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Original Article

VALIDATION OF WARFARIN ANALYSIS METHOD IN HUMAN BLOOD PLASMA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

Objective: Validation of analysis method is important, especially in analyzing narrow-index drugs such as warfarin (WF). This study aimed to obtain a validated method of analyzing warfarin in human plasma according to European Medicine Agency guidelines.

Methods: The optimum conditions for the analysis of warfarin in human plasma using fluorescence detector HPLC with Chiralcel OD-RH column (4.6 x 150 mm i.d., 5µm); Chiralcel OD-RH guard column (4.0 x 10 mm, 5µm), column temperature 45 °C. The mobile phase used was acetonitrile: phosphate buffer pH 2 (40:60), with an isocratic flow rate of 1 ml/min and an injection volume of 20 µl. Excitation and emission wavelengths were 310 and 350 nm (warfarin) and 300 and 400 nm (griseofulvin). The retention time of griseofulvin was 6-7.5 min; R-warfarin was 10-11.5 min; S-warfarin was 14-16 min.

Results: The result of this validation obtained the optimum condition. This method yielded LOD values of 0.0674 ppm (R-warfarin) and 0.0897 ppm (S-warfarin). LOQ values were 0.225 ppm (R-warfarin) and 0.298 ppm (S-warfarin). Linearity at concentrations of 0.2-3 ppm with the line equation y = 0.0705x+0.0704 with R2 = 0.978 for R-warfarin and y = 0.0513x+0.0297 with R^2 = 0.9924 for S-warfarin. 75% of the seven concentrations met the reverse concentration requirements, which were below±15%. This method met the requirements of accuracy and precision within and between runs, selectivity and carryover where the %RSD and %diff values were below±15%

Conclusion: This analytical method can be declared valid and can be used for sample measurement in warfarin pharmacokinetic studies.

Keywords: Warfarin, Validation, HPLC Fluorescence, European medicine agency

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INTRODUCTION

Warfarin is an anticoagulant drug that inhibits the formation of vitamin K. It is used to treat and prevent several cardiovascular and thrombotic diseases. In choosing warfarin as a treatment, several aspects must be considered, including side effects, the therapeutic index, and response variability. The side effects of warfarin include bleeding, hypersensitivity, diarrhea, and decreased hematocrit [1]. Importantly, warfarin has a narrow therapeutic index and significant variability in response between patients. Thus, the administration of warfarin requires knowledge of the pharmacokinetics and pharmacodynamics of this drug [2].

The pharmacokinetic and pharmacodynamic processes of warfarin can be caused by its enantiomers. Warfarin is a racemic drug that has two types of enantiomers, namely R-warfarin and S-warfarin [3]. S-warfarin has an anticoagulant effect 3 to 5 times greater than R-warfarin. In addition, the metabolism and elimination of S-warfarin into its inactive form is also three times faster than R-warfarin [4, 5].

In examining the pharmacokinetic profile of warfarin, several methods can be used, including high-performance liquid chromatography (HPLC). HPLC is considered a gold standard separation technique due to its performance, with excellent selectivity and sensitivity, achieved when coupled with MS, DAD, FLD, or UV detectors compared to spectrophotometric or electrophoresis methods [6]. The detector in HPLC serves to detect sample levels. In measuring warfarin, HPLC with a fluorescence detector is more sensitive than a UV detector. This was seen in the 2015 study by Qayyum *et al.*, which analyzed warfarin with fluorescence detector HPLC to produce an LLOQ of 12.5 ng/ml [7]. Meanwhile, the study of Chua *et al.* in 2019 using UV detector HPLC showed an LLOQ of 100 ng/ml [8]. In addition, HPLC has a wide variety of columns available. One of them is a chiral column, which can separate compounds with two types of enantiomers, such as warfarin [6]. There are several types of commonly used chiral columns, including polysaccharide-based chiral columns such as Chiralcel OD-H, Chiralcel OD-RH, Chiralpak AD, Chiralpak AD-H, and others [9, 10].

In determining the optimal method to use, validation of the method is required. Method validation is carried out to ensure that the method is accurate, specific, reproducible, and resistant in the range of analytes to be analyzed [11]. Validation of bioanalytical methods can follow the International Council of Harmonization (ICH) guidelines from the European Medicines Agency (EMA) [12].

In a study conducted by Putriana *et al.* in 2022, validation of the warfarin analysis method on rat plasma was carried out using a fluorescent HPLC detector. The results obtained are quite good, but there are some drawbacks. With the modification of the method in the study, partial validation was carried out in this study [13].

MATERIALS AND METHODS

Materials

Healthy human blood plasma was obtained from PMI (Indonesian Red Cross). The warfarin standard reference was obtained from Wako. Griseofulvin as the internal standard (IS) was obtained from Kimia Farma. Acetonitrile HPLC grade was obtained from JT Baker. Sterile double distilled water (aquabides) from obtained IPHA Laboratories. Potassium dihydrogen phosphate was obtained from Merck. Phosphoric acid 85% was obtained from UNPAD Central Laboratory. HPLC-grade methanol was obtained from Merck. Nitrogen gas was obtained from AToz Gas.

Preparation of stock solution

Standard warfarin stock solution was prepared in a concentration of 200 ppm with 50% methanol solvent. IS (griseofulvin) stock

solution was prepared in a concentration of 200 ppm with acetonitrile solvent. The stock solution was stored at 4 °C, protected from light. The working solution was freshly prepared before the test from the stock solution.

Optimization of warfarin standard analysis conditions

Optimization was carried out using a standard warfarin solution, concentration 1 ppm and internal standard, 0.5 ppm griseofulvin. The excitation and emission wavelengths were 310 and 350 nm for warfarin and 300 and 400 for griseofulvin. A mobile phase ratio (acetonitrile: phosphate buffer pH 2.0) of (40:60) v/v was used at a rate of 1 ml/min [13]. The column temperature was optimized at 40 °C, 45 °C, and 50 °C. The following parameters were observed and calculated: retention time, resolution (R), capacity factor (k'), theoretical number of plates (N), selectivity (α), and tailing factor (Tf).

Extraction of vitamin K from plasma

The standard solution in plasma was placed in a 2 ml micro-centrifugation tube and 490 µl of cold acetonitrile was added. Then the micro-centrifugation tube was vortexed for 3 min. The tube was then centrifuged at 10000 rpm for 10 min at 5 °C. The supernatant obtained was transferred to another centrifugation tube. The supernatant was then dried under nitrogen flow at 40 °C to obtain a dry residue. The residue was then reconstituted using 70 µl of methanol, and the resulting sample was vortexed for 1 min.

Sample preparation

Samples were prepared by the protein precipitation technique using acetonitrile. 100 µl of blank plasma was added 20 µl of standard warfarin and 10 µl of 0.5 ppm IS (griseofulvin) solution and vortexed. Next, 500 µl of cold acetonitrile was added. Then, it was vortexed and kept in the refrigerator for 15 min. Then, it was centrifuged for 4 min at 13000 rpm at 4 °C. Then, the supernatant obtained was evaporated under nitrogen gas with a sample temperature of±40 °C. Then, reconstitution was done up to 100 µl with mobile phase solution and centrifuged again at 13000 rpm for 4 min. After that, the supernatant was injected into the HPLC system [13].

Optimization and suitability test of warfarin analysis system in plasma

Optimization on plasma was carried out the optimal under conditions for the standard using plasma that had been carried out previously with differences in vortex time of 30, 40, and 60 min. Meanwhile, the system suitability test was carried out on plasma samples with a warfarin concentration of 1 ppm with HPLC conditions being the same as the standard optimization results. Then calculated, retention time, resolution (R), capacity factor (k'), number of theoretical plates (N), selectivity (α), and tailings factor (Tf).

Method validation for the analysis of warfarin in plasma samples

The validation method was conducted following the analytical method validation guidelines from the EMA in 2019. The methods are:

Calibration curve (linearity)

Plasma blanks were prepared with internal standard and warfarin standard. Seven levels of warfarin concentration (0.2, 0.5, 1, 1.5, 2, 2.5, 3 ppm) were prepared. Then, the value of accuracy (% diff) for linearity was calculated. The % diff value of the recalculated concentration of each calibration standard was within±20% of the nominal concentration at LLOQ and less than±15% at all other levels. At least 75% of the calibration standards with at least six levels of calibration standards must meet the above criteria.

Accuracy and precision

Analysis was carried out at 4 levels of QC sample concentrations, namely QC LLOQ, low, medium, and high (0.2, 0.6, 1.5, and 2.25 ppm). Repeatability was tested as many as five replicates per concentration level in one test (within run). While Intermediate Precision was carried out as many as three times testing in two different days (between-run). Then observed and calculate value of accuracy (%diff) and precision (%CV). Accuracy and precision are accepted if values at each concentration level are within<±15% of the nominal concentration, except at the LLOQ, which must be within±20%.

Limit of detection (LOD) and limit of quantification (LOQ)

The blank plasma added warfarin with the lowest concentration (0.2 ppm) where five replications were tested. The LOD value is 3 times the SD value and the LOQ is 10 times the SD value.

Carry-over

Analyzed the blank plasma sample after measuring the highest concentration calibration curve (3 ppm). Then observed the analyte response by calculating the AUC area and calculated the %carry-over. Carry-over is accepted if value<20% of the analyte response at LLOQ and<5% of the standard internal response.

Selectivity

This was performed using six plasma blanks spiked with standard warfarin at the LLOQ concentration of 0.2 ppm as well as internal standards. Measurements on blanks were also performed. The requirement for acceptable results for selectivity is a %diff value<20% and no interference in the blank at the retention time of the analyte and internal standard.

Stability

The stability tests carried out were the stability of the standard working solution, freeze-thaw, and short-term. The standard work stability was carried out with the highest and lowest concentration of warfarin standard solution stored at the same temperature as the analysis temperature for 6 h. Freeze-thaw stability was assessed using plasma samples supplemented with low and high QC concentrations standard warfarin and then stored at-80 °C and after three cycles of freezing and thawing. Short-term stability was carried out using plasma samples supplemented with low and high QC concentrations of standard warfarin that were stored at room temperature for 6 h and compared with fresh samples.

RESULTS AND DISCUSSION

Optimization of warfarin standard analysis conditions

Optimization was carried out to obtain optimal analysis conditions, in accordance with the system before the conditions are used later. In previous studies, optimization of the analytical conditions of warfarin was carried out, but optimization was carried out again because the fluorescent HPLC instrument used was different from the previous study. The results of the test with the stated HPLC conditions with different column temperatures of 40 °C, 45 °C, and 50 °C can be seen in table 1 and the chromatogram can be seen in fig. 1.

Table 1: Result of optimization of column temperatures using warfarin standard with a concentration of 1 ppm

Column temperature	Parameter system suitability test				
	Capacity factor (k') (requirement: 2-10)	Selectivity factor between R and S warfarin (required: >1)	Column efficiency (N) (requirement: >2000)	Resolution (requirement: >1.5)	Symetry factore (TF)
40 °C	k' Griseo = 2.5439	1.4674	N Griseo = 852.404	R and S-WF: 3.2009	TF Griseo = 2.190
	k' R-WF = 4.6650		N R-WF = 2533.225	R-WF and Griseo: 4.5335	TF R-WF =1.017
	k' S-WF = 6.8455		N S-WF = 18456.7512	S-WFand Griseo: 12.0068	TF S-WF = 0.610
45 °C	k' Griseo = 2.3279	1.4582	N Griseo = *12*744.738	R and S-WF: 3.308	TF Griseo = 0.945
	k' R-WF = 4.4733		N R-WF = 4993.488	R-WF and Griseo: 7.6094	TF R-WF = 0.722
	k' S-WF = 6.5229		N S-WF = 9433.489	S-WF and Griseo: 14.8791	TF S-WF = 1.028
50 °C	k' Griseo = 2.2657	1.4539	N Griseo = 1540.359	R and S-WF: 3.2963	TF Griseo = 1.045
	k' R-WF = 4.5804		N R-WF = 4925.646	R-WF and Griseo: 7.1121	TF R-WF = 1.931
	k' S-WF = 6.6593		N S-WF = 9604.0	S-WF and Griseo: 13.6134	TF S-WF = 1.268

*Description: Griseo = Griseofulvin; R-WF = R-Warfarin; S-WF= S-Warfarin

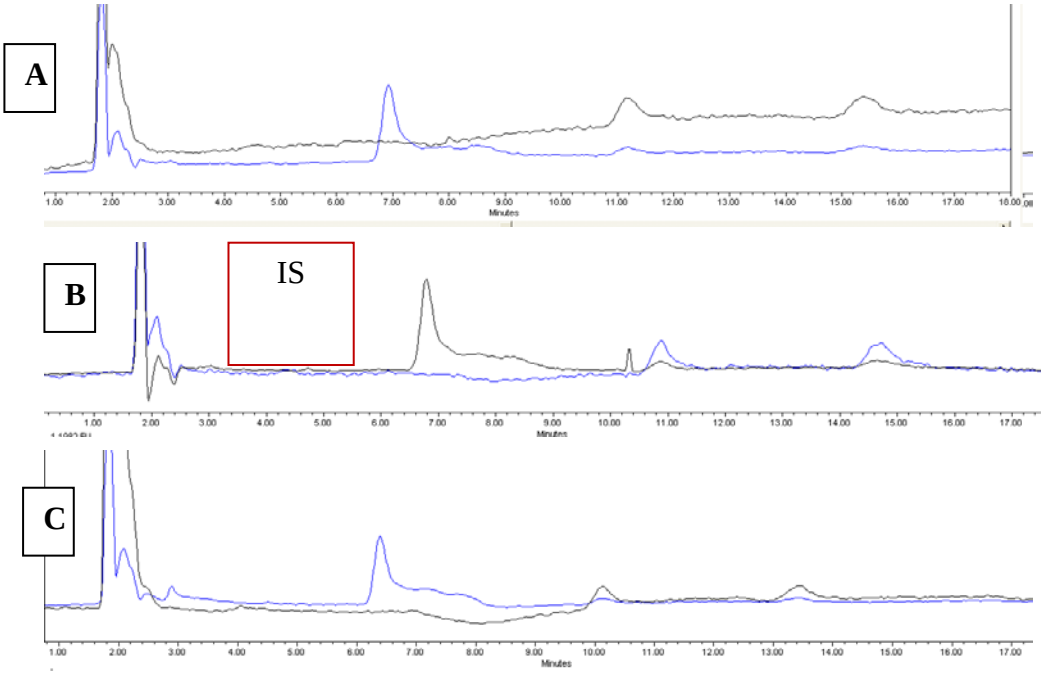

Fig. 1: Chromatogram of warfarin standard with the concentration of 1 ppm and griseofulvin as internal standard. Fig. A is a chromatogram with column temperature at 40 °C; fig. B is chromatogram with column temperature at 45 °C; fig. C is chromatogram with column temperature at 50 °C

From the test results conducted, the column temperature used in this study is 45 °C. In addition, from the optimization results, it can be seen that griseofulvin, R-warfarin, and S-warfarin can separate well, as seen from the resolution and retention time.

Optimization and suitability test of the warfarin analysis system in plasma

After obtaining optimum conditions for the standard, optimization and suitability testing of the system in plasma were carried out. The test results can be seen in table 2. These results show that 60 s produces the largest recovery value. For the system suitability test, the results can be seen in table 3. From these results, it can be seen that all parameters are sufficient.

Method validation for the analysis of warfarin in plasma samples

Calibration curve (linearity)

The calibration curve shows the relationship between the known analyte concentration and the response of the instrument used. The results obtained in this linearity test were obtained to prepare calibration curves; for R-warfarin y = 0.0705x+0.0704 with R^2= 0.978, and for S-warfarin y = 0.0513x+0.0297 with R^2 = 0.9924. The standard curve of R-warfarin can be seen in fig. 2 and that of S-warfarin in fig. 3. The calculation of the reverse concentration was performed, where 75% of 7 is 5.25. Thus, at least five points met the requirements. The results show that six out of seven concentrations met the requirements of<±15%. Thus, it can be concluded that this method has good linearity. The %diff between the reverse concentration and the actual concentration can be seen in table 4.

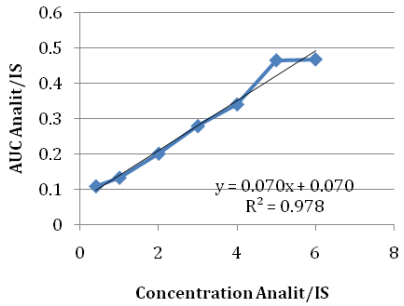

Fig. 2: Calibration curve of R-warfarin for linearity test

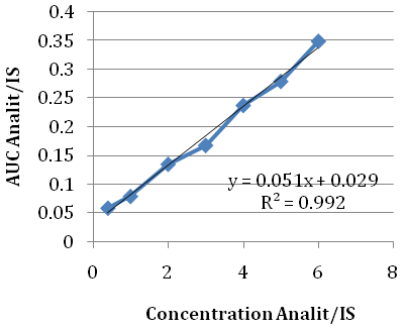

Fig. 3: Calibration curve of S-warfarin for linearity test

Table 2: Result of optimizing vortex time in plasma blank, which contain 1 ppm of warfarin standard

Vortex time (Plasma preparation)	AUC	AUC of standard WF (not in plasma)	%diff	% Recovery
Normal (Initial)	R-WF: 1101039	R-WF: 1783776	R-WF: 38.275%	R-WF: 61.725%
	S-WF: 786422	S-WF: 1617722	S-WF: 51.387%	S-WF: 48.613%
30’	R-WF: 1440702		R-WF: 19.233%	R-WF: 80.767%
	S-WF: 948730		S-WF: 41.354%	S-WF: 58.646%
40’	R-WF: 1697681		R-WF: 4.827%	R-WF: 95.173%
	S-WF: 1431884		S-WF: 11.488%	S-WF: 88.512%
60’	R-WF: 1760607		R-WF: 1.299%	R-WF: 98.701%
	S-WF: 1613032		S-WF: 0.289%	S-WF: 99.710%

Table 3: The result of system suitability tests in plasma with warfarin 1 ppm

Parameter system suitability tests				
Capacity factor (k') (requirement: 2-10)	**Selectivity factor between R and S warfarin (Required: >1)**	**Column efficiency (N) (requirement: >2000)**	**Resolution (requirement: >1.5)**	**Symmetry factor (TF)**
k' Griseo: 2.102	1.46567	N Griseo: 2125.3776	R and S-WF: 5.8624	TF Griseo: 0.898
k' R-WF: 4.5692		N R-WF: 5753.7282	R-WF and Griseo: 8.7635	TF R-WF: 0.869
k' S-WF: 6.6970		N S-WF: 4705.0324	S-WF and Griseo: 2.7962	TF S-WF: 1.078

Table 4: Table of % diff linearity with 7 concentrations of standard warfarin (0.2-3 ppm)

Warfarin	Standard internal		Warfarin		Reverse concentration (ppm)	%diff
	Concentration (ppm)	AUC	Concentration (ppm)	AUC		
R-Warfarin	0.5	6305742	0.2	688438	0.2750	37.5049
	0.5	6763626	0.5	902754	0.4473	-10.5363
	0.5	6176605	1	1246279	0.9317	-6.8269
	0.5	6276778	1.5	1756021	1.4849	-1.0096
	0.5	6029239	2	2057855	1.9214	-3.9317
	0.5	5349487	2.5	2494713	2.8081	12.3252
	0.5	6969688	3	3267702	2.8258	-5.8050
S-Warfarin	0.5	6305742	0.2	370928	0.2839	41.9291
	0.5	6763626	0.5	535435	0.4821	-3.5792
	0.5	6176605	1	833405	1.0256	2.5627
	0.5	6276778	1.5	1052743	1.3452	-10.3182
	0.5	6029239	2	1429694	2.0217	1.0852
	0.5	5349487	2.5	1491338	2.4277	-2.8922
	0.5	6969688	3	2429518	31080	3.6009

Accuracy and precision

Accuracy is a measure that shows the degree of closeness of the analysis results to the actual analyte levels, while precision is a measure of closeness between a series of analyses obtained from several measurements on the same sample. In this study, the accuracy and precision results for within run and between run are quite good. The results shown in table 5 for within run and table 6 for between run and met the requirements.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD and LOQ values obtained were LOD of 0.0674 ppm (R-warfarin) and 0.0897 ppm (S-warfarin). As for the LOQ value, the LOQ is 0.225 ppm (R-warfarin) and 0.298 ppm (S-warfarin).

Carryover

Carryover is a parameter for measured residual analyte from the previous injection. The results obtained in this test are shown in table 7. In these results, carryover met the requirements (<5%), so it can be said that in this method, there was no significant interference with multiple testing.

Selectivity

Selectivity is a parameter that see the ability to separate the analyte in the form of warfarin and griseofulvin from the matrix. For the %diff results in plasma, it meets the requirements where the concentration is<±20%, namely 6.1962% (R-Warfarin) and 4.0301% (S-Warfarin) and there is no interference.

Stability

Stability of the standard working solution

This test shows that the concentration of warfarin will decrease if stored in room temperature. The decrease in concentration can be seen in table 8. The decrease occurs because once warfarin has been dissolved, it is stable for approximately 4 h at room temperature [3].

Table 5: The result of within-run accuracy and precision

Concentration (ppm)	Replication	Calculated concentration (ppm)		Accuracy (%diff)		Precision (%RSD)	
		R-WF	S-WF	R-WF	S-WF	R-WF	S-WF
0.2	1	0.1535	0.2536	-14.2365	6.5963	13.1076	14.0230
	2	0.1446	0.1773				
	3	0.1831	0.2064				
	4	0.1764	0.1969				
	5	0.2000	0.2318				
Average		0.1715	0.2132				
0.6	1	0.5352	0.5831				
	2	0.4706	0.5752	-5.9762	0.9809	11.2164	8.8855
	3	0.6050	0.6974				
	4	0.5781	0.5641				
	5	0.6317	0.6097				
Average		0.5641	0.6059				
1.5	1	1.4723	1.35499	2.3368			
	2	1.5776	1.4271		-6.3666	3.1705	3.2020
	3	1.5894	1.4698				
	4	1.5080	1.3933				
	5	1.52798	1.3773				
Average		1.53505	1.4045				
2.25	1	2.0961	1.5346	0.1723	-11.8519	7.6581	14.0202
	2	2.1366	2.1791				
	3	2.5373	2.2127				
	4	2.2582	2.1147				
	5	2.2412	1.9255				
Average		2.2539	1.9933				

Table 6: The result of between run accuracy and precision with warfarin concentration are 0.2; 0.6; 1.5; 2.25 ppm

Testing day	Concentration (ppm)	Replication	Calculated concentration (ppm)		Accuracy (%diff)		Precision (%RSD)	
			R-WF	S-WF	R-WF	S-WF	R-WF	S-WF
Day 1	0.2	1	0.2257	0.1949	10.7986	6.0353	4.5618	14.231
		2	0.2094	0.1944				
		3	0.2278	0.2469				
	Average		0.22096	0.2121				
	0.6	1	0.61399	0.5994	1.3119	3.3461	2.8482	4.0668
		2	0.5883	0.6482				
		3	0.6213	0.6127				
	Average		0.6079	0.6201				
	1.5	1	1.5215	1.5387	3.3714	2.6259	1.6294	2.0564
		2	1.5628	1.5714				
		3	1.5674	1.5081				
	Average		1.5506	1.5394				
	2.25	1	2.2492	2.2301				
		2	2.2833	2.2726	0.5507	0.0742	0.8083	0.9436
		3	2.2547	2.2523				
	Average		2.2624	2.2517				
Day 2	0.2	1	0.2167	0.2380				
		2	0.2491	0.1843	11.4149	3.0451	10.6928	13.7084
		3	0.2027	0.1959				
	Average		0.2228	0.2061				
	0.6	1	0.6346	0.5764	2.5281	-1.5797	2.7686	4.9767
		2	0.6079	0.6243				
		3	0.60299	0.5708				
	Average		0.6152	0.5905				
	1.5	1	1.5339	1.5198	0.5597	3.0932	2.0582	1.6004
		2	1.5175	1.5688				
		3	1.4738	1.5505				
	Average		1.5084	1.5464				
	2.25	1	2.2572	2.2640	0.1709	0.1803	0.3542	0.4554
		2	2.2447	2.2435				
		3	2.2596	2.2546				
	Average		2.2538	2.2541				

Table 7: The result of carryover for R-Warfarin, S-Warfarin, and IS (griseofulvin)

Parameter	AUC R-warfarin	AUC S-warfarin	AUC IS
LLOQ	408140.2	223682.2	4327710.8
Blanko	195550	31721	3854641
%carry over	0.047912	0.014181	0.0890688

Table 8: The result of stability test of standard working solution

Concentration	0 h		6 h		%diff	
(ppm)	AUC R-warfarin	AUC S-warfarin	AUC R-warfarin	AUC S-warfarin	R-warfarin	S-warfarin
0.2	440858.5	268731.5	231838	137617	-47.4122	-48.7901
3	3135289	2265114	2389730	1187332	-23.7796	-47.5818

Freeze-thaw stability

The results show that freezing and thawing reduced the concentration of the analyte. The decrease in concentration can be seen in table 9.

Short-term stability

The results show that there was a decrease in concentration, but it was quite stable because the decrease was not too large. Changes in concentration are shown in table 10.

Table 9: The result of the freeze-thaw stability test

Concentration (ppm)	Warfarin	No freezing	After 3 freezing cycles	%diff
		AUC	AUC	
0.2	R	474976	444924	-6.32706
	S	255488	226504	-12.7962
2.25	R	2090566	1748558	-16.3596
	S	1467603	1276700	-13.0078

Table 10: The result of short term stability test

Concentration (ppm)	Warfarin	0 h	6 h	%diff
		AUC	AUC	
0.2	R	474976	471387	-0.75562
	S	255488	242171	-5.49901
2.25	R	2090566	1881836	-9.98438
	S	1467603	1283066	-12.574

In validating an analysis method, optimization must be carried out. Optimization is carried out to obtain optimal analysis conditions in accordance with the system before the conditions are used later. In the standard optimization of warfarin, optimization was carried out on a column. When the column temperature rises, the viscosity of the mobile phase decreases so that the flow resistance in the column and the system back pressure become smoother. In addition, an increase in temperature increases diffusion during the chromatographic process. The decrease in mobile phase viscosity and the increase in diffusion cause the resulting peaks to be slimmer and taller, so as to improve peak quality. In addition, back pressure at lower temperatures can accelerate the retention time. However, if the temperature is too high, it can damage the column and the separation is not good [14]. In accordance with this, the column temperature chosen is 45 °C because it produces good chromatograms and parameters and is not too high in temperature.

After optimization of the warfarin standard was carried out, optimization was also carried out on plasma containing warfarin. In the optimization and suitability test of the system in plasma, optimization was carried out on the vortex time. Vortexing is performed to stir and mix compounds and solutions from the sample so that all components can be fused and more extracted. Therefore, the longer the vortexing time, the greater the degree of mixing.

In this study, the plasma was prepared by adding standard warfarin and internal standard in the form of griseofulvin. Griseofulvin was used as an internal standard because griseofulvin has several properties similar to warfarin, including its solubility, as it is practically insoluble in water and similar log P value; the log P value of griseofulvin is 2.18. In addition, griseofulvin can also fluoresce because it has a conjugated cyclic structure [15].

The conditions and methods for the best optimization were also tested regarding the suitability of the system to determine whether these conditions are suitable for testing multiple times on one day. The tests included the capacity factor, which indicates how strongly an analyte can be retained in the stationary phase; selectivity (α), which is the relative retention value of each component by the stationary phase, column efficiency, where an efficient column will produce good resolution, and the resolution value, which is the ability of the column to separate two analytes [11]. The results show good parameter values, so the test conditions used were appropriate.

The appropriate test conditions were then validated. The validation guidelines used were the 2019 EMA (European Medicine Agency) guidelines on bioanalysis validation. The 2019 EMA guidelines are the most recent and were approved by ICH (International Council Harmonization). There are several parameters in the 2019 EMA guidelines. The parameters carried out in this study included linearity, accuracy and precision, selectivity, LOD and LOQ, carryover, and stability.

For the calibration curve, a minimum of six concentrations should be used in accordance with the provisions in EMA 2019. In this study, seven concentrations were used, namely 0.2, 0.5, 1, 1.5, 2, 2.5, and 3 ppm. This concentration range was chosen because warfarin levels in plasma are in the range of 0.5-2.5 ppm [16]. From this linearity, it was found that six out of seven points met the requirements. In the EMA 2019 guidelines, at least 75% meets the requirements. Thus, it can be concluded that the relationship between concentration and AUC is directly proportional and linear [12].

For accuracy and precision, good results were also obtained and met the requirements of both within run and between-run tests. This shows that the analysis results were close to the actual results and there are close results from several tests. The selectivity showed that this method is quite selective for warfarin among the complex matrix. This is also related to the carryover, where the results show the absence of the carryover from the previous test and matrix.

LOD indicates the lowest concentration that can be detected, while LOQ is the lowest concentration that can be quantified. There is no requirement for LOD and LOQ, but the lower the result, the more sensitive the method and instrument. The results show quite good values with LOD of 0.0674 ppm (R-Warfarin) and 0.0897 ppm (S-Warfarin) and LOQ of 0.225 ppm (R-Warfarin) and 0.298 ppm (S-Warfarin).

This stability test was conducted to show the durability of raw warfarin and plasma under certain conditions. From the test results, it was found that raw warfarin was not stable at room temperature for more than 4 h after being dissolved [3]. Warfarin in plasma was quite stable at cold temperatures, but several cycles of cooling and thawing reduced the concentration. This also applied to storage at room temperature.

CONCLUCION

The retention time for griseofulvin was 6-7.5 min; R-warfarin was 10-11.5 min; S-warfarin was 14-16 min. Linearity existed at concentrations of 02.2-3 ppm with y = 0.0705x+0.0704 with R^2 = 0.978 for R-warfarin and y = 0.0513x+0.0297 with R^2 = 0.9924. Based on the results of several validation parameters, this analytical method met the validation requirements with LOD values of 0.0674 ppm (R-Warfarin) and 0.0897 ppm (S-Warfarin) and LOQ values of 0.225 ppm (R-Warfarin) and 0.298 ppm (S-Warfarin). Therefore, it can be concluded that this method can be used for sample measurement in warfarin pharmacokinetic studies.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICTS OF INTERESTS

There are no conflicts of interest

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