ORIGINAL ARTICLE

Validation of warfarin enantiomer analysis method in plasma using high-performance liquid chromatography fluorescence detector

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ABSTRACT

Warfarin (WF) is an anticoagulant commonly used for thromboembolism-related diseases.This study aims to assess the pharmacokinetic profile of WF. The stereospecific interaction of S-and R-WF requires quantification of the enantiomer to determine the pharmacokinetic profile. The analysis method of the enantiomers in plasma is developed using an HPLC fluorescence detector with a Chiralcel OD-RH column (4.6 mm × 150 mm i.d., 5 m) and a Chiralcel OD-RH guard column (4.0 mm × 10 mm, 5 m). The separation is conducted using isocratic with acetonitrile mobile phase: Phosphate buffer, pH 2.00 (40:60 v/v), column temperature 40°C, flow rate 1 mL/min, injection volume 50 L. WF is measured at an excitation wavelength of 310 nm and emission of 350 nm. This method results in limit of detection (LOD) values of 18.6 ng/mL and limit of quantitation (LOQ) of 62.01 ng/mL for R-WF and LOD values of 18.61 ng/mL and LOQ of 62.04 ng/mL for S-WF. The results showed a linearity in concentration between 100 and 2500 ng/mL with r^2 = 0.9969 and r^2 = 0.9991 for R-and S-WF. The validation requirements of selectivity, accuracy, and precision for within and between run with a value of <15% for % relative standard deviation and % diff were achieved. This method can be used in the sample measurement of WF pharmacokinetic studies.

Key words: Fluorescence, validation, warfarin

INTRODUCTION

Warfarin (WF) is a class of anticoagulant drugs widely used to treat thromboembolism-related diseases such as atrial fibrillation, venous thrombosis, and pulmonary embolism. WF also has a narrow therapeutic index, and the variation in response between patients is very high. Therefore, it is challenging to determine the exact dose for each patient since the situation resulted in many cases of drug-related problems in the form of adverse drug reactions.[1,2]

WF exists in two enantiomeric forms, namely R-and S-WF, and differs significantly in pharmacological and metabolic activities.[3] S-enantiomeric has a potency of 3–5 times stronger than the R-WF form in its anticoagulant activity. Furthermore, it is metabolized more rapidly than the R-form by the cytochrome P-450 enzyme group.[3] This stereospecific interaction causes the need for WF enantiomer quantification to determine its pharmacokinetic profile.[4]

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Various methods were developed to analyze WF enantiomers in plasma, and each has its advantages and limitations. The method using High Performance Liquid Chromatography (HPLC) with an ultraviolet (UV) detector for the analysis has a quantification limit value (lower limit of quantification [LLOQ]) limited to 100 ng/ml.[5] Furthermore, the mass spectrometry detector method produced a susceptible method with an LLOQ of up to 1 ng/ml. However, it is too complex to be applied to routine analysis.[6]

The development and validation of the analysis method of WF in plasma using fluorescence detector HPLC were conducted to obtain a simple, sensitive, and valid method in the study of the R-and S-WF pharmacokinetic profile.

MATERIALS AND METHODS

Materials

WF standard reference (Wako), griseofulvin (internal standard [IS]), methanol HPLC grade (Merck), acetonitrile HPLC grade (JT Baker), aquabides (IPHA Laboratories), potassium dihydrogen phosphate, phosphoric acid 85%, vacutainer Na2EDTA, HCl, ethyl acetate, diethyl ether, nitrogen gas, 0.45 µm millipore membrane.

Methods

Optimization of chromatographic analysis conditions

The high-performance liquid chromatography used was the Waters e2695 and 2475 Separation Module with FLR fluorescence detector. The separation was conducted using an OD-RH chiral cell column (4.6 mm × 150 mm i.d.,5 µm) with a Chiralcel OD-RH guard column (4.0 mm × 10 mm, 5 µm). It was set at 40°C, and isocratic was conducted by optimizing the mobile phase of acetonitrile: Phosphate buffer pH 2.0 with a ratio of (65: 35); (40:60); (55:45); and (50: 50) v/v. The maximum excitation and emission wavelengths are determined by conducting tests based on literature (310 and 400 nm) and scanning (310 and 350 nm) wavelengths on fluorescence spectrophotometry.

Sample preparation

Several sample preparations were conducted to determine the appropriate analysis method. The three sample preparation methods are (A) liquid-liquid extraction (LLE) – ethyl acetate, (B) LLE – diethyl ether, and (C) protein precipitation – scetonitrile.

Method validation

The validation was conducted based on the 2009 EMA bioanalysis guidelines. It includes the following parameters: The selectivity was conducted by analyzing WF enantiomer and IS on plasma blanks from six different individuals spiked with standard WF at an LLOQ concentration of 100 ng/ml and an IS of griseofulvin 50 ng/ml.

The LLOQ values and calibration curves were obtained by testing on plasma blank, zero (IS), and plasma samples added with standard WF to 6 concentration levels (0.1; 0.5; 1; 1.5; 2; 2.5 ppm). The LLOQ was obtained from the measurement results at the lowest concentration of the standard curve with five replications. It met the CV requirements and percent diff ± 20% with the LLOQ signal in the sample ×5 signal in the blank.

The accuracy and precision were conducted by testing the sample QC at four concentration levels (LLOQ QC, Low QC, Medium QC, and High QC), namely 0.1; 0.5; 1, and 1.5 ppm. Five samples per concentration level were tested in 1 test for Within Run and Between Run, tested on two different days.

Testing samples evaluated the stability with low and high concentrations, namely 0.5 ppm and two ppm. Furthermore, the stock solution stability test was conducted with a working solution at a concentration of 1 ppm prepared fresh from a 200 ppm stock solution. The test was conducted on days 0, 10, and 21, and Freeze-Thaw sample stability was conducted on plasma samples added with standard WF and stored at − 80°C. Furthermore, the samples were frozen and thawed through 2 cycles of freezing and thawing before being prepared for testing.

RESULTS AND DISCUSSION

WF was detected in the system at an excitation and emission wavelength of 310 nm and 400 nm based on research conducted by.[4] These wavelengths were selected because they were closest to the results of the excitation wavelength scanning performed on a UV spectrophotometer, namely 310 nm. However, they had a low detection sensitivity, where the peak area was too small and could not detect WF at low concentrations.

The excitation and emission with the best fluorescence intensity were obtained at 310 nm and 350 nm. Consequently, they were selected for WF detection in the system, and the suitability test was carried out by varying the ratio of the mobile phase. One ppm standard solution was injected into the system with a column temperature of 40°C, a flow rate of 1 mL/min, and an injection volume of 50 L.

The column temperature used was 40°C, and the temperature was selected because the viscosity of the mobile phase decreases. This will lower the systemic pressure and produce a sharper peak. Furthermore, the thermal and kinetic energies promoted faster molecular movement and sped up sample analysis time. Four variations of the mobile phase ratio were conducted, namely the ratio of acetonitrile: Phosphate buffer pH 2.00 (35:65), (40:60), (45:65), and (50:50) v/v. Table 1 shows the results of the system suitability test.

These results showed that the higher the acetonitrile ratio, the faster the WF elutes. The WF had a pKa value of 5.08 in a nonpolar form with a pH of 2.00. Therefore, it was conducted in a more nonpolar mobile phase with a larger acetonitrile ratio. The condition with the mobile phase ratio of (40:60), (45:55), and (50:50) fulfilled the requirements for each parameter of the system suitability test. Furthermore, the system in these three conditions had a reasonably short analysis time of 10–20 min. Compared to the three, the mobile phase ratio of (40:60) had the best selectivity and resolution values. Therefore, this system was selected to be used in the analysis.

Many studies using fluorescence detectors for WF analysis do not use ISs in their methods. Some used WF-derived compounds such as p-chlorowarfarin as ISs.[4,7,8] The IS used is a readily available drug compound known as griseofulvin, its properties are almost similar to WF, which is practically insoluble in water, and the log *P* value (2.18) is not much different from WF. In addition, griseofulvin has a strong aromatic ring group that has fluorescence ability. Furthermore, multichannel detection at excitation and emission wavelengths of 300 and 400 nm was conducted for griseofulvin detection.[9] Figure 1 shows the results of the separation on the chromatogram.

Method optimization of plasma sample preparation

The results showed that there are no significant differences between the two procedures. The extraction method using diethyl ether has more noise along with the chromatogram than ethyl acetate. Therefore, both procedures show good recoveries in separating analytes from the matrix [% recoveries are reported in Table 2]. The weakness is that during the retention time of R-WF, there was a noise interfering with WF measurements, especially in samples with low concentrations. Therefore, this method cannot be used. Figure 2 and procedure B in Figure 3 showed a comparison of blank plasma chromatograms and WF LLOQ spiked plasma from procedure A.

Procedure C was conducted with the principle of protein precipitation using acetonitrile, which was selected because it is compatible with the mobile phase used in the system. This method showed excellent percent recoveries and precision for S-WF and R-WF. Figure 4 shows the comparison of blank plasma chromatograms and WF LLOQ spiked plasma from procedure C.

Figure 4 shows that Blank plasma chromatogram with protein precipitation-acetonitrile method (a) and WF plasma chromatogram of 100 ng/ml with protein precipitation-acetonitrile method (b). Elution order and retention time: 1 (R-WF, rt: 11.2 min), 2 (S-WF, rt: 15.3 min).

The disadvantage of the protein precipitation method is the lack of effectiveness in removing protein, which was avoided in this procedure by increasing the acetonitrile ratio to 1:5. After adding acetonitrile, the sample was incubated at a low temperature of 4°C and centrifuged. The incubation and centrifugation at low temperatures triggered and accelerated the occurrence of protein aggregation.[10] Therefore, the resulting analyte separation with an efficient matrix was not much different from the LLE method. The sample preparation method with protein precipitation was selected using 1:5 acetonitrile.

Warfarin validation in plasma samples

The WF validation was conducted according to the EMA bioanalysis guidelines 2009.[11] The parameters tested were selectivity, LOD and LOQ, linearity, accuracy, precision, and stability.

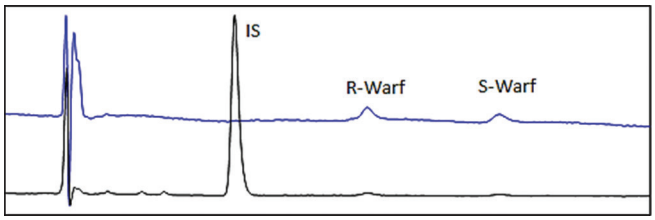

Figure 1: Standard warfarin chromatogram 100 ng/mL and IS 50 ng/mL

Table 1: Results of the suitability test on the warfarin system

Comparison of mobile phase		Warfarin Enantiomer	Retention time (Tr)	Capacity factor (K')	Theoretical plate (N)	Selectivity factor (α)	Resolution (Rs)
Acetonitrile	Phosphate buffer pH 2,00						
35	65	R-WF	18.48	9.82	2140	1.43	3.56
		S-WF	25.76	14.08	1705		
40	60	R-WF	11.67	5.26	3089	1.43	4.58
		S-WF	15.89	7.53	4042		
45	55	R-WF	7.80	3.04	2707	1.42	3.53
		S-WF	10.28	4.32	3006		
50	50	R-WF	5.69	1.99	2143	1.41	3.17
		S-WF	7.26	2.82	3375		

WF: Warfarin

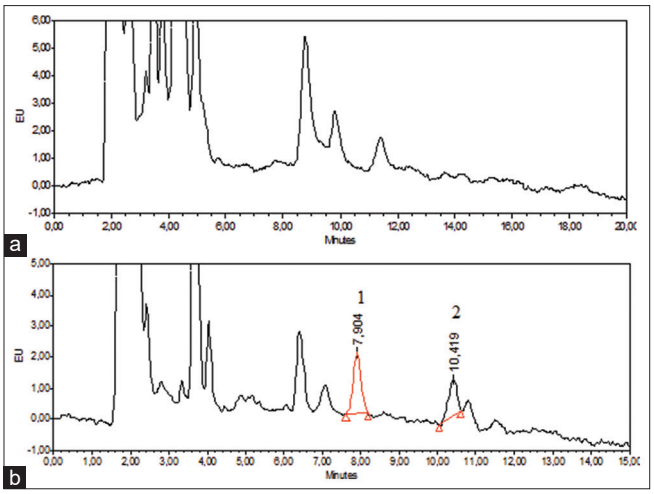

Figure 2: Blank plasma chromatogram with liquid-liquid extraction -ethyl acetate method (a) and Warfarin plasma chromatogram of 100 ng/ml with liquid-liquid extraction -ethyl acetate method (b). Elution order and retention time: 1 (R- Warfarin, rt: 7.9 min), 2 (S- Warfarin, rt: 10.4 min)

Table 2: Sample recovery results

	Sample concentration	Percentage recovery (±SD) *n*=3	
		R-WF	S-WF
Procedure A (LLC-EA)	100 ng/ml	96.69 (±16.5)	64.52 (±14.5)
	2000 ng/ml	84.33 (±4.55)	88.1 (±1.12)
Procedure B (LLE-DE)	100 ng/ml	159.28 (±46.7)	97.98 (±25.23)
	2000 ng/ml	83.29 (±5.3)	95.37 (±9.1)
Procedure C (PP-ACN)	100 ng/ml	90.56 (±3.2)	90.17 (±1.71)
	2000 ng/ml	88.09 (±5.7)	90.70 (±8.35)

SD: Standard deviation, LLC-EA: Liquid-liquid extraction – ethyl acetate, LLE-DE: Liquid-liquid extraction - diethyl ether, PP-CAN: Protein precipitation – acetonitrile, WF: Warfarin

Selectivity

The selectivity parameter indicated the method's ability to separate the analyte and IS from the matrix. It was conducted on six blanks from each different individual. Furthermore, good separation between the analyte and IS was obtained with the matrix. Figures 5 and 6 show the chromatogram of the separation results for WF and IS.

Limit of detection and limit of quantitation

These results showed that the LOD and LOQ values are 18.6 ng/mL and 62.01 ng/mL for R-WF, 18.61 ng/mL, and 62.04 ng/mL for S-WF. The LLOQ value of 100 ng/mL has a % diff of 12.32% and % relative standard deviation (RSD) of 4.04% for R-WF and % diff of 8.77% and % RSD 8.77% for S-WF.

Linearity

Tables 3 and 4 show the test results for the R-WF and S-WF standard curves. The estimated blood concentration of WF in rats with multiple-dose regimens at the Cmax point was ± 700 ng/ml.[6] Furthermore, the ranges allowed a safety margin to detect potential increases in blood drug concentrations due to pharmacokinetic drug interactions.

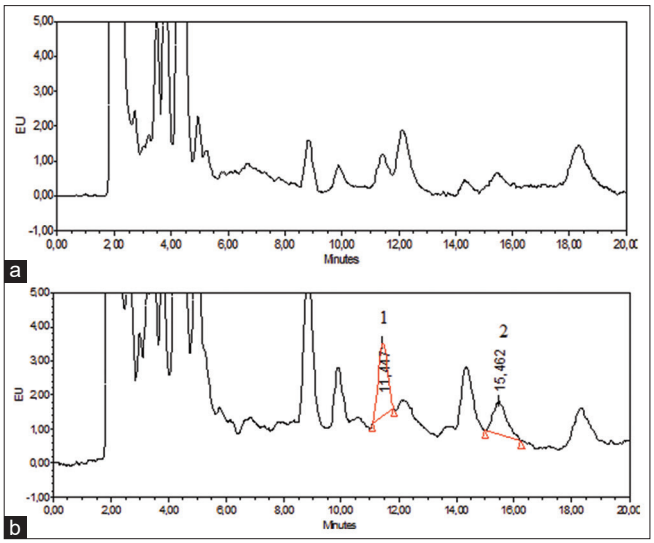

Figure 3: Blank plasma chromatogram with liquid-liquid extraction -diethyl ether method (a) and warfarin plasma chromatogram of 100 ng/ml with liquid-liquid extraction -diethyl ether method (b). Elution order and retention time: 1 (R- warfarin, rt: 11.4 min), 2 (S- warfarin rt: 15,4 min)

The results showed that at 6 points of concentration levels, both on the R-and S-WF curves, the calculated standard concentrations again meet the required average accuracy value within ± 15% of the nominal, including the LLOQ concentration.

The recommended value requirement according to ICH was $r^2 > 0.98$,[11] and the linear regression equation $y = 0.0272x + 0.0107$ with an r2 value of 0.9969 for R-WF and $y = 0.0271x + 0.0116$ with an r2 value of 0.9991 for S-WF were obtained. It was concluded that the linearity parameter meets the requirements, and the linear regression curve is reported in Figure 7.

Accuracy and precision

The accuracy and precision tests were carried out at four concentration levels of 100, 500, 1000, and 1500 ng/ml, each consisting of five samples. The within run test was carried out on the same day, while the between runs were conducted twice on two different days. Tables 5 and 6 show the percent recovery and diff values reported in the QC samples. It was concluded that the accuracy and precision test results had met the requirements.

Stability

The stability of the stock solution and plasma samples were evaluated by measurement at 0, 24, and 168 h, and the stock solution was prepared fresh into a working solution of 1 ppm before being injected into the system. Furthermore, the plasma samples were tested at 200 and

1500 ng/ml concentrations after the two cycles of freeze and thaw at −80°C. The sample stability was evaluated based on comparing the results at a certain time to the test concentration at t = 0. When the percentage of solution concentration at time t was within ±15% of the initial concentration. The stock solution of WF 200 ppm with storage conditions ± 8°C and protected from light can last for 1 week. Meanwhile, there was no change in the measurement results of plasma samples through 2 freeze and thaw cycles.

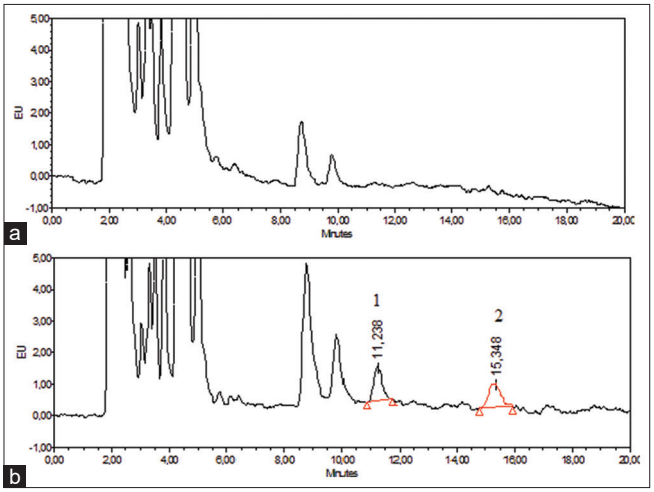

Figure 4: Blank plasma chromatogram with protein precipitation acetonitrile method (a) and warfarin plasma chromatogram of 100 ng/ml with protein precipitation-acetonitrile method (b). Elution order and retention time: 1 (R- warfarin, rt: 11.2 min), 2 (S- warfarin, rt: 15.3 min)

CONCLUSION

This resulted in a LOD value of 18.6 ng/ml, a LOQ of 62.01 ng/ml for R-WF a LOD value of 18.61 ng/ml, and a LOQ of 62.04 ng/ml for S-WF. The linearity in the concentration was between 100 and 2500 ng/ml with the equation y = 0.0272x + 0.0107, r^2 = 0.9969 for R-WF and y = 0.0271x + 0.0116, r^2 = 0.9991 for S-WF. Furthermore, this method fulfilled the requirements of selectivity, accuracy, and precision for within and between runs with a value of

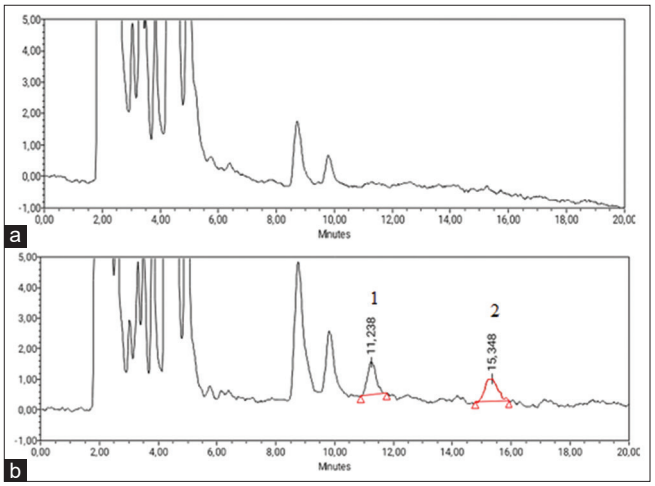

Figure 5: Blank plasma chromatograms at ex 310 nm and em 350 nm (a) and chromatograms at 100 ng/ml warfarin in plasma at ex 310 nm and em 350 nm (b). Elution order and retention time: 1 (R- warfarin rt: 11.2 min), 2 (S- warfarin, rt: 15.3 min)

Table 3: Test results of R-warfarin standard curve

WF concentration	Internal standard		R WF			
	Retention time	Area	Retention time	Area	Concentration calculation results	Percentage difference
0.1	7.113	1,760,325	11.237	113,086	0.09	1.21
0.5	7.141	1,811,331	11.267	544,043	0.53	6.88
1	7.119	1,588,477	11.214	810,772	0.92	7.80
1.5	7.135	2,219,472	11.251	1,882,714	1.54	3.02
2	7.115	2,197,612	11.225	2,472,113	2.05	2.78
2.5	7.126	2,004,346	11.238	2,710,757	2.47	0.97

WF: Warfarin

Table 4: Test results of S-warfarin standard curve

WF concentration	Internal standard		S-WF			
	Retention time	Area	Retention time	Area	Concentration calculation results	Percentage difference
0.1	7.113	1,760,325	15.19	105,059	0.08	11.61
0.5	7.141	1,811,331	15.321	526,465	0.51	2.59
1	7.119	1,588,477	15.279	857,432	0.97	2.90
1.5	7.135	2,219,472	15.3	1,862,598	1.52	1.42
2	7.115	2,197,612	15.276	2,448,272	2.02	1.32
2.5	7.126	2,004,346	15.29	2,710,049	2.46	1.43

WF: Warfarin

Table 5: Test results of R-warfarin precision and accuracy

Concentration (*n*=5)	Within run precision (% RSD)	Within run accuracy (% difference)	Between run precision (% RSD)	Between run accuracy (% difference)
0.1 ppm	4.04	12.32	5.72	8.41
0.5 ppm	6.07	1.45	2.87	1.91
1 ppm	6.42	14.99	14.79	7.224
1.5 ppm	6.80	2.93	5.28	1.415
Average	5.83	7.92	7.16	4.73

RSD: Relative standard deviation

Table 6: Test results of S-warfarin precision and accuracy

Concentration (*n*=5)	Within run precision (% RSD)	Within run accuracy (% difference)	Between run precision (% RSD)	Between run accuracy (% difference)
0.1 ppm	8.77	1.41	5.98	3.64
0.5 ppm	6.39	2.01	6.4	4.48
1 ppm	7.07	12.88	11.68	7.35
1.5 ppm	5.05	3.36	5.49	0.67
Average	6.82	4.91	7.38	4.03

RSD: Relative standard deviation

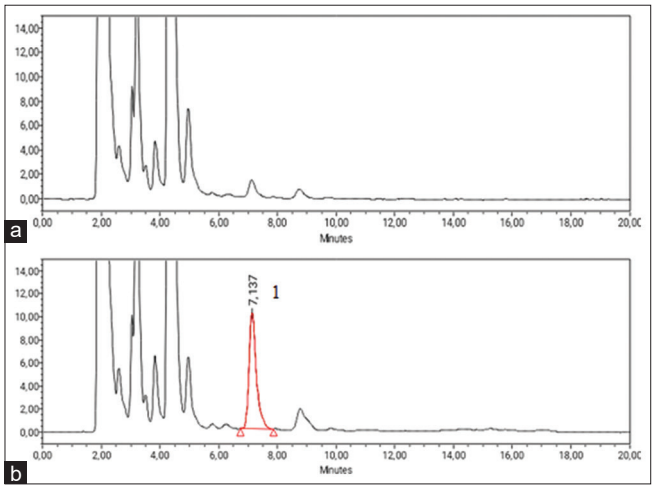

Figure 6: Blank plasma chromatograms at ex 300 nm and em 400 nm (a) and 50 ng/ml IS chromatograms in plasma at ex 300 nm and em 400 nm (b). Elution and retention time: 1 (Griseofulvin, rt: 7.1 min)

%RSD and % diff % <15% and was stable at −80°C storage conditions. This result showed that the analytical method had met the validation requirements. Therefore, it can be used for the sample measurement in WF pharmacokinetic studies.

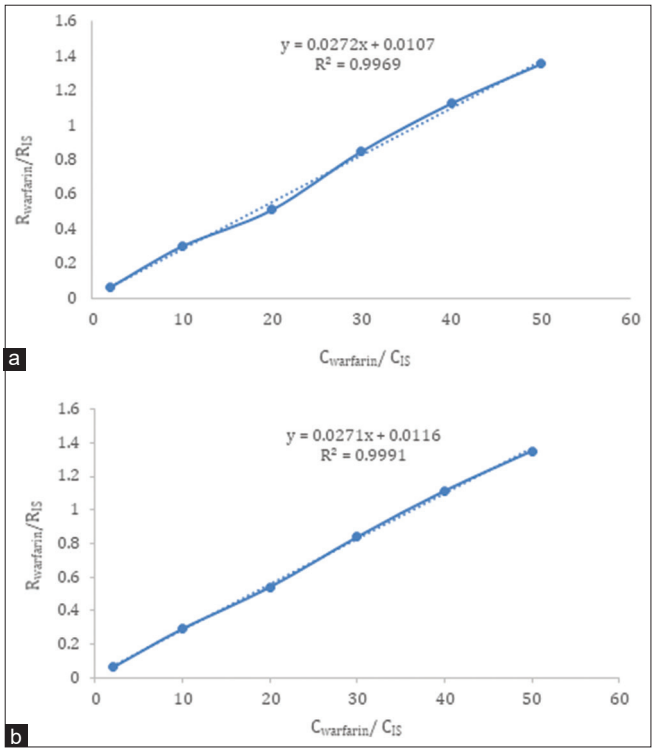

Figure 7: R- warfarin linearity curve (a) and S- warfarin linearity curve (b)

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Obayashi K, Nakamura K, Kawana J, Ogata H, Hanada K, Kurabayashi M, *et al.* VKORC1 gene variations are the major contributors of variation in warfarin dose in Japanese patients. Clin Pharmacol Ther 2006;80:169-78.
2. Lestari K, Diantini A, Rusdiana T, Putri N. Therapy monitoring warfarin in cardiac care patients in a hospital in Bandung. Indones J Clin Pharm 2014;1:282-289.

3. Rusdiana T, Araki T, Nakamura T, Subarnas A, Yamamoto K. Responsiveness to low-dose warfarin associated with genetic variants of VKORC1, CYP2C9, CYP2C19, and CYP4F2 in an Indonesian population. Eur J Clin Pharmacol 2013;69:395-405.
4. Boppana VK, Schaefer WH, Cyronak MJ. High-performance liquid-chromatographic determination of warfarin enantiomers in plasma with automated on-line sample enrichment. J Biochem Biophys Methods 2002;54:315-26.
5. Gong IY, Schwarz UI, Crown N, Dresser GK, Lazo-Langner A, Zou G, *et al.* Clinical and genetic determinants of warfarin pharmacokinetics and pharmacodynamics during treatment initiation. PLoS One 2011;6:e27808.
6. Lomonaco T, Ghimenti S, Piga I, Onor M, Melai B, Fuoco R, *et al.* Determination of total and unbound warfarin and warfarin alcohols in human plasma by high performance liquid chromatography with fluorescence detection. J Chromatogr A 2013;1314:54-62.
7. Maity B, Chatterjee A, Ahmed SA, Seth D. Interaction of the nonsteroidal anti-inflammatory drug indomethacin with micelles and its release. J Phys Chem B 2015;119:3776-85.
8. Malakova J, Pavek P, Svecova L, Jokesova I, Zivny P, Palicka V. New high-performance liquid chromatography method for the determination of (R)-warfarin and (S)-warfarin using chiral separation on a glycopeptide-based stationary phase. J Chromatogr B Analyt Technol Biomed Life Sci 2009;877:3226-30.
9. Wei B, Liang D, Bates TR. Development and validation of a HPLC method to determine griseofulvin in rat plasma: Application to pharmacokinetic studies. Anal Chem Insights 2008;3:103-9.
10. Rosa M, Roberts CJ, Rodrigues MA. Connecting high-temperature and low-temperature protein stability and aggregation. PLoS One 2017;12:e0176748.
11. Timmerman P, Goodman J, Golob M, Haslberger T, Knutsson M, Nelson R, *et al.* European Bioanalysis Forum feedback on draft ICH M10 guideline on bioanalytical method validation during the Step 2b public consultation period. Bioanalysis 2020;12:1-11.