ORIGINAL ARTICLE

Validation of bioanalytical method for quantification of Vitamin K2 (MK-4) in human plasma by high-performance liquid chromatography-ultraviolet

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ABSTRACT

Vitamin K can reduce warfarin's anticoagulant action, causing a variance in response among individuals taking warfarin. Vitamin K comes in two forms, namely Vitamin K1 (phylloquinone) and K2 (menaquinones). Menaquinone-4 (MK-4) is a kind of Vitamin K2 found in meat and dairy products. Analysis of MK-4 levels in human plasma is very useful for patients who receive warfarin therapy. High-performance liquid chromatography (HPLC) can be used for warfarin's bioanalysis, and it must be validated. The purpose of this study was to validate the bioanalytical method for quantification of Vitamin K2 (MK-4) in human plasma according to the 2019 European Medicines Agency (EMA) guideline. Vitamin K2 (MK-4) was extracted using acetonitrile. HPLC with an ultraviolet detector at 245 nm, using a T3 column set at 30°C and an isocratic mobile phase containing methanol: phosphate buffer (95:5) at pH 3, a flow rate of 1 mL/min was used in this study. The warfarin concentration of 0.5–3 μg/mL was used. About 5.50%–17.42% and 6.18%–8.74%, respectively, were the average ranges of percentage coefficient of variation and percentage difference. There was no response at the analyte's retention time in the six blank plasmas and at the analyte's retention time in the blank after the injection of upper limit of quantification, indicates that the procedure was very selective and did not result in any carryover. This bioanalytical method fulfills the parameters of selectivity, accuracy, precision, and carryover based on the 2019 EMA guidelines.

Key words: Bioanalytical method, validation, Vitamin K2 (MK-4)

INTRODUCTION

Vitamin K is one of the essential fat-soluble bioactive compounds needed to regulate body functions. It plays a key role in coagulation by working as a cofactor for the carboxylation process in protein synthesis.[1] Blood-clotting factors (factor II, factor VII, factor IX, and factor X) and natural anticoagulants such as proteins C, S, and Z are among the proteins needed for coagulation. This process occurs as part of the Vitamin K cycle.[2,3]

Warfarin may have an effect on the Vitamin K cycle. One of the enzymes required in the Vitamin K cycle is Vitamin

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K epoxide reductase, which is inhibited by warfarin. As a result, the Vitamin K cycle is inhibited, and the process of forming blood-clotting factors is disrupted. This makes patients on warfarin therapy susceptible to bleeding.[1] However, high Vitamin K consumption may decrease the anticoagulant effect of warfarin.[4] Meanwhile, if the consumption of Vitamin K is too limited, fluctuations in Vitamin K intake can increase and cause instability in the Index Normalized Ratio. Therefore, patients taking warfarin are advised to consume products containing Vitamin K in controlled and not too-low amounts.[5] The interplay of Vitamin K and warfarin makes measuring Vitamin K levels in blood plasma useful for patients receiving warfarin therapy.[6]

Vitamin K exists in two natural isoforms, which are Vitamin K_1 (phylloquinone) and Vitamin K_2 (menaquinone).[1] Vitamin K_2 (VK_2) has several homologous forms that have different numbers of isoprene units, ranging between 4 and 13 units. One of the homologous forms of VK_2 found in blood plasma is menaquinone-4 (MK-4). MK-4 has a side chain of four isoprene units. MK-4 is frequently present in dairy and meat products.[7,8]

Numerous techniques can be used to measure Vitamin K. High-performance liquid chromatography (HPLC) is one of them.[6] The advantages of HPLC are that it only requires a small sample and provides high accuracy and precision. In addition, HPLC is a simpler and cheaper method than LC–MS/MS, especially for routine testing.[9,10] One of the detectors that can be used is an ultraviolet (UV) detector. UV detectors are the most frequently used detectors, have good precision and linearity, and are easy to use.[11,12]

A validated bioanalytical technique is required to determine the levels of Vitamin K in human plasma. Validation is performed to ensure that the analytical method used is specific, accurate, resistant, and reproducible to the range of analytes to be analyzed. The validation of analytical methods for biological samples (bioanalysis) can be carried out based on the 2019 European Medicines Agency (EMA) guidelines.[13] The aim of this study was to validate the bioanalytical method for the determination of MK-4 in human plasma using HPLC–UV.

METHODS

Equipment and materials

The equipment used in this research was an HPLC (Waters e2695 Separation Module) with a UV detector (Waters 2489 UV/Vis Detector) and an Atlantis T3 column (4.6 mm × 150 mm, 3.0 m). The materials used in this study were healthy human blood plasma from the Indonesian Red Cross Society, standard of Vitamin K2 (MK-4) (Merck), acetonitrile HPLC grade (Merck), aquabidest (IPHA), phosphoric acid (Merck), methanol HPLC grade (Merck), and potassium dihydrogen phosphate (Merck).

High-performance liquid chromatography conditions

The stationary phase was a T3 column that was operated at 30°C. Methanol: phosphate buffer (95:5) at pH 3 with isocratic elution was used as mobile-phase system. Before analysis, the mobile phase was freshly prepared, filtered with a 0.45 µM filter membrane, and sonicated to remove gas. The flow rate was set at 1 mL/min, and a UV detector was set at 245 nm for the detection.

Preparation of standard solutions and quality control samples

Vitamin K2 (MK-4) was dissolved in acetonitrile to provide a standard stock solution of Vitamin K2 (MK-4) with a 200 g/mL concentration. Stock solutions were kept at a temperature of −20°C and protected from light. The stock solution of Vitamin K2 (MK-4) was diluted with acetonitrile to create the working solutions, which have concentrations of 8, 16, 24, 28, 32, 40, and 48 g/mL. Blank plasma was prepared from healthy human plasma.

The standard solution in plasma was prepared by spiking the working solution of Vitamin K2 (MK-4) by as much as 10 µL into 150 µL of blank plasma to get a concentration of 0.5, 1, 1.5, 2, 2.5, and 3 µg/mL in a 2 mL microcentrifugation tube. The working solution of Vitamin K2 (MK-4) was spiked into 150 µL of blank plasma to obtain the quality control (QC) samples with a concentration of 0.5, 1.5, 1.75, and 2.5 µg/mL.

Extraction of MK-4 from plasma

In a 2 mL micro-centrifugation tube, the standard solution in plasma was inserted, and 490 µL of cold acetonitrile was added. The microcentrifugation tube was then vortexed for 3 min. The tube was then centrifuged at 5°C for 10 min at 10,000 rpm. The resulting supernatant was transferred to another centrifugation tube. To obtain a dry residue, the supernatant was dried under nitrogen flow at 40°C. The residue was then reconstituted with 70 µL of methanol before being vortexed for 1 min.

Validation of bioanalytical method

Selectivity

Blank plasmas from six different sources were extracted as in the extraction of MK-4 from the plasma process. The sample was then injected into the HPLC system. In addition, the analysis was also performed on the lower limit of quantification (LLOQ) of standard solution (0.5 µg/mL).

Calibration curve and range

The calibration curve was repeated three times by measuring the standard solution in plasma with a concentration of 0.5, 1, 1.5, 2, 2.5, and 3 µg/mL. Plotting the concentration on the X-axis and the area under the curve on the Y-axis were done to create the calibration curve. The linearity was performed.

The calibration curve parameter was determined by calculating the measured concentration of each concentration on the calibration curve. The %diff of the measured concentration for each concentration level was calculated to perform the accuracy.

Accuracy and precision

Standard QC samples were used to assess accuracy and precision at four different concentrations, namely LLOQ, LQC (low QC), MQC (medium QC), and HQC (high QC) (0.5, 1.5, 1.75, and 2.5 g/mL). Each QC sample was measured five replicates for within-run. While for between-run, each QC sample was repeated three times in two different days. Then, the accuracy was determined by calculating the % difference, and precision was calculated by calculating the % coefficient of variation (CV).

Carryover

The analysis was carried out on blank plasma. The blank plasma was extracted as in the extraction of MK-4 from plasma process. The analysis was repeated three times after the injection of upper limit of quantification (ULOQ), 3 µg/mL.

RESULTS

A system suitability test is carried out to ensure that the system used in the analysis works properly. The results of the parameters tested were 8.305 ± 0.056 for capacity factor, 3876.128 ± 780.524 for number of theoretical plate, and 1.003 ± 0.088 for tailing factor. The chromatogram of MK-4 is shown in Figure 1.

Validation of bioanalytical method

Selectivity

The results of the blank measurement in the selectivity test showed that there was no response at the retention time of the analyte (MK-4). The retention time of MK-4 obtained using this method is 17.29 min

Calibration curve and range

The range used in this study was 0.5 µg/mL as the LLOQ, which is the lowest standard concentration, and 3 µg/mL as the ULOQ. A bioanalytical method meets the calibration curve parameter requirements if the %diff of the measured concentration at each concentration level is within ±15% and the %diff for the lowest measured concentration on the curve (LLOQ) is within ±20%. In this study, three calibration curves were obtained. On the first and second calibration curves, the %diff of the measured concentration of all concentration variations met the requirements. In other words, on the first and second calibration curves, 100% of the variation in concentration satisfied the %diff requirement. On the third calibration curve, there was one concentration that did not meet the requirements. However, the third calibration curve was still acceptable because 5 out of 6 (83.33%) concentration variations met the requirements

Accuracy and precision

Accuracy is determined by calculating the %diff of each concentration, whereas the precision is determined by calculating the % CV of each concentration.[13] The %diff and % CV data from each concentration for within-run and between-run tests can be seen in Table 1.

Carryover

The results of the carryover show that there was no response whatsoever to the blank in the retention time of the analyte when the blank test was carried out after the ULOQ test. The chromatogram is shown in Figure 2.

DISCUSSION

In this study, the HPLC–UV detector was used for the analysis because Vitamin K2 (MK-4) has a chromophore group, 2-methyl-1,4-naphthoquinione, which has C = C and C = O bonds, as shown in Figure 3. The wavelength used in this study was 245 nm.

The HPLC system used in this study is a reverse phase system, where the stationary phase used is nonpolar and the mobile phase is polar. The column used in this study is the T3 column. This column provides balanced retention times for polar and nonpolar compounds, gives good peak yields, and has good efficiency for all analytes over a wide pH range. While the mobile phase used was methanol:

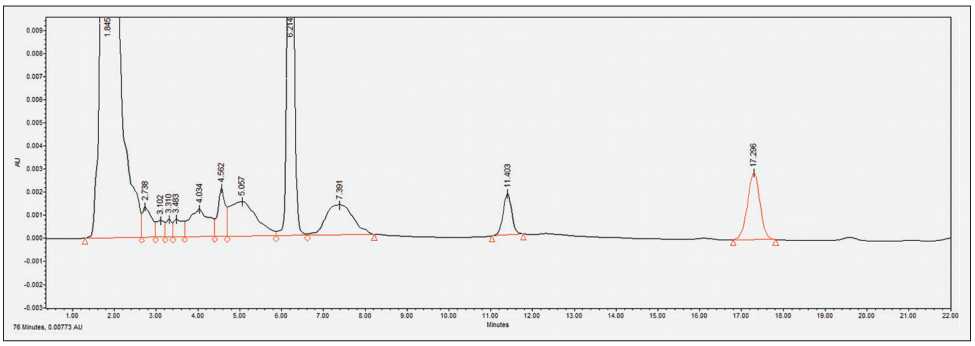

Figure 1: The chromatogram of MK-4

Table 1: Accuracy and precision data

QC samples	Within-run			Between-run		
	Concentration (mean±SD) (μg/mL)	Accuracy (% difference)	Precision (% CV)	Concentration (mean±SD) (μg/mL)	Accuracy (% difference)	Precision (% CV)
LLOQ	0.534±0.093	6.876	17.417	0.525±0.063	4.945	11.962
LQC	1.369±0.1831	−8.747	13.381	1.445±0.125	−3.688	8.624
MQC	1.897±0.276	8.418	14.586	1.959±0.274	11.958	13.974
HQC	2.654±0.146	6.178	5.501	2.538±1.506	1.509	12.271

LLOQ: Lower limit of quantification, LQC: Low-quality control, MQC: Medium quality control, HQC: High-quality control, SD: Standard deviation, CE: Coefficient of variation

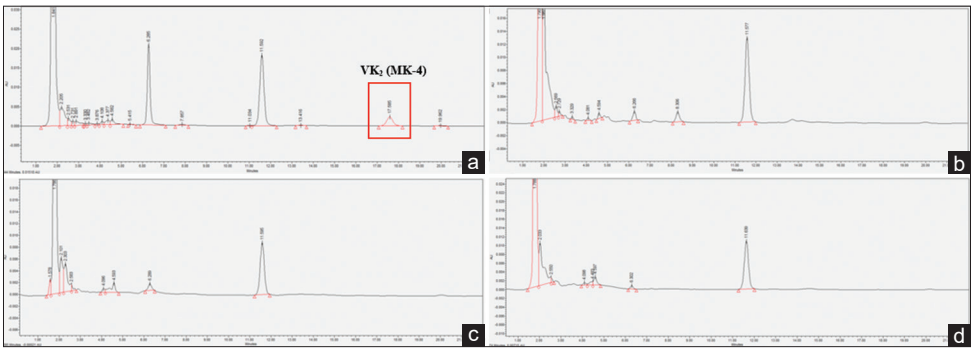

Figure 2: Carryover chromatograms (a) Upper limit of quantification (3 μg/mL); (b) Blank plasma 1; (c) Blank plasma 2; (d): Blank plasma 3

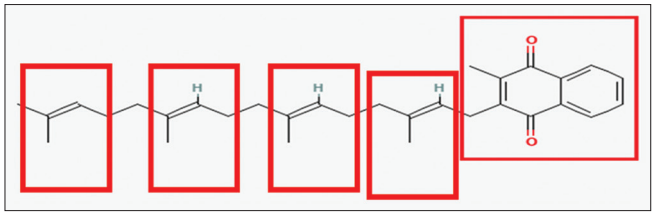

Figure 3: Chromophore in MK-4

phosphate buffer pH 3 (95:5). The composition of methanol in the mobile phase is made higher than that of phosphate buffer so that MK-4 is not too retained on the column so that the retention time obtained is not too long. Reducing the polarity of the mobile phase in the reverse phase, HPLC will shorten the retention time of the analyte.[14]

The flow rate used was 1 mL/min, whereas the column temperature used was 30°C. Increasing the temperature in the column can speed up the separation process so as to speed up retention time and can make the analysis process more efficient. Using this method, the capacity factor, number of theoretical plate, and tailing factor parameters meet the requirement.

Validation of bioanalytical method

Selectivity

The selectivity parameter was evaluated to determine whether the method used could distinguish between the analyte being analyzed and other compounds that might be present in the blank of the biological matrix where their presence could interfere with the analyte response. The response limit detected at the retention time of the analyte is 20% of the response in the LLOQ measurement.[13] The absence of a response in the retention time of the analyte indicates that the analytical method meets the requirements of the selectivity parameter, whereby the method can distinguish the analyte from compounds that may interfere with the analyte response.

Calibration curve and range

The calibration curve describes the relationship between analyte concentration and the response to analyte measurement. Calibration standards are prepared by adding a known amount of analyte to the same blank matrix as the sample matrix. The calibration range is described by the LLOQ, which is the lowest calibration standard concentration, and the ULOQ, which is the highest calibration standard concentration.[13]

In the tests carried out, concentration ranged between 0.5 μg/mL (LLOQ) and 3 μg/mL (ULOQ), with six different concentrations being tested. Based on the results of the %diff calculation for each concentration in the three curves, the number of concentrations that meet the %diff requirements is more than 75%. While, based on the guidelines, 75% of the concentration variations used in the calibration curve parameters must meet the %diff requirements. Based on our results, the tested method meets the requirements of the calibration curve.

In a previous study, the range of MK-4 levels in human plasma was 0.2–4 ng/mL.[9] However, in the current study, the LLOQ was higher than the level of Vitamin MK-4 in human plasma reported in the literature, so to use this

method, it was necessary to concentrate the Vitamin K content of the blood plasma sample to be analyzed.

Accuracy and precision

The accuracy parameter shows the closeness between the measured concentration and the nominal or theoretical concentration. Meanwhile, the precision parameter shows the closeness of the measured concentration between repeated measurements of the same concentration. The concentrations used for the analysis of this parameter are the LLOQ, LQC, MQC, and HQC. LLOQ is the lowest concentration used on the calibration curve, where the concentration of LLOQ must be quantifiable and meet the parameters of accuracy and precision.[15] The LLOQ value obtained in this study was 0.5 µg/mL. The LQC concentration was calculated as three times the LLOQ value, so in this study, the LQC concentration was 1.5 µg/mL. The MQC concentration is 30%–50% of the range of the calibration curve used. In this study, the range was 0.5–3 µg/mL and so the MQC concentration was 1.75 µg/mL, which was 50% of the calibration curve range. Meanwhile, HQC must be at least 75% of the highest concentration used on the calibration curve (ULOQ). In this study, the HQC concentration was 2.5 µg/mL. This concentration exceeded 75% of the ULOQ used, as 75% of 3 µg/mL is 2.25 µg/mL.[13]

The results are accurate if the %diff value is <±15% and the %diff value for LLOQ is <±20%. Meanwhile, the results meet the precision criteria if %CV is <15% and %CV for LLOQ is <20%. Based on the results of the tests that were carried out, the within-run accuracy and precision met the requirements at all concentrations of the QC sample as did the between-run test.

Carryover

Carryover is a parameter that is used to determine whether there is a change in the results due to the presence of analyte residue from the previous injection. A bioanalytical method meets the requirements for the carryover parameter if the response of the blank plasma at the analyte retention time is not more than 20% of the analyte response at the LLOQ concentration.[13] The test results showed that there was no response or peak at the analyte retention time on three blank plasma chromatograms. This shows that there was no analyte residue left from the previous injection that might have interfered with the measurement results.

CONCLUSION

The method of analyzing levels of Vitamin K2 (MK-4) in human plasma using HPLC-UV met the requirements for the validation parameters of selectivity, accuracy, and precision, carryover according to the 2019 EMA guidelines, and obtained the LLOQ of 0.5 µg/mL. However, this method cannot directly detect the concentration of Vitamin K in human plasma because the LLOQ is too high. Therefore, it was necessary to concentrate the MK-4 in plasma, or a more sensitive method for analyzing levels of Vitamin K2 (MK-4) in plasma is needed.

Authors contributions

All the authors have contributed equally.

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Conflicts of interest

There are no conflicts of interest.

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