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Determination of warfarin enantiomers and hydroxylated metabolites in human blood plasma by liquid chromatography with achiral and chiral separation

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Abstract

An assay comprising two simple, selective and isocratic HPLC methods with UV detection was developed and validated for measuring warfarin enantiomers and all five warfarin monohydroxylated metabolites in patient blood plasma. Following liquid/liquid extraction from 1 ml of blood plasma a baseline separation of analytes was achieved on chiral (α_1 acid glycoprotein – AGP) and achiral (C_{18}) column. Both methods were consistent (R.S.D. < 6.9% for warfarin enantiomers and < 8.9% for monohydroxylated metabolites) and linear ($r > 0.998$). The limits of detection were 25 ng/ml for warfarin enantiomers, 25 ng/ml for 4′-, 10-, 6- and 7-hydroxywarfarin, 35 ng/ml for 8-hydroxywarfarin and 50 ng/ml for racemic warfarin. In a clinical study in 204 patients, it was confirmed that the assay is appropriate for evaluation of influences of genetic polymorphisms, demographic factors and concomitant drug treatment on warfarin metabolism.

Keywords: Warfarin enantiomers; Warfarin monohydroxylated metabolites; Human plasma

1. Introduction

Warfarin is a coumarin derivative used as an oral anticoagulant drug in the treatment and prevention of thromboembolism. In addition to narrow therapeutic interval and significant interindividual variability in daily dose requirement, numerous drug–drug interactions often complicate treatment and over- or under-anticoagulation frequently occur [1,2]. Careful monitoring of coagulation by measuring international normalized ratio of prothrombin time (INR) is necessary to tailor the treatment to individual patient, mainly due to complex pharmacokinetics. The asymmetric carbon at position 9 of warfarin gives rise to two enantiomers, *R*- and *S*-warfarin, with distinct pharmacological properties (Fig. 1). Following oral administration of a warfarin racemic mixture concentrations of enantiomers in blood plasma differ because of stereoselective metabolism. The plasma clearance of two to five times more potent *S*-warfarin is approximately twice that of *R*-warfarin [3–5]. The predominant metabolic reaction is hydroxylation at positions 6–8, 10 and 4′ by cytochrome P450 (CYP) leading to a series of inactive monohydroxylated metabolites. Another important route of warfarin metabolic deactivation is reduction of the side chain keto group by carbonyl reductases [6]. Warfarin metabolism is further characterized by a process termed regioselective metabolism and enantiomers considerably differ in sites of hydroxylation and CYP enzymes involved. The more potent *S*-warfarin undergoes substantial 7-hydroxylation although 6- and 4′-hydroxywarfarin are also formed. On the other hand *R*-warfarin is metabolized to all five hydroxywarfarin metabolites, of which 6- and 10-hydroxywarfarin are the most abundant [6]. The reduction of side chain keto group is prevalent in *R*-warfarin. It leads to generation of two inactive diastereomeric alcohols; major (*R*,*S*)-(+)-alcohol and minor (*R*,*R*)-(+)-alcohol [7]. Moreover, warfarin metabolism

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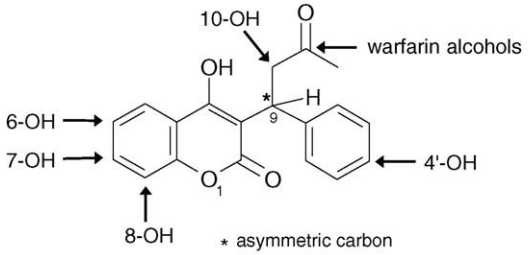

Fig. 1. Chemical formula of warfarin, warfarin alcohol and 4′-, 6-, 7-, 8- and 10-hydroxywarfarin.

depends on presence of specific polymorphisms in the CYP system. Two common single nucleotide polymorphisms in the *CYP2C9* gene are associated with impaired oxidative metabolism of *S*-warfarin. Individual dosing based on pharmacogenetics has the potential to decrease the risk of adverse events. To implement pharmacogenetics in the development of dosing algorithm, studies that would assess contribution of genetic, clinical and demographic factors to variation in metabolism and dose requirement are necessary [8]. This and the fact that anticoagulation with warfarin is increasingly indicated are the reasons for continuing interest in improved methodology for the determination of warfarin enantiomers and metabolites in biological matrices.

Several assays on warfarin enantiomers [5,9–16] and its metabolites [5,11,17–20] have been developed. Mostly they include high-performance liquid chromatography (HPLC) using either ultraviolet or fluorescence detection, although methods where HPLC is coupled with mass spectrometry have also been reported [9,18,19]. Lately chiral beta-cyclodextrin columns have become a powerful tool for separation of warfarin enantiomers [9,10,12]. Furthermore, utilizing cellulose chiral column Takahashi et al. [11] achieved separation of enantiomers of some warfarin metabolites in order to examine stereo- and regioselectivity of CYP mediated metabolism. However, our aim was to develop a method for assaying all five warfarin hydroxymetabolites. Using the method by Zhang et al. [19] separation of four hydroxy-metabolites could be achieved on reversed-phase C_{18} column. Recently, Ufer et al. [18] published alternative method for determination of racemic warfarin and all five monohydroxylated metabolites in plasma utilizing solid-phase extraction. However, both of these methods used mass spectrometry detection and gradient elution and were thus too complex for our application. Moreover, neither of the two methods involved separation of warfarin enantiomers. Utilizing our much simpler method all five warfarin hydroxymetabolites and racemic warfarin can be separated on achiral reversed-phase column coupled with UV diode array detector. Additionally, chiral α_1 acid glycoprotein (AGP) column was selected for separation of warfarin enantiomers due to its ability for coupling with reversed-phase C_{18} column producing achiral/chiral HPLC system [14].

In this paper two separate methods for quantification of warfarin enantiomers and hydroxylated metabolites in patient blood plasma using HPLC with UV detection are presented. The assay was successfully applied in a study of influence of *CYP2C9* polymorphism, demographic factors and concomitant drug therapy on warfarin metabolism and maintenance dose in a group of 204 patients [21].

2. Experimental

2.1. Chemicals

Racemic warfarin sodium and naproxen (I.S.) were supplied from Sigma-Aldrich (Steinheim, Germany). Five warfarin metabolites (6-, 7-, 8-, 10- and 4′-(*R*/*S*)-hydroxywarfarin) and both warfarin enantiomers were purchased from Ultrafine Chemicals (Manchester, UK). Analysis grade diethyl ether, sulfuric acid, hydrochloric acid, potassium dihydrogen phosphate and disodium hydrogen phosphate decahydrate were obtained from Merck (Darmstadt, Germany). HPLC-grade acetonitrile and methanol were from Riedel-de Haën (Seelze, Germany).

2.2. Instrumentation

The Agilent 1100 Series HPLC system (Agilent Technologies) equipped with 1100 model vacuum degasser, binary pump, autosampler, thermostat and UV photodiode-array detector was applied to both of the assays. The system was controlled by Hewlett-Packard 3D ChemStation software. During the analyses the autosampler temperature was kept at 5 °C.

The ODS Hypersil analytical column (5 μm, 100 mm × 4.6 mm i.d., Thermo Hypersil-Keystone, Chesire, UK) coupled with UNIPHASE^{TM} C_{18} guard column (5 μm, 10 mm × 4.6 mm i.d., Thermo Hypersil-Keystone) and with C_{18} column pre-filter (4 mm × 3.0 mm i.d., Phenomenex, Chesire, UK) was used for achiral separation of warfarin and its five metabolites. The column temperature was maintained at 25 °C. The mobile phase consisted of a phosphate buffer (15 mmol/l of KH_2PO_4 and pH adjusted to 3.0 with 1 M HCl), methanol and acetonitrile in the proportion of 52:32:16 (v/v/v). The flow rate was set at 1.2 ml/min. Analytes were detected at different UV wavelengths. For racemic warfarin, 10- and 4′-hydroxywarfarin wavelength was set at 306 nm. Naproxen and 6-hydroxywarfarin were detected at 330 nm. Wavelengths for 7- and 8-hydroxywarfarin detection were 312 and 299 nm, respectively.

For chiral separation of warfarin enantiomers the Chiral-AGP analytical column (5 μm, 100 mm × 3.0 mm i.d., Chromtech, Congleton, UK) in conjunction with Chiral-AGP guard column (5 μm, 10 mm × 3.0 mm i.d.) was used. The column temperature was maintained at 27.5 °C. The flow rate of the mobile phase consisting of a mixture of acetonitrile and phosphate buffer (50 mmol/l, pH 7.0) in the proportion of 15:85 (v/v), was set at 0.9 ml/min. Warfarin enantiomers were detected at 310 nm.

2.3. Study design

In total 204 outpatients on long-term warfarin therapy for more than 6 months with stable INR between 2.0 and 3.5 during the last two visits were enrolled in the study. Warfarin maintenance doses ranged from 0.75 to 20 mg per day. A clinical summary was obtained from records of follow-up visits and a questionnaire. Patients were followed at the University Medical Centre in Ljubljana, Department for Vascular Diseases ($n = 160$) and at the General Hospital Murska Sobota ($n = 44$), Slovenia. An informed consent was obtained from the patients. The study was approved by the Slovenian Ethics Committee for Research in Medicine (no. 60/02/02).

Blood samples were drawn at 12–16 h postdose. Citrated blood samples were centrifuged and blood plasma samples were stored at −20 °C until analyzed.

2.4. Preparation of standard solutions

The stock standard solutions of racemic warfarin (10 μg/ml), five hydroxylated metabolites (10 μg/ml) and internal standard (100 μg/ml) were prepared by dissolving appropriate amounts of the compounds in phosphate buffer solution (pH 7.4). Stock standard solutions were stored in dark at 4 °C. A series of six standard plasma solutions of mixture of racemic warfarin and its five hydroxy metabolites were prepared by adding the appropriate volumes of the stock standard solutions to drug-free human plasma. Standard plasma solutions contained 75, 100, 200, 300, 400 and 600 ng/ml of 4′-, 6- and 10-hydroxywarfarin; 100, 150, 200, 300, 400 and 600 ng/ml of 8-hydroxywarfarin; 75, 100, 300, 500, 750 and 1000 ng/ml of 7-hydroxywarfarin and 150, 200, 500, 1000, 1500 and 5000 ng/ml of racemic warfarin. Additionally, three levels of quality control (QC) samples; 75, 300 and 600 ng/ml for 4′-, 6- and 10-hydroxywarfarin; 100, 300 and 600 ng/ml for 8-hydroxywarfarin; 75, 500 and 1000 ng/ml for 7-hydroxywarfarin; and 150, 1000 and 5000 for racemic warfarin were prepared. Plasma standards and QC samples were aliquoted, stored and treated the same way as patient blood plasma samples. For recovery estimation of the six analytes standard water solutions were prepared by diluting the standard stock solutions in bidistilled water to serve as 100% control.

2.5. Extraction procedure

One millilitre of thawed human plasma sample was mixed with 50 μl of I.S. stock solution and 0.7 ml of 1M sulfuric acid. Consequently 5 ml of diethyl ether was added, vortex-mixed (5 s) then shaken for 60 min at 250 cycles per min in vertical position using Vibromix 403 EVT shaker (Tehtnica, Slovenia) and centrifuged at 600 × *g* for 10 min. The samples were left in a freezer at −20 °C for about 3 h and the diethyl ether layer was decanted from a frozen aqueous layer into a clean tube and evaporated to dryness in a water bath at 45 °C. Samples were reconstituted in 250 μl mixture of acetonitrile in water (25:75, v/v) and two 50 μl aliquots were injected in both methods.

2.6. Assay validation

Selectivity, linearity, linear range, accuracy, precision, recovery, limit of detection (LOD) and quantification (LOQ) were determined according to ICH Q2B [22] and FDA guidance on bioanalytical method validation [23]. To establish specificity of the assay matrix effects were investigated using six independent sources of citrated human blood plasma.

2.6.1. Achiral method

Resolution factors of the adjacent analytes were calculated in order to estimate method selectivity. For each analyte six standard plasma solutions were used for construction of calibration lines. A nonweighted linear regression was applied to calculate slopes and intercepts of the calibration lines constructed as peak area ratios of analytes to I.S. versus the analyte concentration. QC samples of low, medium and high concentrations were injected onto the column in three consecutive days in order to determine accuracy, inter-day repeatability and recovery of each analyte. For intra-day repeatability calculation six determinations of medium concentrations were used [22]. The accuracy was determined as percent ratio of the analyte concentration calculated from the calibration line versus nominal analyte concentration. Extraction recovery was calculated as percent ratio of analyte peak area of extracted plasma QC sample versus analyte peak area of unextracted standard water solution representing 100% recovery. LOD and LOQ were estimated using average slope estimation and the standard deviation of *y*-intercepts of the calibration lines [22].

2.6.2. Chiral method

The same plasma standards were used in validation of the chiral method. Selectivity, linearity, LOD and LOQ were estimated for *R*- and *S*-warfarin. Repeatability and accuracy were calculated using the nonweighted linear regression calibration lines of enantiomers' peak areas versus the analytes concentration. The concentration of the enantiomers was set to the half of the racemic warfarin concentration in standard plasma solutions.

Plasma *R*- and *S*-warfarin concentrations were calculated from the ratio of *R*- to *S*-warfarin response and the corresponding racemic warfarin concentration previously estimated by the achiral method.

2.6.3. Stability

Stability of the analytes was tested according to the FDA guidance on bioanalytical method validation [23]. Freeze and thaw stability was studied by subjecting QC samples at three concentration levels to three freeze-thaw cycles. During each cycle samples were refrozen at −20 °C for 24 h. For demonstration of short-term temperature stability and long-term storage stability, QC samples were kept at ambient tempera-

ture for 6 h and at −20 °C for 15 months, respectively. Post-preparative stability was assessed by keeping QC samples in autosampler at 5 °C for 24 h. Stability of the stock solution of internal standard was validated over a storing period of 72 h in dark at 4 °C.

3. Results and discussion

The aim of this study was to develop a simple and rapid assay for quantitative determination of warfarin enantiomers and all five monohydroxylated metabolites in blood plasma of patients on long-term warfarin therapy in order to study the influence of *CYP2C9* polymorphism, demographic factors and concomitant drug therapy on warfarin metabolism and maintenance dose.

3.1. Assay development

Reversed-phase column was used for separation of racemic warfarin and monohydroxylated metabolites as in most of the previously reported assays [17–19,24]. Zhang et al. [19] and Ufer et al. [18] determined four and five monohydroxylated metabolites, respectively, but both assays used gradient elution and were coupled with mass spectrometer. On the other hand we aimed to develop a simpler method suitable for routine use at the hospital based on short isocratic separation of warfarin and all five monohydroxylated metabolites and UV detection.

With the optimized mobile phase composition baseline separation of all the analytes was achieved. As shown in Fig. 2 analytes eluted in the following order; 4′-hydroxywarfarin (4.8 min), 10-hydroxywarfarin (5.8 min), 6-hydroxywarfarin (6.9 min), 7-hydroxywarfarin (9.3 min), 8-hydroxywarfarin (10.2 min), I.S. (11.1 min) and racemic warfarin (16.0 min). The peaks were identified on the basis of retention times and UV spectra of the individual standard stock solutions. Resolution factors were higher than 1.5 with the exception of 7- and 8-hydroxywarfarin and I.S. (R_S = 1.2). However, due to detection of I.S. at higher UV wavelength (330 nm), at which 8-hydroxywarfarin showed no absorption, its quantification was not affected. As seen from a typical chromatogram of a blank plasma sample (Fig. 2) there are some insignificant interferences in the area between 5 and 6 min and around 10 min, which did not affect analytes quantification.

The chiral AGP column has already been fully utilized in the separation of warfarin enantiomers [14,20,25]. Isopropanol was commonly used as an organic component of the mobile phase. In our assay however, it was replaced with acetonitrile to make the achiral and chiral method more compatible. This way the same procedure for preparation of plasma samples could be used for the two methods. The mean retention times of *R*- and *S*-warfarin were 4.6 and 5.8 min, respectively. With the resolution factor of 1.5 baseline separation of warfarin enantiomers was achieved at all concentration levels (Fig. 2). Retention times of all warfarin metabolites were less than 3 min. Determination of *R*- and *S*-warfarin based on respective peak areas, since I.S. eluted with the solvent peak (1.2 min). Consequently, enantiomers in plasma samples were quantified by the peak area ratio of *R*- to *S*-warfarin and warfarin concentration estimated by the achiral method.

Each sample sequence was run twice. First run was on the achiral column with the runtime of 18 min per sample. In the second step the same samples were injected on the chiral column with the runtime of 8 min per sample. Total runtime of both analyses per sample was therefore, approximately 30 min and up to 50 samples could be analyzed within 24 h.

3.2. Extraction recovery

Liquid–liquid extraction followed the previously reported procedure [9-12]. The extraction recoveries are summarized in Table 1. Compared to the available literature data the recoveries of racemic warfarin and I.S. were found to be lower, 63 versus 80% and 63 versus 88%, respectively, while their variability was similar [10,12]. The intraday relative standard deviation (R.S.D.) of I.S. response ranged from 3.4 to 8.9% (n = 6) and interday R.S.D. was 9.7% (n = 3). Mean recoveries of various warfarin monohydroxylated metabolites differed considerably ranging from 42% for 7-hydroxywarfarin to 90% for 10-hydroxywarfarin. Use of I.S. was therefore, essential for the reliability of this method.

Recoveries of *R*- and *S*-warfarin enantiomers were not thoroughly studied. However, no difference in the recovery of *R*- and *S*-warfarin was observed by comparison of the peak areas of QC samples at all concentration levels. The difference in the recovery of enantiomers was within the experimental error of recovery determination as previously demonstrated [10,12].

Table 1
Extraction recoveries (%) of 4′-, 10-, 6-, 7- and 8-hydroxywarfarin and racemic warfarin from human plasma

Spiked plasma concentration[a]	4′-OH	10-OH	6-OH	7-OH	8-OH	Racemic warfarin
Low	62.0 (2.9)	80.8 (7.4)	86.8 (4.7)	37.6 (2.3)	67.5 (1.9)	58.8 (2.7)
Medium	69.5 (5.4)	89.0 (5.9)	91.8 (5.7)	40.1 (1.7)	76.5 (3.4)	61.7 (1.8)
High	76.8 (3.4)	93.6 (7.1)	92.5 (7.3)	47.3 (4.7)	85.3 (4.3)	67.4 (5.6)
Average	69.3 (7.4)	87.8 (6.5)	90.4 (3.1)	41.7 (5.0)	76.4 (8.9)	62.6 (4.4)

Mean recovery (S.D.) was calculated from three measurements.
[a] Concentration levels are specified in the text.

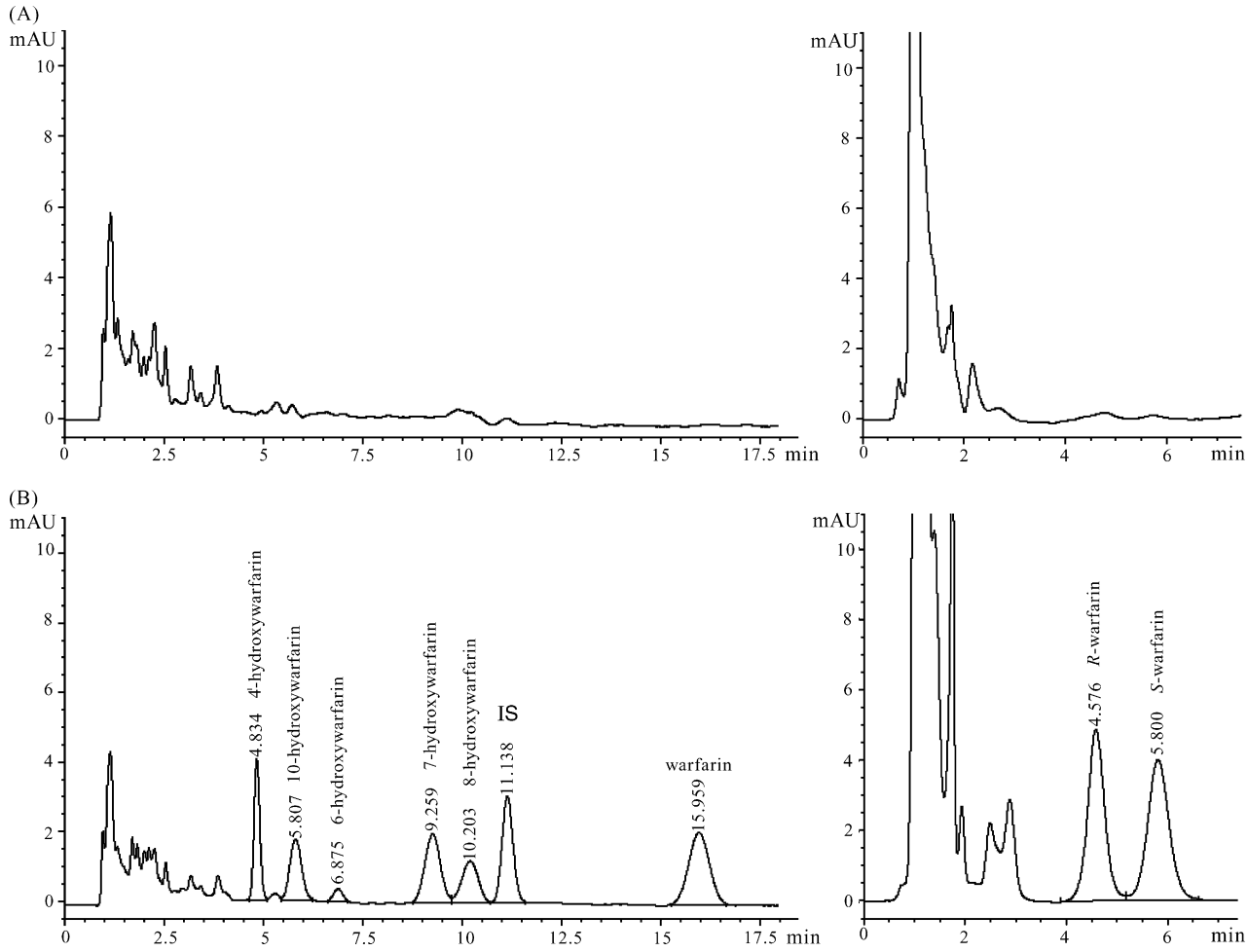

Fig. 2. (A) Chromatograms of extracted blank plasma on achiral column at 306 nm (left) and on AGP chiral column at 310 nm (right). (B) Chromatograms of medium spiked concentration (300 ng/ml for 4′-, 10-, 6- and 8-hydroxywarfarin, 500 ng/ml for 7-hydroxywarfarin and 1000 ng/ml for racemic warfarin) on achiral column at 306 nm (left) and on AGP chiral column at 310 nm (right).

3.3. Assay validation

Both methods were validated, according to the ICH guidance [22], taking into consideration also recommendations from the FDA guidance for industry on bioanalytical method validation [23].

3.3.1. Achiral method

Validation parameters are summarized in Table 2. The lower level of linear range was set to the lowest point possible with acceptable accuracy and repeatability. When analyzing six different sources of human blood plasma, no matrix effect was found to influence determination of 4′-, 6- and 7-hydroxywarfarin, I.S. and racemic warfarin. However, there was some interference observed with 8- and 10- hydroxywarfarin in four of the six blank plasma samples studied, but the area of the interfering peaks was less than 10% of the peak area of these two analytes at the lowest concentration of plasma standards. Linear range for racemic warfarin was set to the expected plasma concentrations in patients on warfarin therapy in maintenance phase according to literature data [5,26,27]. On the other hand, the data on plasma concentration ranges of warfarin metabolites are scarce. In comparison to the method by Ufer et al. [18] where MS detection was used LOQ of monohydroxy metabolites was almost 100 times higher in our method. However, this can be improved by optimizing the extraction procedure by using larger volume of plasma sample and lowering the volume for reconstitution of dried sample or by increasing the injection volume. Although recovery of the extraction procedure for some analytes, especially 7-hydroxywarfarin, is low, validation data demonstrate that the method was consistent and reliable with relatively low values of R.S.D. and bias.

3.3.2. Chiral method

Validation parameters for chiral method are summarized in Table 3 . In contrast to the achiral method accuracy and repeatability were calculated using the calibration lines derived from peak areas of enantiomers versus the analytes concentration and I.S. response was not included in evaluation of these parameters. Despite this fact repeatability and accuracy were within the range of acceptable levels.

Table 2
Method validation parameters and mean equations of calibration curves for the determination of 4′-, 10-, 6-, 7- and 8-hydroxywarfarin and racemic warfarin in human blood plasma

Validation parameter		4′-OH	10-OH	6-OH	7-OH	8-OH	Racemic warfarin
Intraday repeatability (%)[a,c]		1.2 – 4.0	0.9–2.0	1.7–4.0	1.8–2.7	2.3–5.2	1.7–4.0
Interday repeatability (R.S.D.%)[b,c]	Low	3.1	4.8	8.9	6.3	5.6	14.2
	Medium	2.8	3.1	2.0	0.9	3.1	0.5
	High	0.9	0.3	0.8	0.9	1.6	0.6
Accuracy (%)[b,c]	Low	103.2	90.0	99.3	87.3	100.0	96.0
	Medium	98.4	104.4	101.5	99.9	100.2	100.3
	High	100.6	99.6	100.2	100.4	100.5	99.9
Limit of detection (ng/ml)		25	25	25	25	35	50
Linear range (ng/ml)		75–600	75–600	75–600	75–1000	100–600	150–5000
y-Intercept × 10^3 (S.D.)[b]		18 (10)	22 (11)	7.4 (5.0)	41 (12)	95 (11)	29 (18)
Slope × 10^6 (S.D.)[b]		1180 (187)	937 (121)	524 (82)	1083 (116)	1238 (125)	929 (46)
r[b]		0.9993	0.9989	0.9995	0.9989	0.9990	0.9998

R.S.D., relative standard deviation; S.D., standard deviation; *r*, correlation coefficient (mean value); Calibration curves were derived from peak area ratios of analytes to internal standard vs. analyte concentration.
[a] $n = 6$.
[b] $n = 3$.
[c] Concentration levels are specified in the text.

Table 3
Method validation parameters and mean equations of calibration curves for the determination of *R*- and *S*-warfarin in human blood plasma

Validation parameter		*R*-warfarin	*S*-warfarin
Intraday repeatability (%)[a]	500 ng/ml	1.3–4.0	0.5–3.3
Interday repeatability (R.S.D.%)[b]	75 ng/ml	6.7	6.9
	500 ng/ml	5.1	4.6
	2500 ng/ml	0.6	0.8
Accuracy (%)[b]	75 ng/ml	103.2	106.9
	500 ng/ml	99.1	99.2
	2500 ng/ml	100.7	100.7
Limit of detection (ng/ml)		25	25
Linear range (ng/ml)		75–2500	75–2500
y-Intercept (S.D.)[b]		3.94 (2.10)	4.65 (2.03)
Slope × 10^3 (S.D.)[b]		243.5 (12.7)	245.3 (14.0)
r[b]		0.9993	0.9993

R.S.D., relative standard deviation; S.D., standard deviation; *r*, correlation coefficient (mean value); Calibration curves were derived from enantiomers peak areas versus their concentrations.
[a] $n = 6$.
[b] $n = 3$.

No significant matrix effect was observed when examining blank plasma samples from six independent sources.

3.3.3. Stability

The protocol of stability procedure reflected all the possible conditions encountered during sample handling and analysis. Results of stability testing are summarized in Table 4. Among all analytes evaluated, 10-hydroxywarfarin appeared to be less stable than others. Nevertheless, the results indicate that conditions used have little effect on quantification of warfarin and all monohydroxylated metabolites. Stability of extracted samples in autosampler at 5 °C over a period of

Table 4
Stability of 4′-, 10-, 6-, 7- and 8-hydroxywarfarin and racemic warfarin

Stability (%)	4′-OH	10-OH	6-OH	7-OH	8-OH	Racemic warfarin
Freeze and thaw[a]	94.1–104.4	95.9–105.3	96.4–102.0	96.4–102.0	97.0–100.9	97.8–100.7
Short-term temperature[b]	93.9–98.7	90.7–94.5	95.8–99.1	98.1–100.4	97.5–100.8	95.2–100.9
Long-term[c]	99.4–102.4	94.3–108.8	101.7–107.6	107.6–108.9	89.9–100.6	102.5–103.9
Post-preparative[d]	100.4–101.9	96.9–100.0	100.1–101.6	96.9–102.4	102.4–104.8	100.2–104.4

Stability was evaluated in three replicates of low, medium and high quality control plasma samples.
[a] Three freeze (24 h)/thaw cycles.
[b] 6 h at ambient temperature.
[c] 15 months at −20 °C.
[d] 24 h at 5 °C.

24 h permitted overnight sample runs. Evaluating the ratio of *R*- and *S*-warfarin peak area no sizeable difference in the stability of warfarin enantiomers was observed as previously shown [10,12].

Concerning stock solution stability, only I.S. was tested, since plasma standards of warfarin and warfarin metabolites were prepared from freshly made stock solutions. Stock solution of I.S. was found stable 100.2 ± 0.5% (mean ± S.D., $n = 3$) over a period of 72 h in dark at 4 °C.

3.4. Analytical interferences

A list of co-administered medications was obtained for each patient and naproxen was not used by any of the 204 patients involved in the study. Use of naproxen as I.S. therefore appeared acceptable. Nevertheless, in plasma samples of four patients significantly increased response of I.S. was observed. It was suspected that these patients have taken an over the counter naproxen preparations and did not report it at the interview at inclusion in the study. This is a serious weakness of using naproxen as I.S. in assays applied in this type of studies and choice of different I.S. should be taken into consideration as suggested by Ring and Bostick [12].

Some extra peaks with areas comparable to the analytes were observed in chromatograms of some patient plasma samples. A list of co-administered drugs in these patients was compared with the incidence of additional peaks. In chromatograms of plasma samples from all patients concomitantly taking nifedipine ($n = 3$), carbamazepine ($n = 5$), meloxicam ($n = 8$), cyproterone acetate ($n = 2$), bicalutamide ($n = 2$) and fenofibrate ($n = 2$) peaks were observed with similar retention times and UV spectra. The analytical interferences of these drugs are summarized in Table 5. There are 32 commonly used drugs taken by at least five patients listed in Table 5, which were found not to interfere with determination of warfarin and its monohydroxylated metabolites.

3.5. Relevance of the assay for clinical study of warfarin metabolism

In total 204 blood plasma samples from patients in maintenance phase of warfarin therapy were analyzed. In all patients 4′-hydroxywarfarin was undetectable. Plasma concentrations of 6- and 8-hydroxywarfarin were above the limit of quantification in 2 and 3% of the analyzed samples, respectively. Nevertheless, 6-hydroxywarfarin was detectable in 17% of patients. 10- and 7-hydroxywarfarin were quantified more frequently, in 19 and 52% of patients, respectively. Maximal plasma concentrations of 10- and 7-hydroxywarfarin were 460.9 and 372.1 ng/ml. Mean (S.D.) plasma concentrations of 10- and 7-hydroxywarfarin were 145.9 (85.5) and 154.2 (73.1) ng/ml, respectively. Plasma concentrations of warfarin monohydroxylated metabolites can be rank ordered in the following manner: 7-hydroxy > 10-hydroxy > 6-hydroxy > 8-hydroxy > 4′-hydoxywarfarin.

Using the method of Ufer et al. [18] monohydroxylated metabolites would be detectable in many more patients. However, according to the results of our study upper level of the linear concentration range should be at least two to three times higher for 7- and 10-hydroxywarfarin, respectively.

According to Chan et al. [4] 7-hydroxywarfarin and (*R*,*S*)-warfarin alcohol are the major metabolites, which are predominant in human plasma and can be accurately measured. Our results are in accordance with these findings. Additionally, in majority of patients another peak appeared in the chromatogram at 8.5 min, which was well separated from peaks of the analytes. Since only warfarin was taken by all of these patients and UV spectra at the peak top was similar to the UV spectra of the analytes we assume that the peak at 8.5 min belongs to warfarin alcohol. In Fig. 3 chromatograms of a typical patient are presented.

R- and *S*-warfarin were quantifiable in almost all of the evaluated patients. Plasma concentration of *S*-warfarin was below LOQ in only one patient. Mean plasma concentrations (S.D.) of *R*- and *S*-enantiomer were 949.1 (452.8) and 502.9 (251.0) ng/ml, respectively, which is in accordance with the literature data [27]. Large variability in the concentration ratio of the enantiomers (*R*/*S*) ranging from 0.377 to 6.37 with mean (S.D.) value of 2.05 (0.80) was observed.

In this clinical study it was confirmed that the developed assay is appropriate for evaluation of influences of genetic polymorphisms, demographic factors and concomitant drug treatment on warfarin metabolism. Polymorphism in the

Table 5
List of analytically interfering co-administered drugs

Co-administered drug	Achiral column		Chiral AGP column	
	Ret. time (min)	Interference	Ret. time (min)	Interference
Nifedipine	3.5	None	–	None
Carbamazepine	4.5	4′-Hydroxywarfarin	3.0	None
Meloxicam	8.2	None	1.8	None
Cyproterone acetate	10.2	8-Hydroxywarfarin	3.7	*R*-warfarin
Naproxen	11.1	Internal standard	1.2	None
Bicalutamide	12.5	None	–	None
Fenofibrate	29	Subsequent analysis	–	None

Alphabetical list of non-interfering concomitant drugs taken by at least five patients: allopurinol, amiloride, amiodarone, amlodipine, atenolol, bisoprolol, carvedilol, digoxin, diltiazem, doxazosin, enalapril, furosemide, hydrochlorothiazide, indapamide, levothyroxine, losartan, lovastatin, metformin, methyldigoxin, metoprolol, pentoxifylline, perindopril, propafenone, ramipril, ranitidine, simvastatin, sotalol, spironolactone, terazosin, tramadol, trandolapril, verapamil.

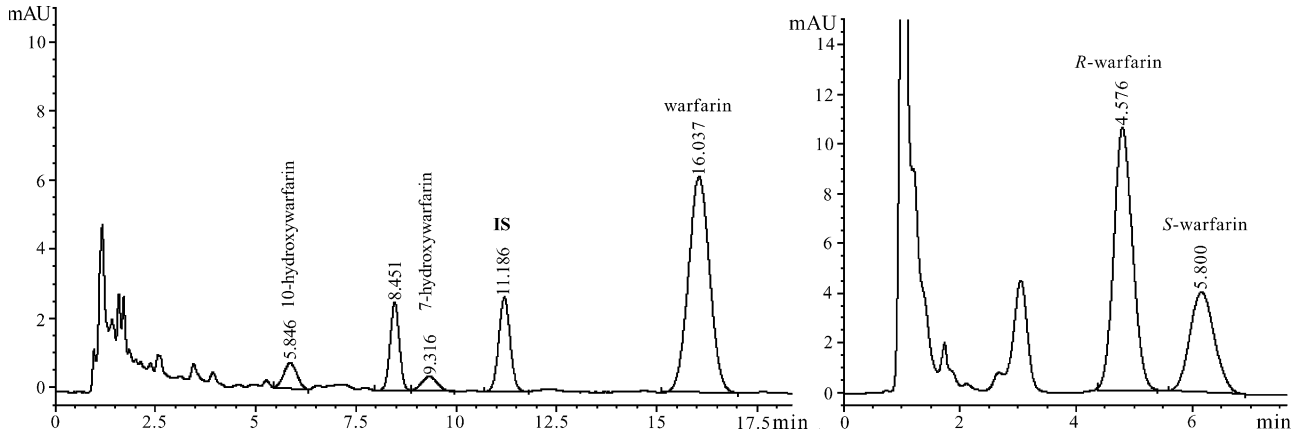

Fig. 3. Chromatograms of a typical patient plasma sample on achiral column at 306 nm (left) and on AGP chiral column at 310 nm (right). The concentration of 10-, 7-hydroxywafarin and racemic warfarin were 100.2, 52.8 (below quantification) and 1662.1 ng/ml, respectively. The *R* to *S* warfarin ratio was 1.96.

CYP2C9 gene leading to impaired metabolism of *S*-warfarin to 7-hydroxywarfarin was found to be the main reason for the observed variability in warfarin daily dose requirement [21].

4. Conclusion

Two simple isocratic HPLC methods with UV detection were developed for measuring warfarin enantiomers and all five warfarin monohydroxylated metabolites in patient blood plasma. With both methods baseline separation of the analytes was achieved. Presented validation data demonstrate that the methods are consistent and reliable with low values of R.S.D. and bias. In a large clinical study it was confirmed that the developed assay is appropriate for evaluation of influences of genetic polymorphisms, demographic factors and concomitant drug treatment on warfarin metabolism.

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