

# Determination of S- and R-warfarin enantiomers by using modified HPLC method

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**Abstract:** Warfarin is a commonly prescribed anticoagulant existing in two enantiomeric forms S- and R-warfarin. Many techniques have been used to analyze warfarin in plasma but less frequently for enantiomeric analysis. One of the HPLC method employed was further simplified and made economical. Method was validated according to ICH guidelines and was found to be sensitive, reliable and less time consuming. For both enantiomers, LLOQ was 12.5ng/mL. The CV% and accuracy for method were in the range of 0.8-14.6% and 92-107% respectively. The recoveries for both enantiomers were in the range of 86-103.8%. Blood samples were collected from 170 stable patients taking warfarin and S- and R-warfarin levels were determined by this method. Majority of subjects were found to have S/R-warfarin ratio of about 1:2 as reported in previous studies due to rapid clearance of S-enantiomer than R-enantiomer. However individual subjects data was suggestive of presence of slow metabolizers of S-warfarin leading to altered S/R ratio. Previous studies have also pointed out CYP2C9 polymorphism being responsible for such inter-individual differences in S-warfarin metabolism. So plasma warfarin S/R ratio may serve as a useful phenotypic test for CYP2C9 polymorphism.

**Keywords:** S-warfarin, R-warfarin, Enantiomers, HPLC, CYP2C9 polymorphism.

## INTRODUCTION

Warfarin has been the most commonly prescribed oral anticoagulant since its approval in 1954 (Ansell *et al.*, 2008; Frueh, 2012). It has been used for prophylaxis and treatment of various venous and arterial thromboembolic disorders. Patients with valvular heart diseases, prosthetic heart valves, atrial fibrillation, myocardial infarction, pulmonary embolism, deep vein thrombosis and recurrent strokes require anticoagulant therapy (Ansell *et al.*, 2008; Campbell *et al.*, 2001; Keeling *et al.*, 2011). Management of warfarin therapy is difficult because of significant intra- and inter-individual variability, low therapeutic index and highly variable pharmacokinetics. Warfarin dose requirement varies considerably among individuals and also in the same person at different time spans due to wide range of factors like age, sex, weight, ethnicity, genetic factors, dietary intake, concurrent diseases and medications (Hirsh *et al.*, 2001; Takahashi *et al.*, 2006; Hines *et al.*, 2011; Lane *et al.*, 2012; Jorgensen *et al.*, 2012). Inadequate or supra-therapeutic anticoagulation may result in substantial morbidity and mortality due to failure to prevent thromboembolism or bleeding complications respectively. The fear of the complications often causes clinicians to avoid prescribing warfarin to patients who are likely to benefit from such therapy (Ansell *et al.*, 2008; Campbell *et al.*, 2001; Gage and Lesko, 2008; Chitsike *et al.*, 2012).

Warfarin (4-hydroxy-3-(3-oxo-1-phenylbutyl)coumarin) exists in two enantiomeric forms, S- and R-warfarin because of presence of centre of chirality at position C9 (fig. 1) (Frueh, 2012; Ring and Bostick, 2000; Boppana *et al.*, 2002; Malakova *et al.*, 2009). Warfarin is administered as a racemic mixture of S- and R-enantiomers. Analysis for detection of warfarin has been carried out in many studies (Huang *et al.*, 2008; Sadrai *et al.*, 2008) but enantiomeric analysis has been less commonly studied because determination of S- and R-warfarin enantiomers needs highly sophisticated analytical methods (Wang *et al.*, 2011; Jin *et al.*, 2012; Wang *et al.*, 2013). Various high performance liquid chromatography (HPLC) methods have been described in literature for determination of warfarin enantiomers in human plasma and these have shown different sensitivities and limitations (Boppana *et al.*, 2002; Malakova *et al.*, 2009; Locatelli *et al.*, 2005). The method used in the present study (Naidong and Lee, 1993) was reliable and reasonably sensitive for accurate estimation of warfarin enantiomers in plasma. The method was modified by using fluorescence detector instead of UV detector making it more sensitive and also naproxen as internal standard was not used as it is a commonly used non-steroidal anti-inflammatory drug and if found in patients' sample, can interfere with results. Various procedures were performed to validate the modified method according to International Conference on

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Harmonization (ICH) Guidelines on validation of analytical procedures (ICH, 2005).

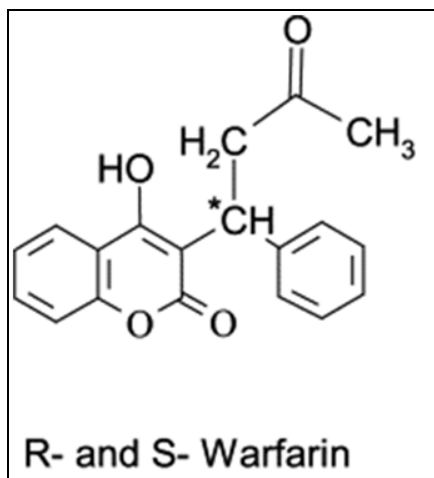


Fig. 1: Chemical structure of warfarin with chiral centre\*

## EXPERIMENTAL

### Instrumentation and Reagents

The High Performance Liquid Chromatography (HPLC) system by Agilent 1100 Series with autosampler and fluorescence detector was used. Chromatographic separation was done on LiChroCART® 250-4 ChiraDex® (250x4 mm, 5 µm particle size) column along with LiChroCART® 4-4 ChiraDex® (4x4mm, 5 µm particle size) guard column provided by Merck Darmstadt, Germany. The chromatograms were recorded on connected computer.

The chemicals and solvents used in this study were of HPLC grade. Acetonitrile, methanol and glacial acetic acid were purchased from Merck Darmstadt, Germany. Sulphuric acid was bought from Reanal Finechemical Pvt Ltd, Hungary. Diethyl ether was purchased from LAB-SCAN Analytical Sciences, Thailand. Triethylamine was bought from Fisher Scientific, USA. Racemic Warfarin (Rac-warfarin), S-warfarin and R-warfarin standards were purchased from Sigma-Aldrich, USA.

## METHODS

### Chromatographic conditions

The fluorescence detector was set at an excitation wavelength of 300 nm and an emission wavelength of 390 nm. The mobile phase consisted of acetonitrile: glacial acetic acid: triethylamine (1000: 3: 2.5, v/v/v). The mobile phase was pumped at a flow rate of 1 ml/min. All analyses were done at room temperature.

### Standards preparation and calibration curve

Separate stock solutions of 1mg/mL concentration of Rac-warfarin, S-warfarin and R-warfarin were prepared by dissolving appropriate amount in acetonitrile. Injection of

pure S-warfarin and R-warfarin were made at start of work to determine the elution order of enantiomers. Rest of analyses were carried out by using Rac-warfarin. The eight working standard solutions of Rac-warfarin were prepared to contain concentrations of 0.125, 0.25, 0.5, 1, 2.5, 5, 10, 25µg/mL each of S- and R-warfarin enantiomers. All solutions were stored in aluminum foil-wrapped bottles to avoid light exposure. The solutions were kept at -20°C for long-term storage.

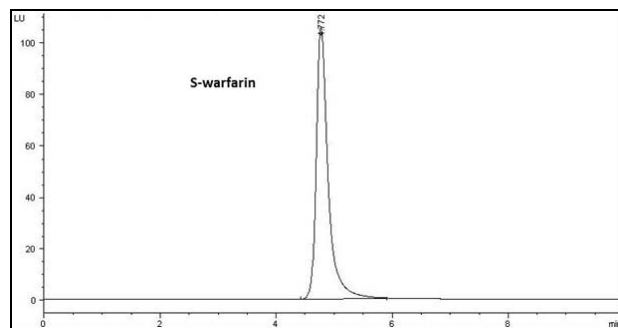


Fig. 2: Chromatogram of pure S-warfarin standard

Calibration standard curves were prepared by spiking 900µL of blank plasma with 100µL of working standards to yield plasma standards containing final concentrations of 12.5, 25, 50, 100, 250, 500, 1000, 2500ng/mL each of S- and R-warfarin. Three levels of quality control (QC) samples with concentrations of 40, 200, 1500ng/mL each of S- and R-warfarin in plasma were also prepared for method validation.

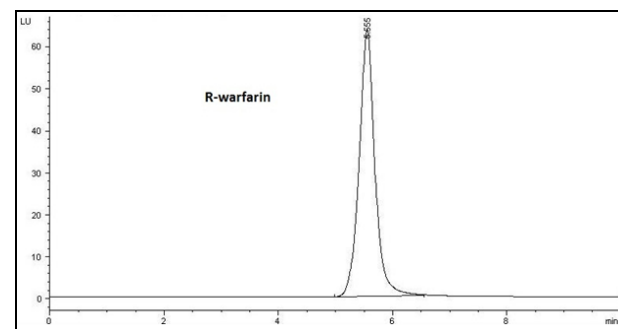


Fig. 3: Chromatogram of pure R-warfarin standard

### Extraction procedure

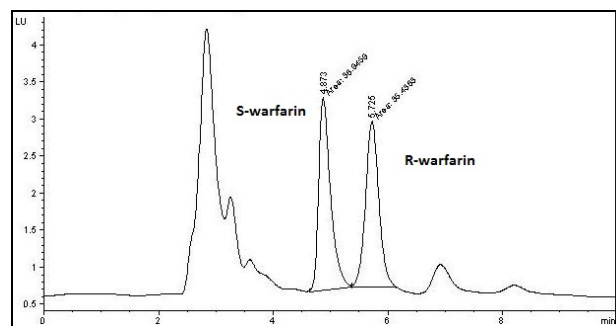
One milliliter of plasma sample was acidified by addition of 700µL of 1N sulphuric acid. After mixing, 3mL of diethyl ether was added to extract S- and R-warfarin. The organic layer was separated and evaporated to dryness under a stream of nitrogen. The residual sample was reconstituted in 300µL of acetonitrile and 40µL was injected onto the HPLC system.

### Method validation

The elution order of enantiomers was assigned by injecting pure S- and R-warfarin standards separately. The identification of two enantiomers in plasma sample spiked with Rac-warfarin was made on the basis of retention

times on chromatograms obtained from plasma samples spiked with pure S- and R-warfarin standards separately.

QC samples were run as replicates of blank plasma spiked with a low concentration (40 $\mu$ g/mL), a middle concentration (200 $\mu$ g/mL) and a high concentration (1500 $\mu$ g/mL) of both S- and R-warfarin enantiomers.



**Fig. 4:** Chromatogram obtained from plasma sample spiked with 200ng/mL standard

Linearity was assessed by calibration curve constructed separately for S- and R-warfarin, using 8 standard solution concentrations covering the range of 12.5–2500 ng/mL for two enantiomers. Standard curves were analyzed in triplicate. The lower limit of quantitation (LLOQ) for both enantiomers was selected as the lowest concentration of the standard curve. The lower limit of quantitation was the lowest concentration of each enantiomer at which the compound peak was identifiable and discrete with suitable precision (coefficient of variation of less than 20%) and accuracy (determined concentration being within 20% variation of the nominal concentration).

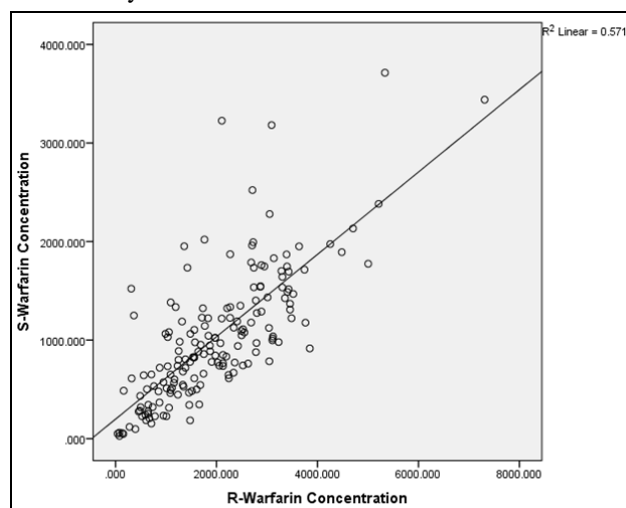
The precision and accuracy of the plasma assay for S- and R-warfarin was determined by running quality control samples for 3 days. Intra-day assay variability was tested with 6 replicates of each quality control concentration run on the same day. Inter-day assay variability was established by running 6 replicates of each quality control concentration for 3 days.

In order to estimate recovery and extraction efficiency, the peak areas obtained with plasma sample spiked with Rac-warfarin were compared with the average peak areas obtained by direct injections of known amounts of the compound in triplicate.

To assure the stability of analyte and the analytical system, quality control samples were run daily at the beginning of each run throughout the period of analysis. Analyte stability was also demonstrated by subjecting the samples of three concentrations to three freeze-thaw cycles.

Benchmark stability was checked by keeping samples at room temperature and running them after 2 hours and

then at 24 hours. To test the stability of the stereo isomers against inter-conversion during analysis, samples containing only one enantiomer were prepared and run immediately and after 24 hours.



**Fig. 5:** Scatter plot showing relationship between R- and S-warfarin enantiomers

#### Clinical application

The study was conducted in accordance with the current Good Clinical Practices (FDA, 1996) and the Declaration of Helsinki (WMA, 2000). The study protocol was approved by Ethical Committee of Centre for Research in Experimental and Applied Medicine (CREAM), Army Medical College, Rawalpindi. A total of 170 stable patients of either sex were recruited for the study. A stable patient was defined as the one whose warfarin dose had been constant for at least three previous clinic visits over a minimum period of three months, and had an INR of prothrombin time within the range of 1.5–3.5. Each subject was evaluated through a detailed medical history, physical examination and laboratory tests. The demographic and clinical data of individuals was collected and those who were suffering from any comorbid disease or taking any concurrent medication which may affect warfarin concentration in plasma were excluded. All subjects gave written informed consent. A 5 mL blood sample was drawn approximately 12 hours after last administered dose of warfarin. Each sample was transferred to heparinized tubes and immediately centrifuged. Plasma was collected and frozen at  $-80^{\circ}\text{C}$  until analysis.

#### RESULTS

Standard calibration curves established to quantify both warfarin enantiomers independently were linear over a concentration range of 12.5–2500 ng/mL for each enantiomer. The equation of calibration using least square regression method was  $y=0.1506x + 5.7997$ ,  $R^2=0.999$  for S-warfarin and  $y=0.153x + 4.2737$ ,  $R^2=0.999$  for R-warfarin.

The retention time for S-warfarin was  $4.8 \pm 0.16$  minutes and for R-warfarin was  $5.7 \pm 0.13$  minutes, making them well resolved (figs. 2, 3 and 4).

The limit of detection (LOD) was 6 ng/mL for both enantiomers. For both enantiomers, LLOQ was 12.5 ng/mL. The CV% and accuracy are given in table 1. The method was shown to be precise and accurate. Accuracy along with intra-day and inter-day variability for quantitation of individual enantiomer are summarized in table 1. Both S- and R- warfarin enantiomers were found to be stable after three freeze-thaw cycles and at room temperature even after 24 hours. The data is summarized in table 1.

The mean recoveries of warfarin enantiomers from plasma assessed at three different concentrations ranged from 86 to 103.8%. The recoveries of S-warfarin at 40, 200 and 1500ng/mL were 94%, 95% and 103.8% respectively and those of R-warfarin were 95%, 86% and 103% respectively.

There was no enantiomer inter-conversion either during extraction or chromatography as none of the unspiked enantiomer was detected on fresh analysis as well as upon re-injection after 24 hours (table 2).

The mean steady-state plasma concentration from analysis of 170 samples was  $1.02 \pm 0.66 \mu\text{g/mL}$  (range 0.028-3.7) for S-warfarin and  $1.97 \pm 1.2 \mu\text{g/mL}$  (range 0.041-5.3) for R-warfarin. A scatter plot was constructed to show the relationship between R- and S-warfarin enantiomers (fig. 5). It showed a strong positive relationship, which was statistically significant ( $r=0.756$ ;  $p=0.000$ ). Plasma S/R ratio for warfarin was  $0.6 \pm 0.5$  calculated from S- and R-warfarin plasma concentrations. There was no effect of age ( $p=0.452$ ) and gender ( $p=0.95$ ) on S/R enantiomers ratio of warfarin.

## DISCUSSION

The method used was simple with single liquid-liquid extraction step. There was no involvement of any complicated device or system as seen in other methods (Wang *et al.*, 2011; Jin *et al.*, 2012; Wang *et al.*, 2013). Both enantiomers were extracted well as observed from the recovery data. The method was found to be reliable as suggested by precision and accuracy figures. Both enantiomers were found to be stable and there was no inter-conversion noticed. The method developed into a more economical, reliable and less time consuming method due to the modifications done in it. The use of fluorescence detector made the analysis more sensitive as there was no interference from the UV absorbent compounds co-eluting with warfarin enantiomers. Internal standard naproxen was not used as it is a commonly used analgesic and can be found in patients' blood leading to

interference in the results. Data obtained from validation procedures confirmed the reliability of method even without the addition of internal standard. Due to early retention times of both enantiomers, the run time for assay was reduced to 10 minutes leading to not only economy of resources but also less time required to analyse a large number of samples.

Warfarin is routinely administered as a racemic mixture of R- and S-enantiomers. After an oral dose, its peak plasma concentration is achieved within 2 hours. The half life of warfarin averages about 36-42 hours. The two enantiomers R- and S-warfarin differ in their metabolism and potency (Kaminsky and Zhang, 1997; Fitzpatrick and O'Kennedy, 2004; Gage and Milligan, 2005). The S-warfarin is metabolized by mainly CYP2C9 and R-warfarin mainly by CYP3A4, CYP2C19 and CYP2C8. At steady state, S-warfarin is present at only half the concentration of R-warfarin because of the rapid rate of metabolism (about 1.5 times faster) of S-enantiomer (Ansell *et al.*, 2008; Kaminsky and Zhang, 1997; Chan *et al.*, 1994; Yamazaki and Shimada, 1997; Henne *et al.*, 1998). The previous studies have shown that S- to R-warfarin ratio in majority of cases were found to be close to 1:2. In our study although the majority of patients showed the ratio of S- to R-warfarin closer to 1:2, there were cases in which this ratio was markedly different, as also observed in other reported studies (Locatelli *et al.*, 2005; Chan *et al.*, 1994; Henne *et al.*, 1998; Hou *et al.*, 2007).

As the clearance of R-warfarin does not vary across different CYP2C9 genotypes as that of S-warfarin varies showing decreased clearance in variant allele carriers, the ratio of S/R warfarin at steady state serum concentration has often been used to estimate the activity of CYP2C9 (Henne *et al.*, 1998; Caraco *et al.*, 2008). The presence of CYP2C9\*2 or CYP2C9\*3 variant alleles leading to slower clearance of S-warfarin, is associated with a significant decrease in the mean warfarin dose. Individuals possessing these allelic variants are at higher risk for serious bleeding as S-enantiomer is around 3-5 times more potent than R-enantiomer and is mainly responsible for warfarin's anticoagulant effect. A number of patients in our study showed increased S/R warfarin ratio suggesting the existence of polymorphic alleles in these patients. This can be further confirmed by the genotyping of CYP2C9 in these patients and strengthening the reported role of CYP2C9 polymorphism in warfarin therapy (Ansell *et al.*, 2008; Hirsh *et al.*, 2001; Takahashi *et al.*, 2006; Gage and Lesko, 2008; Caraco *et al.*, 2008).

## CONCLUSION

The modified method was established to be reliable and economical. The S/R warfarin ratio was found to be

**Table 1:** Intra- and inter-day precision and accuracy of S- and R-warfarin analysis

Assay Validation	S-warfarin				R-warfarin			
	Conc. Added ng/mL	Conc. Found ng/mL Mean $\pm$ SD	Coefficient of Variation (CV %)	Accuracy (%)	Conc. Added ng/mL	Conc. Found ng/mL Mean $\pm$ SD	Coefficient of Variation (CV %)	Accuracy (%)
LLOQ	12.5	13 $\pm$ 1.75	13.4	104	12.5	13.4 $\pm$ 1.97	14.6	107
Intra-day Assay	40	40.4 $\pm$ 2.6	6.5	101	40	42.7 $\pm$ 3.1	7.3	107
	200	201 $\pm$ 1.6	0.8	101	200	190.4 $\pm$ 6.5	3.4	95
	1500	1549 $\pm$ 14	0.9	103	1500	1560 $\pm$ 15	1	104
Inter-day Assay	40	36.6 $\pm$ 3.4	9.3	92	40	37.5 $\pm$ 4.3	11	94
	200	192.3 $\pm$ 9.3	4.9	96	200	185.3 $\pm$ 8.3	4.5	93
	1500	1419 $\pm$ 99	7	95	1500	1404 $\pm$ 117	8.3	94
Freeze Thaw Stability	40	39.9 $\pm$ 1.5	3.7	99.6	40	43.8 $\pm$ 0.8	1.7	109.6
	200	217 $\pm$ 0.9	0.4	108.7	200	222.8 $\pm$ 1.4	0.6	111
	1500	1511.5 $\pm$ 22	1.5	100.8	1500	1580 $\pm$ 25.5	1.6	105
Bench-top Stability	40	40.3 $\pm$ 0.9	2.2	100.7	40	43.4 $\pm$ 1.4	3.2	108
	200	216.6 $\pm$ 1.5	0.7	108	200	222.5 $\pm$ 1.9	0.9	111
	1500	1506 $\pm$ 26.7	1.8	100.4	1500	1575.7 $\pm$ 36	2.3	105

**Table 2:** Analytical stability of single enantiomer

Time (hour)	S-warfarin Spiked Plasma (1000 ng/mL)		R-warfarin Spiked Plasma (1000 ng/mL)	
	S-warfarin ng/mL	R-warfarin ng/mL	S-warfarin ng/mL	R-warfarin ng/mL
0	888.9	ND*	ND*	935.1
24	850.4	ND*	ND*	888.6

\*ND – Not Detected

comparable to reported studies. Data also suggested the presence of inter-individual difference in warfarin metabolism pointing towards the genetic factors.

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