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Novel multi-mode ultra performance liquid chromatography–tandem mass spectrometry assay for profiling enantiomeric hydroxywarfarins and warfarin in human plasma

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ABSTRACT

Coumadin (R/S-warfarin) is a commonly prescribed anticoagulant for over ~20 million Americans. Although highly efficacious, positive clinical outcomes during warfarin therapy depend on maintaining a narrow therapeutic range for the drug. This goal is challenging due to large inter-individual variability in patient response, which has been attributed to diversity in drug metabolism. Warfarin is given as a racemic mixture and evidence suggest differences of R and S-warfarin in their therapeutic activities and metabolism. Previous investigation of warfarin metabolism has been hampered by the inability to quantify the individual enantiomers. To overcome this limitation a multi-mode LC–MS/MS method is reported. This strategy combines phenyl based reverse phase chromatography with chiral phase chromatography prior to quantitation by liquid chromatography tandem mass spectrometry. This approach was made possible through advances in UPLC technology producing narrow peaks suitable for transferring to a second column. The reported method separated individual R and S enantiomers of hydroxywarfarin and warfarin. All four possible isomers of 10-hydroxywarfarin were resolved to reveal unprecedented insights into the stereo-specific metabolism of warfarin. Characterization of the method demonstrated that it is robust and sensitive with inter-day coefficients of error between <7% and a detection limit of 2 nM in sample or 10 fmol on column for each analyte. Individual metabolites may be suitable surrogate biomarkers or predictive markers that predict warfarin dose, adverse interactions, or other important clinical outcomes during anticoagulant therapy. Consequently, the metabolite profiles obtained through this dual phase UPLC–MS/MS method are expected to increase our understanding of the role warfarin metabolism plays in patient response to therapy and yield new strategies to improve patient outcomes.

1. Introduction

Coumadin (R/S-warfarin) is a commonly prescribed anticoagulant for over 20 million Americans during the treatment of atrial fibrillation, deep vein thrombosis and other coagulopathies [1]. Despite its extensive use, warfarin therapy is complicated by a narrow therapeutic range and wide inter-individual response to treatment [2]. This unpredictability derives from a variety of factors including most notably, drug metabolism. Warfarin is given to patients as an equal mixture of R and S enantiomers that undergo extensive metabolism into less active and inactive metabolites [3,4]. The elucidation of these metabolic processes is critical for understanding the mechanisms for patient dose-response relationships during warfarin therapy.

Cytochrome P450s (CYP for specific enzymes) catalyzes the first major step in warfarin metabolism to generate enantiomers of five different hydroxywarfarins (Fig. 1) [3]. CYP2C9 is almost solely (>80%) responsible for S-warfarin metabolism [5], such that S-7-hydroxywarfarin is the major metabolite observed in patient plasma [6] and urine [7]. Minor pathways involve low abundance and/or less efficient P450s including CYP2C19 [8] and CYP3A [5,8,9]. In contrast, CYP1A2, CYP2C19, and CYP3A may contribute significantly to R-warfarin metabolism to yield an array of different R-hydroxywarfarin metabolites [3]. Specific contributions of these P450s toward total R-warfarin metabolism have not been studied in any detail. Their roles in R-warfarin metabolism has been suggested based on the profile of R-hydroxywarfarins present in patient plasma [10]. Elevated 10-hydroxywarfarin levels in all patients signify a common importance for CYP3A enzymes in metabolism. High variability in 6- and 8-hydroxywarfarin levels suggests that

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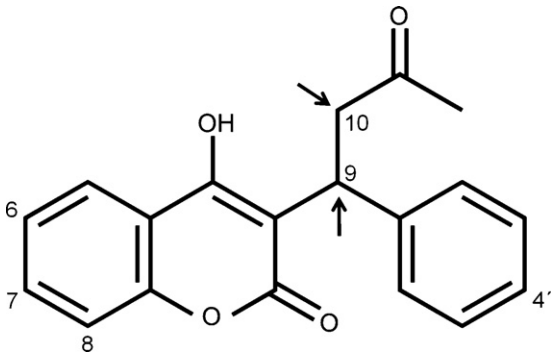

Fig. 1. Sites of warfarin hydroxylation by cytochrome P450s (carbons 4′, 10, 6, 7, 8) and stereo-centers (arrows). Hydroxylation at position 10 introduces a second stereo-center, allowing for four possible isomers (RR, RS, SR, SS).

contributions from CYP1A2 and CYP2C19 vary significantly among patients [3]. Reported drug–drug interactions [11] and genetic studies [12–14] indicate that many of these P450s and others may play a clinical role in patient response to warfarin therapy.

As biological endpoints for metabolic pathways, the profiling of warfarin and its metabolites offers a powerful source of biomarkers for these metabolic pathways and surrogate markers for patient responses to therapy. Consequently, there has been significant interest in developing sensitive methods for monitoring warfarin and its metabolites in plasma and urine. Early efforts with labeled warfarins [15,16] and stereospecific antibodies [17] provided strategies for assessing R- and S-warfarin levels, but required highly specialized reagents. As an alternative, Banfield and Rowland employed a lengthy extraction and chemical derivatization approach to generate diastereomeric esters that could be analyzed by normal phase HPLC and fluorescence detection [18]. That method was the first to report profiling of warfarin and hydroxywarfarin enantiomers present in patient samples. Advances in chiral LC resin development made these chemical modifications unnecessary. While multiple groups resolved R- and S-warfarin [19,20], concurrent separation of all hydroxywarfarin enantiomers has to our knowledge not been reported previously. As a solution, we previously employed two separate, but relatively rapid HPLC–MS/MS based assays to analyze all warfarin and hydroxywarfarin enantiomers present in patient urine samples [7]. Herein, we report a novel multi-mode UPLC–MS/MS method suitable for the accurate quantification of all 14 warfarin and hydroxywarfarin enantiomers in human plasma simultaneously.

2. Material and methods

2.1. Reagents and chemicals

Racemic warfarin, racemic 4′,10,6,7,8-hydroxywarfarins, and deuterated internal standards were obtained from Toronto Research Chemicals (Toronto, Canada). R-warfarin, S-warfarin, and 10-hydroxywarfarin were also obtained from Sigma–Aldrich (St. Louis, MO). Human plasma samples from patients receiving warfarin and blank plasma were purchased from BD Biosciences (San Jose, CA). Only age, sex, and concomitant drug information was provided for each of the samples.

2.2. LC instrumentation and conditions

Hydroxywarfarin and warfarin analytes were quantified by multi-mode ultra high performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) utilizing two commercially available columns with distinctly different stationary phases. The first phase consisted of a phenyl–based reverse-phase chromatography using a Acquity UPLC BEH Phenyl column (2.1 mm × 150 mm 1.7 μm particle column (Waters)), operated at 60 °C. The second phase was chiral chromatography utilizing an Astec Chirobiotic V column [2.1 mm × 150 mm, 5 μm (Supelco)], operated at room temperature (21.6–22.4 °C). The flow rate of 300 μL/min was provided by an Acquity UPLC interfaced with a standard electro-spray ionization source to a Quantum Ultra triple quadrupole mass spectrometer. Warfarin, hydroxywarfarins, and deuterated internal standards were monitored in positive ion mode. Data were acquired in single reaction monitoring (SRM) mode using the ion transitions of 325 to 267 for 4′-hydroxywarfarin, 325 to 179 for 6, 7, and 8-hydroxywarfarin, 325 to 251 for 10-hydroxywarfarin, 309 to 163 for warfarin, 330 to 184 for d_5-8-hydroxywarfarin, and 314 to 168 for d_5-warfarin as previously reported [9].

2.3. Plasma extraction

Human plasma was processed as described previously [21]. In brief, plasma samples (50 μL) [blank, M61, M75, M76, and M80] were spiked with internal standards (10 μL, 60 μM d_5-warfarin and 6 μM d_5-8-hydroxywarfarin 50 mM potassium phosphate pH 7.4) and allowed to equilibrate for ≥12 h at 4 °C. Following equilibration, ice cold 0.2% formic acid in H_2O (190 μL) was added to each sample, followed by ice cold 0.2% formic acid in acetonitrile (1000 μL). Samples were allowed to precipitate at 4 °C for 30 min, followed by centrifugation (10 min at 16,000 × *g*) in a microcentrifuge. The supernatant (1000 μL) was then transferred to a new vial and dried down in a speed vacuum concentrator. Plasma extracts were resolubilized (40 μL) in mobile phase (55% methanol, 45% H_2O with 0.01% formic acid) maintaining a 1:1 ratio with the original volume of plasma.

This extraction method was termed the "metabolomics" method for the current study. In addition to this method, two alternative plasma extraction methods were also explored in an attempt to optimize efficiency and reproducibility. An "ethyl acetate" extraction was also tested, which was accomplished by mixing 50 μL of the plasma sample with 50 μL of ethyl acetate for ~5 min. Following this incubation, 40 μL of the ethyl acetate fraction was dried down and resolubilized in 40 μL of mobile phase for analysis. The third and final method utilized Oasis™ solid phase extraction cartridges. The cartridges were conditioned with 50 mM potassium phosphate pH 7.4 and then loaded with 50 μL of plasma. The cartridges were washed with 1 mL of phosphate buffer and were then eluted with 200 μL of methanol. This methanol sample was then dried down and resolubilized in 50 μL of mobile phase for analysis. To test the relative performance of these three methods, blank human plasma was spiked with warfarin and hydroxywarfarin standards to generate calibration curves ranging from 6.25 to 1000 nM. These samples were split and extracted with the three methods with five independent replicates. The samples were then analyzed by UPLC–MS/MS as previously published [9].

2.4. Microsomal incubations with warfarin enantiomers

Enantiomerically pure R-warfarin and S-warfarin were metabolized by human liver microsomes pooled from 150 donors (HLM150 BD Biosciences) to generate R or S-hydroxywarfarin metabolites, respectively. Stock solutions of R-warfarin or S-warfarin in ethanol were allowed to evaporate to dryness in a microfuge tube and were resolubilized in 50 mM potassium phosphate pH 7.4 for a final concentration of 25 μM or 500 μM in the reaction. The final concentration of microsomal protein was 2 mg/mL. The reaction was initiated by addition of NADPH, for a final concentration of 1 mM, and incubated at 37 °C. The reaction was quenched at 30 min by addition of an equal volume of ice cold 0.4 M perchloric acid. The

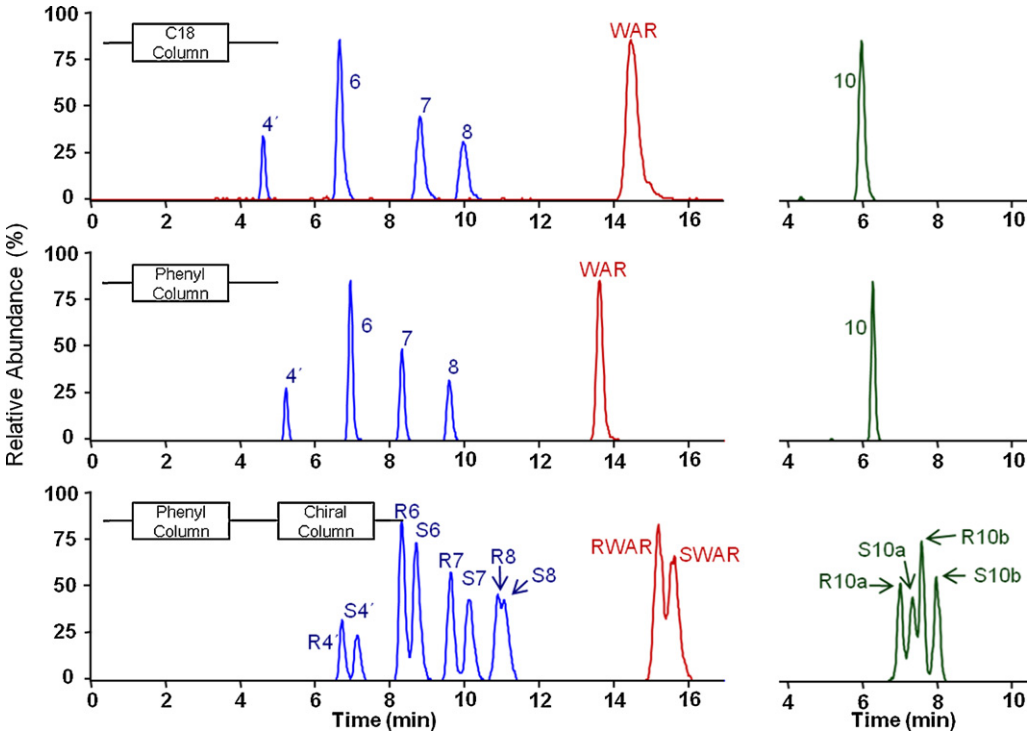

Fig. 2. Representative extracted ion chromatograms of standards (100 nM) with three different column systems. For each column, the flow rate was 300 μL/min with 45% methanol and 55% H_2O with 0.01% formic acid. Hydroxywarfarin standards were separated using a C18 column (top), a UPLC phenyl column (middle) and both the phenyl and chirobiotic columns in-series together (bottom). The extracted ion chromatograms specific for 10-hydroxywarfarin are shown separately (right). The insets symbolize the various system configurations with either one column or both in series. Numbers indicate sites of hydroxylation while R or S signifies stereochemistry e.g. 7 = 7-hydroxywarfarin; S7 = S-7-hydroxywarfarin. 10-Hydroxywarfarin contains two stereo-centers such that four configurations are possible (RR, RS, SR, SS). However, only stereochemistry at carbon 9 could be assigned, therefore peaks were given an "a" or "b" designation based on elution order e.g. R10a = 10-hydroxywarfarin with R stereochemistry at carbon 9 and unknown stereochemistry at carbon 10.

sample was centrifuged at 10,000 × *g* for 10 min and the supernatant was transferred to a fresh vial for analysis by LC–MS/MS.

3. Results

3.1. Characterization of phenyl column chromatography

Initially, separation of hydroxywarfarins was explored using a variety of reverse-phase columns and isocratic conditions (data not shown). This evaluation demonstrated that the phenyl-based column achieved the highest efficiency and selectivity for separation of hydroxywarfarins (Fig. 2, middle). This is likely due to pi-stacking interactions between warfarin ring motifs and the phenyl group on the stationary phase. In comparison with the C18 column (BEH C18 column 2.1 mm × 150 mm, Waters), the peaks were approximately one half as wide at the base with the phenyl column and provided greater separation between 7 and 8-hydroxywarfarin (Fig. 2, top and middle). Varying the isocratic composition of methanol demonstrated that mobile phase compositions with less than 40% methanol resulted in long run times (>20 min) and unacceptably wide peaks. With isocratic compositions up to ~85% methanol, the phenyl column achieved baseline separation of all hydroxywarfarins. Sufficient separation of all hydroxywarfarins was achieved at 45% methanol with a peak resolution of ≥1 min between peaks at the same SRM transition (Fig. 2, middle). Operation at 60 °C significantly reduced the system pressure to ~8500 at a flow rate of 300 μL/min, while maintaining separation of all hydroxywarfarin metabolites. Although this method successfully separated mixtures of hydroxywarfarins into their regio-isomers, each peak represents a mixture of the R and S enantiomers.

3.2. Characterization of chiral column chromatography

The separation of each hydroxywarfarin into its R and S enantiomeric components was investigated under a range of isocratic conditions using the same mobile phases and flow rates as with the phenyl column. The best separation of enantiomers was achieved with 20% methanol, but was maintained up to ~50% methanol. At room temperature (21.6–22.4 °C), each hydroxywarfarin achieved baseline separation into its respective R and S enantiomers with methanol compositions from 45% and below with the exception of 8-hydroxywarfarin which was partially separated at 45% methanol (Fig. 3). Higher column temperatures decreased the separation efficiency of hydroxywarfarin enantiomers. The composition of methanol needed to be approximately 20% to achieve maximum baseline separation for 8-hydroxywarfarin. Unfortunately, lower methanol compositions also led to increased retention times and broader peaks. At 45% methanol, all R-enantiomers had a retention time of approximately 2.1 min while S-enantiomers eluted between 2.3 and 2.8 min (Fig. 3). The operating pressure under these conditions was ~1500 psi. Therefore, sufficient separation on both phases is achieved with 45% methanol enabling in-series combination of both chromatographic systems.

3.3. Dual phase method development

The chromatography of the phenyl and chirobiotic V columns was characterized with identical mobile phases (Sections 3.1 and 3.2) so that the two columns could be incorporated in-series with no additional pumps or switching mechanisms. Each column had a wide range of acceptable percent methanol compositions when operated individually, but, the only common range of isocratic

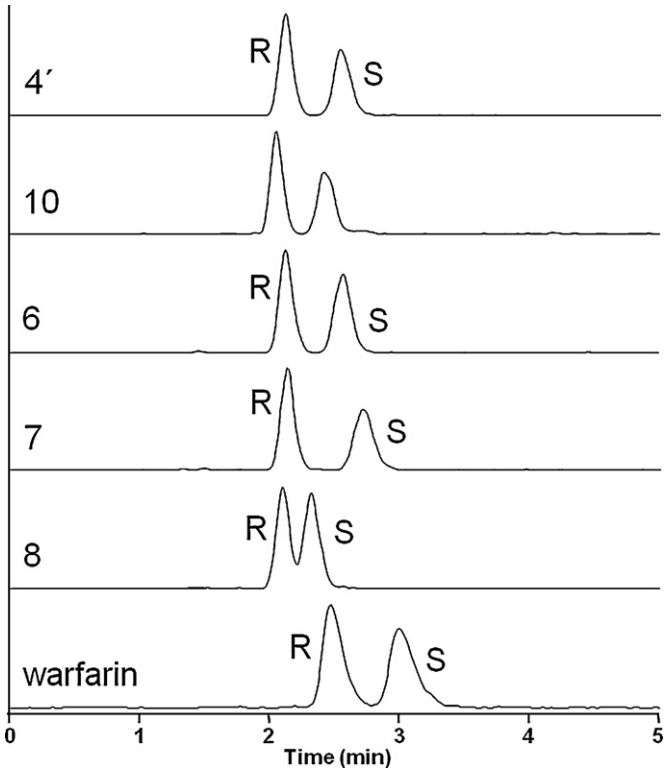

Fig. 3. Racemic standards (100 nM) of each hydroxywarfarin and warfarin were separated into their respective R and S enantiomers with the Chirobiotic V column. For each hydroxywarfarin, the R enantiomer eluted first followed by the S-enantiomer [7]. The method was isocratic with a flow rate of 300 μL/min and 45% methanol operating at room temperature (21.6–22.4 °C). Analytes were detected with the MS/MS experimental conditions as described in Section 2.2. Numbered chromatograms indicate sites of hydroxylation while R or S signifies stereochemistry e.g. 7 = 7-hydroxywarfarin. 10-Hydroxywarfarin contains a mixture of 4 isomers which are not fully resolved by the chiral column alone, but are separated with the dual phase method (Section 3.4).

operating conditions between them was approximately between 40 and 50% methanol. Even at high methanol compositions the phenyl column efficiently separated hydroxywarfarins. On the other hand, the chiral column required low percent methanol compositions to achieve chiral separation. At a composition of 45% methanol, each column achieved sufficient separation with acceptable run times when operated individually. The accompanying flow rate was 300 μL/min. The effect of temperature on separation was another critical factor for successful implementation for the multi-mode chromatography. The phenyl column performed optimally at 60 °C while the chiral column performed best at room temperature as opposed to elevated temperatures.

When the columns were operated in-series (Section 2.2), separation of the hydroxywarfarin mixture into individual enantiomers was achieved (Fig. 2, bottom). The retention time for each hydroxywarfarin enantiomer was approximately equal to the sum of the retention times during characterization of the two columns individually. Each hydroxywarfarin separated into pairs of R and S enantiomers in the same order of elution from the phenyl column (Fig. 2, bottom). The operating pressure with both columns in-series was ~10,000 psi, which was equal to the sum of the operating pressure of each column individually. However, R- and S-8-hydroxywarfarin did not achieve baseline separation under these conditions, but did show distinct peaks.

The most challenging issue in combining the two columns in-series was achieving enough separation on the first column (phenyl) to enable additional enantiomeric separation between the metabolites on the second column (chirobiotic V). Further, peak widths from the first column had to be narrow enough to enable loading onto the second column. Previous attempts at implementing this approach using traditional HPLC columns (≥3.5 μm particles) and HPLC systems were unsuccessful (data not shown). The in-series combination of a traditional HPLC column (C18) with a chiral column generated too much back-pressure at the required flow rates. Further, peaks from a traditional column were too broad to enable loading onto the chiral column. The recent development of UPLC and the highly selective chemistry of the UPLC phenyl column are key technological advancements enabling multi-mode chromatography.

On the first column of novel dual phase UPLC–MS/MS method, the hydroxywarfarin peak widths were approximately 24 s wide at the base and individual hydroxywarfarins were separated by more than 1 min (Fig. 2). This provided sufficient time for an additional chiral separation without interfering with neighboring peaks of the same SRM transition. Therefore, the success of multi-mode chromatography depended on highly efficient UPLC separation in the first phase. For the second phase, a traditional HPLC column was necessary because, to our knowledge, no chiral UPLC columns are available. However, if chiral UPLC columns become available, it may be possible to enhance separation of hydroxywarfarins, simplify the experimental set-up, and achieve shorter run times as long as the increase in pressure can be managed.

3.4. Identification of regio- and stereo-chemistry

To confirm the stereochemistry for analytes in the multi-mode method, we analyzed a mixture of warfarin metabolites obtained by reacting pooled human liver microsomes with R- and S-warfarin, which generated enantiospecific hydroxywarfarin metabolites (Section 2.4). The assignment of regio-chemistry was confirmed by injecting individual racemic hydroxywarfarin standards. The hydroxylated microsomal products of S-warfarin, matched the second peak in each pair of hydroxywarfarins as observed with commercially obtained standards (Fig. 5, middle). The hydroxywarfarin products obtained from reaction with R-warfarin matched the first peak in each pair of hydroxywarfarins (Fig. S2).

Historically, only R and S-10-hydroxywarfarin have been reported as possible metabolites [22]. However, there are four isomeric forms of 10-hydroxywarfarin, because hydroxylation at the 10th position introduces a second chiral center. This fact has previously received much less to no attention in the literature and commercially available 10-hydroxywarfarin is simply labeled (R/S) instead of including all four configurations. Moreover, the isomeric composition of commercial standards varied between vendors making it impossible to assign the stereochemistry for the chiral center at carbon 10 on 10-hydroxywarfarin (Fig. 1). The present multi-mode UPLC–MS/MS method resolves all four of the 10-hydroxywarfarin isomers and suggests that 10-hydroxywarfarin from Sigma–Aldrich contains an equal amount of all four isomers while 10-hydroxywarfarin from Toronto Research Chemicals contained only two of the isomers (Fig. S3). In the absence of standards, we were not able to identify which peaks represented R and S stereochemistry at position 10 and therefore labeled the individual pairs of R and S-10-hydroxywarfatin enantiomers as 10Ra, 10Rb and 10Sa, 10Sb, respectively.

The microsomal incubations with R and S-warfarin (Section 2.4) clearly demonstrate the formation of all four 10-hydroxywarfarin isomers by human liver microsomes (Fig. 4). Incubations with R-warfarin (Fig. S2) produced two product peaks with the 10-hydroxywarfarin specific SRM as observed at 7.06 and 7.74 min at a ratio of 1:10 (25 μM reaction) or 1:18 (500 μM reaction). The presence of two peaks confirms the addition of a second chiral center of 10-hydroxywarfarin (Fig. 4). Similarly, two 10-hydroxywarfarin

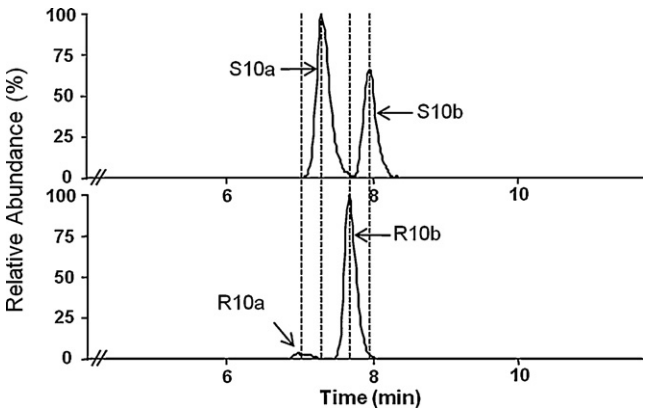

Fig. 4. All four isomers of 10-hydroxywarfarin were generated from reactions of pooled human liver microsomes with either R-warfarin (top) or S-warfarin (bottom) (Section 2.4). For each enantiomer of warfarin, two product peaks were observed, reflecting R or S stereochemistry about the 10th position. Due to the inability to assign stereochemistry, the sequential elution of these isomers are indicated by "a" or "b".

product peaks were observed for incubations with S-warfarin, eluting at 7.37 and 8.03 min at a 1.7:1 (25 μM reaction) or 1.6:1 (500 μM reaction) ratio. These two peaks further confirm the presence of a second chiral center on 10-hydroxywarfarin. For biomonitoring purposes we therefore assigned the 1st and 3rd peaks as 10-hydroxywarfarin metabolites derived from R-warfarin and the 2nd and 4th peaks as 10-hydroxywarfarin derived from S-warfarin. To our knowledge this is the first report of separation and quantitation of all four 10-hydroxywarfarin isomers.

3.5. Comparison of extraction methods, assay linearity, and limits of detection and quantification

The "metabolomics" plasma exaction method was found to be the most reproducible of the tested methods including extraction by ethyl acetate and solid phase extraction (Oasis SPE) and was therefore used for all plasma metabolite analyses in this study. This method extracted hydroxywarfarin and warfarin analytes in a linear fashion ($r^2 > 0.99$). In contrast, the ethyl acetate extraction and Oasis SPE methods varied in both efficiency and linearity (Table S1). Standard curves containing all hydroxywarfarins and deuterated internal standards were prepared in potassium phosphate (50 mM, pH 7.4) and analyzed in triplicate with the assay conditions described in Sections 2.2 and 2.3, thereby accounting for the extraction efficiencies of each analyte. Standards ranged from 0 nM to 1000 nM with the lowest calibrator being 3.1 nM. The method was also linear with r^2 values ≥0.97 (Fig. S1 and Table S1). The limit of detection (LOD) was approximately 10 femtomoles on column with a signal to noise of 10. This allows detection as low as 2 nM in plasma using a 5 μL injection of extracted plasma. The limit of quantification (LOQ) was defined as 5 times the LOD. Metabolite concentrations calculated to be below the limit of quantification were reported as LOQ.

3.6. Quantification of plasma profiles and analysis of inter-day variation

The study was then expanded to the analysis of plasma samples from patients receiving warfarin to demonstrate suitability of the method for *in vivo* biomonitoring. All samples were from males aged 61, 75, 76, and 80 and will be referred to as M61, M75, M75,

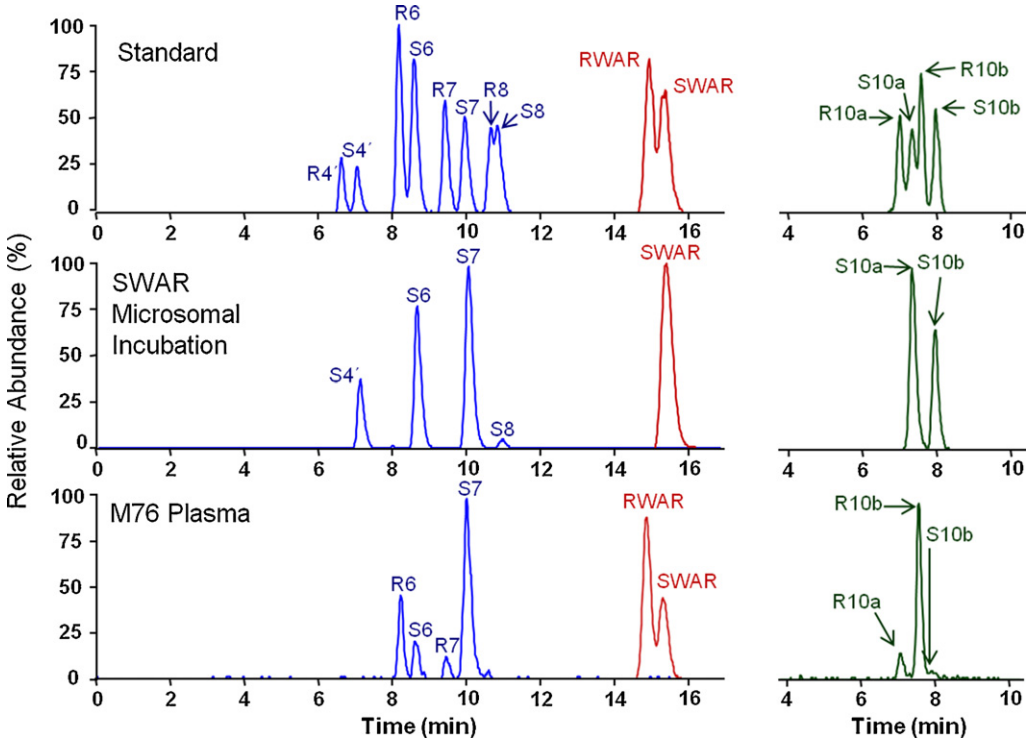

Fig. 5. Representative chromatograms of 100 nM racemic standards (top), enantiomerically pure S-warfarin and S-hydroxywarfarins obtained from a reaction (Section 2.4) with pooled human liver microsomes (middle) and human plasma from a patient receiving warfarin (bottom). Numbers indicate sites of hydroxylation while R or S signifies stereochemistry e.g. S7 = S-7-hydroxywarfarin. Color tracings represent unique SRMs; green represents 325.1/251.1 (10-hydroxywarfarin), blue represents 325.1/267.1 (4′, 6, 7, and 8-hydroxywarfarins) and red represents 309.1/163 (warfarin). For clarity, 10-hydroxywarfarin tracings are shown separately. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
Concentration of plasma hydroxywarfarins across three independent analyses.

Concentration (nM)														
	R4′	S4′	R10a	R10b	S10a	S10b	R6	S6	R7	S7	R8	S8	RWAR	SWAR
M61	LOQ	LOQ	55(5.6)	273(3.7)	LOQ	27(3.4)	57(1.6)	44(4.7)	37(2.2)	452(3.2)	–	–	3865(2.3)	1722(4.1)
M75	–	LOQ	52(2.4)	297(4.7)	LOQ	26(0.2)	98(1.9)	47(2.3)	50(3.5)	343(3.6)	–	–	4833(1.80)	2705(6.2)
M76	LOQ	LOQ	36(1.4)	(2.8)	–	16(3.5)	92(4.9)	51(1.9)	36(4.2)	545(0.8)	–	–	4811(2.3)	3460(2.7)
M80	–	–	13(5.0)	82(4.5)	–	13(4.0)	31(4.7)	13(3.6)	9(6.0)	188(4.1)	–	–	1437(2.7)	689(2.8)

( ) = coefficient of variation [%]. LOQ = lower than limit of quantitation. – = lower than limit of detection. M61, M75, M76, and M80 represent sample from male patients aged 61, 75, 76, and 80 years respectively.

and M80, respectively. Plasma samples were extracted as described in Section 2.3, and analyzed independently on three separate days. Fig. 5 (bottom) shows a representative plasma chromatogram and Table 1 shows the metabolite profiles for the four plasma samples. The coefficient of variation for analytes ranged from 0.2 to 6.2% (Table 1) representing the inter-day variation of the method. The ratio of R to S-warfarin ranged from 1.6 to 2.4 among the plasma samples.

For these patients, the major observed metabolites were R-10-hydroxywarfarins (R10a and R10b) and S-7-hydroxywarfarin indicating the importance of CYP3A [9,22] and CYP2C9 [5], respectively, in warfarin metabolism. Three of the 10-hydroxywarfarin isomers were clearly shown to be present in human plasma (Fig. 5, bottom and Table 1), while one isomer (S10a) was detected but below the limit of quantitation. Together with the microsomal data there is strong evidence for the formation of all four 10-hydroxywarfarin isomers in humans. Future studies will be needed to determine the biological significance of the individual 10-hydroxywarfarin isomers. The total plasma concentration of 10-hydroxywarfarin derived from both R and S-warfarin was higher than S-7-hydroxywarfarin concentrations in M61 and M75. For patients M76 and M80, S-7-hydroxywarafrin was the most abundant metabolite. In addition, R-7-hydroxywarfarin was observed in all four plasma samples, while 4′-hydroxywarfarin was observed in some samples, but was below the limit of quantitation. In these samples, 8-hydroxywarfarin was below the limit of detection.

A comparison between our observations with those by others revealed striking similarities and differences. There was a common agreement on the major metabolites but not the minor ones. In a previous study, only the racemic forms of hydroxywarfarins were quantified among 204 patients undergoing warfarin therapy [6]. Racemic 7- and 10-hydroxywarfarin were major plasma metabolites. In contrast to our results, the authors reported measurable, but variable levels of racemic 8-hydroxywarfarin and the absence of any racemic 4′-hydroxywarfarin among all patient samples. The presence of 8-hydroxywarfarin in the previous study was highly variable and quantitation was possible in only 3% of the patient samples, suggesting that 8-hydroxywarfarin is not a ubiquitous metabolite. The novel multi-mode method allows detection of 4′-hydroxywarfarin in some samples, which was not observed in the study by Locatelli [6]. This is most likely due to the lower detection limit (10 fmol on column) of the new multi-mode method compared to the previous method (3.9 pmols on column). In addition, the previous authors utilized concentrated samples and injected approximately 50 μL with their method, utilizing approximately 40-fold more plasma than the method described here. Alternatively, the apparent variability or absence of hydroxywarfarins may be explained by their subsequent conversion into glucuronides [22]. The resulting conjugated forms of the metabolites are found in patient urine [7], but were not targeted in this study due to the unavailability of standards. However, our data clearly demonstrate that this multi-mode UPLC–MS/MS method is suitable for enantiomeric profiling of warfarin metabolites in human plasma. Analyses of larger study populations are needed to determine the fraction of subjects that form detectable amounts of 8- and 4′-hydroxywarfarins to determine their clinical relevance.

4. Conclusions

A dual phase UPLC method was developed and validated for profiling of specific regio- and enantio-specific hydroxywarfarins and warfarin. The method provides excellent chromatographic separation of warfarin and hydroxywarfarins in 17 min. The analysis of patient samples demonstrated the potential of the method to accurately quantify warfarin and its metabolites present in human plasma with high sensitivity. The presented method marks a significant advancement in the profiling of chiral warfarin and its hydroxylated metabolites. Prior studies have been limited to analyzing either warfarin enantiomers [23] or racemic forms of hydroxywarfarin metabolites [24,25]. Through the novel dual phase UPLC method, it is now possible to effectively assess the widest array of warfarin metabolites for identifying and validating potential biomarkers to metabolic pathways and surrogate markers corresponding to patient responses to warfarin therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2011.03.022.

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