An Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry Method for the Quantification of Vancomycin Requiring Only 2 µL of Rabbit Serum

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Abstract

A highly sensitive ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method was developed for the quantification of vancomycin (VAN) in low volumes of rabbit serum. For each analysis, 2 µL of rabbit serum was precipitated with methanol that contained the internal standard teicoplanin (TEI). The supernatant was transferred into a 384 well-plate, diluted with water, covered with a pierceable silicone mat and 5 µL was analyzed in positive ionization mode. The UHPLC-MS/MS consisted of an Agilent 1290 Infinity UHPLC system connected to an AB Sciex QTrap® 5500 hybrid linear ion-trap triple quadrupole mass spectrometer equipped with a Turbo Spray source. Chromatographic separation was achieved using a Waters Acquity UPLC BEH C18 (1.7 µm, 2.1 mm × 100 mm) column, a VanGuard (1.7 µm, 2.1 × 5 mm) guard column and a mobile phase of water and methanol both containing 5 mM ammonium acetate with 0.1% formic acid. VAN was quantified with multiple reaction monitoring using the transitions of m/z 725.5/144.2, and TEI was monitored at m/z 940.6/316.4. The accuracy, precision, linearity, range and lower limit of quantification (LLOQ) were determined. The accuracy was ≤9.93% and the precision was ≤10.6%. The range was established as 0.1 to 40 µg·mL⁻¹. The LLOQ was 0.1 µg·mL⁻¹ VAN requiring 2 µL of sample with an accuracy of −20.2% and precision of 8.39%. The method was applied successfully to determine the VAN concentrations in rabbit serum after the i.v. administration of VAN.

Keywords

Ultra-High Performance Liquid Chromatography-Tandem Mass Spectrometry, Vancomycin, Rabbit, Serum, Pharmacokinetics
1. Introduction

Vancomycin (VAN) is a glycopeptide antibiotic used to treat severe bacterial infections caused by, for example, methicillin-resistant *Staphylococcus aureus*. If VAN is not dosed properly, it can cause drug resistance, kidney damage, and hearing loss in patients [1] [2]. In the clinical practice, the dosage regimen of VAN is personalized using therapeutic drug monitoring (TDM) and the blood concentration of the drug is monitored closely using an appropriate analytical technique [1] [2].

There are several methodologies available for the measurements of VAN in biological matrices that include radioimmunoassays, fluorescence immunoassays, fluorescence polarization immunoassays [3], particle enhanced turbidimetric inhibition immunoassays (PETINIA) [4], liquid chromatography (LC) [5], and LC-tandem mass spectrometry (LC-MS/MS) [6] [7] [8] [9]. These methods were successfully used to measure VAN in a variety of matrices, but they had their limitations. They either were not sufficiently precise [3], suffered from interference [4], had insufficient sensitivity [5] [6] [8], or required large sample volumes [6] [8] [7] [9]. Large sample volumes are sometimes difficult to obtain, especially in studies where VAN is determined in the samples of small laboratory animals, such as rabbits, where the availability of the biological matrix is limited.

There are assays available in the literature to determine VAN in rabbit serum [10] [11]; however, there have been no attempts to quantify VAN in rabbit serum using LC-MS/MS. The objective of this study was to develop a sensitive and selective ultra-high performance LC-MS/MS (UHPLC-MS/MS) method for the quantification of trace levels of VAN in rabbit serum requiring a low sample volume. The method was applied successfully to measure the VAN concentrations in rabbit serum after the i.v. administration of VAN. The data were compared to results obtained by PETINIA in a previous study (unpublished data). To our knowledge, this is the first UHPLC-MS/MS assay for the quantitation of trace levels of VAN in rabbit serum requiring only 2 µL of sample. The feasibility was investigated to expand the current assay to larger studies in the future where the method is modified and implemented in similar matrices, such as interstitial fluid (ISF) [12].

2. Experimental

2.1. Materials and Reagents

Vancomycin hydrochloride (purity > 80%), teicoplanin (teicoplanins A2, purity ≥ 80%), methanol (HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Vancomycin hydrochloride (USP grade) for the rabbit experiments was from Hospira (Lake Forest, IL, USA). Formic acid (purity 98%) was from Acros Organics (Geel, Belgium), ammonium acetate (purity 97%) from Caledon Laboratories (Georgetown, ON, Canada). Pooled drug-free serum from female New Zealand white rabbits (protein content 63.0 ± 0.3 mg∙mL⁻¹ normalized to
bovine serum albumin) was from the Centre for Comparative Medicine (Vancouver, BC, Canada). Ultra-pure water was prepared in our laboratory using a Milli-Q Synthesis system (Millipore, Billerica, MA, USA).

2.2. Rabbit Experiments

Animal experiments conducted in this study were approved by The University of British Columbia Animal Care Committee (Certificate number A10-0149) and adhered to the guide for the care and use of experimental animals [13]. New Zealand white rabbits (female, 1.75 - 5.5 kg) obtained from Charles River Laboratories (Wilmington, MA, USA) were used in the study. Rabbits were acclimatized for at least 7 days in group pens in a temperature-controlled room under a dark and light cycle of 12 h. They had access to typical diet (i.e., pellets, hay, and vegetables) and water ad libitum before and after the testing procedures. Five i.v. doses of VAN (vancomycin hydrochloride, diluted in sterile sodium chloride 0.9% solution) were administered to the animals (n = 5). One loading dose of 20 mg·mL⁻¹ followed by four maintenance doses of 15 mg·mL⁻¹ of VAN were administered with a dosing interval of 1.5 h. Blood samples were taken from the opposite ear using intraarterial catheters and transferred into serum collection tubes at the following time points: pre-dose at 0, 1.5, 3, 4.5, 6 h (trough concentration), and post-dose at 6.25, 6.5, 7, 7.5, 8, 8.5, 9 h. Clotted blood samples were centrifuged at 1000 × g for 10 min, serum kept at 4˚C until the end of the study period of 12 h, and then frozen at −20˚C until analysis.

2.3. Instrumentation and Chromatographic Conditions

The UHPLC-MS/MS system consisted of an Agilent 1290 Infinity Binary Pump, a 1290 Infinity Sampler, a 1290 Infinity Thermostat, and a 1290 Infinity Thermostatted Column Compartment (Agilent, Mississauga, ON, Canada) connected to an AB Sciex QTrap® 5500 hybrid linear ion-trap triple quadrupole mass spectrometer equipped with a Turbo Spray source (AB Sciex, Concord, ON, Canada). The mass spectrometer was operated in positive ionization mode and data were acquired using the Analyst 1.5.2 software on a Microsoft Windows XP Professional operating platform. Chromatographic separation was achieved using a Waters Acquity UPLC BEH C₁₈ (1.7 µm, 2.1 × 100 mm) column that was protected by a Waters Acquity UPLC BEH C₁₈ VanGuard (1.7 µm, 2.1 × 5 mm) guard column. The columns were maintained at 35˚C and the autosampler tray temperature was maintained at 10˚C. Solvent A was water with 5 mM ammonium acetate (AA) and 0.1% formic acid (FA), and solvent B was methanol with 5 mM AA and 0.1% FA. The mobile phase initial conditions were solvent A (95%) and solvent B (5%) that was ramped to solvent A (5%) by 0.80 min held until 4.0 min and followed by an equilibration with solvent A (95%) and solvent B (5%) for 2 min. The flow rate was 0.2 mL·min⁻¹, the injection volume was 5 µL with a total run time of 6.0 min. Samples were injected from a 384-well plate.
2.3.1. Mass Spectrometric Parameters

The mass spectrometer was operated in multiple reaction monitoring (MRM) mode with the mass spectrometric parameters as follows. Curtain gas, 30 psi, collision gas (CAD), high, ionspray, 5500 V, temperature, 450°C, ion source gas 1, 30 psi, ion source gas 2, 40 psi. Nitrogen gas was used for curtain gas, collision gas, ion source gas 2 (vaporizing gas), and zero air was used for ion source gas 1 (nebulizing gas). Entrance potential was 10 V, resolution was Unit for Q1, Q3, and dwell time was 150 msec for the compounds.

2.3.2. MRM Experiments

VAN was monitored using the MRM transitions as follows. Quantifier transition (declustering potential DP (V), 146, collision energy CE (eV), 21, collision cell exit potential CXP (V), 24) m/z 725.5/144.2, and qualifier transition (DP, 131, CE, 79, CXP, 48) m/z 725.5/100.0. Teicoplanin (TEI), internal standard (IS), was monitored using the MRM transitions as follows. Quantifier transition (DP, 176, CE, 21, CXP, 6) m/z 940.6/316.4, and qualifier transition (DP, 181, CE, 29, CXP, 6) m/z 940.6/298.2. The mobile phase flow was diverted to the waste during the first 2.0 min of the chromatographic run.

2.4. Preparation of Calibration Standards and Quality Controls

A stock solution of 1 mg∙mL⁻¹ VAN was prepared in 50% methanol/water (v/v) then further diluted to a second stock solution of 40 µg∙mL⁻¹ in rabbit serum, and stored on ice. From this solution, nine calibration standards were prepared to yield the concentrations of 0.1, 0.25, 0.5, 1, 5, 10, 20, 30 and 40 µg∙mL⁻¹ VAN. With each calibration curve, a zero sample (containing only IS) was prepared and the samples were stored at 4°C. Quality control (QC) samples as QC-Low 0.3 µg∙mL⁻¹, QC-Mid 12 µg∙mL⁻¹, and QC-High 32 µg∙mL⁻¹ were also prepared and stored at 4°C. TEI stock solution of 1 mg∙mL⁻¹ was prepared in 50% methanol/water (v/v) then further diluted to yield 600 ng∙mL⁻¹ solution in methanol. This solution was stored at −20°C and used as the precipitation reagent. The final concentration of the IS in the analyzed samples was 250 ng∙mL⁻¹.

2.5. Sample Preparation

Ten µL of ice-cold precipitation reagent containing the 600 ng∙mL⁻¹ IS was pipetted into 0.5 mL tubes (Eppendorf, lowBind, Mississauga, ON, Canada) on ice. After adding 2 µL of calibration standards, QC samples, or study serum sample, the tubes were vortexed (1 s, ~3000 rpm), sonicated for 5 min, again vortexed (1 s, ~3000 rpm) and subsequently centrifuged for 2 min at 9000 rpm (Eppendorf, Mississauga, ON, Canada). On ice, 9.7 µL of the supernatant was transferred to a 384 well plate containing 10 µL of Milli-Q water in each well and mixed by repeated aspiration. After a sonication of approx. 10 s, the plate was covered with a silicone mat. A maximum of 31 samples were prepared in one batch and analyzed with UHPLC-MS/MS.
2.6. Method Qualification

2.6.1. Accuracy and Precision

Five replicates of QC-Low, QC-Mid, and QC-High were prepared in rabbit serum and analyzed. Accuracy was expressed as the percentage deviation of the measured VAN concentration against the added concentration according to the following formula: %Deviation = [(measured concentration/added concentration) × 100] – 100. The acceptance criterion for accuracy was %Deviation ±15% for the QC-Low, QC-Mid, and QC-High samples. Precision was expressed as the relative standard deviation (%RSD) of the VAN concentrations of the QC-Low, QC-Mid, and QC-High samples. The acceptance criterion for precision was the %RSD ≤ 15% for the QC-Low, QC-Mid, and QC-High samples. For intra-day accuracy and precision, five replicates of QC-Low, QC-Mid, and QC-High samples were prepared and analyzed on the same day (n = 5). Inter-day accuracy and precision were determined by repeating intra-day experiments for three separate days, and using the combined results the inter-day accuracy and precision were calculated (n = 15).

2.6.2. Linearity and Range

Calibration curves were prepared on separate days (n = 3) as detailed in section 2.4. The curves were constructed by plotting the concentrations of VAN on the x-axis, vs. the chromatographic peak area ratio of VAN to IS on the y-axis. Linear regression analyses were performed using the calibration curve data and a weighting factor of 1/x^2. The range of the assay was established as the linear section of the calibration curve where the correlation coefficient was r ≥ 0.98 for VAN after weighting with 1/x^2.

2.6.3. LLOQ

LLOQ was determined using five replicates of the 0.1 µg∙mL^-1 calibration standard prepared in rabbit serum and analyzed. The mean response (signal-to-noise, S/N), accuracy and precision were determined from the samples. The LLOQ was determined as the lowest concentration level of the calibration curve that met the following acceptance criteria. The mean response of VAN peaks in the samples was at least 5-times the response compared to the blank sample (S/N was calculated using Analyst 1.5.2 software). The VAN peaks were identifiable, discrete, and reproducible, with an accuracy of %Deviation ± 20% and precision %RSD ≤ 20%.

3. Results and Discussion

3.1. Mass Spectrometry, Liquid Chromatography

The precursor ions and the product ions of VAN and TEI were determined by the direct infusion of their approx. 1 µg∙mL^-1 solutions in 50% methanol/water (v/v) into the mass spectrometer. Using Turbo Spray positive ionization mode, the doubly charged precursor ions of VAN m/z 725.5, and m/z TEI 940.6 were
Figure 1. Representative chromatogram of vancomycin (VAN), 5 µg·mL⁻¹, and teicoplanin (TEI), 0.25 µg·mL⁻¹, in rabbit serum. Inset: LLOQ sample of VAN, 0.1 µg·mL⁻¹. The dotted lines indicate the regions used for signal and noise collection, respectively. CPS: counts per second observed. The precursor ions were fragmented by collisionally activated dissociation and the product ions were generated. The mass spectrometry parameters were optimized to achieve the most intense signals. MRM transitions were created and VAN and TEI were quantitated using their quantifier MRM transitions, and their identity confirmed by monitoring their qualifier MRM transitions. The Waters Acquity UPLC BEH C₁₈ 1.7 µm 2.1 × 100 mm column, the mobile phase composition, and gradient programming provided a fast analysis with a run time of 6.0 min. Using the current experimental conditions, the retention times were for VAN, 2.27 min, and for TEI, 2.68 min. A representative chromatogram of VAN (5 µg·mL⁻¹) and TEI (0.25 µg·mL⁻¹) in rabbit serum is presented in Figure 1.

3.2. Method Qualification

3.2.1. Accuracy and Precision
The results for accuracy and precision of VAN are presented in Table 1. Intra- and inter-day accuracy ranged between 2.11% and 9.93%, and between 5.07% and 7.44%, respectively. Intra- and inter-day precision ranged between 2.51% and 10.6%, and between 6.46% and 8.94%, respectively. The method was accurate and precise for the QC-Low, QC-Mid, and QC-High samples with a %Deviation ± 15% and %RSD ≤ 15%, respectively.

3.2.2. LLOQ, Linearity and Range
The LLOQ was determined in five replicate samples at a concentration of
Table 1. LLOQ, accuracy and precision for VAN in rabbit serum.

<table>
<thead>
<tr>
<th>Added concentration (µg·mL⁻¹)</th>
<th>Accuracy (%Deviation)</th>
<th>Precision (%RSD)</th>
</tr>
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<tbody>
<tr>
<td>LLOQ (n = 5) 0.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measured conc. (µg·mL⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QC-Low 0.08</td>
<td>QC-Mid 0.08</td>
<td>QC-High 0.08</td>
</tr>
<tr>
<td>0.30</td>
<td>12.0</td>
<td>32.0</td>
</tr>
<tr>
<td>QC-Low 0.30</td>
<td>QC-Mid 0.30</td>
<td>QC-High 0.30</td>
</tr>
<tr>
<td>0.30</td>
<td>12.0</td>
<td>32.0</td>
</tr>
<tr>
<td>Intra Day 1 (n = 5) 0.27</td>
<td>Intra Day 2 (n = 5) 0.30</td>
<td>Intra Day 3 (n = 5) 0.29</td>
</tr>
<tr>
<td>0.27</td>
<td>12.3</td>
<td>32.2</td>
</tr>
<tr>
<td>0.30</td>
<td>11.9</td>
<td>28.8</td>
</tr>
<tr>
<td>0.29</td>
<td>12.1</td>
<td>32.4</td>
</tr>
<tr>
<td>0.29</td>
<td>12.1</td>
<td>31.2</td>
</tr>
<tr>
<td>%Deviation = [(measured concentration/added concentration) × 100] – 100, %RSD= Relative standard deviation (%RSD = (standard deviation/mean) × 100).</td>
<td></td>
<td></td>
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</tbody>
</table>

0.1 µg·mL⁻¹. The mean S/N value for these samples were > 5 as calculated by the Analyst 1.5.2 software. The VAN peaks were identifiable, discrete and reproducible with an accuracy (%Deviation) −20.2%, and precision (%RSD) 8.39% (Table 1). A representative chromatogram at the LLOQ (0.1 µg·mL⁻¹) in rabbit serum is presented in the inset of Figure 1. The linear range of the assay was established as 0.1 to 40 µg·mL⁻¹ with the regression coefficient (mean ± SD, n = 3) r = 0.99 ± 0.003. This met the acceptance criterion for linearity of r ≥ 0.98 after weighting with 1/x². Calibration curves were evaluated without weighting, and with a weighting factor of 1/x and 1/x². The weighting factor of 1/x² was used in the present study, because it provided the lowest variance of the calibration points, particularly at the LLOQ and it was the simplest model to adequately describe the curve [14]. Furthermore, this weighting factor is often used for bio-analytical assays using LC/MS/MS [15].

3.3. VAN Pharmacokinetics in Rabbits

The developed method was successfully applied to measure serum VAN levels after the i.v. administration of five doses of VAN to rabbits (n = 5). The concentrations of VAN over time are shown in Figure 2. A loading dose of 20 mg·kg⁻¹ VAN was administered at time zero followed by four maintenance doses of 15 mg·kg⁻¹ VAN administered in every 1.5 h. Serum samples were taken shortly before administering the next dose (i.e., at trough levels). After the last dose of VAN, six subsequent samples were taken to investigate the pharmacokinetic profile of VAN in rabbit serum. Trough levels of VAN showed a constant pattern with serum concentrations of 20.9, 21.0, 21.5, and 23.9 µg·mL⁻¹. The highest concentration was 53.5 µg·mL⁻¹ after the last maintenance dose. In a previously unpublished study, a 20 mg·kg⁻¹ VAN i.v. dose was administered to rabbits and the serum samples analyzed using PETINIA (Siemens Dimension Vista® System, Siemens Canada, Oakville, ON, Canada). The VAN serum concentrations obtained with PETINIA were superimposed over the data obtained with the
current UHPLC-MS/MS method and the results were comparable (Figure 2). Using the serum elimination phase data obtained with UHPLC-MS/MS, a non-compartmental pharmacokinetic analysis was performed (Phoenix WinNonlin 6.3, Certara USA Inc., Princeton, NJ, USA) and the results are presented in Table 2.

**Figure 2.** Vancomycin (VAN) concentrations in rabbit serum. Blue circles denote the VAN concentrations after the i.v. administration of five doses of VAN to rabbits (n = 5), and analyzed by UHPLC-MS/MS. Black squares denote the VAN concentrations obtained in a previously unpublished single dose study using PETINIA (Siemens Dimension Vista® System, Siemens Canada, Oakville, ON, Canada). The thin line is not a model fit but serves as a visual aid.

**Table 2.** Pharmacokinetic parameters for VAN in rabbit serum.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Mean ± SD (n = 5)</th>
</tr>
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<tbody>
<tr>
<td>AUC&lt;sub&gt;0-∞&lt;/sub&gt;</td>
<td>h-µg·mL&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>111 ± 32.2</td>
</tr>
<tr>
<td>C&lt;sub&gt;0&lt;/sub&gt;</td>
<td>µg·mL&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>60.2 ± 11.8</td>
</tr>
<tr>
<td>λ&lt;sub&gt;z&lt;/sub&gt;</td>
<td>1·h&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>0.640 ± 0.160</td>
</tr>
<tr>
<td>MRT&lt;sub&gt;0-∞&lt;/sub&gt;</td>
<td>h</td>
<td>1.95 ± 0.820</td>
</tr>
<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt;</td>
<td>mL·kg&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>262 ± 53.4</td>
</tr>
</tbody>
</table>

AUC<sub>0-∞</sub>: Area under the concentration time curve from time zero to infinity, C<sub>0</sub>: Concentration at time zero, determined through back extrapolation of the log linear transform of the first two sampling points post dose, λ<sub>z</sub>: elimination rate constant, MRT<sub>0-∞</sub>: Mean residence time from time zero to infinity, V<sub>ss</sub>: Volume of distribution at steady-state.
3.4. Applicability for Future Studies

The present study reports a novel UHPLC-MS/MS method that is quick, sensitive, accurate and precise for the quantitation of VAN requiring only 2 µL of sample. The matrix used was rabbit serum, but the method may be easily transferable to other matrices, such as dried blood spots or ISF where only low sample volumes are available. The applicability of the method in ISF would be particularly useful, because once the method is transferred and fully validated in ISF, it can be implemented in studies, such as TDM of VAN in ISF [12]. One shortcoming of the present study may be noted as the method was qualified, not fully validated. However, a full validation was not the aim of the current pilot study, but to qualify the method and apply it for PK studies in rabbits. The significance of the current study is that the method qualification results obtained may provide useful information to assess the feasibility of expanding the process to larger studies in the future where the method is fully validated in appropriate matrices, such as ISF, and implement it in studies on TDM of VAN in ISF.

4. Conclusion

A sensitive and selective UHPLC-MS/MS method was developed for the quantification of VAN in rabbit serum requiring 2 µL of sample. The assay was applied for the measurement of VAN in serum samples of rabbits after the i.v. administration of VAN. The results were compared to data previously acquired using PETINIA. The current UHPLC-MS/MS method is superior to the PETINIA technique as it requires only 2 µL of sample compared to the large volumes (50 µL to fill the sample cuvette) required by the immunoassay. Other LC-MS/MS assays determined VAN in human [8] or rat plasma [7], and human serum [6]. While some of these assays achieved good sensitivity, they required large sample volumes and none of them attempted to measure VAN in rabbit serum [2] [7] [16]. Other methods determined VAN in rabbit serum, but the sensitivity of these assays was insufficient [10] [11]. Compared to these assays, the current method has good sensitivity and to our knowledge, it is the first UHPLC-MS/MS method to quantify VAN in rabbit serum using only 2 µL of sample. The method may be easily transferable to other matrices, such as dried blood spots or ISF where obtaining VAN for TDM is only possible in very small volumes [12]. In fact, many experiments involving small laboratory animals, such as rabbits, will benefit from this method that allows for repeated sampling of very small biological samples.

Acknowledgements

This study was supported by the operating grant of the Collaborative Health Research Project, Natural Sciences and Engineering Research Council of Canada, and Canadian Institute of Health Research (CHRPJ 385967).
References


