New liquid chromatography-tandem mass spectrometry method for routine TDM of vancomycin in patients with both normal and impaired renal functions and comparison with results of polarization fluoroimmunoassay in light of varying creatinine concentrations

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ARTICLE INFO

Keywords:
Vancomycin
LC-MS/MS
Fluorescence polarization immunoassay
Therapeutic drug monitoring
Creatinine concentration
Dialyzed patients

ABSTRACT

A new LC-MS/MS method with simple sample extraction and a relatively short period of vancomycin analysis for routine therapeutic drug monitoring was developed and validated. 50 μL serum was precipitated using 20 μL 33% trichloroacetic acid and 0.5 mol/L NH4OH was added to increase pH before analysis. A RP BEH C18, 1.7 μm, 2.1 × 50 mm column maintained at 30 °C and tobramycin as internal standard were used. Mass detection was performed in positive electrospray mode.

The results obtained with LC-MS/MS method were correlated with an FPIA assay (Abbott AxSYM) using mouse monoclonal antibody. Subjects were divided into three groups according to creatinine levels (53.5 ± 19.1, 150.2 ± 48.4, 471.7 ± 124.7 μmol/L) and Passing-Bablok regression analysis and Bland-Altman analysis were used to compare vancomycin concentrations. The results of subjects with both normal and higher creatinine levels correlated very well and the linear regression model equations were near ideal (LC-MSVAN = 0.947 × AbbottVAN + 0.192 and LC-MSVAN = 0.973 × AbbottVAN − 0.411 respectively). Dialyzed patients with the highest creatinine levels showed about 14% greater vancomycin concentration with the FPIA assay (LC-MSVAN = 0.866 × AbbottVAN + 2.127). This overestimation probably due to the presence of the metabolite CDP ought not to be of clinical relevance owing to the wide range of recommended vancomycin concentration.

1. Introduction

Vancomycin is a glycopeptide antibiotic with a strong bactericidal activity used for the treatment of serious infections caused by gram-positive bacteria including methicillin-resistant Staphylococcus aureus. Its main application is in respiratory tract infection, sepsis, endocarditis and surgical prophylaxis during prosthetic implantations. Vancomycin has also become the drug of choice for treating severe gram-positive infections in patients with hypersensitive reactions to penicillins and cephalosporins. Vancomycin must be given intravenously, oral absorption in not possible because of the highly polar groups that contribute to its structure [1].

Therapeutic drug monitoring (TDM) of vancomycin is highly valuable due to the large interpatient and intrapatient variability of pharmacokinetics on one hand, and the correlation between therapeutic failure and low plasma concentration and toxicity and high plasma concentration on the other. Recommended vancomycin trough concentrations are 10–15 mg/L or up to 15–20 mg/L for severe infections and the peak concentration ought to be below 50 mg/L. Higher trough concentrations and peak concentrations can contribute to nephrotoxicity and ototoxicity, especially in combinations with other nephrotoxic and ototoxic drugs [2]. Vancomycin is eliminated mainly through the kidneys. The half-life of the drug is 5–11 h in normal patients and in patients with renal insufficiency may be prolonged to days. When the drug remains longer in the body exposed to the internal body temperature CDP, a degradation product of vancomycin without antibiotic activity, is formed in high concentrations [3]. The degradation product actually consists of two compounds referred as CDP 1M.
(major) and CDP 1m (minor), which are conformers of each other and exist in dynamic equilibrium [4]. Sym et al. supposed that CDP is also formed in prolonged vancomycin treatment but the increase in vancomycin levels in some patients found by immunological methods was not due to an accumulation of the degradation product but to a change in vancomycin pharmacokinetics [5]. Also the presence of CDP during local application of vancomycin was tested and was found in a measurable amount [6]. CDP in concentrations below 1% is present in all marketed vancomycin injections together with other impurities which are structurally close to vancomycin [7,8,9].

Among the methods developed for the quantification of vancomycin in biological fluids, immunoenzymatic techniques such as FPIA or EMIT and chromatographic methods are the most relevant. The former are widely used for TDM in clinical practice due to their high speed and simplicity but there are several problems associated with these techniques. Firstly, there is the lower precision of EMIT assays for higher concentrations and secondly the lower sensitivity of FPIA methods, with an upper limit of quantification of 2 mg/mL and cross reactivity with the degradation product CDP [10]. In particular, in FPIA assays with a polyclonal antibody a high overestimation of vancomycin concentration (> 60% compared with the HPLC method) was found in patients with severe renal impairment [11]. These drawbacks have led to the development of different chromatographic techniques for more precise, sensitive and accurate determination. Vancomycin has been quantified in biological samples using high-performance liquid chromatography methods with ultraviolet detection [3,6,11,12,13,14,15,16], fluorescence detection [1] and recently mass spectrometry detection [17,18,19,20,21,22,23]. The LC-MS/MS methods in particular have enabled using lower amounts of the biological sample with sufficient sensitivity, which is highly important particularly for TDM in children and newborns [21].

The purpose of this study was to develop and validate a new LC-MS/MS method for measurement of vancomycin concentrations for routine TDM with simple sample extraction and an acceptable runtime, and secondly to compare data obtained from three groups of patients divided on the basis of their serum creatinine concentration both with our previously routinely used immunoenzymatic method FPIA (Abbott AxSYM) and newly introduced LC-MS/MS method.

2. Materials and methods

2.1. Chemicals and solutions

HPLC gradient grade water, standards of vancomycin (vancomycin hydrochloride VAN) and tobramycin (TOB), trichloroacetic acid (TCA) and ammonium hydroxide (c. 25% NH3) were purchased from Sigma–Aldrich (Prague, Czech Republic). Methanol, acetonitrile (both HPLC gradient grade), ammonium acetate fractopur and formic acid extra pure were obtained from Merck (Darmstad, Germany). The drug free serum of healthy volunteers was provided by the Blood Centre, University Hospital Ostrava. Quality control samples (TDM control set) in three concentration levels (7.4, 22.6 and 36.0 mg/L) were purchased from Roche.

Trichloroacetic acid used as precipitating agent was diluted with water for HPLC to 33% and 0.5 mol/L NH4OH was prepared from concentrated ammonium solution. A stock solution of vancomycin was prepared in methanol in a concentration of 100 mg/L and calibration standards were gradually diluted with methanol to concentrations of 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0 and 100 mg/L. A solution of tobramycin which was used as an internal standard was prepared in methanol in a concentration of 50 mg/L. Standard stock solutions and calibration standards were stored at –20 °C, and the internal standard solution at 4 °C.

2.2. Preparation of calibration curve and patient samples

The drug free serum (50 μL) was spiked with 50 μL internal standard and 50 μL of calibration standard. 20 μL of 33% trichloroacetic acid and 0.2 mL of water were added. The mixture was vigorously vortex-mixed for 60 s and then 50 μL acetonitrile and 50 μL 0.5 mol/L NH4OH were added and briefly vortex-mixed. After centrifugation for 20 min at 1370 × g and 4 °C, the upper layer was transferred into vials and 10 μL was injected into a chromatographic column. Patient samples and quality control samples were treated in the same way.

2.3. UPLC equipment and mass spectrometric conditions

The UPLC-MS/MS system consisted of a Waters Acuity UPLC instrument (Waters Corp., Milford, MA, USA) connected to a Quatro Micro Api triple quadrupole (Micromass, Manchester, UK) with an Acuity UPLC RP BEH C18, 1.7 μm, 2.1 × 50 mm mm column. Analysis was performed using Buffer A (2 mmol/L ammonium acetate, 0.1% formic acid in 5% acetonitrile v/v/v) and Buffer B (2 mmol/L ammonium acetate, 0.1% formic acid in 95% methanol v/v/v) as follows: 0–1 min A:B 80:20 (v/v), 1.0–1.3 min and 1.3–4.0 min 5:95 A:B (v/v), 4.0–4.1 and 4.1–5.0 A:B 80:20 v/v. The flow rate was 0.4 mL/min and the temperature of the column was maintained at 30 °C. Analytes in the eluate from the UPLC column were introduced into a capillary sprayer and positive ion electrospray ionization was used. The source and desolvation temperature were set at 100 °C and 400 °C respectively.

Nitrogen was used as a desolusion gas with a flow rate of 600 L/h and the capillary voltage was 1.5 kV. Multiple reaction monitoring with high purity argon as a collision gas was used. The specific data of precursor/product ions were 725.1 > 143.9 and 725.1 > 100.1 for vancomycin, and 468.1 > 163 for tobramycin as the internal standard. All data were evaluated using MassLynx 4.1 sofware (Waters Corp Milford, MA, USA).

2.4. Validation of method

Method validation (linearity, precision, accuracy, recovery, LOQ and matrix effect) was carried out following the FDA criteria [24].

Linearity: A least-square calibration curve consisting of 10 points was constructed by plotting the peak area ratio for vancomycin and that for the internal standard against the standard concentration.

Accuracy and precision: To evaluate these parameters, four concentration levels of vancomycin were used: 0.25, 1.0, 10 and 50 mg/L. Ten replicates of each level were analysed within the same day to obtain repeatability and intra-assay precision, and over 5 different days to obtain inter-assay precision and recovery. The precision (coefficient of variation CV) of the method was required to be within 15% and similarly accuracy should not deviate by 15% from the nominal concentration.

Extraction recovery: To determine the extraction recovery, the peak area obtained from the extracted serum samples was compared with the peak area obtained after injection of an equivalent amount of compound dissolved in a precipitation mixture without serum.

Limit of quantification is defined as the lowest concentration with acceptable precision and accuracy (coefficient of variation < 20% and repeatability between 80 and 120%).

Stability: To test autosampler stability a sample control set was prepared and repeatedly analysed during the five following days (one week).

Matrix effect: To investigate a potential ion suppressive effect attributable either to the matrix or co-medication, a batch of samples was tested as follows: Sample A: A precipitation mixture without serum; and sample B: A precipitation mixture with 50 μL of serum. All samples were enriched with vancomycin in two concentrations, 0.1 mg/L and 100 mg/L respectively, and tobramycin as an internal standard. Finally seven different serum samples were used; two drug-free samples and five samples from patients with complex medication taking more than 5 different drugs.
2.5. Method comparison

Vancomycin concentrations in the serum samples were analysed with the new method and with the FPIA immunoenzymatic method (Abbott AxSYM), which had been routinely used previously. The fluorescence polarization immunoassay with mouse monoclonal antibody had declared parameters as follows: intra-assay coefficient of variation 2.6–4.2%, inter-assay coefficient of variation 2.9–4.3% and recovery 98.9–105.5% [25].

2.6. Patients

Serum samples were obtained from inpatients treated in our hospital during the first three months of 2015. Samples were sent to our department during routine TDM of vancomycin in patients with severe gram-positive infections including MRSA resistant ones. Some serum samples were submitted repeatedly. The samples were usually taken before (trough level) and one hour after infusion of vancomycin (peak level), and were sent to the laboratory and immediately measured. On the same day the serum samples were frozen and kept at −20 °C until being analysed with the new LS-MS/MS method.

Patients were divided into three groups according to serum creatinine levels. More details about the cohort of patients are summarized in Table 1.

2.7. Pharmacokinetic analysis

All vancomycin results were analysed using the pharmacokinetic programme MW-PHARM 3.30 based on Bayesian population pharmacokinetics and the patients’ data were released before application of the next vancomycin dose.

2.8. Statistics

Passing-Bablok regression analysis and Bland-Altman analysis were used to study variations of vancomycin concentration between the two different analytical procedures in the three groups of patients. Pearson correlation coefficients were calculated [26,27].

3. Results

3.1. Method validation

An increased volume of 0.5 mol/L NH₄OH (0.01–0.1 mL) was added to the precipitate resulting from 20 μL 33% TCA and correlation with pH and with area ratios of vancomycin and the internal standard are shown in Fig. 1. While pH was changed gradually, the ratios were relatively stable and increased only with the higher amount of NH₄OH. On the basis of these results, 0.05 mL 0.5 mol/L NH₄OH was chosen for further method validation. Chromatograms of vancomycin and the internal standard are shown in Fig. 2 (A: Standard serum sample; B: Example of dialyzed patient with high creatinine concentration).

The linearity of vancomycin was in the range 0.1–100 mg/L. The limit of quantification in the serum was determined as the lowest concentration of the calibration curve. The parameters of intra-assay and inter-assay precision, accuracy and recovery met validation requirements and are given in Table 2. The extraction recovery of vancomycin for four tested concentrations was between 77.8 and 84.4%.

Commercial quality controls in three levels were measured every day with the batch of patients’ samples and their concentrations were in the declared range (mean ± 20%). To test stability, the sample control set was repeatedly analysed during the five following days with coefficients of variation of 9.3%, 7.9% and 6.2% for low, medium and high concentrations respectively.

The matrix effect was tested using two concentrations of vancomycin (the lowest and the highest standard sample) added to the seven serum samples. Two of them were drug-free (the first and the second) and the others were obtained from patients taking more than five drugs but without vancomycin. The results are summarized in Table 3. All data (the areas of vancomycin and IS and their ratios) are within the interval ± 15%.

3.2. Method comparison

Passing-Bablok regression analysis and Bland-Altman analysis were used to compare the vancomycin concentrations in the three groups of patients with different creatinine levels. The results of patients with normal and increased creatinine concentration (Groups 1 and 2) correlated very well and the equations of linear regression model were near to ideal (LC-MSVAN = 0.947 × AbbottVAN + 0.192 and LC-MSVAN = 0.973 × AbbottVAN−0.411) respectively. Diazylated patients with the highest creatinine concentration (group 3) were slightly different and the eq. (LC-MSVAN = 0.866 × AbbottVAN + 2.127) shows that results obtained with the FPIA assay were about 14% higher. All data are summarized in Fig. 3. In Table 4 the distribution of results in favour of the LC-MS/MS method at lower concentrations is shown. Data for three concentration intervals (< 15 mg/L, 15–25 mg/L, > 25) were compared. Examples of the pharmacokinetic analysis of patients with normal and impaired renal function are shown in Fig. 4.

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Characteristic of cohort.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Before/after (dose)</td>
</tr>
<tr>
<td>1</td>
<td>77/41</td>
</tr>
<tr>
<td>2</td>
<td>27/8</td>
</tr>
<tr>
<td>3</td>
<td>25/5</td>
</tr>
</tbody>
</table>

Table 3. Comparison of results.

Group 1 patients with normal serum creatinine concentration (men 64–104 μmol/L and women 49–90 μmol/L). Group 2 patients with serum creatinine concentration above normal creatinine level. Group 3 patients undergoing dialysis or continuous venovenous hemofiltration.
Table 2
Parameters of validation.

<table>
<thead>
<tr>
<th>Concentration added (mg/L)</th>
<th>Extraction recovery (%)</th>
<th>intra-assay</th>
<th></th>
<th>inter-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Found conc. mg/L</td>
<td>± SD</td>
<td>CV%</td>
</tr>
<tr>
<td>0.25</td>
<td>78.1</td>
<td>0.26 ± 0.01</td>
<td>5.1</td>
<td>102.0</td>
</tr>
<tr>
<td>1.0</td>
<td>84.4</td>
<td>1.0 ± 0.03</td>
<td>3.0</td>
<td>100.3</td>
</tr>
<tr>
<td>10.0</td>
<td>84.0</td>
<td>10.0 ± 0.17</td>
<td>1.7</td>
<td>100.3</td>
</tr>
<tr>
<td>50.0</td>
<td>77.8</td>
<td>49.3 ± 1.32</td>
<td>2.6</td>
<td>98.7</td>
</tr>
</tbody>
</table>

A - standard sample in serum
B - sample of dialyzed patient

Fig. 2. Chromatograms of vancomycin and internal standard (tobramycin). A - standard sample in serum. B - sample of dialyzed patient.

Table 3
Matrix effect.

A 0.1 mg/L vancomycin – the lowest standard sample was added (sample without serum is taken as 100%)

<table>
<thead>
<tr>
<th>Vancomycin</th>
<th>Internal standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area</td>
<td>ME</td>
</tr>
<tr>
<td>Without serum</td>
<td>36.4</td>
</tr>
<tr>
<td>Matrix 1</td>
<td>36.9</td>
</tr>
<tr>
<td>Matrix 2</td>
<td>35.5</td>
</tr>
<tr>
<td>Matrix 3</td>
<td>38.0</td>
</tr>
<tr>
<td>Matrix 4</td>
<td>38.3</td>
</tr>
<tr>
<td>Matrix 5</td>
<td>39.0</td>
</tr>
<tr>
<td>Matrix 6</td>
<td>38.7</td>
</tr>
<tr>
<td>Matrix 7</td>
<td>37.0</td>
</tr>
</tbody>
</table>

B. 100 mg/L vancomycin – the highest standard sample was added (sample without serum is taken as 100%)

<table>
<thead>
<tr>
<th>Vancomycin</th>
<th>Internal standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area</td>
<td>ME</td>
</tr>
<tr>
<td>Without serum</td>
<td>30,897.5</td>
</tr>
<tr>
<td>Matrix 1</td>
<td>29,376.7</td>
</tr>
<tr>
<td>Matrix 2</td>
<td>31,014.4</td>
</tr>
<tr>
<td>Matrix 3</td>
<td>27,189.8</td>
</tr>
<tr>
<td>Matrix 4</td>
<td>29,175.7</td>
</tr>
<tr>
<td>Matrix 5</td>
<td>28,660.8</td>
</tr>
<tr>
<td>Matrix 6</td>
<td>28,402.6</td>
</tr>
<tr>
<td>Matrix 7</td>
<td>27,738.2</td>
</tr>
</tbody>
</table>

Area of vancomycin and ME, area of internal standard and ME and ratio of vancomycin/internal standard area (V/IS) and ME.
4. Discussion

A new LC-MS/MS method with simple sample extraction and a relatively short period of analysis in routine TDM of vancomycin was developed and validated. Many extraction procedures for vancomycin have been described but the most effective is precipitation with TCA or trifluoroacetic acid. Cheng et al. [20] tested various concentrations of TCA and found that the best recovery was provided with 15–35% TCA. In our preliminary experiments we found that vancomycin recovery was inversely influenced by the pH of the precipitation solution. The higher the pH of the precipitation solution used, the lower was the yield of vancomycin. Using a precipitation mixture consisting of methanol and acetonitrile with formic acid (pH 4.1), vancomycin extraction recovery was < 20%. Also the retention time of vancomycin is significantly influenced by the concentration of TCA or other acids due to their ion-pairing effect. The pH of extracts prepared with the 33% concentration of TCA was very low, only 1.1. In our experience, the use of such strong acids is not beneficial to the lifespan of chromatographic columns and the chromatographic system in general. For this reason, the NH₄OH solution was tested to increase the pH of extraction solutions.

Table 4

<table>
<thead>
<tr>
<th>Concentration of vancomycin (mg/L)</th>
<th>Analysis</th>
<th>FPIA</th>
<th>LC-MS/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 15 mg/L</td>
<td>83</td>
<td>51</td>
<td>53</td>
</tr>
<tr>
<td>15–25 mg/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 25 mg/L</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Comparison of vancomycin results obtained with LC-MS/MS and FPIA using Passing-Bablok regression analysis and Bland–Altman analysis. Patients with normal and increased creatinine concentrations and dialyzed patients.
extracts before chromatographic analysis. Using 0.05 mL of 0.5 mol/L NH₄OH increased the pH of acidic extracts by 0.5 to 1.6, with extraction recovery between 77.8 and 84.4% in all tested concentrations.

To quantify drugs isotopically labelled standards have been preferred, but in the case of vancomycin compounds of various origins were used as an internal standard. Antibiotics such as cefazolin [3], ristocetin [13], kanamycin B [21] or erythromycin [1] were applied. Phenacetin belonging to a different class of drugs was used [11], or compounds structurally related to vancomycin, such as vancomycin-des-leucine, vancomycin-glycine, norvancomycin or dalbavancin were synthesized [22,23,28,20]. In our study we gradually tested three antibiotics as an internal standard: erythromycin, kanamycin B and tobramycin, which have not been administered along with vancomycin in our hospital. Tobramycin was found to be the most appropriate for this purpose and was finally chosen for method validation.

Quantitative analysis with mass spectrometry can be also complicated by the presence of matrix components, particularly lipids and phospholipids [29] and with commonly used additives such as heparin and K₃EDTA. The application of sampling tubes without any additives should be preferred to reduce matrix effect [30]. In our previous papers we used a mixture of acetonitrile, methanol and a small amount of zinc sulphate for serum precipitation [31]. A post-column infusion experiment confirmed that the matrix effect was negligible at the time when most compounds were eluted [32,33]. Precipitation of serum using the strong TCA, along with the fact that patients treated with vancomycin are often critically ill with a variety of medication led us to test a possible matrix effect more precisely using both the drug-free serum and serum of patients from emergency care units with extensive medication [22]. Two vancomycin concentrations were chosen (the lowest and the highest) and the area of vancomycin and IS in the precipitation mixture with and without a matrix were compared and the ratios were calculated. In all tested serum samples the matrix suppression or enhancement was < 15%.

To test autosampler stability, a sample control set was prepared and repeatedly analysed during the five following days and all concentrations were in the declared range. The stability of vancomycin in the patients’ samples was not the crucial point in our study since samples were analysed immediately after being delivered to the laboratory. The vancomycin results along with data from pharmacokinetic analysis and possible dosage adjustment were released before administration of the next vancomycin dose.

Commercially available immunoassays used to quantify vancomycin in serum or plasma have been compared with HPLC methods both with ultraviolet and mass spectrometric detection since the 1990s. Assays used polyclonal antibody (FPIA assay Abbott) to compare with HPLC provided results, which were significantly higher (up to 40% in patients with renal impairment), probably due to cross-reactions with vancomycin CDP metabolites. These findings have led to an effort improve the quality of immunological methods and since 1992 a monoclonal FPIA assay has been used with coefficients of < 10% in correlation tests [34,35].

Oyaert et al. tested thoroughly correlations between the chromatographic method with mass detection and four different immunoassay reagent systems and pointed out the clinical impact of results obtained with each individual method [22]. Bijleveld et al. correlated vancomycin concentrations obtained with LC-MS/MS and FPIA in children and newborns [21]. Nevertheless, nobody has compared vancomycin concentrations between LC-MS/MS and FPIA in light of varying creatinine
levels and in patients with renal insufficiency. In our group of dialyzed patients with high creatinine concentrations (above 400 μmol/L), Bland-Altman and Passing-Bablok regression analysis showed that the vancomycin concentrations obtained with the monoclonal FPIA assay were about 14% higher compared with the LC-MS/MS method. This increase may be probably due to the presence of the metabolite CDP in patients with renal failure. Approximately one third of samples sent to our department during routine TDM of vancomycin were from patients with increased serum creatinine level and about 16% of those with severe impairment of renal functions. Recommended vancomycin trough concentrations are 10–15 mg/L (15–20 mg/L in severe infections) and the peak concentration up to 50 mg/L. Owing to the relatively wide window of vancomycin concentration along with the fact that dialyzed patients are monitored more often the 14% overestimation by FPIA assay ought not to be of clinical relevance.

5. Conclusions

A new LC-MS/MS method for the determination of vancomycin has been validated and applied for routine TDM. This method used 33% TCA for protein precipitation and 0.5 mol/L NH₄OH was added to increase the pH of acidic extracts before analysis. This procedure increased the lifespan of chromatographic column and enabled to use a common mobile phases without any special additives. The results obtained with this new LC-MS/MS method were correlated with an FPIA assay in three groups of patients. Vancomycin concentrations in patients with normal and slightly increased creatinine level and about 16% of those with severe impairment of renal functions. Recommended vancomycin trough concentrations are 10–15 mg/L (15–20 mg/L in severe infections) and the peak concentration up to 50 mg/L. Owing to the relatively wide window of vancomycin concentration along with the fact that dialyzed patients are monitored more often the 14% overestimation by FPIA assay ought not to be of clinical relevance.

This LC-MS/MS method requires only 50 μL of serum sample which is significant particularly for children and newborns, has simple sample extraction and a short time of analysis. These analytical conditions enable the release of vancomycin results along with pharmacokinetic data and potential dosage adjustments before the administration of the next vancomycin dose.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.cca.2017.04.003.

References


