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Chiral phase analysis of warfarin enantiomers in patient plasma in relation to CYP2C9 genotype

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Abstract

A direct chiral-phase high-performance liquid chromatographic method for measuring the ratio of *S*-warfarin/*R*-warfarin in patient plasma is described. Plasma samples are first extracted using solid-phase C_{18} extraction columns, and the concentrated extracts analyzed using an (*R*,*R*) Whelk-O 1 column with a mobile phase of 0.5% glacial acetic acid in acetonitrile. The resulting chromatography provides baseline resolution of the warfarin enantiomers and internal standard (racemic ethylwarfarin), and is free from interference from other plasma components. Calibration curves were linear (mean r^2 of 0.999 for both enantiomers) over the concentration range 0.25–1.5 μg/ml. The intra-day and inter-day coefficients of variation for analysis of plasma spiked with 0.33 μg/ml *S*-warfarin and 0.67 μg/ml *R*-warfarin (*S*/*R*=0.5:1) was less than 7% for each enantiomer, with an accuracy of more than 93%. Plasma extracts from thirty-one patients homozygous for wild-type CYP2C9*1 provided an *S*/*R* ratio of 0.51±0.15. Two warfarin patients homozygous for the mutant CYP2C9*2 and CYP2C9*3 alleles exhibited elevated *S*/*R* ratios relative to the mean for individuals homozygous for the wild-type CYP2C9*1 allele. This method is suitable for population studies aimed at establishing the effect of polymorphic expression of CYP2C9 alleles on *S*-warfarin elimination in humans.

Keywords: Enantiomer separation; Warfarin

1. Introduction

Warfarin is a widely used oral anticoagulant agent used to treat thromboembolic disease [1,2]. Although warfarin is administered as a racemate, it is the *S*-enantiomer that is chiefly responsible for the anticoagulant effect [3]. The pharmacological activity of *S*-warfarin is terminated by oxidative metabolism catalyzed by the liver enzyme, CYP2C9 [4]. Mutations in the CYP2C9 gene result in expression of wild-type protein, CYP2C9*1, and two allelic variants, CYP2C9*2 and CYP2C9*3 [5–7], which possess altered catalytic properties relative to the wild-type enzyme [7–9]. We reported previously that an individual who was homozygous for CYP2C9*3, and required only 0.5 mg racemic warfarin/day (~1/10th of the average daily dose) to maintain an adequate anticoagulation response, exhibited a plasma warfarin *S*/*R* ratio of 4:1 [10]. This ratio differed greatly from that observed in the plasma of control patients where the *R*-enantiomer predominates at steady state, and so we interpreted these findings as evidence for a pharmacogenetically determined deficiency in the clearance of *S*-warfarin. Historically,

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chiral analysis has played an important role in the area of P450 pharmacogenetics, and indeed discovery of the CYP2C19 polymorphism was dependent on the measurement of urinary ratios of *S*- and *R*-mephenytoin [11]. Conceivably, the plasma warfarin *S*/*R* ratio might serve as a useful phenotypic test for CYP2C9 polymorphism.

In order to fully evaluate the relationship between CYP2C9 genotype and plasma warfarin enantiomer ratio, it will be necessary to examine a large population of patients because no more than 20% of a Caucasian population express either mutant allele [6,7]. Previous methodologies for extracting warfarin from plasma and analyzing the enantiomers by HPLC generally involved multiple liquid–liquid extraction steps followed by derivatization with carbobenzyloxy-L-proline to form diastereoisomers, which were then separated by silica HPLC [12]. In our earlier study [10], we analyzed the warfarin enantiomers by normal-phase chiral HPLC, but we required a preliminary silica HPLC separation to remove interfering contaminants present in the organic plasma extract. Reported here is a method by which solid-phase extracts of plasma warfarin are analyzed directly by reversed-phase chiral HPLC. This provides a rapid and robust assay, free from interference from plasma constituents, which will expedite determination of the *S*/*R*-enantiomer ratio in large patient populations.

2. Experimental

2.1. Materials

Racemic warfarin was resolved using the method previously described by West et al. [13]. Both *S*- and *R*-warfarin standards generated by this method were shown to be >99% optically pure by chiral-phase HPLC analysis (described below). Racemic ethylwarfarin was obtained from Dr. W.F. Trager (University of Washington, Seattle, WA, USA). Solid-phase extractions were performed on plasma samples using Bakerbond Spe C_{18} columns and a Baker-10 extraction system from J.T. Baker (Phillipsburg, NJ, USA). Acetonitrile used for HPLC analysis was Fisher Optima grade (Fisher Scientific, Pittsburgh, PA, USA), and glacial acetic acid was from J.T. Baker.

2.2. Instrumentation

The HPLC system consisted of a Hewlett-Packard 1050 series instrument, equipped with a 1050 model multi-channel pump, autosampler, and variable wavelength UV detector set at 313 nm. Analyte peak area ratios were recorded with a Hewlett-Packard 3396 Series II integrator. Separation of the warfarin enantiomers was performed on a 250×4.6 mm Pirkle covalent (*R*,*R*) Whelk-O 1 chiral column (Regis Technologies, Morton Grove, IL, USA) [14]. The flow-rate of the mobile phase, which consisted of a mixture of acetonitrile–0.5% glacial acetic acid (40:60, v/v), was set at 1 ml/min. Under these conditions, *S*- and *R*-warfarin eluted at 13.6 and 15.8 min, respectively.

2.3. Standard preparation

2.3.1. Warfarin

For generation of standard curves, a racemic warfarin stock solution (1 mg/ml) was prepared by dissolving solid warfarin in water, which was then basified with a small volume (~100 μl) of 1 *M* sodium hydroxide to ensure complete dissolution. A working solution was prepared by diluting the stock solution to 100 μg/ml in water, and this was used to spike human plasma. Final plasma concentrations of racemic warfarin were 0.5, 1.0, 2.0 and 3.0 μg/ml. Stock solutions of *S*-warfarin (1 mg/ml) and *R*-warfarin (2 mg/ml) were also prepared as above. Aliquots of each were mixed together and diluted to provide a stock solution, 1 mg/ml, containing *S*-warfarin and *R*-warfarin in the ratio of 0.5:1, which approximates that expected in human plasma at steady state. This stock solution was diluted to 100 μg/ml and used to spike human plasma (1.0 ml) to final concentrations of 0.333 μg/ml *S*-warfarin and 0.667 μg/ml *R*-warfarin for estimation of inter-day and intra-day assay variability.

2.3.2. Internal standard

An aqueous working solution of racemic ethylwarfarin (100 μg/ml) prepared as above was used as

internal standard (I.S.) to spike human plasma to a final concentration of 1.0 μg/ml.

2.4. Sample preparation for HPLC analysis

Human plasma samples were obtained from patients receiving warfarin therapy for knee and hip replacement surgery at St. Luke's Hospital of Kansas City, MO, USA. Blood was drawn 5–6 days after the initiation of warfarin treatment. For each sample, a 0.3-ml aliquot of plasma was placed in a 10×75 mm disposable glass culture tube to which 2 ml of 1 *M* hydrochloric acid was added. Acidified dilute plasma was then applied to a C_{18} solid-phase extraction column which had been previously primed with 2 ml of methanol and 2 ml of 1 *M* hydrochloric acid. The column was washed with 2 ml of 1 *M* hydrochloric acid prior to elution of warfarin with 2 ml of methanol. The methanolic eluate was concentrated to ~25 μl under a stream of dry N_2 and then diluted with 85 μl of mobile phase. The sample was then transferred to a 0.6 ml microcentrifuge tube and centrifuged at 16 000 *g* for 15 min to precipitate any protein. The supernatant was then placed in a 500 μl tapered HPLC sample vial, and 100 μl injected on column.

2.5. CYP2C9 genotype

DNA was extracted, by standard procedures, from whole blood obtained from the warfarin patients described above. CYP2C9 genotype at codon 144, Arg→Cys (CYP2C9*2), and codon 359, Ile→Leu (CYP2C9*3) was determined using PCR primers and conditions described by Stubbins et al. [6] and Sullivan-Klose et al. [7], respectively.

3. Results and discussion

3.1. Chromatographic separation

Solid-phase extraction of blank plasma provided a chiral-phase chromatography profile free from interfering peaks in the region between 12 and 15 min where the warfarin enantiomers eluted with baseline resolution (Fig. 1A and B). Comparison with authentic resolved standards demonstrated that *S*-warfarin

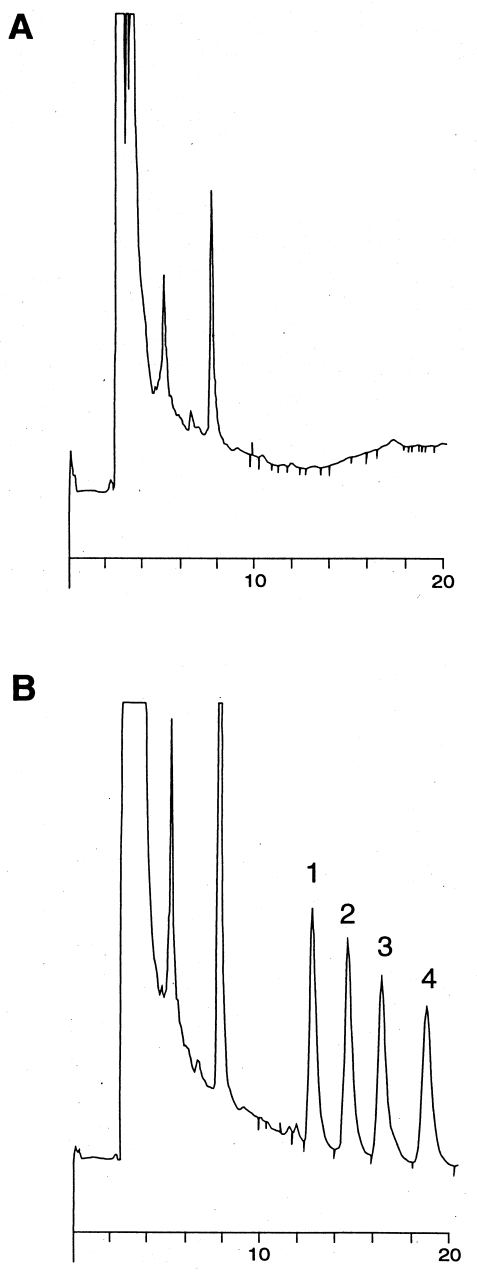

Fig. 1. (A) Chiral analysis of blank plasma after solid-phase extraction; (B) chiral analysis of plasma spiked with 1 μg/ml racemic warfarin and 1 μg/ml racemic ethylwarfarin. Peaks 1 and 2 correspond to *S*- and *R*-warfarin and peaks 3 and 4 correspond to *S*- and *R*-ethylwarfarin, respectively.

Table 1
Accuracy and coefficients of variability for chiral-phase analysis of the warfarin enantiomeric ratio in plasma ($n=8$)

Sample	S/R±S.D.	Accuracy — deviation from nominal[a] (%)	Coefficient of variation (%)	
			Intra-day	Inter-day
Day 1	0.503±0.039	0.6	7.75	
Day 2	0.514±0.020	2.8	3.89	1.53
Day 3	0.500±0.016	0.0	3.20	

[a] Direct chiral-phase analysis of the stock solution in triplicate gave an S/R value of 0.500±0.002.

eluted prior to *R*-warfarin. For our analytical purposes, only the plasma S/R warfarin ratio was needed from each sample, the presence of either enantiomer being sufficient proof of patient compliance. However, where absolute quantitation is required, racemic ethylwarfarin can be used as an I.S., since both enantiomers are resolved under these chromatographic conditions and elute immediately after *R*-warfarin (see Fig. 1).

3.2. Extraction efficiency

The extraction efficiency of racemic warfarin was calculated using human plasma samples spiked to 1 μg/ml of racemic warfarin. Eight extractions were performed as described in Section 2.4, each on a 0.3 ml aliquot of plasma containing 0.3 μg of racemic warfarin. HPLC peak areas that resulted from the extracted samples were compared to peak areas obtained from multiple, direct injections of 0.3 μg standard. The average percentage recovery calculated for *S*-warfarin ($n=8$) was 81.8±6.8%, and the average value for *R*-warfarin ($n=8$) was 79.9 ±6.4%. The same procedure carried out with ethylwarfarin revealed similar recoveries for both enantiomers of the I.S.

Previous methods of recovering warfarin from plasma report extraction efficiencies ranging from 70% to >90% [15–18]. These methods, however, utilize either one or two discrete liquid–liquid extraction steps and are more laborious and time consuming than solid-phase extraction, which has a similar extraction recovery.

3.3. Assay validation

Determination of the variability associated with the measurement of plasma warfarin S/R ratios was assessed by spiking blank plasma to a concentration of 1 μg/ml with a standard solution of 0.5:1 *S*-/*R*-warfarin. Multiple extractions ($n=8$) were then performed on 3 different days, and the results summarized in Table 1. The S/R warfarin ratio, determined directly from the relative detector responses for the two enantiomers, exhibited an intra-day coefficient of variability of less than 7% and an inter-day coefficient of variability less than 2%.

Standard calibration curves were established for the purpose of quantifying both warfarin enantiomers independently. Calibration curves were linear over a concentration range of 0.25–1.5 μg/ml for each enantiomer (0.5–3.0 μg/ml total warfarin). Accuracy, intra-day, and inter-day variability for quantitation of the individual enantiomers are summarized in Tables 2 and 3.

Warfarin would be expected to be administered most commonly with other drug classes used to treat cardiovascular disease, including antiarryhthmics,

Table 2
Accuracy and variability of quantitation of *S*-warfarin in plasma ($n=8$)

Sample	Nominal [*S*-warfarin] (μg/ml)	Calculated [*S*-warfarin] (μg/ml±S.D.)	Accuracy — deviation from nominal (%)	Coefficient of variation (%)	
				Intra-day	Inter-day
Day 1	0.333	0.354±0.022	6.31	6.21	
Day 2	0.333	0.346±0.024	3.90	6.94	1.76
Day 3	0.333	0.342±0.022	2.70	6.43	

Table 3
Accuracy and variability of quantitation of *R*-warfarin in plasma ($n=8$)

Sample	Nominal [*R*-warfarin] (μg/ml)	Calculated [*R*-warfarin] (μg/ml±S.D.)	Accuracy — deviation from nominal (%)	Coefficient of variation (%)	
				Intra-day	Inter-day
Day 1	0.667	0.705±0.040	5.70	5.67	
Day 2	0.667	0.681±0.044	2.10	6.46	1.85
Day 3	0.667	0.701±0.039	5.10	5.56	

ACE inhibitors and diuretics. However, neither amiodarone, lisinopril, isosorbide dinitrate, aspirin, nor furosemide interfered with the assay. Since the chiral selector binds the archetypal CYP2C9 substrate warfarin, it might be expected to retain other drugs commonly recognized as ligands for this enzyme, notably phenytoin, tolbutamide, diclofenac and ibuprofen [19]. However, none of these compounds interfered with enantiomer quantitation.

3.4. Biomedical application of method

DNA isolated from patient blood was genotyped for the CYP2C9 variant alleles and 31 individuals homozygous for CYP2C9*1 identified. Warfarin plasma samples from these individuals were analyzed to determine the ratio of *S*/*R* warfarin in a 'control' population. Values ranged from 0.21:1 to 0.85:1 with a mean value(±S.D.) of 0.51(±0.15):1. A representative chromatogram for a CYP2C9*1 homozygote (*S*/*R*=0.56:1) is shown in Fig. 2A. Plasma from two additional individuals who were homozygous for the CYP2C9*2 and CYP2C9*3 alleles were also analyzed, and the respective chromatograms are shown in Fig. 2B and C. The *S*/*R* ratios were 0.87:1 and 4:1, respectively, for plasma samples obtained from the CYP2C9*2 and CYP2C9*3 homozygotes.

4. Conclusion

Solid-phase extraction of warfarin enantiomers from plasma followed by resolution using reversed-phase chiral HPLC is a rapid and reliable method to determine warfarin *S*/*R* ratios in individual patients. The (*R*,*R*) Whelk-O1 chiral column readily separates the two enantiomers under reversed-phase condi-

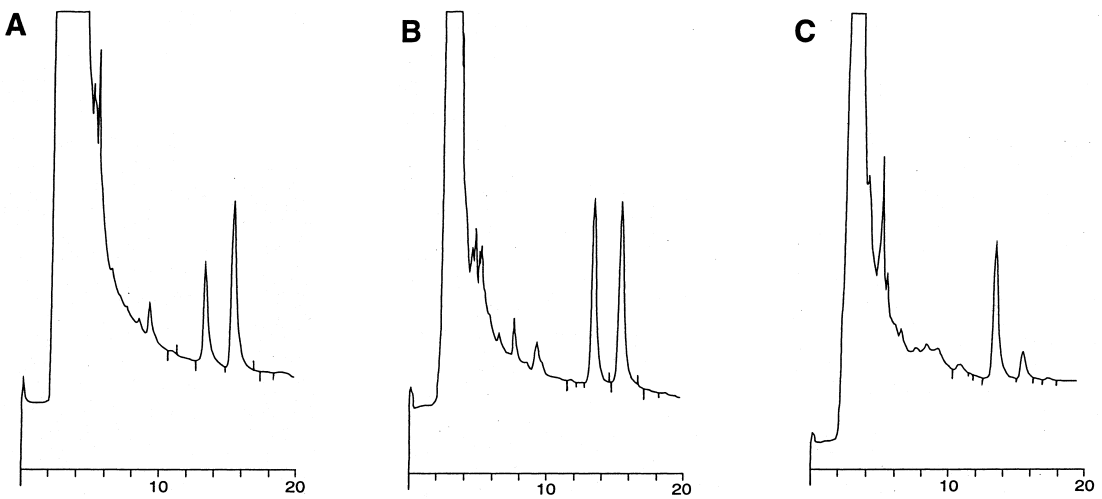

Fig. 2. (A) *S*-/*R*-warfarin ratio (0.56:1) in plasma obtained from a CYP2C9*1 homozygote; (B) *S*-/*R*-warfarin ratio in plasma (0.87:1) obtained from a CYP2C9*2 homozygote; (C) *S*-/*R*-warfarin ratio (4:1) in plasma obtained from a CYP2C9*3 homozygote. *S*-Warfarin and *R*-warfarin elute at 13.6 min and 15.8 min, respectively.

tions, making it unnecessary to derivatize with carbobenzyloxy-L-proline. The preliminary phenotype–genotype data suggest that expression of the CYP2C9*2 and CYP2C9*3 alleles may be associated with a differential effect on the clearance of *S*-warfarin. However, a much larger population study (now ongoing) involving both heterozygotes and additional homozygotes is required to fully evaluate the association between the expression of mutant CYP2C9 alleles and the plasma warfarin *S*/*R* ratio.

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