

Simultaneous determination of warfarin and 7-hydroxywarfarin in rat plasma by HPLC-FLD

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In this study, high-performance liquid chromatography with fluorescence detection (HPLC-FLD) has been used for the first time, for direct determination of warfarin and its major metabolite, 7-hydroxywarfarin, in rat plasma. The simple and sensitive method was developed using Fortis[®] reversed-phase diphenyl column (150 × 4.6 mm, 3 μm) and a mobile phase composed of phosphate buffer (25 mmol L⁻¹)/methanol/acetonitrile (70:20:10, V/V/V), adjusted to pH 7.4, at a flow rate of 0.8 mL min⁻¹. The diphenyl chemistry of the stationary phase provided a unique selectivity for separating the structurally related aromatic analytes, warfarin and 7-hydroxywarfarin, allowing their successful quantification in the complex plasma matrix. The method was linear over the range 0.01–25 μg mL⁻¹, for warfarin and 7-hydroxywarfarin, and was found to be accurate, precise and selective in accordance with US FDA guidance for bioanalytical method validation. The method was sensitive enough to quantify 0.01 μg mL⁻¹ of warfarin and 7-hydroxywarfarin (LLOQ) using only 100 μL of plasma. The applicability of this method was demonstrated by analyzing samples obtained from rats after oral administration of a single warfarin dose, and studying warfarin and 7-hydroxywarfarin pharmacokinetics.

Keywords: warfarin, 7-hydroxywarfarin, high-performance liquid chromatography-fluorescence detection, rat plasma, pharmacokinetics

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Warfarin is the most commonly prescribed anticoagulant in the world (1). It is a vitamin K antagonist used to treat and prevent blood clots and is indicated for the treatment of atrial fibrillation and deep vein thrombosis (2). Warfarin is a racemic mixture of 3-(α -acetylbenzyl)-4-hydroxycoumarin (Fig. 1). The *S*-enantiomer exhibits 2–5 times higher anticoagulant activity than the *R*-enantiomer in humans (3–5). It is absorbed rapidly from gastrointestinal tract following oral administration, reaching peak plasma concentration

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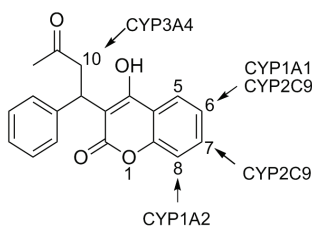


Fig. 1. Warfarin structure showing hydroxylation sites by cytochrome P450.

after 4 h (5, 6). The effective half-life after oral administration ranges from 20 to 60 h with 92 % of the dose excreted in the urine. The half-life of the *R*-enantiomer is longer (37–89 h) than that of the *S*-enantiomer (21–43 h) (6). The two enantiomers are stereoselectively metabolized by hepatic CYP450 microsomal enzymes (7). *S*-warfarin metabolism by CYP2C9 yields 6- and 7-hydroxylated metabolites, whereas *R*-warfarin metabolism by CYP1A1, 1A2 and 3A4 yields 6-, 8-, and 10-hydroxylated metabolites (8–10) (Fig. 1). Overall, 7-hydroxywarfarin is the most predominant metabolite of warfarin in humans (11–13). Several studies indicated that alteration of warfarin pharmacokinetics (PK) following the administration of CYP450 inhibitors can enhance the anticoagulant activity and thus increase the risk of bleeding as a result of elevation of warfarin plasma concentration (14, 15). The likelihood of such interaction is further increased due to the narrow therapeutic index and extensive protein binding (99.83 %) of warfarin (1), in addition to the significant inter-individual variation in drug response. Determination of metabolic ratios of warfarin/7-hydroxywarfarin can provide valuable information about potential interaction of warfarin with drugs or diet at the metabolic level where many warfarin interactions occur. To investigate these interactions, sensitive and selective methods are required for the simultaneous quantification of warfarin and its 7-hydroxywarfarin metabolite in plasma.

Various methods have been reported for the quantitation of racemic warfarin and its main metabolites in biological samples using high-performance liquid chromatography (HPLC) coupled with a variety of detectors including ultraviolet (UV) (16–19), fluorescence (FLD) (19–21) and mass spectrometric detector (MS) (13, 22–26). Although LC-MS/MS methods are very selective and sensitive, the high capital cost of the MS detector presents a major limitation. HPLC-UV is a cheaper option, but it lacks the sensitivity and selectivity required for the analysis of the low plasma levels of warfarin and its metabolites. The inherited selectivity and sensitivity of fluorescence detector were exploited in few studies for the measurement of warfarin and its hydroxylated metabolites in biological samples including urine (19), plasma (20) and microbial cultures (27). Takahashi *et al.* (19) quantified warfarin in human plasma or 7-hydroxywarfarin in urine by HPLC with UV or fluorescent detection. Lomonaco *et al.* (20) developed and validated HPLC-FLD method for simultaneous quantification of warfarin and warfarin alcohols (ketone warfarin). Using post-column pH switching, Lee *et al.* (28) used a normal phase HPLC-FLD method for quantifying racemic warfarin and its major metabolites in plasma. None of the previously described HPLC-FLD methods, however, allowed for direct and simultaneous quantification of warfarin and 7-hydroxywarfarin in plasma.

In this study, we describe the development and validation of the first HPLC-FLD method for simultaneous and direct determination of warfarin and 7-hydroxywarfarin in *in vivo* small samples (100 μL) of rat plasma. Unlike other HPLC-FLD methods, the samples didn't require sample derivatization or post-column manipulation to detect warfarin and 7-hydroxywarfarin in plasma, which is a major advantage for the routine analysis of biological samples. The method was applied successfully to assess the PK of warfarin in rats following oral administration of a single warfarin dose.

EXPERIMENTAL

Materials

Racemic warfarin, 7-hydroxywarfarin and 10-hydroxywarfarin were purchased from Sigma-Aldrich (USA). 4-Hydroxywarfarin, 6-hydroxywarfarin and warfarin alcohol were purchased from Toronto Research Chemicals (Canada). Naproxen (purity 99.2 %) was kindly supplied as a gift from Hikma Pharmaceuticals (Jordan). HPLC grade acetonitrile, ethylacetate and methanol were purchased from Fisher Scientific (USA). Phosphate buffer, potassium monobasic and dibasic, were purchased from Sigma-Aldrich.

Preparation of standard solutions

A stock solution of each warfarin and 7-hydroxywarfarin was prepared by dissolving 5.0 mg of the reference standard in 10 mL methanol in a volumetric flask to produce a concentration of 500 $\mu\text{g mL}^{-1}$. Working solution (50.0 $\mu\text{g mL}^{-1}$) of each, warfarin and 7-hydroxywarfarin, was prepared in methanol after dilution. A stock solution of naproxen, used as an internal standard, was prepared by dissolving 5.0 mg of naproxen in 25 mL methanol to produce a concentration of 200 $\mu\text{g mL}^{-1}$.

The working solutions were used to prepare eight plasma calibration standards within a concentration range 0.01–25 $\mu\text{g mL}^{-1}$ for both warfarin and 7-hydroxywarfarin. Appropriate amounts of the standards were used to spike drug free plasma (blank plasma) to produce the following calibration standard concentrations: 0.01, 0.05, 0.10, 0.50, 1.0, 5.0, 10.0 and 25.0 $\mu\text{g mL}^{-1}$. Quality control (QC) samples were prepared in plasma at four concentration levels for both warfarin and 7-hydroxywarfarin: 20.0 $\mu\text{g mL}^{-1}$ (QC-high), 1.0 $\mu\text{g mL}^{-1}$ (QC-mid), 0.05 $\mu\text{g mL}^{-1}$ (QC-low) and 0.01 $\mu\text{g mL}^{-1}$ (LLOQ). Calibration and quality control standards were stored in propylene tubes at $-40\text{ }^{\circ}\text{C}$.

Instrumentation

Quantification of warfarin and 7-hydroxywarfarin was carried out on a Shimadzu 10A VP HPLC system (Japan) equipped with fluorescence detector adjusted at excitation and emission wavelengths of 310 nm and 390 nm, resp. Data acquisition and integration were performed using a Shimadzu CLASS-VP v. 6.12 software. Chromatographic separation was achieved using Fortis[®] reversed-phase diphenyl column (150 \times 4.6 mm, 3 μm) (Fortis Technologies Ltd., UK) protected by a guard cartridge (4.0 \times 4.6 mm). The mobile phase consisted of phosphate buffer (25 mmol L^{-1})/methanol/acetonitrile (70:20:10, V/V/V) (pH 7.4) and delivered at a flow rate of 0.8 mL min^{-1} in the isocratic mode.

Sample preparation

Plasma samples (100 μL) containing warfarin and 7-hydroxywarfarin were spiked with internal standard solution (20 μL). The samples were acidified by adding 0.05 mol L^{-1} acetic acid (10 μL) and vortex-mixed for 30 s before extraction with ethylacetate (3 mL) for 2 min using a vortex-mixer. After centrifugation at 4000 rpm for 10 min, the upper organic phase (2.7 mL) was transferred into clean glass tube and evaporated under a stream of nitrogen at 40 $^{\circ}\text{C}$. The residue was then reconstituted with 150 μL mobile phase and 50 μL was then injected into the HPLC system for quantifying warfarin and 7-hydroxywarfarin.

Method development

During the method development, different column chemistries were tested with different mobile phase compositions to achieve a chromatographic separation suitable for simultaneous quantification of warfarin and 7-hydroxywarfarin in plasma. Water, methanol and acetonitrile in different ratios were tested as solvents in the mobile phase, with formic acid or phosphate buffer for pH adjustment. The stationary phases tested during method development were C_8 , C_{12} , C_{18} and diphenyl with various particle sizes and column dimensions. The optimized method was then validated in accordance with US Food and Drug Administration guidance for bioanalytical method validation (29).

Analytical method validation

Linearity. – Linearity was evaluated by examining six calibration curves with eight non-zero concentrations of warfarin and 7-hydroxywarfarin (0.01–25.0 $\mu\text{g mL}^{-1}$). Peak area ratios of warfarin or 7-hydroxywarfarin to internal standard was plotted against concentrations of warfarin and 7-hydroxywarfarin using $1/x$ linear regression weighting factor. Coefficient of determination (R^2) equal to or greater than 0.99 was considered as an acceptable evidence of linearity.

Accuracy and precision. – Intra-day accuracy and precision were evaluated by examining plasma samples spiked with warfarin and 7-hydroxywarfarin at four concentration levels (LLOQ: 0.01 $\mu\text{g mL}^{-1}$, QC-low: 0.05 $\mu\text{g mL}^{-1}$, QC-mid: 1.0 $\mu\text{g mL}^{-1}$, and QC-high: 20.0 $\mu\text{g mL}^{-1}$). The run consisted of 24 spiked samples (6 replicates for each concentration) plus calibration standard samples prepared in the same day. Inter-day accuracy and precision were assessed by examining spiked plasma samples of warfarin and 7-hydroxywarfarin at four concentration levels mentioned above, each run in six replicates, on three consecutive days.

Peak areas were calculated and analyzed against a calibration curve that was prepared alongside each batch. To calculate intra-day and inter-day accuracy, the relative error (e_r) at each QC concentration level was estimated. To calculate intra-day and inter-day precision, the relative standard deviation (RSD) at each QC concentration level was determined. e_r and RSD values within $\pm 15\%$, not exceeding 20% for LLOQ, were considered as evidence of acceptable accuracy and precision, resp. (29).

Sensitivity. – Sensitivity was assessed by examining six replicates at LLOQ (0.01 $\mu\text{g mL}^{-1}$). Acceptance criteria for warfarin and 7-hydroxywarfarin were that their response

at the *LLOQ* should be ≥ 5 times their response of the zero calibrator (blank sample) with e_r and $RSD \leq 20\%$. *LLOQ* was defined as the lowest non-zero standard analyte concentration on the calibration curve. Limit of detection (*LOD*) is defined as an analyte concentration resulting in a signal-to-noise ratio of 3:1.

Selectivity. – The method selectivity is the assessment for interference by cross-reacting molecules, concomitant medications and bio-transformed species. The selectivity was assessed by analyzing blank plasma samples from six different animals to show that plasma sources did not contribute to either the analytes or the internal standard responses. Each sample was analyzed for potential interferences from endogenous substances such as endogenous matrix components, metabolites and decomposition products which should be absent at the retention time of warfarin, 7-hydroxywarfarin and the internal standard naproxen. A mixture of standard solutions containing warfarin and its main metabolites (4-, 6-, 7-, 10-hydroxywarfarin and warfarin alcohol) was used to assess if the method is able to distinguish warfarin and 7-hydroxywarfarin from other potential metabolites.

Carryover. – Carryover is the appearance of an analyte in a sample from a previous one. Impact of carryover on the accuracy of the study was assessed by injecting blank samples after a calibration standard sample with the highest concentration. Carryover in the blank sample following the high concentration standard should not be greater than 20 % of *LLOQ* and 5 % for the internal standard.

QC samples were interspersed with study samples before and during processing and analysis. A batch or batches in an analytical run may be rejected if the QC samples fail to meet the acceptance criteria. The total number of QCs was at least 5 % of the total samples analyzed but not less than 6 samples, whichever number is greater. Duplicate QC-low, QC-mid, and QC-high were used in all distinct processing batches within a run. Not less than 50 % of QCs per level should be $\pm 15\%$ of their nominal concentration.

Stability of warfarin and 7-hydroxywarfarin in working standards and in plasma was investigated in previous reports (13, 20, 22).

Pharmacokinetic study

Six male Sprague-Dawley (SD) rats, weighing 150–250 g, were supplied from animal house, Jordan University of Science and Technology (JUST, Irbid, Jordan). The rats were maintained in a clean room at a temperature of $25 \pm 2\text{ }^\circ\text{C}$, with a 12-h light-dark cycle and 50 % relative humidity. The experiments were carried out during the light cycle. The animals were acclimatized to laboratory conditions for a week prior to beginning of the experiments, and they were provided with filtered tap water and a standard rat chow diet *ad libitum*. All experimental procedures were carried out under the approval by the Animal Care and Use Committee (ACUC) of Jordan University of Science and Technology.

The SD rats were fasted 12 h before the beginning of the experiments with access to filtered tap water *ad libitum*. A single dose of a warfarin solution (4 mg mL^{-1} in DMSO) was administered orally to each animal at a dose of 2.0 mg kg^{-1} . Blood samples ($200\text{--}300\text{ }\mu\text{L}$) were collected from the tail instantly before warfarin treatment and at 0.5, 1, 2, 3, 4, 6, 8, 24, 30, 48, 54, 72, 96 and 120 h after warfarin treatment. Blood samples were immediately

treated by centrifugation at 13,000 rpm for 5 min, and 100 μL plasma samples were transferred to sample tubes and kept frozen at $-80\text{ }^{\circ}\text{C}$ until subsequent analysis.

A non-compartmental pharmacokinetic analysis was performed using WinNonlin 5.3 software (Pharsight Co., Mountain View, CA, USA). The non-compartmental pharmacokinetic parameters determined were peak plasma concentration (C_{max}), time to reach peak plasma concentration (t_{max}), terminal half-life ($t_{1/2}$), apparent total clearance (CL/F), apparent volume of distribution of terminal phase (V_d/F), total area under the concentration-time curve AUC_0^{∞} , total area under the first moment curve (AUMC_0^{∞}) and mean residence time (MRT).

RESULTS AND DISCUSSION

Method development and optimization

In order to develop selective and sensitive chromatographic separation for determination of plasma levels of warfarin and 7-hydroxywarfarin *in vivo*, several issues had to be addressed. Although fluorescence detection is more selective than UV, several fluorescent species, such as some warfarin metabolites, are expected to be present in the samples, although at very low concentrations. Since most of these metabolites are isomers to the 7-hydroxy metabolite, a highly selective method is therefore critical for the separation and accurate determination of warfarin and 7-hydroxywarfarin. Moreover, other interferences coming from the complex plasma matrix should be considered during method development.

Initial method development was carried out using a short C_{12} column ($30 \times 2\text{ mm}$, $4\text{ }\mu\text{m}$, Synergi Max RP Phenomenex[®]) and a mobile phase of different ratios of acetonitrile and water. Although several compositions of mobile phase were experimented, warfarin and 7-hydroxywarfarin peaks eluted very close to each other and at early retention times where most of the polar plasma interferences were expected to elute. Therefore, it was decided to use a more retentive C_{18} stationary phase and longer column ($125 \times 4.6\text{ mm}$, 5 mm , LiChrospher Phenomenex[®]) which has resulted in a better separation and retention for both analytes. However, when standards of other metabolites (4-, 6- and 10-hydroxywarfarin in addition to warfarin alcohol) and naproxen, as internal standard, were added to test the selectivity of the separation, some metabolites co-eluted with either warfarin or naproxen. Adjusting the mobile phase conditions or replacing naproxen with alternative internal standards did not produce a better separation.

In order to separate these structurally related analytes within a reasonable run time, we tried a stationary phase with different chemistry and higher selectivity for aromatic compounds like warfarin and its metabolites. Thus, a diphenyl column, with small particle size ($150 \times 4.6\text{ mm}$, $3\text{ }\mu\text{m}$, Fortis[®]) was tested. The phenyl-based stationary phase offers a different selectivity for aromatic compounds due to the π - π interactions between the electron-rich double bonds of warfarin and metabolites and stationary phase-phenyl moieties. A successful separation of all compounds was achieved in 20-min run time, as was demonstrated by injecting a mixture of standard solution of warfarin, 7-hydroxywarfarin and naproxen in the presence of 4-, 6-, 7-, 10-hydroxywarfarin and warfarin alcohol (Fig. 2). The mobile phase used was phosphate buffer (25 mmol L^{-1})/methanol/acetonitrile (70:20:10, V/V/V) (pH 7.4) at a flow rate of 0.8 mL min^{-1} . pH of the mobile phase was chosen based on

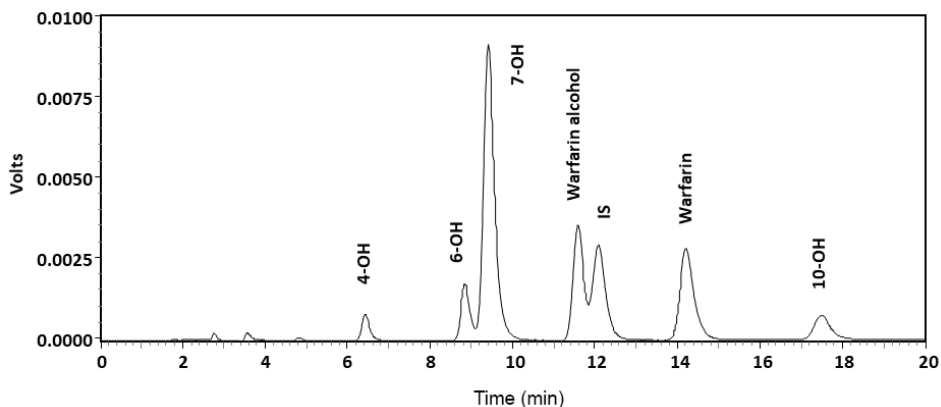


Fig. 2. Representative HPLC chromatogram for a mixture of standards of warfarin ($0.1 \mu\text{g mL}^{-1}$) and its main metabolites (7-hydroxywarfarin: $0.2 \mu\text{g mL}^{-1}$; warfarin alcohol: $0.1 \mu\text{g mL}^{-1}$; 4-, 6- and 10-hydroxywarfarin: $0.05 \mu\text{g mL}^{-1}$). Keys to the peaks: 4-OH – 4-hydroxywarfarin, 6-OH – 6-hydroxywarfarin, 7-OH – 7-hydroxywarfarin, 10-OH – 10-hydroxywarfarin, IS – internal standard (naproxen).

optimization experiments, showing that acidic pH negatively affected the intensity of the fluorescence signal of warfarin and thus pH 7.4 was selected as an optimum pH. Table I gives chromatographic parameters.

Method validation

Linearity. – The method was linear for warfarin and 7-hydroxywarfarin in the concentration range of $0.01\text{--}25 \mu\text{g mL}^{-1}$. R^2 was 0.998 and 0.996 for warfarin and 7-hydroxywarfarin, resp. (Table II). All samples tested and analysed were well within the calibration range of the method for both warfarin and 7-hydroxywarfarin ($0\text{--}25 \mu\text{g mL}^{-1}$). No dilution was required as no samples were above the *ULOQ*.

Accuracy and precision. – The intra-day average relative error (e_r) and RSD were 10.4 and 8.9 % for warfarin, resp., and 10.5 and 7.3 % for 7-hydroxywarfarin, resp. (Table II). Similarly, the inter-day average inaccuracy (e_r) and imprecision (RSD) for warfarin and 7-hydroxywarfarin were 10.1 and 2.5 %, and 10.7 and 4.3 %, resp. (Table II). See Tables III and IV for values at each QC level.

Table I. Chromatographic parameters of the HPLC method

Analyte	Tailing factor	Retention time, t_R (min)	Number of theoretical plates (N)	Capacity factor (k')	Selectivity factor	Resolution
7-Hydroxywarfarin	1.35	9.7	3820	2.33	1.69	2.09
Warfarin	1.23	14.3	4592	3.94		

Table II. Model analytical parameters evaluated for the quantification of warfarin and 7-hydroxywarfarin

Analytical performance		Warfarin	7-Hydroxywarfarin
Accuracy, e_r (%)	Intra-day	10.4	10.5
	Inter-day	10.1	10.7
Precision, RSD (%)	Intra-day	8.9	7.3
	Inter-day	2.5	4.3
Calibration range ($\mu\text{g mL}^{-1}$)		0.01–25	0.01–25
R^2		≥ 0.998	≥ 0.996
LOD ($\mu\text{g mL}^{-1}$)		0.003	0.003
LLOQ ($\mu\text{g mL}^{-1}$)		0.01	0.01

Table III. Intra- and inter-day precision and accuracy of the method for determination of warfarin in rat plasma

QC level	Nominal conc. ($\mu\text{g mL}^{-1}$)	Intra-day (single batch) ^a			Inter-day (three batches) ^b		
		Measured mean conc. ($\mu\text{g mL}^{-1}$)	RSD (%)	e_r (%)	Measured mean conc. ($\mu\text{g mL}^{-1}$)	RSD (%)	e_r (%)
LLOQ	0.010	1.19E-02	9.7	19.0	1.19E-02	4.0	19.0
QC-low	0.050	5.27E-02	11.8	5.4	4.90E-02	3.1	-2.0
QC-mid	1.0	1.13	9.2	13.0	1.08	2.5	8.0
QC-high	20.0	19.0	5.0	-5.0	17.8	0.3	-11.0

^a $n = 6$, ^b $n = 18$

Table IV. Intra- and inter-day precision and accuracy of the method for determination of 7-hydroxywarfarin in rat plasma

QC level	Nominal conc. ($\mu\text{g mL}^{-1}$)	Intra-day (single batch) ^a			Inter-day (three batches) ^b		
		Measured mean conc. ($\mu\text{g mL}^{-1}$)	RSD (%)	e_r (%)	Measured mean conc. ($\mu\text{g mL}^{-1}$)	RSD (%)	e_r (%)
LLOQ	0.010	1.19E-02	8.2	19.0	1.15E-02	2.2	15.0
QC-low	0.050	5.24E-02	12.5	4.8	5.11E-02	5.5	2.2
QC-mid	1.00	1.15	3.9	15.0	1.15	5.0	15.0
QC-high	20.0	19.3	4.8	-3.5	18.0	4.6	-10.0

^a $n = 6$, ^b $n = 18$

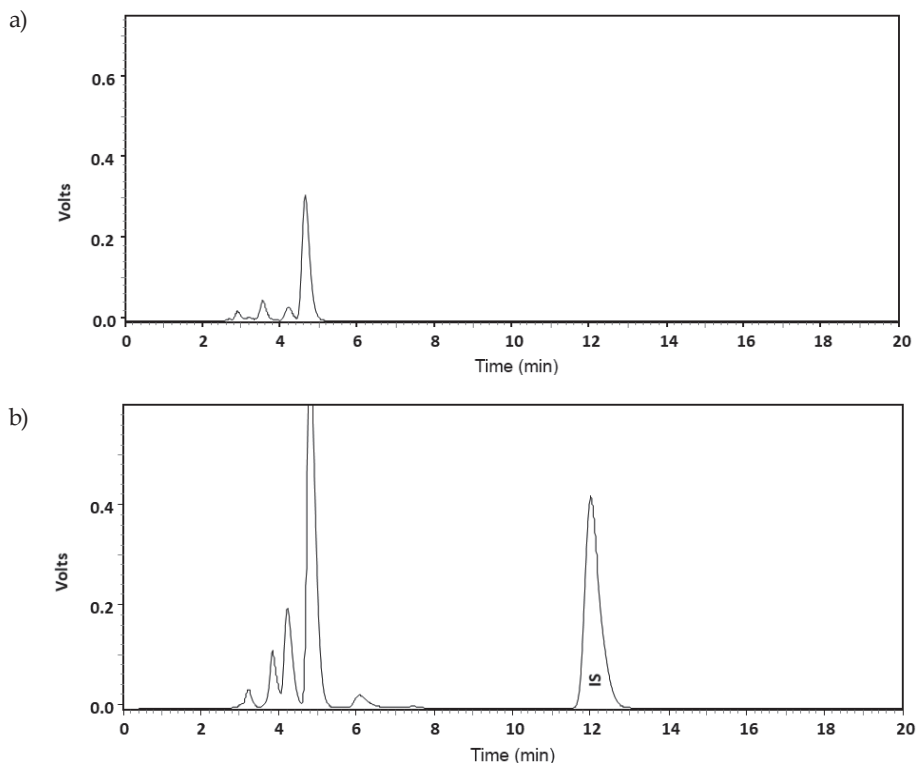


Fig. 3. Representative HPLC chromatogram for: a) blank rat plasma, b) blank plasma spiked with internal standard (IS).

Sensitivity. – LOD and LLOQ were 0.003 and 0.01 $\mu\text{g mL}^{-1}$, resp., for both 7-hydroxywarfarin and warfarin (Table II). The method was found appropriate with RSD and e_r values at LLOQ equal to 4.0–9.7 % and 19.0 %, resp., for warfarin, and 2.2–8.2 % and 19.0 % resp., for 7-hydroxywarfarin (Tables III and IV).

Selectivity. – No endogenous interference from rat plasma was observed at the retention time for warfarin (t_R 14.2 min), 7-hydroxywarfarin (t_R 9.8 min), and IS (t_R 11.8 min) (Fig. 3). The method was able to distinguish warfarin and 7-hydroxywarfarin from other metabolites (Fig. 2). These results demonstrate that the method has the required selectivity to quantify warfarin and 7-hydroxywarfarin in rat plasma.

Carryover. – No analyte or internal standard peaks were observed in the blank samples injected following the highest standard concentration.

Application to the pharmacokinetic study

The validated HPLC-FLD method was applied successfully for simultaneous determination of warfarin and 7-hydroxywarfarin in plasma samples obtained from rats following

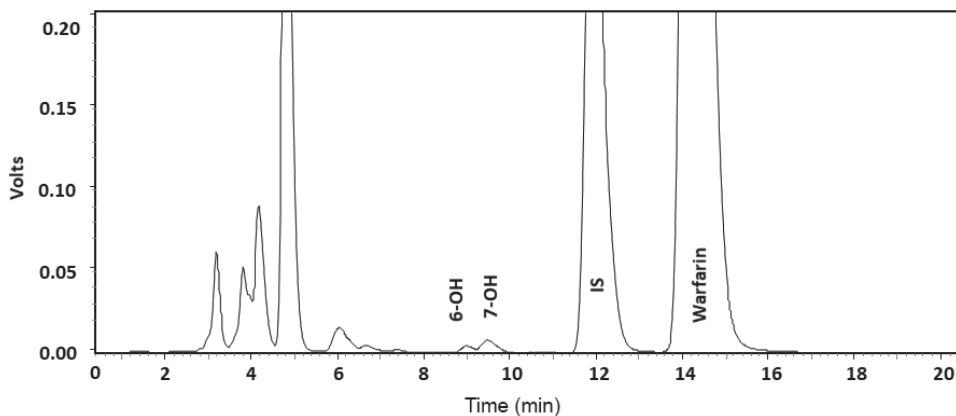


Fig. 4. Representative HPLC chromatogram for warfarin, 7-hydroxywarfarin and internal standard in real plasma sample obtained 24 h after single oral administration of 2 mg kg⁻¹ warfarin. Keys to the peaks: 6-OH – 6-hydroxywarfarin, 7-OH – 7-hydroxywarfarin, IS – internal standard (naproxen).

oral administration of single warfarin dose. Representative chromatogram for a plasma sample collected 24 h post warfarin administration, is shown in Fig. 4. The chromatogram demonstrates the selectivity and sensitivity of the method to analyze real samples and its suitability for performing PK studies of warfarin and 7-hydroxywarfarin in rats. Plasma concentration *vs.* time profiles of warfarin and 7-hydroxywarfarin after oral administration of 2.0 mg kg⁻¹ of warfarin is shown in Fig. 5, and the main PK parameters are outlined in Table V.

It was shown that C_{\max} of warfarin metabolite, 7-hydroxywarfarin, was about 2.3 % of respective C_{\max} of warfarin, with the lower rate of formation/appearance in the systemic

Table V. Pharmacokinetic parameters of warfarin and 7-hydroxywarfarin after single oral administration of warfarin in SD rats^a

Pharmacokinetic parameter	Warfarin ^b	7-Hydroxywarfarin ^b
C_{\max} (µg mL ⁻¹)	16.0 ± 2.4	0.36 ± 0.06
t_{\max} (h)	3.0 ± 0.6	7.0 ± 0.7
AUC_0^∞ (µg h mL ⁻¹)	378.4 ± 66.6	10.3 ± 1.8
$AUMC_0^\infty$ (µg h ² mL ⁻¹)	10236 ± 2770	345 ± 87
MRT (h)	27.0 ± 5.0	33.0 ± 4.0
CL/F (mL h ⁻¹ kg ⁻¹)	6.0 ± 0.9	223.0 ± 35.0
V_d/F (mL kg ⁻¹)	292.0 ± 64.0	6409.0 ± 1832.0
$t_{1/2}$ (h)	32.5 ± 5.0	21.0 ± 6.0

^a Dose of warfarin: 2 mg kg⁻¹.

^b Mean ± SEM ($n = 6$).

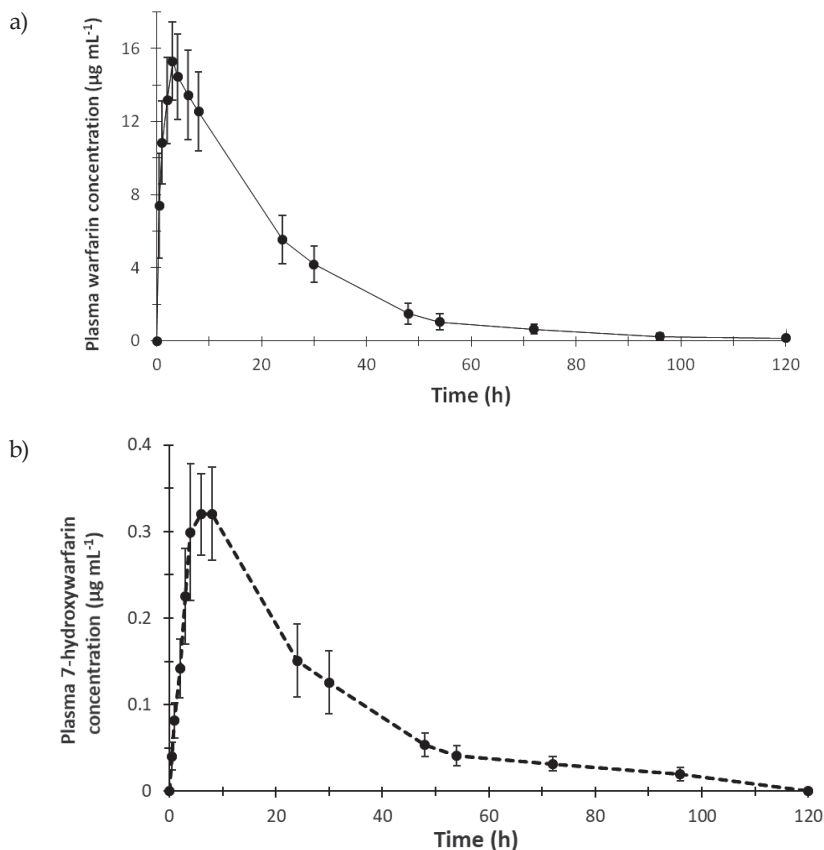


Fig. 5. Plasma concentration *vs.* time profiles of: a) warfarin, b) 7-hydroxywarfarin, following administration of a single oral dose of warfarin (2 mg kg^{-1}). Mean \pm SEM ($n = 6$).

circulation. This was indicated by *ca* 4-h increase in t_{max} compared to warfarin. The extent of warfarin bioavailability, represented by AUC_0^∞ , was shown to be 38-fold higher than that of its metabolite. Shorter terminal $t_{1/2}$ and higher apparent clearance was shown for 7-hydroxywarfarin, which resulted in the 21.9-fold larger apparent volume of distribution when compared to warfarin. These PK results are in agreement with reported PK results for warfarin and 7-hydroxywarfarin in rats (30) and demonstrate the reliability of the HPLC-FLD method for studying PK and metabolism of warfarin in rats.

Comparison of the described method with literature methods (Table VI) highlights the advantage of direct and simultaneous determination of warfarin and 7-hydroxywarfarin without the need for post-column manipulation or laborious sample derivatization which was required by previously published HPLC-FLD methods (28). The simplicity of liquid extraction combined with the isocratic reversed-phase conditions as opposed to normal phase (28), ion pair (27) or gradient elution (25) allows the method to lend itself readily to routine sample analysis such as in pharmacokinetic and animal studies. Moreover, the

Table VI. Summary of existing analytical methods for quantification of warfarin and/or its metabolites in biological samples

Technique	Sample preparation	Mobile phase V/V	Column	Analytes/matrix	LOQ (ng mL ⁻¹)	Ref.
HPLC-FLD	Liquid extraction, protein precipitation and/or ultrafiltration	Phosphate buffer/methanol/acetonitrile 70:25:5 (pH 7.0)	C ₁₈ 150 mm × 4.6 mm, 5- μ m reversed-phase column	Warfarin & warfarin alcohol in human plasma	1.3 for warfarin, 0.7 for warfarin alcohol	20
HPLC-FLD	Liquid extraction	Izopropanol/acetic acid/hexane 18:0.5:81.5	Chiralcel OD column 250 × 4.6 mm, 10 μ m	Enantiomers of 7-hydroxywarfarin in urine	20 for 7-hydroxywarfarin	19
HPLC-FLD	Liquid extraction	Acetonitrile/glacial acetic acid/triethylamine 1000:32.5	ChiraDex [®] column 250 × 4 mm, 5 μ m (LiChroCART [®] 250-4)	Enantiomers of warfarin in human plasma in human plasma	12.5	21
UPLC-MS/MS	Protein precipitation, liquid extraction or SPE	Methanol/H ₂ O/formic acid 55:45:0.01	Acquity UPLC BEH Phenyl column 2.1 mm × 150 mm 1.7 μ m Astec Chirobiotic V column 2.1 mm × 150 mm, 5 μ m	Enantiomers of warfarin and hydroxywarfarins in human liver microsomes	1.0 for all analytes	23
HPLC-UV	SPE	Izopropanol/phosphate buffer 40:60 (pH 7)	C ₁₈ 150 × 4.6 mm, 5 μ m column	Racemic warfarin and 7-hydroxywarfarin in human plasma	100 for both analytes	16
HPLC-UV	Liquid extraction	Izopropanol/acetic acid/hexane 18:0.5:81.5	Chiralcel OD column 250 × 4.6 mm, 10 μ m	Enantiomers of warfarin in human plasma	100 for warfarin	19
HPLC-UV	Liquid extraction	Phosphate buffer (pH 3)/methanol/acetonitrile 52:32:16	ODS Hypersil analytical column 100 mm × 4.6 mm, 5 μ m	Warfarin and hydroxywarfarins in human plasma	150 for warfarin, and 75 for 7-hydroxywarfarin	17
HPLC-MS/MS	Protein precipitation	Ammonium acetate/0.1 % formic acid/40 % acetonitrile	Atlantis [®] C ₁₈ 2.1 × 30 mm, 3 μ m	Racemic warfarin and 7-hydroxywarfarin in human plasma	75 for both analytes	24
HPLC-FLD	Liquid extraction	Phosphate buffer/methanol/acetonitrile (pH 7.4)	C ₁₈ 150 × 4.6 mm, 3- μ m reverse diphenyl column	Racemic warfarin and 7-hydroxywarfarin in rat plasma	10 for both analytes	This work

economy, sensitivity and selectivity of the FLD detection make this method ideal for quantifying warfarin and 7-hydroxywarfarin in complex matrices compared to the less sensitive UV (16, 17, 19) and expensive MS detectors (23–25).

CONCLUSIONS

A new sensitive, selective, accurate and precise HPLC-FLD method has been developed and validated for the quantitation of warfarin and 7-hydroxywarfarin in rat plasma. The method demonstrated adequate sensitivity to quantify $0.01 \mu\text{g mL}^{-1}$ of both warfarin and 7-hydroxywarfarin in only 100 μL plasma. The sensitivity and low instrument capital cost of the fluorescence detector combined with the unique selectivity of the diphenyl stationary phase make the method ideal for carrying out metabolic interaction studies of warfarin in small animals like rats. Quantification of 7-hydroxywarfarin will enable drug-to-drug and drug-to-diet interaction studies investigating altered warfarin metabolism and response because of various CYP2C9 phenotypes.

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