Development and validation of a rapid, simple, and reliable UPLC-MS/MS method for the quantification of vancomycin in human plasma

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Abstract

Vancomycin is a critical antibiotic frequently utilized in clinical settings, with therapeutic drug monitoring (TDM) strongly advised to optimize treatment efficacy and mitigate the risk of adverse effects. However, current methods for measuring vancomycin levels in human plasma are hindered by long analysis times and complicated sample preparations. Thus, this study developed and validated a novel UPLC-MS/MS method for a rapid (with a running time of 3.5 min) and simple analysis of plasma vancomycin. To quantify vancomycin concentration in human plasma, we have developed and validated the UPLC-MS/MS method with high sensitivity, specificity, and accuracy, meeting the strict criteria according to the Food and Drugs Administration (FDA) guidelines for validation biological analysis methods. Vancomycin and atenolol (internal standard) underwent positive electrospray ionization (ESI+) and detection in multi-reaction monitoring (MRM) mode. The selected MRM transitions were m/z 725.66→144.16 for vancomycin and m/z 267.29→189.96 for atenolol. Plasma samples were precipitated using a simple mixture containing acetonitrile, methanol, and formic acid as a pH adjuster. The separation was performed using the Poroshell 120 Phenyl Hexyl Column (4.6 × 150 mm, 2.7 μm) maintained at 25 °C for 3.5 min. Isocratic elution with a mobile phase (methanol and 0.1% formic acid in a 40:60 v/v ratio) at a flow rate of 0.5 mL/min was employed. The method showed linearity (0.1–75 μg/mL) with a coefficient of determination above 0.9994 and a lower limit of quantification at 0.1 μg/mL. Precision, both intraday and interday, was below 10%, and accuracy ranged from 91.70% to 111.57%. System suitability, selectivity, stability, carryover, dilution, recovery, and matrix effect validation results all met acceptable criteria. The established UPLC-MS/MS method is expected to be a rapid, simple, and reliable tool for drug monitoring and pharmacokinetic studies, enhancing patient care during vancomycin administration.

Keywords

human plasma, vancomycin, UPLC-MS/MS, tandem mass spectrometry, therapeutic drug monitoring
**Introduction**

Vancomycin (VAN), a tricyclic glycopeptide compound, exhibits efficacy against a broad spectrum of Gram-positive bacteria, including *Corynebacterium*, *Clostridia*, *Enterococci*, *Staphylococci*, *Listeria*, *Streptococci*, and *Pneumococci*. Currently, VAN is an antibiotic used to treat serious bacterial infections. It is the primary agent in treating bloodstream infections, pneumonia, endocarditis, and osteomyelitis caused by drug-resistant Gram-positive bacterial strains (Rubinstein et al. 2014; Zhang et al. 2023). It is typically used to treat infections produced by methicillin-resistant *Staphylococcus aureus* (MRSA) as well as those who have allergies to cephalosporins or semi-synthetic penicillin (Rubinstein et al. 2014). In recent times, MRSA has become a global concern, with a noticeable increase in its incidence worldwide, and MRSA is the second most common cause of severe community-acquired pneumonia (CAP) in Vietnamese children (2021) (Romero et al. 2021; Tran et al. 2023). In contrast, VAN usage has a serious adverse effect called nephrotoxicity, which has high rates of morbidity and treatment failure (Bellos et al. 2020).

Due to the substantial intra- and interpatient variability in pharmacokinetics, as well as the relationship between toxicity and therapeutic failure at low plasma concentrations, therapeutic drug monitoring (TDM) is required throughout its administration to ensure therapeutic effectiveness and prevent nephrotoxicity (Tsutsuura et al. 2021).

The commonly referenced therapeutic range for monitoring vancomycin typically involves peak and trough plasma concentrations, with suggested levels of 30–40 mg/L for the peak and 5–10 mg/L for the trough (Lundstrom et al. 1995, 2004). Following the updated TDM guidelines for vancomycin published in March 2020, trough-only monitoring, with a target of 15–20 mg/L, is no longer recommended based on efficacy and nephrotoxicity data in patients with serious infections due to MRSA. In patients with suspected or definitively serious MRSA infections, an individualized target of the area under the curve/minimum inhibitory concentration (AUC/MIC) ratio of 400 to 600 (assuming a vancomycin MIC of 1 mg/L) should be advocated to achieve clinical efficacy while improving patient safety (Rybak et al. 2020).

Typically, immunoassays have been used to quantify vancomycin in biological sample matrices; however, these assays have limited sensitivity and accuracy (Oyaert et al. 2015; Brozmanová et al. 2017). Specifically, certain immunoassays have been reported to be interfered with by cross-reacting, such as vancomycin degradation products (Chen et al. 2020). When compared to immunoassays, liquid chromatography (LC) in combination with various detectors can provide improved specificity and sensitivity (Veringa et al. 2016; Brozmanová et al. 2017). Particularly, the UPLC-MS/MS method has made it possible to use less of the biological sample while still maintaining sufficient sensitivity. In addition, vancomycin has a very high molecular weight and limited thermal stability; hence, UPLC-MS/MS seemed the most viable method for this goal.

To the best of our knowledge, there have been almost any investigations in Vietnam using the UPLC-MS/MS technology to quantify vancomycin concentration in human plasma to date. To provide a reliable quantification method with suitable execution time and high sensitivity and specificity, contributing positively to the implementation of vancomycin TDM, we conducted this investigation to develop a method for quantifying vancomycin using UPLC-MS/MS. Subsequently, this enables the establishment of the optimal vancomycin dosage through TDM for each distinct patient group, aiming to improve outcomes and minimize toxicity.

**Materials and methods**

**Chemicals and reagents**

Vancomycin was purchased from Sigma-Aldrich, and atenolol (ATEN) as an internal standard (99.9%) was provided by the Institute of Drug Quality Control Ho Chi Minh City in Vietnam.

Acetonitrile, methanol, and water were of LC-MS grade and supplied by Merck (Darmstadt, Germany). All chemicals or solvents used in sample preparation met analytical standards.

Six batches of human plasma were provided by the Can Tho Hematology Blood Transfusion Hospital in Can Tho (Vietnam) and were kept at -20 °C. The protocol of this study was approved by the Human Investigation Ethics Committee in Biomedical Research of the Can Tho University of Medicine and Pharmacy, Vietnam, code 22.152. SV/PCT-HDDD. The authors hereby declare that all the procedures and experiments used in this study conformed with the ethical standards stipulated in the Helsinki Declaration of 1975, as revised in 2013, as well as national laws (World Medical Association 2013).

**Instruments**

The experiments were performed on the ACQUITY UPLC H-Class PLUS System (Waters, Milford, MA, United States) coupled to Xevo TQD Triple Quadrupole Mass Spectrometry (Waters, Milford, MA, United States) equipped with electrospray ionization (ESI). The data were analyzed by the MasslynxTM version 4.2 software (Waters Corporation, Milford, MA, USA).

**Liquid chromatography and mass spectrometry conditions**

Chromatographic separation was performed on the Poroshell 120 Phenyl Hexityl Column (4.6 x 150 mm, 2.7 μm) at 25 °C. The mobile phase consisted of methanol and 0.1% formic acid (40:60, v/v) with a constant flow rate of 0.5 mL.min⁻¹. The injection volume was 10 μL, and the
total run time was 3.5 min. After the injection of each sample, the needle was rinsed alternately with methanol and water (80:20, v/v).

The ion source was set as ESI in positive mode with multiple reaction monitoring (MRM) modes. Each analyte was monitored in two different transitions, as presented in Table 1. Nitrogen was used as the desolvation gas, under the following conditions: capillary voltage: 4 kV; desolvation temperature: 400 °C, desolvation gas flow: 950 L h⁻¹. The collision gas was argon. Cone voltages and collision energies were optimized for each analyte individually, as presented in Table 1. The dwell time was 1.8 s.

Table 1. The optimized mass spectrometry conditions of VAN and ATEN.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Parent compound (m/z)</th>
<th>Daughter fragment (m/z)</th>
<th>Cone voltage (V)</th>
<th>Collision energy (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAN</td>
<td>725.66</td>
<td>144.16 b</td>
<td>26</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>144.08 b</td>
<td>26</td>
<td>40</td>
</tr>
<tr>
<td>ATEN</td>
<td>267.29</td>
<td>189.96 b</td>
<td>40</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>144.98</td>
<td>40</td>
<td>18</td>
</tr>
</tbody>
</table>

a: qualifier daughter fragment.
b: quantifier daughter fragment.

Sample preparation
After thawing, a 300 µL aliquot of plasma was transferred into a microcentrifuge tube, followed by the addition of 150 µL of the IS working solution. To precipitate the protein, add 500 µL acetonitrile, 450 µL methanol, and 100 µL 0.1% formic acid. It underwent vortex mixing for 1 min. After centrifugation (at 10,000 rpm for 7 min), 500 µL of the supernatant phase was transferred to another tube with the addition of 500 µL formic acid 0.1% and then filtered via a 0.22 µm membrane before being injected into the UPLC-MS/MS system. The flowchart of the extraction procedure is provided in Fig. 1.

Assay validation
The approach was validated in accordance with the US Food and Drug Administration (FDA) bioanalytical method guidelines, encompassing: system suitability, specificity, lower limit of quantification, linearity and calibration curve, inter- and intra-day accuracy, precision, extraction recoveries, stability experiments, matrix effect, carryover, and dilution integrity (FDA 2018).

Results and discussion
Method development
In this study, method development was initiated by meticulously optimizing various critical aspects, including ionization, fragmentation conditions, chromatographic separation conditions, and sample preparation conditions.
Ionization and fragmentation conditions for vancomycin and atenolol were fine-tuned using the Masslynx™ version 4.2 software’s auto-tune function (Waters Corporation, Milford, MA, USA). Subsequently, parameters were carefully confirmed and manually adjusted within the software interface to ensure optimized analytical settings for precise and reliable mass spectrometry analysis. Positive-mode electrospray ionization (ESI) was chosen for its greater sensitivity in detecting both vancomycin and atenolol. To enhance specificity in identifying compound fragments, the MRM mode was employed. This combination ensures heightened sensitivity and improved specificity in detecting and characterizing target compound fragments. Vancomycin’s precursor ion appeared at an m/z ratio of 725.66, representing a doubly charged \([\text{M} + 2\text{H}]^{2+}\) ion. The product ion of vancomycin at the m/z ratio of 144.16 results from the separation of the two parts of the glycoside group. Atenol has been used as an internal standard in several studies (Zhang et al. 2007; Shou et al. 2014). Atenol served as an internal standard to enhance the accuracy, precision, and robustness of quantitation. Table 1 displays optimal mass spectrometry parameters, and Figs 2, 3 depict the mass spectrum plot and fragmentation mechanism for VAN and IS.

Due to vancomycin’s polarity (XLogP3-AA = -2.6), reversed-phase liquid chromatography was employed to achieve separation and reduce retention time (Cheng et al. 2010; König et al. 2013; Lu et al. 2022). The Poroshell 120 Phenyl Hexyl Column (4.6 × 150 mm, 2.7 μm) was chosen for its unique selectivity for aromatic compounds like vancomycin, a characteristic not commonly found in other reversed-phase packings such as C18 and C8. Formic acid served as a pH adjuster, with this study exploring concentrations (0.05%, 0.1%, and 0.2%) as additives to the mobile phases, using methanol and acetonitrile as organic mobile phases (Zhang et al. 2007; Liu et al. 2018; Xiao et al. 2021). The final chromatographic conditions were established with a mobile phase of methanol and 0.1% formic acid in a 40:60 (v/v) ratio, maintaining a constant flow rate of 0.5 mL/min. The injection volume was 10 μL, and retention times for vancomycin and atenolol were 2.58 min and 2.82 min, respectively, within a total run time of 3.5 min. This brief analysis duration is crucial for ensuring efficient application in TDM processes and contributes to cost-saving measures.

The study aimed to devise a straightforward and time-efficient sample processing approach for vancomycin analysis using UPLC-MS/MS in human plasma. While solid-phase extraction (SPE) has proven effective in handling complex biological sample matrices, it often involves multiple steps and increases costs (Javorska et al. 2017). Our sample preparation method prioritizes simplicity, involving protein precipitation, centrifugation, and filtration. Acetonitrile, methanol, and 0.1% formic acid were employed for protein precipitation. Organic solvent precipitants enhance molecule attraction, reduce the dielectric constant of plasma protein solutions, and promote electrostatic protein interactions. Additionally, organic solvents displace organized water molecules around hydrophobic areas on protein surfaces (Polson et al. 2003). After centrifugation, formic acid is added to the supernatant to alter the medium’s pH, influencing the sample’s polarity and separation (Thao Nguyen Ngoc Nha et al. 2020). Adjusting the pH enhances peak shape, ensuring precise and high-quality analytical results. The method demonstrates high recovery rates.

### Method validation

#### System suitability

Tests assessing system suitability are an essential component of liquid chromatographic methods. This guarantees that all equipment, instruments, system operations, and factors affecting chromatography have consistent or reproducible performance with the analysis method being performed. Inject a simulated sample containing a combination of analytes and IS six times in a row for monitoring at MQC.

According to the results, the parameters of analytes and IS after six injections had a relative standard deviation (RSD) of less than 5% for retention time, peak area, peak area ratio, and retention time ratio.
Analytical selectivity

Selectivity was performed by testing blank plasma samples collected from six different lots of plasma with the described procedure to evaluate the absence of interfering peaks at retention times and MRM transitions of VAN and IS. A possible interfering peak is considered a peak with a response higher than 20% of VAN and 5% of IS at LLOQ. According to the results, no interference from human plasma at the retention times of VAN and IS was observed, confirming the selectivity of the assay.

Lower limit of quantification (LLOQ)

The LLOQ of the method was defined as the lowest analyte plasma concentration within the linear range that may be found with a tolerable degree of accuracy and precision. Six QC samples were injected at LLOQ concentrations to conduct the test. The defined LLOQ concentration was found to be 0.1 μg/mL. According to the regulations (allowable limit: 80%–120%, RSD < 20%), the analysis reveals an accuracy of 98.65% and a precision of 2.5% RSD. Furthermore, the S/N ratio of LLOQ was > 5. The lower limit of quantification (LLOQ) concentration achieved in this study surpasses that of previous research (Shi et al. 2018; Stajić et al. 2018; Fan et al. 2019), indicating a heightened sensitivity of the method.

Linearity of calibration curve

UPLC was utilized to evaluate the linearity of the calibration curve with this method using samples with different vancomycin concentrations and identical IS concentrations. Plotting the ratio of the vancomycin peak area to the IS peak area against the vancomycin concentration was the next step. Following a regression test, the significance F-value and P-value of the X-variable (slope) were found. Vancomycin's linearity was evaluated with a correlation coefficient larger than 0.98 between 0.1 and 75 μg/mL of analyte concentration. The specific concentration range and equation for VAN can be found in Table 2. This linear range permits measurement across medications used in various formulations and at various sample intervals, and it is compatible with the therapeutic range in human plasma. This is a crucial starting point for carrying out bioequivalence analyses in further research. Fig. 5 depicts the calibration curve of the VAN.

Precision and accuracy

Accuracy and precision were assessed using blank human plasma samples containing the analytes at four levels of quality control (LLOQ, LQC, MQC, and HQC). Table 3 summarizes the intra- and inter-day precision and accuracy for the VAN from the QC samples. All parameters meet the specified criteria. Chromatograms depicting these analytes and the IS in QC samples are presented in Fig. 4.

Stability

The stability of the analyte in human plasma was assessed under various conditions. Long-term stability was investigated by storing samples at -20 °C for 30 days and 60 days and then subjecting them to three freeze-thaw cycles across two QC concentrations (LQC and HQC). Short-term stability was examined by observing samples at room temperature for 6 hours and at 10 °C in an autosampler for 24 hours. Additionally, the stability of stock standard solutions was tested by analyzing them after 6 hours at room temperature and after storage at -20 °C for 30 days and 60 days. In all cases, the recovery of the analyte fell within the acceptable range of 85% to 115%, with an RSD of less than 15%. Table 4 presents the findings from diverse stability experiments.
Dilution and carryover

Dilution validation involved generating six simulated plasma samples with a concentration twice that of the HQC concentration. Subsequently, these samples were diluted twice with blank plasma, processed, and subjected to analysis. The recovery rate of VAN after two dilutions was 98.4%, with a recovery rate RSD of 3.01%. Both recovery rate and RSD are within permitted bounds (85% to 115%, RSD ≤ 15%). Therefore, the dilution has no effect on the designed sample preparation technique.

Analytes from a prior injection may be detected by performing residual testing. The injections were carried out six times, commencing with the sample possessing the highest concentration (ULOQ) and concluding with the blank sample. As a result, there is little residual sample influence on VAN and IS. The remaining in the blank sample had an impact of 7.51% for VAN and 0.05% for IS when compared to the lower limit of quantification (LLOQ). The percentage values are within permissible bounds (≤ 20% for analytes and ≤ 5% for the internal standard). Fig. 6 provides chromatograms of the blank sample after ULOQ injection.

Table 3. Precision and accuracy of VAN.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Level</th>
<th>QC nominal</th>
<th>Conc (µg/mL)</th>
<th>intra-day (n=6)</th>
<th>inter-day (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Measured Conc (Mean ± SD, µg/mL)</td>
<td>Accuracy (%)</td>
<td>Measured Conc (Mean ± SD, µg/mL)</td>
</tr>
<tr>
<td>VAN</td>
<td>LLOQ</td>
<td>0.1</td>
<td>0.11 ± 0.01</td>
<td>3.93</td>
<td>109.92</td>
</tr>
<tr>
<td></td>
<td>LQC</td>
<td>0.3</td>
<td>0.33 ± 0.01</td>
<td>2.14</td>
<td>111.57</td>
</tr>
<tr>
<td></td>
<td>MQC</td>
<td>18</td>
<td>19.70 ± 1.9</td>
<td>9.63</td>
<td>109.43</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>40</td>
<td>36.68 ± 1.94</td>
<td>5.28</td>
<td>91.7</td>
</tr>
</tbody>
</table>

Conc: Concentration.
Recovery and matrix effects

The extraction recoveries were carried out on samples at LQC, MQC, and HQC concentrations. To determine the extraction recovery, the ratio of peak areas of VAN and IS obtained from pre-extraction and post-extraction spiked plasma samples was calculated.

The matrix effect was performed on samples at LQC and HQC concentrations. Matrix effects were evaluated by comparing the peak areas of VAN and IS obtained from the post-extraction spiked plasma sample to those from the methanol sample. Table 5 summarizes the recovery and matrix effects of vancomycin in human plasma. Both the extraction recovery and matrix effects were within the acceptable range of 85% to 115%. The RSD value of the parameters was <15%.

Table 5. Recovery and matrix effects of VAN in human plasma.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Level</th>
<th>QC nominal</th>
<th>Recovery extraction (n=6)</th>
<th>Matrix effect (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc (µg/mL)</td>
<td>Mean ± SD (%)</td>
<td>RSD (%)</td>
<td>Conc (µg/mL)</td>
</tr>
<tr>
<td>VAN</td>
<td>LQC</td>
<td>0.3</td>
<td>105.51 ± 4.90 4.64</td>
<td>106.16 ± 6.51 6.13</td>
</tr>
<tr>
<td></td>
<td>MQC</td>
<td>18</td>
<td>95.77 ± 5.43 5.67</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>40</td>
<td>103.51 ± 4.13 3.99</td>
<td>108.32 ± 6.22 5.75</td>
</tr>
<tr>
<td>IS</td>
<td>LQC</td>
<td>0.3</td>
<td>94.28 ± 5.92 6.28</td>
<td>100.45 ± 5.79 5.77</td>
</tr>
<tr>
<td></td>
<td>HQC</td>
<td>40</td>
<td>91.53 ± 5.97 6.52</td>
<td>-</td>
</tr>
</tbody>
</table>

The structures of the compounds and matrix samples may have an impact on the percentage of suppression, especially in complex environments and biosamples (Do et al. 2020). In this study, the results indicate that a low matrix effect and high recovery were observed. This suggests that the method is effective in minimizing interference from matrix components while efficiently recovering the analyte from the biological matrix. The impact of the sample matrix on the analytes was reduced in our investigation by employing the internal standard and the protein precipitation technique during the sample processing method.

Conclusion

The rapid, simple, and reliable UPLC-MS/MS method was developed and validated to allow the determination of therapeutic concentrations of vancomycin with a high sensitivity of 0.1 µg/mL. Since it falls within the therapeutic concentration value in patients, the analytical scope of the described approach can be deemed appropriate. The findings obtained validate the applicability of the approach in clinical pharmacokinetic investigations involving vancomycin recipients. To get samples with medication concentrations within the therapeutic range, it can determine the best times to collect samples. Furthermore, bioequivalence studies of generic medication items might benefit from the validation of this methodology.
References


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