with a Chiralcel OD guard column (50×4.6 mm I.D.) was used with a mobile phase that consisted of a 18/0.5/81.5 (v/v) mixture of isopropanol, acetic acid and hexane delivered at a flow-rate of 1 ml/min. Column temperature was maintained at 25°C by a temperature-controlled water bath.

To determine the absolute configuration of the optically active compounds resolved by the chiral HPLC, a Model J-720 spectropolarimeter (Jasco, Tokyo, Japan) with a flow-cell device (1 mm optical path, 19 μl cell volume) was connected with the UV detector in series. The CD spectra for the respective UV peaks were recorded with use of a stop-flow technique to determine peak polarity for each analyte. Peaks corresponding to (*R*)- and (*S*)-enantiomers of warfarin and other metabolites were identified by comparing the retention times and CD spectra of the respective analytes with those of the corresponding authentic standards according to the report of Takatori et al. [15].

2.3. Extraction procedures

For the extraction of warfarin enantiomers from plasma, 1 ml of 2 *M* HCl, 100 μl of diclofenac in a 10/90 (v/v) mixture of isopropanol–hexane solution (equivalent to 1 μg) as an internal standard, and 5 ml of ether were added to a 0.5-ml aliquot of human plasma. The tube was vigorously shaken for 10 min, and then centrifuged at 1200 *g* for 10 min at 4°C. A 4-ml aliquot of the organic layer was removed and evaporated to dryness by a centrifugal vacuum evaporator (VC-96N, Taitec, Tokyo, Japan) for 30 min at 50°C. The residue was reconstituted with 200 μl of 10% (v/v) isopropanol in hexane, and 40 μl of the solution were then injected onto the HPLC system.

For the extraction of 7-hydroxywarfarin enantiomers from urine, a 0.5-ml aliquot of urine, to which had been added 100 μl of the internal standard solution (equivalent to 1 μg of naproxen), was acidified by adding 0.5 ml of 1 *M* acetate buffer (pH 3.0) and extracted with 5 ml of ether–hexane (40:60, v/v). A 4-ml aliquot of the organic layer was transferred to another tube and was alkalinized with 0.5 ml of 1 *M* carbonate buffer (pH 10.2). The mixture was shaken for 10 min and the organic layer was aspirated off. The remaining aqueous layer was re-acidified with 2 ml of 1 *M* acetate buffer (pH 3.0) and back-extracted with 5 ml of ether–hexane (40:60, v/v). A 4-ml aliquot of the organic phase was removed and evaporated. The residue was reconstituted and injected as described above for the plasma samples. In order to examine the existence of glucuronide or sulphate conjugate of 7-hydroxywarfarin, urine samples from a patient on warfarin were hydrolyzed enzymatically. A 200-μl sample of urine was incubated with approximately 1000 units of β-glucuronidase or 25 units of sulphatase at 37°C for 4 h in 0.1 *M* acetate buffer (pH 5.0) according to the method reported elsewhere [16], and 7-hydroxywarfarin concentrations were measured by the method described above.

Calibration curves were constructed by adding known amounts of racemic warfarin to drug-free human plasma (0.5 ml) or racemic 7-hydroxywarfarin to drug-free human urine (0.5 ml) to give final concentrations of 0.1, 0.5, 1.0 μg/ml for each warfarin enantiomer in plasma and 0.02, 0.1, 0.2 μg/ml for each 7-hydroxywarfarin enantiomer in urine.

2.4. Chromatographic interference

Possible chromatographic interference with other therapeutic agents that might be co-administered with warfarin was evaluated based upon the retention times of the respective compounds: the drugs examined were antiplatelet drugs (i.e., aspirin, dipyridamole, ticlopidine, cilostazol and beraprost sodium), cardiovascular drugs (i.e., diltiazem, metoprolol, captopril and disopyramide) and some acidic drugs (i.e., furosemide, ibuprofen, acetaminophen, ketoprofen and indomethacin).

2.5. Plasma protein binding of warfarin enantiomers

Plasma protein binding of warfarin enantiomers was assessed according to the method of Takahashi and co-workers [13,14] with minor modifications. Briefly, plasma was bubbled with 95% O_2:5% CO_2 for 2 min before use to adjust its pH to a range of 7.35–7.45. (*S*)- and (*R*)-[^{14}C]warfarin, which were used for the protein binding study, were resolved from racemic [^{14}C]warfarin by using a similar HPLC method, as described above: radiochemical purity for (*S*)- and (*R*)-warfarin was 98.7 and 98.6%, respectively. To an aliquot of plasma (0.5 ml) 5 μl of (*R*)- or (*S*)-[^{14}C]warfarin (equivalent to 2.04 or 2.19 kBq, respectively) was added and the mixture was incubated for 5 min at 37°C. A portion of plasma (0.4 ml) was then ultrafiltered with Centrifree MPS-3 (30 000 molecular mass cut-off, Amicon, Beverly, MA, USA) at 1260 *g* for 5 min at 37°C. The radioactivity of the remaining portion of plasma (70 μl) and filtrate (70 μl) was determined by liquid scintillation counting (LSC-700, Aloka, Japan). Quenching was corrected for an external standardization method.

In preliminary experiments the ultrafiltration device employed in the present study was shown to have the least adsorption of warfarin enantiomers onto the ultrafiltration membrane and/or device (i.e., <4.9% for (*R*)-warfarin at 0.6 μg/ml and <2.5% for (*S*)-warfarin at 0.8 μg/ml in a phosphate buffer (pH 7.4), respectively) and to give the least variability (C.V.<3.1%) in the unbound fraction (f_u) values for both enantiomers among the four different ultrafiltration devices examined (i.e., Ultrafree C3LGC (Millipore Japan), Centrisart C4 (Sartorius), Centrifree MPS-3 (Amicon) and Spectra/por 2 (Spectrum)).

The concentration-dependency of the plasma protein binding of warfarin enantiomers was assessed by determining the f_u values for each enantiomer over a wide concentration range (i.e., from 0.275 to 10.55 μg/ml) using the method described above. We also investigated whether storage of plasma samples (i.e., freeze and thaw) would influence warfarin binding by comparing f_u values for the respective enantiomers obtained from freshly collected samples ($n=3$) with those obtained from the same samples that had been frozen and thawed 3 times over 14 days.

2.6. Clinical application

To validate the clinical applicability of the present assay method, C_u values for the warfarin enantiomers in plasma in conjunction with anticoagulant response, assessed as INR (international normalized ratio), were determined in a 78-year-old female patient. The patient had been receiving a 4 mg/day dose of warfarin since mitral valve replacement 5 years earlier. Blood samples (5 ml each) were collected into glass tubes containing 19 mg of sodium citrate, 12 h postdose on five different occasions. After centrifugation at 600 *g* for 5 min, separated plasma was stored at −20°C until analyzed. Informed consent was obtained from the patient prior to the study.

2.7. Pharmacokinetic analysis

Based upon the plasma concentrations (C_p) of warfarin enantiomers and f_u determined for each plasma sample, C_u and the unbound oral CL ($CL_{po,u}$) of the warfarin enantiomers were calculated according to the following equations;

$$f_u = \text{filtrate dpm/plasma dpm} \quad (1)$$

$$C_u = C_p \times f_u \quad (2)$$

$$CL_{po,u} = (F \times D/2\tau)/C_u \quad (3)$$

where F is the bioavailability of warfarin and was assumed to be unity, D is the daily dose of the drug and τ is the dosing interval (24 h for the study patient). At steady-state oral dosing, C_u for each warfarin enantiomer is determined by $CL_{po,u}$ for the respective enantiomer. According to the physiological pharmacokinetic modeling [17], $CL_{po,u}$ is defined as being equivalent to intrinsic CL (CL_{int}). Inasmuch as the oral absorption of warfarin is almost complete and more than 98% of the dose absorbed is eliminated by the liver in humans [3], CL_{int} of the drug would largely be equal to the hepatic CL_{int} ($CL_{int,h}$) representing the metabolic activity of the liver to the drug.

3. Results and discussion

This is the first attempt to determine not only

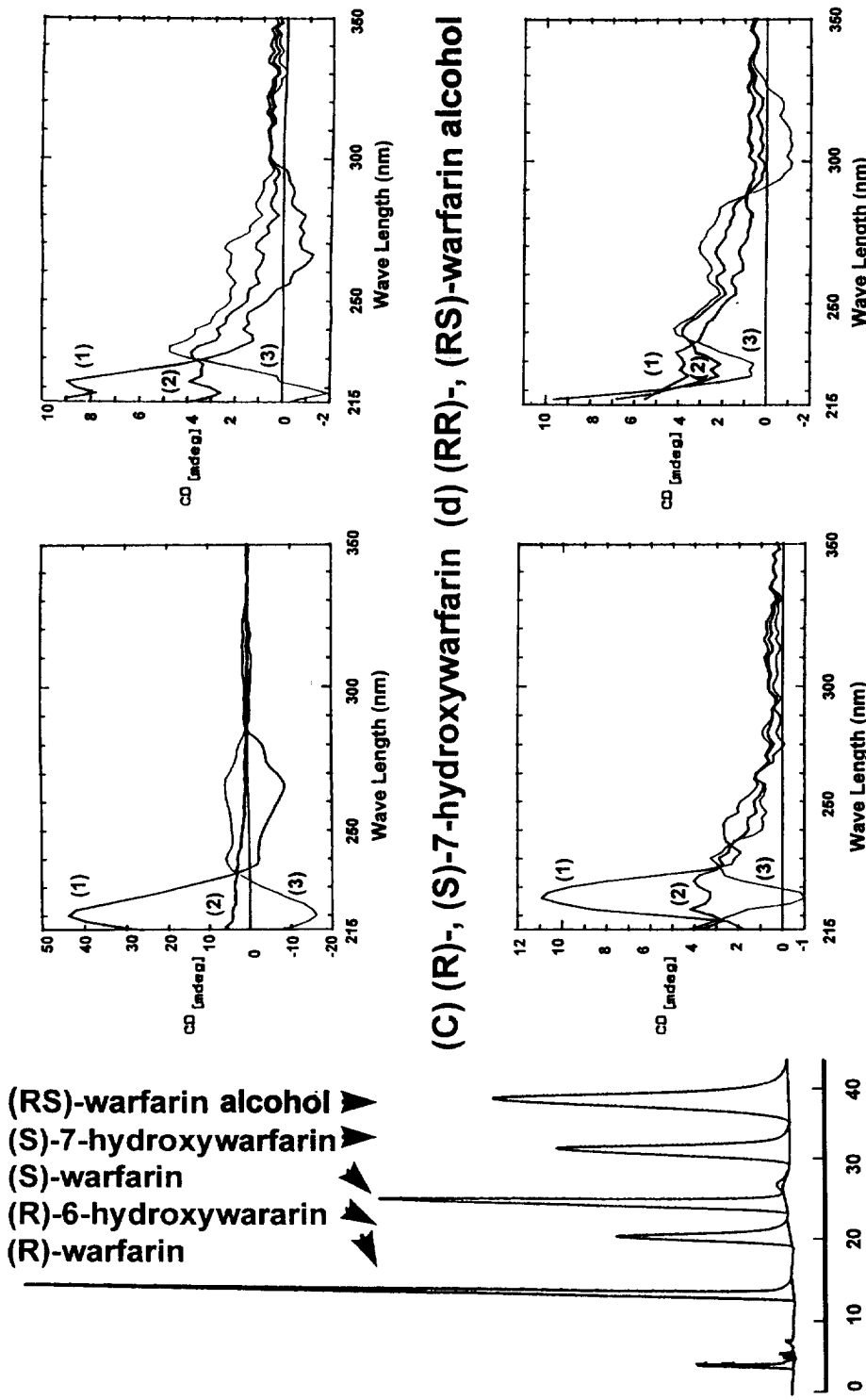

Fig. 1. Representative HPLC chromatogram (left) showing resolution of enantiomers of warfarin and its three major metabolites (i.e., (*R*)-6-hydroxywarfarin, (*S*)-7-hydroxywarfarin and (*RS*)-warfarin alcohol). Peaks corresponding to the respective analytes are indicated by the arrows. Representative CD spectra (right) obtained from the chirally eluted HPLC peaks using a stop-flow technique: (a) (*R*)-warfarin (1), basecline (2) and (*S*)-warfarin (3); (b) (*R*)-6-hydroxywarfarin (1), baseline (2), and (*S*)-6-hydroxywarfarin (3); (c) (*R*)-7-hydroxywarfarin (1), baseline (2), and (*S*)-7-hydroxywarfarin (3); (d) baseline (1), (*RS*)-warfarin alcohol (2), (*RR*)-warfarin alcohol (3), respectively.

concentrations but also the absolute configurations of the enantiomers of warfarin and its major metabolites using a chiral HPLC system coupled with UV/fluorescent/CD detection. Under the present chromatographic conditions the enantiomers of warfarin and its major metabolites (i.e., (*R*)-6-hydroxy-

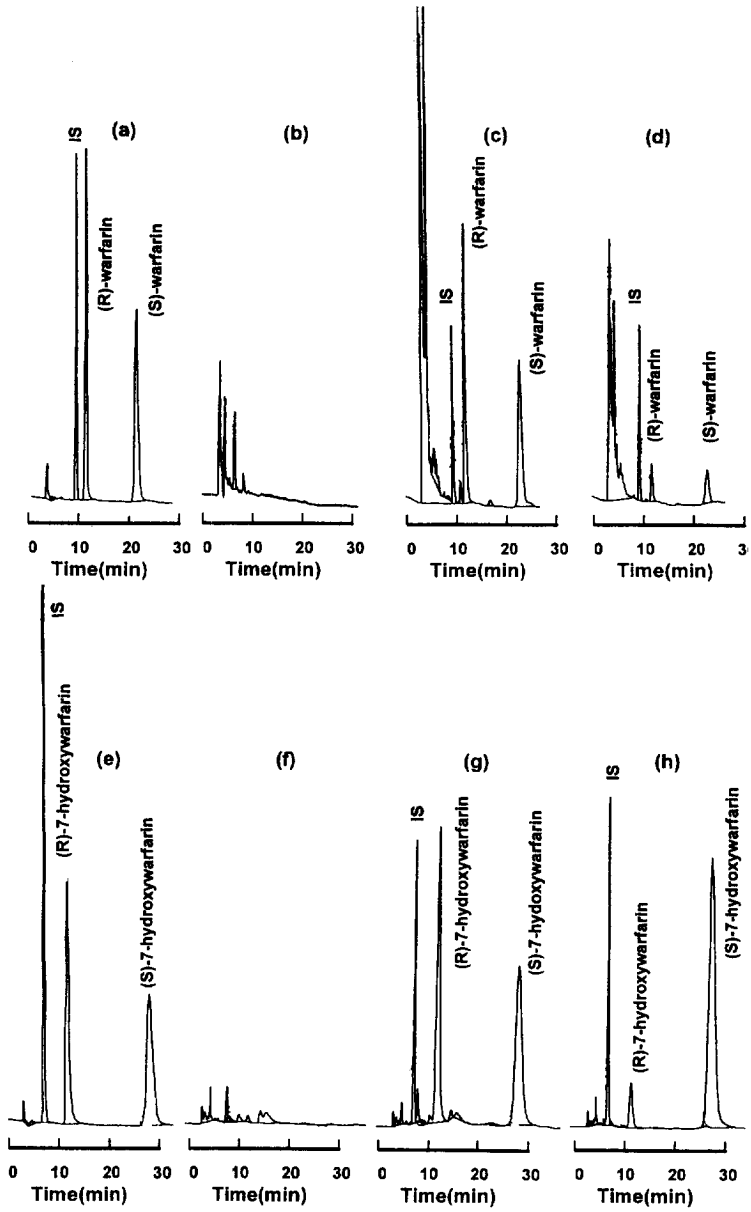

Fig. 2. Representative chromatograms showing resolution of (*R*)- and (*S*)-warfarin and diclofenac (I.S.) in plasma, and (*R*)- and (*S*)-7-hydroxywarfarin and naproxen (I.S.) in urine. Upper panels: (a) standard solution containing 5 μg/ml racemic warfarin, (b) blank human plasma, (c) plasma sample spiked with 10 μg/ml racemic warfarin (corresponds to 1 μg/ml for each enantiomer in plasma) and (d) plasma sample obtained from a patient at 19.5 h after an oral dose of 0.7 mg warfarin. Lower panels: (e) standard solution containing 0.2 μg/ml racemic 7-hydroxywarfarin, (f) blank human urine, (g) urine sample spiked with 1 μg/ml racemic 7-hydroxywarfarin (corresponds to 0.1 μg/ml for each enantiomer in urine) and (h) urine sample obtained from a patient at 14.3 h after an oral dose of 6 mg warfarin.

warfarin, (*S*)-7-hydroxywarfarin and (*RS*)-warfarin alcohol) [6] were well separated in a total assay time of about 40 min (Fig. 1). The resolution factor for warfarin enantiomers in plasma (5.6) and that for 7-hydroxywarfarin enantiomers in urine (4.5) were much greater than those previously reported using different types of chiral HPLC columns [9,18] (Figs. 1 and 2). Although chromatographic peaks for (*R*)-8-hydroxywarfarin and (*R*)-10-hydroxywarfarin are not shown in the figure, they would not interfere significantly with the determination of the above-described compounds according to their retention times (i.e., 12.3 and 18.1 min for (*R*)-8-hydroxywarfarin and (*R*)-10-hydroxywarfarin, respectively).

Because the CD detector was connected with the chiral HPLC–UV system in series, the CD spectra for the respective UV peaks were obtained without time lag with use of a stop-flow technique. The CD spectra corresponding to (*R*)-enantiomers of warfarin, 6-hydroxywarfarin and 7-hydroxywarfarin showed a positive peak polarity relative to the baseline around 220 nm, whereas those corresponding to their respective (*S*)-congeners showed a negative peak polarity (Fig. 1a–c). Both (*RR*)- and (*RS*)-warfarin alcohols exhibited a negative peak polarity relative to the baseline, but the former gave a greater maximum response than the latter (Fig. 1d). These findings indicate that the present HPLC–UV–CD system would provide a useful means to establish enantioselective assay conditions for warfarin and its metabolites and possibly other enantiomeric therapeutic agents as well.

The mean recoveries of warfarin enantiomers from plasma assessed at three different concentrations ranged from 96.9 to 103% and those of 7-hydroxywarfarin enantiomers from urine ranged from 84.9 to 101% (Table 1). The recoveries of the internal standards for plasma (i.e., diclofenac) and for urine (i.e., naproxen) were 102±4.5% and 100±4.8%, respectively. Blank plasma and urine obtained from healthy subjects showed no peaks that might have interfered with the determination of the enantiomers of warfarin and 7-hydroxywarfarin or the internal standards (Fig. 2). According to the retention times obtained, only indomethacin appeared to interfere with the determination of (*R*)-warfarin among the 16 drugs examined (Table 2).

Calibration curves used for the quantification of warfarin enantiomers in human plasma and of 7-hydroxywarfarin in urine were linear with correlation coefficients of >0.997 over the clinically relevant concentration ranges. The lower detection limits, defined as a signal-to-noise ratio of 5, were 20 and 40 ng/ml for (*R*)- and (*S*)-warfarin, respectively, in 0.5 ml plasma, and 2.5 and 4.5 ng/ml for (*R*)- and (*S*)-7-hydroxywarfarin, respectively, in 0.5 ml urine, indicating that the assay sensitivity of the present system would be equal to or somewhat greater than

Table 1
Analytical precision and recoveries for warfarin enantiomers in human plasma and those for 7-hydroxywarfarin enantiomers in human urine

Compound	Concentration (μg/ml)	Coefficients of variation (%)		Recovery (%)
		Within-day	Between-day	
(*R*)-Warfarin	0.1	5.2	4.1	99.7±4.9
	0.5	2.5	3.9	103±3.6
	1.0	3.7	5.0	99.3±4.1
(*S*)-Warfarin	0.1	4.8	9.6	102±4.9
	0.5	3.6	5.9	101±2.0
	1.0	3.6	4.6	96.9±2.6
(*R*)-7-Hydroxywarfarin	0.02	0.9	3.9	97.8±5.3
	0.1	6.9	5.6	84.9±8.6
	0.2	1.0	5.3	93.1±7.0
(*S*)-7-Hydroxywarfarin	0.02	1.4	6.9	101±6.4
	0.1	7.1	7.1	85.9±9.3
	0.2	1.1	6.7	92.1±3.6

Data are mean values±S.D. ($n=5$ each).

Table 2
Retention times of drugs possibly coadministered with warfarin

Drug	Retention time (min)
(*R*)-Warfarin	13.1
(*S*)-Warfarin	25.3
Diclofenac (I.S.)	9.6
Naproxen (I.S.)	9.3
Indomethacin	12.9
Ketoprofen	8.9
Cilostazol	32.0
Aspirin	41.0
Ibuprofen	ND
Disopyramide	ND
Captopril	ND
Diltiazem	ND
Furosemide	ND
Metoprolol	ND
Acetaminophen	ND
Ticlopidine	ND
Dipyridamole	ND
Beraprost sodium	ND

ND, not detected.

those of the previous methods [8,9]. The accuracy of the method assessed at three concentrations showed within- and between-day coefficients of variation (C.V.) of <9.6% for warfarin enantiomers in plasma and of <7.1% for 7-hydroxywarfarin enantiomers in urine (Table 1). After hydrolyzing urine samples from the patient on warfarin with β-glucuronidase or sulphatase, only minimal changes were observed in 7-hydroxywarfarin concentrations (i.e. a 5.7% increase after the β-glucuronidase treatment and a 9.8% decrease after the sulphatase treatment), suggesting that the amounts of 7-hydroxywarfarin eliminated as conjugates are negligible.

Warfarin is highly protein bound mainly to albumin [3]. However, caution must be exercised when establishing experimental conditions for plasma protein binding assays. Because the affinity constant of warfarin for the binding site on albumin changes drastically around pH 7.4 [19], the binding of warfarin to human plasma and serum is pH sensitive. Therefore, plasma samples used for warfarin binding should have their pHs adjusted by bubbling with O_2/CO_2 gas prior to experiment to avoid inadvertent pH increases. The plasma protein binding of warfarin was shown to be concentration independent over a wide concentration range (from 0.275 to 10.55 μg/ml) in accordance with a previous report [20]. Thus, a small amount (about 0.7 μg) of ^{14}C-labeled enantiomers spiked to a plasma sample before ultrafiltration should not alter the original f_u value. In addition, sample storage per se was shown to give no appreciable influence on the f_u values for both warfarin enantiomers: the mean f_u values for (*R*)- and (*S*)-warfarin obtained from fresh plasma samples ($n=3$) (1.2±0.4 and 0.7±0.3%, respectively) were quite similar to the corresponding values obtained from the same samples that were frozen and thawed 3 times (0.8±0.4 and 0.6±0.1%, respectively). Collectively, the f_u values of warfarin enantiomers determined with the above precautions showed a good agreement with those previously reported by ultrafiltration and equilibrium dialysis methods [21].

Clinical applicability of the present method was validated in a patient exhibiting a substantial variability in the anticoagulant response to warfarin (Fig. 3). When the patient showed a large increase in INR under the constant warfarin dose, plasma C_u values of (*R*)- and (*S*)-warfarin increased concurrently by 31 and 59%, respectively, compared with the corresponding preceding values. Furthermore, both INR and C_u returned in parallel to their assumed baseline values within 10 days. In contrast, the f_u values for both warfarin enantiomers remained unchanged throughout the study period. Because (*S*)-warfarin has a 3–5 times greater anticoagulant potency than (*R*)-warfarin [3,4] and the C_u of (*S*)-warfarin showed a greater increase than that of (*R*)-warfarin, we assumed that the temporally augmented anticoagulant effect in the patient would have most likely been attributable to the increase in the C_u of (*S*)-warfarin due presumably to the reduced $CL_{po,u}$ or $CL_{int,h}$ for this enantiomer. However, causative factor(s) responsible for the transient reduction in the hepatic metabolism of warfarin enantiomers remain unknown. The results indicate that the method presented herein would be useful for clarifying the intra-patient variability in warfarin response with regard to the C_u of the pharmacologically more relevant (*S*)-enantiomer.

The hepatic metabolism of warfarin is enantioselective and regioselective. The pharmacologically more active (*S*)-warfarin is metabolized mainly to (*S*)-7-hydroxywarfarin primarily by hepatic cytochrome P-450 (CYP) 2C9 [22]. More recent studies [23,24] suggest that certain types of genetic poly-

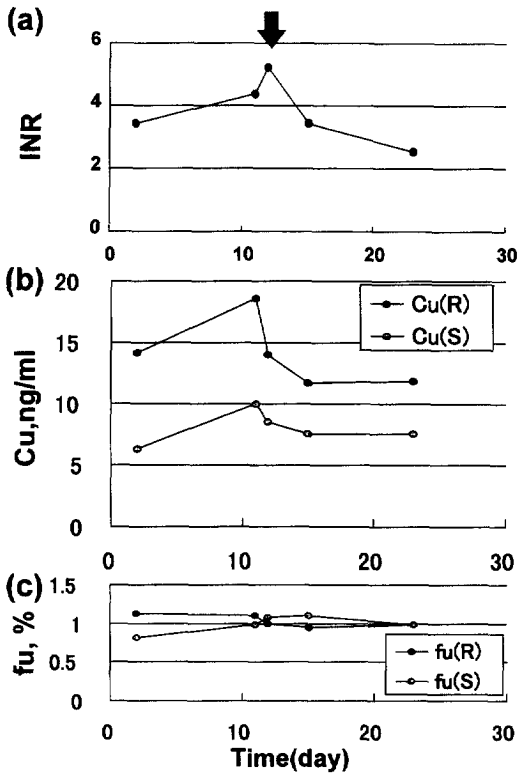

Fig. 3. Time courses of INR (a), plasma unbound concentrations (C_u) of warfarin enantiomers (b) and their respective unbound fractions (f_u) (c) obtained from a patient given a chronic oral dose of warfarin (4 mg/day). Note that an abrupt prolongation of INR from 3.4 to 5.2 was coincident with the increases in C_u values of warfarin enantiomers (i.e., from 14.2 to 18.6 and from 6.3 to 10.0 ng/ml for (*R*)- and (*S*)-enantiomer, respectively), whereas little or no appreciable changes were observed in the f_u values for both enantiomers (i.e., from 1.1 to 1.1% and from 0.8 to 1.0% for (*R*)- and (*S*)-warfarin, respectively). Thus, the unbound oral clearance calculated using the equation described in Section 2 revealed a 29% (i.e., from 106 to 75 ml/min) and 42% decrease (i.e., from 239 to 139 ml/min) for (*R*)- and (*S*)-warfarin, respectively.

morphism of this CYP isoform might be associated with the alteration in the hepatic metabolism of various therapeutically important drugs (e.g., tolbutamide). Therefore, it is of interest to study whether the genetic polymorphism of CYP2C9 would account, at least in part, for a large inter-patient variability in the hepatic metabolism of the warfarin enantiomers. Because the present HPLC method permits determination, not only of plasma warfarin enantiomer concentrations but also those of the major urinary metabolites (e.g., (*S*)-7-hydroxywarfarin), it might also enable evaluation of inter-patient variability in hepatic metabolism in terms of enantioselective and regioselective urinary warfarin metabolite disposition [25].

In conclusion, the present HPLC–UV–CD system coupled with ultrafiltration methodology allows unbound (*R*)- and (*S*)-warfarin in human plasma and their major metabolites in urine to be optically resolved and quantitated. The method is useful for clarifying principal sources(s) responsible for the intra- and inter-patient variability in the anticoagulant response to warfarin with regard to the enantioselective hepatic metabolism of the drug. In addition, because the drug interaction with warfarin is often involved in the enantioselective inhibition in the hepatic metabolism or the displacement at the plasma binding site and a combination thereof, the present method might be valuable for addressing principal mechanism(s) associated with the drug interaction of this anticoagulant.

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