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Original Article

DEVELOPMENT AND VALIDATION OF A BIOANALYTICAL METHOD FOR THERAPEUTIC DRUG MONITORING OF AMIKACIN IN HUMAN PLASMA USING ULTRA-PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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ABSTRACT

Objective: The primary purposes of this research were to develop and validate a novel, accurate, sensitive, and repeatable bioanalytical method for determining amikacin in human plasma employing UPLC-MS/MS.

Methods: The bioanalytical procedure of amikacin involved a BEH C18 UPLC column as a stationary phase, with an employed mobile phase consisting of 0.1% v/v formic acid and acetonitrile (85:15 v/v). The flow rate was set at 0.1 ml/min, and the column temperature was kept at 30 °C. Kanamycin was selected as an internal standard. Amikacin and kanamycin were determined at mass-to-charge ratios (m/z) of 585.9>162.9 and 484.67>162.83, respectively. The amikacin bioanalysis method in the plasma matrix at the optimum separation condition was validated by determination of selectivity, linearity, accuracy, precision, recovery, carry-over, matrix effect, and stability.

Results: The optimum conditions of the sample preparation procedure were obtained through liquid-liquid extraction using trichloroacetic acid, followed by vortex mixing for one minute and centrifugation at 10,000 rpm for five minutes. Ten µl of supernatant was collected and injected into the system. A linear response was achieved in the 1.0-150.0 µg/ml range with R^2 0.9997. Accuracy and precision met the requirements with % differences and coefficient variation at all concentration levels less than 15% and at the LLOQ level (1 µg/ml) less than 20%. The validated analytical method of amikacin in plasma is required for therapeutic monitoring in patients. The data would be valuable for determining or adjusting amikacin doses to enhance patient safety.

Conclusion: A bioanalytical method was developed and validated for determining amikacin in human plasma by UPLC-MS/MS. The method selectivity, linearity, accuracy, precision, recovery, carry-over, matrix effect, and stability were performed.

Keywords: Amikacin, Kanamycin, UPLC-MS/MS, Bioanalytical method, Human plasma, Validation, Therapeutic drug monitoring

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INTRODUCTION

Amikacin is currently the only aminoglycoside class antibiotic that is effective in treating sepsis in Indonesia. In general, amikacin is used with β-Lactam antibiotics to broaden the spectrum in eradicating Multi-Drug Resistance (MDR) pathogenic bacteria such as Pseudomonas aeruginosa. Optimizing the use of amikacin has become medically urgent due to increasing antibiotic resistance [1, 2]. The determination of appropriate loading and maintenance doses of amikacin poses a significant problem for physicians in the Intensive Care Unit (ICU) while managing severe infections. This is a crucial aspect to consider in order to enhance patient survival. Therefore, it is necessary to measure amikacin levels to assist clinical practitioners in setting the right amikacin dose for individual patients, thereby facilitating optimal treatment outcomes and minimizing side effects, especially nephrotoxic and ototoxic [3-5]. Therapeutic Drug Monitoring (TDM) is crucial in managing drugs with a narrow therapeutic index, such as amikacin. The main goal is to ensure that optimal drug concentrations are achieved in the bloodstream, in particular, exceeding the minimum inhibitory concentration (MIC) but not reaching the minimum toxic concentration (MTC) [6].

Methods for the determination of amikacin and other aminoglycoside levels have been developed using microbiological assays, HPLC with photodiode array detector (PDA) UV-Vis spectrophotometer and fluorescence, Fluorimetry, HPLC with catalytic derivatization, spectrofluorimeter, and liquid chromatography-mass spectroscopy (LC-MS). The amikacin sulfate molecule does not have a chromophores group, so it is not suitable to use the UV-visible detectors for qualitative and quantitative-analysis. However, there are amine functional groups that can be derivatized to be detected by PDA and fluorescence detectors [7]. An interest in using Ultra-High Performance Liquid Chromatography (UPLC) in conjunction with high-resolution mass spectroscopy analyzers has grown considerably recently. This may be attributed to several notable advantages, such as enhanced separation efficiency, better sensitivity, and simpler analytical procedures [8].

TDM needs to be carried out in amikacin therapy, but not all hospitals in Indonesia comply with this procedure because it requires high costs and is not covered by insurance [9, 10]. The present study was conducted to develop the TDM procedure for ICU patients at the Central Naval Hospital dr. Ramelan. A bioanalytical technique for measuring amikacin in human plasma using UPLC-MS/MS needs to be developed and validated.

MATERIALS AND METHODS

Instrument

The laboratory equipment used in this study was a UPLC-MS/MS system (Waters, USA) consisted of the Quaternary Solvent Manager Acquity UPLC H-Class for solvent management, the Sample Manager FTN Acquity UPLC for sample handling, the Acquity Column UPLC BEH C18 (100 × 2.1 mm; 1.7 µm) for chromatographic separation,

and the mass analyzer in the form of a triple quadrupole Xevo TQD with Zspray™ ionization source. Data processing is performed using the MassLynx Software, and the computational tasks are carried out on computers (Lenovo, China). The equipment used in this study includes a pH meter (Eutech pH 510), an analytical balance (AND GR-202, Japan), membrane filter 0.2 µm (Whatman, USA), a spectrafuge centrifuge (Labnet International, USA), an ultrasonic stirrer (Elmasonic, Germany), a Freezer capable of maintaining a temperature of-20 °C (GEA, Indonesia), a refrigerator (Samsung, Korea), and Thermo Scientific Vortex Maxi Mix II (USA). For precise liquid handling, an Eppendorf micropipette (Switzerland) was utilized.

Materials

Amikacin (MP Biomedicals, LLC); kanamycin sulfate (Indonesian Pharmacopoeia Comparison Standard); methanol, formic acid, acetonitrile; tricloroacetid acid were obtained from Merck (Darmstadt, Germany); aquabidest (Ikapharmindo, Indonesia); plasma samples (Indonesian Red Cross).

Stock solution

Amikacin or kanamycin, each 20 mg, were precisely weighed and diluted with water to create a stock solution with a 2000 ppm concentration.

Optimization of amikacin analysis used UPLC-MS/MS

The mass spectrometry (Xevo TQD, USA) method was optimized and validated. One ml solution containing amikacin or kanamycin at a concentration of 10.0 µg/ml, was introduced into the reservoir. Subsequently, the standard solution was injected into the mass spectrometry apparatus. During the validation procedure, many parameters are measured, including the tension in the capillary tube, the flow rate of dissolved gas, the flow rate at the cone, the voltage at the cone, the temperature of the source, the energy of ions, the collision energy, and the m/z ratio for each component [11]. By injecting a mixture containing 1.0 g/ml of amikacin and 1.0 g/ml of kanamycin into the UPLC-MS/MS system and observing the chromatographic response, the chromatographic settings were optimized. The optimization process commenced by selecting and evaluating the composition of the mobile phase and then optimizing both the mobile phase and column temperature [12].

Optimization of sample preparation

Sample preparation involved liquid-liquid extraction. A volume of 100 µl of a plasma mixture containing amikacin at a specific concentration was mixed with 50 µl of the internal standard solution, which had a concentration of 100 µg/ml. Subsequently, the mixture underwent extraction using 100 ml of solvents, employing three distinct variations: acetonitrile, formic acid, and trichloroacetic acid [13, 14]. The solutions were vortexed at three various duration (1, 2, and 3 min, respectively) to find optimum mixing. Additionally, centrifugation optimization was conducted for durations of 5, 10, 15, and 20 min at a speed of 10,000 revolutions per minute [15]. A volume of 10 L was then injected into the chromatographic device.

Validation of amikacin in plasma

For the analysis method for amikacin in plasma, LLOQ, selectivity, linearity, accuracy, precision, recovery, carry-over, and stability will all be thoroughly validated [16].

RESULTS AND DISCUSSION

Method development

A volume of 100 µl of plasma was transferred to a 1.5 ml microtube, and a 50 µl aliquot of 100 ppm kanamycin solution was added as the internal standard. The solution was vortexed for 5 seconds. Then 100 µl of 1M trichloroacetic acid and 100 uL of water were added, vortexed for 60 seconds, and centrifuged at 10.000 rpm for 5 min. A total of 150 µl of supernatant was separated and 10 µl was transferred into the UPLC-MS/MS system. This method is less complicated than the previously published study, where the plasma sample was generated by protein precipitation and dilution with heptafluorobutyric acid [13].

Optimization of amikacin using UPLC-MS/MS

The identification of amikacin in mass spectrometry was conducted using positive electrospray ionization with a Multiple Reaction Monitoring approach. Various ionization parameters were employed, including a capillary voltage of 3.5 kV, desolvation rate of 500 L/h, cone flow rate of 0 L/h, cone voltage of 50 V, and a source temperature of 350 °C. The ion energy was 0.2, and the collision energy was 20 V. Amikacin was discovered at m/z ratio 585,9>162,9, while kanamycin was discovered at 484,67>162,83. Table 1 displays an extensive description of the chromatographic conditions.

Table 1: Summary of chromatographs conditions

Parameters	Description
Equipment	Xevo TQD UPLC-MS/MS equipped with an MS photomultiplier detector
UPLC Column	Acquity BEH C18 with length100 mm and diameter 2.1m; particle size: 1.7 µm.
Mobile phase	0.1% formic acid in water–acetonitrile (85:15 v/v)
Flow rate	0.1 ml/min
Injection volume	10 µl
Run Time	7 min
Temperature	30 ℃

Optimization of sample preparation

A volume of 100 µl of plasma was transferred into a 1.5 ml microtube. The microtube was then filled with 50 L of a 100 ppm solution of either kanamycin or amikacin and vortexed for 5 seconds. Afterwards, a volume of 100 µl of a 1M trichloroacetic acid solution is introduced into the experimental system with an equal volume of water. The resulting solution was vortexed for 60 seconds and centrifuged at 10,000 rpm for 5 min [14]. A volume of 150 µl of the supernatant is separated, and 10 µl is injected into the chromatography instrument. Previous research in 2023 has reported similar development in the UPLC-MS/MS method for the TDM of amikacin on neonates plasma with a longer time process, in which mixture was precipitated with 160 µl of cold acetonitrile, extracted with 160 µl of water, and centrifuged for 10 min to get a clean supernatant for LC-MS/MS analysis [17]. These findings confirm that the LC-MS/MS method has become the gold standard for analyzing human plasma amikacin with several sample preparations with the advantage of no derivation step in sample preparation.

Validation of method

The parameters specified in Bioanalytical Method Validation and Study Sample Analysis Validation guidelines were used to validate the method [16]. Results that fulfilled the criteria for each parameter justified the validation of the method used to measure the level of amikacin in human plasma.

Lower limit of quantification

Five plasma samples with an amount of 1 g/ml each were prepared for this experiment. After analyzing the results, the % diff value meets the acceptance criteria below 20% [18]. The quantitation limit value for this method is 1 µg/ml for amikacin with a coefficient of variation (%CV) of 5.19% and an accuracy value (% diff) in the range of-16.92%-(-4.43%).

Selectivity

In the selectivity test, blank plasma from 6 different sources was used. Upon conducting an analysis, a minor interference could potentially arise from endogenous plasma components or chemicals employed in

the amikacin analysis. The interference is noticed during the retention periods of both the analyte and the internal standard. However, it is noteworthy that the percentage interference value in this study remains below 20% of the area corresponding to the LLOQ, and it is also not more than 5% of the area associated with the internal standard. To assess the selectivity of amikacin and kanamycin, the samples were analyzed using a highly selective method based on their m/z ratio. The system was preloaded with the respective m/z values of each molecule. Molecules exhibiting undesirable m/z ratios are eliminated from consideration, while molecules or analytes possessing the intended m/z ratios are directed toward the detector. For the m/z ratio of amikacin, the parent ion is 585.9; the daughter ion is 162.9; for kanamycin, the parent ion is 484.67; and the daughter ion is 162.83.

Linearity

The plot of the peak areas of the analyte versus that of standard is linear within its concentration ranging from 1-150 µg/ml. Calibration curves were generated utilizing a of six concentration range of 1, 5, 10, 25, 50, 100, and 150 µg/ml, including blank and zero samples. Table 2 displays the outcomes of the calibration curves. High linearity was achieved for all calibration curves, as evidenced by a correlation coefficient value of 0.9997. The alternative approach for method development exhibits linearity within ranging from of 0.5–100 µg/ml, with a slightly different value with a correlation coefficient of 0.99843, which shows that the method has a broader concentration level range and better coefficient correlation [17].

Table 2: Linearity of calibration curve

Concentration (µg/ml)	0.00	1.00	5.00	10.00	25.00	50.00	100.00	150.00	R	Slope	Intercept
No.	Measured value (µg/ml)										
1	-0.25	1.19	5.28	10.5	24.64	50.51	97.84	151.29	0.9998	0.0198	0.0067
2	-0.45	0.98	5.15	10.7	24.47	52.1	96.1	151.94	0.9994	0.0207	0.0112
3	-0.27	1.19	5.31	10.51	24.9	49.41	99.36	150.59	1.0000	0.0162	0.0076
Average	-0.32	1.12	5.25	10.57	24.67	50.67	97.77	151.27	0.9997	0.0189	0.0085
SD	0.11	0.12	0.08	0.11	0.22	1.35	1.63	0.67			
CV (%)	0	10.48	1.59	1.09	0.89	2.67	1.67	0.44			

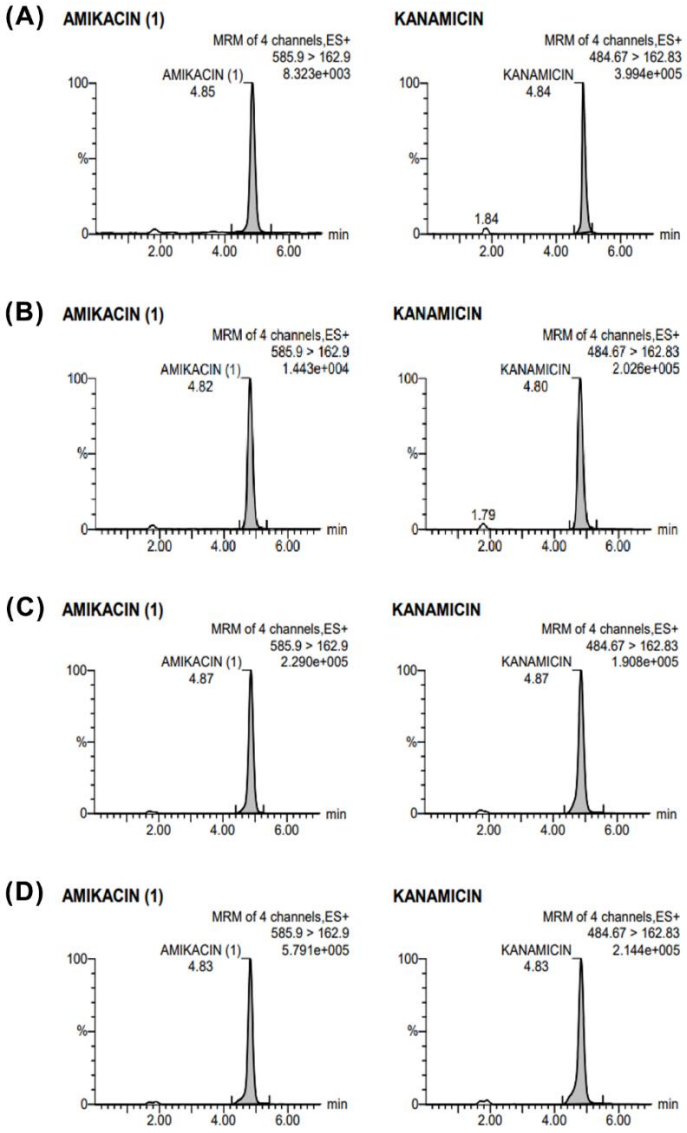

Fig. 1: Amikacin and kanamycin representative UPLC-MS/MS chromatograms in (A) LLOQ; (B) QCL; (C) QCM; and (D) QCH

Accuracy and precision

In intra-batch validation (within-day variation), five replicates of QC samples were evaluated on the same day. QC samples were prepared at four different concentration levels, 1, 3, 60, and 120 µg/ml respectively (fig. 1) [19, 20]. The parameters used to determine the accuracy and precision values were the % differentiation (% Diff) and % coefficient of variation (% CV) values. Precision values (% CV) for QC sample include (13.41%) LLOQ, (4.18%) QCL, (2.32%) QCM and (3.12%) QCH. Meanwhile, the accuracy values were in the range of (-16.48%-18.56%) LLOQ, (3.06%-12.89%) QCL, (-11.46%-(-6.09%)) QCM and (-9.55%-(2.78%)) QCH. The results fulfill the criteria as % diff and % CV<15%.

In inter-batch validation (day-to-day variation), five replicates of QC samples were evaluated on three different days. QC samples were prepared at four concentration levels, namely 1, 3, 60, and 120 µg/ml. The parameters used to determine the accuracy and precision values were the % differentiation (% Diff) and % coefficient of variation (% CV) values. Precision values (% CV) for QC sample include (12.22%) LLOQ, (4.60%) QCL, (6.05%) QCM and (4.53%) QCH. Meanwhile, the accuracy values are in the range of (-16.48%-18.56%) LLOQ, (-4.99%-12.89%) QCL, (-11.46%-6.21%) QCM and (-10.48%-3.15%) QCH. The results fulfill the criteria as % diff and % CV<15%.

Recovery

The recovery values of amikacin in plasma were determined by assessing the peak area ratio of the extracted plasma sample in comparison to the peak area ratio of the unextracted plasma sample at three distinct concentrations. The recovery values for the three concentrations of test samples were within the range with %CV of 2.18% for QCL, 3.14% for QCM, and 7.16% for QCH. The results fulfill the criteria as they have CV values<15%.

Carry-over

The carry-over process is accomplished by inserting a blank sample into the system after injecting the calibration curve's most concentrated solution [21]. According to the experiment's findings, the carry-over value seen in the blank sample does not exceed 20% of the area or peak area of the lower limit of quantification (LLOQ) concentration, and it is also within 5% of the internal standard peak response.

Matrix effect

The matrix effect was used to examine how mass spectrometry could determine the presence of amikacin in plasma. When additional chemicals are present during the ionization of the analyte and the internal standard, in addition to the analyte and the internal standard, the matrix effect occurs. The experiment yielded results indicating that the coefficient of variation (%CV) for matrix effect (ME) at two concentrations, QCL and QCH, using plasma from six distinct sources, was found to be less than 15%.

Stability

Stock solution stability

The stability of the stock solutions of amikacin and kanamycin was investigated during storage at room temperature for 24 h, as well as at a temperature range of 2-8 °C for 37 d. The results showed that the amikacin and kanamycin stock solutions exhibited stability for 24 h when stored at room temperature. Furthermore, these solutions showed stability for 37 d when stored in a refrigerator at a temperature range of 2-8 °C.

Autosampler stability

To evaluate the stability of the samples in the autosampler, two concentrations of 3 µg/ml (QCL) and 120 µg/ml (QCH), with three replicates, were stored in the autosampler immediately after sample preparation. The autosampler stability test showed good results with accuracy values (% diff) (-2.30%)-7.63% and (-3.01%)-(-0.71%) for low and high concentrations. In this case, it was concluded that plasma samples containing amikacin were stable for 24 h if storage in the autosampler.

Short-term stability

The stability of plasma spiked with amikacin at two different concentrations of 3 µg/ml (QCL) and 120 µg/ml (QCH) triplicates were stored at room temperature for 24 h before analysis. Short-term stability test results showed good stability with accuracy values (% diff) in the range of 4.09%-12.23% for QCL and (-0.29%)-3.95%) for QCH. Thus, amikacin in plasma was stable for 24 h of storage at room temperature. The results fulfill the criteria as % diff<15%.

Long-term stability

The 60 d long-term stability test was carried out by analyzing of plasma samples stored in a-20 °C freezer for 60 d. The results showed that the stability met the acceptance requirements as indicated by the accuracy value (% diff) (-5.43%)-4.00% for QCL and (-13.59%)-(-1.49%) for QCH. Thus amikacin in plasma is stable for 60 d of storage in a freezer at-20 °C. The results fulfill the criteria as % diff<15%.

Freeze and thaw stability

The results of the freeze and thaw stability test determination met the requirements as indicated by the accuracy value (% diff) ((-0.70%)-5.23%) for QCL and (-2.45%)-(-1.27%) for QCH after three times freezing and thawing process. The results fulfill the criteria as % diff<15%.

CONCLUSION

A bioanalytical method utilizing UPLC-MS/MS has been successfully developed and validated to quantify amikacin in human plasma. This method is suitable for TDM and can accurately determine the levels of amikacin within the range of 1–150 µg/ml in human plasma.

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AUTHORS CONTRIBUTIONS

Each author has made an equal contribution and approved the completed version of the article.

CONFLICT OF INTERESTS

Declared none

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