

Research Article

A Chiral HPLC-MS/MS Method for Simultaneous Quantification of Warfarin Enantiomers and its Major Hydroxylation Metabolites of CYP2C9 and CYP3A4 in Human Plasma

Ju W¹, Peng K¹, Yang S¹, Sun H², Sampson M² and Wang MZ^{1*}¹Department of Pharmaceutical Chemistry, The University of Kansas, USA²Department of Pharmacy, The University of North Carolina at Chapel Hill, USA***Corresponding author:** Wang MZ, Department of Pharmaceutical Chemistry, The University of Kansas, 2095 Constant Ave, Lawrence, KS 66047, USA**Received:** August 07, 2014; **Accepted:** August 20, 2014; **Published:** August 22, 2014**Abstract**

Warfarin is an oral anticoagulant that requires frequent therapeutic drug monitoring due to a narrow therapeutic window, considerable interindividual variability in drug response, and susceptibility to drug-drug and drug-diet interactions. Enantiomeric separation and quantification of warfarin enantiomers and clinically important major hydroxylation metabolites are essential for drug interaction studies and phenotypic characterization of CYP2C9 and CYP3A4, the major Cytochrome P450 (CYP) enzymes involved in warfarin metabolism. Here, we describe the development and validation of a chiral high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS)-based quantification of R-warfarin, S-warfarin, S-7-hydroxywarfarin (the major CYP2C9 metabolite) and (9R; 10S)-10-hydroxywarfarin (the CYP3A4 metabolite) in human plasma. Simple protein precipitation-based extraction showed good recovery of analytes (82.9 - 96.9%). The developed method exhibited satisfactory intra-day and inter-day accuracy and precision. The lower limits of detection were 0.25nM (or ~0.08 ng/ml) for the warfarin enantiomers and 0.1nM (or ~0.04 ng/mL) for S-7-hydroxywarfarin and (9R; 10S)-10-hydroxywarfarin using only 50 μ L plasma during extraction. The validated method was successfully applied to analyze plasma samples obtained from a healthy human subject who enrolled in a clinical drug interaction study involving warfarin.

Keywords: Warfarin; Chiral separation; HPLC-MS/MS; Hydroxywarfarin; Protein precipitation extraction**Abbreviations**

AUC: Area Under The Plasma Concentration-Time Curve; COD: Coefficient of Determination; CYP: Cytochrome P450; CV: Coefficient of Variation; DMSO: Dimethylsulfoxide; HPLC-MS/MS: High Performance Liquid Chromatography-tandem mass spectrometry; INR: International Normalized Ratio; IS: Internal Standard; LLE: Liquid-Liquid Extraction; LLOQ: Lower Limit Of Quantification; LOD: Limit Of Detection; MRM: Multiple Reaction Monitoring; PPE: Protein Precipitation Extraction; PT: Prothrombin Time; QC: Quality Control; SD: Standard Deviation; SPE: Solid-Phase Extraction; S/N: Signal-to-Noise ratio; ULOQ: Upper Limit Of Quantification

Introduction

Warfarin is the most commonly prescribed oral anticoagulant in North America and is indicated for the prophylaxis and treatment of a number of serious thromboembolic disorders and complications [1]. It elicits an anticoagulant effect by interfering with the synthesis of vitamin K-dependent clotting factors in the liver *via* inhibition of the vitamin K epoxide reductase complex subunit 1 (VKORC1) [2]. Warfarin has a narrow therapeutic index and displays considerable inter individual variability in dose requirements. To achieve and

maintain optimal anticoagulant activity: warfarin efficacy is frequently monitored by the prothrombin time (PT) or the Internationally Normalized Ratio (INR). Despite target INR monitoring and careful dose adjustment: the rate of warfarin-related bleeding events has not diminished [3,4]. Genetic factors including VKORC1: concomitant drugs/diet: and various disease states can impact on the efficacy of warfarin by changing its pharmacokinetics. Thus: sensitive and specific analytical methods to quantify warfarin and its clinically significant metabolites in various matrices: especially human plasma: may help to improve the understanding of warfarin pharmacology and detection of potential warfarin-drug/diet interactions.

Warfarin is a racemic mixture of *R*- and *S*-enantiomers. *S*-warfarin is more potent (3-5 times) pharmacologically than *R*-warfarin [5]: however *S*-warfarin clearance is greater than that of *R*-warfarin. Both enantiomers undergo extensive cytochrome P450 (CYP)-mediated metabolism in the liver. CYP2C9 catalyzes the 6- and 7-hydroxylation of *S*-warfarin: with *S*-7-hydroxywarfarin (*S*-7-OH-warfarin) being the predominant metabolite: and the 4'-hydroxylation of *R*-warfarin [6]. CYP3A4 regio- and stereo selectively catalyzes the 10-hydroxylation of *R*-warfarin to form (9R; 10S)-10-hydroxywarfarin [(9R; 10S)-10-OH-warfarin] [7]. Inhibition and induction of CYP2C9- and/or CYP3A4-mediated warfarin metabolism by co-administered

drugs/diets is the main culprit for many clinically important drug interactions [1]. Therefore: specific quantification of *S*-7-OH-warfarin (or *S*-6-OH-warfarin) and (9*R*; 10*S*)-10-OH-warfarin in plasma will enable phenotypic characterization of CYP2C9 and CYP3A4 catalytic activities during clinical drug-drug and drug-diet interaction studies.

Several analytical methods have been reported to achieve enantiomeric separation of *R*- and *S*-warfarin and/or the stereoisomers of clinically important warfarin metabolites. Separation and quantification of *R*- and *S*-warfarin in human plasma has been demonstrated using various chiral High Performance Liquid Chromatography (HPLC) columns coupled with detection by UV [8-12] or tandem mass spectrometry (MS/MS) [13]. However: chiral separation and quantification of hydroxylated warfarin metabolites has mainly focused on 7-OH-warfarin using a chiral HPLC column coupled with UV [14]; fluorescence [15] or MS/MS detection [16]; or using capillary zone electrophoresis coupled with UV detection [17]. Although specific quantification of the CYP3A4-selective warfarin metabolite: (9*R*; 10*S*)-10-OH-warfarin: in human plasma using MS/MS detection has been reported [18]; analytes were separated by combining phenyl based reverse phase column and chiral column with a complicated sample preparation and a long analytical time (17 min). In addition: previously reported analytical methods for the warfarin enantiomers in plasma involve either extraction using solid phase extraction (SPE) cartridges [8;16;18-20] or phase separation *via* liquid-liquid extraction (LLE) [9-11,13-15]. Extraction of warfarin by Protein Precipitation (PPE) has only been used for microsomal metabolism studies: where protein concentrations are typically much lower (< 2 mg/mL) than in plasma [21,22]. In this study: we aimed to 1) develop a chiral HPLC-MS/MS-based method to quantify the warfarin enantiomers and two stereo isomeric metabolites: *S*-7-OH-warfarin and (9*R*; 10*S*)-10-OH-warfarin: in human plasma for CYP2C9 and CYP3A4 phenotyping with a short chromatographic procedure and 2) determine whether PPE could be used as an alternative extraction method for enantiomeric analysis of warfarin and its major hydroxylated metabolites in human plasma.

Materials and Methods

Materials

R-warfarin: *S*-warfarin: racemic warfarin: warfarin metabolites (4'-: 6-: 7-: 8- and 10-OH-warfarin): and warfarin- d_5 (deuterated phenyl ring; used as internal standard [IS]) were purchased from Toronto Research Chemicals Inc. (North York: ON: Canada). Ammonium acetate and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis: MO: USA). Optima-grade water: acetonitrile: methanol: and acetic acid was obtained from Fisher Scientific (Pittsburgh: PA: USA). Blank human plasma (collected in K_2 -EDTA tubes) was purchased from Innovative Research (Novi: MI: USA). Recombinant human CYP3A4 and CYP2C9 Supersomes™: microsomes prepared from baculovirus-infected insect cells expressing human CYP enzymes and NADPH-cytochrome P450 reductase: were purchased from BD Gentlest (Woburn: MA: USA).

Incubation with recombinant CYP3A4 and CYP2C9 enzymes

The metabolism of *R*- and *S*-warfarin by recombinant CYP3A4 and CYP2C9 was conducted according to a previously published protocol [22] with modifications. Briefly: incubation mixtures (50 μ L)

contained *R*- or *S*-warfarin (10 μ M final concentration): recombinant CYP enzymes individually (50 pmol/mL): and 100 mM phosphate buffer (pH 7.4) containing 3.3mM $MgCl_2$. Reactions were initiated by the addition of NADPH (1 mM final concentration) and allowed to proceed for 30 min at 37°C. Control incubations were conducted in the absence of NADPH. The reactions were stopped with 400 μ L ice-cold methanol-water (7:1: v/v) and vortexed. Following centrifugation to pellet precipitated proteins: the supernatants were dried under nitrogen at 50°C for 45 min using a 96-well micro plate evaporator (Model SPE Dry 96; Biotage: LLC: Charlotte: NC: USA) and the dried sample reconstituted with 250 μ L methanol-water (15:85: v/v) prior to chiral HPLC-MS/MS analysis.

Preparation of calibration standards and quality controls

Individual stock solutions of *R*-warfarin: *S*-warfarin: racemic warfarin: warfarin- d_5 : and each warfarin metabolite (4'-: 6-: 7-: 8- and 10-OH-warfarin) were prepared in DMSO. Working standard mixtures were prepared by mixing and diluting the stock solutions of racemes warfarin:7-OH-warfarin: and 10-OH-warfarin. Calibration standards were prepared by spiking 1.0 μ L working standard mixture into 49 μ L blank human plasma to yield the following final concentrations: 0.25: 0.5: 1.25: 2.5: 5: 12.5: 25: 50: 125: 250: 500: 1250: 2500: and 5000 nM for *R*- and *S*-warfarin: and 0.05: 0.1: 0.25: 0.5: 1: 2.5: 5: 10: 25: 50: 100: 250: 500: and 1000 nM for *S*-7-OH-warfarin and (9*R*;10*S*)-10-OH-warfarin. Quality controls (QCs): prepared separately from individual stock solutions: had final plasma concentrations of 1.25: 2.5: 50: and 1250 nM for *R*- and *S*-warfarin: and 0.25: 0.5: 10: and 250 nM for *S*-7-OH-warfarin and (9*R*;10*S*)-10-OH-warfarin. Calibration standards were prepared in triplicate: while QCs in quadruplicate; all were processed as plasma samples before chiral HPLC-MS/MS analysis.

Sample preparation and HPLC-MS/MS analysis

Following thawing at room temperature: plasma samples were prepared using PPE rather than SPE or LLE. Specifically: plasma samples (50 μ L) were mixed with 400 μ L methanol-water (7:1: v/v) containing 30 nM warfarin- d_5 as the IS. After vortexing for 10 s: the samples were centrifuged at 2250 g for 15 min at 4°C to pellet precipitated proteins. The supernatants were evaporated under nitrogen at 50°C for 45 min. The dried samples were reconstituted with 100 μ L methanol-water (15:85: v/v) prior to chiral HPLC-MS/MS analysis.

HPLC-MS/MS quantification of warfarin and its metabolites was performed on a Waters ACQUITY I-Class UPLC (operated at normal HPLC pressure) coupled to a Xevo TQ-S triple quadrupole mass spectrometer equipped with an electro spray ionization source (Waters Corporation: Milford: MA: USA). Enantiomeric separation of warfarin and its metabolites was achieved with an Astec CHIROBIOTIC™ V Chiral HPLC column (100 mm \times 4.6 mm; 5 μ m; Supelco: Inc: Bellefonte: PA) protected by an ACQUITY column in-line filter (0.2 μ m; Waters Corporation). HPLC mobile phase (A) consisted of 100% (v/v) water with 5mM ammonium acetate (pH4.0: adjusted with acetic acid): while (B) consisted of 100% (v/v) acetonitrile. The initial gradient began with 10% B and was held for 0.2 min. Mobile phase B increased linearly to 40% over 5 min and remained at 40% for 1 min. The system was re-equilibrated with 10% B for 2 min prior to the next injection. A flow rate of 0.8 mL/min was

used throughout the study. The autosampler was set at 6°C and the HPLC column heated at 50°C. The sample injection volume was 10 µL.

The mass spectrometer was operated under negative ion mode for multiple reaction monitoring (MRM) of warfarin and its metabolites. Typical instrument conditions were: capillary voltage: 0.50 kV; source offset: 50 V; desolvation temperature: 500°C; desolvation gas flow: 1000 L/h; cone gas flow: 150 L/h; nebulizer gas flow: 7.0 bar; and collision gas flow: 0.15 ml/min. Analyte-specific instrument parameters (*i.e.*: collision energy; capillary voltage; cone voltage) were optimized prior to analysis using the Intel Start™ auto-tune with infusion of analyte standards. The specific MRM transitions used for quantification were m/z 307.1 → 161.0 for warfarin; m/z 312.2 → 255.1 for warfarin- d_5 ; m/z 323.1 → 177.0 for 6-; 7- and 8-OH-warfarin; and m/z 323.1 → 250.3 for 10-OH-warfarin. Peak area ratios of analyte *vs.* IS were used to generate calibration curves and calculate analyte concentrations in plasma samples.

Method validation

Method selectivity was investigated by comparing HPLC-MS/MS chromatograms from blank human plasma and plasma spiked with standards at the Lower Limit of Quantification (LLOQ). To ensure MRM transitions for warfarin and 6-; 7-; 8-; and 10-OH-warfarin did not cross-detect 4'-OH-warfarin; chromatograms from plasma spiked with 4'-OH-warfarin (200 nM final concentration) also were compared.

The intra-day accuracy and precision of the method were determined by replicate analysis ($n = 4$) of the QCs on the same day. The inter-day accuracy and precision were determined by replicate analysis of the QC on three separate days. Accuracy (%) was defined as the closeness of the average QC concentrations determined by the method to the true concentrations. Precision (Coefficient of Variation [CV]; %) was defined as the spread of the individual measures of multiple QC preparations.

The extraction recovery was determined by comparing peak area ratios of analyte *vs.* IS from QC samples that were spiked with analyte before extraction to ratios from blank plasma extracts spiked with known amounts of pure analyte post-extraction (represents 100% recovery). The extraction recovery was examined at three QC concentrations (2.5; 50; and 1250 nM for *R*- and *S*-warfarin; 0.5; 10; and 250 nM for *S*-7-OH-warfarin and (9*R*;10*S*)-10-OH-warfarin) with quadruplicate samples at each concentration.

Matrix effects during MS/MS analysis were evaluated by comparing peak areas of analyte from blank plasma extracts spiked with known amounts of pure analyte to peak areas from samples prepared in the reconstitution solvent (*i.e.*: methanol-water [15:85: v/v]) with known amounts of pure analyte. The matrix effects also were examined at three QC concentrations (2.5; 50; and 1250 nM for *R*- and *S*-warfarin; 0.5; 10; and 250 nM for *S*-7-OH-warfarin and (9*R*; 10*S*)-10-OH-warfarin) with quadruplicate samples at each concentration. Results were expressed as % signal remaining relative to standards in the reconstitution solvent: which represents 100% signal remaining.

Freeze-thaw stability was evaluated by exposing QCs to three freezing (-80°C) and thawing (room temperature) cycles before

sample preparation. The thermal stability was evaluated by comparing peak areas of standards in methanol-water (15:85: v/v) incubated at 50°C for 50 min (temperature and time needed during drying) to those incubated at 4°C for the same period of time. Sample stability in the autosampler was evaluated by repeat-analysis of the same QCs stored in a thermostatted autosampler (6°C) for 24 h.

The Limit of Detection (LOD) was defined as the lowest concentration of calibration standard that had a signal-to-noise ratio (S/N) greater than 10. The LLOQ was defined as 5 times the LOD with an accuracy of 80-120%: and an imprecision of ≤20%.

Analysis of human plasma samples from a clinical study

Blood samples were obtained from a healthy volunteer who participated in a clinical drug-drug interaction study involving warfarin as the victim drug. The human subject received placebo as the perpetrator drug and a single oral dose of 10 mg warfarin the morning of the study following an overnight fast. Venous blood (5 ml) was collected in K_2 -EDTAVacutainer® tubes (BD Biosciences; Franklin Lakes, NJ; USA) *via* an intravenous line at 0; 0.5; 1; 1.5; 2; 3; 4; 6; 8; 10; 12; 24; 36; 48; 60; 72; 96; 120; 144; and 168 h post-warfarin administration. Within 1 h following collection: blood samples were centrifuged (3000 rpm at 4°C for 10 min) and the resulting plasma samples transferred to pre-labeled cry tubes for storage at -80°C until analysis. The clinical study (ClinicalTrials.gov registry number: NCT01250535) was approved by the Institutional Review Board of the University of North Carolina at Chapel Hill (Chapel Hill: NC; USA). The area under the plasma concentration-time curve (AUC): terminal elimination half-life ($t_{1/2}$): maximum plasma drug concentration (C_{max}): and time to reach C_{max} (T_{max}) were calculated using the trapezoidal rule-extrapolation method and non compartmental analysis (Phoenix Win Nonlin version 6.3; Pharsight; Mountain View: CA; USA).

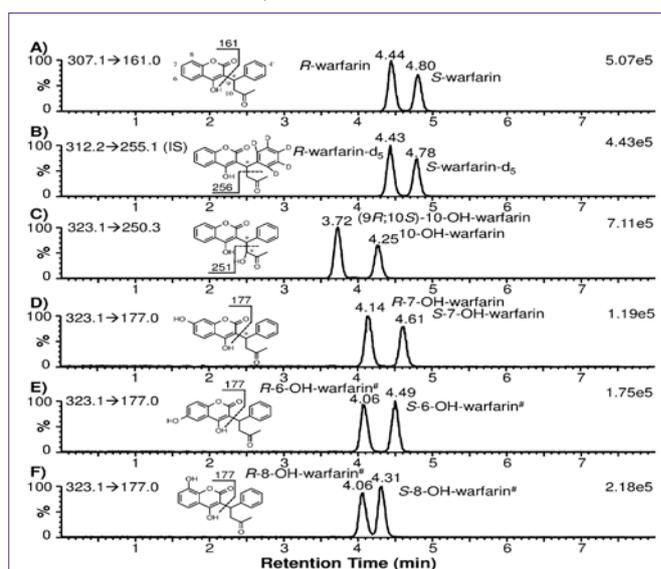


Figure 1: Extracted ion chromatograms of individual warfarin and hydroxylated warfarin standards (200 nM) prepared in blank human plasma. The monitored MRM transitions and signal intensity for the most intense peak are shown in the upper-left and upper-right corners of each chromatogram, respectively. The chemical structure of each analyte also is shown with the postulated fragmentation and fragment mass. * denotes a chiral center; # denotes the postulated stereochemical assignment.

Results and Discussion

HPLC-MS/MS analysis and enantiomeric separation

Enantiomeric separation of warfarin and warfarin- d_5 (as IS) was achieved using a chiral HPLC column. The extracted ion chromatograms showed that both racemic warfarin and warfarin- d_5 standards produced two well-resolved peaks at 4.44 and 4.80 min for warfarin (Figure 1A): and 4.43 and 4.78 min for warfarin- d_5 (Figure 1B). These peaks are distinguishable from the hydroxylated warfarin metabolites due to their characteristic MRM transitions. Using synthetic optically pure *R*- and *S*-warfarin standards: it was determined that *R*-warfarin eluted at 4.44 min and *S*-warfarin at 4.80 min (data not shown). Due to the stereochemical similarity between warfarin and warfarin- d_5 (deuterated phenyl ring; Figures 1A and 1B): it can be postulated that *R*-warfarin- d_5 eluted at 4.43 min and *S*-warfarin- d_5 at 4.78 min. Subsequently: *R*-warfarin- d_5 served as the IS for quantification of *R*-warfarin and (9*R*; 10*S*)-10-OH-warfarin: while *S*-warfarin- d_5 was used as the IS for quantification of *S*-warfarin and *S*-7-OH-warfarin.

To determine CYP2C9 and CYP3A4 phenotypes: it is necessary to measure the formation of *S*-7-OH-warfarin (and/or *S*-6-OH-warfarin) and (9*R*; 10*S*)-10-OH-warfarin: respectively. Since 10-OH-warfarin can be distinguished from the hydroxylated benzopyran metabolites (*i.e.*: 6-: 7-: and 8-OH-warfarin) *via* its characteristic MRM transition (m/z 323.1 \rightarrow 250.3): chromatographic separation of these hydroxylation metabolites is not necessary for HPLC-MS/MS quantification of 10-OH-warfarin. However: 10-OH-warfarin contains two chiral centers (Figure 1c): which results in four possible stereoisomers. The stereochemistry of the 10-OH-warfarin standard

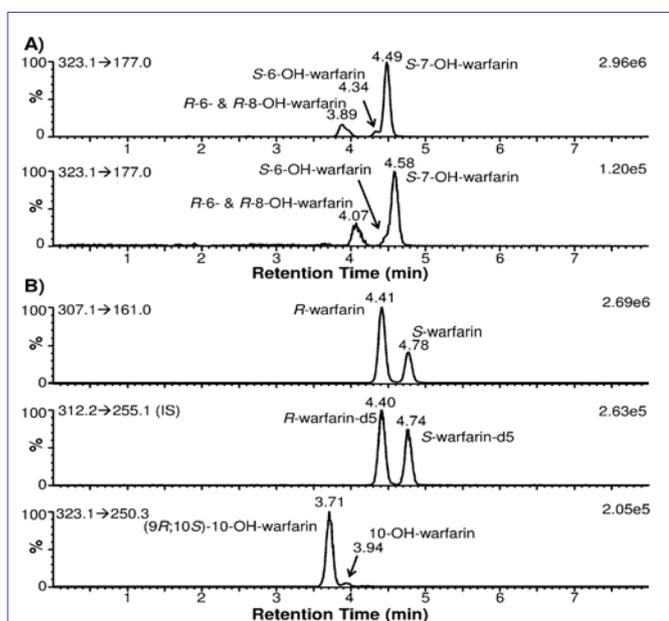


Figure 2: Extracted ion chromatograms of warfarin and its hydroxylation metabolites present in a human subject plasma sample obtained 36 h post-warfarin administration. (A) Chiral separation of *S*-7- from *S*-6-OH-warfarin was dependent upon the batch quality of the chiral HPLC column used. (B) (9*R*; 10*S*)-10-OH-warfarin was the predominant 10-OH-warfarin metabolite. The monitored MRM transitions and signal intensity for the most intense peak are shown in the upper-left and upper-right corners of each chromatogram, respectively.

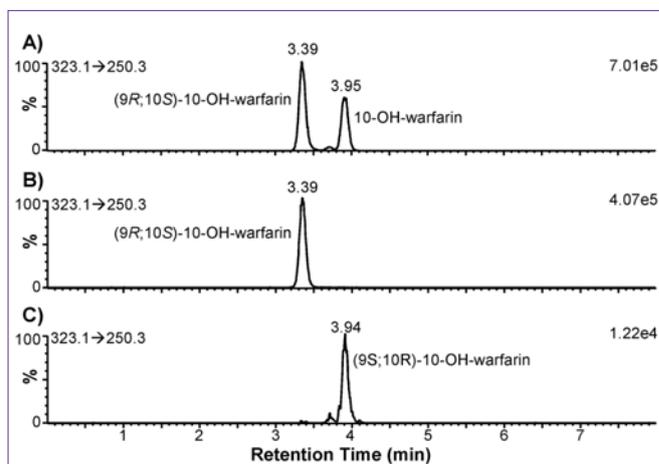


Figure 3: Extracted ion chromatograms of 10-OH-warfarin and its hydroxylation metabolites after 30 min incubation with recombinant human CYP3A4. (A) 10-OH-warfarin standard. (B) *R*-warfarin was used as substrate. (C) *S*-warfarin was used as substrate. The monitored MRM transitions and signal intensity for the most intense peak are shown in the upper-left and upper-right corners of each chromatogram, respectively.

used in this study was not available from the vendor. Upon chiral separation and HPLC-MS/MS analysis: the 10-OH-warfarin standard produced two well-resolved peaks at 3.72 and 4.25 min (Figure 1C) or at 3.39 and 3.95 min (Figure 3A) using a same column of a different batch: corresponding to two of the four possible stereoisomers. It is known that CYP3A4 preferentially catalyzes the formation of (9*R*; 10*S*)-10-OH-warfarin from *R*-warfarin and (9*S*; 10*R*)-10-OH-warfarin from *S*-warfarin [6,7]. When *R*-warfarin was incubated with recombinant human CYP3A4: the predominant 10-hydroxylation metabolite co-eluted with the 3.39-min peak: confirming the identity of the 3.39-min peak as (9*R*; 10*S*)-10-OH-warfarin (Figure 3B). When *S*-warfarin was incubated with CYP3A4: the predominant 10-hydroxylation metabolite co-eluted with the 3.95-min peak: confirming the identity of the 3.95-min peak as (9*S*; 10*R*)-10-OH-warfarin (Figure 3B): the enantiomeric stereoisomer of (9*R*; 10*S*)-10-OH-warfarin. This analysis suggests that the 10-OH-warfarin standard used in this study was a racemic mixture of (9*R*; 10*S*)-10-OH-warfarin and (9*S*; 10*R*)-10-OH-warfarin. Hence it is reasonable to assume they had equal concentration in the racemic mixture: which allowed us to quantify (9*R*; 10*S*)-10-OH-warfarin in plasma samples (Figure 3C).

To ensure specificity: enantiomeric separation of *S*-7- and *S*-6-OH-warfarin from the other hydroxylated benzopyran metabolites (*i.e.*: *R*-7-OH-warfarin: *R*-6-OH-warfarin: and 8-OH-warfarin) should be demonstrated because all of these metabolites have the same MRM transition (m/z 323.1 \rightarrow 177.0): thus making them indistinguishable by MS/MS analysis alone. The extracted ion chromatograms for individual standards of 7-: 6-: and 8-OH-warfarin is shown in Figures 1D-F: demonstrating that the enantiomeric pairs of these hydroxylated benzopyran metabolites were separated from each other. The 7-OH-warfarin standard eluted at 4.14 and 4.61 min (Figure 1D). To determine which peak corresponded to the *S*-7-OH-warfarin metabolite: *S*-warfarin was incubated with recombinant human CYP2C9: as CYP2C9 preferentially catalyzes the formation of *S*-7-OH-warfarin [23]. Results indicated that *S*-7-OH-warfarin

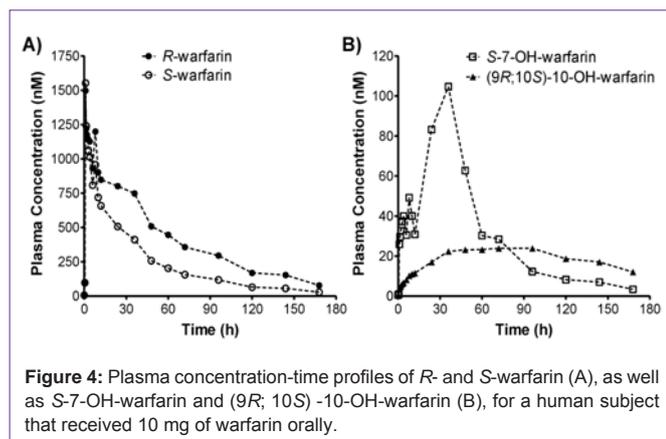


Figure 4: Plasma concentration-time profiles of *R*- and *S*-warfarin (A), as well as *S*-7-OH-warfarin and (9*R*; 10*S*)-10-OH-warfarin (B), for a human subject that received 10 mg of warfarin orally.

eluted at 4.61 min and *R*-7-OH-warfarin at 4.14 min (data not shown). Based on the observed elution order of warfarin and 7-OH-warfarin enantiomers (*i.e.*: *R* proceeds *S*): it can be postulated that *R*-6- and *R*-8-OH-warfarin co-elute at 4.06 min: while *S*-6- and *S*-8-OH-warfarin elute at 4.49 and 4.31 min: respectively (Figure 1E, 1F). Due to minimal HPLC separation of the hydroxylated benzopyran metabolites of *R*-warfarin: they are expected to co-elute if present in human plasma samples when analyzed using our method. Using a longer HPLC gradient (18 min): Zuo et al. [16] achieved a partial separation of these *R*-warfarin metabolites. However: since quantification of these metabolites was not the goal of our study: separation using the longer HPLC gradient was not attempted. The hydroxylated benzopyran metabolites of *S*-warfarin (*i.e.*: *S*-6-: *S*-7- and *S*-8-OH-warfarin) eluted at 4.49: 4.61: and 4.31 min (Figures 1D-F). However: *S*-6- and *S*-7-OH-warfarin were only partially separated. The extent of separation was found to be dependent upon the batch quality of the chiral HPLC column used (Figure 2A). As such: the chiral column with the best separation was used to analyze clinical plasma samples so that *S*-7-OH-warfarin could be specifically quantified.

Calibration standards: accuracy and precision

Warfarin and 7-OH-warfarin used to prepare calibration standards were racemic mixtures of *R* and *S* stereoisomers: and 10-OH-warfarin has been shown to be a racemic mixture of (9*R*; 10*S*)-10-OH-warfarin and (9*S*; 10*R*)-10-OH-warfarin. Calibration standards for *R*-warfarin: *S*-warfarin: *S*-7-OH-warfarin and (9*R*; 10*S*)-10-OH-warfarin were prepared by spiking blank human plasma with working standard mixtures. These calibration standards were then processed in a similar manner as clinical plasma samples: described in Section 2.4 “Sample preparation and HPLC-MS/MS analysis” under Section 2 “Materials and Methods”. The calibration range: LOD: S/N at LOD: representative calibration curves: and coefficient

Table 1: Calibration standards for warfarin enantiomers, *S*-7-OH-warfarin, and (9*R*; 10*S*)-10-OH-warfarin.

Compound	Calibration Range ^a	S/N at LOD	Calibration Curve ^b	COD (R ²) ^b
<i>R</i> -warfarin	0.25–5000 nM	≥13	$-0.0000139022 \cdot X^2 + 0.562725 \cdot X - 0.0504177$	0.99949
<i>S</i> -warfarin	0.25–5000 nM	≥12	$-0.0000138943 \cdot X^2 + 0.564179 \cdot X - 0.0584107$	0.99955
<i>S</i> -7-OH-warfarin	0.1–1000 nM	≥5.4	$0.0000258637 \cdot X^2 + 0.258581 \cdot X - 0.00957948$	0.99967
(9 <i>R</i> ; 10 <i>S</i>)-10-OH-warfarin	0.1–1000 nM	≥18	$0.000118394 \cdot X^2 + 0.6412 \cdot X - 0.0203311$	0.99972

^aCalibration range: LOD-ULOQ

^bRepresentative calibration curves and coefficient of determination

S/N: Signal-to-Noise Ratio; LOD: Limit of Detection; ULOQ: Upper Limit of Quantification; COD: Coefficient of Determination

of determination is summarized in Table 1. The LLOQs obtained by this PPE-based method (using only 50 μ l plasma) were much lower (~10 to 30-fold) than those using an SPE-based HPLC-MS/MS quantification method (using 200 μ l plasma) [16] (Table 2): largely due to the more sensitive triple quadrupole MS instrument used in the current study. The enhanced assay sensitivity and calibration range will be essential for studies with limited sample availability (*e.g.*: capillary micro sampling [24]).

The intra-day accuracy and precision (CV) of QC samples at low: medium and high concentrations were 87.0 to 100.5% and 0.7 to 6.0% (Table 3). The inter-day accuracy and precision of QC samples were 92.3 to 99.5% and 0.4 to 4.9% (Table 3). These values are well within the criteria of 15% bias and 15% CV provided in the US FDA guidance for bio analytical method validation [25].

Extraction recovery: matrix effect and stability

One goal of this study was to simplify the warfarin extraction procedure: preferably avoiding SPE. PPE extraction recoveries of the four analytes in QC plasma samples are summarized in Table 3. They ranged from 82.9 to 95.6% and demonstrated reproducibility (SD < 8.8%). The recovery achieved with PPE is similar to or greater than that obtained with SPE (>80%) [16] or LLE (>73.8%) [14]. Minimal matrix effects were observed for *R*-warfarin: *S*-warfarin and *S*-7-OH-warfarin (Table 4): which all eluted later than (9*R*; 10*S*)-10-OH-warfarin (Figure 1). Significant matrix effects were observed for (9*R*; 10*S*)-10-OH-warfarin (~50% signal suppression; Table 4): but the matrix effects were quite reproducible (CV < 5.5%). Thus: it was important to prepare calibration standards in blank human plasma to mimic the matrix effects of human plasma samples.

The thermal stability of warfarin and its hydroxylated metabolites were evaluated at 50°C for 50 min: which are the temperature and time required to dry down PPE-extracted plasma samples. All four analytes were determined to be thermally stable with the percent remaining ranging from 93.7 to 101.4% (Table 5). The analytes also were stable after three freeze-thaw cycles with the percent remaining ranging from 96.9 to 113% (Table 5).

Application to human plasma sample analytes

The validated PPE-based chiral HPLC-MS/MS method was applied for simultaneous quantification of *R*-warfarin: *S*-warfarin: *S*-7-OH-warfarin: and (9*R*; 10*S*)-10-OH-warfarin in human plasma samples obtained from a clinical drug-drug interaction study involving healthy human subjects. Representative extracted ion chromatograms of warfarin: warfarin-*d*₅: 7-OH-warfarin: and 10-OH-warfarin for a plasma sample collected 36 h post-warfarin administration are shown in Figure 2B. The plasma concentration-time profiles of *R*-warfarin: *S*-warfarin: *S*-7-OH-warfarin: and (9*R*; 10*S*)-10-OH-warfarin were

Table 2: Comparison of chiral LC-MS/MS analytical assays for warfarin and its hydroxylated metabolites formed by CYP2C9 and CYP3A4.

Reported Assays	Analytes Quantified	Extraction (% Recovery)	Analysis Time	Dynamic Range	LLOQ
Zuo et al., 2010 [16]	warfarin enantiomers, stereoisomers of 7-OH-warfarin, and monohydroxylated warfarin metabolites (4-, 6-, 8-, and 10-OH-warfarins)	mixed-mode cation-exchange cartridge (> 87%)	17 min	16.3–4890 nM (5–1500 ng/mL) for warfarin enantiomers 244–586 nM (75–180 ng/mL) for stereoisomers of 7-OH-warfarin	16.3 nM (5 ng/mL) for warfarin enantiomers and stereoisomers of 7-OH-warfarin 0.2 mL of plasma
Jones et al., 2011 [18]	warfarin enantiomers and monohydroxylated warfarin metabolites (4-, 6-, 7-, 8-, and 10-OH-warfarins)	PP by cold acetonitrile with 0.2% formic acid (recovery not reported)	17 min	2–1000 nM for all analytes	10 nM for all analytes 0.05 mL of plasma
Wu et al. (reported herein)	warfarin enantiomers, stereoisomers of 7- and 10-OH-warfarin	PP by methanol-water [7:1, v/v] (83–97%)	6 min	0.25–5000 nM for warfarin enantiomers 0.1–1000 nM for S-7- and (9R;10S)-10-OH-warfarin	1.25 nM for warfarin enantiomers 0.5 nM for S-7- and (9R;10S)-10-OH-warfarin 0.05 mL of plasma

LLOQ: Lower Limit of Quantification.

Table 3: Intra- and inter-day accuracy and precision for warfarin enantiomers, S-7-OH-warfarin, and (9R;10S)-10-OH-warfarin.

Compound	QC Concentration (nM)	Intra-day		Inter-day	
		Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)
<i>R</i> -Warfarin	2.5	94.8	2.2	93.9	0.7
	50	99.2	2.2	97.2	1.8
	1250	98.5	1.0	98.8	1.1
<i>S</i> -Warfarin	2.5	95.1	1.4	94.1	1.0
	50	99.7	2.3	98.1	2.0
	1250	98.9	0.9	99.4	0.4
S-7-OH-warfarin	0.5	87.0	6.0	92.9	4.5
	10	89.6	1.4	92.3	2.0
	250	100.5	1.6	99.5	0.9
(9R;10S)-10-OH-warfarin	0.5	93.2	3.8	96	2.9
	10	93.3	1.4	96.8	2.9
	250	92.3	0.7	99.2	4.9

Table 4: Extraction recovery and matrix effect for warfarin enantiomers, S-7-OH-warfarin, and (9R;10S)-10-OH-warfarin.

Compound	QC Concentration (nM)	PPE Extraction		Matrix Effect	
		Recovery (%)	SD (%)	Remaining Signal (%)	SD (%)
<i>R</i> -Warfarin	2.5	94.4	6.7	96.7	3.4
	50	86.7	3.6	95.2	2.1
	1250	90.2	0.7	96.5	1.0
<i>S</i> -Warfarin	2.5	91.8	7.4	98.8	2.6
	50	86.6	2.9	100.6	2.3
	1250	94.4	5.5	100.5	0.7
S-7-OH-warfarin	0.5	87.7	4.9	89.8	5.0
	10	84.1	1.9	83.5	3.3
	250	87.5	6.9	81.4	1.2
(9R;10S)-10-OH-warfarin	0.5	82.9	8.8	49.2	2.7
	10	88.2	3.3	52.2	1.6
	250	95.6	2.1	50.4	0.7

PPE: Protein-Precipitation Extraction; SD: Standard Deviation.

Table 5: Thermal and freeze-thaw stability for warfarin enantiomers, S-7-OH-warfarin, and (9R; 10S)-10-OH-warfarin.

Compound	QC Concentration (nM)	Thermal Stability		Freeze-Thaw Stability	
		Remaining (%)	SD (%)	Remaining (%)	SD (%)
<i>R</i> -Warfarin	2.5	98.0	3.4	100.9	1.3
	50	100.6	1.7	102.9	1.3
	1250	98.4	1.6	101.9	2.5
<i>S</i> -Warfarin	2.5	98.6	3.2	100.5	1.2
	50	101.4	0.9	101.6	1.2
	1250	98.7	1.5	100.8	2.8
S-7-OH-warfarin	0.5	99.6	6.4	113.0	11.2
	10	93.7	7.4	105.9	4.0
	250	97.6	1.2	112.9	4.2
(9 <i>R</i> ;10 <i>S</i>)-10-OH-warfarin	0.5	97.1	3.0	99.0	8.2
	10	100.0	1.6	102.2	1.3
	250	98.2	2.2	96.9	2.9

SD: Standard Deviation

Table 6: Pharmacokinetics of warfarin enantiomers, S-7-OH-warfarin and (9R; 10S)-10-OH-warfarin in a human subject.

Outcomes	Units	<i>R</i> -warfarin	<i>S</i> -warfarin	S-7-OH-warfarin	(9 <i>R</i> ;10 <i>S</i>)-10-OH-warfarin
C_{max}	nM	1,497	1,552	105	24
T_{max}	h	1	1	36	72
AUC_{last}	nM·h	68,800	39,500	5,100	3,180
$AUC_{0 \rightarrow \infty}$	nM·h	73,800	40,800	5,270	4,470
$t_{1/2}$	h	44	35	35	75

Abbreviations: C_{max} : Maximum Concentration; T_{max} : Time to reach; AUC_{last} : Area under the curve from time zero to the last measurable concentration; $AUC_{0 \rightarrow \infty}$: area under the curve from time zero to infinite time; $t_{1/2}$: terminal elimination half-life

plotted for a human subject that received 10 mg warfarin orally the morning of the study and was monitored for 7 days post-warfarin administration (Figure 4A, 4B). Pharmacokinetic measurements are summarized in Table 6. These values are in agreement with those previously reported for warfarin enantiomers [26].

Conclusion

A simple PPE-based: highly sensitive chiral HPLC-MS/MS analytical method with short retention time has been developed and validated for simultaneous enantiomeric quantification of *R*-warfarin: *S*-warfarin: S-7-OH-warfarin: and (9*R*; 10*S*)-10-OH-warfarin in human plasma. This method is expected to be more time- and cost-effective than SPE- or LLE-based methods. Furthermore: the outstanding sensitivity afforded by the combined high extraction recovery: low matrix suppression effect: and sensitive triple quadruple MS instrument will enable studies with limited sample availability (e.g.: capillary microsampling). Specific quantification of S-7-OH-warfarin and (9*R*; 10*S*)-10-OH-warfarin will enable phenotypic characterization of CYP2C9 and CYP3A4 activities for drug-drug and drug-diet interaction studies involving these two CYP enzymes.

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