Pharmacy Education (2025) 25(2) 82 - 88
https://doi.org/10.46542/pe.2025.252.8288

ICOPMAP SPECIAL EDITION

RESEARCH ARTICLE

UHPLC-MS/MS method optimisation and validation for Artesunate analysis in urine matrices

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Keywords
Artemisinin
Artesunate
Malaria
UHPLC-MS/MS
Urine

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Abstract

Background: Malaria is a significant health threat. Artesunate is an effective treatment for it, but because it binds strongly to haemoglobin, monitoring drug levels in urine is essential to determine its effective concentration. **Objective:** This study aims to optimise and validate the chromatographic conditions and sample preparation for quantifying artesunate in urine using Ultra-High-Performance Liquid Chromatography Tandem Mass Spectrometry (UHPLC-MS/MS). **Method:** Artesunate levels in urine were analysed via UHPLC-MS/MS, with artemisinin as the internal standard. Separation was achieved using a C18 column (Shim-pack XR-ODSIII, 50 mm × 2.0 mm, 1.6 µm) at a column temperature of 30°C. The optimal mobile phase consisted of methanol-0.3% formic acid in water (80:20, v/v), delivered at 0.3 mL/min under isocratic elution. **Result:** Detection utilised a triple quadrupole mass spectrometer in positive electrospray ionisation (ESI+) mode, monitoring transitions at m/z 407.15 → 261.10 for artesunate and m/z 305.00 → 151.10 for artemisinin. Sample preparation involved protein precipitation with 300 µL of methanol, followed by vertexing (one minute) and centrifugation (10,000 rpm, 15 minutes, 4°C). The method achieved a lower limit of quantification (LLOQ) of 2 ng/mL and demonstrated linearity across the range of 2–400 ng/mL. **Conclusion:** The method met 2022 EMA validation criteria, including selectivity, precision, linearity, recovery, accuracy, and stability.

Introduction

Malaria continues to be a significant global health challenge, especially in endemic areas where it remains a serious threat to public health (Julianto, 2017). According to the World Health Organisation (WHO), malaria is one of the most persistent and unresolved national health threats, with millions of cases reported annually. Artemisinin-based combination therapy (ACT) is currently the first-line treatment for uncomplicated malaria due to its high efficacy and rapid action against Plasmodium parasites. (WHO, 2024).

The emergence of partial resistance to artemisinin-based therapies has raised concerns about the long-term effectiveness of ACT (Betty *et al.,* 1996). The WHO has warned that increasing resistance rates could undermine the efficacy of these life-saving treatments, highlighting the urgent need for robust surveillance and monitoring strategies (WHO, 2021).

Artesunate, a semi-synthetic derivative of artemisinin, has been proposed as a safer and more effective alternative, particularly for paediatric patients (Falade *et al.,* 2023). Monitoring artesunate levels in the body could ensure therapeutic concentrations are achieved, thereby inhibiting parasite growth and preventing the development of resistance (Thuy *et al.,* 2008). However, artesunate's strong binding affinity to haemoglobin (Lindegardh *et al.,* 2011) complicates its quantification in blood, necessitating the development of reliable analytical methods for therapeutic drug monitoring as well as pharmacokinetic studies.

Urine sample offers a promising alternative to quantify artesunate levels in the body (Thuy *et al.,* 2008). The measurement requires a highly selective and sensitive bioanalytical method due to the complexity of the

matrix and the low concentrations of the drug. Method validation is a critical step to ensure the accuracy, precision, and reliability of analytical data (Seger & Salzmann, 2020). Ultra-high-Performance Liquid Chromatography coupled with Tandem Mass Spectrometry (UHPLC-MS/MS) offers sensitivity, selectivity, and accuracy in quantifying drugs in complex biological matrices (Prathipati *et al*., 2019). This study aims to address the urgent need for a validated analytical method to quantify artesunate in urine, leveraging the capabilities of UHPLC-MS/MS.

Methods

Chemicals and reagents

Artesunate and artemisinin were purchased from the National Agency of Drug and Food Control (Indonesia). Formic acid, HPLC-grade methanol and acetonitrile were purchased from Merck (Germany). Urine samples were collected from six healthy volunteers (Indonesia).

Instrument and chromatographic conditions

The analysis was conducted using Ultra-High-Performance Liquid Chromatography Tandem Mass Spectrometry (UHPLC-MS/MS) equipped with a C18 Shim-pack XR-ODSIII column (50 mm × 2.0 mm, 1.6 µm) (Shimadzu, Japan). The mobile phase was composed of a combination of methanol and formic acid in water, delivered under isocratic elution. Detection was performed using a mass spectrometer functioning in positive electrospray ionisation (ESI+) mode with multiple reaction monitoring (MRM). The transitions monitored were m/z 407.15 → 261.10 for artesunate and m/z 305.00 → 151.10 for artemisinin as internal standard. The collision energy was set at 20 eV for artesunate and 15 eV for artemisinin. The desolvation temperature, gas flow rate, interface temperature, and heat block temperature were set at 355°C, 10 L/min, 200°C, and 400°C, respectively. The injection volume was 5µL.

Preparation of solutions and standards

Primary stock solutions of artesunate and artemisinin were prepared by dissolving 10 mg of each compound in 10 mL of deionised water, achieving a final concentration of 1000 µg/mL. These stock solutions were subsequently added to urine to create working solutions with concentrations ranging from 2 to 400 ng/mL. Quality control (QC) samples for artesunate and artemisinin were prepared separately using the same method as the working solutions, with concentrations set at 300 ng/mL (high, QCH), 200 ng/mL (medium, QCM), and 6 ng/mL (low, QCL).

Chromatographic condition optimisation

The optimised parameters encompassed the mobile phase combination, its composition, column temperature, and flow rate. Various mobile phase combinations were evaluated, including methanol with 0.2% formic acid in water, methanol with 0.3% formic acid in water, and 0.4% methanol with 0.4% formic acid in water. The tested methanol and formic acid mixtures had volume ratios of 70:30 (v/v), 80:20 (v/v), and 90:10 (v/v). Column temperatures were assessed at 20°C, 30°C, and 40°C, while flow rate variations included 0.3 mL/min, 0.4 mL/min, and 0.5 mL/min. Lastly, a system suitability test was performed.

Sample preparation optimisation

A total of 200 µL of urine sample containing 200 ng/mL artesunate was transferred into a microtube and mixed with a protein precipitation solution containing 100 ng/mL of artemisinin. The protein precipitation solutions tested were 100% methanol, 100% acetonitrile, and a methanol–acetonitrile mixture (50:50). The solution volumes tested were 100, 200, and 300µL. The mixture was vortexed for 1, 3, or 5 minutes and then centrifuged at 10,000 rpm at 4°C for 5, 10, or 15 minutes. The supernatant was injected into the UHPLC-MS/MS system under optimised conditions.

Method validation

The analytical method was validated in accordance with the 2022 European Medicines Agency (EMA) guideline for full validation. The evaluated parameters included recovery, sensitivity, selectivity, linearity, carry-over, accuracy, precision, and stability. The specific parameters validated in this study are outlined below.

Sensitivity

Sensitivity is determined by the lower limit of quantification (LLOQ), which represents the lowest concentration that satisfies precision and accuracy criteria. These criteria include a coefficient of variation (CV) ≤ 20% and a relative difference (%diff) between measured and actual values within ±20%. If these criteria are met, further analysis can be conducted at half the previous concentration (EMA, 2022).

Linearity and calibration curve

The calibration curve was established using at least six concentration levels, along with blank and zero

samples. Each concentration was analysed in triplicate. A linear equation was generated by plotting the peak area ratio (PAR) of the analyte to the internal standard (y-axis) against the corresponding concentrations (x-axis). This equation was used to recalculate calibration standard concentrations, which had to be within ±15% of the actual values, except for the LLOQ, which allowed a deviation of up to ±20% (EMA, 2022).

Selectivity

Selectivity was evaluated by analysing two replicates of LLOQ and blank samples from six different sources. The acceptance criteria specify that interference at the analyte's retention time must not exceed 20% of the LLOQ response or 5% of the internal standard response (EMA, 2022).

Carry-over

Carry-over was evaluated by analysing a blank sample immediately after the upper limit of quantification (ULOQ) concentration. This process was repeated five times. Any interference detected in the blank at the analyte's retention time must be ≤20% of the LLOQ response and ≤5% of the internal standard response (EMA, 2022).

Dilution integrity

Dilution integrity was tested using five replicates of samples at twice the QCH concentration or above the ULOQ, which were serially diluted to fall within the range of the calibration curve. The test was considered acceptable if the bias was within ±15% and the precision (CV) did not exceed 15% (EMA, 2022).

Precision and accuracy

Precision and accuracy were evaluated by analysing LLOQ, QCL, QCM, and QCH in five replicates, both within the same day (intraday) and across different days (interday). The CV for precision in both cases had to be below 15%, while accuracy (bias) had to remain within ±15%, except for the LLOQ, which permitted a deviation of up to ±20% (EMA, 2022).

Recovery

Recovery was determined by comparing the response of extracted samples to blank extracts spiked post-extraction. The test was conducted in triplicate for QC samples. The acceptance criteria required reproducibility, with a CV value not exceeding 15% (EMA, 2022).

Matrix effect

Matrix effects were evaluated by diluting artesunate in urine at QCL and QCH concentrations using six different urine sources, each analysed in triplicate. Artesunate standard dilutions were also prepared in methanol to achieve QCL and QCH concentrations. Samples were processed using the optimised method and analysed via UHPLC-MS/MS. The acceptance criteria required that the %CV and %diff for each concentration did not exceed ±15% (EMA, 2022).

Stability

The stability of stock solutions and urine samples was evaluated by comparing the measurements of samples stored under specific conditions for a set duration with those of freshly prepared samples. Stability testing for urine samples was conducted at two concentrations (QCH and QCL) in triplicate. The results were considered acceptable if the deviation was less than 6% for stock solutions and below 15% for VAMS samples (EMA, 2022).

Results

Chromatographic condition optimisation and system suitability test

The optimal mobile phase combination was determined to be a mixture of methanol and 0.3% formic acid in water, with a composition of 80:20 (v/v) and a flow rate of 0.3 mL/min. The optimal column temperature was found to be 30°C. These optimised conditions were subsequently evaluated for system suitability. The average retention times for artesunate and artemisinin were 1.007 minutes and 0.959 minutes, respectively, with a total run time of 2 minutes. The chromatogram is presented in Figure 1. The coefficient of variation (CV) for the peak area was 0.615% for artesunate and 3.608% for artemisinin. The CV for retention time was 0.400% for artesunate and 0.307% for artemisinin.

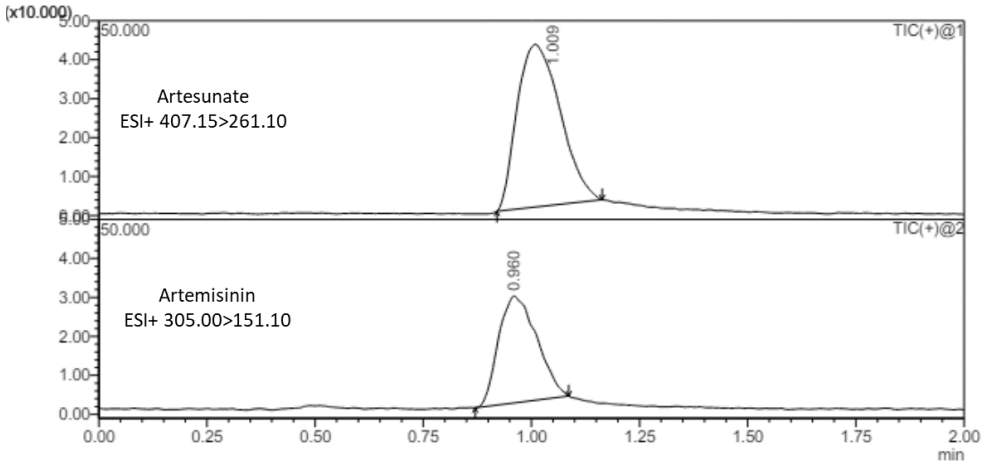

Figure 1: Chromatogram of system suitability test

Sample preparation optimisation

The optimal protein precipitation solution was 100% methanol, with a volume of 300 µL. The optimal vertexing and centrifugation times were determined to be one minute and 15 minutes, respectively.

Sensitivity, calibration curve, and linearity

At a concentration of 1.0 ng/mL, the accuracy (% diff) for artesunate varied from -65.2% to 7.78%, with a coefficient of variation (CV) of 45.58%. In contrast, at 2.0 ng/mL, the %diff ranged from -7.56% to 15.60%, with a CV of 8.32%. Consequently, 2.0 ng/mL was chosen as the lower limit of quantification (LLOQ). The calibration curves exhibited strong linearity over the concentration range of 2.0–400.0 ng/mL, with a correlation coefficient (r) of greater than 0.99. Figure 2 displays chromatograms for the blank, LLOQ (2.0 ng/mL), and upper limit of quantification (ULOQ, 400.0 ng/mL).

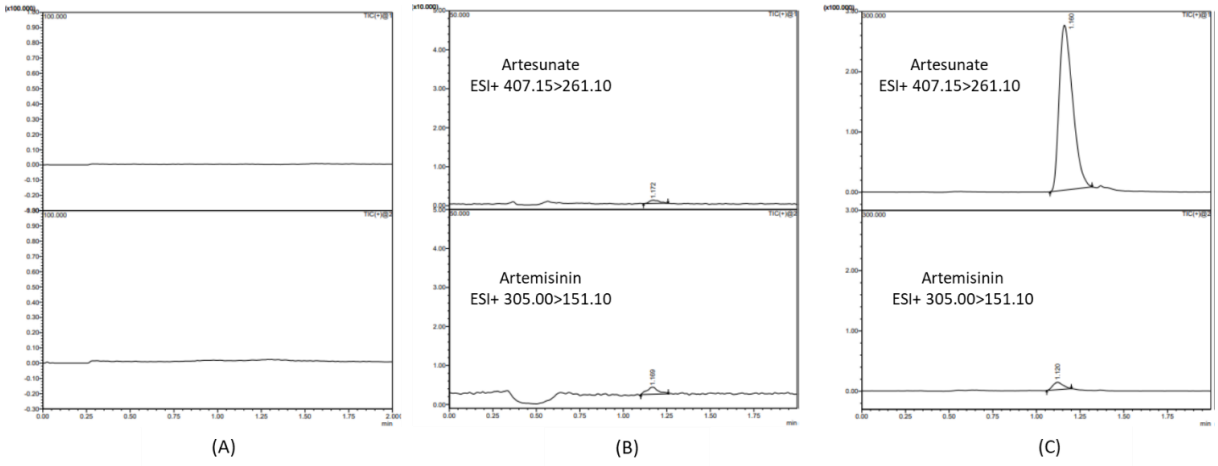

Figure 2: Chromatogram of (A) Blank, (B) LLOQ, and (C) ULOQ samples

Selectivity, carry-over, and dilution integrity

The selectivity and carry-over tests revealed that the interference response at the retention time of artesunate was less than 12.90%, with no interference observed at the retention time of artemisinin. The dilution integrity test yielded a %diff between -14.33% and 12.38%, with a CV below 7.90%.

Precision and accuracy

Intraday and interday accuracy, evaluated over a three-day validation period, showed that the %diff for the LLOQ ranged from -5.91% to 19.41%, with a precision (CV) of less than 9.12%. For the QC samples (low, medium, and high), the %diff spanned from -13.61% to 14.04%, with a precision (CV) below 7.66%. These findings are presented in Table I.

Table I: The result of intraday and interday precision and accuracy

Conc. (ng/mL)	Intraday		Interday	
	Precision (% CV)	Accuracy (% diff)	Precision (% CV)	Accuracy (% diff)
2	9.12	-5.91 to 15.98	4.45	-5.91 to 19.41
6	6.13	-0.67 to 14.04	6.20	-11.67 to 14.04
200	5.75	-2.84 to 11.06	4.74	-10.12 to 12.29
300	7.66	-12.00 to 7.56	0.90	-13.61 to 12.29

Recovery

The mean extraction recovery for artesunate at QCL, QCM, and QCH concentrations was 57.66%, 41.71%, and 59.78%, respectively, with corresponding CV values of 6.84%, 8.08%, and 4.32%. The mean extraction recovery for artemisinin was 63.36%, with a CV of 3.51%.

Matrix effect

The matrix effect, evaluated using six different urine sources, resulted in a %diff ranging from -14.63% to 13.83%, with a CV of less than 9.23%.

Stability

Stability test results indicated that samples remained stable at room temperature (4°C) for six hours, with a %diff ranging from -13.37% to -9.49%. Long-term stability tests conducted over 30 days in a freezer (-80°C) and post-preparation stability tests performed over 24 hours in an autosampler (25°C) also showed a %diff within ±15%. The stability test results are detailed in Table II.

Table II: The result of the stability test

Stability test conditions	QCL (6 ng/mL)		QCH (300 ng/mL)	
	Mean measured conc. (ng/mL) ±SD (n=3)	% diff	Mean measured conc. (ng/mL) ±SD (n=3)	% diff
Short term (25 °C, 6 h)	5.29 ± 0.12	-13.37 to -9.49	340.38 ± 2.15	-11.57 to -7.86
Long term (-80 °C, 30 d)	5.67 ± 0.20	-8.54 to -1.87	288.00 ± 18.3	-10.9 to 0.29
Autosampler (25 °C, 24 h)	5.84 ± 0.30	-5.49 to 3.17	287.83 ± 19.84	-8.02 to 3.58

Discussion

Chromatographic conditions were optimised to achieve the highest peak area, appropriate retention time, and optimal peak shape. The concentration of formic acid in the mobile phase did not significantly affect the retention time of the analyte or internal standard. A concentration of 0.3% formic acid in water, combined with methanol, yielded a greater peak area. In comparison to other concentrations, increasing the formic acid content in the mobile phase, along with adjustments to the flow rate and column temperature, led to a significant reduction in retention time. However, the optimal conditions for achieving the highest peak area were found with a mobile phase composition of 80:20 (v/v), a flow rate of 0.3 mL/min, and a column temperature of 30°C. A system suitability test, with a coefficient of variation (CV) of less than 6%, confirmed that these optimised UHPLC-MS/MS conditions were suitable and reliable for analysis.

The optimisation of sample preparation began with the selection of a protein precipitation solution. Extraction using 100% methanol yielded the maximum peak area. Increasing the methanol volume enhanced the extraction efficiency of the analyte and internal standard, resulting in a larger peak area (Lupo, 2018). Consequently, a volume of 300 µL was selected. The mixture was then vortexed and centrifuged. Vertexing is a critical step that facilitates the transfer of analytes from the urine sample to the methanol phase. Centrifugation separates precipitated proteins and other impurities by applying centrifugal force through rapid rotation of the sample tube. The largest peak area was achieved with vertexing for 1 minute and centrifuging for 15 minutes (Shimadzu, 2024).

The bioanalytical method was validated to confirm its selectivity, sensitivity, accuracy, reproducibility, and applicability for analysing study samples, following the 2022 EMA guidelines. The selectivity evaluation

confirmed that the method could effectively differentiate between analytes, the internal standard, and potential impurities. It met the selectivity criteria, with interference peak areas at the analytes' retention times remaining below 20% of the LLOQ peak area and those at the internal standard's retention time under 5%.

The carry-over test was performed to detect any residual analytes or internal standards from previous injections. Results indicated that the interference peak area at the analyte's retention time was less than 20% of the LLOQ peak area, and the interference at the internal standard's retention time was below 5%. These results aligned with the EMA acceptance criteria.

The sensitivity test established the lower limit of quantification (LLOQ) as 2.0 ng/mL, the lowest concentration with acceptable accuracy (%diff ≤ 20%) and precision (CV ≤ 20%). A concentration of 1.0 ng/mL failed to meet the EMA requirements.

The calibration curve was generated using seven concentration levels (2, 10, 20, 50, 100, 200, and 400 ng/mL), along with blank and zero samples. The curve exhibited linearity, and the %diff of the measured concentrations adhered to the required standards.

A dilution integrity test was conducted to assess the accuracy, precision, and reliability of sample dilution, which is essential for analysing in vivo samples with concentrations above the ULOQ. The results confirmed that the method satisfied the dilution integrity criteria.

Both intraday and interday accuracy and precision met the acceptance criteria, with %diff values within ±20% for the LLOQ and ±15% for QC samples. The CV values were also within limits, not exceeding 20% for the LLOQ and 15% for QC samples. These outcomes confirmed the method's accuracy and precision, complying with EMA guidelines.

Recovery values, with a CV ≤ 15%, demonstrated the reproducibility of the extraction process. Matrix effect results, with a CV ≤ 15% and %diff within ±15%, confirmed that matrix interferences did not affect precision or accuracy.

Stability tests confirmed that stock solutions were stable in a refrigerator (4°C) for 30 days. Artesunate in urine samples remained stable for 6 hours at room temperature (25°C) and for 30 days in a freezer (-20°C). Post-preparation samples also maintained stability in the autosampler (25°C) for 24 hours.

Conclusion

The established bioanalytical method successfully met all complete validation requirements specified in the EMA 2022 guidelines, covering calibration curve, sensitivity, accuracy, recovery precision, selectivity, carry-over, dilution integrity, and stability. The method demonstrated linearity within a concentration range of 2.0–400.0 ng/mL, with a correlation coefficient greater than 0.99.

Acknowledgement

The authors would like to thank the Institute for Research and Community Services, the Republic of Indonesia Defence University, for the research grants received in 2024.

Source of funding

Institute for Research and Community Services, The Republic of Indonesia Defence University.

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