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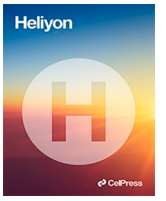

Research article

Development and validation of ivermectin quantification method in volumetric absorptive microsampling using liquid chromatography-tandem mass spectrometry

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ABSTRACT

Background: Ivermectin is a broad-spectrum anthelmintic used to control onchocerciasis from nematode parasites. As an anthelmintic, ivermectin is designed to have high levels in the gastrointestinal tract, so that the systemic intake is relatively low. Due to the very small concentration of ivermectin, a selective and sensitive approach is needed for the analysis of ivermectin in blood. Several methods have been developed using plasma and Dried Blood Spots, but there are still shortcomings due to hematocrit effects. Therefore, this study was conducted to establish a validated ivermectin analysis method with doramectin as the internal standard in using Ultra High-Performance Liquid Chromatography-Tandem Mass Spectrometry.

Methods: Mass spectrometry equipped with triple quadrupole and positive electrospray ionization mode was used to conduct the analysis. For the biological matrix, whole blood was used by Volumetric Absorptive Microsampling and extracted using a protein precipitation technique with a combination of acetonitrile and methanol (1:1). VAMS has some advantages such as not being affected by hematocrit, requires a small and fixed volume of sample, also a more efficient sampling process.

Results: The optimum conditions were achieved with an Acquity® UPLC BEH C18 column (1,7 μm; 2.1 × 100 mm); extracted-flow rate was 0,2 mL/min; mobile phase was 5 mM ammonium formate pH 3.00 and acetonitrile (10:90) with isocratic elution. Multiple Reaction Monitoring (MRM) detection by *m/z* values was 892.41 > 569.5 for ivermectin and 916,41 > 331,35 for doramectin.

Conclusion: The method has been appropriately validated in compliance with the 2018 guidelines laid out by the US Food and Drug Administration. Resulting the minimum detection (LLOQ) was 1 ng/mL with a linear concentration range spanning from 1 to 150 ng/mL.

1. Introduction

Onchocerciasis is a parasitic infection of *Onchocerca volvulus* transmitted through the bite of a black fly [1,2]. This infection causes severe itching and sores, even eye disease [2]. This parasitic infection of *O. volvulus* can cause light insensitivity which leads to blindness. Therefore, another name for onchocerciasis is river blindness [3–6]. Based on WHO, in 2020 it was reported that there were

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more than 21 million cases of Onchocerciasis, almost all of which occurred in 34 countries from the African continent. Due to the high number of cases, annual administration of ivermectin was carried out, aims to reducing the number of microfilariae in infected patients, reducing the transmission of parasitic infections, and preventing blindness and other severe symptoms [3–5]. In African countries, the practice of conducting mass drug administration (MDA) is prevalent, with a primary focus on the utilization of ivermectin as a potent malaria vector control agent, as well as onchocerciasis [14,32]. As for pharmacokinetic activity, ivermectin can be rapidly absorbed orally, metabolized in the liver by CYP3A4, and excreted in feces (98 %) and urine (1 %) [8]. Plasma maximum concentration (20.2 ng/mL in a healthy subject and 54.4 ng/mL a in Onchocerciasis infected subject) can be reached after 5 h due to the fairly long half-life, about 36 h [9,30]. Distribution volume of ivermectin in a healthy subject around 3.1–3.5 L/kgBB [9]. Oral use of ivermectin seldom results in side effects, which are generally mild and ephemeral, for example, momentary episodes of rapid heart rate, flu-like symptoms, and vertigo. While it is theoretically possible for ivermectin to lead to neurological side effects, such as seizures, such cases have been recorded very infrequently [8–11]. Apart from being an antiparasitic against onchocerciasis, ivermectin also has the potential as an antiviral agent [7].

In 2020, the antiviral properties of ivermectin against SARS-Cov-2 was found in-vitro [12]. In this study, ivermectin in vitro was able to inhibit the transfer of ribonucleic acid (RNA) of the SARS-CoV-2 virus five thousand times at a concentration of 5 M within 48 h [11–14]. However, the concentration needed to be able to reduce viral RNA is toxic because very high doses are needed as an antiviral [15,16]. After conducting clinical trials with various stages, ivermectin was not approved by the US FDA as a COVID-19 therapy in humans [17,18].

Analysis of ivermectin in plasma was carried out by Duthaler et al., in 2019. However, the use of this plasma has many drawbacks [19]. The plasma taken by venipuncture technique or taken from a vein. This blood collection is invasive and can cause patient discomfort, also may require the assistance of a phlebotomist [20]. Ivermectin analysis has also been carried out using the DBS biosampling technique or dried blood spot by Schulz et al., in 2018 [1,21]. This DBS method has less risk of infection than venipuncture biosampling. This method only requires a small amount of blood and the collection process is easy [21]. Nevertheless, the DBS technique has its limitations as the homogeneity of the sample may differ based on the hematocrit level or the volume keeps changing. So that it affects the value of drug concentration in the blood [22].

Ivermectin analysis was carried out using the DBS or dried blood spot biosampling technique by Schulz et al., in 2018 [1]. This DBS method has less risk of infection than venipuncture technique because it requires a small amount of blood. But it has their shortages, as the test result could be affected by the hematocrit effect [21–23].

Amid the COVID-19 widespread, the utilize of volumetric absorptive microsampling (VAMS) is suggested [24]. The method of taking blood for the VAMS method is considered very simple, does not require extra costs, can be put at room temperature. The amount of blood drawn is small and can be drawn by patients or volunteers without medical expertise [25]. Hence, the utilize of VAMS as a biosampling technique for checking blood drug levels can diminish the expansion of SARS-CoV-2 infection since the sampling procedure can be easily carried out through self-sampling [24]. Compared to the DBS method, the VAMS method provides accurate volumetric measurements (constant amount) and minimizes the influence of hematocrit on analytical results [23–26]. However, there is still no research that aims for a better result as compares to the previous study of quantitative analysis using VAMS, one of the safest biosampling technique, for ivermectin in Indonesia.

2. Material and methods

2.1. Chemical and Reagents

Ivermectin was obtained from Horster Biotek (India), and doramectin as the internal standard (IS) was acquired from Merck (German). The Indonesian Red Cross in Jakarta, Indonesia provided the whole blood, and Neoteryx® in Torrance, California provided the volumetric absorptive microsampling. Acetonitrile and methanol HPLC grade and manufactured by Merck Co. Ltd. (German), and the ammonium formate was obtained from Sigma Aldrich (Singapore).

2.2. LC-MS/MS instrumentation and settings

The UHPLC-MS/MS system consisted of various components, such as a Quaternary Solvent Manager and a Sample Manager FTN from Waters Acquity® UPLC, a C18 column Acquity™ UPLC BEH (1.7 μm; 2.1 × 100 mm) sourced by an electrospray ionization triple quadrupole mass spectrometer XEVO TQD. All the mentioned items were obtained from Waters (USA). Nitrogen was utilized as an ion source at a temperature of 450 °C, with the gas being released at a rate of 500 L/h. The flow rate was 10 L/h and the positive mode ion spray voltage was set at 3.0 kV, while the cone voltage was set at 32 V. The software Masslynx 4.1 was in charge on operating the system. Ivermectin and doramectin were detected using multiple reaction monitoring (MRM) with m/z 892.41 > 5695 and m/z 916.41 > 331.5, respectively. Subsequently, on a C18 BEH column, the analysis was separated using an isocratic elution method with ammonium format buffer 5 mM (mobile phase A, pH 3), acetonitrile supplemented with formic acid 0.1 % (mobile phase B), and formic acid 0.1 % (mobile phase C). The rate of flow was adjusted to 0.2 mL/min, 10 μL was administered to the system and the duration time was 3.5 min. After loading the samples onto the columns, they underwent a cleansing process with a solution consisting of 30 % acetonitrile to decrease the analyte carry-over [27].

2.3. Stock and working solution preparation

The standard was dissolved in methanol as a solvent to create the stock solution of ivermectin and doramectin, which had a concentration of 1.000 ppm (1 mg/mL). Serial dilutions in methanol were used to create the working solution, which had a concentration of 1 ppm. The freezer was set to −20 °C and used to store all stock and working solutions.

2.4. Calibrators and quality control samples preparation

In order to prepare the calibrators, the working solution should be diluted with whole blood at a concentration of 1–200 ng/mL. Additionally, working solutions were diluted with whole blood to create quality control (QC) samples, whose concentrations were 3 ng/mL for QCL, 60 ng/mL for QCM, and 120 ng/mL for QCH. Standards for calibration and QC samples were kept at −20 °C in the freezer. These solutions are will be used for both calibrations ans QC preparation tests.

2.5. Sample preparation with VAMS

Volumetric absorptive microsampling is one of the safest approach to obtain a dried blood sample for quantitative bioanalysis and mitigate some issues faced in DBS (dried blood sample) sampling. To generate calibration and quality control samples, the VAMS tip absorbed 30 μL of the spiked blood when dipped into it. VAMS that had been dipped was allowed to air dry for 3 h. VAMS that had been dried were obtained by transferring the plastic handle's tips to microtubes. The microtubes were filled with dried VAMS, 600 μL mixture of methanol and acetonitrile (1:1, v/v) and 20 μL of doramectin which had a concentration of 100 ng/mL. The tubes were subjected to vortex mixing for 30 s, proceeded with sonication for 10 min, before being centrifuged for another 10 min at 12,298 g force (10,000 rpm). The supernatant was amassed, followed by evaporation with N_2 gas for 30 min at 40°C. The residue was reconstituted with 150 μL of the mobile phase, followed by mixing vigorously for 30 s, subjected to sonication for 10 min, and centrifuged for 10 min at 12,298 g force (10,000 rpm). Supernatant in the amount of 10 μL were injected to the LC-MS/MS. The schematic diagram of sample preparation shown in Fig. 1.

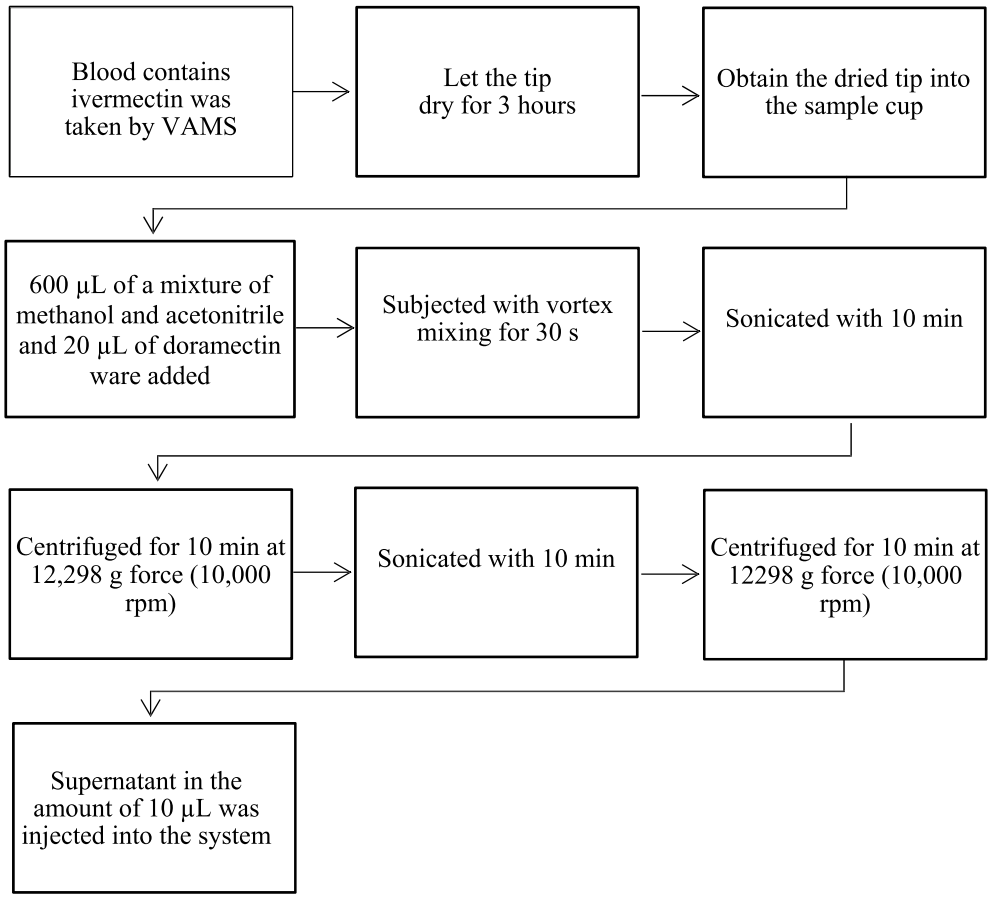

Fig. 1. Sample preparation in schematic diagram.

2.6. Method validation

The validation of this analytical method was in accordance with the Bioanalytical Method Validation guidelines as set forth by the Food and Drug Administration [28,29]. Various factors such as LLOQ, linearity, selectivity, carry-over, precision, accuracy, extraction recovery, the integrity of dilution, matrix effect, and analyte stability were considered.

2.7. Lower limit of quantification (LLOQ)

The limit of quantification was established via the analysis of five replicates of both the sample and blank. The accuracy of LLOQ should exhibit a minimum response that is five-fold higher than that of the zero calibrators [29]. It is recommended that the level of accuracy attained does not surpass 20 % of the nominal concentration, while also not exceeding a coefficient of variation (%CV) of 20 % within the 3 runs consisting of 5 replicates each [28,29]. Half of the tested concentration can be analyzed if the concentration tested meets the requirements.

2.8. Calibration curve

Analysis of a minimum of six concentrations alongside a blank and a zero samples have been used to determine the calibration curve; each concentration was examined 3 times. The generation of the linear equation involved plotting the expected concentration against the peak area ratio of the analyte in relation to the IS [28,29]. The non-zero calibrators should be within 15 % of the expected concentration, while ensuring that the LLOQ is no less than 20 %. A minimum of seven non-zero calibrators or 75 % must fulfill the requirements [28,29].

2.9. Selectivity

By examining duplicates of LLOQ, zero, and void samples obtained from six distinct blood sources, the selectivity was ascertained. The outcome should be free from any interference during the run times of both the analyte and the IS. 5 % interference of the IS and 20 % interference for LOQ should be the maximum for the blank sample response [28,29].

2.10. Accuracy and precision

Precision and accuracy assessments should be performed at a minimum 3 times for both within-run and between-run data by using at slightest 5 replicates various concentration of QC samples, including LLOQ, low, medium, and high per run. To ensure accuracy, the average level of concentration should be accurate to within a 15 % of expected concentration, except for LLOQ, which should be within a 20 % of the expected concentration. The percentage of coefficient of variation (CV) has to be below 15 % whether its within-run or between-run precision, with the exception of the lower limit of quantification which should not exceed 20 % [28,29].

2.11. Recovery

The comparison of the extracted samples with LLOQ and QC samples (low, medium, and high) concentrations to the extracted blank spiked with IS and analyte is being made based on their respective areas. The recovery result does not have to be 100 %, but it has to be reproducible with a CV of less than 15 % [28,29].

2.12. Carry over and matrix effect

Through analyzing the blank along with a high concentration sample, referred to as ULOQ (Upper Limit of Quantification) level, carry over tests were identified. Provided that the analyte and blank yield a response that is within 20 % of the LLOQ, also the IS within 5 %, carry over can be deemed acceptable [28]. To ascertain the matrix effect, the response of the analytes was examined in various blood samples with low (less than three times the LLOQ) and high (up to the ULOQ) concentrations, as well as in the presence of an IS (internal standard). These responses were compared to those achieved through solvent dilution. The acceptance of a matrix effect depends on the CV response percentage not exceeding 15 %, and the normalized matrix factor within a range of 0.8 to 1.2[29].

2.13. Dilution integrity

The accuracy of the dilution (dilution integrity) was evaluated by elevating the concentration of whole blood to twice that of QCH. Each dilution factor underwent at least 5 replicate runs in the analysis. Both %diff and %CV must be less than or equal to 15 % [29].

2.14. Stability

In order to conduct the sample stability test, the concentration levels of QCL and QCH were examined assessed through multiple conditions, such as an auto-sampler stability test, a stock solution stability test, as well as short-term and long-term stability tests. The samples and stock solutions were analyzed for stability over different durations of time, with long-term stability test being done after

storage at −20 °C for 0, 7, 14, and 30 days, and short-term stability being assessed in storage for 0, 6, and 24 h. Analysis of the stability of the autosampler was done both immediately after insertion and 24 h later. Stability was considered acceptable if the %diff for samples was below 15 %, or if the %diff for stock solutions was below 2 % [28,29].

3. Results

3.1. Method development

The primary emphasis of the method development was the refinement of the mobile phase, better validation results, and utilization of VAMS, as there is already a wealth of information available in the literature regarding the chromatography and mass detection profile of ivermectin [27]. Different combinations of mobile phases comprising acid or organic solvents along with buffer were tested. The pairs that underwent analysis were A:B, B:C, and acetonitrile:A, each at a flow rate of 0.2 mL/min and with a ratio of 90:10. The acetonitrile:mobile A 90:10 v/v mixture was selected, as a suitable compromise as the ionization of ivermectin heavily relied on the presence of ammonium formate because the ammonia adducts due to the significantly high intensity of these ions to be detected by mass spectrometry [27]. The specific effect of changes in the composition/proportion/ratio of the mobile phase had changed significantly. The mixture were checked at 5:95 ratio, 10:90 ratio, and 20:80 ratio. The chromatograms shows in Fig. 3. Furthermore, the mass spectrometry signal intensity can be raised by using the positive ESI ionization type in this buffer, enabling the detection of both analytes even at very low concentrations [27]. The result of mass spectrometry scanning (fragmentation mass) of both ivermectin and the internal standard, doramectin, has been provided in Fig. 2. The extraction process flow was adjusted to the standard rate of 0.2 mL/min because as the flow rate increases, so does the pressure within the column. The longevity of the column may be reduced due to the persistent high column pressure, thereby impacting its overall state [20].

3.2. Method validation

3.2.1. Lower limit of quantification (LLOQ)

The LLOQ of ivermectin was identified as 1 ng/mL. The tested concentration meets the LLOQ requirements due to the %diff result was in the range of −8.42 % to −2.5 % and %CV was obtained 2.54 %. The level of concentration at 0.5 ng/mL (half of the concentration) was assessed; however, it failed to meet the standards. As a result, a minimum detectable (LLOQ) concentration of 1 ng/mL was established. Additionally, ivermectin as a drug are 70 % absorbed in plasma [30,31]. Comparing these findings to studies that used DBS, the LLOQ is still improved [1]. The current method yields a lower LLOQ, could be due to the fact that the sample collection is unaffected by hematocrit values. But the LLOQ obtained still could not lower the sensitivity of ivermectin if we compared to the analysis using plasma. The results may be because the ivermectin itself It is highly probable absorbed in plasma for around 70 % [30,31]. The chromatogram of the LLOQ sample is shown in Fig. 3.

3.2.2. Calibration curve

Nine concentration points— including blank, zero sample, and concentration levels of 1, 5, 10, 50, 75, 100, and 150 ng/mL were used in the calibration curve. Correlation coefficients were found to be in the range of 0,9983 to 0,9994, indicating that the curve appears to be linear and the% diff result is acceptable. This linear calibration curve provides accurate and dependable results for the analyte concentration within the specified range. In comparison to previous research, the calibration curves exhibited a broader range, which makes it simpler to quantify ivermectin and potentially obviating the need for sample dilution [32].

3.2.3. Selectivity

In the blank test of the selectivity test from the six blood sources, the interference response of ivermectin ranged from 1.12 % to 2.94 %, and the standard for doramectin ranged from 0.2 % to 0.86 %. Thus the selectivity has met the requirements which indicates that the analytical method is selective for the analytes in the matrix.

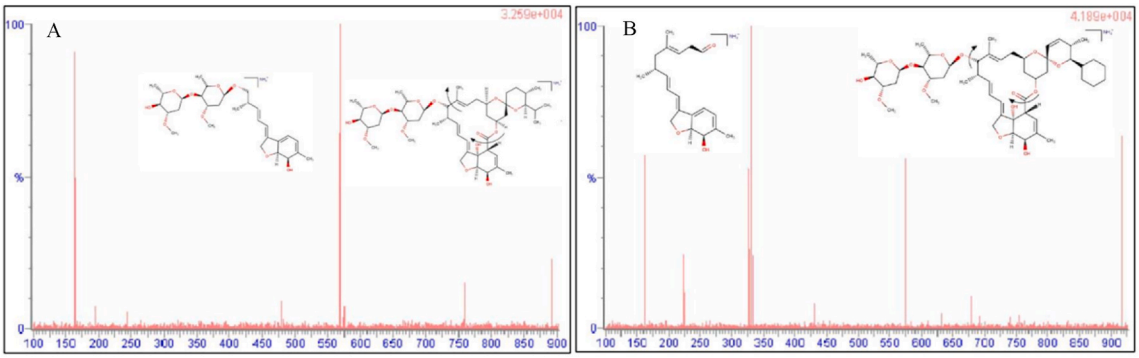

Fig. 2. The mass spectrum fragmentation of (A) ivermectin and (B) doramectin.

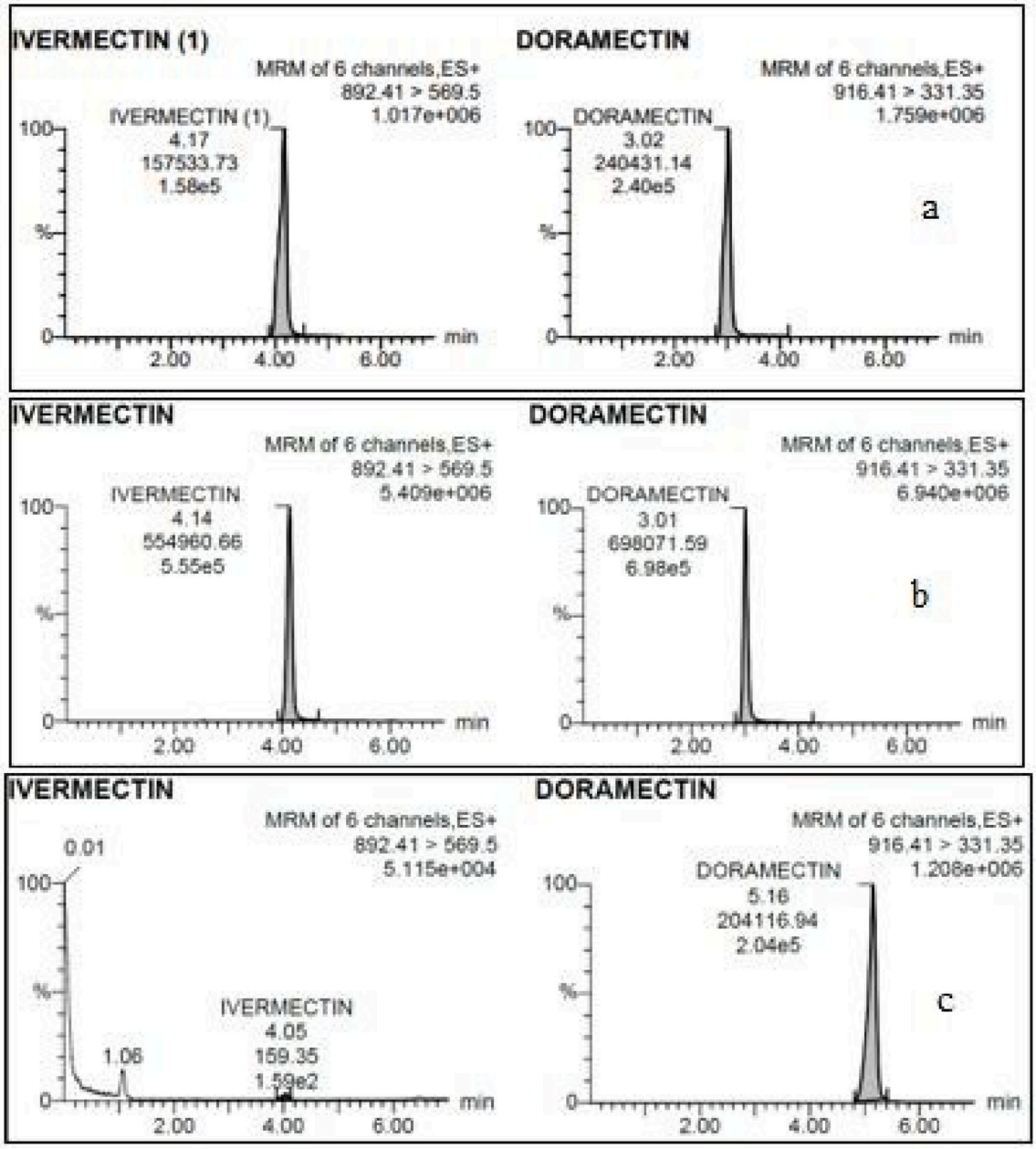

Fig. 3. The Chromatograms of Mobile Phase Composition Optimization as follows (a) 5:95 (v/v), (b) 10:90 (v/v), and (c) 20:80 (v/v) mixture.

3.2.4. Accuracy and precision

Table 1 displays the results for precision and accuracy. For both within-run and between-run tests, all of the data have been found to be accurate. The %diff has not passed 15 % for QC samples and 20 % of LLOQ, as well as the %CV. The chromatograms of QC samples are shown at Fig. 4.

3.3. Recovery and carry over

The test result showed that the percentage recovery was consistent, ranging from 81.29 % to 82.58 %. Therefore, it presumed that the sample preparation method was effective and reproducible. It can be deduced that the extraction method is more effective as the retrieved quantities were more significant when compared to those achieved through DBS. Recovery test was performed in order to show the efficiency of the extraction method. Followed by the carry over test was conducted to see the interference of the analyte and standard on high concentration (ULOQ). The test results and carry over calculations are in the range of 10.8 %–13.49 % and internal

Table 1
Data of Accuracy and Precision of Ivermectin Sample using VAMS.

Nominal concentration (ng/mL)	Day	Within-run			Between-run	
		Mean measured concentration (ng/mL)	CV (%)	% diff range	Mean measured concentration (ng/mL)	CV (%)
LLOQ (1.00)	1	1.10	3.41	3.22–12.03	1.04	9.33
	2	0.92	3.35	−12.83–4.75		
	3	1.12	1.65	9.31–13.86		
QCL (3.00)	1	2.86	2.17	−7.56–2.36	2.9	5.12
	2	2.84	3.72	−9.44–0.64		
	3	2.99	7.18	−8.47–4.7		
QCM (60.00)	1	61.56	5.14	−0.28–8.58	61.04	4.47
	2	59.92	3.19	−3.57–4.76		
	3	61.56	5.14	−0.2.78–8.79		
QCH (120.00)	1	117.99	4.24	−6.91–1.99	114.42	4.15
	2	115.36	2.84	−7.6–1.11		
	3	109.89	0.66	−8.91–−7.38		

standards are in the range of 1.86 %–3.18 %, which has met the acceptance criteria.

3.3.1. Matrix effect

Matrix effect test is should be done when the analysis performed using mass spectrometry. This test aims to know the matrix (blood) will affects the drug sample during analysis. The normalized matrix factor obtained from the concentration of QCL and QCH was 0.89 and 0.92 with %CV obtained 4.11 % and 2.31 %. Consequently, the results met the criteria, indicating that the matrix had no evident impact on the results, which made them reliable. Furthermore, these results indicate that the occurrence of ion intensity suppression can interfere with the process of ionization of compounds [33]. The analytical process can be impacted by reducing ion intensity, which may be due to the ion competing or interfering the analyte from getting charged. This is a typical occurrence when using the Electrospray Ionization (ESI) ionization mode [33].

3.3.2. Dilution integrity

From the results of the dilution integrity test, it was found that all data were included in the conditions of %diff ±15 % and %CV 15 %. As a result, it meets the necessary standards for maintaining the integrity of the dilution process, meaning that results would not be affected by dilution in terms of accuracy and precision.

3.3.3. Stability

The results of the stability test indicated that the stock solution of ivermectin and doramectin 10 ppm remained stable for 24 h when stored at room temperature and for up to 30 days at −20 °C. Meanwhile, the samples in VAMS were viable for up to 24 h stored at room temperature and up to 30 d at freezer temperature (−20 °C). Extracted samples which are ready to be analyzed are stable in an autosampler until 24 h.

4. Discussion

Ivermectin has been used in Africa for many years to help prevent and treat various parasitic infections, such as onchocerciasis. Originally developed as a veterinary drug, it was quickly adapted for human use due to its effectiveness and safety [1]. In recent times, there has been increased interest in the potential of ivermectin to treat other diseases, including COVID-19. Despite some controversy surrounding its use in this context, many African countries continue to rely on ivermectin as an important tool in their medical resources. In the same vein, ivermectin displays potential in both the prevention and treatment of malaria. Although ivermectin is not an independent remedy for malaria, research has indicated that incorporating it in combination therapies can enhance patients' results [27]. The analysis method that we performed was different from the previous studies, which was the combination of mobile phase. We used a buffer solution with smaller and precise concentrations, in which to help maintain the good condition of LCMS system and column. The developed method is using a safest biosampling technique, a great solution of mobile phase to maintain the LCMS at a good state, resulting in a smaller and precise results. The validation results obtained were compared to previous study in order to make sure the development method went well. All other validation parameters were determined from technical data sheets, literature survey, and the experiment itself.

5. Conclusion

The LC-MS/MS method to quantify ivermectin was developed and validated successfully. The validation results have been summarized in Table 2. Comparing the method to earlier methods, it demonstrated improved sensitivity and selectivity using a smaller concentration of mobile phase combination. Therefore, this analytical method can be used for the pharmacokinetics research of ivermectin in healthy individuals with more accurate & smaller amounts of samples.

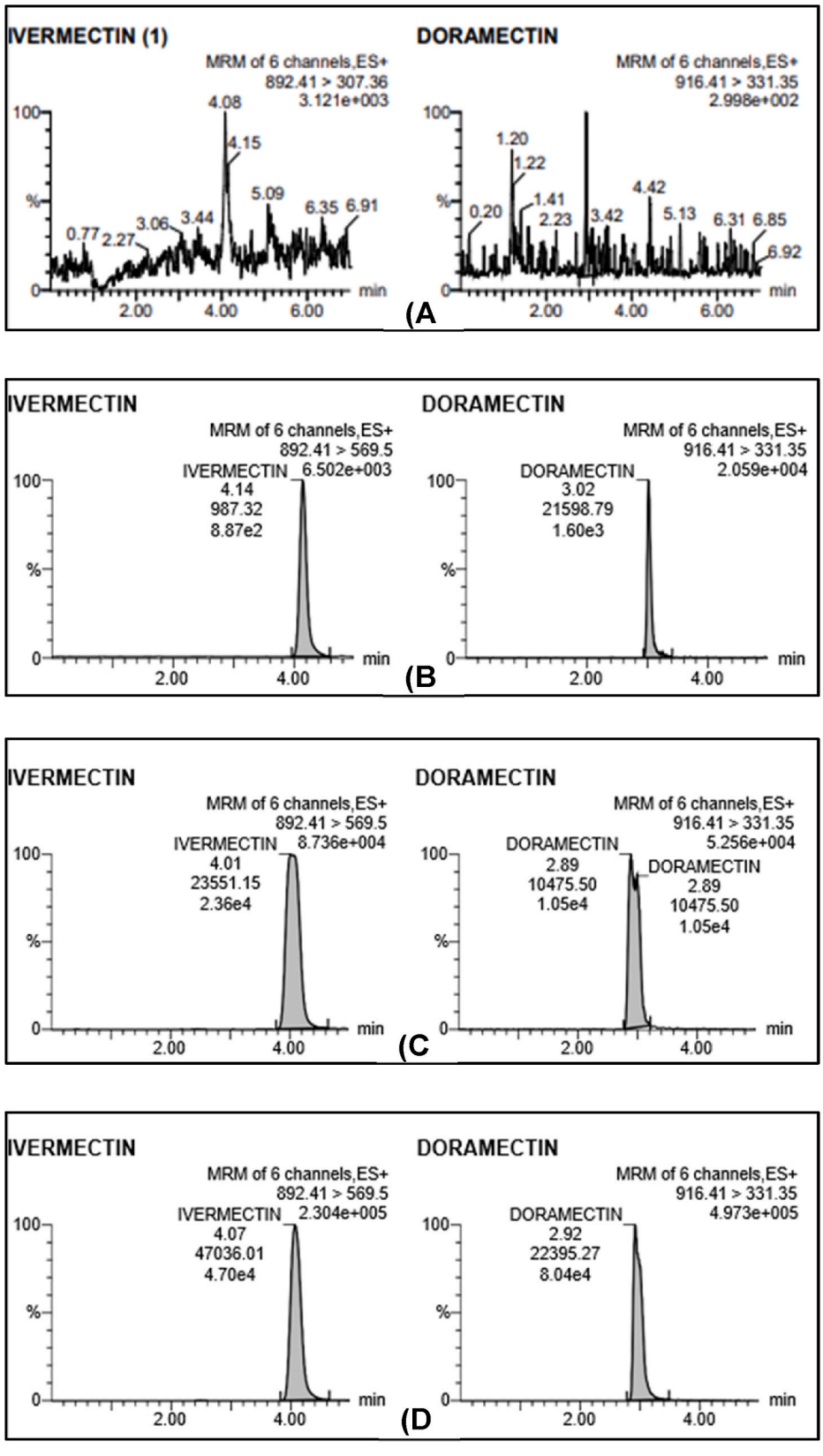

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Fig. 4. The ivermectin and doramectin's LC-MS/MS chromatograms are displayed by (A) blank VAMS; and VAMS spiked with analyte at various concentrations, such as QC samples (B) low, (C) medium, and (D) high.

Table 2
Validation results.

Validation Parameters	Concentrations	Results	Acceptance
LLOQ	1 ng/mL	%diff: 8.42 % to −2.5 % %CV:2.54 %	Accepted
Calibration curve	1–150 ng/mL	%diff: 3.64 %–5.3 % r: 0.9983–0.9994	Accepted
Selectivity	LLOQ and 100 ng/ML (IS)	% interference: 1.12 %–2.94 % % interference of IS: 0.2 %–08.86 %	Accepted
Accuracy and Precision	LLOQ and QC samples	Table 1	Accepted
Recovery	QC samples	% recovery: 81.29 %–81.58 %	Accepted
Carry over	LLOQ and 100 ng/ML (IS)	%CO: 10.8 %–13.49 % %CO of IS: 1.86 %–3.18 %	Accepted
Dilution integrity	½ QCH, QCH, and 2QCH	%diff of ½QCH: 7.67 % to −3.38 % %diff of QCH: 9.5 %–4.13 % %diff of 2QCH: 4.07 %–4.29 %	Accepted
Matrix effect	QCL and QCH	%CV of QCL: 3.63 % %CV of QCH: 1.64 % Normalized matrix factor: 0.89–0.92	Accepted
Stability	LLOQ and QC samples	Stock solution: stable until 24 h at room temperature and 30 d at −20 °C Short-term: stable until 24 h Long-term: stable until 30 d In an autosampler: stable until 24 h	Accepted

Data availability statement

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

CRediT authorship contribution statement

Yahdiana Harahap: Methodology, Investigation, Conceptualization. **Salsabila Salsabila:** Writing – original draft, Formal analysis. **Febrina Amelia Saputri:** Writing – review & editing, Visualization.

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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All authors agreed on the journal to which the article will be submitted, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work. Additionally, all authors participated in the article's conception and design, data collection, analysis, and interpretation, and critical revision for important intellectual content.

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Abbreviations

CO: carry over
CV: coefficient of variation
diff: difference
LLOQ: lower limit of quantification
LC-MS/MS: liquid chromatography tandem mass spectrometry
QC: quality control
QCH: high quality control
QCL: low quality control
QCM: medium quality control
ULOQ: upper limit of quantification
VAMS: volumetric absorptive microsampling