High performance liquid chromatography method for implementation of therapeutic drug monitoring of Vancomycin at Hospital for Tropical Diseases - Ho Chi Minh City Vietnam

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Abstract
The simple, rapid and reliable method of high performance liquid chromatography with diode array detection (DAD) for the quantification of vancomycin in human plasma using trimethoprim as an internal standard has been developed and validated. The drug and IS were extracted by solid phase extraction with ISOLUTE-cartridges C8, IST (50mg/mL) and analyzed on Poroshell 120, C8 EC, 2.7µm, 100mm×4.6mm i.d using a mobile phase of potassium dihydrogen phosphate buffer 25mM, pH 3.0 − acetonitrile (92:8, v/v) at a flow rate of 1mL/min at 40°C. The injection volume was 20µL and the DAD wavelength was set at 236nm. The method was validated over the concentration range of 0.5-100 µg/mL (r2>0.999) with a limit of detection of 0.2µg/mL and limit of quantification of 0.5µg/mL. Intra-assay and inter-assay precision for VAN at three concentrations (low/medium/high) ranged from 1.89 to 6.5% (%CV), and accuracy varied from 99.1 to 103.4%. VAN and TMP (IS) had high recovery (98.5% and 94% respectively). The stability test showed that human plasma containing vancomycin was stable in different conditions. The analytical results obtained by HPLC and FPIA method correlated well (r=0.988, n=18, Pearson correlation test). The method is applied in measuring VAN in human plasma samples of severe infectious patients treated with VAN at Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam.

Keyword: Vancomycin, Therapeutic Drug Monitoring (TDM), HPLC, SPE, FPIA

1. INTRODUCTION
Vancomycin (VAN) is considered the gold standard for treating methicillin-resistant staphylococcal infections, including bacteremia, endocarditis, pneumonia, cellulitis, and osteomyelitis1. Patients who are allergic to penicillin, or patients who have failed to respond to other antibiotics are also treated with VAN. VAN has a narrow therapeutic range, over-dosing results in nephrotoxicity and ototoxicity while under-dosing contributes to ineffective treatment and development of resistance2, 3. Moreover, VAN concentrations vary in different conditions like renal failure, renal support therapies, obesity, liver failure, neutropenia, malignancy and sepsis. Therefore, therapeutic drug monitoring (TDM) has been recommended to guide dosing adjustment of VAN4. Additionally, TDM for VAN has been required in intensive care situations that co-administrate drugs induce important haemodynamic effects5.

Maintaining VAN concentration at the active site above the minimum inhibitory concentration (MIC) during the dose interval

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is assumed to bring about good therapeutic effects. In addition, an AUC/MIC value of 400 has been established as the primary predictive pharmacodynamic parameter for efficacy. While it is not routinely monitored $AUC_{0-24}$ in clinical practice, trough concentrations ($C_{\text{trough}}$) are well correlated with $AUC_{0-24}$ and therefore $C_{\text{trough}}$ are mentioned as an accurate and practical index of monitoring the effectiveness of VAN.

At Hospital for Tropical Diseases (HTD), a priority hospital for infectious patients in southern of Vietnam, the percentage of methicillin-resistant \textit{Staphylococcus aureus} isolated has increased considerably over the past few years from 40% in 2010 to 69% in 2013 and 71% in 2014. The consumption of VAN also augmented dramatically, the defined daily dose per 1000 bed-days (DDD/1000 bed-days) has risen from 2.75 (in 2008) to 8.73 (in 2013) and 9.3 (in 2014). However, despite the need of vancomycin TDM, it had not been applied until this study was set up at HTD in 2014.

In terms of quantification technique, there is a number of methods for VAN quantitative analysis such as biological methods, immunologic methods and chromatographic methods. TDM of vancomycin is usually undertaken by fluorescence polarization immune assay (FPIA), an immunologic method, due to its rapidity and its ease of application. Chromatographic methods like high performance liquid chromatography (HPLC) \textsuperscript{8-10}, ultra-performance liquid chromatographic (UPLC) \textsuperscript{11}, high performance liquid chromatography-mass spectra (LC-MS) \textsuperscript{12} and high pressure liquid chromatography tandem mass spectrometry (LC-MS/MS) \textsuperscript{13, 14}, were also described for measuring VAN. Some papers reported comparison results between different quantification methods of VAN\textsuperscript{14-17}. Some publications showed that the concentrations of VAN as determined by immunologic methods (FPIA and RIA) were slightly higher than those using chromatographic methods (HPLC and LC-MS/MS), and the overestimation of drug concentrations has been observed due to time of sampling, the plasma bilirubin concentration, the presence of VAN metabolites\textsuperscript{18}, the change of VAN pharmacokinetic\textsuperscript{19}, and vancomycin degradation in renal failure or patients on dialysis\textsuperscript{6}.

With the purpose of designing a method concordant with the available facilities at the hospital (HTD), this study focused on developing and validating an isocratic HPLC-DAD method with sample pretreatment on solid phase extraction (SPE). This method applied to measure VAN concentration in samples collected from patients admitted in four main wards of Hospital for Tropical Diseases (HTD) at Ho Chi Minh City, Vietnam: ICU/A/B and Viet-Anh ward.

2. MATERIALS AND METHODS

2.1. Chemicals and materials

All reagents and solvents used were of analytical grade. Potassium dihydrogen phophate ($KH_2PO_4$), phosphoric acid ($H_3PO_4$), acetonitrile (ACN) and Methanol (MeOH) were purchased from Merck. HPLC-water was provided by a Purelab UHQ system (ELGA, Marlow, UK). Phosphate buffer solutions were prepared by dissolving appropriate amounts of potassium dihydrogen phophate in HPLC-water and adjusting the pH with phosphoric acid (Merck Darmstadt, Germany). The reference standards: Vancomycin B [VAN] (93.7% - Lot: SLBD4427V-Sigma) and internal standard [IS] trimethoprim [TMP] (98.5% - Lot: 1132089 13807298-Fluka), were purchased from Sigma-Aldrich Singapore. Blank plasma samples were obtained from a pool of human plasma, supplied by the Blood Transfusion and Haematology Hospital at Ho Chi Minh City.

2.2. Instrumentation

The liquid chromatography system was a Lachrom Elite – Hitachi (Merck) composed of an organizer, an autosampler L-2200, two pumps L-2130, a column Oven L-2350 and a Diode Array Detector (DAD) L-2455. The system was controlled by EZchrom Elite version 3.18 HPLC System Manager Software (Merck–Hitachi Japan). The analysis was performed on a Poroshell 120, C8 EC, 2.7µm, 100 mm×4.6 mm i.d. equipped with fast guard column, C8
Poroshell (Agilent Technologies, USA). The SPE was performed on ISOLUTE-cartridges C8, IST (50mg/1mL) (Biotage AB, Uppsala, Sweden).

The mobile phase consisted of potassium dihydrogen phosphate buffer 25mM (pH 3.0) – ACN (92:8, v/v), filtered (0.2µm, Regenerated Cellulose-Sartorius) and degassed for 30 minutes in a sonic bath (AL 04-12 Advantage Lab, Switzerland). The chromatography was performed at 40°C in 10 minutes at a flow rate of 1.0mL/min. The injection volume was 20µL and the DAD wavelength was set at 236nm. A system suitability test was performed prior to any sequence by injecting six consecutive aqueous standard solutions. The tolerated variation was assessed on area response and retention time with accepted variation of less than 2%.

2.3. Preparation of plasma standards

Stock solutions of TMP (0.5mg/mL) were prepared in MeOH-water (50:50, v/v). The solutions were successively diluted with phosphate buffer 25mM pH 3.0 solution to reach final concentrations of 10µg/mL (WS/TMP) and 1µg/mL (IS/TMP). Stock solutions of VAN (1mg/mL) were prepared in phosphate buffer 25mM pH 3.0 solution. VAN working solution (ranging from 5 to 1000µg/mL) was prepared by further diluted stock solution of VAN with phosphate buffer 25mM, pH 3.0. Plasma calibration curve (CCs) and quality control (QC) were constructed by diluting (1/10) the respective working solutions with blank plasma to give 7 CC points at 0, 0.5, 2.5, 5, 20, 50 and 100µg/mL (VAN) and quality control at low (QCL), medium (QCM) and high (QCH) concentrations of 1.5, 15 and 75µg/mL (VAN).

2.4. Analytical procedure

For sample preparation, 500µL of IS (TMP 1µg/mL in phosphate buffer 25mM pH 3.0) was added into 200µL of thawed plasma sample. The mixture was vortex mixed for 10 seconds and rested for two minutes, then centrifuge at 8000rpm for five minutes. The SPE process was undertaken on the manual SPE system, VAC-Master 20-sample processing Manifold (IST-Biotage, Sweden). The sample mixture was loaded onto the SPE ISOLUTE-cartridges C8, IST (50mg/1mL) (activated and conditioned with 2mL of MeOH, 1mL of water and 1mL ofphosphate buffer 25mM pH 3.0). The SPE cartridge was washed with 1mL of phosphate buffer 25mM pH 3.0, then 1mL of phosphate buffer 25mM pH 3.0-MeOH mixture (97:3, v/v) and dried for two minutes using medium vacuum. Then, VAN and TMP were eluted using 600µL (3×200µL) of phosphate buffer 25mM pH 3.0-MeOH (65:35, v/v) and collected in 4mL glass tubes. Finally, the SPE eluates were transferred to HPLC vials and 20µL was injected into the HPLC system.

2.5. Bioanalytical method validation

The described method was validated in terms of limit of detection (LOD), limit of quantification (LOQ), selectivity, calibration curve, recovery, accuracy, precision and stability according to EMEA and FDA guidelines on the bio-analytical method validation20, 21.

2.5.1. Specificity and selectivity

The ability of the method to differentiate the analytes (VAN and IS/TMP) towards endogenous plasma interferences was investigated by analysis of six different blank plasma samples from six different donors. Potential administered co-medications like ceftriaxone, ertapenem, cilastatin, levofloxacin, meropenem, adefovir, entecavir, paracetamol were also evaluated.

2.5.2. Calibration curve, limit of detection and limit of quantification

The linearity of calibration consisted of seven calibration standards (including a blank sample) and was obtained by calculating the peak-area ratios of VAN to TMP against the corresponding concentrations. The linear regression models were evaluated using data obtained during the assays validation. Back calculations were made to determine concentrations of VAN in the QC validation.
sets and clinical samples. The LOD was determined based on a signal to noise ratio of 3:1. The LOQ was determined based on a signal to noise ratio of 10:1, from the determination of six replicate spiked plasma samples.

2.5.3. Recovery, accuracy and precision

The recovery yields for VAN and IS (TMP) were calculated at QCL, QCM and QCH by comparing the area response of spiked plasma samples to that of unprocessed aqueous solutions. Intra-assay accuracy and precision were determined using four different replicates of QCL, QCM and QCH analyzed within the same day. Inter-assay accuracy and precision were estimated by analyzing four replicates of QC sets on four consecutive days by different analysts. Intra-, Inter- and total-assay precisions were calculated using a single factor analysis of variance (ANOVA). Precision (%) was expressed as the mean relative standard deviation of the concentration area response of analytes/IS. Accuracy (%) was calculated as (estimated concentration/nominal value)×100. The variation of the back calculated concentrations from the nominal concentrations should not be more than 15% in precision and range from 85 to 115% in accuracy.

2.5.4. Stability

The stability study was carried out to test the stability of VAN under different conditions. All of the stability experiments were evaluated by comparing the VAN concentrations area response (analytes/IS) of five different replicates of QCL and QCH against freshly prepared spiked solutions, and corrected from their initial values. Spiked solutions were considered stable if the deviation from the nominal values was within ±15%. The stock solutions of VAN and IS were tested for short term stability at room temperature (RT) for 24 hours and long term stability at -20°C for one month. Heat inactivation process was also investigated. Auto-sampler stability was performed on SPE eluates stored 24 hours. Freeze/thaw stability was studied using three consecutive cycles of freezing (-80°C for at least 24 hours) and gentle thawing at room temperature. Long-term stability of spiked samples was examined after one month at -20°C, and 1, 3 and 6 months storage at -80°C.

2.6 Clinical samples

Clinical samples were collected from patients admitted in four main wards of Hospital for Tropical Diseases (HTD). Blood was drawn, divided in two tubes with different anticoagulants (EDTA and lithium heparin). The EDTA tubes were sent to Choray hospital laboratory for quantitative analysis by fluorescence polarization immuno assay (FPIA) on the AxSYM (Abbott). And the separated plasma in lithium heparin tubes (centrifuged at 2000rpm for 10 minutes) were stored at -80°C at Pharmacology laboratory - OUCRU until analysis by the HPLC method. The clinical samples were analyzed and validated against freshly prepared CCs and QCs. The acceptance criteria on the validation QC sets was <15% for precision and between 85 and 115% for accuracy.

3. RESULTS AND DISCUSSIONS

3.1. Method development

A new HPLC-DAD analytical method for the simultaneous determinations of VAN and TMP (IS) was utilized. Different analytical columns, pH of the buffer, mobile phase compositions, and wavelengths of detection were tested to get the optimum chromatographic condition. Based on six known pK values for vancomycin consisting of 2.18, 7.75, 8.89, 9.59, 10.4, and 12.0, the buffer pH of 3.0 was chosen in association with the high solubility of VAN. With the mobile phase of potassium dihydrogen phosphate buffer 25mM (pH 3.0) – ACN in different ratios (from 5 to 10% of ACN), we found out that 8% of ACN led to reasonable retention time for analytes without interferences and shorten the total analysis time as well. Moreover, the optimal separation
was achieved for a Poroshell 120, C8 EC, 2.7µm, 100mm×4.6mm i.d. column with better peak shapes in comparison with C18 column due to the earlier retention time for VAN. This could be considered an advantage over some previous publications8-10, 12. In the tested wavelength range from 220nm to 280nm, good response signals were achieved for VAN and TMP at 236nm.

Furthermore, sample pretreatment on a solid-phase extraction procedure was proposed. Various types of cartridges (ABN, CBA, C18, C8 cartridges), solutions for column condition and elution were evaluated. ISOLUTE−cartridges C8, IST (50mg/1mL) was selected as it demonstrated the highest recoveries and cleanest samples. The percentage of MeOH in elution solution was also shown to have significant influences on VAN and TMP recoveries. The elution solutions of phosphate buffer 25mM (pH 3.0) − MeOH in different ratios (from 5–40% of MeOH) were tested. Over 35% of MeOH led to broad and malformation peaks while less than 30% of MeOH resulted in low recoveries (<60%) for VAN and TMP. Therefore, 600µL (divided in 3 consecutive times) of the elution solution of phosphate buffer 25mM (pH 3.0) − MeOH (65:35, v/v) was selected.

3.2. Method validation

3.2.1. Specificity and selectivity

The assay did not show any interference with other potential concomitant drugs (see Section 2.5.1). The tested six-blank plasma also did not show any interference in the retention time windows for both compounds (VAN and TMP/IS). The chromatogram showed a clear and good separation, and the retention times of VAN and TMP/IS were approximately 4.3 and 8.5 minutes, respectively (Fig.1).

![Representative chromatograms: (1) human blank plasma, (2) plasma with TMP/IS (1µg/mL), (3) spiked plasma with VAN (15µg/mL, QCM) and TMP/IS (1µg/mL), (4) patient plasma sample with TMP/IS (1µg/mL) at 236 nm.](image_url)
3.2.2. Calibration curve, limit of detection and limit of quantification

The calibration curve was linear over VAN concentration range from 0.5 to 100µg/mL in human plasma. A linear regression model with LSQ non-weighting and log&log scaling was used due to good signal-response. Mean regression coefficient ($r^2$) of all calibration curves was over 0.999 demonstrated the linearity of the method. For 200µL of plasma sample, the LOQ and LOD for VAN were 0.50µg/mL and 0.20µg/mL respectively.

3.2.3. Recovery, precision and accuracy

Four aliquots each at a different concentration (low/medium/high) were analyzed in four different days. Mean recovery of VAN was 98.5% and that of TMP was 94%. The mean recovery of each quality control (QC) was 96.9% for QC low, 99.6% for QC medium, 98.9% for QC high. The SPE with ISOLUTE-cartridge C8, IST (50mg/1mL) brought about higher recovery for both VAN and the IS in comparison with previous methods\textsuperscript{11-13}. Assay results showed acceptable precision with %CV ranges from 1.89 to 6.50% (Table 1). The accuracy of the determinations was also acceptable, ranging from 99.10 to 103.40% (Table 1).

Table 1. Intra-assay, inter-assay and total-assay precision and accuracy of vancomycin in plasma (ANOVA, N=16)

<table>
<thead>
<tr>
<th>Vancomycin</th>
<th>Precision (%CV)</th>
<th>Accuracy (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Inter-assay</td>
<td>Intra-assay</td>
</tr>
<tr>
<td>QC Low</td>
<td>6.30</td>
<td>3.72</td>
</tr>
<tr>
<td>QC Med</td>
<td>3.26</td>
<td>1.89</td>
</tr>
<tr>
<td>QC High</td>
<td>6.50</td>
<td>3.65</td>
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</table>

3.2.4. Stability

The conditions applied in the stability tests simulated situations most likely to be experienced in actual sample analysis. The stability of VAN was confirmed at room temperature (RT-24H), in auto-sampler for 24 hours (EXT-24H), under the three freeze-thaw cycles (C3), in short-term and long-term storage conditions with good results (91.20 - 100.8% for QCL; 92.10% - 100.50% for QCH). However, under the heat process (56°C for 60 minutes) with the purpose of inactivating potential pathogens without denaturing protein, the stability VAN was 80.90% for QCL and 82.58% for QCH, this is outside of the standard range recommended by FDA. Therefore, we suggested that heat inactivation should not be applied in sample preparation process. The detailed results of VAN stability in plasma are presented in table 2.

3.3. Clinical application

The method was applied initially on 18 plasma samples from severe infectious patients treated with VAN at HTD. Concentrations of VAN in the clinical samples are all covered in the CC range of 0.5−100µg/mL (VAN). The maximum value was 37.99µg/mL (sample ID. No. 15), and the minimum value was 2.38µg/mL (sample ID. No.9), measured by the HPLC method.

For the FPIA method, the 18 plasma samples were analyzed on the AxSYM (Abbott). The standard calibrators were 0, 5, 10, 25, 50 and 100µg/mL (VAN). The sensitivity was 2µg/mL (VAN)\textsuperscript{23}. As shown in figure 2(b), the two methods (HPLC and FPIA) correlated well in the determination of VAN plasma levels (correlation matrix-Pearson: 0.988, coefficients of determination
The established equation was: $y = 1.1838x - 1.0255$. In terms of quantitative results, 88.9% (16/18 samples) of concentrations as determined by FPIA were higher than those using HPLC (mean difference = 2.6 µg/mL) (fig. 2(a)).

Figure 2. (a) Vancomycin concentration in patients’ plasma samples measured by HPLC and FPIA; (b) The HPLC–FPIA correlation.

Table 2. Variation of vancomycin concentrations in plasma under different conditions

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Plasma QCL (n=5)</th>
<th>Plasma QCH (n=5)</th>
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</thead>
<tbody>
<tr>
<td>RT-24H</td>
<td>92.05%</td>
<td>94.14%</td>
</tr>
<tr>
<td>EXT-24H</td>
<td>100.8%</td>
<td>98.20%</td>
</tr>
<tr>
<td>Heat 56°C for 60min.</td>
<td>80.90%</td>
<td>82.58%</td>
</tr>
<tr>
<td>C3</td>
<td>99.32%</td>
<td>100.50%</td>
</tr>
<tr>
<td>M1 (-20°C)</td>
<td>95.60%</td>
<td>96.10%</td>
</tr>
<tr>
<td>M1 (-80°C)</td>
<td>95.90%</td>
<td>92.10%</td>
</tr>
<tr>
<td>M3 (-80°C)</td>
<td>96.90%</td>
<td>95.46%</td>
</tr>
<tr>
<td>M6 (-80°C)</td>
<td>91.20%</td>
<td>94.29%</td>
</tr>
</tbody>
</table>

Results in % change = [mean value in stability sample/mean value in reference] × 100. Storage conditions are: RT-24H: Unprocessed samples left at room temperature for 24 hours. EXT-24H: SPE eluates kept in autosampler for 24 hours. C3: third freeze/thaw cycle. M1: Storage for one month at (-20°C) and (-80°C). M3, M6: Storage at -80°C for three months, six months.

4. CONCLUSIONS

The developed and validated HPLC-DAD method enables a reliable simple quantification method of vancomycin for implementing routine TDM and dosing adjustment, applicable to patients with severe infectious treated with VAN at Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam. With only 200µl of plasma sample, the method achieved the LOD of 0.2µg/mL, LOQ of 0.5µg/mL and the optimal separation within 10 minutes of total analysis time.

The VAN concentrations determined by the developed HPLC method demonstrated a significant linear correlation with the values measured by FPIA. Hence, the study suggested the HPLC method could be applicable to both
routine TDM of VAN and other PK/PD studies with added validations for other matrices (e.g. CSF from meningitis patients).

5. ACKNOWLEDGEMENTS
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