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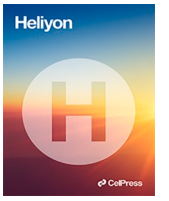

Research article

Determination of warfarin in volumetric absorptive microsampling by liquid chromatography-tandem mass spectrometry

Yahdiana Harahap [a,b,*], Callista Andinie Mulyadi [a], Hary Sakti Muliawan [c], Habibah A. Wahab [d]

[a] Faculty of Pharmacy, Universitas Indonesia, Depok, 16424, Indonesia
[b] Faculty of Military Pharmacy, the Republic of Indonesia Defense University, Bogor, 16810, Indonesia
[c] Department of Cardiology and Vascular Medicine, Faculty of Medicine-Universitas Indonesia Hospital, Depok, 16424, Indonesia
[d] School of Pharmaceutical Sciences, Universiti Sains Malaysia, Pulau, Pinang, 11800, Malaysia

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ABSTRACT

Objective: This study aims to develop and validate bioanalytical method for quantifying warfarin in VAMS samples using liquid chromatography tandem mass spectrometry (LC-MS/MS), directly implementing the method to patients receiving warfarin therapy.
Methods: The UPLC-MS/MS method was developed and optimized, with quercetin as the internal standard. Sample preparation was carried out using protein precipitation with methanol-acetonitrile (1:3 v/v).
Results: Chromatographic separation was achieved using Acquity® UPLC BEH C18 column with 0.1 % formic acid-acetonitrile-methanol (30:69:1 v/v) as mobile phase, in isocratic elution. Multiple Reaction Monitoring (MRM) detection was done using *m/z* values of 307.10 → 161.06 for warfarin and 301.03 → 150.98 for quercetin as internal standard, using Electrospray Ionization (ESI) negative ion source. The clinical application of the bioanalytical method was carried out on 25 patients receiving warfarin therapy at Universitas Indonesia Hospital and warfarin levels were well within the calibration range from 6.05 to 431.39 ng/mL.
Conclusion: A novel method has been developed to analyze warfarin in VAMS samples. This method has been fully validated according to guideline from FDA 2022 and is linear in the range of 5–500 ng/mL and the value of $r \geq 0.9977$, and successfully applied for the analysis of warfarin in VAMS samples of clinical patients.

1. Introduction

Thromboembolism is a hemostasis disorder that occurs due to cardiovascular disease, which can be prevented with anticoagulants [1]. Warfarin is an anticoagulant that exerts its mechanism of action by inhibiting vitamin K epoxide reductase complex sub-unit 1 (VKORC1), which may affect the synthesis of clotting factor II, VII, IC, and X, as well as anticoagulant protein C and S [2,3]. Warfarin has narrow therapeutic index and its use requires close monitoring. Inadequate or excessive anticoagulation effects may result in

* Corresponding author. Faculty of Pharmacy, Universitas Indonesia, Depok, 16424, Indonesia.
E-mail address: yahdiana03@yahoo.com (Y. Harahap).

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life-threatening thromboembolic or bleeding complications [4]. In addition, significant inter-individual variations in warfarin levels have been observed during therapy, causing difficulties in the warfarin therapy management, necessitating routine monitoring during therapy [5].

Therapeutic Drug Monitoring (TDM) of warfarin is usually performed by measuring the International Normalized Ratio (INR) to estimate its pharmacodynamic effect [6,7]. However, clinical testing involving pharmacokinetics and drug interactions generally requires quantitative measurement of warfarin in the bloodstream. Quantitative analysis of warfarin in blood can also be useful in confirming drug resistance, toxicity, or ensuring patient compliance, which are poorly reflected solely by INR value [8]. Better management of warfarin therapy can be achieved through the combination of pharmacokinetic and pharmacodynamic monitoring of warfarin.

Quantitative measurement of warfarin in blood samples have been attempted widely using plasma samples [5,9–11], and serum [12] taken using conventional venous sampling. Although effective, conventional sampling is considered invasive, particularly to patients subjected to frequent blood tests. Microsampling, on the other hand, offers more convenient sampling process through the collection of smaller sample volume and patient-centric sampling [13]. Dried blood spots is a microsampling technique that has been used widely, starting out as a technique for human neonatal screening [14], and further implemented into quantitative bioanalysis of various drugs in blood samples [15–21]. Volumetric absorptive microsampling (VAMS) is a novel dried microsampling approach which is able to overcome limitations of DBS method, namely hematocrit effect and homogeneity issue [22]. Despite its advantages, a number of limited studies in VAMS suggested that this method has been yet to be explored further for its potential to use in clinical and nonclinical studies.

This study aims to develop a LC-MS/MS method for determination of warfarin in VAMS samples using quercetin as internal standard. It is anticipated that the developed and validated method can be employed to assess warfarin therapy in patients, serving as a valuable tool for physicians to monitor the therapy alongside INR testing.

2. Material and methods

2.1. Chemical and reagents

Warfarin was obtained from Indonesian Food and Drug Authority using Indonesian Pharmacopoeia Reference Standards (Jakarta, Indonesia). Internal standard Quercetin was also obtained from Indonesian Food and Drug Authority using Indonesian Pharmacopoeia Reference Standards (Jakarta, Indonesia). Acetonitrile, methanol, and formic acid were purchased from Merck (Darmstadt, Germany). Ultra-pure water was provided by Water Filter System from Sartorius (Göttingen, Germany). Human whole blood used in development and method validation was from The Indonesian Red Cross (Jakarta, Indonesia). Mitra® VAMS® microsampling devices was from Neoteryx (Torrance, USA).

2.2. Preparation of stock and working standard solution

Warfarin and quercetin stock solution were prepared in methanol and acetonitrile, respectively, at 1.0 mg/mL concentration. Warfarin stock solution was diluted in methanol to obtain working standard solution. Calibration standards was prepared by dilution of working standard solution in whole blood to obtain a series of calibration curve in the range of 5–500 ng/mL. Quality Control (QC) solutions were prepared by diluting warfarin stock solution in whole blood, at the concentration of 15 ng/mL (QCL), 250 ng/mL (QCM), and 375 ng/mL (QCH). Quercetin working standard solution was prepared by diluting quercetin stock solution in acetonitrile.

2.3. LC-MS/MS conditions

LC-MS/MS analyses were performed on ACQUITY™ UPLC system (Waters Corp., Milford, USA) coupled with Xevo TQD Triple Quadrupole mass spectrometer (Waters Corp., Manchester, UK) with negative electrospray ionization (ESI-) mode. System control and data acquisition, as well as analysis was performed using MassLynx™ NT4.1 and QuanLynx™ (Waters Corp., Milford, USA). Chromatographic separation was achieved using ACQUITY® UPLC BEH C18 column (1.7 μm, 100 mm × 2.1 mm, Waters Corp., Milford, USA).

The mass spectrometer was set in multiple reaction monitoring (MRM) and electrospray ionization negative (ESI-) mode. The operating conditions were as follows: capillary voltage was 3.5 kV; desolvation gas temperature was 350 °C; desolvation gas flow rate 650 L/h; cone voltage was 34 V for both warfarin and quercetin; collision voltage were 18 and 22 V for warfarin and quercetin, respectively. Transition from precursor into product ion was set at *m/z* value of 307.10 → 161.06 for warfarin and 301.03 → 150.98 for quercetin.

2.4. Optimization of chromatographic separation

Optimization of the chromatographic separation was performed by injecting warfarin and quercetin 1000 ng/mL solution into the LC-MS/MS system using several combinations of mobile phases which comprises of: formic acid 0.1 % – acetonitrile; formic acid 0.1 % – formic acid 0.1 % in acetonitrile; formic acid 0.1 % – acetonitrile – methanol; formic acid 0.2 % – acetonitrile; acetic acid 0.1 % – acetonitrile; acetic acid 0.1 % – acetonitrile – methanol. Further optimization was carried out regarding the composition of the chosen mobile phase combination, and the flow rate of mobile phase (0.1; 0.15; and 0.2 mL/min) to obtain the optimal chromatographic

condition to analyze warfarin in VAMS samples.

2.5. Optimization of sample preparation

Sample preparation was carried out using the protein precipitation method. The VAMS tip which contained 30 μL of blood and had been dried according to the optimum drying time (30, 60, and 120 min) was put into a 1.5 mL Eppendorf tube. A certain volume of extraction solution according to the optimization result and 20 μL of standard quercetin solution in 10 ppm were added to the tube. The tube was shaken with a vortex and sonicated according to the optimum time from the optimization results, then centrifuged for 5 min at 5000 rpm. The supernatant was removed and evaporated, reconstituted, and 10 μL of sample was injected into the LC-MS/MS. Optimization of sample preparation was carried out on the parameters such as sample drying time, type of extraction solvent, volume of extraction solvent, as well as vortexing and sonication time.

2.6. Method validation

The method used in this research was a novel analytical method. Therefore, a full validation was carried out in accordance to Food and Drug Administration (FDA) guideline [23].

2.7. Selectivity

Selectivity is tested by analyzing six individual sources of whole blood that is applied to VAMS devices. The response from the blank sample should be less than 20 % lower limit of quantification (LLOQ) peak area, and less than 5 % peak area for internal standard (IS).

2.8. Linearity

Aliquots of blank whole blood were spiked with warfarin working standard solutions to obtain nine calibration levels of samples containing warfarin in the range of 5–500 ng/mL. Blank, zero, and calibration samples were applied to VAMS devices in three replicates and analyzed. Calibration curves were calculated relating the area ratios from warfarin peaks to the IS peak and with the nominal concentrations of the calibration samples.

2.9. Accuracy and precision, reinjection reproducibility

For accuracy and precision, aliquots of blank whole blood were spiked with warfarin working solutions to obtain four concentrations: LLOQ, QCL, QCM, and QCH, with five replicates per concentration. The samples were extracted and analyzed in three analytical batches to perform within-run and between-run accuracy and precision.

Reinjection reproducibility is assessed by analyzing calibration standards and five replicates of QCL, QCM, and QCH, respectively, and then reinjecting the batch after storage.

2.10. Recovery

Aliquots of blood were spiked with QCL, QCM, and QCH concentrations and applied to VAMS. samples were extracted and injected to the LC-MS/MS system. Standard solutions containing warfarin on QCL, QCM, and QCH were also injected to the LC-MS/MS system. Both assays were performed with three replicates for each concentration.

2.11. Matrix effect

Standard solutions of warfarin were diluted in blood from six different sources to obtain QCL and QCH concentrations, which then applied to VAMS device, and processed before injected into the LC-MS/MS system. The assay was performed with three replicates for each concentration and blood sources.

2.12. Dilution integrity

Warfarin was diluted in whole blood until twice the QCH concentration (exceeding ULOQ) was obtained. This sample was then taken using VAMS and dried. Sample preparation was carried out and the extraction solvent was taken and diluted until it reached half and quarter of the initial concentration. The assay was performed with five replicates for each concentration. Dilutions performed on samples with concentration exceeding upper limit of quantification (ULOQ) should not affect the accuracy and precision of the method.

2.13. Stability

Standard solutions of warfarin were diluted in blood to obtain QCL and QCH concentrations, which then applied to VAMS device. The stability of analyte in VAMS was investigated at room temperature for 24 h, in the autosampler for 12 h after sample processing,

and for long-term storage for 30 days at room temperature after the samples were stored in a seal bag with desiccant. Stability testing was also investigated to the stock solution on room temperature for 24 h, and at −20 °C for 30 days.

2.14. Clinical application of the method

For the clinical application of the method, the study was conducted in outpatient cardiology clinic and inpatient wards at Universitas Indonesia Hospital, Depok, Indonesia. Ethical approval from Universitas Indonesia Hospital Ethics Committee was obtained prior to the initiation of this study, and this study was conducted according to the Declaration of Helsinki. Informed consent was obtained from all the participants enrolled in this study.

A total of twenty-five patients, comprised of 14 females and 11 males, who received warfarin maintenance therapy were recruited. Prior to the recruitment, all the patients were screened according to the inclusion and exclusion criteria. The inclusion criteria were patients using warfarin in the outpatient and inpatient clinics at the Universitas Indonesia Hospital, aged 18–90 years at the time of blood collection, and patients have received a warfarin regimen at a dose of 1–10 mg/day for at least 14 days (has reached steady state conditions). Patients were excluded if they have liver and kidney disorders.

3. Results and discussion

3.1. LC-MS/MS method

The analytical method developed uses mass spectrometry as a detector with Multiple Reaction Monitoring (MRM) mode and a negative mode Electrospray Ionization (ESI) ion source. The analytical method was adapted and modified from the one used by Zhang (2018) which also uses quercetin as internal standard [24]. Warfarin and quercetin detection uses Multiple Reaction Monitoring (MRM) mode, because the analysis is carried out simultaneously for the detection of quercetin and warfarin. Negative ESI is used because it is suitable for compounds that are acidic, or have acidic groups such as carboxylic acids, benzoic acid and its derivatives, phenols, and heterocycles [25].

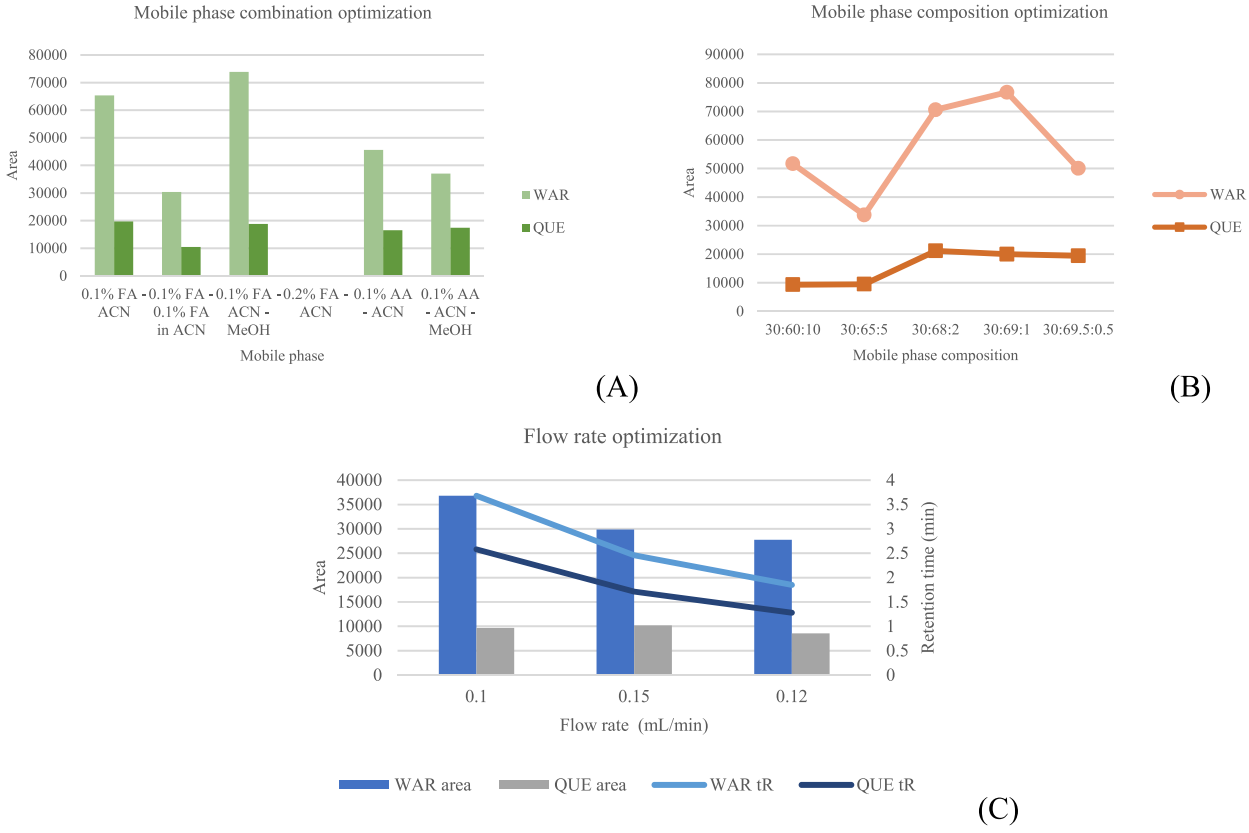

Fig. 1. Result of (A) optimization of mobile phase combination, (B) optimization of mobile phase composition using 0.1 % formic acid-acetonitrile-methanol, and (C) optimization of mobile phase flow rate.

3.2. Optimization of chromatographic separation

The chromatographic optimization was conducted using the solution of warfarin and quercetin. For the first optimization, six combinations of mobile phase were tested, and it is determined that the optimal mobile phase combination was 0.1 % formic acid with acetonitrile and methanol based on the peak area and shape of the peak. 0.1 % formic acid-acetonitrile-methanol and 0.1 % formic acid-acetonitrile gave overall similar response, but 0.1 % formic acid-acetonitrile gave a broader peak, hence the combination of 0.1 % formic acid-acetonitrile-methanol was chosen. The combination was further modified, particularly in the organic phase composition of the mobile phase. Five compositions of the mobile phase were tested, namely 30:60:10 v/v; 35:65:5 v/v; 30:68:2 v/v; 30:69:1 v/v; 30:69.5:0.5 v/v. It was determined that 0.1 % formic acid-acetonitrile-methanol with the composition of 30:69:1 gave the best elution of warfarin and quercetin. The flow rate of the optimal mobile phase was optimized and evaluated based on the peak area and shape, and retention time. Flow rate of 0.2 mL/min gave the sharpest peak and the quickest retention time, but there was a significant decrease of peak areas and the peak was separated poorly. On the contrary, flow rate of 0.1 mL/min shows an increase of peak area, along with a broader peak with notable tailing. 0.15 mL/min was chosen as the final flow rate based on properly separated peak with significant peak area.

The choice of mobile phase plays a role in determining the shape and peak area of the analyte chromatogram, as well as the retention time of the analyte [26]. The selection of the mobile phase is based on the solubility of the analyte and the compatibility with the ionization mode in the analysis. In addition, because this method uses the ESI ionization technique, the volatility and ability of the mobile phase to ionize the analyte is also a consideration. Acetonitrile and methanol as organic solvent has a low surface tension so it can reduce the surface tension of the mobile phase and increase the sensitivity of analysis, though the vaporization enthalpy of acetonitrile is much higher than methanol and thus gives higher response [27]. Addition of weak acid in the composition of the mobile phase may help facilitate ionization of the analyte and increase negative ESI response [28]. Formic acid is usually used in ESI positive method, but studies shown that while it decreases ionic strength, formic acid may also increase signal-to-noise ratio in ESI negative mode [29]. In addition, the mobile phase flow rate can influence the total analysis time, as well as the area and shape of the chromatogram. Fig. 1 shown the result of the chromatographic optimization, which consists of optimization of mobile phase combination (Fig. 1-A), composition of mobile phase 0.1 % formic acid-acetonitrile-methanol (Fig. 1-B), and flow rate ((Fig. 1-C).

Based on the optimization that has been carried out, optimal analysis conditions are determined as follows: detection of analytes and internal standards was carried out using mass spectrometry with negative Electrospray Ionization (ESI) ionization mode with *m/z* values of 307.10 > 161.06 for warfarin; and 301.03 > 150.98 for quercetin as the internal standard. Chromatographic conditions were carried out using a UPLC BEH C18 column as stationary phase, a mobile phase of 0.1 % formic acid in water with acetonitrile and methanol with a composition of 30:69:1, with isocratic elution mode and a flow rate of 0.15 mL/min. This method will be applied during subsequent extraction optimization, validation of the bioanalysis method, and analysis of samples from the subject. Retention time (tR) for warfarin and quercetin were approximately 2.5 and 1.8 min. Blank and sample from patients are shown in Fig. 2A and B. All analytes were separated well, and no significant interference were observed on the retention time of the analytes.

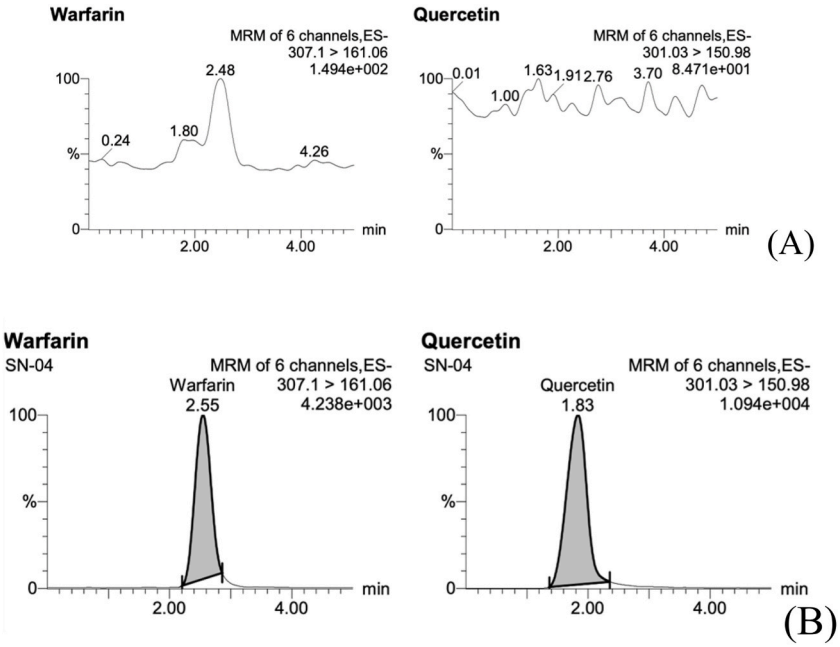

Fig. 2. Representative chromatogram of (A) blank VAMS sample; and (B) VAMS sample from patient who received warfarin therapy.

3.3. Optimization of sample preparation

Optimization of the VAMS sample preparation method was carried out to determine the method that could extract the analyte with the largest yield without affecting the stability of the analyte. The first optimization carried out was of the drying time. The stability of analytes in dried blood samples such as DBS and VAMS should not just be tested in storage after the sample is dried, but also needs to be tested for stability during the drying process [30]. The VAMS samples were dried for 30, 60, and 120 min and analyzed. The result showed that there was a significant increase in peak area with longer drying time, suggesting that blood has not dried fully by 30 and 60 min and thus were unstable in the VAMS samples. Therefore, the optimal drying time for VAMS samples was 120 min.

The optimal extraction solvent must be able to dissolve the analyte, as to increase the recovery from the biological matrix. Acetonitrile and methanol are water-miscible protein precipitants with good efficiency, in the order of acetonitrile > methanol [31]. In addition, the analytes were highly soluble in these two solvents, making it a suitable solvent for the extraction. Optimization of the extraction solvent was carried out using acetonitrile, methanol, and several combinations of these solvents. Methanol-acetonitrile (1:3 v/v) was chosen for the extraction solvent. This combination was optimized further to determine the suitable volume of the extraction solvent. Based from the result of extraction using 500, 750, and 1000 μL of methanol-acetonitrile (1:3 v/v), 500 μL was determined as the optimal volume of extraction solvent. The volume of the extracting solvent influences the amount of analyte that can be withdrawn from the matrix. Furthermore, optimization of vortex (1, 1.5, 2 min) and sonication times (5, 10, 15 min) was also carried out simultaneously. Vortexing and sonication are some of the steps to facilitate the release of analytes from the matrix, thereby increasing efficiency and speeding up the extraction process. This step also denatures and aggregates the protein in the matrix, hence it was followed by centrifugation to separate the particulate from the supernatant [32]. Optimization of sample preparation is carried out sequentially, from the VAMS drying time (Fig. 3-A), followed by type of extraction solvent (Fig. 3-B), volume of the chosen extraction solvent which is methanol-acetonitrile (1:3, v/v) (Fig. 3-C), and optimization of vortex and sonication time (Fig. 3-D).

Based on the optimization that has been carried out, the optimal sample extraction process are as follows: a VAMS tip that contains 30 μL of blood and has been dried for 2 h is inserted into a 1.5 mL Eppendorf tube. 500 μL of methanol-acetonitrile extraction solvent (1:3, v/v) and 20 μL of standard solution in 10 ppm quercetin were added to the tube. The tube was shaken with a vortex for 1.5 min, then sonicated for 15 min. The tube was then centrifuged for 5 min at 10,000 rpm, and the supernatant was evaporated until dry. The residue obtained was reconstituted with 100 μL of methanol-acetonitrile (1:1, v/v), and 10 μL of sample was injected into the LC-MS/MS system.

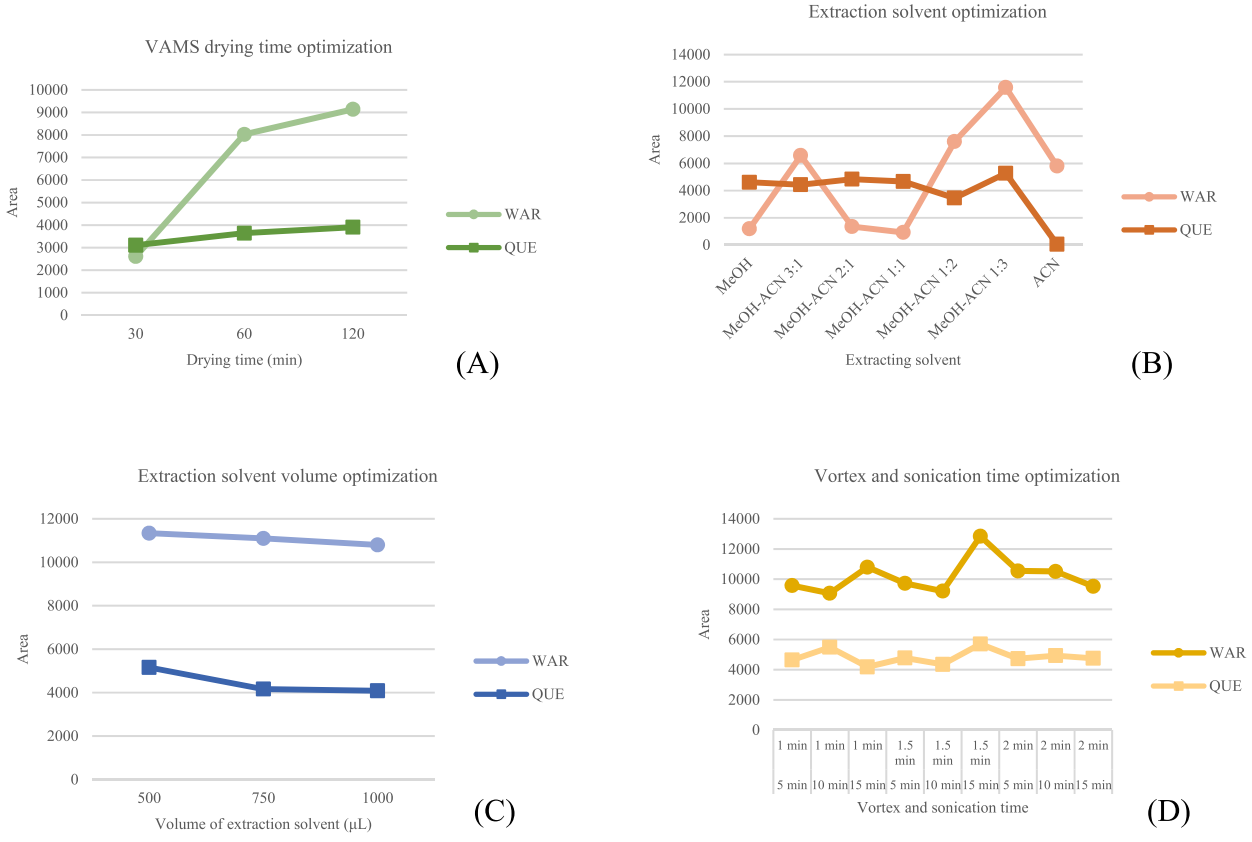

Fig. 3. Result of (A) optimization of VAMS drying time, (B) optimization of extraction solvent composition, (C) optimization of extraction solvent volume using methanol-acetonitrile (1:3, v/v), and (D) optimization of vortex and sonication time.

3.4. Method validation

The recovery test was performed using three replica samples at three concentrations of Validation of the bioanalytical method was carried out in full validation according to the guidelines from FDA 2022. Validation carried out included parameters for determining LLOQ, calibration curve, selectivity, carry-over, dilution integrity, matrix effects, recovery, and stability [23]. Based on the tests carried out, all parameters have met the validation requirements in the guidelines, so the method can be used to analyze samples from patients.

3.5. Selectivity

Selectivity is evaluated to verify that the developed analytical method is able to differentiate analyte and internal standard from possible interferences in the sample. No significant interference was observed in warfarin retention time (<20 %) and internal standards (<5 %) in VAMS samples from six different whole blood sources.

3.6. Linearity

The relationship between peak area ratio of the analyte to the IS with nominal concentration was described using linear regression. The method was linear from 5 to 500 ng/mL. The correlation coefficient for 3 runs was greater than 0.990 in 3 validation runs. The resulting calibration curve can be seen on Fig. 4.

3.7. Accuracy and precision, and reinjection reproducibility

Accuracy and precision was assessed in LLOQ, QCL, QCM, and QCH with five replicates per concentration. The method was accurate with %diff ranging from −18.64 to 6.39 % for LLOQ, and ranging from −13.17 % to 14.31 % for QCL, QCM, and QCH in intra-day and inter-day runs. The CV was 9.65 % for LLOQ, and 6.83 %–9.26 % for QCL, QCM, and QCH. The result of the accuracy and precision testing is described on Table 1.

Reproducibility was assessed by reinjecting five replicates of QCL, QCM, and QCH samples. The accuracy of the reinjected samples was shown by %diff ranged from −10.75 % to 11.35 % during the initial injection, and −14.39 %–14.85 % during the reinjection. The precision was assessed with the CV ranging from 3.23 % to 7.63 % during the initial injection and 2.29 %–8.69 % when reinjected after storage (Table 2). This indicates that the method was accurate and precise, and the processed samples were viable to be reinjected after storage in case of sudden interruption or instrument problems.

4. Recovery

Recovery was not included in validation parameters but should be evaluated to determine the efficiency of sample extraction [33]. Recovery was evaluated in QCL, QCM, and QCH samples, as well as the internal standard. The result ranged from 77.57 to 81. 71 % for QCL, QCM, and QCH samples and 97.50 % for quercetin as internal standard. The CV was 1.31–6.90 %, which indicates that the recovery was consistent during the assay.

4.1. Matrix effect

Matrix effect was evaluated using blood from six different sources which then applied to the VAMS devices. The result showed that the %diff and CV from each matrix and concentration was not greater than 15 %, which indicates that the use of VAMS samples from six different sources of blood did not affect the accuracy and precision of the method. The result of matrix effect is shown on Table 3.

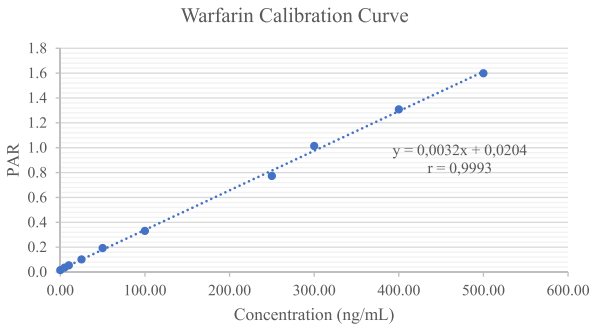

Fig. 4. Calibration curve of warfarin.

Table 1
Within-run and between-run accuracy and precision of warfarin in VAMS samples.

Concentration (ng/mL)	Within-run			Between-run		
	Average conc. (ng/mL)	%diff	CV	Average conc. (ng/mL)	%diff	CV
5.00	4.53	−18.64 to −9.37	5.20	4.52	−18.64 to 6.39	9.65
15.00	14.75	−12.00 to 4.39	7.70	14.84	−13.17 to 12.87	9.26
250.00	262.52	−2.90 to 11.25	6.09	250.15	−12.00 to 14.31	8.46
375.00	373.21	−6.50 to 8.96	8.19	374.42	−9.12 to 8.98	6.83

Table 2
Reproducibility of reinjected QC samples.

Concentration (ng/mL)	Initial injection			Reinjection		
	Average conc. (ng/mL)	%diff	CV	Average conc. (ng/mL)	%diff	CV
15.00	16.70	2.72 to 11.35	3.23	16.16	−0.28 to 14.29	5.92
250.00	252.91	−10.75 to 10.66	7.63	229.12	−14.39 to 5.48	8.69
375.00	356.39	−9.61	6.39	420.34	9.27 to 14.85	2.29

4.2. Dilution integrity

Dilution integrity is evaluated to anticipate samples that exceeded the concentration range of the analysis. For this method, the dilution was performed during the extraction process because this assay used dried VAMS samples. The result showed that the dilution performed on samples with concentration greater than ULOQ did not affect the accuracy and precision of the method, demonstrated by %diff and %CV value from each concentration which did not exceed 15 %. Table 4 described the result of dilution integrity testing.

4.3. Stability

Concentration of the analyte in the samples should not be affected by sample extraction, analysis, and storage conditions. The stability testing was investigated on VAMS samples, processed samples in the autosampler, and stock solution of warfarin and quercetin. The result showed that VAMS samples were stable for 24 h at room temperature which is usually used during sample processing, and for 30 days under the real storage conditions (samples were placed in seal bag with desiccant and stored under room temperature). Post-processing stability was evaluated to ensure that processed sample that was stored on autosampler is stable during the waiting time of the automatic injection. The result showed that the processed samples were stable for 12 h in the autosampler. In addition, stock solution stability was also evaluated to ensure that analyte and internal standard stock solution were stable during the time of analysis. The stock solution of warfarin and quercetin were stable for 24 h in room temperature, and in −20 °C for 30 days.

4.4. Clinical application

To ensure that the bioanalytical method that has been developed and validated can be applied in clinical conditions, the method was applied to patients receiving warfarin therapy. The method was applied to patients in outpatient and inpatient clinics at the University of Indonesia Hospital who received warfarin therapy in their treatment regimen. 25 patients who met the inclusion criteria and were willing to participate in the research was recruited. Blood collection for VAMS samples is carried out from the fingertip using the finger prick method using a sterile disposable lancet. Blood collection for INR examination was carried out from the median cubital vein at the patient's elbow and analysis was carried out at the Clinical Pathology Laboratory of the Universitas Indonesia Hospital.

Based on the results of analysis of 25 VAMS samples, the highest concentration of warfarin was 431.39 ng/mL in patient SN-22, and the lowest concentration was 6.05 ng/mL in patient SN-14. The average measured concentration of warfarin at steady state conditions was 128.17 ng/mL, with a standard deviation of 109.35 and a coefficient of variation of 85.31 %. These concentrations were well with the specified calibration range, namely 5–500 ng/mL. Based on the results of INR analysis from 25 patients, the highest INR value was 9.12 in patient SN-03, and the lowest INR value was 0.90 in patient SN-13. The average INR value of patients was 1.62; with a standard deviation of 1.61 and a %CV of 99.39 %. The result of patients' analysis can be seen on Table 5.

In this study we found a weak and insignificant correlation ($r = 0.211$, $p = 0.311$) between warfarin dose and INR. In addition, tests were also conducted to determine the relationship between dose per day and INR values, as well as warfarin levels in VAMS. The results

Table 3
Matrix effect of warfarin in VAMS samples.

Concentration (ng/mL)	Average conc. (ng/mL)	%diff	CV
15.00	14.42	13.48 to 14.45	7.19
375.00	366.41	−9.11 to 9.15	6.59

Table 4
Dilution integrity of warfarin in VAMS samples.

Concentration (ng/mL)	Average conc. (ng/mL)	%diff	CV
750.00	809.62	5.51 to 10.17	1.65
375.00	371.53	−11.82 to 7.68	7.81
187.50	180.64	−11.36 to 1.54	5.45

of the correlation test between warfarin dose and INR value resulted in a value of $r = -0.032$ and $p = 0.879$; hence it can be concluded that the correlation between warfarin dose and INR value is very weak and there is no significant relationship. Meanwhile, the results of the correlation test between warfarin dose and warfarin levels in VAMS showed a value of $r = 0.524$ with $p = 0.007$; thus, indicating that there is a moderate and significant correlation between warfarin dose and warfarin levels in VAMS. The results obtained in this study correspond to the results in previous research. Studies on plasma generally show a weak correlation between plasma levels and INR [4,8,34], while a study using DBS shown a moderate correlation [35]. Apart from that, previous studies also showed a weak correlation between warfarin dose and INR values. The study using DBS shown that the DBS levels is correlated with warfarin dose, which also agree with the moderate correlation observed in this study. The high variability of individual responses to warfarin is one of the factors that may cause the weak correlation, which may also induce difficulties in therapy due to the lack of a fixed dose for warfarin maintenance doses [36].

Warfarin is a drug with a narrow therapeutic index which requires close supervision to prevent side effects related to subtherapeutic or supratherapeutic doses of warfarin [2]. The pharmacokinetics and pharmacodynamics of warfarin are influenced by genetic and environmental factors. Genetic polymorphisms in warfarin metabolizing enzymes like CYP2C9, or the VKORC1 enzyme which affects warfarin pharmacodynamics, can influence warfarin response [37]. In addition, environmental factors that can influence therapy are drug interactions, either with other drugs or with food and supplements [6]. Each patient receiving warfarin therapy has a different dose-effect ratio, in which the anticoagulant effect and blood concentration of warfarin does not depend on the dose taken by patients [38]. Therefore, in cases where sudden and unnatural changes in INR values were observed, checking warfarin levels in VAMS samples can be used to confirm whether the cause of changes in INR values is related to pharmacokinetics or pharmacodynamics. The patient's history of previous medical conditions, medications, food, and lifestyle needs to be explored further to determine the cause of changes in the patient's therapeutic response before genomic testing is carried out on the patient.

Plasma is the most widely used biological matrix to analyze warfarin [5,8–11,39]. However, plasma sample collection requires blood taken from venipuncture, which was considered invasive and requires larger volume of sample. Assistance from a qualified technician is needed for sample collection, and storage of the plasma samples should met certain conditions [40]. Microsampling was developed to overcome these limitations posed by conventional blood sampling. DBS is a microsampling method which consists of spotting a small volume of blood in paper, which then dried before analyzed. Analysis of warfarin in DBS samples has been conducted in DBS samples of patients [35], and in DBS samples for pharmacokinetics study in rats [37]. DBS has been associated with hematocrit effect and the issue of homogeneity of the sample, which can affect the analysis. A newer microsampling technique, VAMS, is able to

Table 5
Results of patients' analysis.

Patient	Duration of therapy	Dose per day	Measured concentration (ng/mL)	INR
SN-01	2 months	2 mg	149.71	1.2
SN-02	4 years	2 mg	49.04	1.3
SN-03	14 days	2 mg	196.26	9.12
SN-04	1 month	2 mg	82.85	1.14
SN-05	4 years	2 mg	81.08	1.01
SN-06	2 months	2 mg	71.61	1.25
SN-07	3 years	2 mg	43.70	1.02
SN-08	2 years	2 mg	209.56	1.51
SN-09	10 months	2 mg	118.25	1.4
SN-10	2 years	2 mg	8.18	1.25
SN-11	1 month	2 mg	90.15	1.16
SN-12	1 year	2 mg	46.53	0.97
SN-13	3 years	3 mg	12.18	0.9
SN-14	13 months	1 mg	6.05	0.98
SN-15	6 months	1 mg	148.55	1.41
SN-16	5 months	2 mg	140.05	1.08
SN-17	1 months	2 mg	124.01	1.27
SN-18	6 months	2 mg	156.84	1.01
SN-19	1 years	2 mg	62.94	1.11
SN-20	14 days	2 mg	36.49	1.98
SN-21	3 years	2 mg	364.96	1.75
SN-22	3 years	5 mg	431.39	1.37
SN-23	6 months	2 mg	58.87	1.37
SN-24	1 month	2 mg	218.30	2.73
SN-25	1 years	2 mg	296.71	1.19

eliminate the concerns associated with DBS.

To the knowledge of the authors, no study has been conducted to perform analysis of warfarin in VAMS samples before. The result of this preliminary study may serve as a basis for clinical validation, before introducing warfarin VAMS analysis as a complement to INR testing for monitoring of warfarin therapy.

5. Conclusion

A novel method has been developed for quantification of warfarin in VAMS samples with quercetin as the internal standard using LC-MS/MS. The method was linear, accurate, and precise in the range of 5–500 ng/mL. Full validation has been performed and the result has met the requirements according to the guideline from FDA 2022. The method has been successfully applied to monitor warfarin level in VAMS samples from 25 patients, resulted in warfarin VAMS concentration ranged from 6.05 ng/mL to 431.39 ng/mL. Weak and insignificant correlation was observed between warfarin VAMS concentration and INR, as well as between warfarin dose and INR value. Moderate correlation was observed between warfarin VAMS concentration and dose. Further studies should be conducted in a larger number of samples and also take pharmacokinetic and pharmacodynamic factors into consideration, to assess the potential of additional warfarin VAMS monitoring in clinical setting, in order to gain better understanding of warfarin therapeutic response.

Ethics statement

This study was reviewed and approved by the Research Ethics Committee of Universitas Indonesia Hospital, Depok, Indonesia with the approval number: S-025/KETLIT/RSUI/III/2023.

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Data availability statement

Data included in article/supplementary material/referenced in article.

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Yahdiana Harahap: Writing – review & editing, Methodology, Conceptualization. **Callista Andinie Mulyadi:** Writing – original draft, Formal analysis. **Hary Sakti Muliawan:** Writing – review & editing, Supervision. **Habibah A. Wahab:** Writing – review & editing, Validation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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